Human Assisted Reproductive Technology

Future Trends in Laboratory and Clinical Practice



EDITED BY
David K. Gardner
Botros R. M. B. Rizk
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CAMBRIDGE
UNIVERSITY PRESS

CAMBRIDGE UNIVERSITY PRESS
Cambridge, New York, Melbourne, Madrid, Cape Town,
Singapore, São Paulo, Delhi, Tokyo, Mexico City

Cambridge University Press
The Edinburgh Building, Cambridge CB2 8RU, UK

Published in the United States of America by Cambridge University Press, New York

www.cambridge.org
Information on this title: www.cambridge.org/9781107001121

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First published 2011

Printed in the United Kingdom at the University Press, Cambridge

A catalog record for this publication is available from the British Library

Library of Congress Cataloging in Publication data
Human assisted reproductive technology : future trends in laboratory and clinical practice / edited by David K. Gardner, Department of Zoology, University of Melbourne, Australia, Botros R. M. B. Rizk, Division of Reproductive Endocrinology and Infertility, University of South Alabama College of Medicine, and University of South Alabama In Vitro Fertilization Laboratory, Mobile, AL, USA, Tommaso Falcone, Cleveland Clinic Foundation, Cleveland, OH, USA.
p. ; cm.
Includes bibliographical references and index.
ISBN 978-1-107-00112-1 (hardback)
1. Human reproductive technology. 2. Infertility – Treatment. I. Gardner, David K., 1954 – editor. II. Rizk, Botros R. M. B., 1958 – editor. III. Falcone, Tomasso, 1953 – editor. IV. Title.
[DNLM: 1. Reproductive Techniques, Assisted. 2. Infertility – therapy. WQ 208]
RC889.H83 2011
616.6′9206–dc22

2010046887

ISBN 978-1-107-00112-1 Hardback

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To Bob Edwards for his insight and wisdom, and for his support of a great many scientists and clinicians from the very beginnings of human IVF. A true pioneer and gentleman.

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Preface

Approximately 1% of all children born in the developed world are now conceived through in vitro fertilization (IVF). Since the birth of Louise Brown in 1978, there has been a growing list of new technologies developed for assisted conception, such as intracytoplasmic sperm injection and embryo biopsy, with an estimated four million IVF children born world-wide. Assisted Reproductive Technologies (ART) represent an increasingly important means of infertility treatment. It is, therefore, fitting that in 2010 the Nobel Prize for Physiology or Medicine was awarded to Professor Robert Edwards for the development of human IVF. This Prize not only acknowledges the phenomenal dedication and contribution of our friend and colleague Bob Edwards, but also reflects the significance in which Reproductive Medicine is now held.

The twenty-first century has been witness to further remarkable developments in the field of ART. Developments in patient management and stimulation continue to be important determinants in the outcome of ART. Optimizing the patient for a successful IVF cycle sometimes requires surgical intervention. Modern surgery often implies the use of sophisticated technology such as computer-enhanced or robotic surgery.

In the embryo laboratory there has been the introduction of the "OMIC" technologies, more advanced culture procedures for follicles, gametes, and embryos, along with exciting developments in microfluidic culture and analysis. These are genuinely exciting times, and the clinical developments and new research described in this volume will become a part of the main fabric of human IVF procedures in the years to come. Ultimately, such improvements will greatly assist in the move towards single embryo transfer for the majority of patients seeking infertility treatment. The days of multiple gestations should soon be confined to the annals of human infertility treatment.

Acknowledgments

Given the breadth of expertise required to perform optimized IVF, we approached leaders in both the medical and scientific community to share their insight into what our field will look like in the near future. We are most grateful for their generosity of time and intellect to make this book a reality. In such a volume it is not possible to cover all new developments and so we consider this edition as one of several possible windows into the future. We would also like to thank Nick Dunton, Nisha Doshi and Jo Endell-Cooper at Cambridge University Press for their professionalism and commitment to this project.

Chapter 1

Use of robotics in reproductive surgery

Michelle Catenacci, Carrie Bedient, and Tommaso Falcone

Introduction

With increasing interest surrounding minimally invasive procedures, robotic-assisted laparoscopic surgery is becoming more prominent in gynecological surgery. Laparoscopy offers several benefits over laparotomy including shorter hospital stays and quicker return to normal activities. Patients undergoing laparoscopy also report less pain and utilize fewer narcotics postoperatively. Decreased blood loss and overall improved cosmesis are other attractive aspects of laparoscopic surgery. Despite the advances, however, limitations to laparoscopy do exist. Laparoscopic surgery requires a longer learning curve compared to laparotomy. Limitations exist with the degree of instrument movement and loss of depth perception [1]. Technology has made steps in overcoming the limitations of traditional laparoscopy with the development of robotic-assisted laparoscopic surgery.

Robotic surgical tools first appeared in operating rooms in the mid 1980s with the Programmable Universal Machine for Assembly (PUMA) 560 (Unimation), a device used to perform brain biopsies. Other early robotic models include the Automated Endoscopic system for Optimal Positioning (AESOP, Computer Motion) and the EndoAssist (Armstrong Healthcare). These two systems consist of simple robotic arms designed to hold and position laparoscopic cameras. Computer Motion then combined three AESOP robotic arms to create the ZEUS surgical system. The ZEUS utilizes a master and slave model of a robotic system with the surgeon seated at a master console controlling the slave robotic arms. This system improved upon some of the difficulties of traditional laparoscopic surgery with improved ergonomics, increased degree of instrument movement, development of three-dimensional (3D) imaging and provided a steady camera [2]. Of the previously mentioned robotic devices, the ZEUS was the first robotic system utilized in gynecological surgery [3]. The ZEUS disappeared from the commercial market, replaced by the robotic system used currently in gynecological surgery: the da Vinci immersive telerobotic system (Intuitive Surgical Inc.).

Robotic hardware

The da Vinci surgical system consists of three components: a surgeon's console, a patient-side cart with four interactive arms, and a vision cart. The surgeon operates via the surgeon's console positioned outside of the operating field. The surgeon sits in the console and possesses a 3D view of the operative field while controlling the camera and all interactive arms docked in the patient. The surgeon's fingers are placed in the master controller, and the system translates his or her hand movements into real-time movements of the surgical instruments docked in the patient-side cart (see Figure 1.1). The system allows for seven degrees of motion in the instruments that mimic the dexterity of the human hand and wrist (see Figure 1.2). The patient-side cart docks near the patient with one camera arm and two or three surgical arms positioned in the patient. It may be docked either between the patient's legs or in a side docked position next to the patient, an arrangement which gives improved access for uterine manipulation.

The surgeon experiences several benefits while utilizing the da Vinci surgical system. Improved ergonomics are achieved with the surgeon more comfortably seated at the surgeon's console. This prevents physician fatigue that might otherwise develop during lengthy laparoscopic procedures. Three-dimensional vision at the surgeon's console and enhanced contrast and magnification improve the visual field. The surgical system

Human Assisted Reproductive Technology: Future Trends in Laboratory and Clinical Practice, ed. David K. Gardner, Botros R. M. B. Rizk, and Tommaso Falcone. Published by Cambridge University Press. © Cambridge University Press 2011.

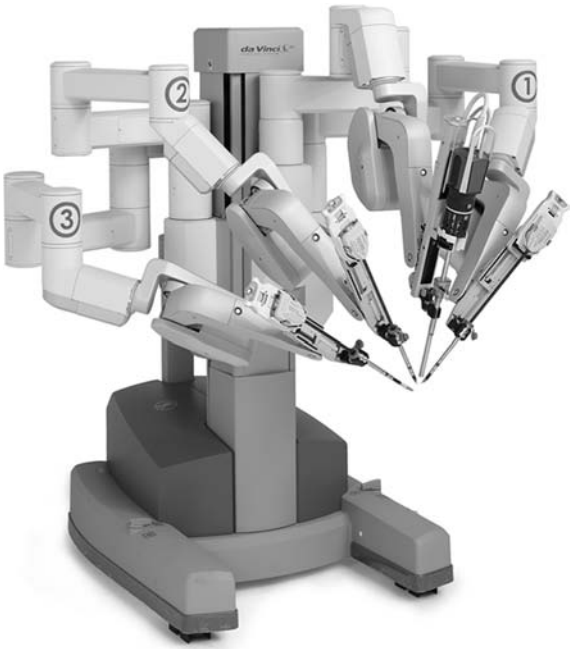


Figure 1.1. The patient side cart of the da Vinci Robotic System (courtesy of Intuitive Surgical).



Figure 1.2. The da Vinci endowrist device (courtesy of Intuitive Surgical).

also reduces hand tremor thereby improving surgical precision. Additionally, the fulcrum effect seen in traditional laparoscopic procedures disappears with the robotic system due to the fact that the robotic instruments move in the direction of your hands rather than the opposite direction.

Drawbacks to the da Vinci surgical system do exist. First, the significant amount of space taken up by the system limits its use to only larger operating rooms. Another negative aspect is the lack of tactile feedback, requiring the surgeon to rely on visual cues instead. At 1.5 million dollars each, the high cost of the robotic unit prevents widespread distribution. Also, the surgical system does not eliminate the need for operative assistants. Each surgery requires an assistant to change the robotic instruments. Also, a traditional laparoscopic port must be placed for use, through which the assistant retracts, suctions fluid and passes suture. Despite these limitations of the surgical system, the popularity of this device continues to increase in several surgical specialties including gynecology.

Furthermore, a learning curve exists for any new technology. This is not only true for the surgeon and surgical assistants, but also includes a learning curve for operating room support staff. Lehihan *et al.* reported the learning curve at their institution, studying primarily laparoscopic hysterectomies. They found that it took 20 cases for the OR team to be able to set the robot up for surgery in 45 minutes or less and it took 50 cases to improve this time to 35 minutes or less. Robot console time and total operative time became consistent after 50 cases with approximately 50 minutes needed for console time and 90 minutes needed for total operative time [4]. The technical challenges of this newer technology can be overcome with its repeated use and the aid of well-trained support staff.

Robotic-assisted laparoscopic surgery in gynecology

Use of robotics in general gynecology

After the initial emergence of the robot as a technology to assist minimally invasive surgery, more and more investigators started to amass data with respect to

operative outcomes using this new innovation. No randomized clinical trials have been published, but many physicians have begun to publish case reports and retrospective comparisons on procedures done in general gynecology, urogynecology, gynecological oncology, and reproductive surgery.

Magrina *et al.* performed a retrospective comparison of 85 patients who underwent robotic adnexectomy with 91 patients who underwent similar laparoscopic surgery. Findings included statistically significant longer operating time for the robotic group, but not necessarily clinically significant, with a difference of approximately 10 minutes. There was no significant difference in blood loss, intraoperative, or postoperative complications between each group. Surgeon preference in this study gravitated towards laparoscopic removal of large masses due to greater ease of manipulation as well as trocar placement and drainage of masses. These authors also noted that staging could be performed more easily with a laparoscopic case because of the greater access to the upper abdomen that is not obtained without significant repositioning in a robotic case [5].

A different study retrospectively reviewed 100 patients undergoing total hysterectomy laparoscopically (pre access to the robot) compared with 100 patients undergoing hysterectomy robotically. Among the outcomes examined, operative time was also found to be longer overall in robotic cases, this time by 27 minutes. When the final 25 robotic cases of the series were compared with the time for laparoscopic cases, however, the robotic cases were actually shorter by 13 minutes. Time spent at the robot console also significantly decreased from an average of 105 minutes for the first 25 cases down to 49 minutes for the last 25 cases in the series, arguing for a significantly shorter learning curve for robotic surgery as compared to laparoscopic surgery. Another factor demonstrating a shorter learning curve with robotics is that the rates of abandonment of a minimally invasive procedure were higher in the pre-robotic group as compared to the robotic group (11% vs. 0%) as were intraoperative conversions to laparotomy (9% vs. 4%). The majority of conversions (both preoperative and intraoperative) were related to uterine size, with the authors noting the robotic group could tolerate a much larger uterine size before requiring conversion (1214 g vs. 259 g). Mean blood loss was almost twice as much for the laparoscopic group, although again this is potentially not clinically important (113 ml vs. 61 ml). Length of stay was 1.6 days for the laparoscopic group, compared with one day for the robotic group. Complications were minimal for each group. Of note, vaginal hysterectomies were also included in this study with surprisingly low numbers (6% of hysterectomies pre-robotics and 2% post-robotics), which may indicate that this study population was skewed towards laparoscopy [6].

Boggess *et al.* reported cases of 152 patients undergoing non-oncological hysterectomy with more complex pathology. Findings included operating times of 122 minutes, similar to Magrina *et al.* Surgeries were noted to take longer if the uterus weighed more than 250 g or if residents or fellows were involved. Estimated blood loss was 79 ml, length of stay was one day, and there were no conversions to open cases. They concluded robotic hysterectomy was possible while also achieving minimal blood loss, short hospital stay, and low complication rate. This study compared their robotic data with other major series reported by the time of publication, finding that operative time tended to be lower than other published reports, estimated blood loss was similar, as was length of hospital stay [7].

With any new technology come problems, and organized information regarding consistent complications is lacking. Kho *et al.* examined patients who had a vaginal cuff dehiscence after robotic hysterectomy (either simple or radical), trachelectomy, or upper vaginectomy. They found 4% of these patients experienced a vaginal cuff dehiscence, noted to be a full thickness separation of anterior and posterior vaginal cuff. This is a distinctly larger percentage of patients with this complication compared to hysterectomies by other means (abdominal and vaginal approaches). In this series, the colpotomies had been performed using monopolar coagulation, and the vaginal cuff was closed using nonlocking running sutures of polyglactin secured with absorbable clips. Average time to dehiscence was 6 weeks, and the precipitating factor was coitus in 10 of the patients [8]. Only one other study has shown a similar increased rate of vaginal cuff dehiscence with traditional total laparoscopic hysterectomies compared to abdominal or vaginal hysterectomies [9]. In fact, many reports and randomized clinical trials on laparoscopic hysterectomy do not show this increased rate of dehiscence [10]. Reasons for the potential increase in dehiscence rates for robotic or traditional laparoscopic hysterectomies may be related to delayed wound healing secondary to thermal dissection methods and other techniques used specific to laparoscopy. The true significance of this is unclear at this time as the Cochrane review by Johnson *et al.* comparing different surgical

approaches to hysterectomies does not mention vaginal cuff dehiscence in their meta-analysis [10]. Larger prospective trials are needed to further address this potential complication.

Use of robotics in urogynecology

Urogynecologists have also started to adopt the new robotic technology, finding somewhat similar results. Three studies have examined short-term outcomes, long-term outcomes, and feasibility of robotic-assisted sacrocolpopexy. Considerable laparoscopic skill is required to complete a sacrocolpopexy with conventional laparoscopy, particularly given issues with visualization, suture placement, and managing intraoperative complications, especially bleeding. Inability to master these difficulties results in laparotomy. Ideally, the robot facilitates knot tying with the seven degrees of freedom the articulating instruments provide. For a case series of 77 patients undergoing da Vinci assisted laparoscopic sacrocolpopexy, conventional laparoscopy assesses the abdominal cavity, and when adequate visualization is obtained the robot is engaged. The remainder of the procedure is completed using the robot. Results showed complications including one conversion to laparotomy for bleeding (1.3%), seven patients with mesh erosion (9.1%), as well as other complications including cystotomy, enterotomy and ileus, each of which were 7% or less. No patients required transfusion, and mean hospital stay was only 2 days. In this series operative time also decreased from 188 minutes in the first 36 cases to 155 minutes for the last five cases. Assessment one year postoperatively showed approximately 5% of patients had pelvic pain, prolapse symptoms, or new incontinence, and 10% of patients had dyspareunia. Ninety-four percent of patients reported they would be willing to undergo the procedure again and were satisfied with results, which, combined with intraoperative results, indicates feasibility of robotic sacrocolpopexy [11].

Other published studies looking at robotic sacrocolpopexies have also shown similar promising results. A retrospective review of colpopexy done either robotically or abdominally for vaginal vault prolapse found robotic surgeries were associated with decreased blood loss (103 ml vs. 255 ml) and shorter length of hospital stay (1.3 days vs. 2.7 days) but also longer operating time by 100 minutes. The authors commented that when only colpopexies were analyzed, without other concurrent procedures, the blood loss was 69 ml in the robotic group compared with 412 ml in the abdominal group. Very minimal differences in POPQ scores were noted at the 6 weeks postoperative visit [12]. An additional urogynecology group investigated long-term outcomes of robotic sacrocolpopexy. In the course of their investigation, they noted time to complete the entire procedure robotically decreased by almost two hours after becoming accustomed to the technology. Only one conversion to laparotomy occurred due to dense adhesions prohibiting laparoscopic dissection. These patients were discharged on postoperative day one except for a single patient who left on postoperative day two. Minimal immediate postoperative complications were noted. At one year follow-up 95% of patients had no further problems. These authors concluded robotic sacrocolpopexy may offer the same benefits of long-lasting repair as the corresponding abdominal procedure and may benefit a population of patients who would otherwise be unable to undergo abdominal sacrocoplpopexies due to its lengthy operation and recovery [13].

Use of robotics in gynecological oncology

The gynecological oncologists perhaps have the most to gain by successful adoption and adaptation of robotic technology to previously existing procedures, given that so many oncological surgeries are done abdominally with significant morbidity and mortality. We will briefly review the use of robotics in gynecological oncology here.

Several studies have compared robotic-assisted radical hysterectomies to laparotomic procedures, reporting benefits of robotic-assisted radical hysterectomies. Significantly less blood loss and fewer complication rates have been noted when comparing robotic-assisted cases radical to open cases. Additionally, postoperative hospital stay is markedly decreased in the robotic group. In one study, postoperative hospital stay decreased from six days in open procedures to one day in the robotic-assisted procedures. As can be expected, longer operative times compared to open procedures have been found, but operating times have been shown to decrease with surgeon experience [14].

Both traditional laparoscopic radical hysterectomies and robotic-assisted radical hysterectomies have demonstrated benefits over open procedures in these comparison studies. A significant decrease in the length of postoperative stay and intraoperative blood loss for the laparoscopic and robotic groups

as compared to the laparotomy group has been demonstrated [14]. Intraoperative and postoperative complication rates have been found to be similar or decreased in the robotic groups compared to open procedures. Additionally, comparable or superior lymph node sampling has been noted in patients undergoing a robotic-assisted procedure compared to an open one [15, 16]. When comparing traditional laparoscopic to robotic-assisted laparoscopic radical hysterectomies, operative time, length of hospital stay, blood loss, or bladder catheterizations have been found to be similar in some reports [17], while other studies have shown traditional laparoscopic surgery to be significantly longer than robotic-assisted [15]. This is probably due to the fact that as experience with the robotic system is increased, efficiency is gained, and the procedures can be performed in a timelier manner.

Endometrial cancer staging and hysterectomy is another area of gynecological oncology that has utilized robotic technology. Several studies have looked at the advantages and disadvantages of the robot for these procedures. Findings of decreased intraoperative blood loss and decreased hospital stay for robotic-assisted cases compared to open procedures have again been demonstrated for patients in this setting [18]. Boggess *et al.* compared patients undergoing hysterectomy and staging for endometrial cancer via three approaches: robotically, laparoscopically, and abdominally. Longest operating times were noted in the traditional laparoscopic group, followed by the robotic-assisted group then the abdominal group (213 min vs. 191 min vs. 146 min). Length of stay was similar for the robotic and laparoscopic groups (1 day, 1.2 days) in comparison to the longer hospital course after abdominal surgery (4.4 days). Blood loss was also lower in the robotic group compared with both laparoscopic and abdominal groups (74 ml, 145 ml and 266 ml respectively). Postoperative complications occurred more frequently in the abdominal group (29%) with wound separation and readmission for ileus being the most common [19].

The learning curve for robotic-assisted endometrial cancer staging and hysterectomy has also been investigated by Seamon *et al.* They found that the majority of robotic cases that needed to be converted to an open procedure occurred within the first 50 procedures. The authors concluded the major learning curve in performing robotic endometrial cancer staging was within the first 20 cases. They also noted that operative time increased approximately 8 minutes for every increase of 1 unit of body mass index (BMI), as did the likelihood of conversion to laparotomy [20].This group further analyzed their cases to detail the outcomes of obese patients undergoing endometrial cancer staging, an area of particular interest given the relationship between obesity and endometrial cancer. A retrospective chart review matched laparotomic and robotic staging procedures for endometrial cancer with patients with a mean BMI of 40. Each group had similar lymph node counts. The robotic procedure resulted in a lower estimated blood loss (109 vs. 394 ml), lower blood transfusion rate (2% vs. 9%), shorter length of stay (1 vs. 3 days), and fewer complications than the laparotomic procedures. The laparotomic procedures were significantly shorter than robotic procedures (143 vs. 228 minutes). The collective findings indicate that patients who are more likely to develop endometrial cancer based on their obesity may be more viable candidates for minimally invasive surgical treatment [21].

Minimal information has been published regarding ovarian cancer, although conceivably many of the advantages found with endometrial and cervical cancer could be applied to techniques managing ovarian cancer. Similarly, little information is available regarding use of the robot during pelvic exenteration. Schneider comments briefly on the feasibility of laparoscopic or robotic pelvic exenteration, noting that the most difficult portion of laparoscopic exenteration is the urinary diversion. Few studies have been reported using the robot for this specific purpose; however, he reviews two case series of urinary diversion which suggest the possibility of being able to perform an exenteration robotically. More research needs to be done to further explore robotic assistance with this procedure [22].

Use of robotics in reproductive surgery

One area in reproductive surgery in which robotic assistance has been utilized is in tubal ligation reversal surgery. Tubal sterilization is a popular permanent method of contraception. However, many women have been later plagued with regret over the decision to undergo surgical sterilization. A large, prospective, multi-center study in the United States found the cumulative probability of regret 14 years out from tubal sterilization to be 12.7%. The findings were even more dramatic for women who underwent the procedures at age 30 or younger. The 14 year cumulative probability of regret in the younger age group was found to be 20.3% [23].

Women with regrets from tubal sterilization generally have two options available to them: in vitro fertilization (IVF) or surgical reanastomosis. Tubal anastamosis is traditionally performed though a minilaparotomy incision utilizing microsurgical techniques. Traditional laparoscopic surgery became an option for some patients desiring this procedure, and has been shown to give equivalent pregnancy rates when compared to anastomosis done by laparotomy [24]. However, traditional laparoscopic instruments are not ideal for tubal anastomoses due to the precise microsurgical suturing that is required for success of this procedure. Robotic technology with its seven degrees of freedom and superior magnified visualization can facilitate the technically challenging microsurgical suturing.

Initial reports of robotic tubal anastomosis involved the Zeus robotic system (Computer Motion Inc.). The pilot study by Falcone *et al.* described the use of the Zeus robotic system for 10 patients desiring sterilization reversal. Nineteen fallopian tubes were successfully anastomosed and postoperative hysterosalpingogram demonstrated patency in 17 of the 19 tubes [4]. Five of the 10 patients subsequently achieved pregnancy within one year of surgery, carrying their pregnancies to term [25]. As previously stated, the Zeus robotic system is no longer commercially available.

Similar to the Zeus system, initial case series describing successful tubal anastomosis have been reported utilizing the current surgical robotic system on the market, the da Vinci system (Intuitive Surgical, Inc.) [26]. Furthermore, comparisons of tubal ligation reversals done with the assistance of the da Vinci system to those performed with traditional laparotomy have also been reported in the literature. Pregnancy rates have been found to be statistically similar between the two techniques with no difference in ectopic rates. Potential advantages noted in patients undergoing robotic tubal anastomosis include a decreased use of post-op analgesics and a shorter time to recovery. Specifically, a statistically significant decreased time to return to work has been found in patients undergoing robotic tubal anastomosis compared to patients who had laparotomy. However, total operative time and anesthesia time for the robotic procedures were found to be statistically longer when compared to the procedures done with an open technique. Additionally, operative costs with the robot are significantly increased [27, 28]. Continued advances in surgical robotic technology will help overcome these current limitations of robotic-assisted laparoscopic tubal anastomosis.

Another area in reproductive surgery in which the utilization of surgical robotics has been explored is in laparoscopic myomectomies. Myomas can be found in a variety of places within the pelvis, most commonly intracavitary, intramural, or serosal. The size and location of fibroids can cause a variety of symptoms for patients, and many patients may be asymptomatic. Frequently, fibroids are discovered in asymptomatic women during an infertility evaluation. Previous studies have demonstrated decreased pregnancy outcomes when myomas distort or obstruct the uterine cavity [29]. It is generally recommended to remove these distorting myomas to improve fertility outcomes. In many cases myomas that distort or obstruct the cavity can be removed by hysteroscopic resection. Not all, however, will be suitable for hysteroscopic resection and will need to be removed by laparotomy or laparoscopy. Specifically, myomas that are mostly intramural cannot be removed hysteroscopically. Controversy exists on the fertility effects of subserosal and intramural fibroids that do not distort or obstruct the endometrial cavity. A recent meta-analysis conducted a systematic review of the literature to better answer this question. This review by Sunkara *et al.* analyzed studies that compared IVF outcomes of patients with non-distorting intramural myomas to control patients. The analysis found that for patients with a non-distorting myoma there was a significant decrease in clinical pregnancy rates by 15% per IVF cycle and a significant decrease in live birth rate by 21% when compared to controls. No significant difference was found in implantation rate or miscarriage rate [30]. The decreased pregnancy rate and live birth rate found in this study would seem to support removal of these lesions prior to IVF. However, the meta-analysis did not address how the removal of these myomas would influence IVF outcomes. There are limited studies that look at the affects of myomectomies on fertility. One study that did look at fertility outcomes in patients before and after abdominal myomectomies for subserosal or intramural fibroids did find a significant decrease in pregnancy loss after myomectomy from 69% to 25% and a significant increase in live birth from 31% to 75% [31]. This would again support removal of non-distorting myomas for improved fertility outcomes. However, this was a relatively small and retrospective study. Prospective, randomized trials are lacking and are needed to clarify this controversial topic.

Furthermore, for patients who will undergo a myomectomy for intramural or subserosal myomas, a

decision needs to be made on the appropriate surgical approach. Criteria used by our institution to select cases that are appropriate for intramural laparoscopic myomectomy include: uterine size of 15 cm or less, dominant fibroid is 15 cm or less, a total of five fibroids or less that need removal, and fibroid location excludes the broad ligament or cervix. MRI is generally done preoperatively to assess the number, size, and location of myomas. Recently, cases done by robotic-assisted laparoscopic myomectomies have been increasing and criteria for robotic-assisted and traditional laparoscopic myomectomies should be identical. Comparing robotic-assisted laparoscopic myomectomies to traditional abdominal myomectomies, Advincula *et al.* found patients undergoing a robotic-assisted approach to have significantly less blood loss during surgery. In their study of 58 patients, the transfusion rate in patients undergoing open myomectomies was 6.9% compared to zero blood transfusions in the robotic-assisted laparoscopic cases. Postoperative complication rates were also less in the robotic arm of this study. Similar to the studies looking at robotic-assisted tubal anastomosis, the other advantage to robotic-assisted myomectomy was an overall decrease in hospital stay compared to the laparotomy group. Disadvantages noted were increased overall cost and operative time [32].

More recently, two retrospective reviews comparing traditional laparoscopic myomectomies to robotic-assisted laparoscopic myomectomies have been reported. In the study by Nezhat *et al.*, 15 robotic-assisted cases were compared to 35 traditional laparoscopic cases. This study found similar operative blood loss, complications, and hospital stay between the two groups. Hospital charges and operative time were again found to be significantly greater in the robotic-assisted group [33]. A slightly larger retrospective study by Bedient *et al.* also compared traditional and robotic-assisted laparoscopic myomectomies. After adjustment for uterine size, number of fibroids, and size of the largest fibroid, no significant difference in operative times was found between the two study arms. Intraoperative blood loss and surgical complications were also not significantly different after adjustment of data was performed. The authors noted that the robotic arm of the study had significantly fewer uterine incisions when compared to the traditional laparoscopic group, thought to be due to the improved instrument articulation in the robotic procedures. A potential benefit from this could be a decreased incidence of future uterine rupture [34].

One study looked at the impact of BMI on surgical outcomes in patients undergoing robotic-assisted laparoscopic myomectomies. This retrospective cohort study of 77 patients divided into five BMI groups found no intraoperative complications in the obese or morbidly obese patients. Additionally, they did not find any significant difference in procedure time, estimated blood loss, or hospital stay between the five BMI groups [35]. This study failed to find any evidence that increasing BMI worsened surgical outcomes in patients undergoing robotic-assisted laparoscopic myomectomies. Similar to the data on obese patients in the gynecological oncology literature, the minimally invasive technique of robotic-assisted myomectomies may give superior outcomes in the obese and morbidly obese patient population. Head to head trials of the surgical approaches should be done to verify this likely benefit.

There are other potential uses of robotic-assisted laparoscopic surgery in the field of reproductive medicine. For example, women needing to undergo pelvic radiation therapy are in danger of losing ovarian function. Ovarian transposition can be done to move the ovaries high above the field of radiation, giving protection to future ovarian function. The use of the da Vinci surgical system to perform this procedure has been described in the literature [36]. In the future, robotic-assisted laparoscopic surgery could be used for uterine transplant surgery. Currently experimental, this potentially complex procedure may benefit from the technical advantages of robotic-assisted surgery.

The future of robotic surgery

As technology continues to improve, advances will be made in the current robotic system that will further enhance its use. One limitation previously mentioned that exists in the robotic system is its lack of tactile feedback. This can be particularly frustrating when working with delicate tissue or fine suture material. With the current system, surgeons must adapt to visual cues alone. The ability to have tactile feedback would be a great improvement to the system and lessen the learning curve of robotic surgery.

Another limitation of the system is its large size. The three components of the robotic apparatus are very bulky and may not fit in some operating rooms. Ideally, future models will be decreased in size. One way to accomplish a size reduction of the system

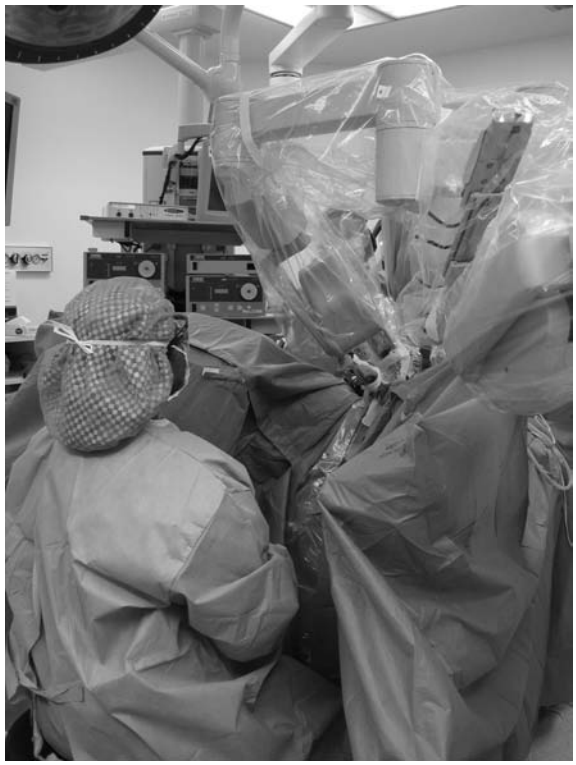


Figure 1.3. Side-docking of the patient side cart of the da Vinici Robotic System allowing improved assistant patient access for uterine manipulation.

would be to have rooms with the robotic system structurally incorporated. Permanent robotic operating suites with the arms attached to the ceiling of the operating room would help decrease some of the bulkiness. This will give better access to the patient for the assistant and may make docking easier and more efficient. Another way to achieve improved access to the patient is the incorporation of side-docking (see Figure 1.3). As previously mentioned, this new docking technique places the patient side cart next to the patient instead of between her legs. This gives easy access for uterine manipulation during procedures. Until robotically incorporated rooms are developed, side-docking can be used for improved patient access.

Other improvements of the robotic system include the addition of telesurgery technology and single-port systems. The Zeus robotic system was developed with telesurgery technology and has been used for this purpose [3]. The current da Vinci surgical system, however, is not available for telesurgery. The ability to perform surgery from a remote location can have a significant impact on patient care and access to care and should be incorporated into future robotic models. Single-port robotics is another area of robotics that is just being developed. Laparoendoscopic single-site surgery (LESS) is gaining in popularity for its minimally invasive nature and improved cosmetics (see Figure 1.4). The incorporation of robotics with LESS will facilitate

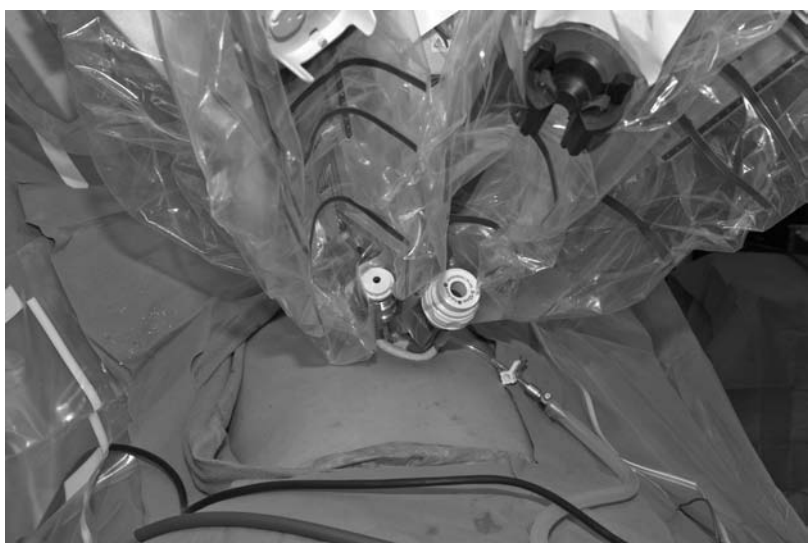


Figure 1.4. Robotic-assisted laparoendoscopic single-site surgery; only one trocar is required.

complex procedures. However, instrument arms will need to be improved upon to truly embrace this technique. Creating arms that are smaller and have greater intracorporal flexibility and articulation will be needed for single-port robotics.

Conclusion

Robotic surgery is emerging as a viable option for gynecological surgeons in general gynecology, urogynecology, oncology, and reproductive surgery. Feasibility has been demonstrated in all areas of gynecology. In each area the benefits of traditional laparoscopy translate well to robotically assisted laparoscopy. Decreased blood loss, shorter hospital stays, and faster recovery with lower morbidity are noted as compared with laparotomy. In many cases robotic surgery takes as long as laparoscopy. However, multiple studies demonstrate a rapid learning curve in which operative time steadily decreases, even becoming shorter than laparoscopy, some approaching laparotomy. Studies have demonstrated that few conversions to laparotomy were needed using the robotic technology. Furthermore, improved outcomes were noted on obese patients when robotic procedures were done instead of traditional laparoscopy. This finding alone opens many doors for obese patients previously considered poor candidates for minimally invasive surgery. More studies need to be done, particularly prospective randomized trials to demonstrate definitive benefit, or lack thereof, of the robotic technology as compared with laparoscopy and laparotomy. As institutions and surgeons become more familiar with the technology, hopefully these results will become available to develop the incorporation of robotic technology in gynecological surgery.

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Chapter 2

Removal of hydrosalpinges and uterine leiomyoma to improve IVF outcome

Eric S. Surrey

Introduction

A critical component of a successful IVF cycle is the transfer of embryos into an environment in which implantation is not compromised. Failure to do so can reverse all of the benefits of optimizing stimulation, embryo culture environment as well as embryo selection and transfer techniques. Surgical intervention to optimize an IVF cycle should be considered in cases where the evidence clearly points to improved outcomes. Unfortunately few randomized controlled trials exist and much of the data are retrospective. However, we are faced with this clinical dilemma often. If the abnormality involves the uterine cavity it seems logical to proceed to removal of the abnormality. However, in cases of tubal disease and myomas that do not involve the uterine cavity directly intervention is more controversial. In this chapter, we shall address the controversies surrounding the impact and surgical management of hydrosalpinges and uterine leiomyoma on IVF cycle outcome.

Hydrosalpinx

Evidence accumulated over the last 15 years suggests that either unilateral or bilateral hydrosalpinges may exert deleterious effects on IVF cycle outcome [1–9] (Table 2.1). A meta-analysis encompassing 1004 patients with hydrosalpinges and 4588 control patients with tubal factor infertility but without hydrosalpinges in 14 studies showed significant decreases in pregnancy, implantation, and delivery rates in the hydrosalpinx groups (OR 0.64, 0.63, 0.58, respectively) [10].

A variety of hypotheses have been proposed to explain this effect. The accumulation of fluid of any source within the endometrial cavity has been shown to impair embryonic implantation [11]. In addition, hydrosalpinx fluid may have a direct embryotoxic effect and may also inhibit fertilization [12–15]. This deleterious effect may be mediated by the presence of inflammatory cytokines present within hydrosalpinx fluid [16, 17]. Others have shown that the endometria of women with hydrosalpinges expressed significantly less $\alpha v\beta_3$ integrin, a presumptive marker of endometrial receptivity, than fertile controls [18]. Implantation may also be inhibited by diminished endometrial expression of HOXA-10, purported to be a regulator of endometrial receptivity, in the presence of hydrosalpinx fluid [19].

If retrograde flow of hydrosalpinx fluid is the cause of this deleterious effect, then prophylactic salpingectomy or tubal occlusion performed prior to IVF-ET should enhance outcomes. On the other hand, if damage from this fluid to the uterine environment is irreversible, then such surgery would have no beneficial effect. Strandell and colleagues published the results of a prospective randomized trial of 192 patients with hydrosalpinges who underwent salpingectomy or no intervention prior to IVF-ET and also began an actual treatment cycle [20]. The salpingectomy group experienced significantly increased delivery rates with a trend towards increased clinical pregnancy rates. A Cochrane review including three randomized trials similarly reported that the likelihood of live birth was significantly higher after salpingectomy in patients with hydrosalpinx (OR: 2.13, 95% CI: 1.25–3.65) [21]. No significant differences in implantation, ectopic pregnancy, or miscarriage rates were noted.

The way in which salpingectomy acts to improve IVF outcome has not been shown conclusively. Bildirici *et al.* proposed that salpingectomy may restore $\alpha v\beta_3$ integrin expression, thus enhancing endometrial receptivity [22]. Others have demonstrated a reduction of endometrial lymphocyte clusters after salpingectomy, purportedly reflecting suppression of natural killer cell

Human Assisted Reproductive Technology: Future Trends in Laboratory and Clinical Practice, ed. David K. Gardner, Botros R. M. B. Rizk, and Tommaso Falcone. Published by Cambridge University Press. © Cambridge University Press 2011.

Table 2.1. Hydrosalpinx and IVF: pregnancy (PR) and implantation (IR) rates

		IR/transfer (%)		PR/transfer (%)	
1st author	Ref.	Hydrosalpinx	No hydrosalpinx	Hydrosalpinx	No hydrosalpinx
Strandell	1	NR[a]	NR	13.2 (12/91)	26.0 (74/285)
Kasabji	2	7.7 (59/796)	11.7 (83/710)	18.4 (43/234)	31.4 (70/223)
Anderson	3	2.9 (8/273)	10.3 (221/2152)	27.0 (20/91)	35.6 (265/744)
Vandromme	4	4.2 (8/190)	13.4 (36/269)	11.3 (7/62)	31.6 (30/95)
Katz	5	3.9 (17/434)	11.5 (643/5577)	16.8 (16/95)	36.8 (467/1268)
Sharara	6	9.8 (43/437)	12.6 (50/396)	24.5 (25/101)	33.7 (30/89)
Akman	7	5.0 (2/40)	10.4 (30/289)	7.1 (1/14)	24.5 (24/98)
Blazar	8	NR	NR	39.0 (26/67)	45.0 (81/180)
Murray	9	2.8 (5/167)	15.8 (189/565)	8.5 (4/47)	38.6 (56/146)

All differences statistically significant.
[a] NR = not reported.

activation [23]. Seli and coworkers reported that endometrial leukemia inhibitory factor (LIF) expression, a proposed marker for endometrial receptivity, is suppressed in the presence of a hydrosalpinx but increased after tubal resection [24].

It is possible that not all hydrosalpinges exert the same negative effect on IVF outcome. Several groups have reported that only large hydrosalpinges, visible on ultrasound, resulted in reduced implantation and pregnancy rates [20, 25]. Thus, the question of whether only larger hydrosalpinges should be removed has not been resolved, although it would be unfortunate to await cycle failure before making the decision to remove a less distended but functionless tube.

Salpingectomy could theoretically result in a decrease in ovarian perfusion given that a portion of the blood supply to the ovary is derived from the branches of the uterine artery and the mesosalpingeal vascular arcade [26]. Ovarian blood flow and antral follicle counts have been shown to be reduced on the ipsilateral side of women who had undergone unilateral salpingectomy [27]. Gelbaya and coworkers have demonstrated compromise in ovarian response to gonadotropins without an effect on pregnancy rates in patients who had undergone prior salpingectomy [28]. However, others have shown no such ill effects [29, 30]. Minimizing the extensiveness of resection of the mesosalpinx can only be beneficial in preserving blood supply.

Proximal tubal occlusion requires less surgical dissection and less potential for interrupting blood supply while still eliminating retrograde flow of hydrosalpingeal fluid into the endometrial cavity. In a controlled trial, Surrey and Schoolcraft noted that laparoscopic proximal occlusion and transection of the affected fallopian tube resulted in similar ovarian response and cycle outcome as in patients with hydrosalpinges who had undergone salpingectomy as well as patients in two other control groups – those with non-occlusive tubal disease and those who had undergone prior bilateral tubal ligation for sterilization [31] (Table 2.2).

Two other investigative teams have demonstrated similar outcomes. Murray *et al.* reported that implantation and ongoing pregnancy rates after embryo transfers performed in 15 women with hydrosalpinges who had undergone proximal tubal occlusion were similar to those of 23 women who had undergone salpingectomy, although response to ovarian stimulation was not addressed [9]. Stadtmauer and coworkers reported that proximal occlusion yielded significantly improved outcomes in comparison to patients with untreated hydrosalpinges [32]. A trend towards higher pregnancy and implantation rates in comparison to those treated with salpingectomy was noted. It is important to be aware of the fact that transection of the tube too close to the cornua may also increase the risk of an interstitial pregnancy after embryo transfer, a potentially devastating complication [33].

Less invasive approaches have also been attempted. A small body of literature describes the hysteroscopic

Table 2.2. Salpingectomy vs. proximal tubal occlusion in hydrosalpinx patients: effect on IVF cycle outcome

Group:	I	II	III	IV
Parameter:	**Hydrosalpinx: salpingectomy**	**Hydrosalpinx: proximal tubal occlusion**	**Tubal disease without hydrosalpinx**	**Bilateral tubal ligation for sterilization**
Cycles	35	17	37	15
Age (mean ± SEM)	35.1 ± 0.7[c]	35.4 ± 1.0[a,b]	35.6 ± 0.7	38.2 ± 1.0
E_2 day of hCG administration (pg/ml)	2555 ± 219[a,b]	2366 ± 282[a,b]	2925 ± 259	2479 ± 281
(Mean± SEM)				
Clinical pregnancy/embryo transfer procedure (%)	16/28 (57.1[a])	7/15 (46.7)[a,b]	18/34 (52.9)	7/12 (58.3)
Implantation/embryos transferred (%)	21/78 (26.9%)[a]	8/49 (16.3%)[a,b]	23/108 (21.3%)	12/36 (33.3%)

[a] P = not significant (vs. Groups III or IV).
[b] P = not significant (vs. Group I).
[c] $P < 0.05$ vs. Group IV.
Modified from Surrey and Schoolcraft [31].

placement of an occlusive microinsert into the proximal portion of the fallopian tube prior to IVF in patients with a hydrosalpinx [34]. Although this approach is clearly less invasive, it is an off-label use of the device which is approved for sterilization only. In addition, hysteroscopic evidence that the "tail" of this device is no longer visible within the endometrial cavity would be necessary before considering embryo transfer. This typically takes several months to accomplish. The use of a radiofrequency occlusive transcervical device may also be beneficial and would have the theoretic advantage of avoiding the presence of foreign body within the endometrial cavity [35]. Clearly, more clinical data are required before a recommendation can be made.

Antibiotic therapy has been attempted as well. Sharara *et al.* reported a trend towards reduced implantation and pregnancy rates which did not reach statistical significance in women with hydrosalpinges in whom antibiotic therapy was administered prior to an IVF cycle [36]. Others have shown that such therapy resulted in outcomes which were no different than untreated controls [37]. This approach, which has not been addressed in a randomized trial, should theoretically only have an impact on acutely or chronically infected tubal fluid, but might not address the embryotoxic effects of hydrosalpingeal fluid derived from a non-infectious etiology.

An alternative approach would be to drain hydrosalpinges prior to cycle initiation or at the time of oocyte aspiration under ultrasound guidance. This option has met with mixed results [38, 39]. Although this technique would reduce the overall volume of hydrosalpingeal fluid, drainage would eliminate neither its source nor its ability to flow into the endometrial cavity even in reduced amounts. Indeed, Bloechle and colleagues have described reaccumulation of hydrosalpinx fluid within three days of aspiration performed at oocyte retrieval, which would precede the time of embryo implantation [40].

In summary, the deleterious impact of large hydrosalpinges on IVF outcome and the benefit of mechanical interruption of the tube in overcoming this effect have been well shown. Whether all tubes which are distally occluded should be treated has not been demonstrated as conclusively, although such an approach would certainly be logical. In addition, it would appear that ruling out hydrosalpinx prior to initiating an embryo transfer cycle in patients who have not had a recent tubal evaluation or who have experienced unexplained prior IVF cycle failure is an approach that would be prudent to consider.

Uterine leiomyoma

The effect of uterine leiomyomata on IVF cycle outcome is more controversial.

It is reasonable to assume that submucosal leiomyomas which distort the endometrial cavity would affect implantation. However, the mechanism by which intramural lesions not associated with cavitary distortion might exert a deleterious effect is less clear. A variety of hypotheses have been proposed to explain this effect, including inherent changes in the uterine environment which could negatively impact sperm transport and implantation as a result of a decrease in uterine perfusion, abnormal local expression of growth factors, or aberrant gene expression [41–43].

In a recent literature review and analysis of four previously published studies, Pritts *et al.* reported that women with submucosal lesions experienced significantly lower ongoing pregnancy/live birth (RR 0.31; 95% CI: 0.19–0.85, $P < 0.001$) and implantation (RR 0.283; 95% CI: 0.12–0.65, $P = 0.003$) rates than fertile controls [44]. Although there were no differences in clinical pregnancy rates in comparison to controls when fibroids were not distorting the uterine cavity, significantly lower ongoing pregnancy/live birth (RR: 0.78; 95% CI 0.69–0.88, $P < 0.001$) and implantation (RR: 0.792; 95%CI 0.86–0.901, $P < 0.001$) were noted based on an analysis of 16 studies. It is important to note that this review did not specifically address IVF-ET outcomes.

The impact of uterine leiomyomata specifically on the outcome of assisted reproductive technologies has been evaluated by others with conflicting results. In one study, pregnancy and implantation rates in 141 IVF cycles performed on patients with leiomyomata were lower than age-matched controls only in the group of patients with endometrial cavitary distortion [45]. Stovall and coworkers noted that patients with both intramural and subserosal myomas undergoing assisted reproductive technologies experienced a significant reduction in the likelihood of achieving a live birth (RR = 0.68, 95%, CI = 0.47–0.98) [46]. Submucosal leiomyomas were excluded from the analysis. Eldar-Geva and colleagues stratified patients by leiomyoma location and noted that the presence of either intramural or submucosal leiomyomas greatly reduced both implantation and pregnancy rates in comparison to matched controls [47]. Subserosal lesions alone exerted no impact. In a more recent prospective trial, Hart *et al.* evaluated 106 women with intramural fibroids diagnosed by ultrasonography, hysteroscopy, or sonohysterography without commenting on the degree of cavitary distortion [48]. The presence of fibroids produced significantly negative effects on clinical pregnancy, implantation, and ongoing pregnancy rates.

Surrey and colleagues described a significant decrease in implantation rates in women with intramural leiomyomata but hysteroscopically normal endometrial cavities under 40 years of age undergoing IVF in comparison to age matched controls [49]. Trends towards lower clinical pregnancy and live birth rates, which failed to reach statistical significance, were also reported. Regression analyses revealed that neither leiomyoma size nor volume correlated with the likelihood of implantation.

Not all investigators have shown that intramural fibroids exert a deleterious effect. Yarali and Bukulmez reported results of a case-controlled study of 73 women with intramural fibroids and 35 women with subserosal fibroids undergoing IVF and ICSI and noted no adverse effect on either clinical pregnancy or implantation rates [50].

It is difficult to compare outcomes from these various trials due to significant variations in patient selection. Mean sizes and numbers of leiomyomata vary amongst the studies as does the way in which the uterus is evaluated. The degree of normal intervening myometrium between the fibroid and degree of distortion of the endometrial cavity are rarely described. In addition, differences in outcomes among various IVF laboratories represent a significant confounding variable.

Although it would be logical to assume that excising clinically relevant fibroids would enhance IVF cycle outcome, this issue has not been extensively evaluated. In the aforementioned analysis, Pritts *et al.* reported that when myomectomy was performed on submucosal lesions, ongoing pregnancy and live birth rates were similar to those of infertile women without fibroids (RR 1.128; 95% CI 0.959–1.326) [44]. Resection of intramural lesions resulted in outcomes no different than women with fibroids left *in situ*. However, this analysis did not solely address patients who were undergoing IVF or specifically control for the size of the fibroids in question.

In a recent case controlled trial, Surrey *et al.* reported on the effect of myomectomy on IVF outcomes in patients with leiomyomata considered to be clinically significant, i.e. submucosal or intramural tumors with associated endometrial cavitary distortion or direct impingement upon the cavity [51]. Patients

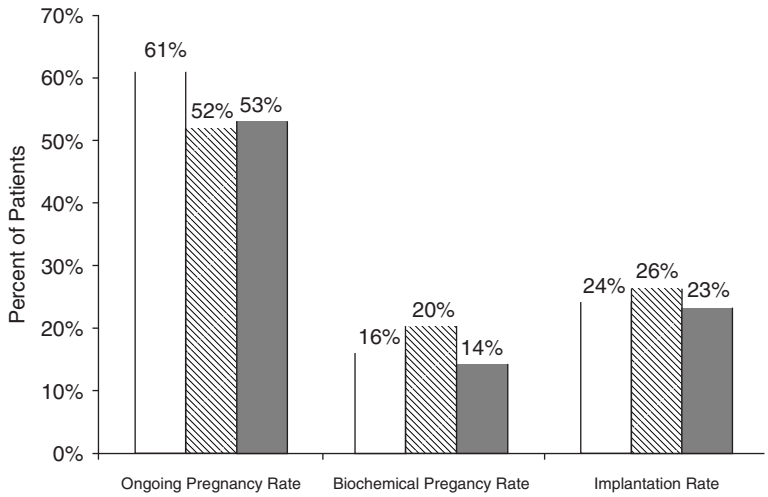


Figure 2.1. Cycle outcomes of patients undergoing IVF with their own oocytes after myomectomy (Surrey *et al.* [51]). Reprinted with permission from the American Society for Reproductive Medicine. □, Group A, hysteroscopic myomectomy; ▩, Group B, myomectomy at laparotomy; ■, Group C, controls without fibroids.

who underwent pre-cycle myomectomy experienced ongoing pregnancy and implantation rates which were similar to controls without leiomyomata. The outcome for patients with smaller submucosal lesions resected hysteroscopically was the same as for those with large submucosal lesions with a significant intramural component or those intramural lesions which directly impinged upon or distorted the endometrial cavity resected at laparotomy (Figure 2.1). Similar conclusions were reached after a separate analysis of patients undergoing oocyte donation. An appropriately designed prospective randomized trial would clearly provide more definitive answers, although recruitment would be challenging.

Evaluation of the uterine cavity by hysteroscopy or sonohysterography should be a routine part of the pre-cycle evaluation. The accuracy of routine ultrasound evaluation and hysterosalpingography is more limited. Oliveira and colleagues performed hysteroscopy on patients who had a normal hysterosalpingogram and had failed two prior IVF cycles despite transfer of good-quality embryos [52]. A significant percentage (48%) were noted to have abnormal findings at hysteroscopy, including leiomyoma, polyps, or adhesions. Once these abnormalities were surgically corrected, the patients subsequently underwent a third IVF cycle resulting in a 50% clinical pregnancy rate. If these individuals had undergone hysteroscopy and surgical correction of abnormalities prior to their first IVF cycle, one could conjecture that the number of failed cycles would have been reduced.

In summary, it appears that leiomyomata that distort the endometrial cavity have an impact on cycle outcome. The effect of intramural leiomyomas has not been uniformly demonstrated due to variations in patient selection. However, consideration should be given to resection of submucosal fibroids and intramural lesions that distort or directly impinge upon the endometrial cavity prior to IVF. On the other hand, the benefit of resecting other asymptomatic intramural or subserosal lesions with a normal uterine cavity has not been demonstrated and may be associated with risks which outweigh any theoretic benefits.

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Chapter 3

Ovarian endometriomas: effect on IVF outcome

Gabriel de la Fuente and Juan A. Garcia-Velasco

Introduction

Endometriosis affects approximately 10% of the female population in their fertile years and 10–25% of patients requiring assisted reproduction treatment (ART)[1]. Ovarian endometriomas are a common form of the disease and may be present in up to 30–40% of women with endometriosis [2].

The presence of ovarian endometrioma is a common and specific manifestation of the disease, and may arise as a consequence of metaplasia of the coelomic epithelium or invagination of the ovarian cortex after local implantation of the endometrium on the ovarian surface [3].

The influence of endometriotic ovarian cysts on the results of IVF cycles is still a matter of debate. Assisted reproduction is widely used to manage sterility associated with ovarian endometriomas as it may enhance the low fecundity associated with advanced endometriosis.

Various studies have evaluated the success of IVF in patients with endometrioma, and some studies have reported beneficial effects [4] while others have reported poor outcomes [5, 6].

This lack of consensus on the outcome of assisted reproduction in patients with ovarian endometriomas is due to multifactorial reasons, because IVF outcome can be affected by selection criteria, patient management, size of the endometrioma, prior surgical therapy, stimulation protocols, and other factors. Besides, most of the studies in the literature have investigated IVF outcomes in patients with resected endometriomas.

Therefore two different clinical scenarios must be considered; patients with endometriomas who have not undergone previous ovarian surgery and patients who have been operated on for endometriomas before IVF. Findings from these two groups may be of particular interest because they allow one to distinguish the effects related to the presence of the endometriomas from those related to surgery.

Further variables that should be considered are bilaterality, dimension of the cysts, and the specific surgical technique used.

Unfortunately, the vast majority of available studies have not taken into consideration this distinction and little information can be found on the effect of endometriomas per se on IVF outcomes.

Nowadays there is convincing evidence that ovarian responsiveness after ovarian cystectomy is reduced and the number and quality of oocytes retrieved are at least not improved [7].

Endometriosis and ovarian responsiveness

IVF treatment in the words of the ESHRE Special Interest Group (SIG) on Endometriosis appears to be appropriate in patients with advanced endometriosis, which is frequently associated with adhesions, ovarian endometriomas, and tubal obstruction. However, the IVF pregnancy rate in this population compared with control groups has also been a topic of debate (ESHRE guidelines).

In a meta-analysis Barnhart *et al.* indicated that pregnancy rates are lower in women undergoing IVF treatment with endometriosis than in women with tubal infertility [8]. The review included 22 studies, consisting of 2377 cycles in women with endometriosis and 4383 in women without the disease. After adjusting for confounding variables, there was a 35% reduction in the chance of achieving pregnancy with IVF in women with endometriosis (OR 0.63; CI 0.51–0.77). Other outcome parameters, such as fertilization rate,

Human Assisted Reproductive Technology: Future Trends in Laboratory and Clinical Practice, ed. David K. Gardner, Botros R. M. B. Rizk, and Tommaso Falcone. Published by Cambridge University Press. © Cambridge University Press 2011.

implantation rate, and number of oocytes retrieved were also significantly lower in women with endometriosis compared to those with tubal factor infertility. These data suggested that the presence of endometriosis affects reproductive success rates after IVF.

However, it has to be noted that endometriosis does not adversely affect pregnancy rates in some large databases [9]. Since the inclusion of confounding factors in the systematic review strengthened the negative association between endometriosis and IVF outcome, this might explain the findings in the large databases.

Based on the Centers for Disease Control (CDC) data, similar pregnancy and live birth rates have been reported when comparing couples with diagnosis of tubal factor infertility, ovulatory dysfunction, endometriosis, male factor infertility, or unexplained infertility [10].

Witsenburg *et al.* (2005) reported a 63.2% cumulative live birth rate (CLBR) in patients with endometriosis treated with IVF and ICSI in a cohort study [11]. These findings are also in contrast to those from the meta-analysis by Barnhart *et al.* [8]. The observed differences may be explained by the patient population used to make the observation. The diagnosis of endometriosis in large registries is less reliable than those in more precise, specific studies.

Endometriomas and ovarian responsiveness

Ovarian responsiveness to hyperstimulation plays a crucial role in determining the success rate of IVF. Former studies as happened with general endometriosis had reported an adverse effect of ovarian endometriomas on oocyte and embryo quality and implantation rates [5, 12, 13]. In women developing few follicles despite the use of elevated dosages of gonadotropins, the prognosis is worse [14].

In this regard, it is noteworthy that ovarian responsiveness is reduced in the presence of ovarian endometriomas [15, 16]. In a recent meta-analysis, Gupta *et al.* assessed the effects of ovarian endometrioma on fertility outcomes with assisted reproduction techniques. Analysis of the six studies involved showed that the number of developing follicles and the number of retrieved oocytes are lower in affected women when compared with controls who were unaffected. Specifically, fewer follicles developed and fewer oocytes were retrieved in these women [15].

The harmful effect of endometriomas on ovarian responsiveness is further supported by studies focusing on women with monolateral disease and comparing responsiveness to hyperstimulation in the affected and in the contralateral intact gonad of the same patient [16–18]. Collectively, these studies strongly support a marked reduction in the number of developing follicles and retrieved oocytes in the endometrioma-affected ovaries. Conversely, the potential impact of this endometrioma-related reduced responsiveness on other IVF parameters is less recognized.

In their meta-analysis, Gupta *et al.* reported an OR for clinical pregnancy rates in women with the disease of 1.07 (95% CI 0.63–1.81), showing that endometriomas may result in a decrease in the number of retrieved oocytes, but that overall fertility outcomes are not affected [15].

One of the hypotheses to explain this contrasting result is that the damage could be mainly quantitative rather than qualitative. In other words, in contrast to women whose ovarian reserve has naturally declined, fewer oocytes but of unaffected quality might be retrieved in affected women. Interestingly, in the single available prospective study comparing responsiveness of ovaries operated for endometriomas to contralateral intact gonads of the same patient, Ragni *et al.* documented a marked reduction in the number of developing follicles and in the number of retrieved oocytes, but the fertilization rate and rate of good-quality embryos were similar in affected and unaffected ovaries [17]. Even though only 38 subjects were included, this study suggests that the excision of endometriomas is associated with a quantitative but not a qualitative damage to ovarian reserve.

Another hypothesis that could explain these results is that ovarian endometriomas are mostly monolateral. Both gonads are involved only in 19–28% of cases [19]. The contralateral intact ovary may adequately compensate for the reduced function of the affected one. In this context, studies that have specifically focused on women with bilateral endometriomas should be considered more informative.

In a large study including 68 women operated on for bilateral endometriomas Somigliana *et al.* showed a statistically significant reduction in the chances of success compared with unaffected controls [19]. The odds ratios (OR) for clinical pregnancy and delivery in the study group were 0.34 (95% CI 0.12–0.92) and 0.23 (95% CI 0.07–0.78) respectively, compared with

controls. In these patients, despite the use of higher doses of gonadotropins, the numbers of follicles ($P < 0.006$), oocytes retrieved ($P < 0.024$), and embryos obtained ($P < 0.024$) were significantly lower.

Collectively, insights emerging from these observational studies support the conclusion that ovarian responsiveness is modified in affected gonads. Although the injury could be more quantitative than qualitative, in some cases the insult can be so relevant that no or only few oocytes are retrieved. In this regard, it is noteworthy that Ragni *et al.* documented a failure in the growth of codominant follicles in 34% of operated ovaries [17]. This issue may be assumed to be particularly relevant in women with bilateral disease. In fact, bilateral disease with laparoscopic removal of endometriomas from both ovaries has a 2.4% risk of premature ovarian failure [20]. Busacca *et al.* studied the frequency of post-surgical ovarian failure in 126 patients operated on for bilateral ovarian endometriosis. Ovarian failure was documented in three cases, corresponding to a rate of 2.4%. This shows that patients who had been operated on for bilateral endometriomas have a low but definite risk of premature ovarian failure occurring immediately after surgery.

Endometrioma-related impact on ovarian responsiveness

There are currently insufficient data to clarify whether the endometrioma-related damage to ovarian responsiveness precedes or follows surgery.

Elucidation of this point is of utmost interest since it would strongly impact on the decision of whether to operate on women with endometriomas and who are selected for IVF. At present, there appears to be evidence supporting both an endometrioma-related injury and a surgery-mediated damage. The relative importance of these two insults remains to be clarified.

Endometrioma-mediated damage

Data regarding responsiveness to ovarian stimulation during IVF in unoperated gonads are scanty. The vast majority of studies regarding the impact of endometriomas on IVF outcome have focused on previously operated women.

Thus, they cannot clarify whether the damage is caused by the development of an endometrioma or by its surgical removal. Maneschi *et al.* assessed the ovarian cortex surrounding ovarian benign neoplasms in 44 women who underwent surgery (13 mature teratomas, 9 benign cystadenomas, and 32 endometriomas). There were reduced follicular numbers and activity antecedent to surgery in endometriomas when compared with teratomas or benign cystadenomas, suggesting that the gonadal damage is at least partly caused by the presence of an endometrioma per se [21].

Morphological patterns similar to those of the normal ovarian cortex were observed in the cortical tissue surrounding mature teratomas, benign cystomas, and endometriomas in 92%, 77%, and 19% ($P < 0.01$) of specimens, respectively, and a regular vascular network was observed in 84%, 78%, and 22% ($P < 0.01$). Microscopic endometriosis was observed surrounding the endometrioma in the stroma of 82% of specimens, which may cause a distortion of the physiological function of the ovary.

Moreover, in a rabbit model, Kaplan *et al.* showed that endometrial implants in the ovaries decreased the number of ovulation points and that this reduction was primarily related to periovarian adhesions [22].

The functional consequences in humans of this finding have been poorly investigated.

One of the studies addressing this point evaluated whether the space-occupying effect of an endometrioma, rather than endometriosis itself, affects results in IVF [23]. Eighty-five patients with endometriomas who underwent IVF treatment without surgery were compared with 83 patients with simple ovarian cysts. The presence of an endometriotic cyst during the IVF cycle was demonstrated to be associated with a lower embryo quality and implantation rate, although pregnancy success was unaffected. This adverse effect may suggest that the poor ovarian responsiveness associated with endometrioma is also due to the result of the disease itself and not only because of the presence of a cystic mass.

To clarify whether the presence of ovarian endometriomas is associated with a reduced responsiveness to ovarian hyperstimulation, Somigliana *et al.* assessed 36 unoperated women with monolateral endometriomas who were selected for IVF [24]. The numbers of codominant follicles (>15 mm) in affected and contralateral unaffected gonads were 3.0+1.7 and 4.0+2.2, respectively ($P = 0.01$). This difference corresponded to a 25% (95% CI 6–44) reduction in the number of developing follicles in the affected ovaries [24]. This shows that the number of codominant follicles

developing in affected gonads was significantly reduced when compared with the contralateral intact ovaries of the same patients. This conclusion is further supported by the observation that this effect is dependent on the size and number of the cysts. In women with larger endometriomas, this difference was indeed more evident. A trend for a poorer response was also observed in patients with more than one cyst.

The pathogenic mechanisms behind this are unknown. It may be speculated as described by Maneschi *et al.* that the inflammatory reaction typically associated with the presence of an endometrioma may play a role. Alternatively, the presence of an expanding ovarian cyst per se may mechanically damage the ovarian tissue or disturb the vascularization of the organ. Besides the impaired function of the ovary may be due, at least in part, to other forms of the disease such as superficial implants and adhesions which typically co-exist with ovarian endometriomas.

Ovulation and ovarian reserve in the endometrioma-affected ovary

Unfortunately, data on natural cycle ovulation in unselected women with ovarian endometriomas are very limited. Most studies evaluated ovarian response after stimulation in ART treatment, but there is little information concerning the frequency of ovulation from the affected ovary in non-stimulated cycles.

Horikawa *et al.* investigated the rate of ovulation in 28 infertile women with monolateral endometriomas and found a 34.4±6.6% ovulation rate in the affected gonad when it is assumed that the frequency of ovulation occurred equally in each ovary with the same ovarian reserve [25].

Benaglia *et al.* in a larger prospective study assessed the ovulation rate in women with unilateral endometriomas who had not undergone previous surgery [26]. Ovulation occurred in the affected ovary in 22 of the 70 women recruited (31%; 95% CI 22–43%); this difference was statistically significant ($P = 0.002$). These results suggest that the presence of an endometrioma has a detrimental impact on ovarian physiology. In fact, the rate of ovulation in healthy and affected ovaries is, respectively, about 2:1; similar to the data reported in the smaller study by Horikawa *et al.*

The ovulation rate in the affected ovary was 35% when only one endometrioma was present, and 19% when two or more cysts were detected. These studies support the role of endometriotic cysts per se in the reduction of ovarian responsiveness in IVF cycles.

Damage to the ovarian reserve associated with endometriomas may vary according to the affected side. It appears that the left ovary is less vulnerable than the right one, as the ovulation rate is significantly reduced only when the cyst is right-sided (41 vs. 21%). This finding may at least partly explain the consistently demonstrated asymmetry in lateral distribution of ovarian endometriotic cysts [27].

Anti-Mullerian hormone (AMH) is a very useful predictor of the number of early antral follicles and ovarian response to stimulation. Garcia-Velasco *et al.* evaluated AMH follicular fluid concentrations in three clinical groups: (a) women with endometriosis and a visible endometrioma in one of the ovaries, (b) the same women with endometrioma-free contralateral ovary, and (c) women without endometriosis [28]. Interestingly, when the three groups were evaluated individually, the concentration of AMH in follicular fluid obtained from the ovaries where a large endometrioma was present was significantly lower than in the other two groups: (a) 4.1 ± 2.7 ng/ml in the ovaries with the endometrioma, (b) 4.9 ± 2.6 ng/ml in the contralateral ovary in the same patients, and (c) 6.2 ± 3.0 ng/ml in control patients ($P = 0.039$). Considering that severe endometriosis affects oocyte quality, these findings suggest that intrafollicular AMH concentration may be related to oocyte competence. Recently, it has been reported that AMH concentrations are associated with the fertilization rate [29]. In agreement with this, intrafollicular AMH levels were 3.42-times higher in those follicles that contained fertilized eggs when compared to those from non-fertilized eggs. However, this seemed to be a weak predictor of pregnancy potential. Therefore follicular fluid AMH concentrations may be an indicator not only of oocyte quantity but also of the quality of granulosa cell function in women suffering from endometriosis.

According to these data, it is thought that the physiological mechanisms leading to ovulation are abnormal in ovaries with endometriomas [26].

Surgery-mediated damage

The ovulation rate has been repeatedly shown to be reduced in operated gonads compared with contralateral intact gonads [25, 30]. Moreover, data from IVF cycles consistently showed a decreased ovarian responsiveness to hyperstimulation in previously

operated ovaries [17]. Conversely, the surgical treatment of ovarian endometriomas does not seem to impair IVF success rates [15, 16, 19, 31].

Tsoumpou *et al.* [31] evaluated the effect of surgical treatment of endometrioma on IVF outcomes, including clinical pregnancy rate and ovarian stimulation response. This meta-analysis included five studies that compared women who had surgical treatment for endometrioma with women with untreated endometrioma. There were no significant differences in pregnancy and clinical pregnancy rate per cycle between women who underwent surgery and those who received no treatment for endometrioma; the OR for pregnancy rate per cycle was 0.92 and the OR for clinical pregnancy rate per cycle 1.34. As described by Gupta *et al.* [15] there was a reduction in the number of oocytes retrieved with a weighted mean difference of 1.53 (95% CI 0.17–3.23) in the post-surgery group; however, this was not statistically significant.

One of the studies included in this meta-analysis was the restrospective study by García-Velasco *et al.* assessing the effect of surgery on endometriomas before IVF on fertility outcomes [32]. Of 189 women with endometriomas who underwent IVF, 56 women proceeded directly to IVF and 133 first underwent conservative ovarian surgery. A similar number of mature oocytes were obtained in both groups and there were no significant differences in fertilization rates, embryos obtained per cycle, or embryos transferred. However, a significant difference in the total doses of gonadotropins required was noted, as well as in peak E_2 levels on the day of hCG, in favor of women who had proceeded directly to IVF.

The rate of severe ovarian damage following surgery of endometrioma was recently studied by Benaglia *et al.* measuring the rate of ovaries remaining silent when stimulated after surgery [33]. The mean numbers of follicles in the operated and contralateral gonads were 3.4+2.4 and 5.7+3.0, respectively ($P < 0.001$). This difference corresponded to a mean 42% reduction in the number of follicles (95% CI 28–58%). Absence of follicular growth was observed in 12 operated ovaries of the 93 studied. This event never occurred in the contralateral gonad ($P < 0.001$).

This result is in line with the data reported by Busacca *et al.* in women who were operated for bilateral ovarian endometriomas [20].

The reduced responsiveness of affected ovaries cannot indeed be used to infer a deleterious impact on the rate of success of IVF. In fact, as mentioned earlier, a recent meta-analysis on this point reported a decreased folliculogenesis in women with ovarian endometriomas but failed to document an impact on pregnancy rate [15]. The results of the Gupta *et al.* meta-analysis are also consistent with the results reported by the Demirol *et al.* prospective randomized trial [34]. In this study patients were randomized to either ICSI directly or ovarian surgery followed by ICSI. Patients in the second group had longer stimulation and lower oocyte numbers but the fertilization, implantation, and pregnancy rates did not differ between the two groups. To date, this is the only published RCT on this issue.

However, one needs to be cautious when attributing the diminished response of the post-surgery ovaries only to the surgical injury to the ovary, as it has been shown ovaries with endometriotic cysts already exhibited reduced numbers of follicles and vascular activity compared with other types of benign cysts. Furthermore, it is difficult to identify the impact of endometriomas per se on IVF outcomes because the majority of cases are associated with concomitant peritoneal disease.

Conservative laparoscopic surgery of ovarian cysts with well-defined ovarian capsules (e.g. teratomas and benign cystadenomas) very seldom (6%) shows healthy ovarian tissue being removed next to them [35]. In contrast, in more than 50% of the endometriomas removed, primordial follicles are found, probably due to the very adherent capsule and to technical difficulty in the removal. This is in line with the most commonly accepted pathogenic theory positing that the endometrioma is a pseudocyst with the inverted ovarian cortex being its wall [36]. In this regard, it is not surprising that removal of the cyst will inadvertently also include removal of healthy ovarian tissue with primordial follicles.

The damage inflicted by surgery to ovarian responsiveness may be due not only to the removal of healthy tissue by laparoscopic stripping, but also to surgery-related local inflammation or vascular compromise following electrosurgical coagulation during hemostasis [19].

The employment of electrocoagulation may have an important detrimental effect on the remaining primordial follicle pool [7].

The pathogenic mechanisms may reduce ovarian reserve and function afterwards by damaging ovarian stroma as well as ovarian vascularization. Interestingly, power Doppler imaging of the ovaries after

laparoscopic surgery for large endometriomas has shown a decreased ovarian stromal blood flow, suggesting a permanent insult to the gonad [37]. More information is required to clarify whether the damage is transitory or permanent.

Ovulation in the post-surgery ovary

Most studies investigating the surgical adverse effect on the affected ovary evaluated ovarian response after stimulation in ART treatment without addressing the frequency of ovulation in the affected ovary in non-stimulated cycles.

Horikawa *et al.* observed a 16.9±4.5% ovulation rate in the affected gonad after surgery, which was significantly lower than that before surgery [25]. This study demonstrated that the presence of an endometrioma reduces the ovulation rate from affected ovaries without surgical treatment. But surgery seems to cause a further reduction of the ovarian reserve, as evidenced by the diminished ovulation rate of the affected ovary after surgery.

There is further evidence supporting surgery-mediated damage. Chang *et al.* studied the impact of cystectomy on ovarian reserve by measuring AMH levels before and after surgery [38]. Twenty women with benign ovarian masses participated (13 endometrioma, 6 mature teratoma, and 1 mucinous cystadenoma).

Median AMH level was 2.23 ng/ml before operation, but reduced to 0.67 ng/ml at the first week postoperatively and then increased to 1.50 ng/ml in the third month. The serum AMH level after 3 months postoperatively was recovered to about 65% of the preoperative level. Interestingly the serum AMH level at postoperative 1 week was more decreased in endometrioma compared with non-endometrioma patients (33.9% vs. 69.2% of preoperative level).

Although the statistical power of the study was diminished by the small number of study subjects the results suggest that ovarian reserve could be reduced after laparoscopic cystectomy.

In conclusion, severe ovarian damage may occur in gonads operated for ovarian endometriomas. At present, there is evidence suggesting that this damage may both precede and follow surgery, but the relative importance of these two pathogenic mechanisms has yet to be fully clarified.

This point is of utmost relevance since the demonstration that surgery may be mainly responsible for the damage would strongly caution against systematic surgical removal of these lesions. In particular, a more conservative attitude would have to be considered in women with bilateral cysts, in those with small asymptomatic lesions and in those selected for IVF.

Treatment prior to IVF

Ovarian endometriotic cysts respond poorly to medical therapy. Medical treatment is moderately effective in improving pain but ineffective in improving fertility in women with endometriosis [39]. Medical treatment may prevent further growth of the cyst or reduce the size, although shrinkage does not imply a reduction of the endometriotic tissue but mainly a diminution of the chocolate fluid within the cyst [40].

Thus, medical therapy by itself should not be considered in infertile women with endometriomas.

In contrast, it has been suggested that pituitary suppression with the administration of GnRH analogs for a few months prior to IVF may increase the success rate in women with endometriomas. The hypothetical beneficial effects may derive from the induced amenorrhea, a similar endocrine environment to hypogonadotropic hypogonadism, or to the effects of GnRH analogs on aromatase expression or on uterine NK cells, but this is all still speculative.

Over the last few years, there is cumulative evidence supporting the view that ovarian endometriomas may develop from ovulatory events.

Because ovulation and endometrioma development appear strongly associated, maintenance of ovulatory function in spite of the presence of endometriosis may favor cyst formation. The transformation of a corpus luteum into an endometrioma has been recently documented in 11 cases by Vercellini *et al.* [41]. Jain and Dalton also reported the transformation of a follicle into an endometrioma in 12 cases[42]. Moreover, oral contraceptives have been shown to be extremely effective in preventing endometrioma recurrence [43], thus indirectly supporting a critical role of ovulation in the pathogenesis of these cysts.

A recent meta-analysis on this subject showed that a 3–6 month treatment period with GnRH analogs prior to an IVF cycle improved the odds of clinical pregnancy in women with endometriosis by 4-fold [44]. As the results were extracted from only 165 patients and 78 pregnancies, they should be interpreted with caution. Moreover, the three trials included in the meta-analysis did not specifically focus on women with endometriomas. These results

encourage further randomized trials to finally determine whether or not there is a benefit from this combined approach.

Down-regulation with GnRH agonists in assisted reproduction may be beneficial in patients with endometriomas; the mechanism of action proposed is a reduction in cytokine concentrations. Gonadotropin agonist pituitary desensitization may lead to reduced interleukin-6 production by the endometriotic cells [45]. Moreover, expression of secretory leukocyte protease inhibitor in tissue and peritoneal fluid of patients with endometriomas was also reduced by GnRH agonist treatment [46]. In conclusion, GnRH agonists may result in the partial involution of endometriomas, wherein the actions of GnRH analogs are mediated through modulating the concentrations of cytokines such as interleukin-6 or secretory leukocyte protease inhibitor.

An alternative to surgery in some cases might be ultrasound-guided aspiration of ovarian endometriomas, a procedure first proposed by Aboulghar *et al.* [47]. Whether it is just cyst aspiration or aspiration plus *in situ* irrigation or injection with a sclerosing agent, the published evidence is not very compelling. Ultrasound-guided aspiration has been described as an option for managing large ovarian endometriomas that are unlikely to respond to medical treatment [48]. Dicker *et al.* demonstrated that the aspiration of endometriomas improves the ovarian response with an increase in number of oocytes available for retrieval and an increased number of embryos available for transfer [49].

Finally, for those patients who decline surgery, or in whom surgery is contraindicated, cyst aspiration may facilitate oocyte retrieval, although the rates of disease recurrence are high. Hormonal suppression therapy following aspiration may reduce the recurrence rates [7].

Conclusions

Ovarian endometriomas are a common and specific manifestation of the disease endometriosis. Knowledge in the field of endometrioma-associated injury to the ovarian reserve is still elusive, and research should be pursued. Nevertheless, based on current available evidence, some conclusions can be drawn. First of all, most of the published information indicates that ovarian endometriomas have an adverse effect on the follicle number and oocytes retrieved in IVF cycles but not on pregnancy outcomes. Data indicate that IVF enhances the low fecundity that may be associated with endometriomas. Patients with ovarian endometrioma may also benefit from IVF due to pituitary down-regulation caused by GnRH analogs.

Laparoscopic excision of endometriomas may be associated with quantitative damage to the ovarian reserve. This damage, however, is, at least in part, already present at the time of surgery. Therefore, women undergoing laparoscopic cystectomy must be counseled that surgery for ovarian endometrioma may result in a decrease in the number of retrieved oocytes, even though overall IVF fertility outcomes are not affected. Surgery should be envisaged in specific circumstances, such as to treat concomitant pain symptoms which are refractory to medical treatments, or in the presence of large cysts. Whether or not the deleterious effect associated with the presence of the endometriomas before surgery is transient or permanent remains to be clarified.

Overall, surgical removal of ovarian endometriotic cysts prior to IVF does not offer any additional benefit in terms of fertility outcomes. We thus recommend generally proceeding directly to IVF to reduce time to pregnancy and to avoid potential surgical compromise to the ovary.

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Chapter 4

Impact of uterine cavity abnormalities on IVF and pretreatment cavity evaluation

Steven F. Palter

Introduction

Many IVF programs employ a group of pretreatment screening evaluations in an attempt to maximize procedure success rates. Perhaps the most important factor contributing to success after the production of high-quality embryos is ensuring a receptive adequate uterine environment to maximize the potential for implantation. Human embryo implantation is a three-stage process (apposition, adhesion, and invasion) involving synchronized cross-talk between a receptive endometrium and a functional blastocyst [1]. Successful implantation requires a receptive endometrium, a functional embryo and synchronized cross-talk between the endometrium and embryo [2].

Both in nature and during IVF treatment implantation remains a paradoxically inefficient process in humans. Implantation failure can be the result of uterine factors or embryonic factors [1–4]. Both developmental secretory factors and an adequate gross structure of the uterus are required. Most programs have used hysterosalpingography as an initial evaluation of the infertile woman and have relied upon it to also assess the normalcy of the uterine cavity. More recently transvaginal ultrasound has been employed to further evaluate the uterine cavity. Technological advances have allowed the incorporation of direct visualization of the cavity with hysteroscopy in both the office and traditional operating room environment. Increasingly, structural abnormalities of the uterus have been recognized as significant factors limiting IVF success. This chapter will examine the evidence and methods of uterine cavity evaluation and the structural abnormalities that may compromise IVF success.

Methods of evaluating the uterine cavity before IVF: to screen or not to screen?

In the past, structural evaluation of the uterine cavity was often performed only in cases of IVF implantation failure. In a 1997 survey of board-certified reproductive endocrinologists 96% performed HSG routinely in the infertility evaluation whereas only 55% routinely performed ultrasound and 53% hysteroscopy [5]. A 2000 European working group on the ideal infertility evaluation recommended only HSG for evaluating the uterine cavity [6].

More recently, many groups now employ routine universal uterine cavity evaluation prior to IVF treatment initiation. The methods of cavity evaluation available include 2D and 3D ultrasound, saline infusion sonography (sonohysterography, SHG), hysterosalpingography (HSG), gel-infusion sonohysterography, and office or operating-room direct visualization of the cavity with hysteroscopy using carbon dioxide, saline, or other fluids for distention. The use of magnetic imaging resonance (MRI) with fluidic cavity distention and "virtual hysteroscopy" via multislice CT volumetric reconstruction are investigational methods of uncertain potential.

In one of the early studies of the use of diagnostic hysteroscopy prior to IVF in patients with a previously normal HSG, the benefit of treating the pathology found was uncertain so results were not communicated to the IVF treatment group and they were untreated [7]. Twelve of 16 patients had uterine abnormalities identified including small uterine septa, small submucous fibroids, uterine hypoplasia, and cervical ridges associated with a significantly lower pregnancy rate in this group.

Human Assisted Reproductive Technology: Future Trends in Laboratory and Clinical Practice, ed. David K. Gardner, Botros R. M. B. Rizk, and Tommaso Falcone. Published by Cambridge University Press. © Cambridge University Press 2011.

Other studies of the utility of examining the uterine cavity in patients with failed IVF followed and all showed high rates of identifying pathology. Oliveira *et al.* examined the uterine cavity of 55 women with two failed IVF cycles with good-quality embryos and a normal HSG within the prior 12 months using hysteroscopy [8]. Twenty-five (45%) patients had abnormal endometrial findings including polyps, myomas, endometritis, and adhesions that were not seen on the previous HSG. Pregnancy (50% vs. 20%) and implantation (19% vs. 5.5%) rates were significantly higher in a third subsequent IVF cycle in patients who were treated for uterine abnormalities than in patients who had normal uterine cavities on hysteroscopy. The authors concluded that the incidence of pathological findings on hysteroscopy is high in patients with repeated failures of IVF-ET. Sala *et al.* similarly identified an intrauterine structural defect with hysteroscopy in 18% of 100 women with two previously failed IVF cycles despite good quality embryos [9].

Next the routine use of uterine cavity evaluation with non-invasive SHG pre-IVF was advocated [10]. This early study found intracavitary lesions in 11.1% of 72 examinations and lesions visualized were confirmed and treated by hysteroscopy. No statistically significant difference was observed in the pregnancy outcome for patients undergoing IVF who had sonohysterography compared with that for patients undergoing IVF during the same period who previously had a uterine evaluation by a different method. Yauger *et al.* were able to reduce IVF cycle cancelations due to midstimulation observed endometrial polyps to 0.5% via the use of routine SHG evaluation [11].

Tur-Kaspa *et al.* examined the relative frequency of uterine structural and intracavitary abnormalities when SHG was used as a universal first-line diagnostic tool in patients with infertility and abnormal uterine bleeding [12]. Of 600 women with infertility, 16.2% had intracavitary abnormalities, including polyps (13.0%), submucous fibroids (2.8%), and adhesions (0.3%). Significantly, more (39.6%) of the 409 patients with abnormal bleeding had such intracavitary abnormalities. Developmental Mullerian uterine anomalies were more common in the infertility group found in 20% vs. 9.5% of women with abnormal bleeding.

Gera *et al.* extended this concept of universal screening with SHG pre-IVF to patients undergoing frozen thaw embryo transfer cycles [13]. In this group of women when previously cycled and who had uterine cavitary evaluations in the past a high incidence of uterine abnormalities was still identified prior to frozen thaw ET cycles. Uterine abnormalities were found in 11/36 patients (30.5%), which included uterine septum (9.0%), endometrial polyp (45.4%), intramural fibroid with normal cavity (9.0%), cystic endometrial changes (9.0%), cervical stenosis (18.1%), and calcification with normal cavity (9.0%). The authors recommended reevaluation if more than 1–2 years had passed since initial uterine evaluation; however, this time period was empiric and the optimal screening interval remains to be determined. However, the clinical utility of cavity evaluation is supported by a diverse body of evidence.

What is the optimal method to evaluate the uterine cavity?

Unfortunately, HSG has been shown to be relatively inaccurate for the evaluation of the uterine cavity. In fact, HSG has been shown to have poor sensitivity and specificity for the uterine cavity as early as the 1980s [14–17]. Prevedourakis *et al.* evaluated 323 women with infertility via HSG and hysteroscopy. In 20% similar abnormalities were observed with a global correlation of 74.8%; however, HSG had false positive results in 11.7% and false negative ones in 13.3% [16]. Golan *et al.* similarly evaluated 464 infertile women with both tests and also found poor results with HSG. Compared to hysteroscopy the sensitivity of HSG was 98%, but its specificity only 15%, the positive predictive value 45%, little better than a coin flip, and negative predictive value 95%. On hysteroscopy a normal uterine cavity was found in 53% of the cases with a filling defect and in 56% of those with uterine wall irregularity on HSG [15]. Even when experts evaluate the same HSG study there is considerable disagreement on whether a lesion is seen in the uterine cavity! When a group of fertility specialists were shown the same exact HSG there was very poor agreement between them on the normalcy of the uterine cavity with a kappa for agreement of only 0.345 [18].

The gold standard method for the evaluation of the uterine cavity is direct visualization with hysteroscopy. An added advantage is that structural abnormalities identified can be treated in the same setting. Wang *et al.* employed both HSG and hysteroscopy in the evaluation of patients with infertility and demonstrated superiority of hysteroscopy. The HSGs were interpreted as abnormal in 63% of patients. Of patients identified at HSG as having a normal uterine cavity hysteroscopy identified a false negative rate of 35.4%.

Of patients with abnormal HSGs there was a false positive rate of 15.6%. The sensitivity of HSG was 80.3% in revealing intrauterine abnormality and its specificity was 70.1%. In 35.0% the findings of HSG differed from those of hysteroscopy [17]. Soares *et al.* [114] took the comparison one step further and directly compared SHG, HSG, ultrasound, and hysteroscopy. Sonohysterography had the same diagnostic accuracy as the gold standard for polypoid lesions and EH, with no equivocal diagnosis. Hysterosalpingography showed a sensitivity of 50% and a PPV of 28.6% for polypoid lesions and a sensitivity of 0% for EH. Transvaginal sonography had both sensitivity and PPV of 75% for polypoid lesions and EH. For uterine malformations, SHG had a sensitivity of 77.8%, whereas TVS and HSG both had a sensitivity of 44.4%. Sonohysterography and HSG had a sensitivity of 75% in the detection of intrauterine adhesions and respective PPVs of 42.9% and 50%. Transvaginal sonography showed sensitivity and PPV of 0% for this diagnosis. They found sonohysterography had the same diagnostic accuracy as the hysteroscopy for polypoid lesions while HSG only had a sensitivity of 50% and a PPV of 28.6%. Transvaginal sonography had both sensitivity and PPV of 75% for polypoid lesions. For Mullerian abnormalities SHG had a sensitivity of 77.8%, whereas TVS and HSG both had a sensitivity of 44.4%. Sonohysterography and HSG had a sensitivity of 75% in the detection of intrauterine adhesions and respective PPVs of 42.9% and 50%. Transvaginal sonography showed sensitivity and PPV of 0% for this diagnosis. While hysteroscopy remains the gold standard SHG closely approaches it for screening but suffered from a high false positive rate.

One factor only identifiable with ultrasound and not via HSG or hysteroscopy is the appearance of intracavitary fluid during stimulation for IVF. The association of such fluid with hydrosalpinges is well established as is their association with lowered IVF success rates. Akman *et al.*, however, demonstrated that intracavitary fluid accumulation is actually more common in patients with PCOS than tubal factor infertility but that the fluid only had a detrimental effect in the tubal factor patients [19].

Newer evaluation techniques

HSG requires the use of iodinated contrast agents and is contraindicated in patients with sensitivity to these compounds. While shellfish allergy has been shown to be primarily directed at protein components of the seafood and not iodine itself, some patient do exhibit true contrast allergies. In these cases gadolinium, an MRI contrast agent, has been successfully employed [20, 21].

Perhaps the gold standard of the future will be a micro-pan-endoscopic evaluation of the entire female reproductive system allowing direct visualization of tubes, uterus, and ovaries, as well as pelvic pathology. Such an approach was shown to be feasible and tolerable [22]. Even more fanciful is the ability to perform a "virtual hysteroscopy" entirely via computer-reconstructed 3D imaging. Preliminary feasibility studies have been done using ultrasound or now a high-speed multislice CT scanner which captures a volume of area incorporating the entire uterus in a matter of seconds. Next a high-power computer reconstructs the images and can give any possible view of surface and deep pathology [23, 24]. Such technology is revolutionizing colon screening for structural abnormalities and may replace colonoscopy as a primary screening test [25–32].

Endometrial polyps

One of the most commonly encountered uterine cavity abnormalities is endometrial polyps. Polyps are benign localized overgrowth of the endometrial lining. They can be often mistaken grossly or at imaging studies for pedunculated myomas; however, the two differ in tissue of origin and consistency. While commonly associated with abnormal uterine bleeding or early pregnancy wastage, the association with infertility or implantation failure at IVF is less clear. Potential mechanisms by which they might interfere with implantation include disordered development of the receptive endometrium, ease of trauma with resultant intrauterine bleeding, abnormal local growth factor environments, mechanical interference with implantation or endometrial blood flow, or interference with sperm or embryo transport.

The gold standard method of diagnosis is hysteroscopy. The hysteroscopic appearance of a polyp is shown in Figure 4.1 and the sonographic appearance at SHG in Figure 4.2. Hysteroscopy identifies polyps in women with otherwise unexplained infertility 16.5–26.5% of the time [33, 34]. If a polyp is identified at hysteroscopy it can also be directly removed in the same procedure using either biopsy forceps, scissors, a resection loop, wire snare, or bipolar electrode. These methods are preferred over blind curettage, which may frequently miss or incompletely remove lesions. A newer hysteroscopic morcellator device has been

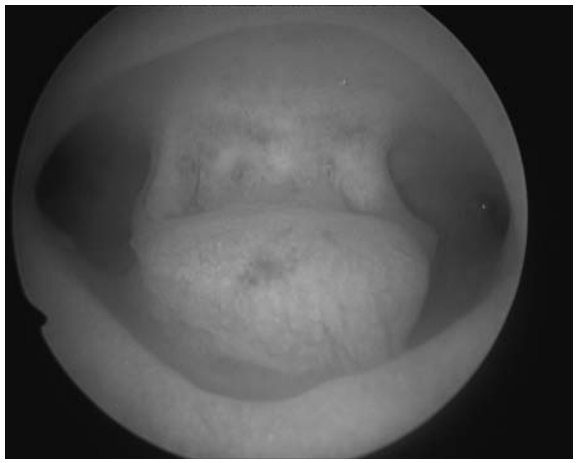


Figure 4.1. Intracavitary uterine polyp as seen on hysteroscopy. See colour plate section.



Figure 4.2. Intracavitary uterine polyp as seen on sonohysterography.

developed as a modification of an arthoscopy shaver and has been suggested to decrease operative time. In patients with uniformly thickened endometrial linings visualization at hysteroscopy may be limited and the procedure may be aided by pretreatment with progestins or oral contraceptive pills to thin the lining [35]. The generalized appearance of diffusely polyploid endometrial lining also has been associated with poorer reproductive outcome and miscarriage [36]. Most polyps do not have a vascular stalk. In some cases polyps may exhibit endometrial hyperplasia or carcinoma confined to the tip and pathological evaluation of the resected tissue is recommended.

The occurrence of polyps has been positively correlated with BMI. Reside *et al.* found that patients with polycystic ovary syndrome (PCOS) had a higher number of endometrial polyps, but the difference was not statistically significant (28.9% vs. 18.3%) [37]. When comparing the patients according to BMI, patients with BMI ≥ 30 had a statistically significantly higher number of endometrial polyps versus BMI < 30 (52% vs. 15%). Obesity correlated with the occurrence, size, and multiplicity of polyps [37]. One group identified a higher incidence in patients with endometriosis [33].

One trial examined the effect of polyps on pregnancy in cycles with gonadotropin supraovulation and IUI [38]. Patients were randomized to either hysteroscopic complete resection or biopsy with IUI in cycles beginning 3 months later. Potential effects on implantation at IVF might be inferred from their results. The clinical pregnancy rate was 63% in the polypectomy group compared with 28% in the control group (RR ¼ 2.3; 95% CI 1.6–3.2) corresponding with a number needed to treat (NNT) to achieve one additional pregnancy of 3 [39]. Survival analysis shows that after four cycles the pregnancy rate was 51.4% in the study group and 25.4% in the control group. Surprisingly, pregnancies in the study group were obtained before the first IUI in 65% of cases. The rest were obtained over the four-cycle period of supraovulatory cycles. This corresponds to a spontaneous pregnancy rate of 29% in the polypectomy group versus 3% in the control group [39]. When results were compared by polyp size no significant difference in benefit was seen in the range of 5 to 20 mm.

Although traditionally polyps are not commonly regarded as sole causes of infertility three other non-randomized studies have similarly found high rates of spontaneous pregnancy in women with infertility after simple polypectomy. Varasteh *et al.* found a pregnancy rate of 78.3% after polypectomy compared with 42.1% in those without polyps while Spiewankiewicz *et al.* reported a pregnancy rate of 76% in infertile patients after polypectomy [40, 41]. Shokeir *et al.* reported a 50% pregnancy rate after polypectomy [42].

Results of direct studies of the impact of polyps on IVF outcome have been less consistent. Lass *et al.* divided 83 women with ultrasonographically identified endometrial polyps <2 cm in diameter into two groups before oocyte retrieval [43]. Forty-nine were untreated and 34 underwent polypectomy immediately after oocyte retrieval followed by embryo cryopreservation and replacement in a subsequent thaw cycle. No significant difference was seen in pregnancy rates between the groups; however, their study design of cryopreservation in the treatment group is an unacceptable confounder.

A study of 33 women with small ultrasonographically identified polyps ranging in size from 5 to 12 mm found no differences in implantation or miscarriage rates in IVF when compared to women with sonographically normal cavities [44]. This size limitation, however, only includes very small polyps and may include some cases of otherwise normal polypoid endometrium. Similarly Isikoglu *et al.* found that small polyps <1.5 cm do not adversely affect IVF outcome [45]. Based on these studies some have suggested removing only those polyps >2 cm in size whereas others still advocate removal of any space-occupying lesion identified, especially if in office small-caliber hysteroscopy without the need for anesthesia is available.

Polyps <1 mm in size have been called "micropolyps" although their significance on implantation and differentiation from the normal variant remain unproven. Cicinelli *et al.* [115] identified such "micropolyps in 11% of women undergoing fluid distention hysteroscopy and found a high correlation with chronic subclinical histologically defined endometritis (OR 124.2, CI 50.3–205.4). Sensitivity, specificity, positive, and negative predictive values were 54%, 99%, 94%, and 89%. Johnston-MacAnanny *et al.* [116] studied the association of chronic endometritis with implantation failure in IVF patients. In a retrospective analysis they frequently found chronic endometritis in patients with failed IVF implantation who underwent diagnostic endometrial biopsies. Unfortunately, antibiotic treatment was ineffective in raising implantation rates in subsequent cycles.

Whether polyp location is significant was investigated by Atsushi *et al.*, who examined both the frequency polyps were located in different anatomic locations in the uterus and the impact of location on pregnancy outcome after treatment. The distribution of polyps in the uterus was found to be: uterotubal junction, 8.0%; posterior uterine wall, 32.0%; anterior uterine wall, 15.4%; lateral uterine wall, 9.2%; and multiple, 35.4%. The pregnancy rate after surgery at the uterotubal junction was significantly higher than that of other locations while the posterior wall polyp was the most frequent [46].

Müllerian anomalies

Müllerian anomalies are structural developmental abnormalities of the female reproductive system. They are classified in accordance with their mechanism of formation into lateral and vertical fusion defects, partial formation, and obstructive and non-obstructive variants of each [47]. The ASRM staging system further classifies these abnormalities into hypoplastic/agenesis, unicornuate, didelphus, bicornuate, septate, arcuate, and DES-related and is shown in Figure 4.3 [48].

To better understand these requires an understanding of normal development. The Müllerian or parmesonephric ducts arise as a paired set of tubes in the seventh gestational week. These ducts elongate and then fuse medially from the Müllerian tubercle moving cephalad. This is then followed by the disappearance of the separating walls between the fused ducts. In simple terms, failure of fusion leads to the duplication defects and failure of disappearance of the wall leads to septal defects. In normal development the unfused cranial portion becomes the fallopian tubes and the fused lower portion the uterus and proximal vagina. Recent reports have suggested that the fusion wave may proceed from the central portion simultaneously cephalad and caudad leading to combined proximal and distal abnormalities [49].

Approximately 1–2% of women have congenital abnormalities of the Müllerian system [50, 51]. However, the incidence as diagnosed by ultrasound

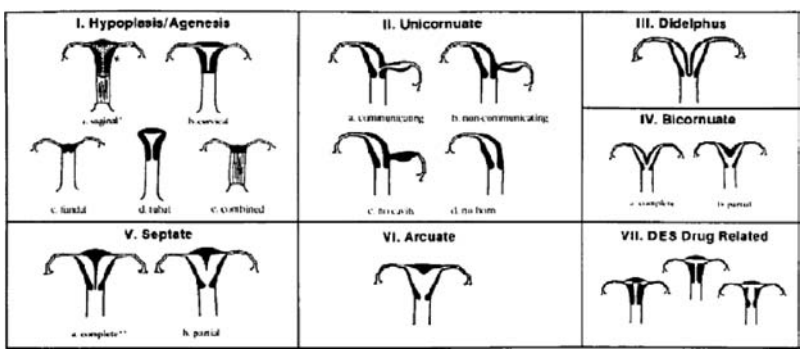


Figure 4.3. ASRM Müllerian anomaly classification system.

in women with recurrent pregnancy loss is three fold higher [52].

The septate uterus

The septate uterus is the most common Müllerian anomaly, the one associated with the worst reproductive outcome, and the one most amenable to hysteroscopic correction [49, 51, 52]. Uterine septae form from incomplete absorption of the intervening tissue as the two Müllerian ducts fuse. High rates of both first-trimester (25%) and midtrimester (6%) losses have been described. Loss rates greater than 50% have been described in other studies. The most common theory is vascular compromise as a result of the fibrous tissue of the septum compromising fetal development [49, 51, 52], although many large septae have been shown via biopsy and MRI to contain a muscular component [53, 117].

Traditionally uterine septae were considered causes of miscarriage but not infertility per se [54–56]. Retrospective studies which looked at the outcome of ART treatments in women with untreated septae versus controls without septae found both a difference and no difference. [57, 58]. Mollo *et al.* performed a prospective controlled trial that demonstrated a benefit in conception with septum treatment in patients with otherwise unexplained infertility [59]. This study showed both pregnancy rate (38.6% vs. 20.4%) and live birth rate (34.1% and 18.9%) were significantly higher in the group treated with septum resection versus controls who were women with unexplained infertility but without septae. Survival analysis showed that the probability of a pregnancy in the 12-month follow-up was also significantly higher in the treatment arm. The corresponding fecundity (10-week pregnancy) rates were 4.27 and 1.92 person-months in women who had undergone metroplasty and in women with unexplained infertility. Once the repair is performed a retrospective study found no benefit to the traditional 10–12 week waiting period after metroplasty versus immediate IVF in the first 9 weeks post-op [60].

Preoperative evaluation of the septum

It is crucial to make an accurate diagnosis of septate versus bicornuate uterus prior to hysteroscopic repair to avoid inadvertent fundal perforation of the indented central segment of the bicornuate uterus.



Figure 4.4. Uterine septum as seen on sonohysterography.

Hysterosalpingography is unable to differentiate these two conditions nor is hysteroscopy, as neither provides a view of the external surface contour of the uterine fundus [61]. MRI has been the gold standard method of diagnosis but this is becoming replaced by high-resolution ultrasound, especially 3D reconstructed coronal views which provide a similar image. [50, 53, 61–63]. The addition of fluid instillation into the uterine cavity with 3D sonohysterography (SHG) is now replacing MRI since it is simple, low-cost, and provides excellent visualization of both the internal and external uterine anatomy [64]. Figure 4.4 demonstrates duplicated endometrial cavity on SHG which is more easily identified via reconstructed coronal plane imaging in Figure 4.5. Figure 4.6 shows the appearance of the septum at hysteroscopy.

Surgical approach to the uterine septum

Hysteroscopic resection has replaced the older metroplasty procedures for the uterine septum [65–74]. If the diagnosis of septum is not confirmed by preoperative imaging studies then laparoscopic guidance is suggested. In routine cases we have replaced the use of laparoscopic guidance with transabdominal guidance when necessary [49, 75]. However, unlike cases of Asherman's syndrome, this is rarely if ever required for septae. Various methods have been used to resect the septum including laser, scissors, and wire loop or electrode. We prefer to use cold scissors and avoid electrosurgery to theoretically reduce thermal and cautery damage to the cut edges. The resection is continued cranially until the cavity is flush across the fundal portion and the hysteroscope can be moved from one tubal ostium to the other without hitting residual



Figure 4.5. Uterine septum as seen on sonohysterography. 3D coronal reconstructed view.

septal tissue. Studies have shown that small residual septae of less than 1 cm do not adversely affect reproductive outcomes [76]. The resection is more accurately described as an incision rather than an excision or resection since no tissue is actually removed. When the procedure is performed correctly the septal tissue will retract and flatten as it is incised. The majority of the septal tissue is fibrous and avascular. In all cases the use of an automated fluid management system to record fluid absorption is recommended.

If electrosurgery is used, specialized needle or knife electrodes are recommended. It is not recommended to straighten curved wire loop electrodes as this produces areas of weakness. Utmost care must be taken when incising the septum as this will often require forward movement of the electrode, increasing the risk of inadvertent perforation. Any case of perforation with an activated electrode requires exploration to exclude electrosurgical injury to the bowel.

In cases where the septum extends to involve the cervical canal traditional practice has recommended

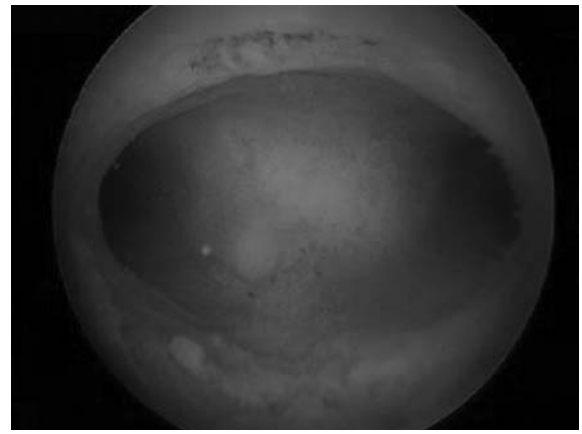


Figure 4.6. Uterine septum as seen on hysteroscopy. See colour plate section.

not fully excising the cervical portion of the septum. The hysteroscope is inserted on one side of the septum and cautery or scissors used to incise above the lowermost portion. This approach has been recommended to reduce the risk of subsequent cervical incompetence; however, there is no direct proof of this and some have fully resected the cervical component without complication.

The arcuate uterus

The arcuate uterus occurs when there is a mild extension from the uterine fundus caudally. While some view this as a minimal extension septum, it is probably a normal variant in many women. In general, the tissue is normal muscular myometrium and not fibrous like a septum. Both normal and adverse reproductive outcomes have been reported [50, 61, 62]. Most of these studies used older methods of diagnosis and suffer from methodological limitations. Treatment, when indicated, is performed in the same fashion as for a uterine septum.

Space-occupying lesions: myomas

Myomas are the most common benign tumor of the female reproductive system and are found by ultrasound in more than 70% of women by age 50 [77]. The classic classification system describes myomas based upon their location within the uterus (submucosal, intramural, and subserosal). Submucosal myomas may be further classified according to the extent to which they impinge upon the uterine cavity. The European Society for Hysteroscopy Classification System for

Submucosal Myomas describes myomas based upon the relative amounts of intramural and intracavitary tissue [78, 79]. A type 0 myoma is wholly contained inside the endometrial cavity and is also referred to as a pedunculated myoma. Type I myomas have <50% intramural extension and are amenable to hysteroscopic resection. Type II myomas have more than 50% intramural extension and in general are more amenable to transabdominal excision approaches. Hysteroscopic resection of these is more likely to result in incomplete removal, persistence of symptoms or adverse effects on fertility, and regrowth. Hysteroscopy remains the most accurate method of assessing the relative intracavitary versus intramural components of type 0–I myomas. The assessment of this via ultrasound or sonohysterography is highly dependent upon scanning in the appropriate perpendicular planes to the uterine axes.

Intracavitary myomas and infertility

It is generally recommended that myomas which distort the cavity be removed prior to conception. A type 0 myoma (wholly within the cavity) is most easily resected with either resectoscope or via transection of its pedicle. Since these may be vascular, electrosurgical energy to achieve hemostasis is recommended to be available. In general, myomas are primarily resected in an operating room setting. Since fluid absorption can be rapid and unpredictable the use of an automatic system to monitor fluid balance is recommended especially when using non-isotonic distension media [80].

Type I myomas, where a portion grows in the intramural portion of the uterus, are more difficult to resect. These are resected using the electrosurgical resectoscope and both unipolar and bipolar versions can be used. These are traditionally resected in the operating room environment and the use of an automatic fluid monitoring system is again recommended [80]. Various techniques have been advocated to address the intramural component of the myoma. With partial resection, only the portion that extends beyond the myometrium is resected down to flush with the uterine wall. While technically simpler, regrowth of the myoma is a significant risk. In addition, if interruption of normal blood supply to the surface endometrium is a factor, this method will not restore normal anatomy. An alternate technique is to remove the distention media once the myoma is resected flush with the uterine wall. After a short period of waiting the uterus is allowed to contract upon itself. In some cases, the intramural portion will be herniated into the cavity as it is pushed by myometrial contractions and will now be amenable to further resection. A case report suggested that intraoperative injection of prostaglandin F-2-alpha increased the expulsion of the intramural portion [81]. Most recently, a dissection technique has been described [82]. Here, an incision is made over the endometrial portion of the myomas and it is then enucleated as if the surgeon was performing an abdominal myomectomy. Some have advocated this procedure with electrosurgical electrode dissection while others have done so with CO_2 distension using a purely dissection technique or using a combined resection and grasper technique [83]. A hysteroscopic resection device modeled on an arthroscope shaver has been recently approved for use and also may allow better resection of type I myomas. This device is purely mechanical without electrosurgical current. It morcellates the myoma and mechanically shaves it while simultaneously removing the chips. In both the OR and office setting, entry of the hysteroscope in cases of cervical stenosis can be added by the use of oral or transvaginal misoprostol but not mifepristone [84, 118–120].

Large intramural myomas without an intracavitary component remain controversial. There are no absolute figures on exact size–risk relationships and clinical judgment is recommended. The largest meta-analysis of 106 studies concluded that myomas which distort the endometrial cavity have a deleterious effect on fertility [85]. Several recent studies have questioned the relationship of small intramural myomas to pregnancy loss or implantation failure [86, 87]. A large prospective study of more than 400 women found that the presence of even small intramural myomas not distorting the cavity had a significant negative effect on pregnancy at IVF (odds ration 0.46) [86]. In terms of overall reproductive outcome at IVF, a retrospective study found that patients who undergo precycle myomectomy have IVF cycle outcomes that are similar to controls with regard to ongoing pregnancy, implantation and early pregnancy loss. Outcomes were similar for myomas resected via hysteroscopy regardless of intramural components or if resected at laparotomy. It is possible that the amount of normal myometrial tissue between the myoma and the cavity may be another significant predictor.

Asherman's syndrome

Asherman's syndrome (AS; intrauterine synechiae) was first described by Heinrich Fritsch in 1894 and

then further characterized by Asherman in 1948 [88]. In this classic paper he described the radiological appearance of the uterine cavity with adhesions seen at HSG. While generally considered to be a rare disorder it may be more common than initially suspected [89]. In this study, 40% of women who had a delivery or miscarriage complicated by retained placental fragments for more than 24 hours developed hysteroscopic evidence of adhesions. It has recently been suggested to potentially exist in a more mild form in the asymptomatic eumenorrheic infertile woman [90].

The clinical features of AS are poorly described. Classically, it is most commonly associated with secondary amenorrhea especially following postabortal curettage. Many authors have postulated the potential etiological role of infection [91]. Unfortunately, no consensus exists regarding the optimal treatment regimen and treatment outcomes reported are generally poor [92–98].

We performed an international registry study of 297 women with AS to determine the clinical features and treatment outcomes [99]. In this study, reproductive outcome was extremely poor and pregnancy losses were extremely common. Pretreatment, only 61% of patients were able to achieve a live-born child. First-trimester losses were the most common (49% of all pregnancies, 42% of patients). Even more ominous, second- and third-trimester losses were four times more common than would be expected (8.5% of all pregnancies). The vast majority of patients presented with hypo- or amenorrhea but infertility and pregnancy losses were also common.

Classification of Asherman's syndrome

There are several classification systems for Asherman's syndrome although none directly correlates with outcomes [91]. We prefer to list the location of each area of scar and normal endometrium and quantify the percentage of normal cavity. Scar is described as vascular or vascular with a descriptor for density. Areas of normal-appearing proliferative endometrium are described. The two most clinically useful scoring systems are the European Society of Hysteroscopy (ESH) system and the American Society for Reproductive Medicine (ASRM) scoring system. The ESH system is based upon the thickness of the adhesive bands, the patency of the tubal ostia, and the amount of the cavity obliterated. Since this is a fertility-based system a large emphasis is placed upon tubal ostial patency. The ASRM system is based upon the amount of cavity obliterated, the density of the adhesion and the menstrual pattern. While successful pregnancies have been reported, even with small residual cavities the risk of adverse obstetrical outcomes increases [91, 92, 101, 102–107].

Treatment of Asherman's syndrome

We also devised and described a comprehensive diagnostic and treatment algorithm for patients with AS [100]. The mean age of patients treated was 33.8 years and mean median gravity and parity were 2/0. More than 1/3 had previous attempted surgical repair. Surgical correction of the cavity was possible in almost all cases. In our referral practice for Asherman's syndrome all patients undergo a preoperative 2D and 3D sonohysterographic evaluation of the uterus with particular attention paid to the location of areas of apparent scar versus intact proliferative lining and focal hematometria if present. In some cases pretreatment with estrogen may induce proliferation of otherwise non-visible endometrial lining tissue and aid targeting these areas in the operating room.

We perform all significant lysis of intrauterine adhesion procedures in the operating room under general anesthesia. We use transabdominal ultrasound guidance during these procedures to ensure complete lysis of adhesions [108]. Adhesiolysis is performed using semiflexible micro-scissors without the use of intrauterine energy to minimize the risk of further adhesion formation or devascularization.

Several methods have been used to reduce the incidence of postoperative adhesion reformation. The most commonly used method is the intrauterine placement of a barrier device. There are no currently FDA-approved devices for this indication. Off-label, the IUD, Foley catheter, and intrauterine splint (designed for short-term management of hemorrhage) have been used. Direct comparisons of the Foley catheter and the IUD have shown superior efficacy with the balloon [109]. In 110 cases treated with either method and retrospectively analyzed, 81.4% of the patients using the Foley had restoration of normal menstruation compared with 62.7% in the IUD group. Persistent post-treatment amenorrhea and hypomenorrhea occurred less frequently in the Foley catheter group (18.6%) than in the IUD group (37.3%). The conception rate in the catheter group was 33.9%, also greater than in the IUD group [109].

At the completion of the procedure an 8–12 French foley catheter with its distal tip cut flush to the balloon was inflated in the cavity under ultrasound guidance. It is inflated under transabdominal ultrasound guidance at the completion of the lysis of adhesions procedure to just fill the cavity with minimization of pressure. There have been anecdotal reports of intraoperative uterine rupture when the balloon catheter was overfilled without ultrasound guidance. During the immediate postoperative period the patient is placed on a broad-spectrum antibiotic and estrogen to help stimulate endometrial re-proliferation [110].

The potential utility of bioabsorbable barriers similar to those used at laparoscopy has been recently suggested in pilot studies only [111–113]. Both spray-gel and cross-linked hyaluronic acid have been shown potential utility in these pilot studies with reduction in de novo and reformed adhesions [111–113]. We have presented preliminary evidence from both a human case series and a non-human primate model of AS suggesting that lower segment obstruction to menstrual outflow could cause a secondary reflex down-regulation of the global endometrial lining which might represent a novel AS variant [90].

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Chapter 5

Sperm retrieval techniques

Sandro C. Esteves and Ashok Agarwal

Introduction

Two major breakthroughs occurred in the area of male infertility only 2 to 3 years apart [1–3]. The first was the development of intracytoplasmic sperm injection (ICSI) for the treatment of male factor infertility due to severely abnormal semen quality [1]. The second was the extension of ICSI to azoospermic males and the demonstration that spermatozoa derived from either the epididymis or the testis were capable of normal fertilization and pregnancy [2, 3]. Azoospermia is defined as an absence of spermatozoa in the ejaculate after centrifugation. This condition, which is found in 1–3% of the male population and approximately 10% of infertile males, results in infertility but does not necessarily imply sterility [4]. In the case of azoospermia, two totally different clinical situations exist, i.e. obstructive and non-obstructive azoospermia. In obstructive azoospermia (OA), spermatogenesis is normal but a mechanical blockage exists in the genital tract, somewhere between the epididymis and the ejaculatory duct, or the epididymis and vas deferens are totally or partially absent. Causes of OA may be acquired or congenital. Acquired OA may be due to vasectomy, failure of vasectomy reversal, post-infectious diseases, surgical procedures in the scrotal, inguinal, pelvic, or abdominal regions, and trauma. Congenital causes of OA include cystic fibrosis, congenital absence of the vas deferens (CAVD), ejaculatory duct or prostatic cysts, and Young's syndrome [4]. Non-obstructive azoospermia (NOA) comprises a spectrum of testicular histopathology resulting from various causes that include environmental toxins, medications, genetic and congenital abnormalities, varicocele, trauma, endocrine disorders, and idiopathic. In both OA and NOA, pregnancy may be achieved through assisted reproductive techniques, i.e. in vitro fertilization associated with ICSI [4–5].

Several sperm retrieval methods have been developed to collect epididymal and testicular sperm for ICSI in azoospermic men. Either percutaneous (PESA) [6] or microsurgical epididymal sperm aspiration (MESA) [2] can be successfully used to retrieve sperm from the epididymis in men with obstructive azoospermia. Testicular sperm aspiration (TESA) can be used to retrieve sperm from the testes in men with OA who fail PESA as well as in those with NOA [7]. Testicular sperm extraction (TESE) using single or multiple open biopsies [8, 9] and more recently microsurgery (micro-TESE) are indicated for men with NOA [9–12]. Sperm can be easily obtained from infertile men with OA for ICSI whereas individuals exhibiting NOA have historically been the most difficult to treat. It is out of the scope of this chapter to provide a step-by-step laboratory description of the commonly used methods for PESA/TESA/TESE sperm processing and identification of viable immotile sperm for ICSI. However, as a general rule, processing of surgically retrieved spermatozoa should not only ease the selection of the best-quality spermatozoa for ICSI but also optimize the fertilizing ability of these often compromised specimens, particularly in the cases of NOA and after the freeze-thawing process.

This chapter describes surgical methods for retrieval of epididymal and testicular spermatozoa in men with obstructive or non-obstructive azoospermia. Sperm retrieval rates using different methods and in several clinical conditions are also presented, as well as clinical outcomes of ICSI using testicular and epididymal sperm.

Human Assisted Reproductive Technology: Future Trends in Laboratory and Clinical Practice, ed. David K. Gardner, Botros R. M. B. Rizk, and Tommaso Falcone. Published by Cambridge University Press. © Cambridge University Press 2011.

Step-by-step description of surgical techniques

Percutaneous sperm retrieval techniques

Anesthesia

Percutaneous sperm retrieval is carried out under local anesthesia only or in association with intravenous sedation. In both cases, a 10 ml solution of 2% lidocaine is injected around the spermatic cord near the external inguinal ring. In cases where intravenous anesthesia is used, local injection of the anesthetic is performed after patient unconsciousness is achieved.

Percutaneous epididymal sperm aspiration (PESA)

Indications

PESA is indicated in obstructive azoospermia cases only.

Technique

- After anesthetic blockade of the spermatic cord, epididymis is stabilized between the index finger, thumb and forefinger while the testis is held with the palm of the hand.
- A 13-gauge needle attached to a 1 ml tuberculin syringe is inserted into the epididymis through the scrotal skin. Loupe-magnification is used to avoid injuring small vessels seen through the skin (see Figure 5.1A).
- Negative pressure is created and the tip of the needle is gently moved in and out within the epididymis until fluid enters the syringe. The amount of epididymal fluid obtained during aspiration is often minimal (~0.1 ml), except in cases of CAVD, in which 0.3–1.0 ml may be aspirated.
- The needle is withdrawn from the epididymis and the aspirate is flushed into a 0.5–1.0 ml 37°C sperm medium.
- The tube containing the epididymal aspirate is transferred to the laboratory for microscopic examination. PESA is repeated at a different site of the same epididymis (from cauda to caput) and/or at the contralateral one until adequate number of motile sperm is retrieved. If PESA fails to retrieve motile sperm for ICSI, TESA is performed at the same operative time (see Figure 5.1B).

Testicular sperm aspiration (TESA)

Indications

TESA may be performed in either OA or NOA cases. In OA, TESA is carried out after a failed PESA, but may be also used as a primary retrieval procedure in cases of absent epididymis or intense epididymal fibrosis. In NOA, TESA may be used as a diagnostic tool

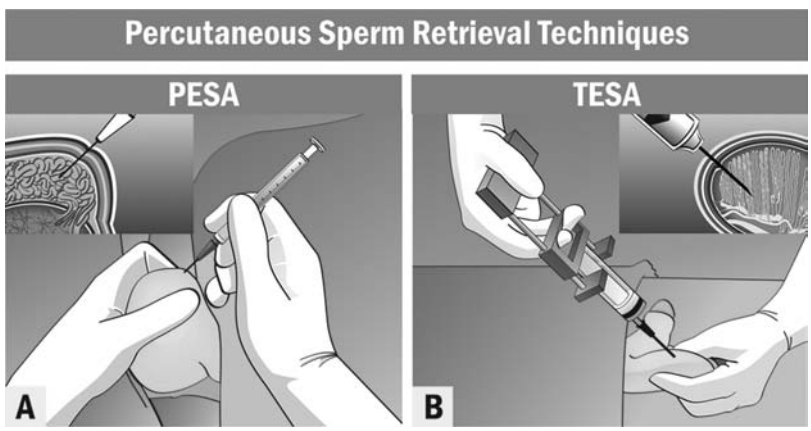


Figure 5.1. Percutaneous sperm retrieval techniques. (A) Percutaneous epididymal sperm aspiration (PESA). Epididymis is stabilized between the index finger, thumb, and forefinger. A needle attached to a tuberculin syringe is inserted into the epididymis through the scrotal skin, and fluid is aspirated. Aspirate is flushed into a tube containing HEPES-buffered sperm medium and sent for microscopic examination. (B) Testicular sperm aspiration (TESA). A 20-ml needled-syringe connected to a holder is percutaneously inserted into the testis. Negative pressure is created and the tip of the needle is moved within the testis to disrupt the seminiferous tubules and sample different areas. A piece of testicular tissue is aspirated, and a forceps is used to remove the seminiferous tubules that exteriorize from the scrotal skin. The specimen is flushed into a tube containing sperm medium, and the tube is transferred to the laboratory for processing and examination. See colour plate section.

to obtain testicular parenchyma for histology analysis and sperm search previous to the ICSI cycle. Also, it is indicated for sperm retrieval in cases of favorable prognosis, such as the ones with a previously successful TESA attempt or those with testicular biopsy result showing hypospermatogenesis.

Technique

- After anesthetic blockade of the spermatic cord, epididymis is stabilized between the index finger, thumb, and forefinger while the anterior scrotal skin is stretched.
- A 23 gauge needle attached to a 20 ml syringe is connected to a syringe holder and is inserted through the stretched scrotal skin into the anteromedial or anterolateral portion of the superior testicular pole, in an oblique angle towards the medium and lower poles (see Figure 5.1B). Loupe-magnification is used to avoid small vessels seen through the skin.
- Negative pressure is created by pulling the syringe holder while the tip of the needle is moved in and out within the testis in an oblique plane to disrupt the seminiferous tubules and sample different areas. When a small piece of testicular tissue is aspirated, the needle is gently withdrawn from the testis while the negative pressure is maintained. A pair of microsurgery forceps is used to grab the seminiferous tubules that exteriorize from the scrotal skin, thus aiding in the removal of the specimen.
- The specimen is flushed into a tube containing 0.5–1.0 ml warm sperm medium, and is transferred to the laboratory for microscopic examination. TESA or TESE may be performed at the contralateral testis if insufficient or no sperm are obtained.

Microsurgical sperm retrieval techniques

Anesthesia

Microsurgical sperm retrieval may be performed under either local anesthesia in association with intravenous sedation or epidural anesthesia. In the case of the former, which is our preference, a 10 ml solution of 2% lidocaine is injected around the spermatic cord near the external inguinal ring. Operating microscope and microsurgery technique are used throughout the procedures (see Figure 5.2).

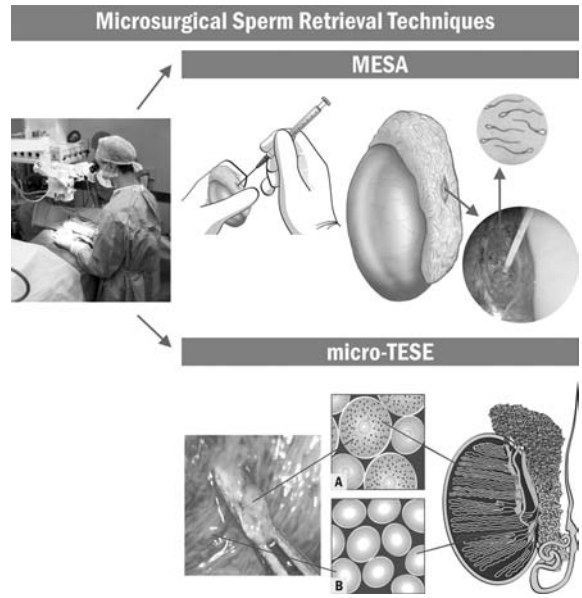


Figure 5.2. Microsurgical sperm retrieval techniques. Operating microscope and microsurgical technique are used throughout the procedures. Microsurgical epididymal sperm aspiration (MESA): after exposure of testis and epididymis, a dilated epididymal tubule is dissected and opened. Fluid is aspirated, diluted with sperm medium, and sent to the laboratory for examination. Microsurgical testicular sperm extraction (micro-TESE): after testis exteriorization, a single and large incision is made in an avascular area of the albuginea to expose testicular parenchyma. Microdissection of seminiferous tubules is carried out to identify and remove large tubules that are most likely to contain germ cells and active spermatogenesis (see photograph at ×40 magnification indicating enlarged (A) and non-enlarged (B) seminiferous tubules). Enlarged tubules may contain active spermatogenesis, as illustrated in the transversal section of a histopathology specimen (A). Non-enlarged tubules are more likely to contain no active spermatogenesis (B). Excised testicular specimens are washed in a well-dish containing sperm media to remove blood clots and are sent to the laboratory for processing and examination. See colour plate section.

Microsurgical epididymal sperm aspiration (MESA)

Indications

MESA is indicated in obstructive azoospermia cases only.

Technique

- After anesthetic blockade of spermatic cord, the anterior scrotal skin is stretched and the skin and tunica vaginalis are infiltrated with 2 ml of 2% lidocaine. A transverse 2 cm incision is made through the anesthetized layers, and the testis is exteriorized.
- The epididymis is examined and its tunica is incised. An enlarged tubule is dissected and opened with sharp microsurgical scissors.

- Fluid exuding from the tubule is aspirated with a silicone tube or blunted needle attached to a 1 ml tuberculin syringe. The aspirate is flushed into a 0.5–1.0 ml 37°C sperm medium.
- The tube containing the epididymal aspirate is transferred to the laboratory for microscopic examination. MESA is repeated at a different site of the same epididymis (from cauda to caput) and/or at the contralateral one until adequate number of motile sperm is retrieved. If MESA fails to retrieve motile sperm, TESA or TESE may be performed at the same operative time.

Microsurgical testicular sperm extraction (micro-TESE)

Indications

Micro-TESE is indicated in NOA cases only.

Technique

- After anesthetic blockade of spermatic cord, the anterior scrotal skin is stretched and the skin and tunica vaginalis are infiltrated with 2 ml of 2% lidocaine. A transverse 2 cm incision is made through the anesthetized layers, and the testis is exteriorized.
- A single, large, mid-portion incision is made in an avascular area of the tunica albuginea under 6–8× magnification, and the testicular parenchyma is widely exposed.
- Dissection of the testicular parenchyma is carried out at 16–25× magnification searching for enlarged seminiferous tubules (more likely to contain germ cells and eventually normal sperm production). The superficial and deep testicular regions may be examined, if necessary, and microsurgical-guided testicular biopsies are performed by removing enlarged tubules (see Figure 5.2B). If enlarged tubules are not seen, then any tubule different than the remaining ones in size is excised [10]. If all tubules are identical in appearance, random micro-biopsies (at least three at each testicular pole) are performed.
- Each excised testicular tissue specimen is placed at the outer-well dish containing sperm media. Specimens are washed grossly to remove blood clots and are sent to the laboratory for processing and search for sperm.
- Albuginea and scrotal layers are closed using non-absorbable and absorbable sutures, respectively.

Conventional testicular sperm extraction (TESE)

Indications

Single or multiple open testicular biopsies may be taken to obtain sperm in both OA and NOA, but TESE is used mainly in cases of NOA. In OA, TESE may be used after failed PESA or TESA. In NOA, TESE may also be used as a diagnostic tool to obtain testicular parenchyma for histology analysis and search of sperm previous to the ICSI cycle.

Anesthesia

TESE may be performed under either local anesthesia with or without intravenous sedation or epidural anesthesia.

Technique

- After anesthetic blockade of spermatic cord, the anterior scrotal skin is stretched and the skin and tunica vaginalis are infiltrated with 2 ml of 2% lidocaine. A transverse 2 cm incision is made through the anesthetized skin, cremaster, and parietal tunica vaginalis. Conventional TESE is carried out without magnification.
- A small self-retaining eyelid retractor is placed to improve exposure of the tunica albuginea, since the testis is not exteriorized.
- The tunica albuginea is incised for approximately 1 cm. Gentle pressure is made on the testis to extrude testicular parenchyma.
- A fragment of approximately 5×5×5 mm is excised with sharp scissors and placed promptly in sperm culture media (see Figure 5.3). Specimen is sent to the laboratory for processing and microscopic examination.
- Albuginea is closed using non-absorbable sutures. Procedure may be repeated if multiple biopsies are selected for preference.

Clinical outcomes

Out of 2136 males seeking infertility evaluation at our tertiary Center in Brazil from 2002 to 2009, 142 (6.6%) and 176 (8.2%) had obstructive and non-obstructive azoospermia, respectively, and underwent sperm retrieval for either diagnostic or therapeutic purposes. In this section, we present success rates of percutaneous and microsurgical techniques both in OA and NOA, and clinical outcomes of ICSI using fresh epididymal and testicular spermatozoa.

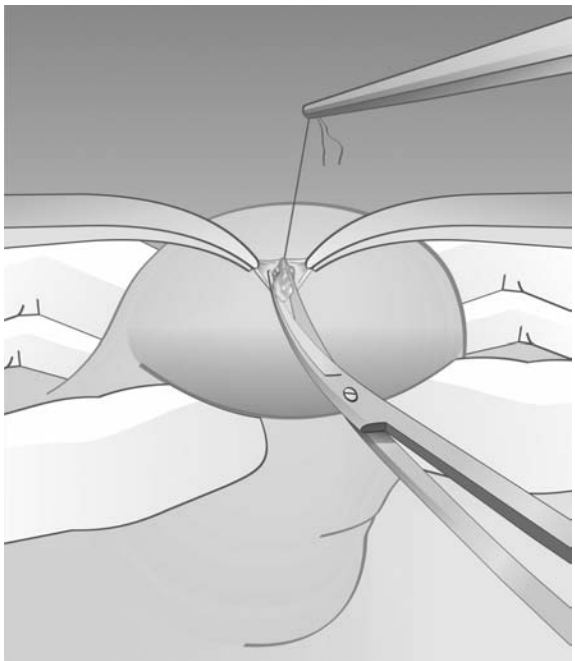


Figure 5.3. Testicular sperm extraction (TESE). Illustration of TESE using a single open biopsy. A 2-cm skin incision is made to allow opening of scrotal layers down to the albuginea. Testicle is not exteriorized from scrotum. A small 0.5-cm incision is made in an avascular area of the albuginea to expose testicular parenchyma. A fragment of approximately 5×5×5 mm is excised and sent to the laboratory for processing and examination. Additional fragments may be taken from the same incision or from different testicular poles using multiple incisions. See colour plate section.

Sperm retrieval rates

PESA and TESA were highly effective methods for retrieving sperm in the group of men with OA. Successful sperm retrieval (SRR) was achieved in over 85% of the cases using PESA, but more than one aspiration was required in several cases. In cases of failed PESA, TESA was adequate to obtain sperm in practically all cases. Motile spermatozoa was obtained in approximately 73% of the cases after the first or second PESA aspirations, and TESA was carried out as a rescue procedure after failed PESA in about 14% of the individuals (see Table 5.1). Successful sperm retrieval using percutaneous techniques appears to be independent of the cause of obstruction, since SRR rates did not differ among groups (see Table 5.1). In the group of men with NOA, SRR rates were in the range of 50–70% in most etiology-specific causes of NOA (see Table 5.2). Testicular histopathology results were predictive of sperm collection using both TESA and micro-TESE. According to our data involving 176 individuals, overall SRR rates by TESA were 64.4%, but only 20.7% and 33.3% in cases of Sertoli-cell only and maturation arrest, respectively. On the other hand, SRR by TESA was 100% and 82.3% in NOA men presenting with either hypospermatogenesis on testicular histology or a history of a previous successful TESA attempt. Using micro-TESE, overall SRR rates were 52.3%, but higher than TESA in cases of maturation arrest and Sertoli cell-only (see Table 5.2). The overall sperm retrieval rates (SRR), defined as successful surgical collection of spermatozoa, were significantly higher in the OA group (SSR = 97.9%; $n = 139/142$) compared to NOA (SSR = 61.9%; $n = 109/176$) ($P < 0.001$). The chances of retrieving spermatozoa were markedly increased in couples whose male partner had obstructive rather than non-obstructive azoospermia (OR 43.0; 95% CI 10.3–179.5).

ICSI outcomes using epididymal and testicular spermatozoa

ICSI outcomes in men with OA seem to be independent of the cause of obstruction. In the group of men with OA, fertilization and live birth rates were not different in individuals who had vasectomy / failed reversal, CBAVD, or infection as the cause of obstruction (see Table 5.3). Either epididymal or testicular spermatozoa retrieved from men with OA exhibited similar reproductive potential (see Table 5.4). Fertilization rates by ICSI using spermatozoa from men with OA and NOA were 62.5% and 51.1%, respectively ($P < 0.01$). The overall pregnancy rates, defined as the live birth rate (LBR) per transfer, were 40.2% (41/102) and 25.0% (22/88) in groups of OA and NOA, respectively ($P = 0.03$). The chances of achieving a live birth by ICSI (OR 1.93; 95% CI 1.04–3.61) were increased in couples whose male partner had obstructive rather than non-obstructive azoospermia, indicating that the reproductive potential of infertile men undergoing ART is related to the type of azoospermia.

Expert commentary

Obstructive azoospermia

The adoption of strict criteria to diagnose OA is crucial for obtaining high success retrieval rates in the

Table 5.1. Sperm retrieval rates (SRR) of PESA and TESA in obstructive azoospermia (AO) according to the number of aspiration attempts and the cause of obstruction

	Presence of Motile sperm by PESA: *n* (%)	Cumulative Successful retrieval rate: *n* (%)
By retrieval attempt: *n* (%) – 130 (100.0%)		
1st PESA attempt	66/130 (50.8)	–
2nd PESA attempt	29/64 (45.3)	95/130 (73.1)
3rd PESA attempt	10/35 (28.6)	105/130 (80.7)
4th PESA attempt	7/25 (28.0)	112/130 (86.1)
Rescue TESA after failed PESA	16/18 (88.9)	128/130 (98.4)
By cause of obstruction: *n* (%) – 142 (100.0%)[a]		
CAVD – 30 (23.1%)	21/30 (70.0)	30/30 (100.0)[a]
Vasectomy/failed reversal – 64 (49.2%)	40/64 (62.5)	61/64 (95.3)[a]
Post-infection/EDO/trauma – 48 (36.9%)	31/36 (81.6)	48/48 (100.0)[a]
Overall SRR: *n* (%)	92/130 (70.8)	139/142 (97.9)[a]

Source: Androfert.
CAVD, congenital absence of vas deferens; EDO, ejaculatory duct obstruction.
[a] PESA+TESA: in 12 cases of post-infectious OA, TESA was performed as the first choice due to intense epididymis fibrosis. In three of them, no sperm was obtained after TESA.
Chi-square test was used to compare SRR rates among groups stratified by the cause of obstruction. Results were not significantly different; $P < 0.05$ considered significant.

Table 5.2. Sperm retrieval rates (SRR) in 176 non-obstructive azoospermic men according to the surgical method of collection stratified by histopathology results and cause of azoospermia

	Presence of testicular spermatozoa: *n* (%)
By method: *n* = 176 (100.0%)	
TESA (*n* = 90); overall SRR, *n* (%)	58/90 (64.4)
Hypospermatogenesis	26/26 (100.0)
Maturation arrest	2/6 (33.3)
Sertoli-cell only	6/29 (20.7)
Not available[a]	24/29 (82.3)
Micro-TESE (*n* = 86); overall SRR, *n* (%)	45/86 (52.3)
Hypospermatogenesis	19/19 (100.0)
Maturation arrest	7/12 (58.3)
Sertoli-cell only	13/39 (33.3)
Not available	6/16 (37.5)
By cause of NOA: *n* = 176 (100.0%)	
Varicocele (*n* = 66)	45/66 (68.2%)
Genetic (*n* = 12)[b]	6/12 (50.0%)
Cryptorchidism (*n* = 19)	12/19 (63.1%)
Idiopathic (*n* = 63)	33/63 (52.4%)
Radiotherapy/chemotherapy (*n* = 6)	3/6 (50.0%)
Orchitis/gonadotoxin/endocrine (*n* = 10)	10/10 (100.0%)
Overall SRR: *n* (%)	109/176 (61.9)

Source: Androfert.
[a] Cases with previous successful TESA attempt.
[b] Includes cases of Klinefelter syndrome and AZFc Y-chromosome microdeletions.

range of 90–100% using percutaneous techniques. Using PESA, our approach is to perform the first aspiration at the corpus epididymis, and proceed to the caput if necessary, since aspirates from the cauda are usually rich in poor-quality senescent spermatozoa, debris, and macrophages. Most cases of PESA failures are not necessarily technical failures because immotile spermatozoa are found. However, in certain cases of epididymal fibrosis due to multiple PESA attempts or post-infection, PESA may be ineffective to retrieve sperm. In these cases, PESA can be attempted at the contralateral epididymis or TESA can be applied successfully if there is spermatogenesis in the testes. Routinely, procedures are performed under local anesthesia, with or without intravenous sedation. Percutaneous sperm retrieval techniques can be performed both for diagnostic and for therapeutic purposes. In the latter, sperm retrieval is often carried out on the same day as oocyte retrieval or the day before. Patients are discharged one hour later and can return to normal activities the same day. Oral analgesics are prescribed but pain complaints are minimal. The most common complication is fibrosis at the aspiration site. Other potential complications include hematoma, bleeding, and infection, but they are rare [6]. Some authors claim that MESA allows the collection of larger and cleaner quantities of sperm than PESA [2], but this debate seems trivial.

Table 5.3. ICSI outcomes according to the cause of obstructive azoospermia (AO)

	CAVD	Vasectomy/failed reversal	Infection/other
Number of cycles: *n* = 145	32	59	54
Female age in years: mean ± SD	31.4±5.0	32.6±6.2	32.9±5.9
2PN fertilization rate: mean (%)	64.1%	65.3%	59.3%
Cleavage rate: mean (%)	98.9%	98.8%	99.1%
Top-quality embryo for transfer: mean[a] (%)	44.9%	57.9%	49.4%
Number of embryos transferred: mean	2.9	2.6	3.0
Clinical pregnancy rate per transfer: *n* (%)	16/29 (55.2%)	26/59 (44.0%)	23/53 (43.4%)
Miscarriage rate: *n* (%)	5/16 (31.2%)	7/26 (26.7%)	3/23 (13.1%)
Live birth rate per transfer: *n* (%)	11/29 (37.8%)	19/59 (32.2%)	20/53 (37.7%)

Source: Androfert.
CAVD, congenital absence of vas deferens.
[a] 7–9 blastomeres of similar size, and grades I or II cytoplasmic fragmentation on the day of embryo transfer (day 3).
One-way ANOVA and Chi-square test were used to compare laboratory and clinical parameters among groups. Results were not significantly different; $P < 0.01$ considered significant.

Table 5.4. ICSI outcomes using spermatozoa retrieved from men with obstructive (AO) and non-obstructive azoospermia (NOA)

	Obstructive azoospermia		Non-obstructive azoospermia
Source of sperm for ICSI	**Epididymal**	**Testicular**	**Testicular**
Number of cycles: *n* = 107	93	14	95
Female age in years: mean ± SD	32.6 ± 5.3	32.1 ± 5.4	33.1 ± 5.7
2PN fertilization rate: %	66.0[b]	56.6[b]	51.1[c]
Cleavage rate: %	99.4	95.7	96.7
Top-quality embryo rate for transfer:[a] %	51.9	48.9	46.1
Cycles with embryo transfer: *n* (%)	88 (94.6)	14 (100.0)	88 (92.6%)
Number of embryos transferred: mean	2.8	3.3	2.7
Clinical pregnancy rate per transfer: *n* (%)	45/88 (51.1)[d]	7/14 (50.0)[d]	28/88 (31.8)[e]
Miscarriage rate: *n* (%)	11/45 (24.4)	1/7 (14.3)	6/28 (21.4)
Live birth rate per transfer: *n* (%)	34/88 (38.6)[f]	6/14 (42.8)[f]	22/88 (25.0)[g]

Source: Androfert.
Values expressed as means for fertilization, cleavage, and embryo quality rates.
[a] 7–9 blastomeres of similar size, and grades I or II cytoplasmic fragmentation on the day of embryo transfer (day 3).
One-way ANOVA and Chi-square test were used to compare laboratory and clinical ICSI parameters between OA and NOA groups, and between epididymal and testicular sperm in OA group. Statistically significant results were obtained only for fertilization ([b×c]$P < 0.001$), clinical pregnancy ([d×e]$P = 0.008$), and live birth rates ([f×g]$P = 0.03$) between NOA and OA groups; $P < 0.05$ considered significant.

In our series of 142 men with OA, the cumulative successful retrieval rate after PESA and/or TESA was higher than 95%, and an adequate number of motile sperm for cryopreservation was obtained in approximately one-third of the cases (35/112). Clinical outcomes of ICSI using PESA or TESA-derived spermatozoa were not different, indicating that sperm fertility potential is independent of the source in OA. Moreover, we have demonstrated that ICSI outcomes using fresh epididymal and testicular

spermatozoa retrieved from men with OA are comparable to those obtained with ejaculated sperm [13]. Although the cryopreservation rate after PESA is not high, repeated aspirations can be carried out in men with OA with minimal morbidity and lower cost compared to MESA. In rare circumstances, we perform MESA for sperm retrieval in OA men with coagulation disorders.

Non-obstructive azoospermia

The best sperm retrieval technique in NOA is yet to be established. To date, no randomized controlled trial has compared the efficiency of these strategies and thus current recommendations are based on cumulative evidence provided by descriptive, observational, and controlled studies. The efficiency of TESA for retrieving spermatozoa in NOA varies from 10% to 30% [14], except in the favorable cases of men with previous successful TESA or testicular histopathology showing hypospermatogenesis. In such individuals, SRR rates by TESA are in the range of 70–100% [12, 15, 16]. In a recent systematic review the mean reported SRR for TESE was 49.5%. TESE with multiple biopsies resulted in higher SRR than fine-needle aspiration, a variation of TESA, especially in cases of Sertoli-cell-only (SCO) syndrome and maturation arrest [17]. In NOA, current evidence suggests that micro-TESE performs better than conventional TESE or TESA in cases of SCO, where tubules containing an active focus of spermatogenesis can be identified. Micro-TESE also appears to be the safest technique regarding postoperative complications. Proper identification of testicular vessels under the tunica albuginea is made prior to the placement of an incision into the testis. The use of optical magnification and microsurgery techniques allows the preservation of intratesticular blood supply, as well as the identification of tubules more likely to harbor sperm production [10–12, 18]. Therefore, the efficacy of sperm retrieval is improved while the risks of large tissue removal are minimized. Excision of large biopsy samples in conventional TESE has been shown to impair testosterone production [18]. Tissue removal in micro-TESE is often 50–70-fold less than conventional TESE [8–10], and the small amount of tissue extracted facilitates sperm processing.

The clinical outcomes of ICSI using testicular sperm extracted by TESA or micro-TESE in NOA are significantly lower than those obtained with either ejaculated or epididymal/testicular sperm from men with OA [13]. From the limited data available, it is suggested that the sperm retrieval technique itself has no impact on ICSI success rates [17]. Our data indicate that testicular spermatozoa of men with severely impaired spermatogenesis have decreased fertility potential, and may have a higher tendency to carry deficiencies such as the ones related to the centrioles and genetic material, which ultimately affect the capability of the male gamete to activate the egg and trigger the formation and development of a normal zygote and a viable embryo [13].

Predictive factors for retrieving sperm in non-obstructive azoospermic men

Testicular spermatozoa can be obtained in most etiology-specific causes of NOA, such as varicocele, cryptorchidism, orchitis, and genetic, endocrine, and gonadotoxic-induced cases [5, 10, 19–24]. In genetic-related NOA, such as Y-chromosome infertility and Klinefelter syndrome (KS), pregnancies may be achieved by ICSI in males with retrievable testicular sperm [22–24]. The presence or absence of retrievable sperm in azoospermic men with Y-chromosome infertility varies depending on the specific microdeletion. In partial and complete AZFc deletion azoospermic patients, testicular sperm can be found in approximately 70% of the cases. In contrast, the chance of finding sperm in azoospermic men with complete AZFa or AZFb deletions is unlikely [22]. If a successful pregnancy is obtained, male offspring will harbor the same deletion as their father, with a high risk of male infertility. In NOA men with KS, sperm are found in approximately 50% of the cases on testicular exploration. Pregnancy rates by ICSI range from 30% to 50% and children who have been born have a normal karyotype [23]. It has been recently demonstrated that germ cells in men with KS are euploid, 46,XY, and thus can form normal, haploid gametes [24].

Although not absolute, testicular histology is still considered the best predictor for successful sperm retrieval in NOA men. The probability of obtaining sperm varies according to the testicular histopathology results [12, 16], as also shown by our own data (Table 5.2). However, even the combination of histology and FSH levels provides only a "fair" prediction model for sperm retrieval (accuracy of 0.74) [25], and

testicular sperm can be collected even in the more adverse histopathology pattern. FSH levels have also been used as a marker of testicular reserve, but it has been recently demonstrated that normal FSH levels in NOA men are not predictive of SRR [11, 26]. Serum FSH reflects the global spermatogenic function, but in cases of diffuse maturation arrest, adequate control feedback from germ cells and Sertoli cells exists despite the absence of sperm production [26]. Sperm can be retrieved from testicles of men with elevated serum FSH, and SRR rates appear to be correlated with the technique of sperm retrieval rather than with FSH levels. Significantly higher retrieval rates (~60%) were reported using micro-TESE compared to random multiple testicular biopsies in NOA men with elevated FSH levels [11, 27].

The importance of surgical and medical treatment prior to sperm retrieval in NOA men has been recently highlighted. It has been suggested that treatment of clinical varicoceles prior to sperm retrieval significantly increased the chance of testicular sperm collection by micro-TESE in a group of NOA individuals with clinical varicoceles [19]. In this retrospective study, SRR rates were 53% and 30% in the treated and untreated men, respectively (OR: 2.63; 95% CI 1.05–6.60, $P = 0.03$). Medical therapy (aromatase inhibitors, clomiphene, or human chorionic gonadotropin) prior to micro-TESE was also shown to enhance sperm retrieval success rates in Klinefelter syndrome men who responded to medication by increasing serum testosterone to more than 100 ng/dL from baseline [28].

Tips and pitfalls

Our approach is to perform TESA only in the favorable prognosis cases mentioned before. If TESA fails, however, we neither perform a second aspiration in the same testis, at the same operative time, nor convert it to an open procedure to avoid the risk of hematoma and testicular injury. Extensive bleeding is often seen during a rescue TESE after a failed TESA. Therefore, enlarged seminiferous tubules are difficult to identify even using the operating microscope. On these occasions, we opt to perform TESA or TESE at the contralateral testis. For NOA patients without previous diagnostic testicular biopsy or TESA attempt, our choice is to perform sperm extraction using micro-TESE. Selection of spermatozoa from a smaller population of contaminating testicular cells allows more ease and greater speed for sperm pick-up and injection process, as well as alleviating contamination and blockage of the injection needle with cells and debris. It is far less technically demanding and labor-intensive to extract spermatozoa from small-volume specimens than large pieces of testicular tissue that must be dissected, red-blood cells lysed, and the rare spermatozoa searched for in a tedious fashion under an inverted microscope. TESE sperm processing may be incredibly labor-intensive and the searching process may miss the rare spermatozoa within a sea of seminiferous tubules and other cells. TESE/micro-TESE may be scheduled either for the day of oocyte collection and ICSI or the day before. In a previous study, we observed that optimal fertilization by ICSI using surgically retrieved sperm is obtained when the time frame from hCG administration to microinjection does not exceed 44 h [29]. Testicular tissue sperm processing, searching, and selection of viable spermatozoa for ICSI may take several hours in NOA cases. Our laboratory takes approximately 11.6 minutes to handle a single testicular spermatozoon from processing to microinjection in NOA, but only 5.5 minutes in OA. In other words, the average time required to perform ICSI in a standard NOA treatment cycle involving 8–12 metaphase-II oocytes is approximately 2 hours. For these reasons, we elect to perform micro-TESE the day before oocyte collection when a busy next-day IVF laboratory workload is anticipated.

The concept of cryopreservation may be used in association with sperm retrieval procedures. Epididymal and testicular spermatozoa can be cryopreserved using protocols routinely used for ejaculated sperm [30, 31]. Some centers prefer to retrieve and intentionally cryopreserve sperm for future use. This strategy offers the advantage of avoiding ovarian stimulation when no sperm is obtained from testicular specimens. If sperm is found and frozen, thawing can be done at any time, thus obviating the need to organize two operations (oocyte and sperm retrieval) on the same day. Also, cryopreservation may be an interesting tool to preserve left-over specimens that would be discharged after ICSI, especially if the treatment cycle does not result in a pregnancy. Future ICSI attempts may be carried out without repeated surgical retrievals. We routinely freeze excess motile epididymal spermatozoa which are not needed for the current ICSI cycle. Most often, motile sperm will be available after thawing in such cases, and ICSI outcomes using motile

fresh or frozen epididymal sperm seem not to differ [16, 30, 31]. If only immotile spermatozoa are obtained, a method for selecting viable sperm for ICSI may be used, since it has been observed that conventional seminal parameters have little or no influence in ICSI outcomes, except when only immotile spermatozoa are available [32]. Methods for selecting immotile viable sperm for ICSI, such as hyposmotic swelling [33, 34], sperm tail flexibility [35, 36], or motility stimulant sperm challenge tests [37–40] are available, but results are limited for cryopreserved specimens. Cryopreservation of testicular sperm is also advisable, especially for men with NOA, who often require multiple ICSI attempts to conceive but may not have an adequate number of sperm available for repeated retrieval attempts. However, post-thaw testicular sperm are often immotile or exhibit only a twitching motility, and ICSI results using immotile testicular sperm tend to be poorer than with fresh ones [31]. Different strategies can be developed according to the results of each group. If freezing of surgically retrieved specimens provides results similar to those with the use of fresh sperm, then the use of frozen specimens would be preferable. If not, fresh specimens are preferable. Currently, our cryopreservation technique for surgically retrieved sperm is the standard liquid nitrogen vapor method using TEST-yolk buffer and glycerol as cryoprotectants [41, 42]. Epididymal specimens are concentrated by washing before freezing, and testicular sperm are freed from the testicular parenchyma, i.e. testicular homogenates are frozen. Recently, it has been demonstrated that human spermatozoa can be successfully vitrified, and this strategy may be of interest for preserving small quantities of surgically retrieved gametes [43].

Conclusions and key points

In OA, sperm production is normal and gametes can be easily retrieved from epididymis or testicle in most cases, irrespective of the technique. PESA or TESA are simple and efficient methods for retrieving epididymal or testicular spermatozoa in men with OA. For NOA, TESE with or without magnification is the preferred approach, and sperm can be retrieved in approximately 60% of the cases. The use of microsurgery during TESE may improve the efficacy of sperm extraction with significantly less tissue removed, which ultimately facilitates sperm processing. Testicular histology results, if available, may be useful to predict the chances of retrieving sperm in men with NOA. However, sperm can be obtained even in the worst-case scenario except in cases of Y chromosome infertility with complete AZFa and/or AZFb microdeletions. In both OA and NOA, the sperm retrieval technique itself seems to have no impact on ICSI success rates. The main goal of PESA/TESA/TESE sperm processing is the recovery of a clean sample containing motile sperm. Such specimens are more fragile, and often compromised in motility, as compared to those obtained from ejaculates. Laboratory techniques should be carried out with great caution not to jeopardize the sperm fertilizing potential. Surgically retrieved spermatozoa can be intentionally cryopreserved for future use. Spare left-over specimens that would be discharged after ICSI can also be cryostored. Different strategies can be developed according to each group's results. If freezing of surgically retrieved specimens provides results similar to those with the use of fresh sperm, then the use of frozen specimens would be preferable. If not, fresh specimens are preferable. The reproductive potential of infertile men undergoing ART is related to the type of azoospermia. The chances of retrieving spermatozoa and of achieving a live birth by ICSI are increased in couples whose male partner has obstructive rather than non-obstructive azoospermia.

Glossary

Azoospermia.	Absence of spermatozoa in the microscopic examination of the seminal fluid after centrifugation on at least two separate occasions.
Cryopreservation.	The freezing process for storage of gametes or gonadal tissue at ultra-low temperature.
ICSI.	Intracytoplasmic sperm injection: a procedure in which a single spermatozoon is injected into the oocyte cytoplasm.
MESA.	Microsurgical epididymal sperm aspiration: a microsurgical procedure used to aspirate spermatozoa directly from the epididymal tubules for use in an ICSI procedure.

Micro-TESE.	Microdissection testicular sperm extraction: a microsurgical procedure used to dissect the seminiferous tubules within the testis in an attempt to identify areas of sperm production and extract spermatozoa for use in an ICSI procedure.
PESA.	Percutaneous epididymal sperm aspiration: a procedure in which a needle is inserted into the epididymis to retrieve spermatozoa for use in an ICSI. procedure.
Sperm processing.	Laboratory techniques used to remove contaminants (cellular debris, microorganisms, red blood cells, etc.) and to select the best-quality spermatozoa to be used in conjunction with assisted reproduction technology.
TESA.	Testicular sperm aspiration: a procedure in which a needle is inserted into the testicle in order to retrieve spermatozoa for use in an ICSI procedure.
TESE.	Testicular sperm extraction: operative removal of testicular tissue in an attempt to collect sperm for use in an ICSI procedure.

Acknowledgments

The authors would like to acknowledge Sidney Verza Jr., Christina Prudencio and Bill Seol for their assistance in data collection, and Mrs. Fabiola Bento for editorial and text revision.

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Chapter 6

New concepts in ovarian stimulation

Ying Cheong, Nick Brook, and Nick Macklon

Introduction

Ovarian stimulation is central to many fertility treatments. The goal of ovarian stimulation is to induce ongoing development of multiple dominant follicles and to mature many oocytes to improve chances for conception with in vivo (empirical ovarian stimulation with or without intrauterine insemination) or in vitro. This approach of interfering with physiological mechanisms underlying single dominant follicle selection is usually applied in normo-ovulatory women. This must be differentiated from ovulation induction, which aims to induce monofollicular development and ovulation in anovulatory women. Ovarian stimulation enables the retrieval of many cumulus-oocyte complexes, and this allows for inefficiencies in subsequent oocyte maturation, fertilization in vitro, embryo culture, embryo selection for transfer, and implantation. Multiple embryos, if required, can be transferred in the great majority of patients, and spare embryos may be cryopreserved to allow for subsequent chances of pregnancy without the need for repeated ovarian stimulation and oocyte retrieval. This paradigm has formed the basis of clinical practice since the early days of IVF. However, increased understanding of the intricacies of the follicular development and selection processes has been critical to the development of many of the new developments in ovarian stimulation in clinical practice. In this chapter these clinically related physiological aspects of ovarian stimulation are reviewed, and the resulting new concepts of ovarian stimulation are discussed.

The endocrinology of follicular development and ovulation

Follicular growth

Follicular growth, which begins when primordial follicles emerge from their resting state, occurs continuously from the fifth and sixth month of intrauterine life until the menopause [1] and it occurs over several stages. Chronologically, the stages are (1) initial recruitment of resting primordial follicles, (2) the development of pre- and early antral follicles, (3) cyclic recruitment of preantral follicles and (4) selection of a single dominant follicle. The process of folliculogenesis starts when follicles leave the pool of resting follicles into the growth phase. The number of resting follicles in the pool is predetermined during fetal life, and usually reaches about 6–7 million by 20 weeks of gestation.

The initial recruitment (primary recruitment) of primordial follicles in the ovaries occurs as a random and continuous process every month. Around 1000 primordial follicles grow every month in the adult ovary but the great majority will undergo atresia. The exact mechanism underlying the initiation of growth of the primordial follicles is not well understood and would appear to be under para- and autocrine controls. The early phases of follicular development are considered to be largely FSH independent. Evidence suggesting that FSH is not essential for the initiation of follicular growth includes the fact that the process occurs in hypophysectomized animals, although pituitary-derived factors (not entirely FSH) are probably important in the development of oocytes given that hypophysectomized fetal Rhesus monkeys were observed to be oocyte depleted [2, 3].

After the initial recruitment, follicles enter a growth phase where the granulosa cells expand and the oocyte increases in size. It is estimated that it takes several months for the initially recruited follicles to develop into the preantral stage. It is during this stage of development that FSH receptors are identified on the granulosa cells. At this stage, follicular growth can be stimulated by the presence of FSH, although its absence has no detrimental effect on the follicle. In contrast to the earlier phases of follicular development,

Human Assisted Reproductive Technology: Future Trends in Laboratory and Clinical Practice, ed. David K. Gardner, Botros R. M. B. Rizk, and Tommaso Falcone. Published by Cambridge University Press. © Cambridge University Press 2011.

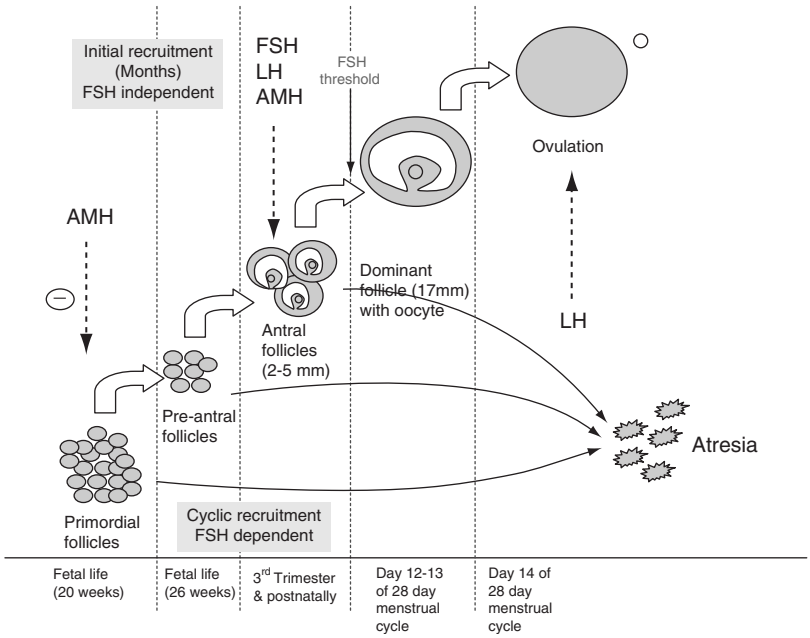


Figure 6.1. Pictorial illustration of the process of folliculogenesis.

FSH is an absolute requirement for the development from the antral follicle stage (2–5 mm in diameter). Due to the demise of the corpus luteum during the late phase of the menstrual cycle, E_2, inhibin A and progesterone (P) levels fall. This results in an increased frequency of pulsatile GnRH secretion, causing a rise in the FSH levels at the end of the luteal phase. Only those antral follicles that are at a more advanced stage of development (2–5 mm in diameter) during this inter-cycle rise of FSH (the so-called threshold for ovarian stimulation) gain gonadotropin dependence and continue to grow while the others undergo atresia. The normal number of follicles available for cyclical recruitment is usually around 10 per ovary [1, 4].

After the initial rise, the level of FSH reaches a plateau and finally decreases during the mid- to late follicular phase, suppressed by ovarian inhibin B and E_2 negative feedback. An inhibin B level rise appears to limit the duration of an FSH rise. Decreasing FSH levels close the so-called FSH *window*, a period of time that is crucial for the selection of a single dominant follicle from the cyclical recruited cohort. The dominant follicle will continue to grow as its sensitivity to FSH increases. Whilst the transient increase in FSH creates a short 'window' for the selection of a single dominant follicle, in ovarian stimulation cycles a prolonged FSH administration at this phase of the cycle will increase the length of time for this 'window' to allow for the selection of multiple follicles. Hence, the administration of small doses of FSH over a prolonged period of time is sufficient to override this single dominant follicle selection process as opposed to the administration of large doses of FSH during the entire follicular phase [5] (Figure 6.1).

Mature follicles also express LH receptors. Elegant studies have revealed that the mature follicle alters in sensitivity from predominantly FSH to include LH [6, 7]. Indeed the leading follicle can continue to develop under LH stimulation alone, whereas smaller follicles undergo atresia due to the insufficient support from decreasing FSH concentrations and relative insensitivity to LH. This provides a potential therapeutic opportunity to manipulate the later stages of follicle development by switching from FSH stimulation to predominantly LH activity. In recent years this approach has been investigated in clinical trials, and these data are reviewed later in the chapter.

Ovulation and corpus luteum function

The corpus luteum is regulated predominately by LH. The mid-cycle surge of LH stimulates the resumption of meiosis and oocyte maturation, and initiates the process of formation of the corpus luteum from the follicle (luteinization). The pulsatile nature of LH secreted by the anterior pituitary gland during the

luteal phase of the menstrual cycle promotes the normal function and lifespan of the corpus luteum. In the event of implantation, human chorionic gonadotropin (hCG) secreted by the blastocyst extends the lifespan of the corpus luteum into early pregnancy until the placenta shifts over to the production of hCG. The corpus luteum is highly dependent on LH from the early luteal to the mid luteal phase and suppression of LH support for 72 hours during this critical time period induces the irreversible loss of luteal structure [8], and LH or hCG (not FSH) replacement sustains this function [9]. The active prevention of the pulsatile decline of LH during the luteal phase does not appear to prevent the regression of the corpus luteum [10]; thus it would appear that it is the rising levels of LH/hCG that are the most potent stimulus for the extension of the lifespan of the corpus luteum.

The local hormonal milieu may modify the corpus luteal function through progesterone receptors in the granulosa cells in the follicle, estrogen receptor-beta and/or androgen receptors [11–13]. More relevant to the ovarian stimulation process is the role of LH/hCG in the regulation of angiogenic factors such as vascular endothelial growth factor (VEGF) which is essential in the regulation of normal angiogenic processes in the corpus luteum; it is proposed that the over-expression, increased bioavailability, or change in the ratio of angiogenic factors, notably VEGF-A, is a cause of ovarian hyperstimulation syndrome (OHSS), a serious side effect of ovarian stimulation characterized by intravascular volume loss and extravascular fluid accumulation [14].

Endometrial receptivity

During the follicular phase, estrogen secreted by the developing follicle stimulates the production of two estrogen receptors ER-α and ER-β on the endometrium. ER-α exists predominantly in both glands and stroma and is significantly down-regulated in the luteal phase whilst ER-β only exists in the endothelium [15]. In terms of progesterone receptors (PR), this is most prominent in glandular epithelium in the proliferative phase and is undetectable in the mid-luteal phase. In contrast, stromal cells have high levels of PR in the follicular and luteal phases. The human progesterone receptor exists in two isoforms, PR-A and PR-B, with their peak expression on the endometrium being during ovulation. In the endometrial glands, PR-A and PR-B are both expressed in the follicular phase but only PR-B persists through the mid- and late luteal phases of the cycle. In the endometrial stroma, PR-A predominates throughout the cycle, suggesting its importance in the luteal phase. The down-regulation of epithelial PR coincides with the opening of the window of implantation and uterine receptivity for embryo implantation and histologically delayed endometrium is associated with failure of such PR down-regulation [16, 17].

Ovarian aging

The total number of oocytes declines continuously after birth such that by the time of menarche, only a quarter of a million follicles remain. During reproductive life, follicle depletion occurs at the rate of about 100 per month by either atresia or entry into the growth phase; this phase increases from 35 years of age to menopause where the stock of resting follicles falls to less than 1000 per ovary. From puberty until the menopause, only about 400 follicles are destined to achieve full maturation. A large degree of variability exists in the rate of follicle pool depletion between the ages from 40 to 60 years old. Hence, the chronological age of a woman is only loosely associated with the actual ovarian act.

Identification of markers that are specific and sensitive to ovarian aging may enable the more accurate prediction of response to ovarian stimulation. FSH has been the most widely used ovarian reserve marker until recently. However, there is large inter-individual variation in the follicular phase FSH concentrations in the normo-ovulatory cycle. Furthermore, quantity of oocyte (follicle numbers) is not equivalent to quality (competence of oocyte). Young patients with a high FSH, indicative of poor ovarian reserve, demonstrate lower numbers of growing oocytes but can achieve good ongoing pregnancy rates [18]. Some authors have suggested that day 3 E_2 will add predictive value to FSH alone; and elevated E_2 on day 3 tends to indicate poor response to stimulation for ART even in the presence of a normal FSH concentration [19]. Others have examined the role of specific clinical parameters such as that of antral follicle count and ovarian volume in predicting ovarian reserve and response to stimulation [20–22]. However, the assessment of these parameters can be subjective and alone lacks sensitivity and specificity as a diagnostic test.

Several authors have attempted to use E_2, inhibin A, B, and/or FSH in combination with various clinical

parameters to estimate ovarian reserve and ovarian response to stimulation. A decrease in inhibin B precedes the fall in inhibin A and the rise in perimenopausal FSH. Inhibin B is produced by developing antral follicles although inhibin B alone appears not to be as predictive of ovarian reserve as a combination of FSH, inhibin B, and ultrasound characteristics combined in a logistic model [23]. Another approach regarding the assessment of ovarian reserve uses high-resolution transvaginal ultrasound scanning to count small "antral" follicles of 2–10 mm diameter. Antral follicle count (AFC) correlates with oocyte yield in IVF and with the various biochemical markers. Drawbacks include the test being operator-dependent and requiring attendance at the ultrasound facility, and recent studies have not shown superiority over AMH measurement [24, 25]. Combinations of these various markers have been evaluated and may offer better prediction of oocyte yield after stimulation.

Recently, anti-Mullerian hormone (AMH) has been studied as a marker for ovarian aging. AMH is a member of the TGF-beta superfamily and is produced by the granulose cells from about 36 weeks gestation to the menopause [26]. AMH is expressed most in the preantral and small antral follicles. AMH-deficient mice have ovaries that deplete of primordial follicles significantly quicker than AMH non-deficient mice. This is mainly due to the absence of AMH, which normally has an inhibitory role in the cyclic recruitment of follicles by decreasing the sensitivity of the follicles to FSH [27]. AMH also correlates well with ovarian parameters such as ovarian volume and antral follicle count and age as well as FSH and inhibin B [28]. Furthermore, its concentrations in the serum are non-menstrual cycle dependent. Further information about how AMH is involved in the physiology of folliculogenesis is required before scientists can design possible mechanisms to manipulate the development of the ovarian follicles.

Several studies have shown that AMH is increased in women with polycystic ovaries (PCOS) compared with controls [29–31]. AMH measurements have been found to offer a relatively high specificity and sensitivity (92 and 67% respectively) as a diagnostic marker for PCOS [32]. As is discussed later in this chapter, recent studies have indicated that AMH may be a more reliable and robust predictor of ovarian response during ovarian stimulation [33](see Towards individualized protocol).

Preparations used for ovarian stimulation

Gonadotropins

Human urinary preparations of LH and FSH (human menopausal gonadotropin, hMG) were used for ovarian stimulation in the early 1960s. The initial preparations were very impure with many contaminated proteins, with less than 5% of the proteins being bioactive. However, since the early 1980s, improved purification techniques have enabled the production of purified urinary FSH (uFSH) by the use of monoclonal antibodies. Demand for gonadotropin rose significantly worldwide during this time, and the supply of postmenopausal urine could no longer be guaranteed. The advent of recombinant DNA technology has enabled large-scale production of human recombinant FSH (recFSH). The technology allows for the transfection of human genes encoding for the common α subunit and hormone specific β subunit of glycoprotein hormone into Chinese hamster ovary cell lines, enabling large-scale production of recFSH independent of the supply of human postmenopausal urine, and addressing concerns about batch-to-batch consistencies. Because of its purity, recFSH can now be administered by protein weight rather than bioactivity, and so-called 'filled-by-mass' preparations are now in clinical use. Hence, the use of gonadotropin has developed over a number of decades from preparations with hMG (containing both LH and FSH bioactivity), followed by purified uFSH and more recently recFSH, rec LH, and recH hCG. A new long-acting FSH, corifollitrophin alfa, has recently been introduced into clinical practice (see section Urinary versus recombinant gonadotropins).

GnRH analogs

Early studies demonstrated that pituitary down-regulation could be induced by the continued administration of GnRH [34]. This induces an initial stimulation of gonadotropin release (the so-called flare effect) followed by a down-regulation due to the clustering and internalization of the pituitary receptors. This resolved a number of issues associated with poor results related to hMG treatment alone, where a premature LH peak occurred in 20–25% of cycles due to the positive feedback activity by high serum E_2 levels during the mid-follicular phase of the stimulation cycle. Induced pituitary down-regulation resulted in

a significant reduction in the cancelation rate and improved the overall IVF outcome in the 1980s. Furthermore, the approach with GnRHa co-treatment facilitated scheduling of IVF and timing for oocyte retrieval.

Although GnRH antagonists were developed soon after, the low potency of the first two generations of drugs, and associated anaphylactic responses due to histamine release, delayed their clinical introduction until the third generation were shown to be safe and efficacious in IVF. Whilst the widely employed GnRH agonist long protocol requires a prolonged period of down-regulation (usually 2 weeks) followed by high-dose FSH stimulation to induce multiple follicular growth, the immediate action of GnRH antagonists mean that they can be administered during the mid-to-late follicular phase to prevent premature luteinization. This avoids unpleasant 'menopausal' side effects associated with pituitary down-regulation, and allows the endogenous inter-cycle FSH rise to be utilized for follicle stimulation. The cyclic recruitment and the initial stages of dominant follicle selection can proceed within the natural cycle and the use of exogenous FSH for inducing multiple follicle growth can be restricted to the mid-to-late follicular phase, as in certain mild stimulation protocols [35]. Hence the overall length of stimulation is shorter than with conventional IVF. Other advantages of the GnRH antagonists over agonist include the absence of the 'flare effect,' which may cause ovarian cyst formation, and, in turn, lower oocyte quality, fertilization rate, number of oocytes retrieved, and embryo quality [36].

From physiology to therapy: current concepts in ovarian stimulation

Current concepts: long protocol

The long ovarian stimulation protocol combining GnRH agonist with exogenous gonadotropin administration has been the most popular treatment regime for the past 20 years. While a variety of different protocols based on GnRHa treatment have been described, few studies have been performed to determine optimal or GnRHa preparation. In the long protocol, GnRH agonist is usually administered during the luteal phase in the preceding cycle and is continued until hCG administration. In the short protocol GnRH agonist therapy is started on day 2 of the stimulation cycle, with the aim of utilizing the 'flare' effect of the GnRHa as an additional initial stimulus for follicular recruitment. However, a meta-analysis comparing the long versus the short protocol revealed that although the long protocol required more gonadotropins, it yielded more eggs and a higher pregnancy rate [37]. The long protocol, generally commenced during the luteal phase of the previous cycle, is advantageous in that initiation of gonadotropin stimulation can be delayed, allowing scheduling of IVF cycles with no clear adverse effect on outcomes. However, starting treatment at this phase of the cycle cannot completely exclude a concurrent early pregnancy.

GnRH agonist versus antagonist

The first meta-analysis published, comparing outcomes following co-treatment with antagonist versus agonist based on five multicenter RCTs, concluded that the GnRH antagonist was as efficient as GnRHa for preventing a premature LH surge but the probability of clinical pregnancy rates was shown to be lower than in the GnRHa group, although there was no difference in the live birth rate. Although this 5% lower clinical pregnancy rate was probably of marginal clinical significance, the study generated a significant amount of concern and resulted in a lower acceptance of GnRH antagonists in ovarian stimulation in IVF [38]. Data from the German national IVF registry suggested that this regime was only used in patients with a poorer prognosis – those who were older and had undergone more unsuccessful IVF cycles [39]. However, systematic reviews published later have shown no differences in the live birth rate [40]. The non-significant difference in the live birth rate should not be unexpected, because no difference between the two analogs has been demonstrated in terms of embryo [41, 42] or endometrium quality [43]. However, there may be a learning curve associated with adoption of GnRH antagonist regimens, which would account for the relative poor performance of the antagonist protocol in the early years. More recent randomized studies comparing the agonist and antagonist protocol have shown no significant differences in pregnancy outcomes, although the application of GnRH antagonist in ovarian stimulation for IVF was also associated with a significantly lower probability of OHSS associated with hospital admission.

While more is to be learned regarding the optimal protocol for GnRH antagonists, current evidence supports the use of that shown in Figure 6.2 [44]. This

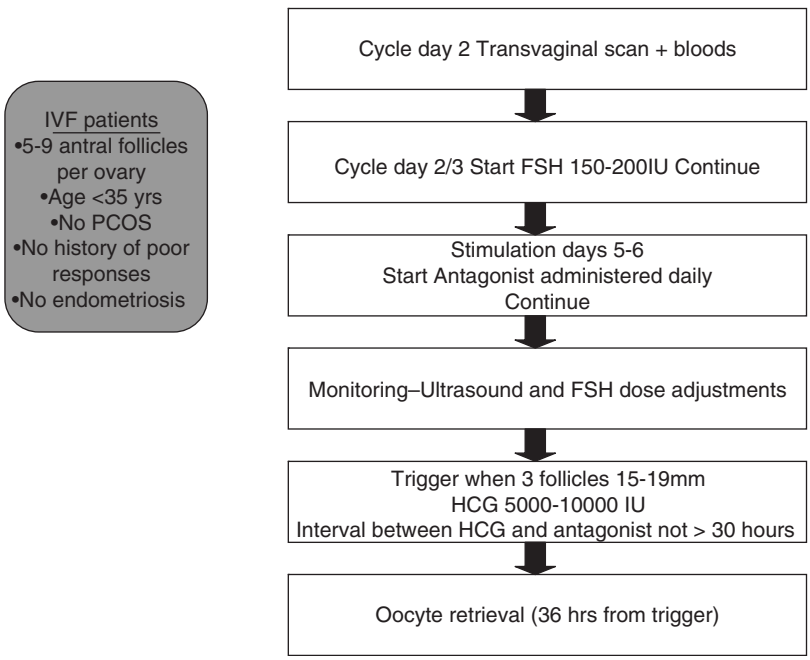


Figure 6.2. A suggested GnRH antagonist protocol for normal responders (Adapted from Devroey *et al.* [44].)

protocol is recommended for patients who are expected to be normal responders: patients with 5–9 antral follicles per ovary, age <35 years, no PCOS, normal menstrual cycle, no history of poor responses, and no pelvic pathology.

Urinary versus recombinant gonadotropins

Preparations of gonadotropins available for ovarian stimulation were initially hMG (containing both LH and FSH bioactivity), followed by purified urinary FSH and more recently recFSH and recLH. A comparative assessment of IVF outcome in patients treated with hMG versus recombinant FSH (rFSH) under a long GnRH agonist protocol has been performed in several randomized controlled trials (RCT), which were summarized in the most recent systematic reviews and meta-analyses [45, 46]. Ten randomized controlled trials were included (n = 2937), the live birth rate was found to be significantly higher with hMG (OR 1.20, 95% CI 1.01–1.42) versus r-FSH, but OHSS rates (OR 1.21, 95% CI 0.78–1.86) were not significantly different.

However, only one RCT has thus far evaluated IVF outcome stimulated with hMG versus rFSH in GnRH antagonist cycles [47]. In this study of 280 women, the number of oocytes retrieved was significantly reduced in patients treated with hMG (mean ± SD 11.3 ± 6.0) compared with those treated with rFSH (14.4 ± 8.1). However, live birth rates were not significantly different between patients randomized to receive hMG versus those randomized to receive rFSH (34.3% vs. 31.4%, respectively, 95% CI: –8.1 to + 13.7) [47].

The selection of a gonadotropin during ART also depends on several other important considerations. In terms of tolerability, recFSH preparations show some advantage over urinary products, allowing the patient to administer in a safer subcutaneous manner. A more important consideration is that of the theoretical risk of transmission of prion proteins, which have been identified in human urine. Although the risk is now considered very low, it has influenced policy regarding the use of urinary versus recombinant gonadotropins in certain countries.

Evolving concepts in ovarian stimulation

The objectives of ovarian stimulation in ART are evolving. More focus is now placed on quality of the patient's experience. Whilst the end point of traditional IVF was previously pregnancy, there is a shift in modern ART towards enhanced patient experience as the primary outcome. The following section will highlight some of the new emerging concepts in ART.

Milder treatment regimes

Traditional IVF stimulation regimes are associated with aggressive use of gonadotropins to stimulate the development of a large number of follicles. These traditional regimes are often complex, expensive, extend over a prolonged period of time, and require intensive monitoring. A retrospective analysis of 7422 women who underwent oocyte retrieval after long protocol IVF (GnRHa) showed that overall the highest pregnancy rates per embryo transfer and per started cycle were observed when 13 oocytes were obtained (31 and 28% respectively) [48]. The starting doses for treatment normally ranged between 100 and 300 IU/d and are often adjusted according to individual patient response. Several randomized controlled trials have failed to demonstrate improvements in outcome when higher doses of FSH are used, even in poor-response patients [49, 50–53]. Hence, using large doses of gonadotropins to stimulate the development of more oocytes does not increase the pregnancy rate but may in fact increase patient discomfort, side effects, and serious complications such as OHSS. There is also increasing evidence that ovarian stimulation and excessive response may be detrimental to oocyte and embryo quality [54, 55]; furthermore, profound stimulation also has a detrimental effect on luteal-phase endocrinology and in turn impacts on endometrial receptivity [56, 57].

Increasing recognition of the detrimental effects of conventional profound stimulation regimens has led to a change in the paradigm for ovarian stimulation in IVF. Milder regimens are being adopted, due to the reduced patient burden, the need for fewer embryos as single embryo transfer becomes established into clinical practice, and possible benefits on embryo quality (Table 6.1). Key to the development of milder stimulation protocols has been the introduction of GnRH antagonists which allow for the initiation of the IVF treatment cycle in a normal menstrual cycle with an undisturbed recruitment of a cohort of follicles during the early follicular phase. This approach enables the endogenous inter-cycle FSH rise to be utilized rather than suppressed, resulting in a reduction of gonadotropins required. The treatment cycles are thus shorter and not associated with hypoestrogenic side effects related to GnRHa down-regulation and reduced cancelation rates [58] (see section on GnRH analogs).

However, one of the concerns for many IVF practitioners is that such a regime has a lower oocyte yield and thus poorer pregnancy rates. A recent meta-analysis combining three studies with a total of 592 first treatment cycles showed that the mild stimulation protocol resulted in a significant reduction of retrieved oocytes compared with conventional ovarian stimulation (median 6 versus 9, respectively, $P < 0.001$) [59]. Optimal embryo implantation rates were observed with five oocytes retrieved following mild stimulation (31%) versus 10 oocytes following conventional stimulation (29%) ($P = 0.045$). It would appear that the modest number of oocytes obtained after mild ovarian stimulation is not a reflection of poor ovarian response and the authors claimed that "the fear of reducing the number of oocytes retrieved following mild ovarian stimulation appears to be unjustified" [59].

Milder stimulation regimes have been shown to produce proportionally more chromosomally normal embryos and further analysis suggests that the increased chromosomal abnormalities observed after conventional IVF are mainly due to an increased incidence of mitotic segregation errors resulting in chromosomal mosaicism [60].

Until recently, IVF clinicians have striven to achieve a high pregnancy rate at all cost. However, over the years, the concept of success as defined by a high pregnancy rate has given way to a more holistic approach to the management of the IVF patient and their journey through their treatment. One key

Table 6.1. Considerations related to different approaches in ovarian stimulation

Current ovarian stimulation approaches
Aiming for maximum number of oocytes • Time-consuming • High costs • Much patient discomfort • Short-term complications and OHSS • Long-term health consequences uncertain • High drop-out rates • Supra-physiological steroid levels • Emphasize maximum pregnancy rate per cycle
Mild stimulation approaches • Less complex • Less time-consuming • Cheaper • Reduce complications, discomfort, drop-out
Effects on oocyte and embryo quality (better) • Emphasize maximum chances for healthy baby per treatment at reasonable cost, patient discomfort, complications

With permission from Verberg *et al*., *Hum Reprod Update* 2009 [59].

criterion of success in IVF treatment is the achievement of a low multiple-pregnancy rate, as mortality rates are increased up to seven-fold in twins and up to 20-fold in triplets. Prematurity and low birth weight also have serious long-term health consequences. Furthermore, the economic burden for society is significantly increased in higher-order multiple pregnancies [61, 62]. Widespread concern about the medical, social, and economic consequences of multiple higher-order pregnancies has prompted the development of strategies aimed at prevention of this iatrogenic complication, shifting the definition of success in IVF therapy from that of pregnancy rate per treatment cycle towards that of achieving a healthy singleton child per started course of treatment.

One of the strategies to reduce multiple-pregnancy rates is to transfer fewer embryos, but the fear remains among may practitioners that the success rate will decline. Data from the United Kingdom demonstrated that despite the reduction of embryos transferred from three to two, there was not only no reduction in birth, but fewer multiple pregnancies and associated morbidity [63]. Data from the European IVF-Monitoring Consortium have shown a continuing trend toward transferring fewer embryos. The number of cycles involving three or more embryo transfer has fallen from 49% in 1999 to 24% in 2005 [64, 65]. Despite these trends, similar to the UK data, there was no reduction in the pregnancy rate per retrieval; rather there has been a slight increase of clinical pregnancy per retrieval from 24% in 1999 to 27% in 2005. In a recent prospective randomized study, the use of a mild stimulation protocol combined with single embryo transfer resulted in the same number of live births over a period of 12 months as a conventional strategy combined with dual embryo transfer, while reducing costs, patient burden, and the the number of twin pregnancies [66].

A meta-analysis including seven studies comparing single embryo transfer (SET) with multiple embryo transfer found that there was no statistically significant difference in cumulative live birth rates (CLBR) after a single fresh cycle of double embryo transfer (DET) versus two fresh cycles of SET (OR 1.23, 95% CI 0.56 to 2.69, $P = 0.60$). The live birth rate (LBR) per woman in a single fresh treatment was higher following DET than SET (OR 2.10, 95% CI 1.65 to 2.66, $P < 0.00001$). However, the difference in CLBR after DET and those after SET followed by transfer of a single frozen thawed embryo (1FZET) was not statistically significant (OR 0.81, 95% CI 0.59 to 1.11; $P = 0.18$) [66–72]. The results of this review indicated that although the use of SET in a fresh IVF cycle results in lower live birth rate, subsequent transfer of a frozen embryo can provide outcomes that are not significantly different compared to one fresh cycle of DET. The results have been clearly demonstrated in younger women although data from older women are lacking.

Two main factors constitute the bulk of treatment burden in IVF, that is, the length of treatment and the side effects. The need for simplified treatment approaches, which will lessen the treatment burden of IVF, is self-evident. Patient experience is likely to influence treatment success. Emotional distress should be considered an important negative side effect associated with IVF treatment. This side effect is commonly reported by patients undergoing IVF treatment [73]. Patients undergoing GnRHa down-regulation have been found to have increased symptoms of depression compared to controls [74]. Levels of stress at baseline have been significantly associated with the pregnancy rate, the live birth rate, and birth weight [75]. Furthermore, the psychological burden of treatment is cited as the most common reason for discontinuing assisted conception treatment [76].

One of the most significant and high-risk side effects of ovarian stimulation is OHSS. Coasting and cryopreservation are the two methods currently used to prevent OHSS. However, recently, cabergoline used in low doses for 8 days from the day of hCG administration was shown to reduce the rate of OHSS compared with placebo [77]. In a meta-analysis of four studies, the incidence but not the severity of OHSS was found to be reduced by cabergoline treatment, without reducing pregnancy rates [78].

A further development which promises to further reduce the burden of ovarian stimulation is the introduction of a long-acting FSH preparation which greatly reduces the number of injections required during an IVF treatment cycle. Corifollitropin α is a recombinant fusion protein composed of FSH and the carboxy terminal peptide (CTP) of the hCG β-subunit which has a two-fold longer elimination half-life and an almost four-fold extended time interval to peak serum concentration than recFSH preparations. This allows a single injection of corifollitropin α to initiate and sustain multiple follicular growth for up to 7 days. Furthermore, after corifollitropin α injection, peak FSH activity is reached in 2 days compared to that of recFSH in 4–5 days. A recent multicenter

"double-blind double dummy" randomized controlled study comparing corifollitropin α and recFSHin a GnRH antagonist protocol reported no difference in the pregnancy rate of the corifollitropin α treatment group compared to the recFSH treatment group [79]. This preparation will become available for the treatment of women with an AFC of less than 20, who are co-treated with GnRH antagonist, as data from GnRH agonist co-treatment studies remain sparse.

Role of LH in stimulation regimes

Studies with recombinant gonadotropins with no LH activity in women with hypogonadal hypogonadism demonstrated that LH is not required for follicular development to the pre-ovulatory stage [80]. However, the debate regarding the benefits of LH for oocyte maturation and quality continues. LH activity can be provided in the form of: (i) human menopausal gonadotropins (hMG) containing urinary-derived LH activity, (ii) recombinant LH (rLH), (iii) human chorionic gonadotropin (hCG), and (iv) recombinant hCG (rhCG). A recent meta-analysis assessed the benefits of the addition of recLH to rec FSH during ovarian stimulation in IVF cycles [81]. No statistically significant differences in live birth rates were observed between patients who received recLH and those who did not. Based on these data, the addition of recLH during the follicular phase does not seem to increase the probability of pregnancy in patients treated with recFSH and GnRH analogs for the general population undergoing IVF. However, a Cochrane systematic review suggested that certain subgroups of patients with very low endogenous LH activity may benefit from the addition of LH [82].

It has been suggested that LH-induced androgen production prior to ovarian stimulation might lead to an increased follicular recruitment as intra-ovarian follicular androgens can promote the aromatase activity of antral follicles [83]. The potential role of LH activity in this context during early folliculogenesis was investigated in a recently published RCT [84]. In this randomized study 146 women were treated in a long course high-dose GnRH agonist (Decapeptyl, 4.2 mg s.c.) protocol and were randomized to receive r-hLH (Luveris, 300 IU/day) for a fixed 7 days, or no r-hLH treatment. This was followed by a standard r-hFSH stimulation regime (Gonal-F, 150 IU/day). The LH treatment was associated with increased small antral follicles prior to FSH stimulation ($P = 0.007$), and an increased yield of normally fertilized (2 PN) embryos ($P = 0.03$) but no difference in the ongoing pregnancy rate was observed. Although more studies are required, at present rLH pretreatment of patients undergoing ovarian stimulation with the use of GnRH agonists and rFSH does not seem to increase the probability of ongoing pregnancy.

In recent years, several groups have focused on the potential significance of late follicular-phase LH levels for clinical IVF outcome. Based on classical principles, both LH and FSH are required for adequate ovarian estrogen biosynthesis and follicle development. Theca-cell derived androgen production (under LH control) is mandatory as a substrate for the conversion to estrogens by FSH-induced aromatase activity in the granulose cells. It has been shown that during the mid-to-late follicular phase, FSH induces LH/hCG receptor expression in granulosa cells of large follicles [85]. A number of studies have indicated that excessively suppressed late follicular-phase LH concentration may be detrimental to IVF outcome. Six studies evaluated the association between endogenous LH levels during ovarian stimulation and the likelihood of ongoing pregnancy beyond 12 weeks in normo-ovulatory patients treated for IVF with GnRH analogs [86]. They showed no evidence that low LH levels on day 8 of stimulation reduced ongoing pregnancy rates.

The demonstration of expression of LH receptors by follicles in the late follicular phase has led to a number of workers advocating the substitution of FSH with the administration of hCG during the mid-to-late follicular phase of ovarian stimulation for IVF [6, 87–89]. In one study, hCG (200–300 IU) was administered concurrently with a discontinued or reduced dosage of FSH (75 IU) when the leading follicle reached approximately12–14 mm in diameter [6]. Final oocyte maturation was then triggered when the follicles reached 18 mm. Although these studies did not show any differences in clinical pregnancy rate, the total dose of recFSH required for ovarian stimulation was significantly decreased in the low-dose hCG group. Evidence thus far suggests that hCG could partially or completely substitute the role of FSH during mid-to-late stages of the follicular phase in an ovarian stimulation cycle, without compromising pregnancy rates and leading to a significant reduction in the cost of IVF cycles.

Final trigger for oocyte maturation

The introduction of GnRH antagonists has enabled the concept of using GnRH agonists for triggering oocyte maturation by inducing an endogenous LH surge to be investigated. This more physiological approach promises to reduce the risk of OHSS known to be associated with the administration of hCG to trigger final oocyte maturation. The GnRH agonist displaces the GnRH antagonist from the receptor and initiates a 'flare up effect' seen typically in the use of GnRH long protocol. Moreover, the luteal phase steroid concentrations may approximate more closely to the physiological range with possible benefits for improving endometrial receptivity [90]. Initial studies showed the resultant LH peak to be short lived [91] raising concerns that the early luteal phase may be inadequately supported by this regimen. Two early prospective randomized controlled trials reported poor reproductive outcomes when GnRHa was used to trigger ovulation in IVF/ICSI treated women, in part due to increased early pregnancy loss [92, 93]. It was suggested that the decreased clinical pregnancy rate observed was due to a luteal-phase defect and poorer endometrial function despite luteal-phase support with progesterone and E_2 due to the shorter half-life (24–36 hours) and lower amplitude of the GnRH agonist induced endogenous LH surge compared to that of a natural cycle (48 hours) [94, 95]. This was supported by good birth rates in the frozen-thawed embryo replacement cycles in the cycles where GnRHa has been used as a trigger for oocyte maturation [96]. More recent studies have addressed how best to support the luteal phase when GnRHa is used as a trigger.

Several studies have examined the role of usage of hCG concurrently with GnRHa as ovulation trigger compared to hCG (10 000 IU) alone as the ovulation trigger as a means to support the luteal phase. A small dosage of hCG (1500 IU) has been used as a supplementary dosage after GnRHa administration as a trigger for final oocyte maturation [97, 98]. Whilst both groups showed similar miscarriage, ongoing pregnancy, and delivery rates when compared to 10 000 hCG ovulation induction cycles, no OHSS cases were seen in the GnRHa group.

Current evidence seems to support the fact that the luteal phase in IVF cycles, with final oocyte maturation triggered by GnRHa, can be rescued by the use of LH activity, resulting in reproductive outcome comparable to that of hCG-triggered final oocyte maturation.

Luteal-phase support

The luteal phases of all stimulated IVF cycles are abnormal. The main cause for this defect in the luteal phase in stimulated IVF cycles is related to the multifollicular development during ovarian stimulation. As supra-physiological concentrations of steroids are secreted by a high number of corpora luteum during the early luteal phase, the LH release is inhibited via negative feedback actions at the hypothalamic-pituitary axis. Although it is generally accepted that luteal-phase supplementation in IVF cycles improves the outcome, there is no general agreement on the best protocol to be used.

In a meta-analysis by Nosarka *et al.*, hCG appeared to be associated with higher pregnancy rates than progesterone, but at the expense of a significantly increased risk of the development of OHSS [99]. Over the years, progesterone has replaced hCG for luteal-phase support. The effectiveness of progesterone as luteal support for IVF compared to placebo has been confirmed in a meta-analysis showing an odds ratio of delivery rate of 5.5 (95% CI 1.3–35.5) [100].

While consensus has been achieved regarding the use of vaginal progesterone supplementation as first-line therapy for luteal support, a number of controversies remain. Many centers administer progesterone for several weeks, despite studies showing that this has no benefit over a shorter period [101].

Discussion also continues regarding the value of adding estrogen support, and more recently GnRH agonists in the luteal phase [102]. A recent meta-analysis examining the role of adding estrogens to progesterone in luteal-phase support included four RCTs ($n = 5587$ patients) and showed no statistically significant differences between patients who received a combination of progesterone and estrogen for luteal support when compared with those who received only progesterone, in terms of positive hCG rate (RR 1.02, 95% CI 0.87–1.19), clinical pregnancy rate (RR 0.94, 95% CI 0.78–1.13), and live birth rate (RR 0.96, 95% CI 0.77 1.21) per woman randomized. Therefore the current available evidence suggests that the addition of estrogen to progesterone for luteal-phase support does not increase the probability of pregnancy in IVF [102].

GnRH agonist was recently suggested as a novel luteal-phase support that may act at the level of pituitary gonadotrophs, the endometrium, and the embryo itself [103]. It was postulated that GnRH agonist may support the corpus luteum by stimulating the secretion of LH or

by acting directly on the endometrium. While one RCT showed a higher pregnancy rate in the GnRH agonist group [103], a later study showed no such benefit [104]. There is a need for future larger RCTs to delineate the role of GnRH agonist as a luteal-phase support.

Role of adjuvant treatments

Androgens have been proposed as having a crucial role in steroid production within the ovary, the keystone for effective hormonal control of ovulation, implantation, and subsequent pregnancy [105]. Androgens exert their effect on the granulosa cells regulating aromatase (P450arom enzyme) activity within the follicle [106]. This enzyme converts androgens to estrogen, a vital controlling step in the recruitment and development of ovarian follicles.

Androgens have seemingly a dual stimulation/inhibition role dependent on the stage of the follicular recruitment cycle. In small follicles, stimulation by androgens is apparent while a negative effect is seen in pre-ovulatory follicles. In some studies on primates androgens have been shown to increase the number of FSH receptors in the granulosa cells, making them more sensitive to FSH stimulation, but other animal studies have not demonstrated this. However, there is evidence that high androgen levels found in women with PCO are associated with an excess antral follicle count and over-sensitivity to FSH stimulation during ovarian induction with FSH. It has been suggested that androgen supplements could enhance the ovarian response in women undergoing ART (IVF/ICSI), with the potential of reducing the need for excessive FSH dosage, reducing the risk of ovarian hyperstimulation syndrome, and optimizing the response in women with a history of poor ovarian response in the ART cycle.

Commercially available forms of transdermal testosterone supplements provide a nominal 2.5 mg/day delivery rate and have been shown to enhance follicular development if given between days 1 and 5 of ART stimulation in some studies [107]. However, overall there is no clear evidence that these treatments should be implemented to all patients undergoing ART.

DHEA (dehydroepiandrosterone) is an essential substrate (prehormone) for follicular testosterone, and therefore a prehormone for estrogen within the follicle. A number of studies have suggested that short-term (up to 8 weeks) DHEA supplementation prior to ART stimulation in women with evidence of poor ovarian function may enhance ovarian function, improve oocyte yield, improve oocyte and embryo quality, spontaneous pregnancy rates in women with a poor prognostic history, pregnancy rates in IVF, time to pregnancy, and cumulative pregnancy rates in women undergoing IVF [108, 109]. The mechanism of action of DHEA is not absolutely clear but it may act by stimulation of production of insulin-like growth factor (IGF-1). IGF-1 regulates follicular development and the granulosa cells' response to FSH at the pre-antral stage. In mice studies it has been shown to have an effect on steroidogenesis, oocyte maturation, fertilization, and embryo development [108]. DHEA via IGF-1 has been shown to reduce the apoptotic rate within the ovary and in addition reduce the aneuploidy rate (and miscarriage rate) by reducing the disturbances in chromosome alignment on the meiotic spindle of oocytes. DHEA supplements can be purchased over the counter in the UK. It is a mild male hormone that if given in doses of less than 100 mg a day has few side effects. Short/medium side effects include acne, deepening of the voice, and increased facial hair. Long-term side effects have not been demonstrated. However, there is a theoretical risk that as DHEA is a precursor to androgens there could be an increase in any androgen-related malignancy, but this evidence has not been substantiated in practice. In summary, DHEA supplementation could enhance the clinical outcomes for women undergoing ART. It is a safe drug that has few side effects and potentially has substantial impact not only on the infertile population but also on those suffering from related fertility problems such a recurrent miscarriage. However, there are currently no randomized controlled study data that would support the introduction of supplementation in all women undergoing ART.

Growth hormone supplementation has been shown to potentially enhance clinical outcomes in ART. The mechanism by which it enhances the outcomes is indirectly via IGF and directly on the ovary itself. IGF production requires GH priming. IGF-2 (the primary IGF found in the human ovary) has been found to stimulate steroid genesis and proliferation of the granulosa and theca cells. GH enhances the IGF (1 and 2) production, which then has a direct effect on enhanced follicular development. It enhances fertilization rate potentially via enhanced follicular fluid levels of IGF. Uncontrolled studies and a recent Cochrane analysis on women who have previously poorly responded

suggested that GH supplementation shows a small but significant improvement in live birth rates in women. However, no randomized controlled trials looking at the impact of GH supplements on the clinical outcomes on women undergoing IVF are yet available and the present data do not support the generalized implementation of GH supplements in women undergoing ART.

Aromatase inhibitors (AI) block aromatase (P450arom enzyme) activity within the follicle, inhibiting estrogen production. Letrozole, a third-generation selective aromatase inhibitor, has been used in women undergoing treatment for post-menopausal breast cancer, but has started to be used in women undergoing ART. The advantage of using AIs is that they can reduce the negative feedback of the estradiol on the pituitary gonadotropin production, enhancing ovarian function without blocking the estrogen receptors. Their use could reduce the effect of supraphysiological levels of estrogen on the endometrium, follicle, oocyte, and embryo. Third-generation AI could have distinct advantages over other ART treatments. They are specific in inhibiting the aromatase enzyme without significant inhibition of the other steroidogenesis enzymes. They can be taken orally, have 100% bioavailability, are rapidly cleared from the body, have few side effects, are well tolerated, and are inexpensive. They can be used as an alternative to clomiphene citrate and have been shown to potentially optimize monofollicular growth in women undergoing ovarian induction [110]. They also reduce the total amount of FSH required in women undergoing ART, achieving controlled ovarian hyperstimulation without any anti-estrogenic effects. It has also been suggested that women with a history of poor ovarian response could benefit from AI use. In some women there is an enhanced ovarian response with greater number of mature follicles and improved clinical outcomes. However, concerns have been raised regarding the safety of these drugs. These include the potential effect of the low estrogen on the developing follicle and oocyte, the effects of accumulated androgens on the follicle/oocyte maturation, and an increased risk of birth defects from children conceived following the use of AIs. As a result of this concern the manufacturer has instructed clinicians to avoid use in women who are pregnant or are going to get pregnant. Although more recent data from larger studies have demonstrated the safety of aromatase inhibitors in this context, the use of AI use in ART remains off license.

Metformin is an oral biguanide insulin-sensitizing agent that acts by inhibiting hepatic glucose production without causing hypoglycemia or increasing insulin production [111]. In women with a history of polycystic ovaries metformin has been shown to decrease insulin resistance, and insulin levels, as well as testosterone and LH levels in these women that have previously been elevated. By reducing insulin resistance and insulin levels the hyperandrogenism that is associated with this should decrease. In theory the hyperinsulinemia has a direct effect on ovarian steroidogenesis by stimulating androgen production from the thecal cells. The elevated levels can also inhibit IGF-1 binding protein production by the liver, which increases the bioavailability of IGF-1, which can cause a rise in follicular androgen production. Metformin has been shown to reduce hyperinsulinemia and hyperandrogenemia in women with PCO, resulting in restoration of normal menses and increasing spontantoeus conception rates. In women with clomiphene-resistant PCOS, studies have shown that those women receiving metformin developed significantly fewer large follicles, produced less estradiol, and had fewer cycles canceled due to excessive follicular development. In women with hyperinsulinemic polycystic ovarian syndrome there is evidence that pre- and concurrent treatment with metformin while undergoing ART will improve clinical outcomes including fertilization and pregnancy rates while reducing the cycle cancelation rate and risk of ovarian hyperstimulation syndrome. However, a recent Cochrane review does not support the co-adminstration of metformin in women with PCOS undergoing ART, as the evidence overall does not show an improvement in clinical outcomes of live birth, clinical pregnancy, and miscarriage rates. In addition there was no significant effect on ovarian stimulation, or embryological parameters. It did, however, reduce the OHSS rate by 75%. Therefore routine use of metformin in women undergoing ART and demonstrating hyperinsulinemia should not be advocated.

Towards individualized protocols

In assisted conception, unsuccessful treatment cycles are often due to a suboptimal individual response to treatment. Hence there has been great interest in identifying factors which enable the optimal individual dose to be determined for each patient.

Fine-tuning of the FSH dosage can be achieved by adding specific patient markers such as smoking

status, ovarian ultrasound features, and age into a scoring system. This system was shown to improve pregnancy outcome compared with fixed dosing [112]. With regard to whether and how the dose should be increased for poor responders and decreased with over-responders it is still unclear. Although PCOS is considered to be a major risk for OHSS, a meta-analysis of women with PCOS undergoing IVF suggested only a trend towards higher OHSS rate [14]. Similarly, increasing the dosages for women who are deemed poor responders (obese, older women, and previous failed response) is not well supported by research evidence. The CONSORT study utilizes a dosing algorithm that individualizes recFSH doses (starting from 37.5 IU recFSH) according to patient characteristics (basal FSH, body mass index, age, and antral follicle count) [113]. Overall, a median of 9.0 oocytes were retrieved (8.5, 8.0, 10.0, 12.0, and 8.0 in the 75, 112.5, 150, 187.5 and 225 IU groups respectively). Clinical pregnancy rates/cycle started were 31.3, 31.1, 35.3, 50.0, and 20.0%, respectively (overall, 34.2%). Two patients had severe OHSS. The authors concluded that individualized dosing in increments of 37.5 IU of recFSH is possible through the use of the CONSORT dosing algorithm to achieve a good rate of oocyte retrieval and pregnancy achieved using this individualized approach.

Recently, there has been increased interest in the use of AMH to help predict dosing regimes. Seifer *et al.* [124] first reported that a higher AMH level on day 3 was associated with a greater number of oocytes retrieved. Since then, a number of retrospective and prospective studies have demonstrated similar findings [114–118]. A recent meta-analysis [24] of 13 studies reporting on AMH and 17 on AFC showed that in terms of predicting poor response and non-pregnancy, there was no significant difference in terms of the predictive value of AMH over antral follicle counts (ACF). The advantage of AMH over any menstrual cycle dependent predictor marker is its low inter- and intra-cycle variability. La Marca *et al.* first demonstrated that AMH measured during any time of the menstrual cycle predicted a reasonable response for ovarian stimulation cycles [33]. This has since been confirmed by a larger cohort study [117].

However, whilst serum AMH measurements may be effective in predicting response, they have not been shown to predict the likelihood of achieving pregnancy after ART. To date, only one study has reported on the use of AMH and its correlation with increased live birth rate [119]. Other studies examining the AMH and its ability to distinguish pregnancy and non-pregnancy ovarian stimulation cycles have reported disappointing results [120–123].

Given that the use of AMH can predict response, albeit that it cannot predict outcome, one line of treatment strategy has emerged whereby AMH alone (excluding age or BMI of patient) is utilized to provide individualized treatment. Nelson *et al.* [117] demonstrated that aggressive dosing of patients who have AMH < 5 pmol/l was safe whilst that of the normally suggested 150 IU FSH dosage for women with an AMH >15 pmol/l led to a high incidence of OHSS. This dosing regime is associated with reduced treatment burden, cycle cancelation, and a trend towards more cycle efficacy. However, these data derive from a non-randomized study, and future well-designed studies will be required to confirm the cost/benefit and clinical efficacy of such a regime. There may well also be substantial benefit combining AMH testing with protocols in mild stimulation treatment strategies as described by Popovic-Todorovic *et al.* [112], so that ovarian stimulation regimes can be tailor-made for patients according to their needs.

Conclusion

Recent advances have seen the development of several new concepts of ovarian stimulation highlighted in this chapter. However, ultimately the most important criteria to judge the success or failure of treatment will be significantly associated with patients' experience. Patients' experience can be marred by the treatment burden, exposure to high risk, and psychological distress. A mild stimulation regime with a GnRH antagonist regime has an equivalent live birth rate to a conventional IVF stimulation regime, and has advantages of tolerability and safety.

Looking into the future, ovarian stimulation in ART will progress into a new era where treatments will be more patient journey focused and holistic compared to the traditional IVF where success was defined by the achievement of pregnancy alone. Despite impressive development in ovarian stimulation preparations and regimens, the principal determinant of outcome from ovarian stimulation remains the patient herself. In order to further improve outcomes, the focus should shift from the treatment to the patient, and the optimization of her health and factors known to influence outcome, prior to commencing therapy.

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Chapter 7

GnRH antagonists in ART

Efstratios M. Kolibianakis and Georg Griesinger

Introduction

Throughout the history of in vitro fertilization (IVF), inhibition of premature luteinizing hormone (LH) surge has remained an integral part of ovarian stimulation [1]. Failure to suppress LH surge inevitably leads to cycle cancelation. For that purpose, gonadotropin releasing hormone (GnRH) analogs have been used since 1984 [2].

In contrast to GnRH antagonists, GnRH agonists are characterized by a lack of immediate suppression of endogenous gonadotropins, requiring a long pretreatment period prior to initiation of gonadotropin stimulation. Despite this significant disadvantage, they were used exclusively to control endogenous LH secretion until the end of the 1990s, since they were the only clinically available analog. Routine use of GnRH agonists made it possible to schedule oocyte pick-up, and was associated with an increase in the probability of pregnancy compared to cycles in which no suppression was used [3].

It is thus not surprising that clinicians were educated to consider as a necessary burden some of the well-known disadvantages of GnRH agonist use, such as the long pretreatment period, the probability of cyst formation, the associated estrogen-deprivation symptoms, and the not-infrequent occurrence of ovarian hyperstimulation syndrome (OHSS).

It was also predictable that following the availability of GnRH antagonists, which are devoid of the above disadvantages, GnRH agonist utilization would decrease. Currently, approximately one-third of IVF cycles are performed using GnRH antagonists [4].

The availability of GnRH antagonists did not only offer clinicians an alternative to GnRH agonists but, more importantly, has led to the development of new concepts aiming to increase safety and simplicity in ovarian stimulation. These include the modified natural cycle [5], mild IVF [6], the use of GnRH agonist for triggering of final oocyte maturation with elective cryopreservation in patients at risk of developing OHSS [7], the administration of antagonists during the luteal phase for management of severe OHSS [8–10], as well as control of endogenous LH with GnRH antagonists in intrauterine insemination (IUI) cycles [11].

Approximately a decade following GnRH antagonist introduction their use has been refined and for several practical questions there are now data available, guiding clinicians to achieve pregnancy rates equal to that offered by GnRH agonists, while lessening the burden that couples frequently experience during their attempts to become parents [12].

Scheme of GnRH antagonist administration

Administration of GnRH antagonists can be performed by either a single dose [13] or by using a daily scheme [14]. Administration of a single-dose antagonist is effective in suppressing endogenous LH for 4 days [15]. If the criteria to trigger final oocyte maturation have not been met by the end of this time period (which was the case for ~10% of patients in a large phase III trial) [16], daily antagonist dose can be administered accordingly.

Apparently, the single-dose administration is patient friendlier compared to the daily dose, since it is associated with a decreased number of antagonist injections. However, theoretically it might result in unnecessary antagonist administration, if the criteria to trigger final oocyte maturation are met before the effective period of 4 days is over. In this respect, although daily antagonist necessitates multiple injections, it allows using the minimally necessary dose of antagonist in a given treatment cycle.

Human Assisted Reproductive Technology: Future Trends in Laboratory and Clinical Practice, ed. David K. Gardner, Botros R. M. B. Rizk, and Tommaso Falcone. Published by Cambridge University Press. © Cambridge University Press 2011.

Until today only two comparative RCTs between the two schemes of antagonist administration have been published, including 215 patients [17, 18]. Stratified analysis of these two trials shows no difference in the probability of clinical pregnancy (rate difference [RD]: −2% in favor of the daily dose protocol; 95% CI: −16 to +11). Although these data suggest the absence of a clinically significant difference in the probability of pregnancy, they are based on a small number of patients and thus are not conclusive. Nevertheless, the majority of published antagonist studies have been performed with the daily dose protocol.

Optimal dose of GnRH antagonist

Three RCTs were performed to establish the dose under which GnRH antagonists should be used in IVF. The optimal dose was 0.25 mg [19] for the daily dose scheme and 3 mg for the multiple dose scheme [15, 20].

Higher antagonist doses for daily administration have been associated with very low LH levels and a lower probability of pregnancy [19]. Moreover, they lead to detectable antagonist in circulation by the day of embryo transfer [19], which has been suggested to be detrimental for embryo implantation [21, 22].

Timing of GnRH antagonist administration

Antagonist administration was performed in the initial comparative trials between GnRH agonists and GnRH antagonists with a fixed scheme, starting on day 6 of stimulation. Optimization of this fixed antagonist protocol, based on data regarding endogenous LH control [23], has recently moved antagonist initiation to an earlier time point, e.g. to day 5 of stimulation [24].

In the flexible antagonist scheme, antagonist is started when an LH surge is likely to occur. Since there is significant heterogeneity between individual treatment cycles, different criteria have been used to guide antagonist initiation, which are based on either ultrasound [25] and/or hormonal criteria [26].

Fixed compared to flexible antagonist initiation is a simpler protocol that requires less monitoring. On the other hand, it might lead to unnecessary antagonist administration, since in a proportion of patients an LH surge is unlikely to occur on day 5 of stimulation due to absence of follicular development.

Both the fixed (day 6 of stimulation) and the flexible protocol (using different criteria for antagonist initiation) have been compared in four RCTs, the results of which have been summarized in a meta-analysis that did not show a significant difference in clinical pregnancy rates between the two protocols (OR 0.7; 95% CI 0.47 to 1.05) [27]. However, all studies showed the same direction of effect, which was not in favor of the flexible protocol.

Is duration of GnRH antagonist administration important for the quality of embryos?

Currently, it appears that the duration of antagonist administration does not affect embryo implantation potential. Data supporting this notion stem from studies in which GnRH antagonist was started on day 1 of stimulation with excellent results [28, 29] and studies in which duration of antagonist administration in the fresh cycle had no impact on the probability of pregnancy following transfer of thawed embryos [30].

Should we assess E_2 and progesterone level at initiation of stimulation?

In the long agonist protocol, low estradiol levels are used to confirm down-regulation, which is a prerequisite for the initiation of gonadotropin administration. However, no information is present regarding the prognostic significance of E_2 and progesterone levels at initiation of stimulation in the short agonist protocol, where, similarly to GnRH antagonists, stimulation starts in the early follicular phase.

In GnRH antagonist cycles, there are data to suggest that progesterone levels should be within the reference range for the follicular phase prior to initiation of stimulation. In patients with elevated progesterone on day 2 of the cycle, when gonadotropins were commenced 1 or 2 days later and only after progesterone levels were normalized, progesterone levels were significantly higher during the follicular phase, compared with patients with normal progesterone levels at cycle initiation, while the probability of pregnancy was significantly lower [31]. Although this issue has not been explored in further studies, a fraction of normally cycling women appear to have elevated progesterone at the onset of menstruation, and it is likely that in the presence of elevated progesterone levels the probability of pregnancy is decreased. Thus, postponing the initiation of ovarian

stimulation to the next menstruation is one option to consider.

On the other hand, elevated estradiol levels on day 2 of the cycle are indicative of an ovarian cyst, and thus serum estradiol levels should be assessed prior to initiation of stimulation.

Should FSH starting dose be determined differently in an antagonist compared to an agonist cycle?

Despite several attempts, there is currently no universally accepted algorithm which will objectively determine the starting dose of FSH for an individual patient in an IVF cycle [32–34]. Thus, the so-called "standard dose" of 150 IU is an arbitrary, although well-accepted, notion [35].

Two studies have been performed in antagonist cycles to determine whether a higher (200 IU or 225 IU) than the "standard" (150 IU) dose is beneficial for the probability of pregnancy [36, 37]. The available data do not suggest that pregnancy rates are increased by using a higher than the "standard" dose of FSH (odds ratio for clinical pregnancy 0.81; 95% CI 0.51 to 1.28), though it has to be mentioned that pregnancies resulting from the transfer of frozen embryos were not taken into account.

When should FSH be started?

Since the introduction of GnRH antagonists in clinical practice and in most trials published, initiation of stimulation is performed on day 2 or day 3 of the menstrual cycle [24, 38, 39]. This allows for some flexibility by scheduling the initiation of stimulation. However, there are no RCTs to test whether IVF outcome is dependent on the timing of initiation of stimulation. Data from the ENGAGE trial [24] (personal communication) suggest that such an intervention (starting on day 2 or 3 of menses) does not alter the probability of pregnancy. A later initiation of stimulation on day 5 of the cycle has been performed in the so-called "mild stimulation protocols" [40], the target of which is increased safety and decreased drug consumption [41, 42].

When is the optimal time for triggering of final oocyte maturation?

Several criteria have been used to determine the timepoint for triggering final oocyte maturation. These criteria are based on ultrasound and/or hormonal data [43], which are, however, not evidence-based. There are few studies in agonist or in antagonist cycles which have attempted to define the optimal time for triggering final oocyte maturation. Perhaps, after all, this is not a feasible task, since these criteria are usually dependent on the target of ovarian stimulation, the availability of cryopreservation, and the quality of the laboratory that will culture the resulting embryos. These parameters may differ considerably between IVF centers.

Nevertheless, in both agonist and antagonist cycles there are data to suggest that prolongation of the follicular phase is associated with a decreased probability of pregnancy [43, 44]. In antagonist cycles a decreased pregnancy rate has been observed by prolonging the follicular phase for two days as soon as three or more follicles of 17 mm or more in diameter are present at ultrasound. However, data from the ENGAGE trial [24] suggest that prolongation by one day might not affect the probability of pregnancy (personal communication), which allows for some flexibility in scheduling oocyte pick-up.

Is the need for luteal-phase support different between agonist and antagonist cycles?

Luteal-phase support is needed in both antagonist and agonist cycles [45], since the major reason for the very low LH levels observed in the luteal phase as well for the abnormalities in the endometrium development is gonadotropin stimulation and not the type of GnRH analog used for LH suppression. Micronized progesterone is frequently used for luteal-phase support while the addition of E_2 appears not to be beneficial for pregnancy rates [46].

Evidence for efficacy of GnRH antagonists from meta-analyses

The interest in the effectiveness of GnRH antagonist in IVF has generated more than 30 comparative RCTs. Moreover, soon after antagonist introduction three conflicting meta-analyses were published [47–49], which were followed a few years later by two updated meta-analyses [23, 50]. The latter were still conflicting in terms of the statistical significance of the difference observed in the probability of pregnancy between the

two analogs. However, they were in line regarding the magnitude of effect. This was 2.7% and 4% respectively; thus less than the 5% that is often considered as a clinically significant difference in IVF. Therefore, as the best estimate, the probability of pregnancy appears to be independent of the type of analog used for endogenous LH suppression.

GnRH antagonists in special populations

Based on the existing published data on the use of GnRH antagonist in patients with polycystic ovarian syndrome (PCOS) that have been summarized in a recent meta-analysis [51], no differences appear to be present when GnRH antagonist multiple-dose protocol and GnRH agonist long protocol are compared ($P < 0.01$) with the exception of a significantly shorter duration of stimulation in the antagonist-treated patients.

In poor responders, the type of analog used to suppress premature LH surge does not appear to influence the outcome of treatment with the exception of the number of cumulus-oocyte complexes, which is significantly higher ($P = 0.05$) in the GnRH antagonist multiple-dose protocol as compared to GnRH agonist long protocol [51].

It should be noted that for both poor responders and PCOS patients, sample sizes are currently small, even after pooling several RCTs, and thus the power to detect subtle differences is limited.

Do GnRH antagonists increase the safety of ovarian stimulation?

Compared to GnRH agonists GnRH antagonists offer a similar probability of pregnancy, a shorter duration of stimulation, and a more rational way of performing ovarian stimulation, in which the analog used to suppress premature LH surge is administered only for the time period when it is necessary. However, probably their more important advantage is the increase in safety of the IVF procedure.

GnRH antagonists lead to approximately 50% lower probability of hospital admission due to ovarian hyperstimulation syndrome (OHSS) as compared to GnRH agonist stimulation [23, 52]. Moreover, they can be combined instead of hCG with GnRH agonist for triggering final oocyte maturation in cases at high risk for OHSS and virtually eliminate the occurrence of this dreadful syndrome [53]. In these cases, all resulting embryos are cryopreserved and embryo transfer is deferred for a future cycle [54]. Finally, GnRH antagonists can be reinitiated in the luteal phase when severe OHSS is diagnosed to enhance luteolysis [8–10].

Co-interventions during GnRH antagonist treatment

Oral contraceptive pill (OCP) pretreatment

Oral contraceptive pills have been used to program IVF cycles in which antagonists were used. Currently, there are six RCTs evaluating OCP pretreatment, including 1343 patients. The probability of ongoing pregnancy is decreased after OCP pretreatment (relative risk: 0.80; 95% CI 0.66 to 0.97; $P = 0.02$; rate difference: −5%, 95% CI: −10% to −1%; $P = 0.02$). Moreover, OCP pretreatment was shown to significantly increase duration of stimulation (weighted mean difference [WMD]: +1.35 days; 95% CI +0.62 to +2.07; $P < 0.01$) and gonadotropin consumption (WMD: +360 IU; 95% CI +158 to +563; $P < 0.01$) [55].

LH addition

LH addition has so far been tested in the general population in two RCTS including 176 patients, which were part of a larger meta-analysis evaluating recombinant LH addition during ovarian stimulation [56]. Due to the small number of patients no solid conclusions can be drawn; however, in both studies minimally (although not statistically significantly) higher pregnancy rates were observed in patients who received FSH only.

Towards simplification of ovarian stimulation

The introduction of GnRH antagonists was a breakthrough in ovarian stimulation, allowing for a safer and simpler approach to IVF treatment. However, even with GnRH antagonists, treatment still requires approximately 10 days of FSH injections, which represent a significant burden to the patient.

The need to simplify further ovarian stimulation led to the development of long-acting FSH. Long-acting FSH replaces 7 days of daily FSH injections with a single dose administered on day two or three

of the cycle. Long-acting FSH has been developed for use in the GnRH antagonist protocol and accordingly the label of the product will not include GnRH agonist protocols. Currently, available data suggest a similar efficacy of long-acting FSH compared with daily FSH [24].

Apparently, the transition from a traditional agonist/daily FSH protocol to an antagonist/long-acting FSH protocol represents an organizational challenge for both patients and doctors. Ultimately, a simple and safe ovarian stimulation procedure should aim the effort of achieving high cumulative pregnancy rates at a less demanding and risky treatment.

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Chapter 8

Pharmacogenetics of ovarian stimulation in the twenty-first century

Valeria Pugni and Manuela Simoni

Ovarian stimulation

Different types of assisted reproduction technique (ART) procedures are available depending on the indication (male factor, female factor, or a combination of both). In cases of chronic anovulation, ovulation induction can be achieved by means of clomiphene and hCG or FSH and/or hMG and hCG, followed or not by intrauterine insemination. In cases of normally ovulating women, the indications for IVF are infertility due to extra-ovarian factors (tubal damage or endometriosis), male factor, or failure to conceive after 12 cycles of successful ovulation induction or after six cycles of intrauterine insemination, and unexplained infertility. The basic steps in an IVF treatment cycle include ovarian stimulation, oocyte retrieval, fertilization, embryo culture, and embryo transfer. The current protocols for ovarian stimulation in IVF involve the use of GnRH analogs (antagonist or agonist) and FSH (or hMG) and hCG. The most common complications associated with IVF treatment are failure to respond adequately to gonadotropin stimulation (poor response), problems experienced as a consequence of ovarian hyperstimulation, the risk of multiple pregnancy, the surgical risks associated with oocyte collection, and the possibility of ectopic pregnancy [1].

It is well known that there is a high variability of the clinical outcome in women undergoing controlled ovarian hyperstimulation (COH) and, in particular, two opposite situations should be avoided: on one hand poor ovarian response and, on the other hand, ovarian hyperstimulation syndrome (OHSS) [2]. The most common reasons for IVF failure are cycle cancelation (up to 10% of treatment cycles are abandoned before oocyte collection), failure to collect oocytes (about 1%), failure of fertilization (about 5%), and failure of implantation. Approximately 15–20% of IVF pregnancies do not result in a live birth [1]. Ongoing pregnancy per transfer rates are usually 26–33%.The cumulative live birth rate after six cycles of IVF can reach 51–72% [3]. However, despite careful monitoring, up to 33% of IVF treatments have been reported to be associated with mild forms of OHSS, and severe OHSS has been reported in 3–8% of IVF cycles [4].

Many efforts have been made in order to improve the IVF success rate and reduce complications. The ovarian response to COH is associated with well-known individual features such as ovarian reserve, chronological age, AMH levels, ovarian volume, ovarian Doppler score, and smoking habits [5]. A combination of four of such factors (baseline serum FSH, BMI, age, antral follicular count) was assessed in an algorithm to calculate the exact FSH dose for each single woman candidate for assisted reproduction [6]. However, none of these approaches was completely successful. Probably most of the differences in individual COH response that cannot be justified by these known factors are linked to genetic variability among subjects. Discovering the genetic variants associated with ovarian response to gonadotropins is an important step towards individualized pharmacogenetic protocols of ovarian stimulation.

Genetic polymorphisms

The human genome is 99.9% identical in all individuals. More than 90% of the genetic variability is caused by the presence of single nucleotide polymorphisms (SNPs). The remaining less than 10% is due to insertions, deletions, tandem repeats, and microsatellites [7]. A genetic variation is defined as polymorphism when it is common in a specific population: by definition a polymorphism occurs in more than 1% of individuals belonging

Human Assisted Reproductive Technology: Future Trends in Laboratory and Clinical Practice, ed. David K. Gardner, Botros R. M. B. Rizk, and Tommaso Falcone. Published by Cambridge University Press. © Cambridge University Press 2011.

to the same group, while a mutation is rare, with a frequency less than 1% [5]. A minority of SNPs occur in exons (protein-coding regions of a gene), the rest are intronic [7]. Polymorphisms can modify gene function by changing biochemical properties of the gene product (protein) or by modifying the activity of the promoter, or altering the stability/metabolism/degradation of mRNA resulting in a larger or smaller amounts of protein [8].

Pharmacogenetics

Genetic differences among subjects are probably the main cause of individual drug response: the effects of a pharmacological therapy administered uniformly to a population can vary in terms of clinical response, side effects, and adverse events. Pharmacogenetics is the science that describes the relationship between genetic variability and drug response and studies how to tailor pharmacological therapy to the genetic features of the individual patient and how to improve desired actions and minimize side effects [5].

The study of polymorphisms or other clinical biomarkers has already been applied in medicine, for example in the use of imatinib mesylate in chronic myeloid leukemia (in the BCR-ABL-positive tyrosine kinase genotype) or trastuzumab in breast cancer (if positive for the expression of the receptor tyrosine kinase HER2/neu), or to predict toxicity in response to the anticancer drug irinotecan (linked to a genetic variant of the UDP-glucuronosyltransferase 1A1 enzyme UGT1A1) or in the development of cancer vaccine oncophage [9].

Pharmacogenetics of ovarian stimulation

Different genes have been studied in relation to the characteristics of the normal ovarian cycle or different individual responses to COH (Table 8.1).

The FSH receptor gene (*FSHR*) is crucial in follicular maturation: inactivating *FSHR* mutations almost always leads to amenorrhea and activating mutations can cause a spontaneous ovarian hyperstimulation syndrome or predispose to iatrogenic OHSS [10]. These mutations are very rare and self-eliminating because of the fundamental role of FSH in human reproduction [5].

The *FSHR* gene is located on chromosome 2 p21-p16, consists of 10 exons and nine introns [11] and contains about 2010 SNPs. A common SNP is located within the core promoter region of the gene at position −29 [8]. An initial study investigated whether differences in gene transcription in vitro result from this SNP but was unable to show any correlation between the nucleotide at position −29 and FSH activity. In contrast, recent in vitro studies showed that the A allele at position −29 of the *FSHR* gene is associated with reduced transcriptional activity. Moreover, clinical studies revealed that the subjects with AA genotype at position −29 required the highest amount of exogenous FSH for ovulation induction, and estradiol concentrations the day before hCG administration were significantly lower compared with the GA genotype. The numbers of pre-ovulatory follicles and retrieved oocytes were lowest in the subjects with AA genotype. These results indicate that the AA genotype at position −29 may be associated with the poor ovarian response [12]. Given the contrasting results between these two reports more studies are necessary to clarify the role of the −29 SNP in *FSHR* transcription and activity.

Five SNPs are located in exon 10, at codons 307, 329, 524, 665, and 680. Only four of these cause an amino acid substitution [8]. p.Ala665Thr and p.Arg524Ser are not well characterized with respect to frequency and ethnic distribution, while more information is available about the prevalence of p.Ala307Thr (rs6165) and p.Ser680Asn (rs6166) [8]. The two codons codifying for the amino acids 307 and 680 are in linkage disequilibrium, i.e. during recombination they are linked to each other in a way that does not follow a casual pattern. This generates four allelic variants, which are found with a specific frequency in a given population. Therefore, if one polymorphism occurs at one site, the nucleotide at the second site is usually exchanged as well, resulting in the two most common allelic variants: Ala307/Ser680 and Thr307/Asn680, the latter representing about 55% of the alleles in the Caucasian population [13]. Each allelic variant can be found in homozygosity or in heterozygosity: as a result, nine different combinations are possible [10]. Exon 10 encodes the intracellular and the transmembrane domain, and the C terminal end of the extracellular domain of the FSHR; therefore it is involved in signal transduction, but is not fundamental for ligand binding [8]. Residue 307 is located in the region that connects the hormone-binding domain to the transmembrane domain and that varies among three glycoprotein hormone receptors. Residue 680 is located

Table 8.1. Clinical studies on association of genetic polymorphisms and ovarian stimulation: summary of the literature

Study	Population origin	Sample size (cases/ controls)	Treatment	Locus	Genetic markers	dbSNP ID	Main findings
Georgiou *et al.* [27]	Greece	100/100	hMG	ESR1	c.453–397G > T (PvuII) c.261G > C (BstUI)	rs2234693 rs746432	*ESR1* T allele correlated with pregnancy rate during IVF. Follicle:oocyte ratio altered
Sundarrajan *et al.* [28]	Korea	200/200	Urinary FSH	ESR1	c.453–397G > T (PvuII) c.261G > C (BstUI)	rs2234693 rs746432	*ESR1* T allele associated with implantation (pregnancy outcome). Follicle and oocyte numbers lower in CC homozygotes
Perez Mayorga *et al.* [16]	Germany	161/–	Urinary FSH/ Recombinant FSH	FSHR	p.Ser680Asn	rs6166	Ser680 allele related to FSH basal levels and elevated gonadotropin requirement during COH
Sudo *et al.* [31]	Japan	58/–	hMG	FSHR	p.Ser680Asn	rs6166	Ser680 allele related to FSH basal levels and elevated gonadotropin requirement during COH
de Castro *et al.* [30]	Spain	102/–	Recombinant FSH	FSHR	p.Ser680Asn	rs6166	Ser680 allele associated with poor response and COH cycle cancelation
de Castro *et al.* [29]	Spain	170/–	Recombinant FSH	FSHR ESR1 ESR2 CYP19	p.Ser680Asn c.453–397G > T (PvuII) 39*G > A *19C > T	rs6166 rs2234693 rs4986938 rs10046	Ser680 allele associated with poor response during COH, *FSHR*, *ESR1* and *ESR2* trigenic epistatic interactions observed in relation to COH outcome
Daelemans *et al.* [18]	Belgium	37/–	hMG	FSHR	p.Ser680Asn	rs6166	p.Ser680Asn marker associated with severity of OHSS symptoms
de Castro *et al.* [22]	Spain	213/–	GnRHA (triptorelin)	FSHR ESR1 ESR2 CYP19	p.Ser680Asn c.453–397G > T (PvuII) 39*G > A *19C > T	rs6166 rs2234693 rs4986938 rs10046	*CYP19* rs10046 marker associated with partial pituitary suppression during COH
Behre *et al.* [17]	Germany	93/–	Various	FSHR	p.Ser680Asn	rs6166	Ser680 homozygotes present lower estradiol levels after FSH stimulation
Achrekar *et al.* [12]	India	50/100	Recombinant FSH	FSHR	c.–29 G > A	Rs1394205	AA genotype at the –29 position required the highest amount of exogenous FSH for ovulation induction

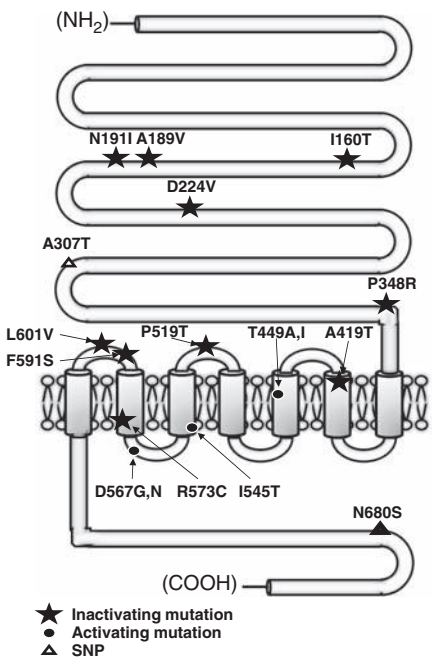


Figure 8.1. Residue 680 is located in another region that is highly variable in these receptors, the C terminal region of the intracellular domain.

in another region that is highly variable in these receptors, the C terminal region of the intracellular domain (Figure 8.1) [8].

There is no difference in the fertility potential of women carrying Ala307/Ser680 or Thr307/Asn680 but these polymorphisms influence the dynamics of the normal ovarian cycle. The similar frequency of these two polymorphisms in the human species implies that both are compatible with normal fertility and no evolutionary advantage is clearly evident at this point of human evolution [5].

The ovarian cycle is characterized by a discrete FSH "threshold": serum concentrations of FSH have to be higher than a certain level to maintain follicle growth, otherwise follicles become atretic. Duration of FSH increase is more important than magnitude in determining how many follicles will mature. FSH also promotes differentiation and growth of granulosa cells in tertiary follicles, induces the production of estradiol and aromatase, and the acquisition of LH receptors [14]. FSH exerts all these actions via FSHR, which in women is exclusively expressed in granulosa cells [14]. To better understand the significance of *FSHR* gene polymorphisms and their influence on reproductive function and response to controlled ovarian hyperstimulation, the majority of the studies take into accout only homozygous carriers of Ser680 (20% of the female population [13]) or Asn680, and not heterozygous Ser680/Asn680.

Greb *et al.* studied the dynamics of menstrual cycle in 29 eumenorrheic women carrying homozygous Ser680 or homozygous Asn680 genotype in the *FSHR* gene. LH levels were similar in the two groups, while Ser680/Ser680 women showed higher FSH levels both in late luteal and in follicular phase. Luteal levels of estradiol, progesterone, and inhibin A decreased earlier in the Ser680/Ser680 women and this was correlated to a longer length of the follicular phase, while the dynamics of luteolysis were similar. These women show a significantly longer duration of the menstrual cycle (median values of 29.3 days vs. 27.0 days). No significant differences in estradiol levels during follicular phase could be demonstrated, suggesting that in Ser680/Ser680 women higher FSH levels are necessary to reach the same estradiol levels [14]. Estradiol production of granulosa cells depends on the availability of androgen substrate, which is LH-dependent, and FSH stimulates the expression of LH receptors [15]. It is possible that *FSHR* in Ser680/Ser680 women could be less effective in inducing LH receptors or aromatase expression [5]. However, more studies are necessary to verify this hypothesis. Follicular maturation was affected only before the selection of the dominant follicle, but its growth was similar, as soon as the dominant follicle was selected [14].

FSHR sensitivity to FSH is obviously fundamental in COH. At present the FSH dosage for a single patient candidate for IVF is individualized taking into account parameters of ovarian reserve. A high variability in ovarian response to exogenous FSH can be observed and this variability is only in part explained by known individual factors such as, for example, the presence of a polycystic ovary syndrome (predisposing to a high response) or ovarian aging (associated with a low response) [5]. Some studies have been conducted to determine how *FSHR* polymorphisms can affect the necessary FSH dosage, independently and in association with already known clinical features, and to know in advance the optimal FSH dose for each patient. Changing gonadotropin dose during ovarian stimulation according to the number of recruited follicles and estradiol concentration on days 7–8 is problematic. Increasing the dose often results in a heterogeneous cohort of follicles because large follicles continue to grow while the FSH threshold of non-growing small follicles will be passed. On the other hand, decreasing

the dose could result in a diffuse follicle atresia and poor oocyte quality [5].

In a retrospective study Perez Mayorga *et al.* suggested that the number of FSH ampoules needed during COH could be predicted from a linear combination of *FSHR* genotype and serum FSH levels, an index of ovarian reserve [16]. This hypothesis was further investigated in a prospective study. Behre *et al.* examined 93 Caucasian women homozygous Ser680/Ser680 or Asn680/Asn680. All selected volunteers presented a normal menstrual cycle and no ovarian pathologies. Starting from the hypothesis that Ser680/Ser680 women would have responded worse than Asn680/Asn680 to an equal dose of exogenous FSH, the authors divided the patients into three groups: (I) Ser680/Ser680 women treated with a traditional dose of FSH (150 U/day); (II) Ser680/Ser680 women treated with a dose of FSH augmented of 50%, equal to 225 U/day; and a group (III) of Asn680/Asn680 women treated with a traditional dose of FSH (150 U/day), because they were supposed to have a good response. The FSH dose was kept constant until the patients reached a follicle size of at least 17 mm and, regardless of estradiol levels, ovulation was induced with hCG [17]. The authors found that estradiol levels on the day of hCG administration were significantly lower in group I than in group III, while they were similar between group II and group III, suggesting that increasing the FSH total dose was necessary to reach the same stimulation FSH levels in Ser680/Ser680 women compared to Asn680/Asn680 women. No differences in number of follicles, retrieved oocytes, fertilization rate, cumulative embryo score, and pregnancy rate were seen, although these parameters were not primary end points of the study. None of the 93 women experienced severe OHSS. It was concluded that the decreased sensitivity of the *FSHR* homozygous Ser680/Ser680 compared to estradiol production can be overcome by a 50% increased dose of FSH and that Asn680/Asn680 patients, if stimulated with the same dose of FSH, could develop inadequate endometrial maturation and be at greater risk of OHSS, compared to Ser680/Ser680 women.

Daelemans *et al.* demonstrated that the Ser680 allele was significantly more represented in women presenting iatrogenic OHSS but the Asn680 variant was significantly associated with the severity of this syndrome [18]. Moreover, the existence of mutations causing a spontaneous ovarian hyperstimulation syndrome underscores the importance of the *FSHR* gene in OHSS [2].

Overbeek *et al.* studied the *FSHR* SNPs in polycystic ovary syndrome (PCOS) patients, in particular in relation to response to clomiphene citrate [19]. Clomiphene citrate is the first-line treatment for infertility caused by PCOS: it is a competitive antagonist of estradiol and its blockade eliminates the negative feedback on the hypothalamus. As a consequence, increased quantities of LH and FSH are secreted and this increase in gonadotropins stimulates follicle growth and ovulation. Ovulation is generally restored in 80% of treated patients, while 20% of them do not ovulate after the maximal dose of clomiphene citrate (150 mg) [20]. Several individual factors (free androgen index, BMI, cycle history, androgen levels, ovarian volume, insulin and glucose levels, leptin) have been studied as predictors of response to the therapy [21], but they are insufficient to know in advance whether or not a patient will ovulate [22]. In 193 PCOS patients (92% of them Caucasian) de Castro *et al.* found no significant differences in distribution of *FSHR* polymorphisms compared to the general population. Differently from the general population, however, basal FSH levels were not significantly different between the three groups (Asn680/Asn680, Asn680/Ser680, Ser680/Ser680). The authors studied the clinical response to therapy with clomiphene citrate in relation to the presence of these polymorphisms and found that significantly more patients homozygous for Ser680 did not ovulate after three cycles of increasing clomiphene doses (50 mg/day for 5 days, then raised to 100 and 150 mg) and were therefore defined as clomiphene-resistant. The authors suggested that while the hypothalamus-pituitary-ovarian axis of normally ovulating women is able to adjust to higher levels of FSH in relation to a minor sensitivity to FSH of the ovarian *FSHR* in Ser680/Ser680 carriers, in PCOS patients this feedback is compromised: basal FSH levels, although within normal values, are too low to induce follicle maturation and in Ser680/Ser680 carriers it is even harder to overcome this higher FSH threshold with clomiphene therapy [22].

In summary, the literature reports an important role of the *FSHR* SNPs in influencing FSHR sensitivity to endogenous or exogenous FSH: this sensitivity has a prominent role in determining the duration of the menstrual cycle and basal FSH levels in normally ovulating women, the FSH dose necessary to achieve a good response in COH, the incidence and severity of iatrogenic OHSS, and the response to clomiphene.

FSH effects are related to granulosa cell proliferation, oocyte maturation, and estrogen synthesis through

the activation of the *CYP19* aromatase gene [2]. The *CYP19* (P450arom) gene is located in the chromosome 15q21.2 region and is composed of a 30 kb coding region and a 93 kb regulatory region: the regulatory region contains 10 tissue-specific promoters that are alternatively used in various cell types. Each promoter is regulated by a distinct set of regulatory sequences in DNA and transcription factors that bind to these specific sequences [23].

Estrogens are directly involved in follicle growth, maturation, or oocyte release [24], so estrogen receptor (ESR) genes are good candidates for evaluating SNPs in relation to COH response. Estradiol binds two intracellular receptors: receptor α (ERα) and receptor β (ERβ) encoded respectively by *ESR1* and *ESR2* genes. *ESR1* is on chromosome 6, whereas *ESR2* is on chromosome 14 [25]. The *ESR1* gene has been related to many estrogen-related traits [26].

Georgiu *et al.* examined polymorphisms of *ESR1* after using *PvuII* and *BstUI* restriction enzymes in 100 Greek women candidates for IVF. The authors found that *Pp* and *PP* genotypes of *PvuII* showed a higher follicle/oocyte number ratio, if compared to the *pp* genotype; the *PP* genotype was also associated with a lower pregnancy rate, so they suggested that these different alleles might also affect embryo implantation during IVF embyo transfer. However, *PvuII* and *BstUI* markers of *ESR1* were not related to the overall number of follicles or oocytes obtained during COH [27].

Sundarrajan *et al.* examined Korean women and confirmed the findings regarding pregnancy rate, but found that *PP* carriers showed lower numbers of follicles and oocytes, smaller follicle size, and lower number of embryos retrieved during COH, in addition to higher estrogen levels at the end of the COH cycle. In this study, patients did not differ from fertile controls in the frequency of the *PvuII* polymorphism, suggesting that different alleles do not cause infertility, but could have an effect on the quality of follicles and oocytes [28]. None of these studies presented data about OHSS.

De Castro *et al.* found no correlation of *ESR1 PvuII* markers and poor or high response, estrogen levels, or numbers of oocyte or follicles obtained during COH in Spanish women. Moreover they found no association of the *ESR2* gene with poor or high response to gonadotropins [29]. The same group proposed that COH outcome could be influenced by the interaction of several genes, in a multifactorial model [30]. They found evidence of an interaction among *FSHR*, *ESR1*, and *ESR2* in relation to COH outcome: in particular, the association between *FSHR* Ser680, *ESR1 PvuII* p and *39G *ESR2* was linked to a low response to COH, even in heterozygous women. An association with COH outcome of *ESR1* and *ESR2* or of *CYP19* aromatase allelic form has not been found yet [29].

In another study, the same authors analyzed the same loci in relation to pituitary suppression with triptorelin, a gonadotropin-releasing hormone agonist, in 213 premenopausal women. This is a good model to study the regulation of estrogen production via *CYP19* aromatase, because some treated women present only partial estrogen suppression after GnRH agonist treatment [22]. They found out that the C allele of the rs10046 marker of the *CYP19* aromatase locus is associated with poor pituitary suppression and a higher number of days to reach pituitary suppression, if compared with T allele carriers.

In summary, the literature does not report unequivocal results about the role of *ESR1* and *ESR2* or of *CYP19* aromatase allelic form in determining response to COH. It is probable that response to COH is influenced by several clinical and genetic factors, acting together in a polygenic model. More studies are necessary to clarify the interaction between different SNPs of different genes.

Future perspectives

Pharmacogenetics is rapidly changing our approach to drug therapy, allowing the individualized treatment of patients depending on their genetic background. In the field of infertility treatment, a pharmacogenetic, stratified approach to COH is already pursued empirically in many centers, based on the partially contradictory results reported in this chapter. In particular, the *FSHR* polymorphism at codon 680 seems to be very promising as a predictive factor, although properly conducted, prospective studies with independent confirmation and large numbers of patients are necessary before this marker can enter clinical practice based on solid evidence. The studies conducted until now demonstrate that ovarian stimulation efficacy and efficiency depend on a number of factors, some of which can be easily assessed without resorting to genetic testing. Identifying and validating genetic markers such as the *FSHR*, *ESR1*, *ESR2* etc., and including them in an extended algorithm to choose the gonadotropin dose might improve success and reduce side effects. However, such an approach requires

clinical validation, and well-controlled multi-center, longitudinal studies should be performed to acquire the necessary evidence. Since genetic testing is a relatively simple and inexpensive procedure, its usefulness to optimize ovarian stimulation should be carefully assessed.

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Chapter 9

Non-invasive diagnosis of endometriosis with proteomic technologies

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Endometriosis affects between 10 and 15% of all women. Among infertile women, the prevalence of endometriosis is 20 to 40% and increases to 20 to 70% in women with chronic pelvic pain. On the other hand, a large proportion of women with endometriosis may be asymptomatic. It has been estimated that there has been a 7 to 9 year delay between the onset of symptoms and the establishment of the diagnosis. The current gold standard for diagnosis of endometriosis is laparoscopy. In addition to its expense, laparoscopy may be associated with surgical and anesthetic complications.

A non-invasive test for endometriosis would eliminate the risks associated with operative laparoscopy. It would also be suitable for adolescent patients, who frequently encounter an excessive delay in diagnosis. An ideal test should be able to distinguish between early and late endometriosis and fertile from infertile women. Early treatment may offer the window to prevent excessive pelvic adhesions, which are frequently associated with endometriosis-associated infertility in advanced cases.

There are many new "omics" approaches that may lead to effective diagnosis. This chapter will focus on the non-invasive approaches which largely concentrate on proteomics analyses. The examination of tissue changes using any "omics" technique must still be considered invasive as tissue will have to be taken under surgical approaches and this is not suitable for the screening of a population. Thus changes such as mutation in a genome and protein production in cells by the measurement of mRNA are not considered suitable for a routine diagnostic test. Of the remaining approaches, the analysis of proteins in body fluids has received considerable attention. The proteins present in such fluids are referred to as the proteome and the study of these as proteomics.

Routine protein analyses

In a routine clinical test, methods such as ELISA and RIA (radioimmunoassay) are routinely used for the measurement of protein levels. Most diagnostic biomarkers, or the few that exist, are protein-based and almost exclusively samples in plasma or serum. The best-known examples of these are PSA (prostate specific antigen) for prostate cancer and CA 125 for ovarian cancer. Both of these tests are not that reliable. CA 125 is a well-accepted protein biomarker for ovarian cancer but it is also released under many other conditions such as endometriosis. It is highly unreliable for the determination of ovarian cancer and has been ruled not suitable for diagnosis but is useful for following a diagnosed patient for the signs of reoccurrence or metastasis. CA 125 is also highly associated with endometriosis and research has focused on the association of this marker with other proteins in an effort to improve its predictive ability. Targeted tests such as CA 125 may be routinely applied as they have been established and the necessary antibodies and reagents produced. In order to discover better biomarkers for endometriosis, a comparison of the protein profiles of endometriosis patients versus healthy controls must be performed and the changes noted.

Proteomics of tissue samples as compared to body fluid samples

There are many reports that examine the changes in the protein production (e.g. mRNA analyses via RT-PCR) of cellular samples, either directly from tissue or via the growing of human cell isolates in laboratory incubators. While mRNA will reveal changes in the production of all proteins in the cells, these changes do not always parallel the levels of active proteins in a

Human Assisted Reproductive Technology: Future Trends in Laboratory and Clinical Practice, ed. David K. Gardner, Botros R. M. B. Rizk, and Tommaso Falcone. Published by Cambridge University Press. © Cambridge University Press 2011.

cellular system. However, the direction of change as distinct from the amplitude of the change is normally in agreement. These results can reveal proteomic changes that may be pursued in non-invasively obtained samples. With tissue, histological staining may show changes in protein production and location in cells and tissue but these may not relate to any changes of protein in body fluids. Proteins in tissues and cells are fixed in these systems, and are largely intracellular proteins in the cytosol, nucleus, and other organelles. These proteins are not normally secreted from the cells unless cell lysis occurs. Other proteins are generated from the genome with signal sequences that direct them to the cell membrane or are secreted in the extracellular environment. Secreted proteins are often post-translationally modified, in particular by glycosylation. These proteins are then released into the interstitial environment where they may enter body fluids such as the blood system and, in the case of endometriosis, be detected in endometrial, peritoneal, or follicular fluids. Secreted and shed proteins have the highest probability of becoming biomarkers of any disease. Proteins that are classically secreted from the cell can be predicted from their signal sequence and software such as SignalP on the CBS servers (http://www.cbs.dtu.dk/services/) can perform these predictions.

A signal sequence may direct the protein to the membrane, and the extracellular portion of these proteins may become biomarkers if they are shed by the actions of enzymes in the extracellular environment. Other proteins may be secreted by non-classical methods and the CBS SecretomeP software estimates this probability. Secreted and shed proteins have the probability to become biomarkers of any disease. The secretome of cells grown under culture may be collected using an optimized method. To do this is now a well-accepted approach [1, 2]. Here the cells are grown under serum-free conditions overnight and the media collected containing the secretome free from the serum proteins. This reveals one of the current limitations of proteomics in that proteins are present at highly different levels, spanning as much as ten orders of magnitude. The removal of serum from these cells provides easy access to the secretome profile without other interfering proteins. While this approach may be useful for the examination of changes in cells grown under different conditions or from different patients, such as we have used for breast cancer [2], it may not resemble the profile of protein released from endometriosis tissue in patients. Thus, it is important to measure samples that are obtained from a patient, preferably by an accepted and non-invasive approach.

Sources of non-invasive samples for endometriosis diagnosis

There are multiple body fluid samples that can be considered for endometriosis, not all non-invasive. Blood-based protein biomarkers (serum or plasma) have received the most attention and are preferred for standard clinical methods such as ELISA. Endometriosis tissue is rich in blood vessels, creating a high likelihood that secreted proteins will be drained into and detected in the blood system. Because of its contact with endometrial tissue and confined location, peritoneal fluid (PF) is an ideal source for diagnostic markers but is not routinely available except as a result of laparoscopic collection. It is also not diluted with secreted proteins and substances from the rest of the body, such as albumin, which can trap other proteins such as biomarkers. Proteomic analysis suffers from the presence of high levels of blood proteins that obscure the biomarkers, and depletion methods can remove the biomarker proteins. Samples of follicular fluid (FF) can be collected by either laparoscopy or ultrasound-guided transvaginal aspiration; endometrial fluid (EF) can be collected by simple aspiration via catheter. An alternative source of endometrial fluid may be collected as part of a normal Pap sample taken for cervical cancer diagnosis. That test targets the cells that are sampled but with the natural flow of mucus from the fallopian tubes and uterus exiting via the cervix, the potential for the sampling as part of a routine Pap analysis is an exciting prospect. Urine represents another possible non-invasive source for diagnosis but may largely be useful for metabolomic studies. There are only two reports of endometrial biomarker studies using urine as the source. Potlog-Nahari *et al.* studied vascular endothelial growth factor-A and determined it was not suitable for detecting endometriosis, and Tay and Chua found that serum CA 125 levels were better than urine for prediction [3, 4]. Urine as a potential non-invasive fluid is largely unstudied.

Proteomic analyses for endometriosis diagnosis

Proteomics is still a relatively new art and its power is increasing every day along with the equipment and techniques available. The dynamic range of protein in plasma spans at least ten orders of magnitude, causing

many marker proteins to be highly diluted and difficult to detect over the background of normal proteins. In most proteomic approaches, mass spectrometry (MS) plays a part, even a critical part, in the analyses. There is often an enrichment technique and/or separation technique up front to increase the power and sensitivity of the approach. Apart from the discussion of SELDI to follow, almost all MS analyses involve the digestion of the protein into peptides using specific enzymes. The most common of these is trypsin, which cuts at arginine and lysine residues, both of which have basic side chains, which assists in the MS analyses. Electrospray ionization (ESI) is the most common method for sample introduction into the MS. In this a liquid containing the sample (peptides) is sprayed into the source of a mass spectrometer fitted with an ESI source. There is a potential (typically 2–5 kV) between the spray tip and the entry to "leak" into the mass spectrometry vacuum system. The liquid is drawn to a Taylor cone and the electric field at the tip causes the liquid to become a very fine charged mist. Within the MS the solvent is removed, leaving dry charged peptides to enter the MS analysis system. Especially for tryptic digests, most peptides form multiple charged species. There are multiple MS analyzers available and these determine the mass divided by the charge (m/z) of these peptides. The mass accuracy of modern instruments has improved and peptides are often measured within a definable 1–2 ppm. In addition, most proteomics analyses involve MS/MS analyses where individual peptide ions are activated or collided with gas. This results in fragment ions that provide sequence information, largely resulting from fragments occurring along the peptide backbone. There are multiple database search engines that can assign peptide sequences based on a database search and link these to proteins covered by multiple peptides. These search engines include Mascot from Matrix Science (http://matrixscience.com) and Sequest from ThermoElectron (San Jose, CA). The number of proteins detected is dependent on the pre-separation methods used before the MS (such as liquid chromatography, LC). Changes in proteins may be determined by the position in the search list (higher score, more protein) and other approaches.

There are multiple other approaches for differential analysis of proteomic samples. These are stable isotopic label approaches or label-free approaches. The approaches that use stable isotopic labels involve reaction of proteomes from different conditions with isotopically labeled reagents. Isotope codes affinity tags (ICAT) have a heavy and light form and allow the comparison of just the cysteine peptides. After labeling, the proteomes are mixed. The newer iTRAQ approach has up to eight reagents, all of which have the same molecular weight and label each peptide in a proteome. All eight proteomes may be mixed and peptides from each have the same molecular weight. However, on fragmentation (MS/MS) marker ions are detected at low m/z, which allows the direct quantification of the amount of each peptide in each proteome that was mixed. All label approaches limit the number of comparisons and are not suitable for the analysis of many individual patients.

Since each is run separately on the MS instrument label-free methods are not limited by the number of samples analyzed, as samples are almost always eluted from a LC into the ESI source of the MS. In order to compare samples, a time alignment of the LC profiles must be performed to allow for any chromatographic variation. Only after that can the runs be compared between patient samples and replicate analyses. Methods using label-free approaches are currently under optimization in many facilities, including our own, called DifProWare. We align the chromatography by the exact elution time of peptides from major proteins present in the sample using a single selected run as the standard elution profile. Then all the aligned peptides that elute are computed into a time/mass/intensity spreadsheet for every peptide in each sample based on the MS data acquired. The MS/MS data that were co-acquired in the run are combined and used later to assign peptide sequences and protein names. Data from every sample and LC-MS run are then aligned by mass and time within allowed small tolerances and a combined spreadsheet of the aligned data is produced that shows a single line per peptide followed by the intensity of this peptide in every run. We can currently process up to 400 data files at a time and generally analyze all samples in triplicate. The final spreadsheet adds the MS/MS analyses and attempts to assign all peptides detected, using MS/MS data from all of the runs to increase coverage. Not all peptides detected are hit by the MS/MS sequencing in each run and this is somewhat random for the lesser peptides. Using multiple runs provides significant increased coverage, especially if the protein or peptide is significantly elevated in a single sample. However, a peptide needs only to be identified if it shows a statistical difference. The output is ideally set up for statistical analysis.

Normalization of data sets can be a problem. With a serum or plasma proteome, the distribution of the major proteins is relatively constant and multiple approaches are suitable for normalization of the data intensities which may represent variations in sample preparation and MS sensitivity. In samples that may have variable contaminations from blood and other proteomic sources, this is more difficult. This is true of PF, FF and EF samples. We have analyzed a significant number of PF samples and are using ratios of similar proteins or those that contrast the over-expression of potential biomarkers associated with the disease. We have yet to see a good solution to this problem but statistical methods can be used to help with the normalization.

MALDI and SELDI analyses

MALDI (matrix assisted laser desorption ionization) may also be used as an alternative to ESI. This involves desorption of the proteins or peptides from a surface using a laser which is tuned to a matrix molecule that the sample is dissolved in. Most MALDI instruments use a nitrogen laser which has a 334 nm emission and the matrix is selected that has a maximum absorption close to that value. For peptides, MALDI gives largely singly charged species as compared to the multiply charged peptide ions in ESI. With total proteomic analyses, such as may be involved in biomarker discovery, a protein fractionation technique is usually employed prior to protein digestion and MALDI. Such methods include gel electrophoresis, liquid chromatography, and offline molecular weight separation devices. When MALDI is used with gel electrophoresis (especially 2D gel) there are very few proteins under most spots and peptide molecular weight data may only be collected and used to identify the protein(s) under the spot. In more complex cases where multiple proteins are in a MALDI sample, instruments such as the MALDI TOF/TOF (Applied Biosystems) may be used to obtain both peptide molecular weight and sequence information to identify the proteins present. In this way MALDI parallels the information that may be obtained from ESI approaches. Two-dimensional gel electrophoresis (2D gel) is a very common analytical tool in proteomics analyses and is designed to reveal differences in protein expression across different proteomic samples. Proteins are first separated by isoelectric point (pI) and then by molecular weight. The plates may be manually inspected but are normally scanned, and the images compared by software that may adjust axes to allow for the differences in the plates. The density of the spots indicates the level of protein present. In-gel digestion of proteins (digestion in the gel spots and extraction of the resulting peptides) can be a time-consuming task if many proteins need to be analyzed. This process can be automated and there are robotic systems available now that can scan gels looking for changes, cut out the spots selected, automatically prepare the in-gel digests, and spot these onto a MALDI plate with the selected matrix. For weaker spots it is always advisable to remove gel spots adjacent to the spots of interest to provide a background scan of the area, and human keratins from dust in the air and skin are major contaminants. With MALDI, it is normal to derive protein identification from the analyses. A pictorial of the MALDI and SELDI methods is shown in Figure 9.1.

A variation of this, SELDI, has been used in endometriosis biomarker research. SELDI (surface enhanced laser desorption ionization) uses a special surface on which the proteins adsorb, and these surfaces may be varied to help select special features of the sample. For instance, surfaces may be anionic, cationic, hydrophobic, or metal affinity. The sample (e.g. plasma) is then applied as a solution and allowed to adsorb to the surface. In general, there is no digestion and intact molecules, including proteins and metabolites, may attach. The non-adsorbed sample is removed and the plate washed to leave only the adsorbed compounds. A matrix is applied to the surface and the sample desorbed with a laser in the same way as with MALDI. Just as in MALDI the matrix may be varied and some will provide more lower molecular weight features while others are better for larger molecules. In general, the data are collected into time bins (time of flight in the MS represents mass) and accumulated (many laser shots) until a satisfactory signal is obtained. The image obtained is processed for features that may be associated with endometriosis as compared to that from controls. Thus, bins and sets of bins that show decreased/increased abundance in the diseased state are identified and used for disease detection. It is important to note that all mass spectra are acquired as mass divided by the charge on the molecule. In most MALDI and SELDI spectra the charge is one but larger proteins such as serum albumin at 66 kDa will show a significant doubly charged state along with the singly charged state. In general, it is not possible to tell the charge state in SELDI spectra although most authors describe their features as mass peaks and report the size in daltons. The term protein is generally used but most of the reported masses (for endometriosis) are larger

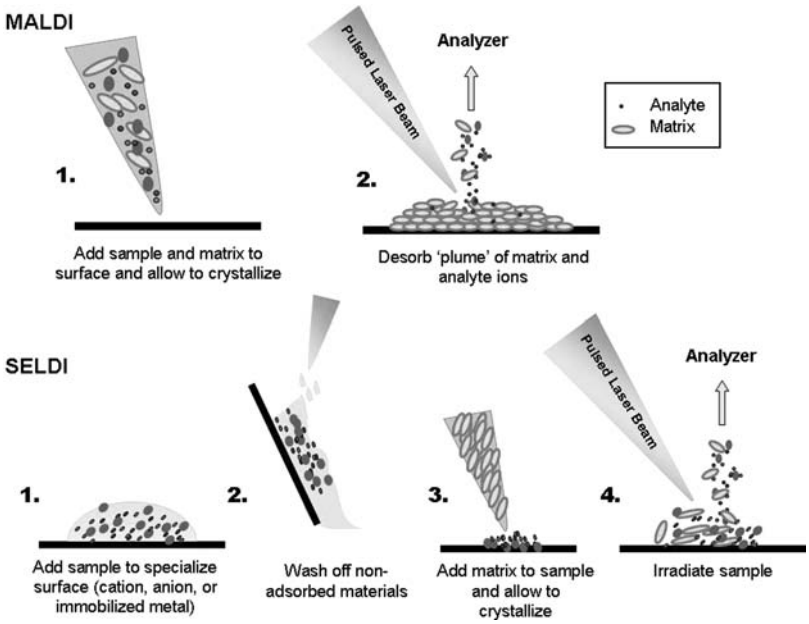


Figure 9.1. Many of the proteomic analyses in endometriosis studies have involved the use of MALDI (matrix assisted laser desorption ionization) and SELDI (surface enhanced laser desorption ionization). Both methods involve the application of a sample to a surface and the addition of a light (generally UV) absorbing matrix. The laser strikes the surface and releases and ionizes the sample which is then analyzed. For SELDI, in general intact proteins from a sample are applied to a special surface (e.g. anionic) and the sample adsorbs. The non-adsorbed material is removed and the surface washed. For MALDI, the sample may be prepared, cleaned, and probably enzyme-digested offline and then applied to the surface. In SELDI features are identified that are often not able to be associated with particular proteins, whereas in MALDI, with digestion, the proteins are often identified. See colour plate section.

peptides with "masses" below 8 kDa where multiple charging is unlikely. They may come from proteins through degradation and could be possibly considered as metabolites. In general, there is no identification of a "protein" peak from SELDI spectra although extensive work can be performed later to possibly provide identification. It can be also considered that the multiple peaks found may originate from the same protein. Another problem with SELDI and the computational methods used to identify/select the features is that there are many more bins than patient samples and there is always a solution. To judge the results, features that are visually apparent are important and any study must do a blinded validation using a different set of patients. Feature-based approaches have been the subject of criticism resulting from its use for ovarian cancer screening.

There have been several papers, especially in the past 2 years, showing the results of SELDI analyses of endometriosis and control samples, mainly in serum/plasma. A selection of these are discussed. In these publications, SELDI is being used as a screening technique for the disease and some of the results appear promising. Selection of controls is critical and, for instance, infertile patients who do not have endometriosis may show features of the infertility cause, rather than endometriosis. Using SELDI, Jing *et al.* compared 59 patients with endometriosis to 31 without and 30 healthy controls [5]. They found peaks at 5830 Da and 8865 Da that gave a blinded sensitivity of 86.67% and specificity of 96.77%, significantly higher than CA 125 for detecting endometriosis. Wolfler *et al.* used 51 endometriosis and 39 controls, processing the data with multiple algorithms to identify the features [6]. They grouped patients by diagnosis and selected five features for each, some of which overlapped. Their sensitivity and specificity were much lower and they concluded that screening of serum proteins by SELDI is not a "quick fix" diagnostic test. Seeber *et al.* selected six proteins (features) that were able to diagnose 55% of patients with 99% accuracy [7, 8]. They combined this with four protein determinations based on results in their previous study and improved the predictions to 73% of subjects with 94% accuracy. Another study by Zhang *et al.* reported results using nine down- and 15 up-regulated protein peaks for their determination and obtained a high sensitivity of 91.7% and specificity of 95.8% using a blinded set representing one-quarter of their patients and controls [9]. The protein peaks identified were grouped in two regions: small (<8.2 kDa) and large between 34 and 47 kDa. Wang *et al.* selected five protein peaks to provde 91.7% sensitivity and 90.0% specificity, and then examined endometrial tissue and discovered a different set of masses [10, 11].

Analysis of endometriosis by 2D gel

Two-dimensional gel analyses have traditionally been used for the differential analysis of proteomes and

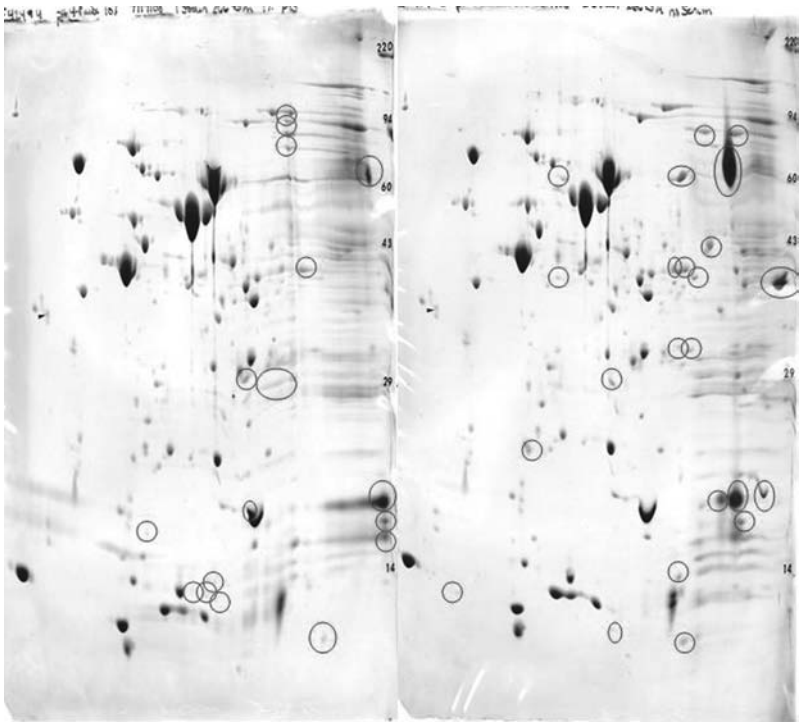


Figure 9.2. Differential gel digests can be used to identify biomarkers in endometriosis and there are many reports of their use in endometriosis biomarker research. Two or more gels representing different conditions (e.g. healthy versus endometriosis) are run and compared, and the differing spots noted as shown in the figure. These spots may then be excised and in-gel digested for protein identification by MALDI or ESI (electrospray ionization). An alternative involves the labeling of each proteomic preparation with differing dyes. The samples are then combined and run on the same gel and the proteomic changes compared using the differing coloured densities.

differences may be observed and selected visually (see Figure 9.2) or using image analysis of scanned gels. For instance, Zhang *et al.* [12] observed differences in 13 spots from serum that correlated with endometriosis. Mass spectrometry can assist in the identification of these spots using in-gel digestion and analysis of the extracted peptides using MS or MS/MS. Of the 13 spots, 11 were identified as known proteins and three showed significant changes in expression with endometriosis. These were vimentin, beta-actin, and ATP synthase beta subunit but further work was needed to verify these as biomarkers. Ferrero *et al.* examined PF by 2D gel and identified nine protein spots that were elevated in endometriosis [13]. These were two isoforms of serotransferrin, one isoform of complement C3, serum amyloid P-component, alpha-1-antitrypsin and clusterin, along with three spots that were not identified. It was noted that most of the proteins were involved with the immune response. EF has also been studied and showed 31 proteins related to cell signaling, cell death, and cell movement, which showed significant changes in expression [14]. Of these, 14–3–3 (signal transduction) and moesin (cytoskeletal structure) were confirmed in a separate group of patients.

Multidimensional protein and peptide separations

There are a variety of alternative strategies for the analysis of proteomics samples other than gel-based approaches and some of these are shown in Figure 9.3 and discussed below. The input of peptides to almost all ESI analyses is by liquid chromatography (LC). This assists the MS to acquire better individual MS profiles of the peptides and increases the coverage. It also enhances the acquisition of MS/MS data by providing the instrument more time to select and acquire peptide sequence data. The longer the run and if the instrument is trained to avoid duplicating acquisition of the same peptide, increased depth of peptide coverage is obtained. To further increase coverage, a multidimensional approach may be used that pre-separates the peptides or protein in the sample into fractions. While protein fractionation may be used followed by digestions of each fraction and MS analysis, MuDPIT is the most commonly applied approach [15] and involves the offline or online collection of fractions separated by ion exchange, each of which is then analyzed by LC-MS/MS. The disadvantage of this approach is the number of fractions that need to be run for each sample and the need to pool

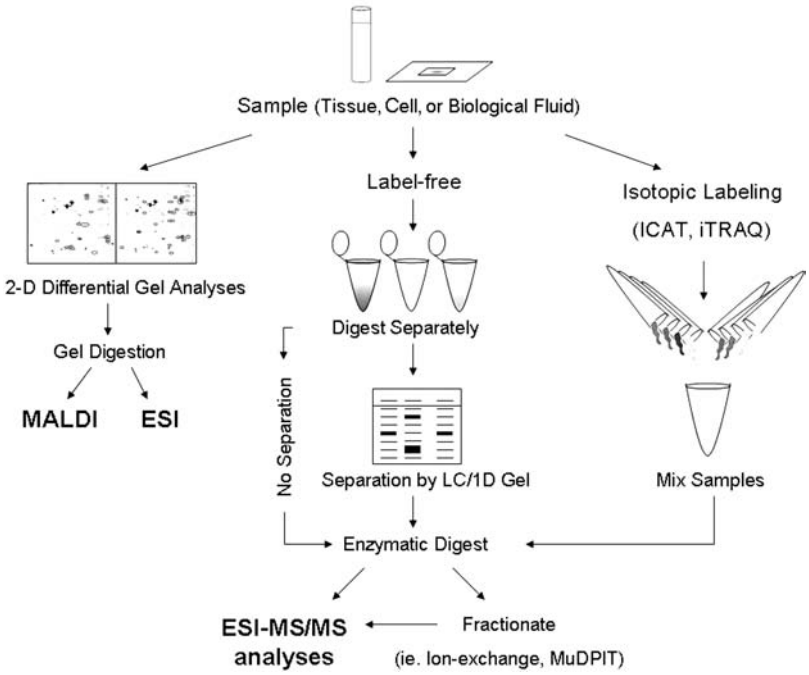


Figure 9.3. Proteomic samples may be analyzed in labeled or unlabeled approaches. 2D gel analyses are common and proteins that alter may be identified by MS after in-gel digestion. While total proteomic digests may be analyzed as a single fraction, many groups pre-fractionate the sample by multiple methods such as liquid chromatography and 1D gel separations at the protein stage. Samples are then digested where they can also undergo a pre-fractionation using a method such as MuDPIT (multidimensional protein identification technology) where the peptides are pre-fractionated on an ion exchange column. Final analysis is generally by LC-MS/MS where peptides are separated on a reverse-phase column prior to ESI ionization and sequence-based identification. Methods such as iTRAQ rely on the labeling of each sample which is then mixed and analyzed with MS/MS often using a multidimensional pre-fractionation such as MuDPIT. The ratio of ions from each labeled form of the peptides may be directly compared to yield relative quantitative analyses of each peptide (and thus proteins) across all samples. See colour plate section.

samples to make this feasible. Pooling of samples has dangers and we have observed that a single sample containing a high level of a protein may skew the analysis of a combined sample. For instance, we have observed selenium binding protein significantly elevated in select gynecological secretions that are from cancer patients, but it is not found in normal controls. We do not consider this as a biomarker and it may be the result of the consumption of selenium supplements by these individuals. Thus methods that require pooling of samples may produce false leads and this may also apply when limited numbers of analyses are possible, such as with the iTRAQ 4- or 8-label approach.

Targeted proteomic analysis of serum and plasma

The difficulty in performing plasma proteomics can be observed in the results of the HUPO plasma proteome initiative. While as many as 9000 proteins were detected, a small percentage of these were reproducibly detected across all collaborating laboratories analyzing the same samples. With the very high abundance of the major blood proteins, coupled with the dilution of any biomarker in the whole blood volume, plasma, and serum are difficult discovery targets for the detection of endometriosis, especially in early stage. The most common approach is to deplete the major proteins using depletion kits and columns that feature a mixed antibody bed. Commonly the top 12–14 proteins are depleted but a new antibody kit is available that depletes the top 20. The co-removal of biomarker proteins needs to be considered because they can become depleted during this process. As a result, almost all protein analysis in blood uses routine clinical analyses such as ELISA.

There are many reports which target the levels of protein in plasma or serum, tested either singly or in combination. The predominant aim is to find a better marker for endometriosis diagnosis than CA 125 by the examination of either a single protein or cohorts of proteins. Table 9.1 provides a partial list of these and recent references with their conclusion. In many cases multiple proteins were examined, such as the adhesion molecules sICAM, sVCAM, and E-selectin, the interleukins IL6, IL8, and IL-12, and markers of oxidative stress. For some of these, PF/EF/FF analyses have also been performed but these will be discussed later. Unfortunately the conclusion to most of these blood analyses is that CA 125 remains the best predictive marker for endometriosis although combinations of other proteins may be helpful. However, recent reports

Table 9.1. Potential biomarker proteins for the prediction of endometriosis from serum and plasma and the current status.

Protein measured	Results reported	Reference
CA 19–9	May be a useful marker when used in conjunction with CA 125	[18–20]
Soluble CD163	Not useful as marker	[21]
CEA carcinoembryonic antigen	Not useful as a marker alone; possibly useful with other proteins	[20]
IL-6	Promising marker, especially for the analysis of mild to moderate disease	[18, 22, 23]
IL-8	Plasma levels significantly higher than controls	[18]
IL-12	Elevated in severe endometriosis	[24]
sICAM-1, soluble intercellular adhesion molecule-1	Does not discriminate endometriosis. Detected in late stage	[25, 26]
sVCAM-1, soluble vascular cell adhesion molecule-1	Detected in late stage	[26]
E-selectin	Not statistically significant	[26]
C-reactive protein	Significantly elevated over controls	[18]
Complement C3a	No difference in levels	[27]
Follistatin	92% sensitivity and 92% specificity as marker of ovarian endometrioma	[28]
Insulin-like growth factors 1 and 3	No significant differences	[29, 30]
Leptin hormone	May be useful as part of a panel of markers	[8]
Monocyte chemotactic protein	Significantly elevated in endometriosis and may be used with other markers	[7, 22, 31]
Osteopontin	Possible useful marker	[32]
Soluble tumor necrosis factor receptor-1	Possible biomarker during the follicular phase of the cycle	[29]
Tumor necrosis factor-α	No statistical difference in endometriosis	[22]
Urocortin	May be useful for the differential diagnosis of ovarian endometrioma	[33]
Markers of oxidative stress	Not consistent with staging or progression of the disease	[34]

do show that some cytokines, especially IL-6, may be promising markers, especially for mild to moderate staging. The paper by Seeber *et al.* possibly summarizes the current status of the analyses by stating in its title "Panel of markers can accurately predict endometriosis in a subset of patients" [7].

In contrast to proteomic analyses, circulating cell-free DNA has been suggested as a potential plasma or serum marker for endometriosis. In research by Zachariah *et al.*, cell-free DNA from 500 µl of plasma and serum and 19 endometriosis patients was compared with normal controls and mild cases [16]. The DNA was amplified using the real-time multiplex polymerase chain reaction (RT-PCP) and was identified in endometriosis patients with 70% sensitivity and 87% specificity. Like most new tests, this is still not better than CA 125, which is reported to have a positive predictive value of 92.9% at the cutoff value of 30 U/ml [17].

Peritoneal fluid as a discovery proteome

The collection of peritoneal fluid is invasive and one that can only be performed during surgery. However, this sample is an excellent source for the discovery of changes in protein expression in the peritoneal cavity as a result of endometriosis, especially in advanced stages as it spreads within this space. Ferrero *et al.* have published several papers examining the proteome of peritoneal fluid, using the comparative analysis of 2D gels with Phoretix 2D software. Protein spots that were determined to have changed along with normalization proteins were in-gel digested and identified using MS [13, 35–37]. In the first report, they examined proteins that had a pI of 4–7 with masses between 10 and 190 kDa. Of the 98 spots consistently observed, they found that 11 of these had significantly higher

expression in endometriosis and two had lower expression. The proteins were identified and included four isoforms of α2-Heremans Schmidt glycoprotein, three isoforms of α1-antitrypsin, one isoform of S100-A8, and one of apolipoprotein A-1. The isoforms of antitrypsin increased significantly with severe disease but this protein can be considered a stress response protein and not be specific to endometriosis. S100-A8 also showed expression levels that correlated with severity. They further looked at the correlation of proteins with the ASRM endometriosis staging using PF from 109 women and analyzed a total of 470 protein spots. Three of these were higher in stage I/II and nine higher in stage III/IV. For stage I/II, the proteins were a haptoglobin alpha chain isoform, α1b-glycoprotein, and an unknown protein. For later stages, isoforms of α1-antitrypsin, α1b-glycoprotein, S100-A8, and serotransferrin were determined to be markers. It was noted that most of these proteins were related to inflammation and immune response. They further examined fertile and infertile women with endometriosis and again observed that certain isoforms of proteins distinguished the patient classes.

These continuing studies by Ferrero *et al.* are the most extensive performed on the PF proteome. There are multiple other reports of proteins that show altered expression in endometriosis and these have largely been analyzed by targeting the protein(s) using more traditional methods such as ELISA. As is true of most biomarkers, proteins showing increased expression are considered the most promising biomarkers. Using a selection of 105 patients submitted to laparoscopy, Fairbanks *et al.* demonstrated that the increased expression of IL-12, but not IL-18, previously observed in blood were also observed in PF [24]. The levels correlated with severity of the disease and were most marked in advanced stages. They suggested that this may be related to induction of the Th1 immune response. In other studies, the soluble form of CD44 was observed to be higher in endometriosis [38], and ferritin levels, which may reflect iron metabolism in the peritoneal cavity, were elevated [39]. Annexin I protein is over-expressed in the eutopic endometrium of patients with endometriosis as compared to controls and Li *et al.* went on further to show that this protein was detected in the PF at increased levels. They suggested the protein may make the pelvic environment "permissive" to the adherence and implantation of endometriosis cells [40]. While levels of proteins may increase and correlate with increased blood levels, Florio *et al.* observed that their promising urocortin serum marker was not detectable in PF [33]. In contrast to the increased levels of many proteins, in a study of 147 women (77 with endometriosis) both serum and peritoneal fluid levels of interferon-γ-induced protein-10 (CXCL10) were reduced in endometriosis and it was suggested that this "indicate[s] an impaired immune activity" in endometriosis [41]. A number of other reports have focused either on selected individual proteins or groups of protein such as the cytokines. Macrophages, including those from PF, are highly metabolically active immune cells that produce and secrete a host of different cytokines and other growth factors and may be the source of many proteins studied in PF. PF from endometriosis patients shows a significantly increased concentration of activated macrophages. The proteins shed from PF macrophages have also been investigated as sources of markers. Thus, a positive correlation to endometriosis was observed for the levels of α- and β-estrogen receptors, differentiation markers, and pro-inflammatory cytokines, including TNF-α, IL-6, and IL-1β in a study involving 30 endometriosis patients and 22 controls [42]. The same cytokines have been examined and used by a number of other groups [43–47]. There are mixed conclusions and it has been suggested that these factors may better distinguish fertile from infertile women rather than endometriosis diagnosis. Many of these proteins are those reported as potential markers in less invasively obtained fluids.

The follicular fluid proteome

There are a few reports on the measurement of levels of proteins in follicular fluid, maybe due to the difficulty of sampling. However, FF may be significantly enriched in proteins that are transported down from the ovaries and thus be an ideal proteomic source for discovering markers of ovarian endometrioma, although no reports of this use have been observed. Proteins that have been reported to be elevated in FF have some similarity to PF and include cytokines (especially IL-6), TNFα, and epithelial neutrophil-activating peptide 78 [48, 49].

The endometrial fluid proteome

There are two reports of extensive analysis of the endometrial fluid proteome and the changes that occur in endometriosis, both published in 2009. In the first, 2D

gel analyses were performed using three groups: control subjects and early-stage and late-stage endometriosis patients [14]. The analyses focused on almost 400 protein spots that were present in ≥70% of the samples. Of these, 31 showed at least a two-fold difference in expression in a three-way comparison between groups. A panel of 22 markers could distinguish early-stage endometriosis and 10 distinguished advanced-stage from those without endometriosis. The majority of proteins selected in these panels were related to cell motility, signal transduction, cell cycle regulation, and cytoskeletal structure. Amongst the list of proteins selected, names common to previous analyses include glycodelin, annexin-A1, and ferritin. As previously reported in 2D gel analyses, select isoforms of biomarker proteins were observed to change, such as with moesin and the 14–3–3 protein family. The differences in expression of these two proteins were confirmed with a second group of endometriosis patients.

The second report by Casado-Vela *et al.* was much more extensive and used complementary analyses to increase the proteomic coverage [50]. An extensive analysis of the proteome of EF from the secretory phase of the menstrual cycle was performed. Samples were analyzed by 2D gel electrophoresis followed by the digestion and MS to provide the identification of 607 protein spots. The proteomes were also separated on a preparative 1D gel which was sliced into 29 sections after running, and the slices in-gel digested with trypsin. The tryptic digests were then analyzed on two LC-MS/MS instruments. The final result is a reference EF proteomic database containing a total of 803 proteins which may become a rich source of proteins for further examination as potential biomarkers. Its aim was to catalog the EF proteome rather than to discover proteins that are differentially expressed in endometriosis.

The potential of cervico-vaginal fluid (CVF)

With the gentle mucus flow through the uterus and out the cervix and vagina, CVF may be an excellent biomarker source that is already sampled during gynecological visits. There are already a number of proteomic analyses of CVF that pave the way for biomarker discovery [51–55], but there appear to be no reports that have focused on the use of this fluid for endometriosis research.

Post-translational modifications (PTMs)

There are many reports of isoforms, including those previously discussed in this chapter, of specific isoforms of proteins showing changes that are consistent with the endometriosis diagnosis. This is especially observed in 2D gel analyses where lines of spots are assigned to the same protein. These are caused by PTMs on the proteins such as glycosylation and phosphorylation. These modifications can alter the action of a protein; phosphorylation changes are associated with activation and deactivation, especially in terms of protein-protein interactions. Glycosylation can change in disease and aberrant glycosylation is listed as a hallmark of cancer. With the similarity of endometriosis and tissue invasion and metastases, does glycosylation change in endometrial secretions and tissues? Changes in glycosylation profiles in endometriosis have been observed, largely based on lectin binding/profiling [56–58]. It has also been reported that autoantibodies associated with endometriosis may be formed against specific carbohydrate epitopes [59, 60]. There is a major effort to improve proteomic methods for the analysis of glycosylation and as methods improve, the detailed changes associated with endometriosis will be revealed and this could lead to future, highly specific biomarkers based on some of the current leads.

Summary

There are many excellent reviews of proteomics and biomarkers in endometriosis [20, 61–69]. However, the current opinion in gynecological practice is that, while not ideal, CA 125 is still the best indicator. However, many new endometriosis biomarker proteins are being identified and some reported have sensitivities and specificities better than CA 125. It is hoped that these will bring about a routine screening for endometriosis and detection in very early stages where treatment options are optimal. While panels of 10, 20, and more markers are also being suggested, it is unlikely these will be accepted unless the individual markers are associated with specific pathologies or stages of the disease.

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Chapter 10

Antiangiogenic agents for endometriosis

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Introduction

Endometriosis is a common, benign, estrogen-dependent, chronic gynecological disorder associated with pelvic pain and infertility and pathologically defined by the ectopic presence of both endometrial glands and stroma outside the uterine cavity.

The prevalence of endometriosis approaches 14% in the general female population; 20% to 40% in infertile women [1], 6% to 18% in women undergoing sterilization [2], and 15% to 70% in patients with chronic abdominal pain [3–6]. However, a large proportion of women with the disease may be asymptomatic, which may lead to an underestimation of the number of cases.

This entity is an estrogen-determined disease and therefore affects almost exclusively and is most commonly diagnosed in women of reproductive age, although times for diagnosis can be very long because of variability in symptoms and signs and confusion with other disorders.

Symptomatic disease may cause prolonged suffering and disability, negatively affecting health-related quality of life [7, 8]. Surgery is currently the only diagnosis option, by direct visualization, and also it is often considered the best treatment alternative in women with symptomatic endometriosis for improving the pain symptoms [9], but only provides temporary relief and symptoms recur in up to 40–50% of women after 5 years [10]. Therefore, a combination of surgery and medical therapy is the approach employed in almost every case with endometriosis. Endometriosis continues to remain a significantly under-treated disease [11].

The two principal aims of current pharmacological treatments are the pain relief, amelioration of infertility, or both. The main problem is that medical therapies for pain are, in general, not useful for infertility and surgery is usually used to treat infertility associated with endometriosis, even if the analysis of the non-randomized trials does not completely support this [12–14]. During surgery, some harm can also be induced, such as removal of healthy ovarian cortex with reduction of the ovarian pool and subsequent increased risk of earlier menopause [15, 16].

Estrogen production plays an important key role in endometriosis and it is found in high concentrations in endometriotic lesions [17]. Due to the estrogen-dependency of endometriosis, the most common agents that have been used to treat the disease are drugs able to suppress ovarian function and limit growth and activity of endometriosis and associated pain, including gonadotropin-releasing hormone agonists (GnRHa), oral contraceptives, androgens, progestagens [18, 19], and most recently aromatase inhibitors [17].

GnRHa are currently the most common medical therapy for endometriosis, reducing the estrogenic pattern in these patients, but they have a brief effect due to the endometriosis recurrence at the resumption of menstruation after the end of treatment. GnRHa are generally well tolerated, and are effective in relieving the symptoms of endometriosis; however, the low estrogen state that they induce is associated with adverse effects including acceleration in bone mineral density loss [20].

The advantage of oral contraceptives over other hormonal treatments is that they can be taken indefinitely and it has been reported that they are more effective in treating dysmenorrhea than GnRHa [21]. Androgens are used for endometriosis treatment inducing endometrium atrophy. Danazol inhibits the steroid hormone production from the ovaries, followed by a hypoestrogenic state contributing to an

Human Assisted Reproductive Technology: Future Trends in Laboratory and Clinical Practice, ed. David K. Gardner, Botros R. M. B. Rizk, and Tommaso Falcone. Published by Cambridge University Press. © Cambridge University Press 2011.

inhibition of eutopic and ectopic endometrial growth [22]. Danazol and GnRHa have a similar effectiveness in reducing endometriosis-related symptoms and endometriotic lesions growth [23]. The main problem of this treatment is its potential androgenic side effects including weight gain, breast atrophy, hot flashes, and hirsutism [24, 25] and its action is effective in stages of minimal endometriosis [26, 27].

Progestins, which have been in use for many years, have been shown in several randomized controlled trials to provide equivalent efficacy to danazol or GnRHa for the relief of endometriosis-associated pain [28]. However, it cannot be excluded that the systemic effect remains essential for the therapeutic success. In this regard, establishment of anovulation and of a steady hormonal environment with consequent amenorrhoea may be revealed to be more important than the specific type of steroidal environment achieved in terms of estrogen and/or progestin serum levels or drug-associated androgenic activity [29].

Aromatase inhibitors are able to reduce or eliminate endometriotic implants and associated pain by interfering in the estrogen biosynthetic pathway. They are commonly used in combination with a GnRHa, progestin, or oral contraceptive because they present fewer side effects and may be administered long term or for repeated courses [30].

The absence of any wholly successful medical or surgical therapy and the unacceptable side effects provoked by long-term medical treatments and the epidemiological and social impact of endometriosis make it absolutely necessary to continue the research in this field to understand the mechanisms involved in endometriosis progression and to discover new treatments. It is well known that endometriosis patients present alterations of inflammatory and immune responses, angiogenesis and apoptosis, facts that enhance the survival and replenishment of endometriotic tissue [31–35]. Of particular interest is the angiogenic potential of the human endometrium. Endometriotic lesions are larger in areas with a rich blood supply [36] and it is well known that the establishment of a new blood supply is essential for the survival of ectopic endometrial implants and the development of endometriosis [37]. Women with endometriosis present a peritoneal environment with increased angiogenic activity. Moreover vascular endothelial growth factor (VEGF), one of the most important regulators of endometrial angiogenesis, is expressed in endometriotic lesions, especially in the red ones, which is a consequence of this being the most active and fastest-growing type of lesion [38, 39]. All these findings suggest that targeting the angiogenic process, whose presence in endometriotic implants is essential for their establishment and development [36], could be a novel therapeutic approach to endometriosis in humans.

Angiogenesis and endometriosis

Angiogenesis involves the formation of new blood vessels released by pre-existing vessels. The endometrium of women with endometriosis has an increased capacity to proliferate, implant, and grow in the peritoneal cavity. The relationship between endometriosis and angiogenesis has been studied in ex vivo and in vitro animal models analyzing the revascularization process of experimental endometriosis lesions [40] and demonstrating that an adequate angiogenic response is critical for the successful survival and growth of endometrial tissue in ectopic locations [41, 42].

The continuous angiogenic stimulus and impulses for vascular remodeling to meet the needs of developing endometriotic tissue are guaranteed by the chronic inflammatory environment and the innate properties of the human endometrium [41, 42]. The existence of an imbalance between pro- and antiangiogenic growth factors in peritoneal fluid from endometriosis patients has been reported [40, 43].

Endometrial angiogenesis is regulated by several factors, the VEGF family being particularly important [44]. It is widely believed that VEGF is the main stimulus for angiogenesis and increased vessel permeability in this disease [33]. Originally identified as vascular permeability factor (VPF), VEGF is also a potent selective endothelial mitogen and survival factor, which delays or reverses senescence of endothelial cells [45–48]. The human VEGF gene has been mapped to chromosome 6p12 and is made up of eight exons [49]. Exons 1–5 and 8 are always present in VEGF mRNA, whereas the expression of exons 6 and 7 is regulated by alternative splicing. This phenomenon produces various VEGF isoforms. In humans, five different VEGF mRNAs have been detected encoding the isoforms VEGF121, VEGF145, VEGF165, VEGF189, and VEGF206 [50]. The isoforms VEGF121 and VEGF165 appear to be mainly involved in the process of angiogenesis [51]. Five endothelial cell-specific receptor tyrosine kinases, Flt-1 (VEGFR-1), KDR/Flk-1 (VEGFR-2), Flt4 (VEGFR-3), Tie and Tek/

Tie-2, have so far been described, which possess the intrinsic tyrosine kinase activity essential for signal transduction [52, 53]. The receptor Flk-1/KDR appears to be mainly involved in regulating angiogenesis and vasculogenesis [54, 55].

The high angiogenic profile of human proliferative endometrium tissue was demonstrated by quantitative RT-PCR and confirmed at protein level, revealing that VEGF-A is the predominant angiogenic factor [56]. Moreover it has been recently shown that deep endometriosis has a higher VEGF expression than other lesions [57]. Given its potent angiogenic function, it has been proposed that inhibition of VEGF may be an attractive novel therapeutic approach for the treatment of endometriosis [58].

Targeting angiogenesis in endometriosis

Several substances have been demonstrated to exert an effect on the shed endometrial tissue with the use of experimental approaches that have been designed to block this critical step in the development of the disease. Several antiangiogenic agents, most of them with cytotoxic properties, have been successfully tested in experimental models of endometriosis [55, 59–65] inhibiting new vessel formation. These compounds target specifically the endothelial cells without penetration in the tissues.

The first approach was the use of a soluble truncated receptor that antagonizes VEGF and anti-VEGF-A antibody. The administration of both substances in experimental endometriotic lesions showed a significant decrease of endometriotic explants and vascular destruction [60]. Other antiangiogenic agents were tested in a heterologous endometriosis mouse model. Nap *et al.* [56] demonstrated that avastin (a specific VEGF A inhibitor) and other general efficient angiogenesis inhibitors (TNP-470, endostatin, anginex) significantly decreased the number of endometriosis lesions and blood vessels compared to the untreated animals. One of these agents, the endostatin [61], as well as two synthetic fragments of the endostatin molecule [66] were subsequently evaluated in vitro and in vivo in mice, showing that although the growth of experimental endometriotic lesions and endothelial migration associated with the angiogenic process were inhibited when the treatment was initiated immediately after surgical transplantation, endostatin therapy was ineffective when being applied in established lesions [61].

Park *et al.* [67] confirmed the usefulness of anti-VEGF treatments through the administration of immunopurified antibody blocking VEGF receptor (anti-Flk1 antibody) in Rhesus monkeys. After the administration of this compound, an inhibition of explants formation was observed.

Eicosanoid ligands of peroxisome proliferator-activated receptor-gamma (PPAR-g), a VEGF regulator, were tested in human endometrial cell cultures [68]. It was observed that PPAR-g ligands repressed VEGF gene expression, suggesting that agonists of this nuclear receptor might be exploited pharmacologically to inhibit pathological vascularization in endometriosis [68]. Thiazolidenedione, a PPAR-g agonist, was subsequently evaluated in a baboon endometriosis model showing an inhibition of endometrial VEGF gene expression and a reduction of surface area of experimentally induced endometriotic lesions [69].

The use of angiogenesis inhibitors may also have some disadvantages. They require chronic administration and are likely to be particularly favorable in early-stage disease as they are prone to prevent recurrence after surgery as well as interfering with new vessel formation, conferring a preventive effect. Endometriotic lesions diagnosed at early stages have not yet progressed beyond the superficial lesion, which is usually highly angiogenic [36, 70].

Several anticancer drugs with antiangiogenic potential have been found to have a detrimental effect on reproductive function in both animal models and patients [71, 72]. However, the most important concern remains the risk of teratogenic effects associated with antiangiogenic therapies in case of pregnancy and the side effects of these drugs. VEGFR-2-mediated endothelial cell signals are critical to maintain the functionality of luteal blood vessels during pregnancy [72]. Treatment with TNP-470 was found to completely inhibit embryonic growth [71]. Thus, other alternatives able to target VEGF without affecting those relevant functions must be sought.

In humans, some of these agents with antiangiogenic properties such as TNP-470, avastin (a human anti-VEGF antibody), vitaxin (a humanized anti-avb3-integrin antibody), and batimastat (a MMP inhibitor) have been tested in oncology patients, with the aim of inhibiting the tumor growth or metastasis prevention by blocking the angiogenic process. However, only bevacizumab, a humanized VEGF-neutralizing antibody that neutralizes activators of angiogenesis [73], has been approved [74] and tested, producing

satisfactory results [75] for the treatment of certain defined cancer indications. However, the systemic administration of these agents inhibits other important physiological processes, inducing severe side effects [76].

Dopamine agonists in endometriosis

The studies in experimental oncological models achieved by Basu *et al.* [77] demonstrated that dopamine agonists (DA) have an antiangiogenic effect promoting the VEGFR-2 endocytosis in endothelial cells, preventing the VEGF- VEGFR-2 union and avoiding receptor phosphorylation and signal cascade (Figure 10.1).

These agents have been employed in the prevention of ovarian stimulation syndrome in animals, showing that cabergoline, a DA, reduced ovarian hyperpermeability and ascites formation without affecting corpus luteum angiogenesis and function by dephosphorylation of the VEGFR-2 [78]. When these drugs were administered in humans, ascites, hemoconcentration, and ovarian perfusion were also reduced [79].

Cabergoline is currently used for the suppression of breast-feeding and hyperprolactinemia treatment [80–83] without increased risk of spontaneous miscarriage, premature delivery, multiple pregnancy, or congenital abnormalities in pregnant patients [83, 84].

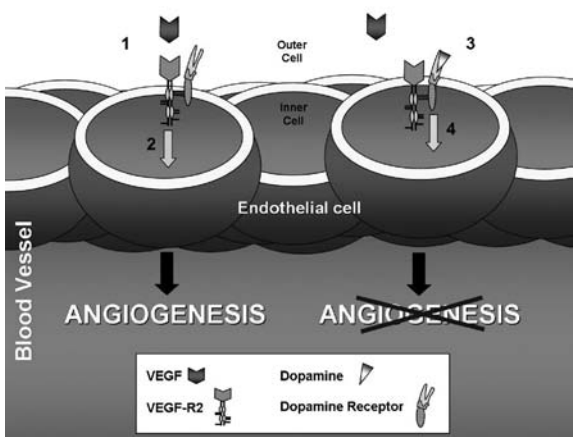


Figure 10.1. (1) During the angiogenesis process VEGF binds to VEGFR-2 located on the epithelial cell surface. (2) The VEGF-VEGFR-2 binding promotes receptor phosphorylation and initiates the angiogenic signal cascade. (3) The dopamine neurotransmitter binds to the dopamine receptor-2 (Dp-r2). (4) Dopamine-Dp-r2 binding promotes VEGFR-2 endocytosis in endothelial cells and prevents VEGF-VEGFR-2 binding. As a consequence, there is no receptor phosphorylation and the angiogenesis signal cascade is inhibited. See colour plate section.

Based on the aforementioned findings we undertook a series of studies to analyze the role of cabergoline in inhibition of neoangiogenesis in endometriosis [64]. Human endometrium fragments were stuck into the nude mouse peritoneum wall by *n*-butyl-ester cyanoacrylate adhesive. Three weeks later, time enough for the revascularization of endometrium implants on the mouse peritoneum, cabergoline was administered at different doses during 2 weeks.

After treatment, the animals were sacrificed and the experimental lesions were recovered. Macroscopic lesions were observed on the peritoneal wall in all experimental groups, the endometriotic lesions of untreated animals presenting a rich vascular net (Figure 10.2A); however, cabergoline-treated animals presented pale-coloured lesions revealing a less developed vascularization (Figure 10.2B, C).

It is well known that the inhibition of the angiogenic process and the vascular destruction affect tissular integrity, so after cabergoline treatment the tissular status was studied in all the samples by histological techniques. Optical microscopy revealed that the endometriotic lesions of untreated mice presented a high cellular stroma and a histological aspect of complete reorganization and structure (Figure 10.2D), while in treated lesions a lax stroma with lost cellularity and organization was observed (Figure 10.2E, F). When these findings were quantified by morphometric analysis, a significant difference among the groups in the ratio of glands/stroma was observed, showing fewer glands in mice treated with different doses of cabergoline than in controls, and demonstrating that cabergoline treatment produced a decrease in the amount of endometrial glands. Histologically an endometriotic lesion is defined as the presence of both glandular and stromal elements. Thus, when the active lesion number was compared among the experimental groups, a significant decrease in the percentage of active lesions in cabergoline-treated mice with respect to the untreated mice was observed.

The proliferative status of endometriotic cells was investigated and quantified by immunohistochemistry and morphometry techniques, using the Ki-67 labeling index, with which the total antigen expression per square micrometer (proliferation index) was measured (Figure 10.2G–I). The proliferation index was significantly lower in mice treated with low and high doses of cabergoline than in untreated animals.

To test whether cabergoline inhibited blood vessel formation in established lesions, the new and old or

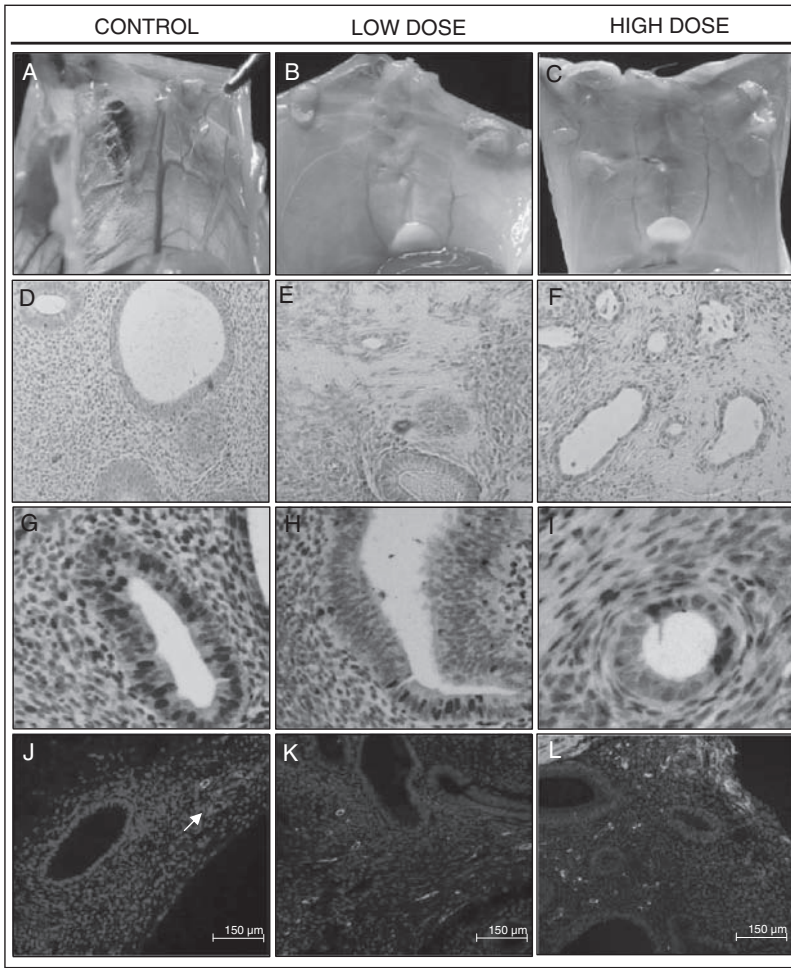


Figure 10.2. Untreated mice lesions (A) show a rich vascular net compared to the lesions of the cabergoline-treated mice (B, C) which present a white aspect and a less developed vascularization. Histological studies reveal a gland's decrement in cabergoline-treated lesions as well as an unstructured stroma (E, F), while the lesions from untreated mice present a complete organization and normal tissular structure (D). The number of positive Ki-67 cells was higher in the lesions from untreated mice (G) compared with the lesions from cabergoline-treated mice (H, I), demonstrating that cabergoline significantly decreased the proliferation index. The lesion's vascularization was analyzed by confocal microscopy (J–L). Modified from Novella-Maestre *et al.* [64]. See colour plate section.

mature blood vessels were detected and quantified by immunofluorescence and morphometry techniques (Figure 10.2J–L) and different angiogenic factors were also studied at the molecular level (Figure 10.3). A significant difference among groups in the ratio newly formed/mature blood vessels was observed, and a significant decrease of proangiogenic factors in the lesions from the mice treated with the DA was also shown (Figure 10.3), indicating that cabergoline treatment was clearly associated with a decrease in the amount of newly formed blood vessels and consequently with the blocking of the angiogenic process. At the protein level, the VEGFR-2 phosphorylation degree was significantly lower in the experimentally induced lesions from cabergoline-treated animals, confirming that the molecular mechanism by which cabergoline exerts its antiangiogenic action is induction of VEGFR-2 endocytosis, preventing VEGF binding and receptor phosphorylation.

The results of this study demonstrated that DAs such as cabergoline have an antiangiogenic effect in the treatment of established experimentally induced endometriotic lesions by blocking the VEGF system.

Recent research consistently associates the use of cabergoline and pergolide (another dopamine agonist) for the treatment of chronic conditions, such as Parkinson's disease, hyperprolactinemia, and restless leg syndrome, with an elevated incidence of cardiac valve regurgitation [85, 86]. In the context of endometriosis, it would be important to explore the use of other dopamine agonists, which may not carry the same side-effect profile.

Based on the results obtained in endometriosis treatment with cabergoline in the rodent model [64]

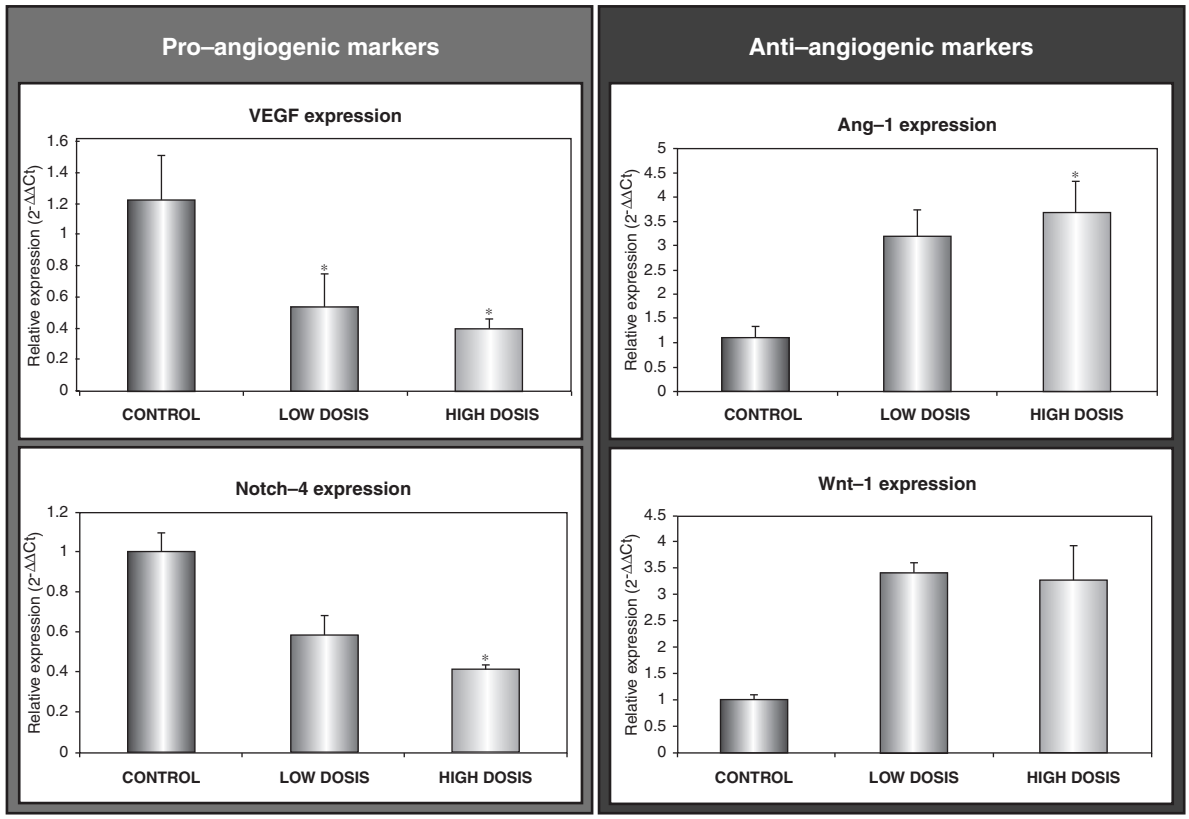


Figure 10.3. Pro- and antiangiogenic markers were analyzed by quantitative RT-PCR TaqMan technology. Cabergoline-treated lesions presented a significantly lower expression of proangiogenic factors VEGF and Notch-4 as compared to controls while expression of antiangiogenic factors Ang-1 and Wnt-1 was clearly up-regulated in the lesions treated with cabergoline as compared to controls (mean ± SEM) (*$P < 0.05$). Modified from Novella-Maestre *et al.* [64].

and the difficulties in employing cabergoline for chronic diseases such as endometriosis, we have finished a pilot study in humans that basically confirms the findings in rodents. This would represent an important advancement in the treatment of the disease and deserves to be further explored.

Conclusions

Medical treatment of endometriosis is a necessary step in the management of the disease due to its high rate of recurrence and different clinical situations. So far, all the efforts have been directed towards the estrogen-dependency of the disease. However, other relevant pathophysiological features are currently being developed. One of them is interfering with the angiogenesis process, which is critical for the establishment of endometriosis implants. Several strategies taken from oncological developments have been assayed in experimental models of endometriosis with success. However, these experiments showed that targeting VEGF, the main player in angiogenesis, is effective, but the clinical application of these medications to treat endometriosis is questionable, due to their side effects and interference with relevant physiological functions, such as implantation. We developed the hypothesis that dopamine agonists, which interfere with VEGF/VEFGR2 binding, may also be effective in the treatment of endometriotic implants and have the advantage of being safe, including in pregnancy. For this reason, experiments with different dosages of cabergoline were established. We observed reduction in the size and extent of the implants; impaired neovascularization and cellular proliferation, and down-regulation of genes involved in the angiogenic process, showing that dopamine agonists represent an interesting real alternative in the medical treatment of endometriosis.

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Chapter 11

New concepts in the management of polycystic ovary syndrome

Roy Homburg

Introduction

Polycystic ovary syndrome (PCOS) is associated with approximately 75% of the women who suffer from infertility due to anovulation [1, 2] and is frequently diagnosed for the first time in the infertility clinic. The majority of women with anovulation or oligo-ovulation due to PCOS often have clinical and/or biochemical evidence of hyperandrogenism. Almost all these women will have a typical ultrasonic appearance of the ovaries [3].

The exact mechanism causing anovulation associated with PCOS is not known and the excess of small antral follicles, hyperandrogenemia, hyperinsulinemia and dysfunctional feedback mechanisms have all been implicated. There are, however, a number of strategies to restore ovulation, most of them reliant on increasing FSH concentrations, either endogenously or exogenously or reducing insulin levels. These include medical therapies, clomiphene citrate (CC), aromatase inhibitors (AIs), metformin, and low-dose gonadotropin therapy or surgically by way of laparoscopic ovarian drilling. Here I will describe in detail treatment with CC, AIs, gonadotropins, and metformin followed by a discussion on the management of women with PCOS undergoing IVF. Laparoscopic ovarian drilling is dealt with elsewhere in this volume.

Weight loss

Just as obesity expresses and exacerbates the signs and symptoms of insulin resistance, then loss of weight can reverse this process by improving ovarian function and the associated hormonal abnormalities [4, 5] and may alone induce ovulation and pregnancy. Loss of weight induces a reduction of insulin and androgen concentrations and an increase in SHBG concentrations. For obese women with PCOS, a loss of just 5–10% of body weight is enough to restore reproductive function in 55–100% within 6 months of weight reduction [4–6]. Weight loss has the undoubted advantages of being effective and cheap with no side effects and should be the first line of treatment in obese women with anovulatory infertility associated with PCOS.

Clomiphene citrate (CC)

Mode of action

Clomiphene citrate (CC) is a long-established first-line treatment for women with PCOS who have absent or irregular ovulation. Paradoxically, its anti-estrogen action in blocking estradiol receptors in the hypothalamus invokes the negative feedback mechanism inducing a change in GnRH pulse frequency, release of FSH from the anterior pituitary and consequent follicular development and estradiol production.

Dose

Clomiphene has been given in a dose of 50–250 mg per day for 5 days starting from any of days 2–5 of spontaneous or induced bleeding. The minimum starting dose may be raised in increments of 50 mg/day each cycle until an ovulatory cycle is achieved. Little advantage is gained in using a daily dose of more than 150 mg, which seems to significantly increase neither the ovulation rate nor follicular recruitment. The, approximately, 20% who remain resistant to CC (i.e. remain anovulatory) are thus identified in three cycles.

Results

A compilation of data collected from the literature [7] is presented in Table 11.1. The notable features are an

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Table 11.1. Results of treatment with clomiphene citrate: a collection of published data [7]

	No. of patients	Ovulation	Pregnancy	Abortion	Live birth
McGregor *et al.* 1968	4098	2869	1393	279	1114
Garcia *et al.* 1977	159	130	64	16	48
Gysler *et al.* 1982	428	364	184	24	160
Hammond, 1984	159	137	67	10	57
Kousta *et al.* 1997	128	113	55	13	42
Messinis *et al.* 1998	55	51	35	4	31
Imani *et al.* 2002	259	194	111	11	98
Total (%)	**5268 (100)**	**3858 (73)**	**1909 (36)**	**357 (19)**	**1550 (29)**

ovulation rate of 73%, a pregnancy rate of 36%, and a live birth rate of 29%.

Clomiphene failures

Patients who do not respond to clomiphene are likely to be more obese, insulin resistant, and hyperandrogenic than those who do respond [8]. A course of six ovulatory cycles is usually sufficient to know whether pregnancy will be achieved using CC before moving on to more complex treatment as approximately 75% of the pregnancies achieved with clomiphene occur within the first three cycles of treatment [9] and pregnancies are rarely achieved following six ovulatory cycles.

Although ovulation is restored in approximately 80%, pregnancy is achieved in only about 35–40% of patients who are given clomiphene [9–11]. There are several possible explanations for this "gap." Clomiphene induces a discharge of LH as well as FSH so those with high basal LH levels are less likely to respond and conceive with clomiphene treatment [12]. However, the most probable factor involved in this large discrepancy between ovulation and pregnancy rates in patients treated with clomiphene is its anti-estrogenic effect at the level of the endometrium and cervical mucus. While the depression of the cervical mucus, occurring in at least 15% of patients, may be overcome by performing intrauterine insemination (IUI), suppression of endometrial proliferation, unrelated to dose or duration of treatment but apparently idiosyncratic, indicates a poor prognosis for conception in my experience when endometrial thickness remains < 8 mm.

Monitoring

Ultrasound evaluation of follicular growth and endometrial thickness on day 11–14 of the cycle is justified by the identification of those who are not responding or have depressed endometrial thickness and is helpful in the timing of natural intercourse or IUI. Although this monitoring implies added expense, this is neutralized by the prevention of protracted periods of possibly inappropriate therapy and delay in the inception of more efficient treatment. In our recent study comparing two large groups of ultrasound-monitored or non-monitored clomiphene-treated cycles, those that were monitored yielded significantly better pregnancy and live birth rates [13].

Adjuvant treatment

Co-treatment with several proposed adjuvants has been advocated in an attempt to produce improved results from clomiphene treatment. The addition of an ovulation-triggering dose of hCG, 5000–10 000 IU, is only theoretically warranted when the reason for a non-ovulatory response is that the LH surge is delayed or absent despite the presence of a well-developed follicle. Although the routine addition of hCG at mid-cycle seems to add little to the improvement of conception rates [14, 15], it is very usefully given when an ultrasonically demonstrated leading follicle attains a diameter of 18–24 mm, for the timing of intercourse or IUI.

Dexamethasone, 0.5 mg/day at bedtime, as an adjunct to clomiphene therapy, suppresses the adrenal androgen secretion and may induce responsiveness to clomiphene in previous non-responders, mostly hyperandrogenic women with PCOS with elevated

concentrations of dehydroepiandrosterone sulfate (DHEAS) [16, 17]. Although this method meets with some success, medium to long-term glucocorticoid steroid therapy often induces side effects including increased appetite and weight gain, which is counterproductive for women with PCOS.

The combined treatment of clomiphene with metformin is dealt with below.

Aromatase inhibitors

Aromatase inhibitors (AIs) are potent, non-steroidal compounds that suppress estrogen biosynthesis by blocking the action of the enzyme aromatase, which converts androstendione and testosterone to estrogens. The AIs letrozole (Femara, Novartis) and anastrozole (Arimidex, Zeneca) have mainly been employed for the treatment of postmenopausal women with advanced breast cancer. Given orally, they are almost free of side effects.

Mode of action

The efficient estrogen-lowering properties of the aromatase inhibitors temporarily release the hypothalamus from the negative feedback effect of estrogen so inducing an increased discharge of FSH [18]. Although the end result of an increased discharge of FSH is common to both aromatase inhibitors and clomiphene citrate (CC), the differences in their mode of action confer several theoretical advantages to aromatase inhibitors used for ovulation induction. (1) Aromatase inhibitors have no effect on estrogen receptors and therefore no deleterious effect on cervical mucus or endometrium. (2) Aromatase inhibitors do not block hypothalamic estrogen receptors and, therefore, the negative feedback mechanism remains intact. This enables regulation of the FSH discharge when estrogen is produced and should reduce the prevalence of multiple follicle development and, consequently, of multiple pregnancies when compared to CC. (3) The half-life of the aromatase inhibitors is about 2 days, much shorter than that of CC [18, 19].

Indications

Indications for the use of aromatase inhibitors in ovulation induction are virtually the same as for CC, i.e. for women with absent or irregular ovulation associated with normal concentrations of endogenous estradiol and FSH (WHO Group II, hypothalamic-pituitary dysfunction). A very large majority of these cases are associated with PCOS. In addition to treatment-naive patients, a trial of treatment with aromatase inhibitors has been suggested for patients who are CC resistant. Preliminary results have shown that this may be a worthwhile step before proceeding to gonadotropin therapy [20].

Dose

Letrozole has been given in a dose of 2.5–5 mg per day for 5 days starting on days 2, 3, 4 or 5 of the cycle, similar to CC. In the case of failure to ovulate on these doses as much as 7.5–10 mg a day has been administered [21] but 5 mg/day would appear to be the optimal dose [22]. Letrozole has virtually no side effects. The optimal dose of anastrozole for ovulation induction has yet to be determined.

Evidence

Recent RCTs have compared the results of ovulation induction for treatment-naive women with PCOS using CC (100 mg/day) with letrozole, 2.5–5 mg/day [23–26]. Two recent meta-analyses of studies comparing pregnancy rates with CC and letrozole have demonstrated slight [27] or clear [28] superiority of letrozole with pregnancy rates ranging from 15–33% per patient and around 16% per cycle. In general, both the number of mature follicles and estradiol levels were lower with the use of letrozole. The multiple-pregnancy rate with letrozole, recorded only in an uncontrolled series, was 0.2% [21].

In a study of women with CC-resistant PCOS given letrozole, an ovulation rate of 54.6% and a pregnancy rate of 25% were achieved, suggesting that this treatment be tried before proceeding to more sophisticated treatments in this group of patients [20].

Although anastrozole (1 mg/day for 5 days) has also been shown capable of inducing ovulation and pregnancy, letrozole has been shown to be more successful in CC-resistant patients [29]. However, it is very possible that this dose of anastrozole was not optimal for this indication [30].

Safety

Initial unsubstantiated fears regarding possible teratogenic effects of AIs have largely been quashed by the reporting of a significantly lesser incidence of both

minor and major congenital anomalies in a very large group of women who conceived using letrozole compared with those who used CC [31]. Despite this, the use of aromatase inhibitors for ovulation induction remains off-label in many countries. Hopefully this limitation will be rescinded in the near future.

As the use of aromatase inhibitors in the treatment of infertility is still in its infancy, many questions still remain to be answered. Trials with aromatase inhibitors for ovulation induction have, reasonably, mimicked treatment with CC, being administered on day 3–7 of the cycle, and optimal doses have not been fully established. It is a little too early to enthuse optimistically about the chances of letrozole and anastrozole to become standard treatment for ovulation induction. However, now the initial studies have been completed, there is enough evidence for optimism to encourage further serious trials for this potentially valuable, simple, and inoffensive treatment.

Metformin

Mode of action

Metformin is an oral biguanide, well established for the treatment of hyperglycemia, that does not cause hypoglycemia in normoglycemic patients. In women with PCOS, metformin is said to lower fasting insulin concentrations but also probably acts directly on theca cells and attenuates androgen production. The sum total of its actions is often a decrease in insulin and androgen levels and, consequently, a resulting improvement of the clinical sequelae of hyperandrogenism. Although oligomenorrhea improves in some women with PCOS, significant numbers remain anovulatory [32]. Metformin does not produce consistent significant changes in BMI or waist-to-hip ratio but the degree of improvement in ovulation frequency is mainly achieved with weight reduction through life-style modification and there is no difference between metformin and placebo in this regard [33]. The improvement has been estimated to represent one extra ovulation every five woman-months [34].

Dose

Metformin for ovulation induction is given in a dose of 1500–2500 mg a day in two or three divided doses. Gastro-intestinal side effects are not uncommon.

Evidence

For induction of ovulation, two randomized controlled trials indicate that metformin does not increase live birth rates above those observed with CC alone, in either obese or normal weight women with PCOS [35, 36]. In fact, the larger of these two trials [35] demonstrated a selective disadvantage to metformin compared to CC and no apparent advantage to adding metformin to CC, except perhaps in those with CC resistance. Clomiphene resulted in higher ovulation, conception, pregnancy, and live birth rates compared to metformin, while the combination of both drugs did not result in a significant benefit. Disappointingly, addition of metformin did not decrease the incidence of miscarriage, which in fact was higher in the metformin group. Furthermore, metformin treatment conferred no additional advantage when administered to newly diagnosed women with PCOS [36]. An ESHRE-ASRM consensus meeting therefore concluded that insulin sensitizers should not be used as first-choice agents for induction of ovulation in women with PCOS, while their administration does not appear to decrease the incidence of early pregnancy losses [37]. In addition, data so far do not confer any advantage to the use of thiazolidinediones over metformin [38].

Safety

Although data to date suggest that metformin is safe during pregnancy, there is no definitive indication for its use during pregnancy. It does not seem to affect the miscarriage rate and although there have been suggestions that continuing metformin during pregnancy may be protective against complications such as hypertension and gestational diabetes [39], currently, this strategy is not widely practiced.

Low-dose gonadotropin therapy

Principle

The aim of the chronic low-dose step-up protocol is to obtain the ovulation of a single follicle. Unlike the conventional protocol, the low-dose protocol employs a dose of gonadotropin that is not supra-physiological but reaches the threshold for a follicular response without exceeding it and thereby produces monofollicular rather than multifollicular ovulation. This practically eliminates the occurrence of OHSS and reduces multiple pregnancies to less than 6% [40].

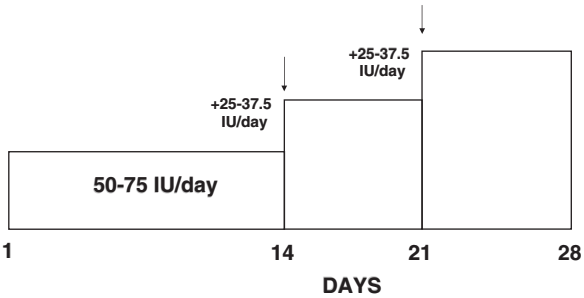


Figure 11.1. A recommended scheme for the first cycle of low-dose, step-up, FSH administration.

Table 11.2. Results of treatment of clomiphene resistant patients with low dose, step-up FSH [7]

No. of patients	841
No. of cycles	1556
Pregnancies (% patients)	320 (38%)
Fecundity/cycle	20%
Uniovulation	70%
OHSS	0.14%
Multiple pregnancies	5.7%

Regimen

The classic chronic low-dose regimen (Figure 11.1) employs a small starting dose in the first cycle of treatment, usually 50–75 IU of FSH which remains unchanged for 14 days [41]. If this does not produce the criteria for hCG administration, a small incremental dose rise of 25–37.5 IU is used every 7 days until follicular development is initiated. Even smaller incremental dose rises of 8.3 IU have been employed with similar results [42]. The dose that initiates follicular development (at least one follicle > 10 mm) is continued until the criteria for giving hCG are attained. hCG should not be given if three or more follicles > 15 mm diameter are seen in order to minimize the chance of a multiple pregnancy. The majority of patients on a low-dose protocol develop a single large follicle meeting hCG administration criteria within 14–16 days without any change in the initial dose for 14 days [40]. In the relatively unusual case (often in very obese women) where a treatment cycle is abandoned after 28–35 days due to lack of response, a larger starting dose may, of course, be employed in a further attempt.

Evidence

A compilation of reported results from the literature [40], using a chronic low-dose protocol identical to that described above, is presented in Table 11.2. The prominent features include a remarkably consistent rate of uniovulatory cycles of around 70% in each series. The pregnancy rates of 40% of the patients and 20% per cycle are acceptable judging from past experiences with conventional therapy and taking into account that many of the patients composing these series received only one cycle of therapy. However, the justification for the adoption of the chronic low-dose protocol may be seen in the extraordinarily low prevalence of OHSS and a multiple-pregnancy rate of 5.7%.

Variations

Some centers reduce the initial no-dose-change time to 7 days from 14 days in order to shorten the duration of treatment but this may be at the expense of an increased occurrence of multiple follicular ovulations and, consequently, multiple pregnancies [40]. On the basis of physiological principles concerning concentrations of FSH in a natural ovulatory cycle, a step-down protocol has been suggested starting with 150 IU of FSH for 5 days, raising the dose by 37.5 IU every 3 days if necessary, until a follicle of 10 mm is obtained. The daily dose is then reduced by 37.5 IU every 3 days until the criteria for giving hCG are reached [43]. However, although pregnancy rates are similar and FSH is given for a shorter duration with step-down, the low-dose step-up has a lower rate of overstimulation, double the rate of monofollicular ovulation and a higher ovulation rate and is, therefore, preferred by the majority of centers [44].

From the largest published series of chronic low dose step-up therapy [45], the comparison of a starting dose of 75 IU with that of 52.5 IU for an initial 14-day period with an incremental dose rise of 37.5 IU or 22.5 IU respectively demonstrated a pregnancy rate/patient, uni-ovulatory cycle rate, and miscarriage rate slightly in favor of the smaller starting dose. In a further series, no difference, other than a slight saving in FSH requirements, was found between the use of a starting dose of 37.5 or 50 IU [46].

There is now sufficient evidence to demonstrate that low-dose, step-up gonadotropin therapy should be preferred to the now outdated conventional therapy

for anovulatory patients and particularly for those with PCOS. Small starting doses in the first cycle for a 14-day initial period without a dose change and then a small incremental dose rise if required seem to give the best results.

In vitro fertilization (IVF)

The anovulation associated with PCOS is not an indication per se for IVF. When anovulation is the sole cause of infertility, the vast majority of women with PCOS will ovulate and conceive following ovulation induction therapy. Those who fail to do so very often have an additional factor, whether it be male, mechanical or endometriosis which may have gone undetected. While results of IVF for women with PCOS are generally satisfactory compared with those with normal ovaries, ovarian stimulation protocols must be adapted accordingly to avoid the major pitfall of ovarian hyperstimulation syndrome (OHSS) in these women.

Results

Women with polycystic ovaries will almost inevitably yield more oocytes than those with normal ovaries. However, the fertilization rate of the oocytes from polycystic ovaries is almost equally inevitably lower. The resultant yield of embryos available for transfer is thus often similar and there is little difference in the outcome of IVF for women with normal or polycystic ovaries, whether calculated per started cycle, egg retrieval, or embryo transfer [47].

Complications

Compared with ovulation induction for PCOS, the multiple-pregnancy rate can be better controlled in IVF by regulating the number of embryos transferred. However, ovarian stimulation for IVF in women with polycystic ovaries is fraught with the danger of OHSS. The first step in avoiding this complication is knowing the diagnosis beforehand and applying an appropriate soft stimulation. The further avoidance of OHSS, whether by cancelation of the cycle, coasting, albumin administration etc., is dealt with in Chapter 13. All are particularly applicable to PCOS.

Protocols

The optimal stimulation protocol for women with PCOS undergoing IVF is still debated. However, most would agree that a milder stimulation protocol should be employed for women with polycystic ovaries. For the first cycle at least, it is recommended that the starting dose of gonadotropins should be lower than that usually employed for women with normal ovaries in order to avoid possible over-stimulation. We have found that, for the first cycle, a starting dose of 150 IU of gonadotropins is often adequate and even less may be used. Whether the gonadotropins used are urinary or recombinant FSH, hMG, or a combination does not seem to unduly influence the outcome and usually depends on local circumstances. Although milder stimulation protocols are generally recommended, the combination of CC with hMG has produced inferior results in our hands.

The main bone of contention has been the choice between GnRH agonist or antagonist for these women. For many years the long GnRH agonist protocol has been the standard choice. However, with increasing experience with the use of the GnRH antagonist, some advantages have become apparent. The antagonist protocol is shorter and therefore more "patient friendly." It requires less gonadotropin stimulation than with the agonist and this reduces the danger of overstimulation and cancelation of the cycle or OHSS [48].

The use of the antagonist also offers the alternative of substituting the hCG triggering of ovulation and maturation of oocytes with one shot of a GnRH agonist in patients who have been over-stimulated. This will almost entirely rule out the occurrence of OHSS and avoids cancelation of the cycle. Although it has been reported that pregnancy rates are significantly lower using this form of triggering [49], this can apparently be overcome by heavy supplementation of the luteal phase [50].

Pretreatment with oral contraceptives before both GnRH agonist and antagonist cycles is favored in many centers and is said to improve outcome for patients with PCOS [51].

The use of metformin in IVF cycles for women with PCOS has been examined in two well-conducted trials. When given as co-treatment, starting from the first day of GnRH agonist treatment in a long protocol, compared with placebo, metformin did not change any of the accepted IVF outcome factors other than the live birth rate, which was doubled! It also produced a lesser incidence of OHSS [52]. A further similar study using a longer duration of metformin administration also showed a doubling of the pregnancy rate in

patients with a relatively normal body mass index [53]. Although further confirmation is needed, this may be a good indication for the use of metformin.

While the freezing of embryos and their replacement in a further cycle will avoid the danger of OHSS, the emergence of in vitro maturation (IVM), particularly for patients with polycystic ovaries, may be the future treatment. This subject is dealt with in Chapter 17 but it is worth mentioning here that excellent results have been achieved using IVM with no stimulation at all [54].

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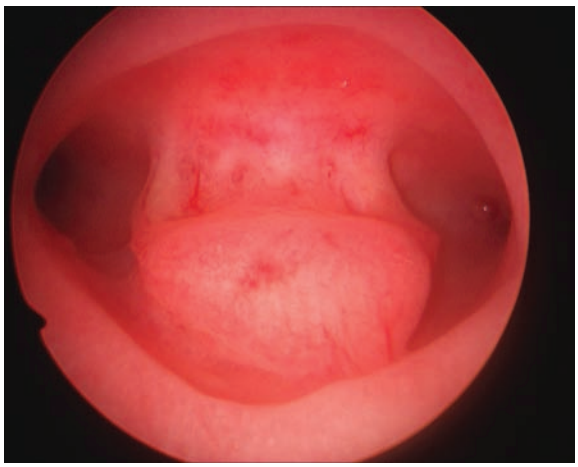


Figure 4.1. Intracavitary uterine polyp as seen on hysteroscopy.

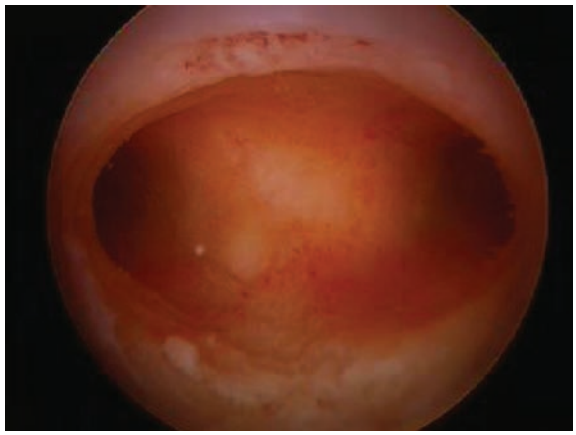


Figure 4.6. Uterine septum as seen on hysteroscopy.

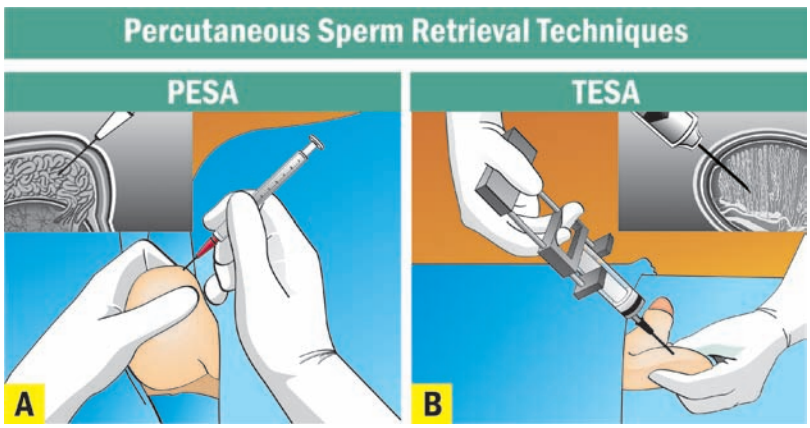


Figure 5.1. Percutaneous sperm retrieval techniques. (A) Percutaneous epididymal sperm aspiration (PESA). Epididymis is stabilized between the index finger, thumb, and forefinger. A needle attached to a tuberculin syringe is inserted into the epididymis through the scrotal skin, and fluid is aspirated. Aspirate is flushed into a tube containing HEPES-buffered sperm medium and sent for microscopic examination. (B) Testicular sperm aspiration (TESA). A 20-ml needled-syringe connected to a holder is percutaneously inserted into the testis. Negative pressure is created and the tip of the needle is moved within the testis to disrupt the seminiferous tubules and sample different areas. A piece of testicular tissue is aspirated, and a forceps is used to remove the seminiferous tubules that exteriorize from the scrotal skin. The specimen is flushed into a tube containing sperm medium, and the tube is transferred to the laboratory for processing and examination.

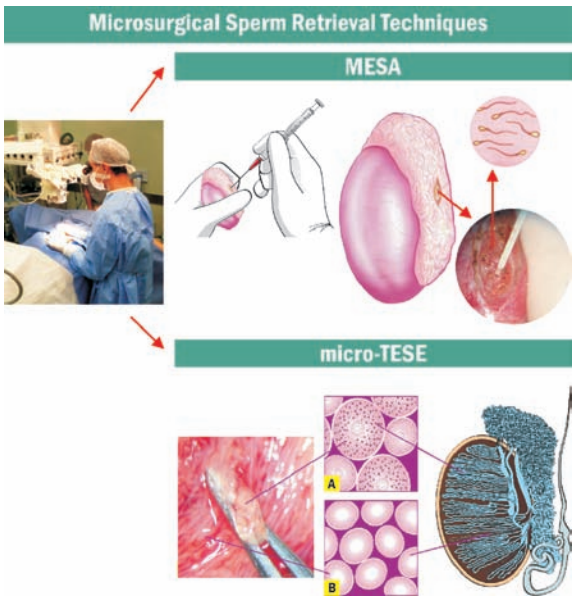


Figure 5.2. Microsurgical sperm retrieval techniques. Operating microscope and microsurgical technique are used throughout the procedures. Microsurgical epididymal sperm aspiration (MESA): after exposure of testis and epididymis, a dilated epididymal tubule is dissected and opened. Fluid is aspirated, diluted with sperm medium, and sent to the laboratory for examination. Microsurgical testicular sperm extraction (micro-TESE): after testis exteriorization, a single and large incision is made in an avascular area of the albuginea to expose testicular parenchyma. Microdissection of seminiferous tubules is carried out to identify and remove large tubules that are most likely to contain germ cells and active spermatogenesis (see photograph at ×40 magnification indicating enlarged (A) and non-enlarged (B) seminiferous tubules). Enlarged tubules may contain active spermatogenesis, as illustrated in the transversal section of a histopathology specimen (A). Non-enlarged tubules are more likely to contain no active spermatogenesis (B). Excised testicular specimens are washed in a well-dish containing sperm media to remove blood clots and are sent to the laboratory for processing and examination.

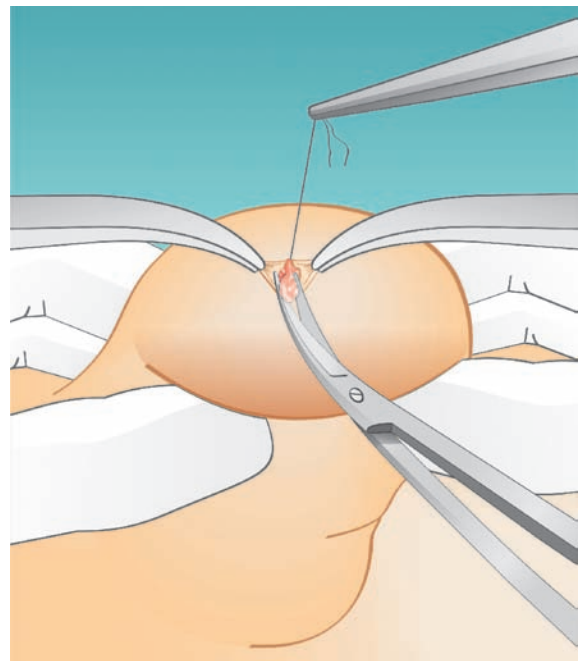


Figure 5.3. Testicular sperm extraction (TESE). Illustration of TESE using a single open biopsy. A 2-cm skin incision is made to allow opening of scrotal layers down to the albuginea. Testicle is not exteriorized from scrotum. A small 0.5-cm incision is made in an avascular area of the albuginea to expose testicular parenchyma. A fragment of approximately 5×5×5 mm is excised and sent to the laboratory for processing and examination. Additional fragments may be taken from the same incision or from different testicular poles using multiple incisions.

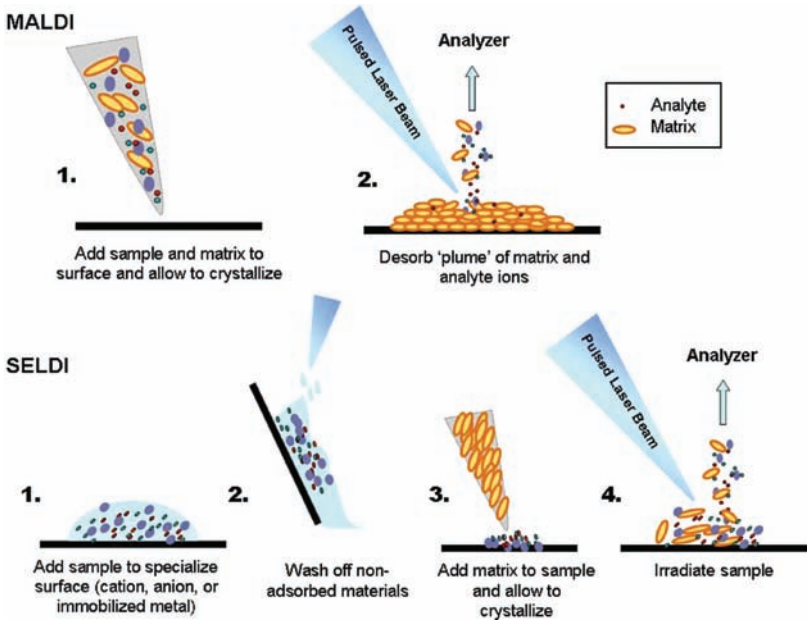


Figure 9.1. Many of the proteomic analyses in endometriosis studies have involved the use of MALDI (matrix assisted laser desorption ionization) and SELDI (surface enhanced laser desorption ionization). Both methods involve the application of a sample to a surface and the addition of a light (generally UV) absorbing matrix. The laser strikes the surface and releases and ionizes the sample which is then analyzed. For SELDI, in general intact proteins from a sample are applied to a special surface (e.g. anionic) and the sample adsorbs. The non-adsorbed material is removed and the surface washed. For MALDI, the sample may be prepared, cleaned, and probably enzyme-digested offline and then applied to the surface. In SELDI features are identified that are often not able to be associated with particular proteins, whereas in MALDI, with digestion, the proteins are often identified.

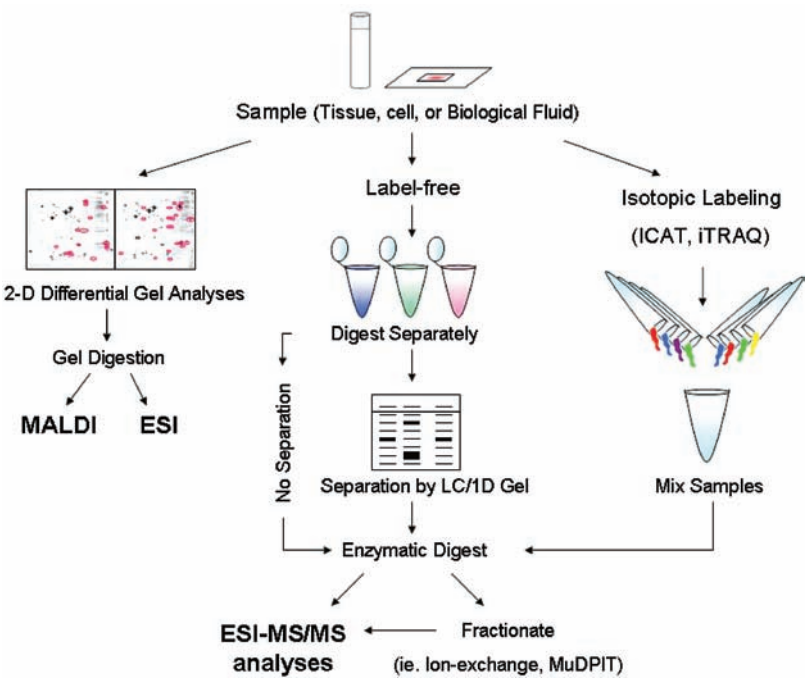


Figure 9.3. Proteomic samples may be analyzed in labeled or unlabeled approaches. 2D gel analyses are common and proteins that alter may be identified by MS after in-gel digestion. While total proteomic digests may be analyzed as a single fraction, many groups pre-fractionate the sample by multiple methods such as liquid chromatography and 1D gel separations at the protein stage. Samples are then digested where they can also undergo a pre-fractionation using a method such as MuDPIT (multidimensional protein identification technology) where the peptides are pre-fractionated on an ion exchange column. Final analysis is generally by LC-MS/MS where peptides are separated on a reverse-phase column prior to ESI ionization and sequence-based identification. Methods such as iTRAQ rely on the labeling of each sample which is then mixed and analyzed with MS/MS often using a multidimensional pre-fractionation such as MuDPIT. The ratio of ions from each labeled form of the peptides may be directly compared to yield relative quantitative analyses of each peptide (and thus proteins) across all samples.

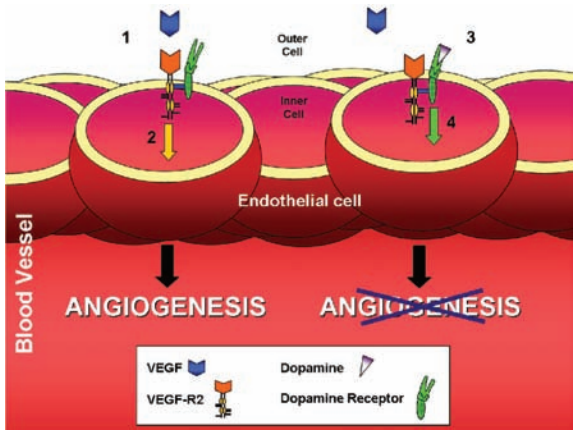


Figure 10.1. (1) During the angiogenesis process VEGF binds to VEGFR-2 located on the epithelial cell surface. (2) The VEGF-VEGFR-2 binding promotes receptor phosphorylation and initiates the angiogenic signal cascade. (3) The dopamine neurotransmitter binds to the dopamine receptor-2 (Dp-r2). (4) Dopamine-Dp-r2 binding promotes VEGFR-2 endocytosis in endothelial cells and prevents VEGF-VEGFR-2 binding. As a consequence, there is no receptor phosphorylation and the angiogenesis signal cascade is inhibited.

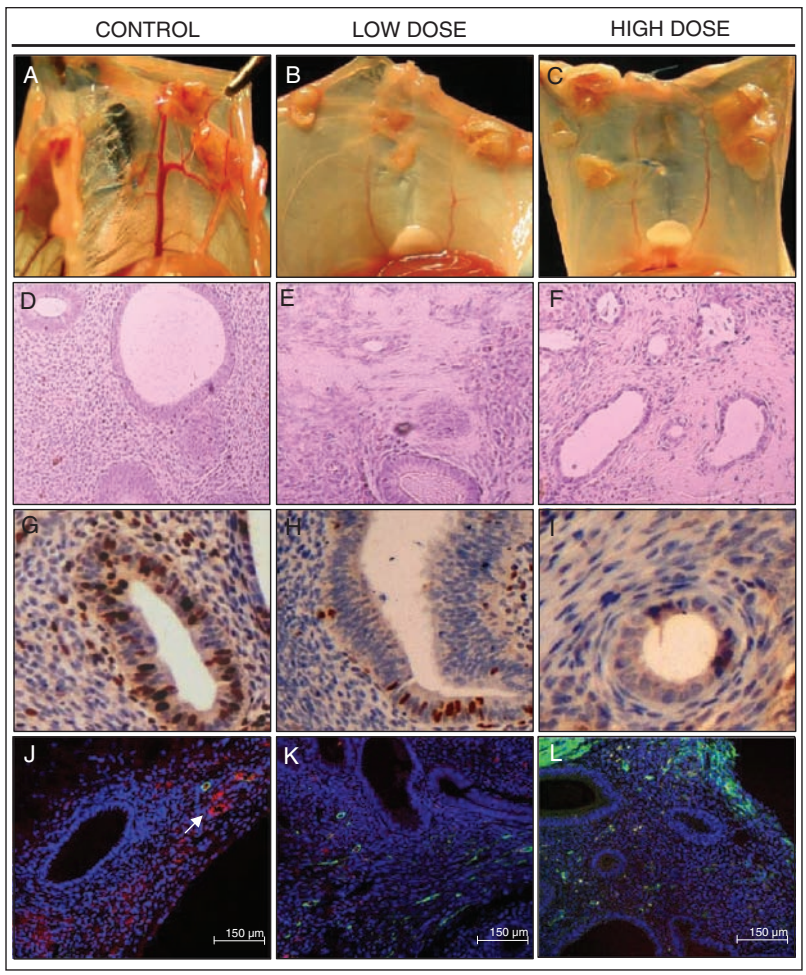


Figure 10.2. Untreated mice lesions (A) show a rich vascular net compared to the lesions of the cabergoline-treated mice (B, C) which present a white aspect and a less developed vascularization. Histological studies reveal a gland's decrement in cabergoline-treated lesions as well as an unstructured stroma (E, F), while the lesions from untreated mice present a complete organization and normal tissular structure (D). The number of positive Ki-67 cells was higher in the lesions from untreated mice (G) compared with the lesions from cabergoline-treated mice (H, I), demonstrating that cabergoline significantly decreased the proliferation index. The lesion's vascularization was analyzed by confocal microscopy (J–L). Modified from Novella-Maestre *et al.* [64].

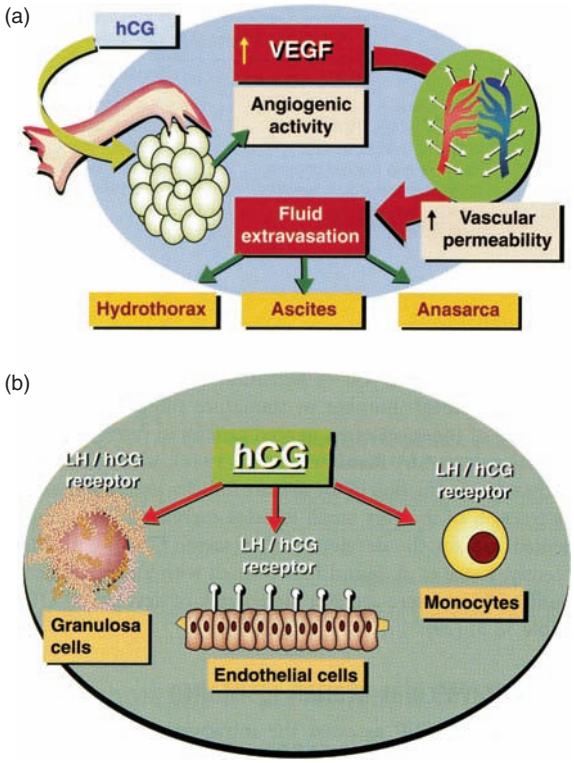


Figure 13.5. Pathophysiology of ovarian hyperstimulation syndrome. Reproduced with permission from: Rizk B, Rizk CB, Nawar MG, Garcia-Velasco JA, Sallam HN. Ultrasonography in the prediction and management of ovarian hyperstimulation syndrome. In: Rizk B, ed. *Ultrasonography in Reproductive Medicine and Infertility*. Cambridge: Cambridge University Press; 2010: 299–312.

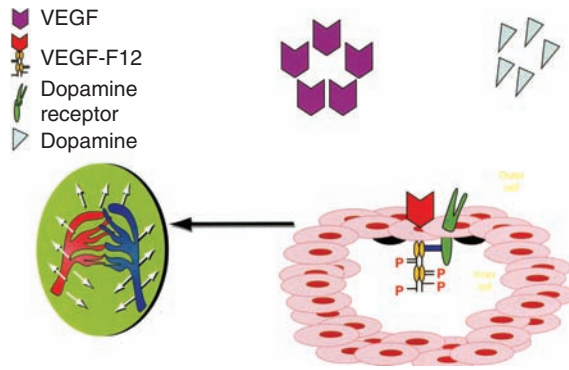


Figure 13.13. Molecular mechanism of dopamine agonist on vascular permeability. Reproduced with permission from: Rizk B, Rizk CB, Nawar MG, Garcia-Velasco JA, Sallam HN. Ultrasonography in the prediction and management of ovarian hyperstimulation syndrome. In: Rizk B, ed. *Ultrasonography in Reproductive Medicine and Infertility*. Cambridge: Cambridge University Press; 2010: 299–312.

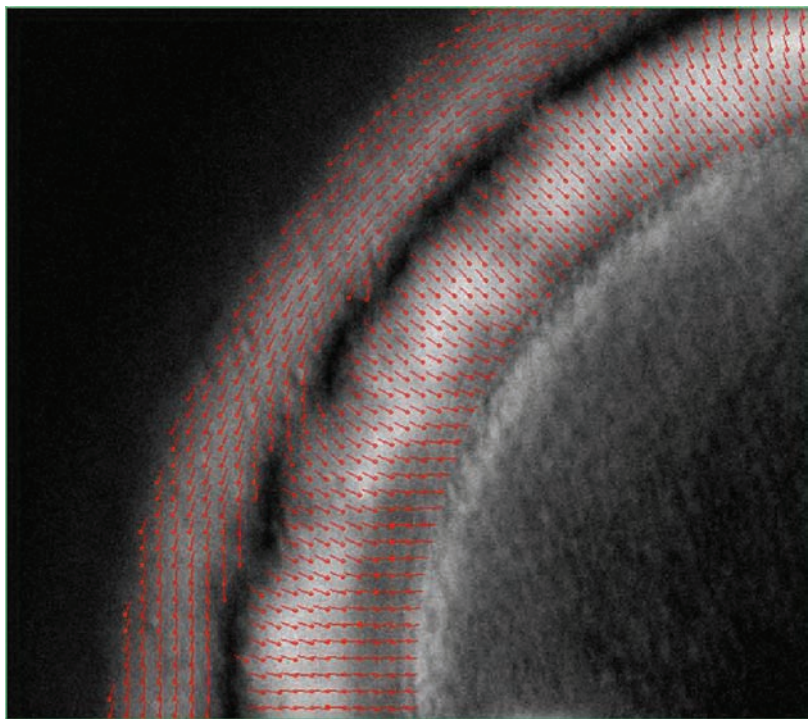


Figure 18.10. The ZP is composed of a lattice of intermediate filament zona pellucida (ZP) proteins organized into three layers. The small red arrows demonstrate the azimuth. The image's gray scale reflects the level of retardance, or molecular order. Filaments of the inner layer orient radially and are highly ordered, as shown by high retardance. Outer layer filaments orient tangentially and have intermediate levels of retardance. Middle layer filaments orient randomly and have minimal retardance. Alterations in the zona are linked to failed or abnormal fertilization, poor embryo development, and implantation.

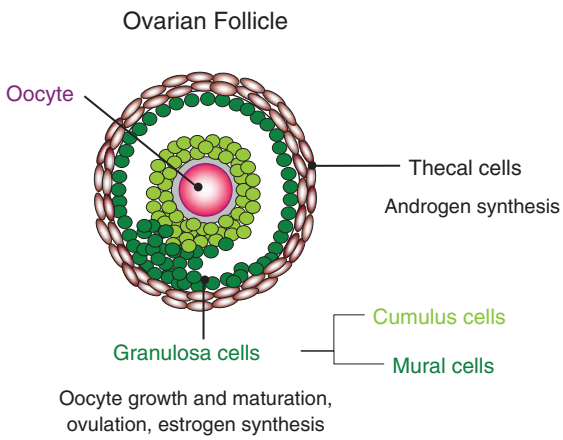


Figure 19.1. Schematic of human ovarian follicle.

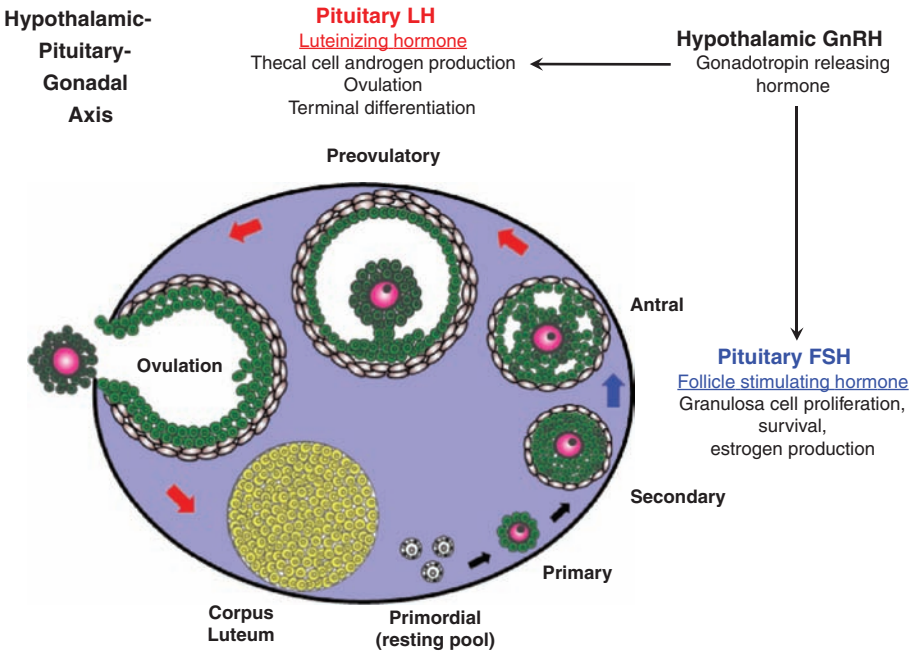


Figure 19.2. Schematic of folliculogenesis.

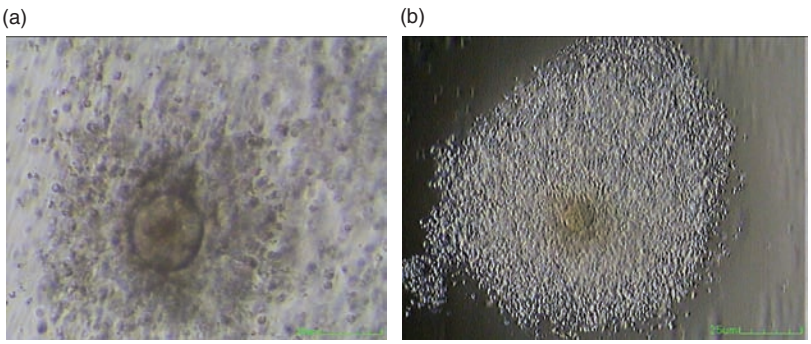


Figure 19.4. Photomicrograph of mature human oocyte with radiant dispersal of cumulus cells.

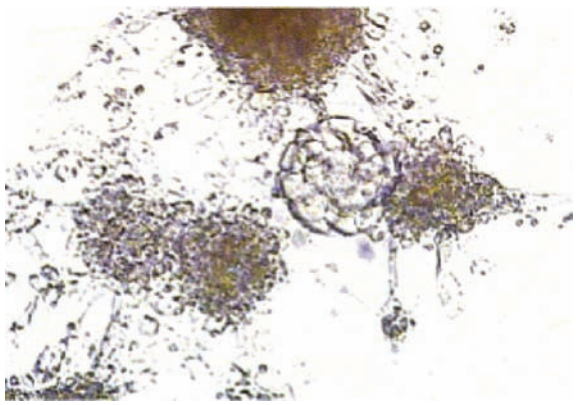


Figure 19.5a. Day 3 embryo completely immobilized by the encroaching expanding cumulus cell colonies. Interlacing dendritic processes are pictured.

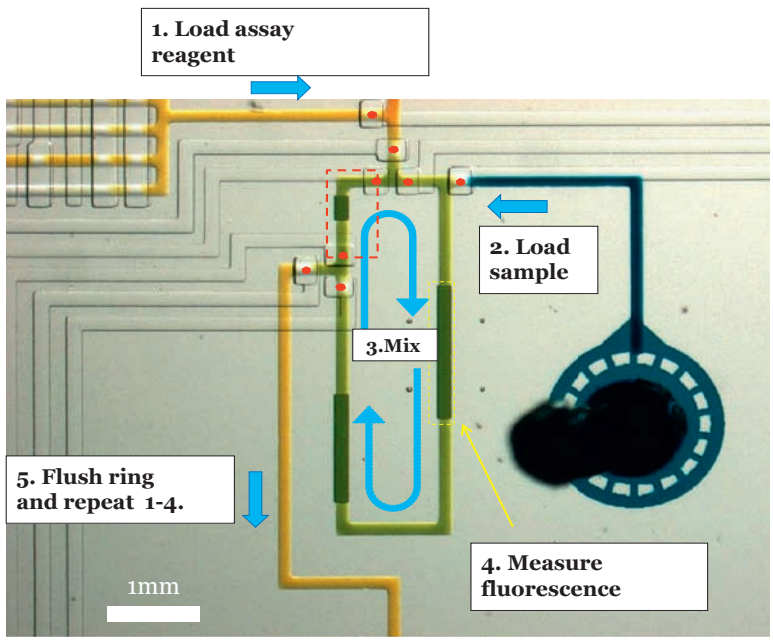


Figure 21.4. Microanalytical device for determining the concentrations of metabolites in culture media. This photomicrograph shows a section of a microfluidic device that we developed in collaboration with the laboratory of Dr. Todd Thorsen [86]. Above the image is a multiplexor with 10 input channels for automated loading of assay cocktail and standards. To the right is a sample loading port where approximately 100 nl of culture medium is loaded manually. By automatically activating pneumatic valves (marked with red dots) in defined sequences, 9/10 of the mixing circuit with assay cocktail and 1/10 of the ring with sample (section of the mixing ring outlined with the red dashed line) is filled. Sequential firing of the valving within the mixing circuit then mixed the samples and then NAD(P)H fluorescence of the fluid within the measurement chamber (highlighted by dashed yellow lines) was determined using an automated fluorescence microscope equipped with a photomultiplier tube. Nine measurements (glucose, lactate, and pyruvate) were determined automatically using this sequence. The sample port and mixing circuit were then flushed clean, another culture medium sample was loaded and the sequence was repeated. To see this procedure in real time, we refer readers to the supplementary video file included with Urbanski *et al* [86].

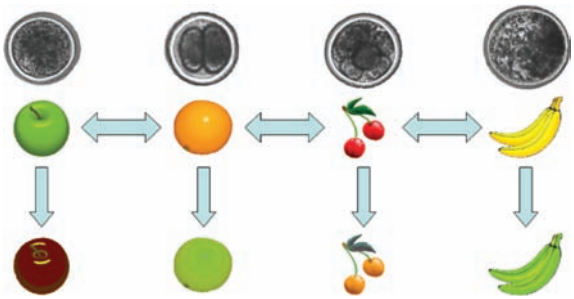


Figure 24.1. Challenges associated with contrasting transcriptomes across embryo stages. Preimplantation development is fraught with dramatic changes in cell number and size as well as very distinct intervals in terms of nuclear activity. As a consequence, the RNA composition fluctuates greatly between developmental stages. Sample processing can thus profoundly impact the physiological relevance of the downstream transcriptomic data. It is expected that within-stage comparisons (green apple vs. red apple) will be less prone to the introduction of methodological biases than inter-stage comparisons (apple vs. orange).

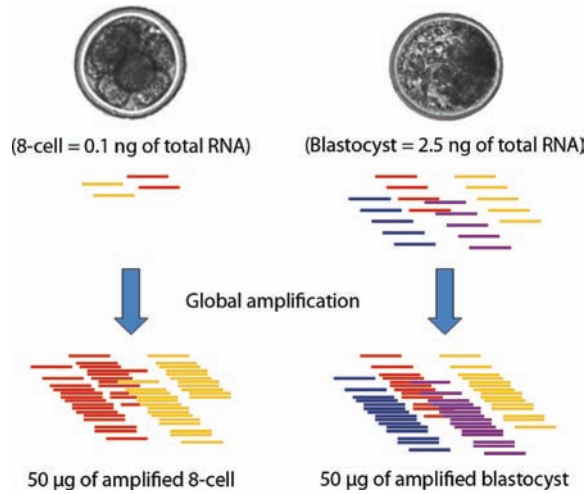


Figure 24.2. Loss of physiological representation following sample amplification. Due to the unique nature of preimplantation development, additional care must be taken to ensure sample processing does not result in a loss of physiological relevance. The global amplification step most often yields a constant output regardless of the RNA content of the input sample. This proportionally skews the downstream gene expression data.

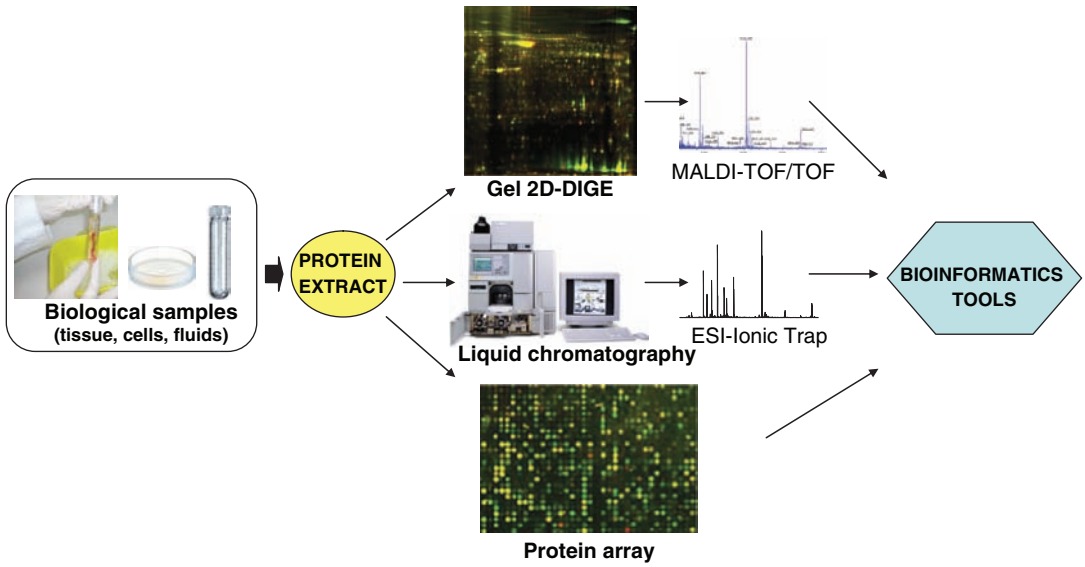


Figure 26.1. Strategies of proteomic analysis. Proteins are extracted from biological samples, fractionated optionally, separated, and analyzed by differential techniques. In the gel-based methods (up), different protein samples are labeled with different fluorescent dyes, and are then mixed together. Next, proteins are separated into two-dimensional difference gel electrophoresis (2D-DIGE) according to their isoelectric point and molecular weight. Gels are scanned by laser scanners, while those spots corresponding to proteins with a differential expression are identified. Finally, these proteins are identified by mass spectrometry (MALDI-TOF/TOF). In the chromatographic separation methods (center), the extracted proteins, protein fraction (SELDI) or one-dimensional gel bands (SDS-PAGE) are digested enzymatically, while the peptidic mix is separated by liquid chromatography (HPLC). Usually, peptides are analyzed and identified by mass spectrometry, typically electronebulization (ESI), and coupled with an ionic trap. Other methods are based on protein arrays (down). These arrays are membranes that contain a certain number of pre-absorbed antibodies that correspond to different proteins. Finally, all the information obtained by different methods is analyzed using bioinformatics tools.

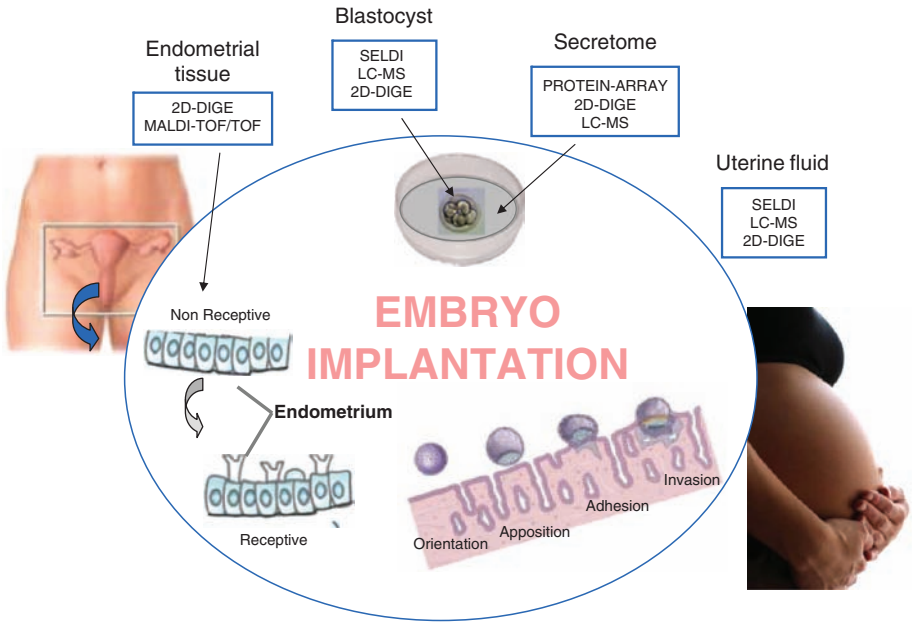


Figure 26.2. Area of use of the proteomics technologies in the human embryo implantation analysis. Different proteomics approaches can be used for the study of the embryo implantation process. The identification of differentially expressed proteins will allow us to understand this complex biological process and to use them as key interceptive markers to prevent embryo implantation, also as markers of endometrial receptivity and embryo viability or to identify causes of diseases.

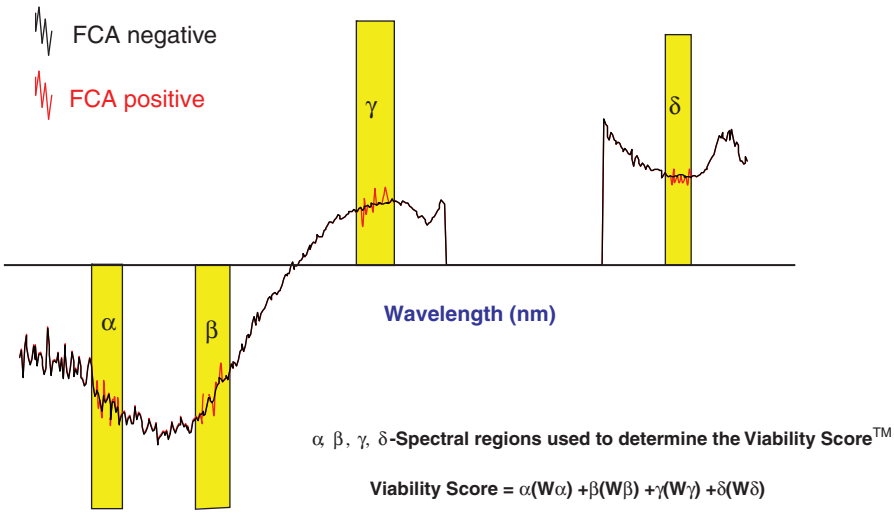


Figure 27.6. Training spectra used to distinguish between embryo culture media giving rise to positive and negative fetal cardiac activity (FCA). Spectra leading to positive and negative FCA outcomes are compared to search for areas of differences in the near infrared spectral range. In this example four wavelet regions discriminate between the positive and negative samples a, b, c, and d. Once found the wavelet regions are used to create a formula that generates a Viability Score™ whereby the higher the score the closer the spectra resemble that of one that leads to a pregnancy. With permission of Molecular Biometrics.

Chapter 12

Adjuvant therapy to increase implantation rates

David Meldrum

Several medications have been found to increase implantation rates with IVF when given as adjuncts to FSH stimulation of the ovaries in preparation for oocyte retrieval. These adjuncts act by: (a) increasing the response and synchrony of resting follicles so that more embryos will be available to select the best for transfer; (b) modifying the levels of luteinizing hormone (LH) or LH-like activity during oocyte maturation; (c) directly stimulating granulosa cell health and therefore the quality of the eggs and embryos; (d) influencing the receptivity of the endometrium; (e) increasing blood flow to the ovary and/or uterus to improve their function. Some adjuncts may act at more than one level. We will not review effects on implantation or the type of FSH chosen, or the use of progesterone and estrogen for luteal support, because these issues are covered elsewhere.

We have pointed out in an editorial in Fertility and Sterility [1] that although these adjuncts are approved as safe for other uses, they are "orphan indications" for the uses we are going to discuss. The reader should refer to that editorial for recommendations of how a clinician can decide when to adopt these new treatment modalities in the absence of approval by regulatory agencies and development and promotion by the pharmaceutical industry. Recommendations have also been made regarding informed consent.

Leuprolide acetate (LA)

LA has been used for IVF for over 20 years in the USA without a specific approval for that indication. Adjunctive use of GnRH agonists reduced the rate of premature ovulation from over 20% to almost zero. For that reason alone, it was suggested that it should be used routinely for IVF [2]. However, it also suppresses LH throughout follicle maturation, which has now been clearly shown using GnRH antagonists to benefit implantation [3]. In that study the investigators found that suppression of LH by a GnRH antagonist increased implantation, although the statistical analysis and power was not adequate to make the conclusion that profound LH suppression was beneficial, or even that it was not deleterious. The adverse effects of higher LH levels may be in part through accelerating endometrial maturation [4] or it may be also due to an effect of higher LH levels on the oocyte itself. A meta-analysis found that the likelihood of a successful implantation using LA was increased almost two-fold [5], although that was probably an overestimate due to the inclusion of studies where the control group not given LA also received clomiphene citrate. GnRH agonists also synchronize the follicular cohort, resulting in more embryos to choose the best for transfer and more embryos for cryopreservation, resulting in more pregnancies from those additional embryos.

Oral contraceptives (OCs)

Although GnRH agonists can be used to schedule cycles and synchronize the follicular cohort, pronounced side effects can occur during extended ovarian suppression. By using OC overlapping with agonist, symptoms can be minimized by varying the duration of OC rather than the agonist for scheduling purposes. Initially there was concern that ovarian response might be reduced [6], but it is now realized that at least 5 days must elapse following OC to allow for endogenous gonadotropins to recover before starting the ovarian stimulation [7]. Biljan *et al.* reported that OC pretreatment actually reduced the amount of gonadotropin required for COH and therefore appeared to improve synchronization of the follicular cohort over agonist alone [8]. In that study the overlap

Human Assisted Reproductive Technology: Future Trends in Laboratory and Clinical Practice, ed. David K. Gardner, Botros R. M. B. Rizk, and Tommaso Falcone. Published by Cambridge University Press. © Cambridge University Press 2011.

of OC and GnRH was only 1 day, whereas even a 7 day overlap has been associated with an improved response [9]. OC pretreatment also dramatically reduces cyst development [8, 9], even with only a 1 day overlap [8]. It may be important that hMG was used in both of these studies showing improved ovarian response with OC pretreatment, thus ensuring adequate levels of LH following the oral contraceptive suppression.

Luteal estrogen

Luteal estrogen was first used for scheduling of controlled ovarian hyperstimulation (COH) for intrauterine insemination [10], but it has subsequently been reported to also synchronize the follicles and improve the response to COH [11]. Luteal estrogen has also been reported to improve COH in poor responders [12]. Stimulation has generally been started on the day after the last estrogen dose, although overlapping the estrogen for 3 days in poor responders did not appear to impair the ovarian response [12].

Metformin (MET)

A prospective, randomized trial in women with PCOS having IVF showed significantly higher rates of ongoing pregnancy per cycle and per transfer with MET versus placebo [13]. A case-control study also reported an increase in the pregnancy rate and a highly significant increase of embryo quality with MET [14], but in a meta-analysis of the five small randomized trials published to date, the 29% increase of the pregnancy rate observed was not statistically significant [15]. There were only about 200 subjects in each group. Because individual studies have reported significantly improved outcomes, and such small numbers make it difficult to assess the true effect, we must assume that MET increases implantation rates in these women and, as yet, the most accurate estimate would be 29%. A large meta-analysis of trials adding MET or placebo to clomiphene citrate (CC) for ovulation induction has found significant increases of ovulation and pregnancy with MET [16], with the greatest effect in CC non-responders and obese women (Table 12.1). Ovulation induced with MET has been associated with decreased testosterone (T) levels and marked increases of glycodelin levels during the luteal phase in women with PCOS [17]. Uterine blood flow is reduced in PCOS and both MET and blockade of the effect of T by flutamide increase uterine blood flow in PCOS women [17, 18]. HOXA-10, required for implantation, is suppressed in PCOS by T, and that effect is blocked by the T antagonist, flutamide [19]. It should be noted that most clinical studies on the use of metformin in PCOS have not been based on demonstrated insulin resistance, although the criteria for the diagnosis of PCOS have varied. Meta-analysis of five trials has also found that MET decreased the incidence of ovarian hyperstimulation syndrome (OHSS) (OR 0.21, CL 0.11–0.41, $P < 0.00001$) in women with PCOS having IVF [16]. Because the upper 95% confidence limit was 0.41, the minimum expected benefit is an astounding 59%. Insulin is one of the principal factors that stimulates the production of vascular endothelial growth factor (VEGF) by luteinized granulosa cells [20] and insulin is reduced by MET [17].

Table 12.1. Odds ratios (95% confidence limits)) of ovulation and pregnancy in women treated with CC and MET compared with placebo. CC-resistant women and women with BMI over 30 had significant and very high odds of ovulation and pregnancy with CC/MET compared with placebo, whereas the ORs for non-obese patients were not significant

OR (CL)	All	CC-resistant	BMI > 30	BMI < 30
Ovulation	4.4 (1.9–10)	5.1 (1.4–18)	10.9 (2.7–44)	1.8 (0.6–5.2)
Pregnancy	2.7 (1.5–5.0)	9.6 (3–31)	3.7 (1.2–11.2)	2.7 (0.96–7.6)

Based on these observations, MET appears to improve implantation in PCOS women by improving uterine blood flow and the implantation factors, glycodelin, and HOXA-10. These effects may also reduce the chance of miscarriage, particularly if MET is continued into pregnancy [21], although no prospective randomized study has been done to absolutely verify that benefit. The dramatic effect in reducing OHSS would itself be sufficient reason to routinely administer MET to all PCOS women having IVF.

Growth hormone (GH)

GH was initially investigated for increasing ovarian response. Subsequent meta-analysis of randomized trials in poor responders did not confirm an effect on ovarian response, but the pregnancy and birth rates were noted to be increased by approximately three- and four-fold respectively compared with placebo [22]. Subsequently, a randomized trial of GH in

Table 12.2. In poor-responding women over age 40, adjuvant growth hormone led to marked and significant increases of the implantation rate (IR) and delivery rate (DR) per embryo transferred. The delivery rate per cycle was increased over five-fold. The number of embryos with more than six cells almost doubled. The higher follicular fluid (FF) and peak circulating estradiol levels (pg per ml) with GH confirmed a positive effect on granulosa cell function and consequently on oocyte and embryo quality [23]

	Placebo	GH	*P*
Peak serum estradiol	912 ± 129	1523 ± 208	0.01
FF estradiol	578 ± 85	921 ± 98	0.01
>6 cell embryos	1.4 ± 0.09	2.6 ± 1.1	NS
IR (%)	1.7	6.2	0.05
Birth per embryo (%)	1.1	5.2	0.05
DR per cycle (%)	4	22	0.05

poor-responding women over age 40 showed approximately four- and five-fold increases of the pregnancy and delivery rates, with a strong trend toward better embryo quality (Table 12.2) [23]. Their poor responder status was clearly indicated by a peak level of estradiol that was 912 pg per ml (SD 129) in the control group, in spite of stimulation with 600 IU of gonadotropins. The subjects had a very poor prognosis with further IVF, with a mean age of 42 and a mean of almost three previous IVF attempts, and yet they achieved a delivery rate per cycle of 22% (Table 12.2). Follicular fluid estradiol levels were also significantly increased. In this latter study the GH was given at a dose of 8 units daily from day 7 of stimulation until oocyte retrieval. Although one might be tempted to start the GH according to the size of leading follicles, it is possible that slower-responding patients may have benefited by receiving the GH from earlier in the progress of follicular growth. In a study of mini-dose LA together with GH, a 25% rate of heartbeat per transferred embryo was achieved in poor responders with a mean of almost three failed cycles, consistent with the degree of benefit reported in these randomized trials [24]. In that study the GH was given throughout the ovarian stimulation, although at a lower dose.

Increased apoptosis has been reported in the granulosa cells of older women having IVF. GH and its intermediary, IGF-1, are two of the most well-characterized factors known to reduce apoptosis and improve the health and proliferation of granulosa cells [25], which are crucial to the nourishment of the maturing oocyte. These effects explain the finding that the circulating level of estradiol is about 50% higher with GH in poor responders (Table 12.2) [23]. Because of this higher estrogen level, clinicians must be cautious not to administer hCG too early if they are accustomed to using the estradiol level in determining when to trigger final oocyte maturation. The improved health of the granulosa cells in turn yields better oocyte and embryo quality. This can be seen in a strong trend toward improved embryo morphology (Table 12.2) [23]. Although larger studies are required for a more accurate estimate of the degree of benefit achieved, GH appears to be the single most important agent currently available to improve successful implantation in poor responders.

Dexamethasone (DEX)

Over 25 years ago, DEX was first reported to increase the response to CC in women with PCOS compared with placebo [26]. Later studies in women resistant to CC reported a high response rate, even when the levels of adrenal androgens were normal [27, 28]. DEX was given in those trials daily at a dose of 0.5 mg. Recently there have been two further randomized trials in women failing to ovulate with up to 150 to 250 mg of CC [29, 30]. The ovulation rate increased four- to five-fold and the pregnancy rate per cycle increased eight- to 10-fold (Table 12.3). The dose of DEX was 2 mg but given only during the 5 days of CC and for the following 5 days (day 5–14 for women receiving CC days 5 through 9). hCG was routinely given to induce ovulation. The mean BMI in both studies was 29–30, indicating that the majority of subjects were not obese. Although responders had the same mean BMI as non-responders, they tended toward a lower waist-hip ratio and had a significantly lower rate of hirsutism. Also, in a large randomized trial of DEX during stimulation for IVF, a dramatic decrease of canceled cycles from 12.4 to 2.8% was noted, and the implantation and pregnancy rates were higher in spite of inclusion of those poor-prognosis women going to egg retrieval [31]. The ratio of cortisol to cortisone in follicular fluid has been reported to strongly correlate with IVF success [32, 33]. These studies clearly show that the ovary is more responsive when DEX is given together with stimulation, and implantation rates also appear to be increased. Because research on glucocorticoids has been outside of the traditional realm of research by reproductive endocrinologists, the role of

Table 12.3. In two randomized trials of women failing to ovulate on 150 to 250 mg of CC, DEX revealed four- to five-fold increases of ovulation and eight- to 10-fold increases of pregnancy [29, 30]. The majority of women were not obese (BMI <30)

Study	No. of subjects	BMI (mean)	Ovulation (%)	Pregnancy (%)
[29] Placebo	111	29.8	20	4.2
[29] DEX	119	30.3	88	40.5[a]
[30] Placebo	40	29.6	15	5
[30] DEX	40	29.4	75[b]	40[c]

[a] Cumulative conception rate $P < 0.0001$; [b] ovulation per cycle $P < 0.001$; [c] pregnancy per cycle.

Table 12.4. In a randomized trial, addition of recombinant LH in GnRH antagonist cycles pretreated with OC (confirmed with the author) was associated with significantly increased fertilization, mature oocytes, high-quality embryos and implantation, and a significant reduction of biochemical pregnancies [38]

%	OC/Ant	OC/Ant/rLH	*P*
Fertilization	71	83	< 0.05
MII oocytes	71	80	< 0.05
Grade 1 embryos	3	17	< 0.05
Biochemical	28	10	< 0.05
Implantation	15	35	< 0.05

glucocorticoids in the follicle has not been extensively studied. Further research in this area could yield important clinical applications.

Human chorionic gonadotropin (hCG)

hCG has been widely used as an LH surrogate to induce ovulation for many years. Following the development of recombinant FSH for ovarian stimulation, we proposed that small doses of hCG could be used to supply LH activity during ovarian stimulation so that use of urinary human menopausal gonadotropins (hMG) would be unnecessary [34]. Most of the LH activity in hMG is from the approximately 10 units of hCG in each 75 IU vial, but the amount of hCG and LH bioactivity varies from batch to batch and among suppliers. hCG has the advantage of providing a consistent LH-like effect, but a sufficient volume must be used so that a given dose can be accurately delivered. Compounding pharmacies can easily make up these small doses. A single dose of 50 IU of hCG raises the level of bioactive LH/hCG to normal in the mid to late follicular phase in women suppressed with a potent GnRH antagonist [34]. Because hCG has a long half-life, a daily dose of 20–30 IU would provide similar LH-like activity. A dose of 50 IU of hCG has been used successfully with hypogonadotropic hypogonadism, allowing pure FSH to be used for follicle growth [35]. Because the new gonadotropin pens allow small increases of FSH dose, one can then achieve a more controlled ovarian response in these difficult cases. However, because of their low endogenous LH levels, these patients do require full luteal support with either low doses of hCG or both estrogen and progesterone [36].

Small doses of hCG, such as 20 IU, have been successfully used to provide LH activity in women receiving the GnRH antagonist for oocyte retrieval who have been pretreated with OC and therefore have low levels of endogenous LH [37]. Recombinant LH has been shown to increase the pregnancy rate with oocyte donors given OC/antagonist (Table 4, OC use confirmed with the author, but not mentioned in the article) [38]. Either hMG or low-dose hCG would be expected to provide this same benefit.

Low-dose aspirin (ASA)

Low dose ASA was first shown in a large, placebo-controlled trial [39] to increase ovarian response, pregnancy outcome, and ovarian and uterine blood flow using 100 mg daily in a population residing in a large metropolis. Those investigators started the ASA with the onset of mid-luteal agonist and it was continued through into early pregnancy. Low-dose ASA is thought to increase blood flow by changing the balance of vasoconstricting thromboxane relative to vasodilating prostacycline. Ovarian blood flow has been reported to correlate with ovarian response and blood flow surrounding the mature follicle has been

correlated with embryo quality and implantation [40]. It is not known how long ovarian blood flow must be increased to influence ovarian response. Subsequently meta-analyses have been published [41, 42], combining remarkably heterogeneous trials with the conclusion that the ovarian response and pregnancy rate are not increased. In two of the trials the ASA was started on the same day ovarian stimulation was begun and in one trial the ASA was stopped at the time of hCG (the most important time for maximal blood flow may be between hCG and egg retrieval during which time meiosis resumes). The patients who would be expected to have the most benefit would be those under more stress and with resulting vasoconstriction, yet patient populations in various trials have varied widely from a small city in Scandinavia to environments closer to that of the original trial. Two trials were on patients having frozen embryo cycles and receiving egg donation where an effect on embryo quality would not be seen and the hormone levels are entirely different from the original trial. A further trial was in poor responders and another was in women with an endometrium refractory to usual doses of estrogen. In a very large trial not included in the meta-analyses, the ASA was started only with embryo transfer, yet a significant increase in the pregnancy rate was observed. In spite of such heterogeneity, a re-analysis of published trials by the Division of Epidemiology of the National Institutes of Health [43] concluded that the clinical pregnancy rate was increased and "there is no reason to change clinical management and discontinue the use of aspirin." The platelet-inhibiting effect of ASA may also reduce the chance of a thrombotic event with COH and OHSS, and a recent study found a marked reduction in severe OHSS, which further extends the therapeutic benefit of this adjunct [44]. Meta-analyses can only disprove a positive therapeutic result from a large well-designed trial if the studies included in the meta-analysis all reproduced the protocol that had been used in the original trial, which is far from the case with this adjunct. The positive benefit of low-dose ASA must stand until a large number of similar trials show that the benefit is not sufficient to justify its use. The ancillary finding of a lower incidence of severe OHSS may make further studies moot.

Androgens and androgenic drugs

Balash *et al.* reported the use of trans-dermal testosterone for 5 days preceding ovarian stimulation in poor responders, with a marked increase in the number of follicles and peak estradiol, and an increase of circulating IGF-1 [45]. Although the patients' prior cycles were used as the control, therefore potentially biasing the study by regression to the mean, their inclusion of a second control cycle with an identical poor response would argue for a true treatment effect. These investigators subsequently have published a randomized trial that confirmed the increased ovarian response with this adjunct [46]. Letrozole, which increases androgen within the ovary by blocking conversion to estrogen, has also been reported to be associated with an increased ovarian response to gonadotropins, increased follicular fluid testosterone, and a marked increase of implantation compared with women not given letrozole [47]. Barad and Gleicher have reported that giving dehydroepiandrosterone (DHEA, a precursor for testosterone in the ovary) before and during IVF in poor responders was associated with an increase in the number of oocytes, embryos and the rate of clinical pregnancy compared with retrospective controls [48]. Androgens increase FSH receptor activity, and well-controlled studies in the primate have reported increased ovarian response using a comparable amount of testosterone to that used in Balash's study. Granulosa cell androgen receptor mRNA in the primate has been reported to correlate positively with proliferation and negatively with apoptosis of granulosa cells [49]. Androgens increase IGF-1 [46] and therefore may act on granulosa cells in a way similar to growth hormone. Although letrozole causes fetal abnormalities in animals, such an effect has not been demonstrated in humans where the drug is stopped about 10–12 days before embryo transfer [50]. These studies suggest that androgens increase ovarian response and that concomitant increases of IGF-1 may improve oocyte and embryo quality and therefore implantation in poor responders. However, further randomized trials are necessary to confirm these findings.

Summary

Both GnRH agonists and antagonists have dramatically improved IVF implantation per started cycle because they virtually always prevent ovulation prior to oocycte retrieval, but also because they reduce the levels of LH during COH. GnRH agonists, OC, and estrogen pretreatment help to synchronize the

follicular cohort resulting in an improved ovarian response. With more mature oocytes, there are more embryos to choose the best for transfer. MET increases implantation in PCOS women having IVF and dramatically reduces the incidence of OHSS in these women. GH markedly increases implantation in poor-responding women having IVF. Small doses of hCG can logically be used to provide LH activity allowing use of pure FSH products and the pen devices that deliver graduated FSH doses. Low-dose ASA increases ovarian response and implantation, and reduces the incidence of severe OHSS. Finally, androgens and drugs such as letrozole that increase androgens may prove to be useful agents to increase ovarian response in poor responders.

None of the adjuncts we have discussed has been officially approved for the purposes described, but they are all considered safe for other indications. In the absence of involvement of regulatory agencies and the pharmaceutical industry in development and promotion of these "orphan indications," a physician is left to decide as to when to adopt these treatments. We have published a recommended means of evaluating the evidence and making a clinical decision, as well as recommendations regarding informed consent [1]. It is clear that major benefits for IVF and ovulation-induction patients would not have accrued if we as clinicians had not adopted useful adjuncts because the pharmaceutical industry had not expended the resources necessary to obtain regulatory approval. Unless some accelerated pathway to approval of these orphan adjuncts is established, as has been done for orphan drugs, physicians are left with making a clinical decision as to what is in the best interests of their patients.

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Chapter 13

Ovarian hyperstimulation syndrome

Botros R. M. B. Rizk

Introduction

Ovarian hyperstimulation syndrome is the most serious iatrogenic complication of ovulation induction [1–5]. OHSS is characterized by bilateral, multiple follicular, and theca-lutein ovarian cysts (Figure 13.1) and an acute shift in body fluid distribution resulting in ascites (Figure 13.2) and pleural effusion [3] (Figure 13.3). The incidence of OHSS has been estimated at 20–33% for mild cases, moderate cases of OHSS are estimated at 3–6%, and 0.1% and 2% for severe cases [6–18]. While mild OHSS is of no clinical relevance, severe OHSS, characterized by massive ovarian enlargement, ascites, pleural effusion, oliguria, hemoconcentration, and thromboembolic phenomena, is a life-threatening complication.



Figure 13.1. Bilateral enlarged cystic ovaries. Reproduced with permission from: Rizk B, Rizk CB, Nawar MG, Garcia-Velasco JA, Sallam HN. Ultrasonography in the prediction and management of ovarian hyperstimulation syndrome. In: Rizk B, ed. *Ultrasonography in Reproductive Medicine and Infertility*. Cambridge: Cambridge University Press; 2010: 299–312.

OHSS classifications

OHSS may be moderate or severe in severity, early or late in onset (Figure 13.4) [19, 20], spontaneous or iatrogenic in etiology. Early OHSS presents 3 to 7 days after the ovulatory dose of hCG whereas late OHSS presents 12 to 17 days after hCG. Early OHSS relates to "excessive" preovulatory response to stimulation, whereas late OHSS depends on the occurrence of pregnancy, is more likely to be severe, and is only poorly related to preovulatory events. Most cases of OHSS are iatrogenic following gonadotropin stimulation. Rarely, OHSS may be spontaneous, occurring as a result of mutations in the follicle-stimulating hormone receptor resulting in stimulation of the FSH receptor by chorionic gonadotropin that is abundant in early pregnancy.

Aboulghar and Mansour [21] reviewed the classifications used for OHSS over the last four decades (Table 13.1). The most recent classification was introduced in 1999 by Rizk and Aboulghar [5]. They classified the syndrome into only two categories, moderate and severe, with the purpose of categorizing patients into more defined clinical groups that correlate with the prognosis of the syndrome. The new classification can be correlated with the treatment protocol and prognosis.

The mild degree of OHSS, used by most previous authors, was omitted from the new classification, as this degree occurs in the majority of cases of ovarian stimulation and does not require special treatment. The great majority of cases of OHSS present with symptoms belonging to the category of moderate OHSS. In addition to the presence of ascites on ultrasound, the patient's complaints are usually limited to mild abdominal pain and distension and their hematological and biochemical profiles are normal.

Human Assisted Reproductive Technology: Future Trends in Laboratory and Clinical Practice, ed. David K. Gardner, Botros R. M. B. Rizk, and Tommaso Falcone. Published by Cambridge University Press. © Cambridge University Press 2011.

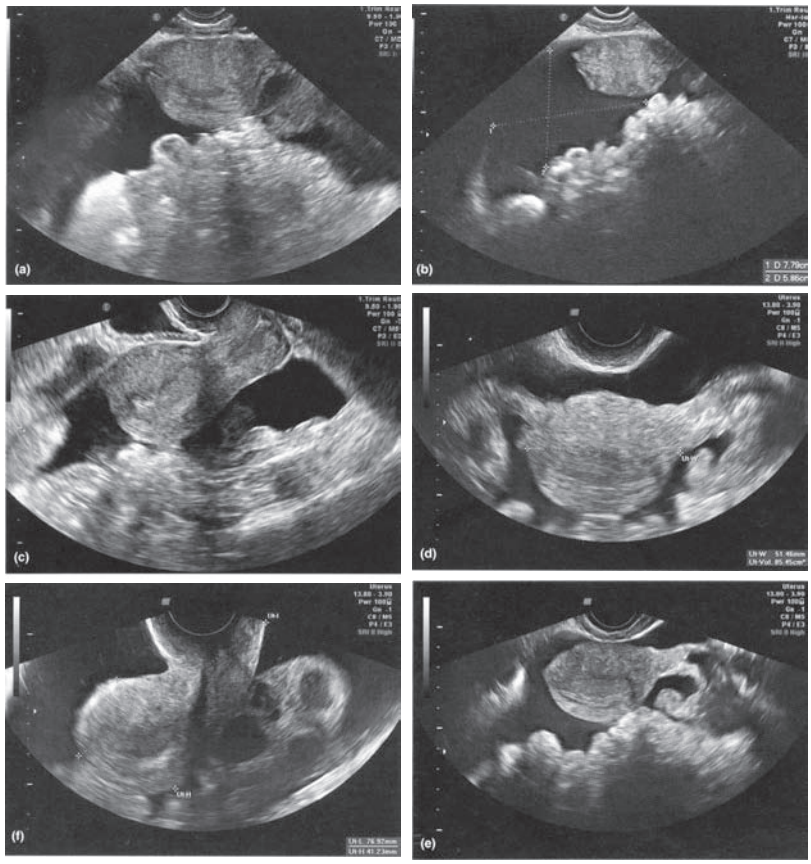


Figure 13.2. Ascites in severe ovarian hyperstimulation syndrome. Reproduced with permission from: Rizk B, Rizk CB, Nawar MG, Garcia-Velasco JA, Sallam HN. Ultrasonography in the prediction and management of ovarian hyperstimulation syndrome. In: Rizk B, ed. *Ultrasonography in Reproductive Medicine and Infertility*. Cambridge: Cambridge University Press; 2010: 299–312.

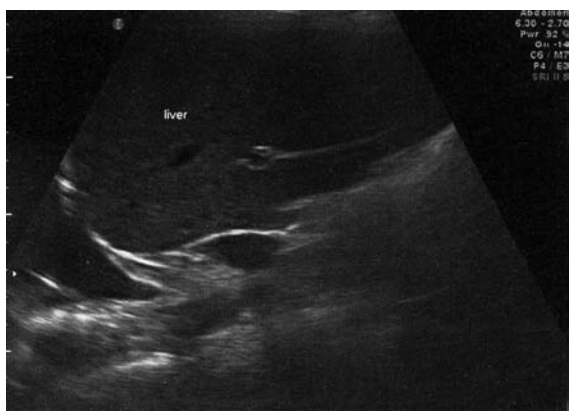


Figure 13.3. Right pleural effusion in OHSS. Reproduced with permission from: Rizk B, Rizk CB, Nawar MG, Garcia-Velasco JA, Sallam HN. Ultrasonography in the prediction and management of ovarian hyperstimulation syndrome. In: Rizk B, ed. *Ultrasonography in Reproductive Medicine and Infertility*. Cambridge: Cambridge University Press; 2010: 299–312

- **Moderate OHSS**:
  - Discomfort, pain, nausea, abdominal distension, no clinical evidence of ascites, but ultrasonic evidence of ascites and enlarged ovaries, normal hematological and biological profiles; can be treated on an outpatient basis with extreme vigilance.
- **Severe OHSS**
  - Grade A: dyspnea, oliguria, nausea, vomiting, diarrhea, abdominal pain; clinical evidence of ascites plus marked distension of abdomen or hydrothorax; ultrasound scan showing large ovaries and marked ascites, normal biochemical profiles; can be treated as inpatient or outpatient depending on the physician's comfort, the patient's compliance, and medical facility.
  - Grade B: all symptoms of grade A, plus massive tension ascites, markedly enlarged ovaries, severe dyspnea and marked oliguria; biochemical changes in the form of increased hematocrit, elevated serum creatinine, and liver dysfunction; would be treated in an inpatient hospital setting with expert supervision.

Table 13.1. Classifications of ovarian hyperstimulation syndrome (1967–1999): reproduced with permission from Aboulghar MA, Mansour RT. Ovarian hyperstimulation syndrome: classifications and critical analysis of preventive measures. *Hum Reprod Update* 2003;9:275–89

Study	Mild	Moderate	Severe	
Rabau *et al.* (1967)	Grade 1: estrogen > 150 µg and pregnanediol > 10 mg 24 h. Grade 2: + enlarged ovaries and possibly palpable cysts. Grade 1 and 2 were not included under the title of mild OHSS	Grade 3: grade 2 + confirmed palpable cysts and distended abdomen. Grade 4: grade 3 + vomiting and possibly diarrhoea	Grade 5: grade 4 + ascites and possibly hydrothorax	Grade 6: grade 5 + changes in blood volume, viscosity, and coagulation, time
Schenker and Weinstein (1978)	Grade 1: estrogen > 150 (µg/24 h and pregnanediol > 10 mg 24 h. Grade 2: grade 1+ enlarged ovaries, sometimes small cysts	Grade 3: grade 2 + abdominal distension. Grade 4: grade 3 + nausea, vomiting, and/or diarrhea	Grade 5: grade 4 + large ovarian cysts, ascites and/or hydrothorax	Grade 6: marked hemoconcentration + increased blood viscosity and possibly coagulation abnormalities
Golan *et al.* (1989)	Grade 1: abdominal distension and discomfort. Grade 2: grade 1 + nausea, vomiting and/or diarrhea, enlarged ovaries 5–12 cm	Grade 3: grade 2 + ultrasound evidence of ascites	Grade 4: grade 3 + clinical evidence of ascites and/or hydrothorax, and breathing difficulties	Grade 5: grade 4 + hemoconcentration, increase blood viscosity, coagulation abnormality, and diminished renal perfusion
Navot *et al.* (1992)			Severe OHSS: variable enlarged ovary; massive ascites ± hydrothorax; Hct > 45%; WBC > 15 000; oliguria; creatinine 1.0–1.5; creatinine clearance ≥ 50 ml/min; liver dysfunction; anasarca	Critical OHSS: variable enlarged ovary; tense ascites ± hydrothorax; Hct > 55%; WBC ≥ 25 000; oliguria; creatinine ≥ 1.6; creatinine clearance < 50 ml/min; renal failure; thromboembolic phenomena; ARDS
Rizk and Aboulghar (1999)	Discomfort, pain, nausea, distension, ultrasonic evidence of ascites and enlarged ovaries, normal hematological and biological profiles	Grade A: dyspnoea, oliguria, nausea, vomiting, diarrhea, abdominal pain, clinical evidence of ascites, marked distension of abdomen or hydrothorax, US showing large ovaries and marked ascites, normal biochemical profile	Grade B: grade A plus massive tension ascites, markedly enlarged ovaries, severe dyspnea and marked oliguria, increased hematocrit, elevated serum creatinine, and liver dysfunction	Grade C: complications as respiratory distress syndrome, renal shut-down, or venous thrombosis

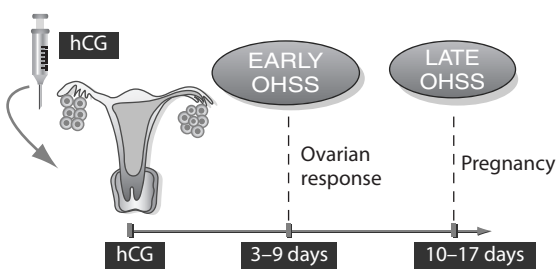


Figure 13.4. Classification of ovarian hyperstimulation syndrome early and late. Reproduced with permission from: Rizk B, Rizk CB, Nawar MG, Garcia-Velasco JA, Sallam HN. Ultrasonography in the prediction and management of ovarian hyperstimulation syndrome. In: Rizk B, ed. *Ultrasonography in Reproductive Medicine and Infertility*. Cambridge: Cambridge University Press; 2010: 299–312.

- Grade C: OHSS complicated by respiratory distress syndrome, renal shut-down, or venous thrombosis is critical; would be treated in an intensive care setting.

Pathophysiology of OHSS

Ovarian hyperstimulation syndrome is characterized by bilateral ovarian cystic enlargement and third-space fluid shift. The ovaries are noted to have a significant degree of stromal edema, interspersed with multiple hemorrhagic follicular and theca-lutein cysts, areas of cortical necrosis, and neovascularization. The second pathological phenomenon is that of acute body fluid shifts, resulting

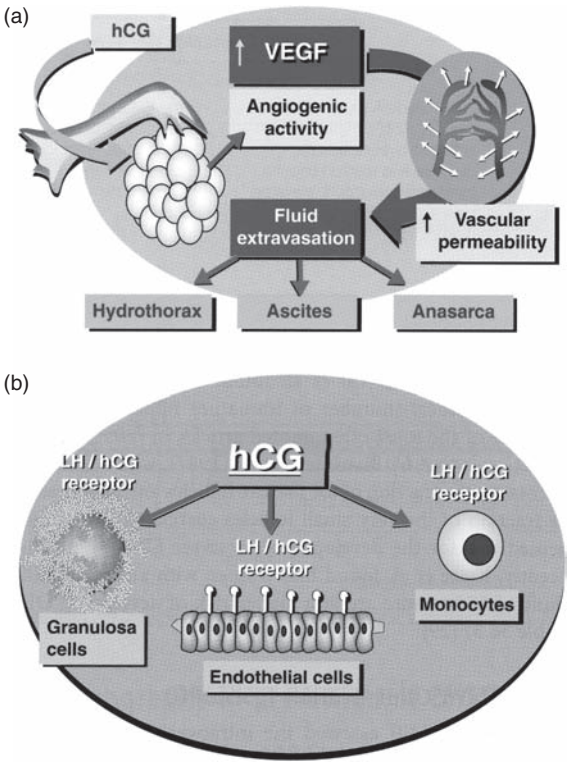


Figure 13.5. Pathophysiology of ovarian hyperstimulation syndrome. Reproduced with permission from: Rizk B, Rizk CB, Nawar MG, Garcia-Velasco JA, Sallam HN. Ultrasonography in the prediction and management of ovarian hyperstimulation syndrome. In: Rizk B, ed. *Ultrasonography in Reproductive Medicine and Infertility*. Cambridge: Cambridge University Press; 2010: 299–312. See colour plate section.

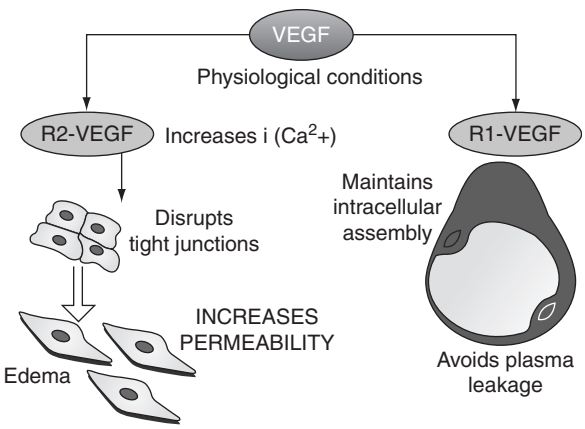


Figure 13.7. Vascular endothelial growth factor receptors and vascular permeability. Reproduced with permission from: Rizk B, Rizk CB, Nawar MG, Garcia-Velasco JA, Sallam HN. Ultrasonography in the prediction and management of ovarian hyperstimulation syndrome. In: Rizk B, ed. *Ultrasonography in Reproductive Medicine and Infertility*. Cambridge: Cambridge University Press; 2010: 299–312.

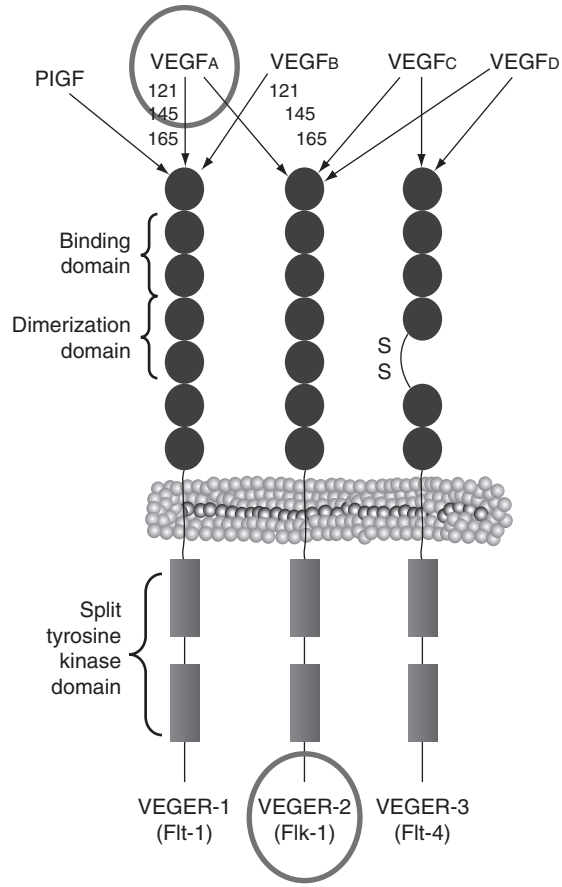


Figure 13.6. Vascular endothelial growth factor receptors. Reproduced with permission from: Rizk B, Rizk CB, Nawar MG, Garcia-Velasco JA, Sallam HN. Ultrasonography in the prediction and management of ovarian hyperstimulation syndrome. In: Rizk B, ed. *Ultrasonography in Reproductive Medicine and Infertility*. Cambridge: Cambridge University Press; 2010: 299–312.

in ascites and pleural effusion. Most investigators believe that these fluid shifts are the result of enhanced capillary permeability. Rizk *et al.* [11] and Pellicer *et al.* [22] investigated the role of vascular endothelial growth factor as a mediator for the capillary permeability and fluid leakage (Figures 13.5–13.7). The VEGF production is dependent on human chorionic gonadotropin stimulation that is administered to trigger ovulation or during the early part of the pregnancy.

Prediction of OHSS

Can OHSS be accurately predicted?

Prediction of OHSS is the cornerstone of prevention. Prediction is based on identifying the characteristics of the patients who would be high responders as well

Table 13.2. Prediction of OHSS

History and physical 1. OHSS in a previous cycle 2. Polycystic ovarian syndrome 3. Young patients 4. Low body mass index 5. Hyperinsulinism 6. Allergies
During ovarian stimulation 1. High serum estradiol, rapid slope of E_2 and absolute value 2. Ultrasonography a. Baseline PCO pattern b. PCO pattern of response to GnRH before gonadotropins c. Large number of follicles > 20, on each ovary 3. Doppler low intraovarian vascular resistance
Outcome of ART cycles 1. Conception cycles 2. Multiple pregnancy

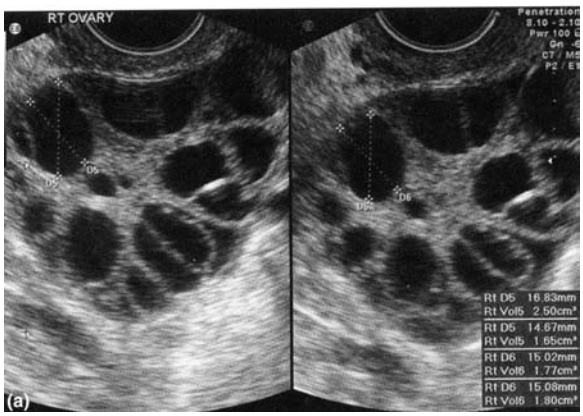


Figure 13.8. Polycystic ovaries at the beginning of an IVF cycle. Reproduced with permission from: Rizk B, Rizk CB, Nawar MG, Garcia-Velasco JA, Sallam HN. Ultrasonography in the prediction and management of ovarian hyperstimulation syndrome. In: Rizk B, ed. *Ultrasonography in Reproductive Medicine and Infertility*. Cambridge: Cambridge University Press; 2010: 299–312.

as the use of ultrasonography and estradiol assessment (Table 13.2). Rizk *et al.* studied the role of ultrasonography before, during, and after ovarian stimulation and is the key to early prediction and successful prevention (Figures 13.8–13.10) [12]. Rizk and Aboulghar emphasized the role of estradiol measurement in the detection of high responders who are at risk for severe OHSS [6].

Prediction of patients at risk of severe OHSS based on characteristics

Rizk studied the epidemiology of OHSS [10]. The characteristics of patients at risk have been investigated by three groups of investigators from Belgium, Egypt, and

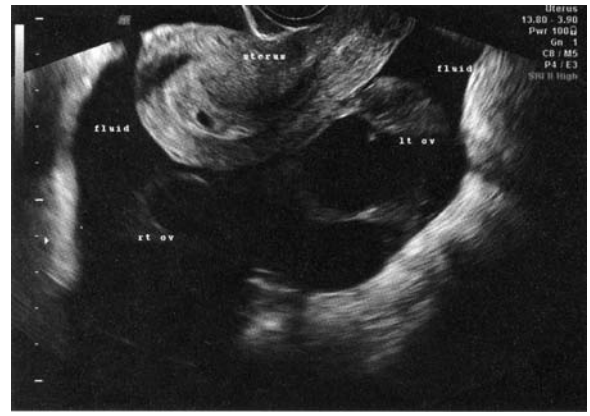


Figure 13.9. Ascites in moderate ovarian hyperstimulation syndrome in early pregnancy. Reproduced with permission from: Rizk B, Rizk CB, Nawar MG, Garcia-Velasco JA, Sallam HN. Ultrasonography in the prediction and management of ovarian hyperstimulation syndrome. In: Rizk B, ed. *Ultrasonography in Reproductive Medicine and Infertility*. Cambridge: Cambridge University Press; 2010: 299–312.



Figure 13.10. Twins associated with OHSS. Reproduced with permission from: Rizk B, Rizk CB, Nawar MG, Garcia-Velasco JA, Sallam HN. Ultrasonography in the prediction and management of ovarian hyperstimulation syndrome. In: Rizk B, ed. *Ultrasonography in Reproductive Medicine and Infertility*. Cambridge: Cambridge University Press; 2010: 299–312.

Israel [23–27]. The characteristics found to increase the risk of OHSS include younger age [28] (Table 13.3), low BMI [28] (Table 13.4), previous history of OHSS, and polycystic ovarian syndrome (PCOS) [28–30] (Tables 13.5, 13.6), allergy [31], hyperinsulinism, or insulin resistance (Table 13.7) [32].

Prediction of OHSS by FSH Receptor Genotype

Rizk investigated the role of FSH receptor mutations and polymorphisms in the development of OHSS [16].

Table 13.3. Age and OHSS

Author	Patients with OHSS (age in years)	Controls (age in years)	*P* value
Navot *et al.* (1988)	27.8 ± 3.6	31.5 ± 5.7	< 0.05
Lyons *et al.* (1994)	29.7 ± 1.8	33.9 ± 0.15	< 0.05
Delvigne *et al.* (1993)	30.2 ± 3.5	32.0 ± 4.5	< 0.05
Enskog *et al.* (1999)	30.2 ± 0.7	32.5 ± 0.2	< 0.05

Table 13.4. BMI and OHSS

Author	Number of patients with OHSS	Number of control subjects	*P* value
Papanikolau *et al.* (2006)	23.13 ± 0.8	23.05 ± 0.1	NS
Delvigne *et al.* (1993)	22.0 ± 3.4	21.9 ± 3.2	NS
Enskog *et al.* (1993)	23.2 ± 0.92	23.0 ± 1.6	NS

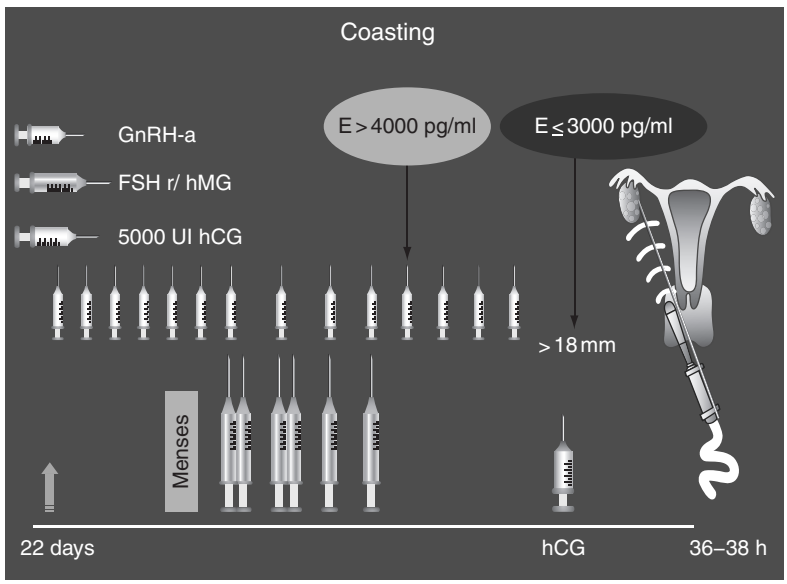


Figure 13.11. Coasting for prevention of ovarian hyperstimulation syndrome. Reproduced with permission from: Rizk B, Rizk CB, Nawar MG, Garcia-Velasco JA, Sallam HN. Ulttrasonography in the prediction and management of ovarian hyperstimulation syndrome. In: Rizk B, ed. *Ultrasonography in Reproductive Medicine and Infertility*. Cambridge: Cambridge University Press; 2010: 299–312

Mutations in FSH receptors could be activating resulting in OHSS or inactivating resulting in sterility [33–36]. To date, 744 single nucleotide polymorphisms have been identified in the FSH receptor gene. Genetic studies of the FSH receptor mutations have increased the expectations that OHSS could be predicted based on the FSH receptor genotype (Table 13.8). Two common SNP within exon 10 of the human FSHR gene result in two almost equally common allelic variants exhibiting Thr or Ala at position 307 in the hinge region, and Asn or Ser at codon 680 of the intracellular domain. Clinical studies have demonstrated that p.N680S polymorphism determines the ovarian response to FSH stimulation in patients undergoing IVF treatment. Patients with the Ser^{680} allele need more FSH during the stimulation phase to reach the serum estradiol concentrations of Asn^{680} patients. A study investigating women with normal, mono-ovulatory menstrual cycles revealed that the Ser^{680}/Ser^{680} genotype leads to higher FSH serum concentrations and a prolonged cycle.

Jun *et al.* investigated the association between FSH receptor (FSHR) gene polymorphism at position 680 and the outcomes of IVF embryo transfer in Korean women [37]. This study included 263 patients with polycystic ovary syndrome, endometriosis, or a previous history of ovarian surgery. Following extraction of genomic DNA, the FSHR polymorphism at position 680 was determined by PCR analysis. The FSHR genotype distribution was 41.8% for Asn/Asn, 45.6% for Asn/Ser, and 12.5% for Ser/Ser FSHR genotype groups. Although there was no difference among

the three genotype groups in terms of the age and infertility diagnosis of study subjects, the basal concentrations of FSH (day 3) were significantly different (mean ± SEM: 5.7 ± 0.3 IU/l, 6.0 ± 0.3 IU/l, and 8.2 ± 0.9 IU/l for Asn/Asn, Asn/Ser, and Ser/Ser groups, respectively). The Ser/Ser group required a higher dose of gonadotropin for ovarian stimulation and showed lower serum estradiol concentrations at the time of hCG administration than the other two groups, although these differences did not reach statistical significance. The numbers of oocytes retrieved were different for the three groups (9.6 ± 0.6, 10.2 ± 0.6, and 7.9 ± 0.8 for Asn/Asn, Asn/Ser, and Ser/Ser groups, respectively). More interestingly, the clinical pregnancy rate was significantly higher in Asn/Asn, compared with the others (45.7 vs. 30.5%, $P = 0.013$). In conclusion, the homozygous Ser/Ser genotype of FSHR polymorphism at position 680 may be associated with a reduced ovarian response to ovarian stimulation for IVF embryo transfer, while Asn/Asn genotypes showed a higher pregnancy rate. The potential association of the S^{680} allele with poor responders to ovarian stimulation for IVF led to the hypothesis that the N^{680} allele could be associated with the hyperresponders, i.e. patients at risk of iatrogenic OHSS. Daelemans *et al.* found no statistically significant differences between the IVF control population and the OHSS patients in allelic or genotypic frequencies. However, a significant enrichment in allele 680 was observed as the severity of OHSS increased ($P = 0.034$). The results of this study also suggested that the genotype in position 680 of the FSH receptor cannot predict which patient will develop OHSS, but could be a predictor of severity of OHSS symptoms among OHSS patients [36].

Table 13.5. PCOS and OHSS

Author	Patients with OHSS	Controls	*P* value
Smitz *et al.* (1990)	50% (5/10)	None	<0.0001
MacDougall *et al.* (1993)	63% (5/0)	None	<0.0001
Delvigne *et al.* (1993)	37% (38/256)	15% (38/256)	<0.0001

Table 13.6. OHSS in PCOS patients compared with normo-ovulatory patients: Modified with permission from Smitz *et al. Hum Reprod* 1990;5:933–7

	OHSS (*n* = 10)	Normal cycles (*n* = 40)	Significance
Number of days before sensitization	30.2 ± 6.0	21 ± 7.0	$P < 0.01$
Days of hMG stimulation	9.6 ± 1.7	12.3 ± 2.5	$P < 0.01$
Number of ampoules of hMG used	21.9 ± 6.9	39.2 ± 14.2	$P < 0.001$
Preovulatory E_2 concentration (ng/l)	3735.0 ± 1603	1634 ± 492	$P < 0.001$
Number of oocytes retrieved	19.1 ± 10.3	7.5 ± 4.2	$P < 0.001$

Table 13.7. Insulin resistance to predict OHSS in PCOS: modified with permission from Felghesu *et al. J Clin Endocrinol Metab* 1997;82:644–8

	Normoinsulinemic (*n* = 21)	Hyperinsulinemic (*n* = 31)	*P* value
Mean total dose of HMG ± SD (IU)	1395 ± 472	1507 ± 727	NS
Mean dose/BMI ±SD (IU/BMI)	57.7 ± 18.7	54 ± 18	NS
Ovulation rate (*n*/cycle)	85.7% (18/21)	83.8% (26/31)	NS
OHSS rate (*n*/cycle)	23.8% (5/21)	64.5% (20/31)	<0.05
Pregnancy rate (*n*/cycle)	28.5% (6/21)	16% (5/31)	NS
Abortions (*n*/pregnancies)	16.6% (1/6)	20% (1/5)	NS

Table 13.8. Genetic predisposition to predict OHSS: modified with permission from Daelemans *et al. J Clin Endocrinol Metab* 2004;89:6310–15

	Allelic frequencies		Genotypic frequencies		
	A	T	AA	AT	IT
Caucasian controls	40% (78)	60% (118)	17% (17)	45% (44)	38% (37)
IVF controls	48% (121)	52% (131)	25% (31)	47% (59)	28% (36)
OHSS patients	55% (41)	45% (33)	30% (11)	51% (19)	19% (7)
P value	NS	NS	NS	NS	NS

Prediction of OHSS by bone morphogenic protein-15

BMP-15 appears to be associated with mechanisms of infertility and super-fertility in a dosage-sensitive manner [38, 39]. BMP-15 protein is a member of the transforming growth factor-β (TGF-β) superfamily of proteins. This superfamily controls many aspects of development by binding and activating two types of Trans-membrane serine/threonine kinase receptors and SMAD (signaling mothers against decapentaplegic homolog) proteins to regulate cellular functions. Moron *et al.* [39] performed a genetic association study of ovarian stimulation outcome in 307 unrelated women with normal ovarian function who underwent ovarian stimulation using recombinant FSH. Four single nucleotide polymorphisms located at the BMP-15 gene were analyzed in order to investigate the role of this gene in relation to ovarian stimulation outcome. The results support the hypothesis that BMP-15 alleles predict over-response to recombinant FSH and ovarian hyperstimulation syndrome in humans. Further studies of BMP-15 may help to further confirm this hypothesis and be able to predict patients who are at risk for OHSS [38, 39].

Treatment factors that influence the incidence of OHSS

Iatrogenic factors that increase risk of OHSS include use of gonadotropins in long gonadotropin releasing hormone agonist protocols (Table 13.9). The duration of OHSS is longer and its expression is more severe when pregnancy ensues, especially when there are multiple concepti (Table 13.10) [10].

How to predict OHSS during ovarian stimulation

Follicular monitoring by ultrasound

Ovarian volume and the number of antral follicles are highly associated with the development of OHSS in assisted reproduction (Tables 13.11, 13.12). The number, size, and pattern of distribution of the follicles are important in the prediction of OHSS. Tal and coworkers found a positive correlation between the mean number of immature follicles and OHSS [40]. The diagnosis of polycystic ovaries at ultrasound examination (the necklace sign) improved the prediction of OHSS to 79% in the Belgian multicenter study. Blankstein and colleagues stated that a decrease in the fraction of mature follicles and an increase in the fraction of very small follicles correlated with an augmented risk for the development of severe OHSS [41].

Danninger and associates studied the baseline ovarian volume prior to stimulation, to investigate whether it would be a suitable predictor for the risk of OHSS [42]. They performed three-dimensional volumetric ultrasound assessment of the ovaries prior to ovarian stimulation and on the day of hCG injection. There was a significant correlation between the baseline ovarian volume and the subsequent occurrence of OHSS. The authors suggested that volumetry of the ovaries could help to detect patients at risk. Kwee *et al.* demonstrated an elegant correlation between the number of basal follicles and ovarian hyper-responsiveness (Table 13.12) [43].

Predictive value of serum estradiol concentration in severe OHSS

The predictive value of serum estradiol concentration has been studied in patients with very high estradiol levels and a large number of oocytes. Asch *et al.* reported severe OHSS in five of 13 patients whose estradiol levels were above 6000 pg/ml on the day of hCG. The sensitivity was 83%, and specificity was 99%; however, the positive predictive value was only 38% [44]. When the number of oocytes retrieved was over 30, the sensitivity was 83%, the specificity was 67%, and the positive predictive value was only 23%. The proportion of patients who had higher than 6000 pg/ml of estradiol and more than 30 oocytes retrieved was not given but analysis

Table 13.9. OHSS using different GnRH agonist/gonadotropin protocols: reproduced with permission from Rizk and Smitz, *Hum Reprod* 1992;7:320–7

Reference	Study group	Incidence Of OHSS %	GnRH agonist used	Dose	%OHSS pregnant	hMG regimen	Luteal support
Golan *et al.* 1988	143 cycles, 117 patients	8.4	D-Trp 6	3.2 mg, long acting	83	Started with 3 ampoules	2500 IU hCG every 72 h and adjusted to estradiol
Belaisch-Allar and De Niyzib 1989	304 embryo transfers	5.9	D-Trp 6 Or D-Ser (TBU) 6	NM	NM	NM	2500 IU hCG (151patients), or placebo (153 patients) randomized
Buvat *et al.* 1989	171 embryo transfers	1.8 (moderate)	D-Trp 6	short-acting	NM	NM	3 × 1500 IU hCG or 400 mg progesterone p.o. daily
Herman *et al.* 1990	36 embryo transfers	1.4	D-Trp 6	3.2 mg long-acting	80	Started with 3 ampoules and adjusted to estradiol	2500 IU every 3rd day or placebo (18 patients)
Forman *et al.* 1990	413 cycles	1.9 Severe	D-Trp 6 or D-Ser (TBU) 6	3.75 mg long-acting injection 100 ng/day or nasal spray 100 ng/day	88	Started with 2 ampoules and adjusted to estradiol	Didrogesterone 30 mg/day p.o. or 2500 IU hCG every 72 h
Smitz *et al.* (1990)	1673 cycles	0.6 Severe	D-Ser (TBU)6	600 ng daily nasal spray	70	Started with 2 ampoules hCG and adjusted to estradiol	1500 IU every 72 h or progesterone vaginal or i.m.
Rizk *et al.* (1993)	1562 cycles	1.3 severe	D-Ser (TBU) 6	Subcutaneous injection 200 µg/day or nasal spray 500 µg/day	57	Started with 2 ampoules hCG and adjusted to estradiol	2000 IU on days 2 and 5 or progesterone 200 mg/day vaginal suppository

predicted an 80% chance of developing OHSS. Morris *et al.* studied 139 IVF cycles using two assisted reproductive techniques; oocyte donor ($n = 72$) and IVF ($n = 67$) in which the estradiol level was above 4000 pg/ml, oocyte number > 25 or both were elevated. There were no cases of severe OHSS in the oocyte donor group and six in the IVF group. Among 10 patients with estradiol concentrations > 6000 pg/ml and > 30 oocytes retrieved, only one had OHSS (10%). It therefore appears that the prediction of OHSS could be very variable based on these two studies from California in the early 1990s. While there are not large numbers of patients who meet these criteria in any IVF center, it is reasonable to say that the prediction of OHSS in such patients would be in between those two predictions [45].

Prevention of ovarian hyperstimulation syndrome

Rizk in 1993 [14] suggested a "Ten Commandments" for the prevention of OHSS. These consisted of identifying patients at risk, use of treatment of other than gonadotropins for PCOS patients such as metformin and ovarian diathermy, and use of low doses and GnRH antagonists when gonadotropins were necessary (Table 13.13). A second "Ten Commandments" addressed the secondary prevention of OHSS and included withholding or delaying hCG, follicular aspiration, switching to IVF with cryopreservation of all embryos, and progesterone for luteal-phase support (Table 13.14) [10]. Other measures unique to prevention

Table 13.10. OHSS and conception cycles: modified with permission from Rizk and Aboulghar. In: Brinsden P, ed. *A Textbook of In-vitro Fertilization and Assisted Reproduction*, 2nd edn. Carnforth: Parthenon; 1999: 131–55

Author	Year	Incidence (%)	Number of patients	Multiple pregnancies
Rabau	1967	42	6/14	2/6
Schenker and Weinstein	1978	40	10/25	5/10
Tulandi	1984	34.6	10/29	4/10
Golan	1988	91	10/11	1/10
Borenstein	1989	35	14/39	3/14
Herman	1990	80	4/5	¼
Forman	1990	88	7/8	2/7
Smitz	1990	70	7/10	3/7
Rizk	1991	57	12/21	5/12

Table 13.11. Ovarian volume and OHSS: modified with permission from Danninger *et al. Hum Reprod* 1996;11:1597–9

	OHSS	Controls	*P* value
Number of patients	8	86	
Days of stimulation	10.5 ± 2.5	10.5 ± 1.8	NS
Estradiol (pg/ml)	2439 ± 1350	937 ± 636	0.00001
No. of follicles	23.3 ± 4.3	13.8 ± 7.5	0.0025
No. of oocytes	164 ± 26	5.0 ± 3.0	0.00001
Cycle length	34.1 ± 5.8	28.7 ± 2.2	0.00001
Body wt before stimulation	55.4 ± 3.8	62.8 ± 11	0.011
Body wt after stimulation	54.3 ± 4.5	62.9 ± 10.7	0.03
Ovarian volume (ml)	13.2 ± 5	8.9 ± 3.7	0.035

Table 13.12. Antral follicle count and OHSS: modified with permission from Kwee *et al. Reprod Biol Endocrinol* 2007;5:9–18

Total AFC	Sensitivity	Specificity	PPV	Accuracy
>10	0.94	0.71	0.36	0.76
>12	0.88	0.80	0.44	0.81
>14	0.82	0.89	0.58	0.88
>16	0.47	0.96	0.67	0.88
>18	0.29	0.98	0.71	0.87

Table 13.13. Primary prevention of OHSS: the Ten Commandments: reproduced with permission from: Rizk B. *Ovarian Hyperstimulation Syndrome: Epidemiology, Prevention and Management*. Cambridge: Cambridge University Press; 2006

1. Prediction of OHSS from history, exam, and ultrasound
2. Laparoscopic ovarian drilling in PCOS patients
3. Metformin in PCOS patients
4. Octreotide in PCOS patients
5. Low-dose gonadotropins in PCOS patients
6. GnRH antagonist protocol
7. Recombinant LH to trigger ovulation
8. GnRH agonist to trigger ovulation
9. In vitro maturation of oocytes
10. Replacement of only one embryo

of OHSS are: use of GnRH antagonists instead of agonists to prevent premature LH surge, decrease in the dose of hCG, use of LH or GnRH agonist in place of hCG for triggering ovulation, administration of albumin, use of glucocorticoids, and administration of dopaminergic drugs.

GnRH antagonist as an alternative to the long agonist protocol

Al-Inany and Aboulghar, in a Cochrane review, compared the efficacy of GnRH antagonist to the long agonist protocol in assisted conception [46]. In comparison with the long GnRH agonist protocol, there was no statistically significant reduction in the occurrence of severe OHSS (RR 0.50; OR 0.79; 95% CI 0.22–1.18), however, there were significantly fewer pregnancies with GnRH antagonist (OR 0.79; 95% CI 0.63–0.99). Ludwig *et al.* compared the two GnRH antagonists cetrorelix and ganirelix to the long protocol [47]. A significant reduction of OHSS was observed in cetrorelix studies (OR 0.2; 95% CI 0.10–0.54) but no reduction for ganirelix (OR 1.13; 95% CI 0.24–5.31). The pregnancy rate in the cetrorelix studies was not significantly different from the long GnRH agonist protocol (OR 0.91; 95% CI 0.68–1.22). The pregnancy rate in the ganirelix protocols was significantly lower compared to the long GnRH agonist protocol (OR 0.76; 95% CI 0.59–0.98).

Table 13.14. Secondary prevention of OHSS: the Ten Commandments: reproduced with permission from: Rizk B. *Ovarian Hyperstimulation Syndrome: Epidemiology, Prevention and Management*. Cambridge: Cambridge University Press; 2006

1. Withholding hCG +/– continuation of GnRH-a/GnRH antagonist
2. Coasting or delaying hCG: currently most popular method
3. Use of GnRH-a to trigger ovulation
4. Follicular aspiration
5. Progestesterone for luteal phase
6. Cryopreservation and replacement of frozen-thawed embryos at a subsequent cycle
7. Dopamine agonist
8. Albumin, administration at time of retrieval
9. Glucocorticoid administration
10. Aromatase inhibitors

In two recent meta-analyses comparing the outcome of GnRH agonist versus antagonist, both showed that the incidence of OHSS was significantly reduced in the antagonist protocol [48, 49]. Al-Inany *et al.* showed that the incidence of OHSS associated with hospital admission was significantly lower in the antagonist group (OR 0.46; 95% CI 0.26–0.82; $P = 0.01$) [48].

From the above data, it seems logical that patients with a history of OHSS and patients who are considered at high risk of developing OHSS would have a lower risk of OHSS if treated by GnRH-antagonist protocol.

Canceling cycles to avoid OHSS

Withholding hCG used to be the most common method used to prevent OHSS in patients predicted to be at high risk of developing OHSS [6]. Rizk and Aboulghar analyzed the estradiol criteria for withholding hCG to prevent OHSS and observed a wide range reported over 20 years from 800 pg/ml to 4000 pg/ml [6]. Nearly all authorities recommend withholding hCG when estradiol is ≥ 4000 pg/ml. Equal or more important criteria for canceling cycles than estradiol levels are the number of 8–10 mm follicles that may have acquired LH receptors.

Coasting or delaying hCG administration

Coasting or delaying hCG administration to prevent OHSS is currently the most popular method for prevention of OHSS (Figure 13.11) [15].

Start coasting when serum estradiol is above 4500 pg/ml, estradiol production is above 150 pg per follicle, 16–18 mm, and the number of mature follicles is greater than 15 but less than 30.

Monitor estradiol on a daily basis. Do not skip any days to avoid a sudden unexpected drop.

Trigger ovulation using 5000 IU of hCG or GnRH agonist, 0.2 mg, when estradiol concentrations fall below 3500 pg/ml

Abandon the cycle if estradiol concentration rises to above 7000 pg/ml and the number of follicles is over 30. If coasting takes over 4 days, the outcome is usually poor.

Decrease in hCG dosage

Several clinical trials have been published in the literature regarding the impact of the dose of hCG on the occurrence of OHSS [50, 51](Tables 13.15, 13.16). The use of a very low dose of hCG, 2000 IU, would definitely impact the number of mature oocytes that can be retrieved; however, there seems to be no difference in the incidence of severe OHSS when patients are given 5000 IU of hCG or 10 000 IU hCG.

GnRH agonist as an alternative to hCG to trigger ovulation

Alternatives to hCG to trigger ovulation include GnRH agonists, native GnRH, and recombinant LH. Although the incidence of OHSS is lowered, the ongoing pregnancies in IVF cycles are also reduced when ovulation has been triggered by GnRH agonist or recombinant LH rather than urinary or recombinant hCG [7].

Griesinger *et al.* [52] performed a meta-analysis which showed that the use of GnRH agonist to trigger final oocyte maturation in IVF, where inhibition of premature LH surge is achieved with GnRH antagonists, yields a number of oocytes capable of undergoing fertilization and subsequent embryonic cleavage which is comparable to that achieved with hCG. However, the likelihood of an ongoing clinical pregnancy after GnRH-agonist triggering was significantly lower as compared with standard hCG treatment (OR 0.21; 95% CI 0.05–0.84; $P = 0.03$).

Recombinant LH to trigger ovulation

hCG is a promoter of OHSS whereas an endogenous LH surge rarely causes OHSS. The idea of using leuteinizing hormone is an old idea, not a new one. The only recent development is the development of

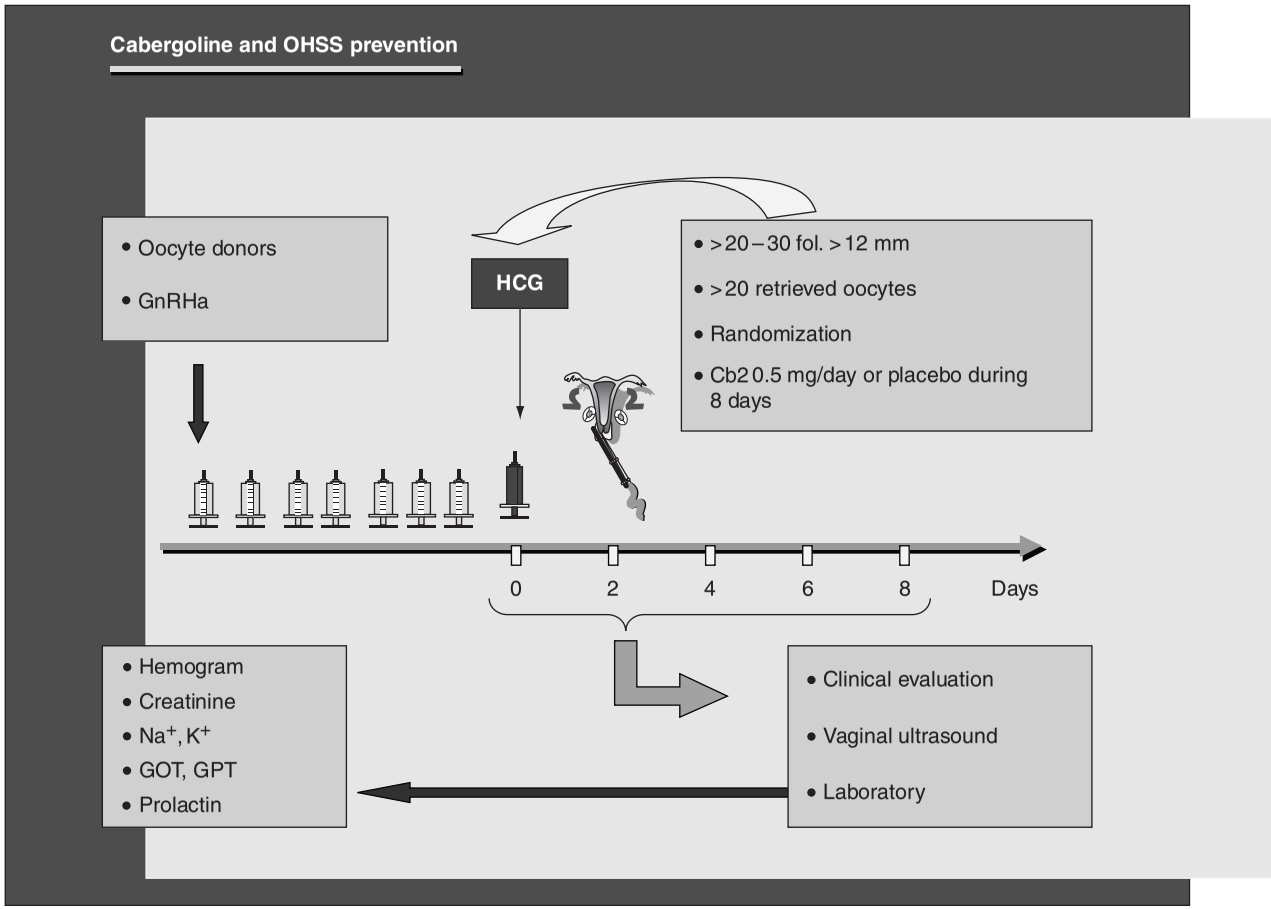


Figure 13.12. Cabergoline and prevention of ovarian hyperstimulation syndrome. Reproduced with permission from: Rizk B, Rizk CB, Nawar MG, Garcia-Velasco JA, Sallam HN. Ultrasonography in the prediction and management of ovarian hyperstimulation syndrome. In: Alvarez C, Marti-Bonmati L, Novella-Maestre E. Dopamine agonist cabergoline reduces hemoconcentration and ascites in hyperstimulated women undergoing assisted reproduction. J *Clin Endocrinol Metab* 2007;**92**:2931–7.

Table 13.15. Decreasing the dose to prevent OHSS in GnRH agonist cycles: modified with permission from Tsoumpou *et al. Reprod Biomed Online* 2009;19(1):52–8

Abdalla *et al.*, 1987	RCT	Age: 24–43 years, normal ovulatory cycles	I VF–ET, long or HMG protocol	Pure FSH or HMG	2000 or 5000 or 10 000 IU	ND	Successful oocyte recovery OR, PR
Wikland *et al.*, 1995	Prospective cohort	Age: ≤ 39 years, BMI: ≤ 28 kg/m^2	1VF/ SUZI–ET, long protocol	Pure FSH	5000 or 10 000 IU (i.m. or s.c.)	P 300 mg tds for 2 weeks (PV)	hCG serum concentration OR, cleaved oocytes, FR, PR, MR, LBR
Prien *et al.*, 2000	Retrospective cohort study	Age < 39 years	IVF–ET, long protocol	HMG	5000 or 10 000 IU	ND	OR, PR Serum P pre- and post hCG
Schmidt *et al.*, 2004	Retrospective cohort study	High responders E_2 2500 pg/ml	IVF/ICSI–ET, long protocol	r-FSH	3300 or 5000 IU depending on E_2 concentrations	P 50 mg from evening after OR until viable pregnancy	OR, FR, PR, CPR, OHSS

Table 13.16. Decreasing the dose to prevent OHSS in GnRH antagonist cycles: modified with permission from Tsoumpou *et al. Reprod Biomed Online* 2009;19(1):52–8

Detti *et al.*, 2007	Retrospective cohort study	ND	IVF/ ICSI–ET, long protocol	r-FSH	5000 or 10 000 or 15 0001U depending on E_2 concentrations and number of follicles	ND	hCG concentrations after hCG trigger, OR
Kolibianakis *et al.*, 2007	RCT	PCO, age: < 39 years Day 3 FSH < 10 IU/l, E_2 < 80 pg/ml *P* < 1.6 ng/ml, no endometrioma	IVF/ ICSI–ET, long protocol	r-FSH	2500 or 5000 or 10 000 IU	P 200 mg TDS from day 1 after OR until 7 weeks of gestation	OR, FR, IR, CPR MR, OHSS; cancelation rate

recombinant LH. In the experimental animal model, the capacity of LH, FSH, and hCG to trigger ovulation was compared and their effects on vascular permeability and the expression of VEGF in the ovaries were studied. Immature female rats were stimulated with 10 IU of pregnant mare serum gonadotropin (PMSG) for 4 days and thereafter ovulation was triggered by using 10 IU of hCG, 10 IU of FSH, 10 IU of LH, 60 IU of LH, or normal saline. All the hormones utilized were equally effective at triggering ovulation, and significantly different from the control injection. However, only 10 IU of LH resulted in a significantly lower vascular permeability and VEGF expression. A lower dose of LH (10 IU vs. 60 IU) prevented the undesired changes in vascular permeability and the risk of OHSS. However, clinicians are encouraged to determine the optimal dosage of LH needed to trigger ovulation in women and at the same time to abolish OHSS. This is a very important issue because the clinical trials of recombinant LH have also suggested a lower OHSS rate associated with a lower pregnancy rate. The success in determining the optimal dose or doses of LH could be a significant step towards eliminating OHSS while retaining the pregnancy rate [10].

Intravenous albumin

How could intravenous albumin prevent OHSS? First, it acts to sequester vasoactive substances released from the corpora lutea. Secondly, albumin also serves to sequester any additional substances which may have been synthesized as a result of OHSS. Finally, the oncotic properties of albumin serve to maintain intravascular volume and prevent the ensuing effects of hypovolemia, ascites, and hemoconcentration.

The effectiveness of human albumin administration in prevention of severe OHSS was reviewed using the Cochrane "menstrual disorders and subfertility group"

literature search strategy [53]. Only randomized controlled trials comparing the effect of human albumin with placebo or no treatment were included. Seven randomized controlled trials were identified, five of which met the inclusion criteria and enrolled 378 women: 193 in the albumin group and 185 in the control group. There was a significant reduction in the occurrence of OHSS in the albumin group (OR 0.28, 95% CI 0.11–0.73); the relative risk was 0.35 (95% CI 0.14–0.87) and the absolute risk reduction was 5.5. For every 18 women at risk for severe OHSS, albumin infusion may save one case of OHSS. Based on this review, there is a clear benefit from the administration of intravenous albumin at the time of oocyte retrieval. However, a new Cochrane database review that will be soon published found albumin to be of no effect in preventing OHSS.

Hydroxyethyl starch solution

Hydroxyethyl starch (HES) is a synthetic colloid, glycogen-like polysacharride which is derived from amylopectin [10]. It has been used as an effective volume expander and is available in several types of molecular weight with different chemical properties. Several small studies suggested a beneficial effect of HES in decreasing OHSS and indicate that HES should be further investigated [10]. In one study, 1000 ml of 6% HES was used at the time of oocyte retrieval and an additional 500 ml was given 48 h later in 100 patients considered at high risk of OHSS, with estradiol > 11 000 pmol/l and 20 follicles or more. The outcome was compared with that of a historical control group of 82 patients without any prophylactic measure. Seven cases of severe OHSS and 32 cases of moderate OHSS occurred in the control group, compared with two cases of severe and 10 cases of moderate OHSS in the HES group ($P < 0.05$ and $P < 0.001$ respectively). In a prospective, randomized trial HES and placebo were evaluated in patients with estradiol levels > 1500 pg/ml and ten follicles on the day of hCG. The dose of HES was 1000 ml after oocyte retrieval. In the HES group, there was one case of moderate OHSS and no severe cases, compared with six cases of moderate OHSS and one case of severe OHSS in the control group.

Glucocorticoid administration

The pathophysiology of OHSS suggests the involvement of an inflammatory mechanism during the development of the fluid leakage associated with OHSS. Therefore, investigators hypothesized that glucocorticoids could possibly prevent OHSS in patients at high risk. Rizk [8] and others found no protective effect of intravenous glucocorticoid.

Laparoscopic ovarian drilling in PCOS patients

Laparoscopic ovarian drilling has been used successfully for prevention of OHSS in patients with polycystic ovaries. Both ovarian diathermy and laser vaporization have been used immediately prior to the commencement of ovarian stimulation in patients at risk of OHSS. There are no particular advantages for any power modality. Ovarian diathermy has been performed in either one or both ovaries. Transvaginal ovarian drilling has also been reported in patients with PCOS undergoing assisted reproductive technology. It has been suggested that transvaginal ovarian drilling has been effective in improving IVF results in difficult cases and is less invasive and expensive when compared with laparoscopic ovarian drilling. The technique is relatively straightforward: a triple puncture laparoscopy is usually performed and the ovary is grasped by the ovarian ligament. A variety of instruments have been used for many years with tremendous variation in the literature regarding the three variables involved, which are the number of ovarian punctures, the duration of diathermy, and the wattage used. Generally no fewer than four and no more than 10 punctures to a depth of 4–10 mm on each ovary should be made. Fewer than four punctures on each ovary results in lower pregnancy rates but more than 10 may cause ovarian damage. The best results are typically seen in slim patients with high serum concentrations of LH.

Metformin

Tang *et al.* [54] performed a randomized, placebo-controlled, double-blinded study between 2001 and 2004. Patients with PCOS undergoing IVF/ICSI treatment using a long GnRH-agonist protocol were randomized to receive 850 mg metformin or placebo tablets twice daily from the start of the down-regulation process until the day of oocyte collection. A total of 101 IVF/ICSI cycles were randomized to receive metformin ($n = 52$) or placebo ($n = 49$). Both the clinical pregnancy rates beyond 12 weeks' gestation per cycle (metformin 38.5%, placebo 16.3%, $P = 0.023$) and per embryo transfer (metformin 44.4%, placebo 19.1%;

$P = 0.022$) were significantly higher in those treated with metformin. Furthermore, a significant decrease in the incidence of severe OHSS was observed (metformin 3.8%, placebo 20.4%; $P = 0.023$), and this was still significant after adjustment for body mass index, total recombinant FSH dose, and age (OR 0.15; 95% CI 0.03–0.76; $P = 0.022$). In conclusion, short-term co-treatment with metformin for patients with PCOS undergoing IVF/ICSI cycles does not improve the response to stimulation but significantly improves the pregnancy outcome and reduces the risk of OHSS.

In a systematic review by Moll *et al.* [55] for IVF, it was found that metformin led to fewer cases of OHSS (RR 0.33; 95% CI 0.13–0.80).

Dopamine agonists in the prevention of OHSS

Vascular endothelial growth factor (VEGF) secreted by the hyperstimulated ovary acting via the VEGF receptor 2 is a major cause of OHSS and its specific inhibition prevents increased vascular permeability (Figures 13.12 and 13.13) [56–60]. Ovarian gene expression in animals subjected to different regimens of stimulation, including control animals and stimulated rats developing OHSS, were compared. VP was measured 48 hours after hCG and mRNA from ovaries was extracted to perform gene expression profiles in macroarray filters containing 14 000 genes. Gene expression showed 80 up-regulated and seven down-regulated genes in OHSS as compared to mild stimulation and controls. Up-regulated genes were grouped in five families: cholesterol synthesis, VEGF signal transduction, prostaglandin synthesis, oxidative stress process, and cell cycle regulation. The down-regulation of tyrosine hydroxylase (TH, enzyme responsible for dopamine synthesis) was considered a characteristic of OHSS as well [56–60].

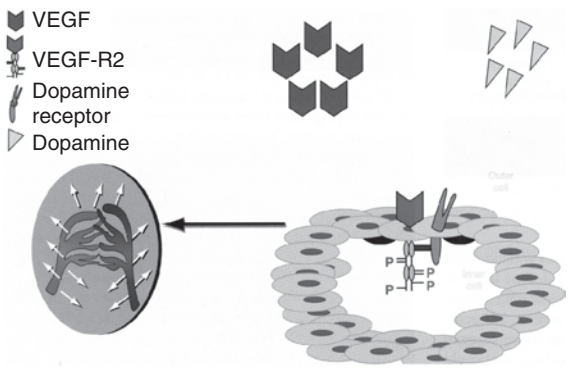


Figure 13.13. Molecular mechanism of dopamine agonist on vascular permeability. Reproduced with permission from: Rizk B, Rizk CB, Nawar MG, Garcia-Velasco JA, Sallam HN. Ultrasonography in the prediction and management of ovarian hyperstimulation syndrome. In: Rizk B, ed. *Ultrasonography in Reproductive Medicine and Infertility.* Cambridge: Cambridge University Press; 2010: 299–312. See colour plate section.

Targeting the up-regulated genes could compromise basic cellular or physiological processes, such as E_2 and P4 production and ovulation, or will block unspecific secondary signal transduction pathways shared by many molecules. The focus was the down-regulated genes, with the idea that they could act in the opposite way to the up-regulated genes, that is, as a natural inhibitors of the angiogenic processes that then needed to be enhanced or up-regulated [56–60].

Tyrosine hydroxylase, the key enzyme responsible for dopamine synthesis, was down-regulated, suggesting that maybe dopamine could act as an antiangiogenic factor in the ovary, so a deficit in its production after PMSG + hCG administration in the OHSS animal model could be involved in the increased VP, which characterizes the syndrome. In fact, dopamine administration could decrease VP in in vitro and in vivo cancer models by decreasing VEGFR-2 phosphorylation, which is the first step in VEGF downstream signaling leading to the iVP after VEGF binding to this receptor. Although the mechanism by which dopamine is able to decrease VEGFR-2 phosphorylation still remains unknown, the administration of dopamine to the OHSS rats may become as effective, inhibiting iVP as it had been decreasing angiogenesis in the cancer models [58].

Dopamine receptor 2 (D-r2) agonists, used in the treatment of hyperprolactinemia including pregnancy, inhibit VEGFR-2-dependent VP and angiogenesis when administered at high doses in animal cancer models. To test whether VEGFR-2-dependent VP and angiogenesis could be segregated in a dose-dependent fashion with the Dp-r2 agonist cabergoline (Cb2), a well-established OHSS rat model supplemented with prolactin was used. A 100 μg/kg low-dose Dp-r2 agonist reversed VEGFR-2-dependent VP without affecting luteal angiogenesis through partial inhibition of ovarian VEGFR-2 phosphorylation levels. No luteolytic effects (serum progesterone levels and luteal apoptosis unaffected) were observed. Cb2 administration also did not affect VEGF/VEGFR-2 ovarian mRNA levels [58].

Alvarez *et al.* performed a pilot study designed to analyze whether cabergoline administration, as an intervention for preventing OHSS, affects the outcome of assisted reproduction treatment. The study was

retrospective with the end-points of implantation and ongoing/term pregnancy rates. Women ($n = 35$) at risk of OHSS (20–30 follicles developed and >20 oocytes collected) took a daily oral dose of 0.5 mg cabergoline for 8 days, beginning on the day of hCG administration. They were matched with controls treated during the same period and who were similar with respect to age, number, and quality of the embryos replaced, embryonic stage at transfer, and sperm quality. No difference was detected between the groups in fertilization, implantation, or pregnancy rates. Administration of cabergoline in order to prevent OHSS is safe and does not appear to affect assisted reproduction outcome.

The study by Alvarez *et al*. is the first well-designed randomized double-blinded study that investigated the role of cabergoline in the prevention of OHSS. The study showed that cabergoline significantly lowered hematocrit, hemoglobin, and ascites on day 4 and day 6 after treatment, as compared with placebo. However, the study showed a significant reduction in the incidence of moderate OHSS only in the cabergoline arm. No difference was noticed in the incidence of severe OHSS. Although cabergoline was effective in prevention of moderate OHSS, it did not affect estradiol concentration.

Busso *et al*. reported a randomized, double-blinded, parallel group, dose-finding, placebo-controlled multicenter study assessing the effect of three oral doses (50 μg/day, 100 μg/day, 200 μg/day) of the non-ergot-derived dopamine agonist quinagolide in comparison with placebo in preventing moderate/severe early OHSS in IVF patients. A total of 182 IVF/ICSI patients with 20–30 follicles ≥ 10 mm and serum estradiol ≤ 6000 pg/ml at the end of stimulation were included [59]. Quinagolide/placebo was initiated at least 2 h before hCG administration (250 μg s.c.) when two or more follicles of ≥ 18 mm were observed and continued for 17–21 days until the day before the serum β-hCG test. The study showed that moderate/severe early OHSS was reported in 23% of the placebo patients, and 12%, 13%, and 4% in the quinagolide groups, 50, 100, 200 μg/day, respectively [59].

Cryopreservation of all embryos for future transfer

In a Cochrane review by D'Angelo and Amso, 17 studies were identified, two of which met the inclusion criteria [61]. One study was included where cryopreservation was compared with IV albumin administration and one study was included where elective cryopreservation of all embryos was compared with fresh embryo transfer. When elective cryopreservation of all embryos was compared with fresh embryo transfer, no difference was found in all the outcomes examined between the two groups. This review showed that, at present, there is insufficient evidence to support routine cryopreservation and also insufficient evidence to determine the relative merits of IV albumin versus cryopreservation.

In vitro maturation of immature oocytes

In vitro maturation of immature oocytes presents a potential alternative for the fertility treatment of these patients. In patients at risk of OHSS, hMG, or hCG priming was performed and the blastocyst stage was reached without any manifestation of OHSS [62–64].

Treatment of ovarian hyperstimulation syndrome

The clinical course of OHSS depends on its severity, whether complications have already occurred and the presence or absence of pregnancy [6, 15–17]. The clinical management involves dealing with electrolytic imbalance, neurohormonal and hemodynamic changes, pulmonary manifestation, liver dysfunction, hypoglobulinemia, febrile morbidity, thromboembolic phenomena, neurological manifestations, and adnexal torsion (Figure 13.14). The general approach will be adapted to the levels of severity. Specific approaches such as paracentesis and pleural puncture should be carefully performed when necessary. Medical management requires familiarity with the disease. Many of the problems that occur happen as a result of lack of realization by internists and medical specialists that the syndrome is different from similar presenting medical syndromes. A better understanding of the underlying pathophysiological mechanisms will help in refining the management.

Outpatient management for moderate OHSS

Based on the classification of Rizk and Aboulghar [5], moderate OHSS will be followed up by regular telephone calls daily at least and twice weekly office visits. The assessment at the office includes pelvic ultrasound, complete blood count, liver function test and coagulation profile. The patient should be instructed

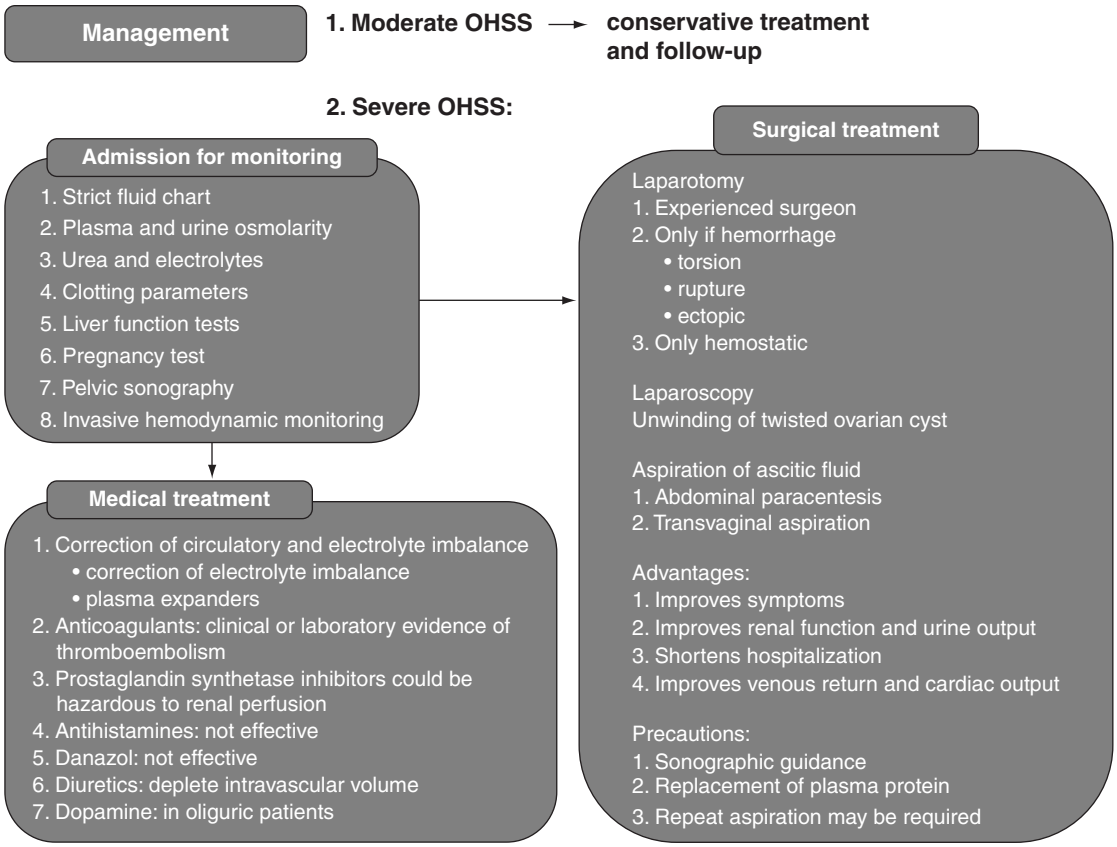


Figure 13.14. Management of OHSS. Reproduced with permission from: Rizk B, Rizk CB, Nawar MG, Garcia-Velasco JA, Sallam HN. Ultrasonography in the prediction and management of ovarian hyperstimulation syndrome. In: Rizk B, ed. *Ultrasonography in Reproductive Medicine and Infertility*. Cambridge: Cambridge University Press; 2010: 299–312.

to report to the hospital if she develops dyspnea, if the volume of urine is diminished, or upon development of any unusual symptoms such as leg-swelling, dizziness, numbness, and neurological problems.

Outpatient management of severe OHSS

The question of whether severe OHSS should be managed on an outpatient basis depends on the classification and definition of severity, comfort of the physician, and compliance and reliability of patients. OHSS grade A is treated by aspiration of ascetic fluid, administration of intravenous fluids, and evaluating all biochemical parameters on an outpatient basis.

In-hospital management of severe OHSS

Patients with severe OHSS grade B or C are admitted to hospital for treatment. Indications for hospitalization are shown in Table 13.17. Hospitalization should be considered if one or more of these symptoms or signs are present [64]. Great caution is required in all grades because complications can suddenly occur.

Clinical and biochemical monitoring in hospital

The patient's general condition requires regular assessment, with documentation of vital signs, together with daily weight and girth measurement. Strict fluid balance recording is needed, particularly of urine output.

Biochemical monitoring should include serum and electrolytes, renal and liver function tests, a coagulation profile, and blood count. Serum and urinary osmolarity and urinary electrolyte estimation may be required as the severity of the disease process increases. Respiratory compromise and/or significant deterioration of renal function require evaluation of blood gases and acid-base balance. The frequency of these investigations will depend on the severity of the condition.

Table 13.17. Indications for hospitalization of patients with severe OHSS: reproduced with permission from: Rizk B. *Ovarian Hyperstimulation Syndrome: Epidemiology, Prevention and Management*. Cambridge: Cambridge University Press; 2006, Chapter 8, p. 201

1. Severe abdominal pain or peritoneal signs.
2. Intractable nausea and vomiting that prevents ingestion of food and adequate fluids
3. Severe oliguria or anuria
4. Tense ascites
5. Dyspnea or tachypnea
6. Hypotension (relative to baseline), dizziness, or syncope
7. Severe electrolyte imbalance (hypernatremia, hyperkalemia)

Ultrasonographic examination provides accurate assessment of ovarian size and the presence or absence of ascites, as well as pleural or pericardial effusions. Also, it will help in the diagnosis of intra- or extra-uterine pregnancy as well as multiple or heterotrophic pregnancy. Chest X-ray will also provide information on the presence of hydrothorax. Assay of β-hCG will help to diagnose pregnancy as early as possible.

Invasive hemodynamic monitoring (central venous pressure and pulmonary artery pressures) may be needed under certain circumstances in which volume expanders are being employed.

Medical treatment

Circulatory volume correction

The main line of treatment is correction of the circulatory volume and the electrolyte imbalance [7, 9]. Every effort should be directed towards restoring a normal intravascular volume and preserving adequate renal function. Volume replacement should begin with intravenous crystalloid fluids at 125–150 ml/h. Normal saline and lactated Ringer have been successfully used. Plasma colloid expanders may be used if necessary. One concern with using plasma expanders is that the beneficial effect is transitory before their redistribution into the extravascular space, further exacerbating ascites formation. Albumin, dextran, mannitol, fresh frozen plasma, and hydryoxyethyl starch (HES) have also been used. HES has the advantage of a non-biological origin and high molecular weight (200–1000 kDa vs. 69 kDa for albumin. Abramov *et al.* compared the efficacy of hydroxyethyl starch and human albumin for the treatment of 16 patients with severe OHSS [65]. They observed a higher urine output, fewer paracenteses, and shorter hospital stays with hydroxyethyl starch solution (Table 13.18). Gamzu *et al.* compared hydroxyethyl starch 10% and Haemaccel and found no clinical advantage for the hydroxyethyl starch [66].

Table 13.18. Controlled cohort study to compare the efficacy of 6% HAES and human albumin for treatment of severe OHSS: reproduced with permission from Abramov *et al. Fertil Steril* 2001;75(6):1228–30

Variable	Human albumin group ($n = 10$)	Hydroxyethyl starch group ($n = 6$)
Baseline characteristics		
Age (years)	28.1 ± 8.9	29.4 ± 7.4
Estradiol level on day of HCG	6164 ± 1418	9080 ± 2450
Administration (pg/ml)		
Ascites (%)	10 (100)	6 (100)
Pleural effusion (%)	2 (20)	1 (17)
Gastrointestinal symptoms (%)	4 (40)	6 (100)
Ovarian diameter	10.7 ± 3.4	10.5 ± 3.8
Oliguria	1 (10)	1 (16)
Hematocrit (%)	42 ± 3.6	43 ± 7
Daily fluid intake (ml)[a]	3300 ± 310	3150 ± 2170
Patient outcome		
Daily urine output[b]	2557 ± 1032	3580 ± 1780
Abdominal paracentesis (%)	8 (80)	2 (33)
Total fluid aspirated per patient (ml)	2300 ± 230	1930
Pleurocentesis (%)	2 (20)	0
Hospital stay (days)	19 ± 8.2 /emphasis>	15.7 ± 5.7
Conception	7 (70)	4 (67)
Miscarriage	2 (28)	1 (17)
Congenital malformation	0	0

Values are means ± SD or percentages of patients.
[a] Including oral and intravenous hydration.
[b] During the first five days of hospitalization.

Table 13.19. Electrolyte disorders in OHSS: modified with permission from Delvigne *et al. Hum Reprod* 1993;8:1361–6

Type of disorder	No. (%)
Hemoconcentration	91 (71.1%)
Electrolytic imbalance	70 (54.6%)
K	31 (24.2%)
Na	29 (22.7%)
Cl	5 (3.9%)
HCO_3	5 (3.9%)
Elevated transaminases	33 (25.8%)

Electrolyte replacement

Appropriate solutions will correct electrolyte imbalances (Table 13.19). A cation exchange resin may rarely be needed in cases of potassium-level disturbance. Sodium and water restriction has been used but others found no change in the patient's weight, abdominal circumference, or peripheral edema when sodium and water were restricted. Therefore, salt and water restriction are not widely advocated [10].

Anticoagulant therapy

Anticoagulant therapy is indicated if there is clinical evidence of thromboembolic complications or laboratory evidence of hypercoagulability [16, 17]. Venous thrombosis is the most common serious complication of OHSS [67]. Preventative treatment with heparin should be used whenever there is a thromboembolic risk. In cases of severe OHSS, the following situations are recognized as indicating an increased risk of thromboembolism: immobilization, compression of pelvic vessels by large ovaries or ascites, pregnancy coagulation abnormalities, and hyperestrogenemia. Prevention using mobilization and antithrombosis stockings is insufficient as thrombosis may occur at all localizations and may be systemic in nature.

Anticoagulant prophylaxis

Prophylaxis with heparin remains debatable. First, there are no randomized studies proving its efficacy in preventing thromboembolic complications during severe OHSS. Second, in some clinical scenarios, thromboembolism still occurs despite giving heparin [10]. Despite these reservations Rizk [10] recommended giving heparin or Lovenox for patients with severe OHSS. The incidence of deep vein thrombosis is markedly increased in patients with Leiden factor V mutation, protein C & S deficiency, and antithrombin III deficiency [68, 69]. Leiden factor V mutation occurs in 4% of Northern European women. Patients should be questioned about a history of personal or familial thrombosis, and if positive should be tested for Leiden factor V mutation. If OHSS occurs, patients with Leiden factor V mutation should be placed on prophylactic heparin.

Duration of anticoagulation

The duration of anticoagulant administration is debatable. Some investigators have reported late thrombosis up to 20 weeks post transfer and many investigators are in favor of maintaining heparin therapy for many weeks [18]. Severity of OHSS must be separated from risk of thromboembolism because intrinsic coagulopathy may trigger the problem even in moderate cases. However, those who have followed a more liberal policy for prophylaxis have had to deal with operating on ruptured ectopic pregnancies in anticoagulated patients. Therefore, thromboembolism will remain a more difficult complication to prevent and may complicate the outcome.

Antibiotic treatment

Infections are not uncommon in the setting of treatment of OHSS because of frequent catheterizations, venipuncture, transvaginal aspiration of ascitic fluid, and pleural drainage. Furthermore, hypoglobulinemia is present in severe cases. Preoperative antibiotic prophylaxis is highly recommended. Some authors suggested the administration of immunoglobulins for the treatment of infections associated with severe OHSS. However, this intervention still awaits further evaluation.

Diuretics

Diuretic therapy without prior volume expansion may prove detrimental, by further contracting the intravascular volume, thereby worsening hypotension and its sequelae [9]. Diuretics will increase blood viscosity and increase the risk of venous thrombosis. Diuretic use should be restricted to the management of pulmonary edema [9].

Dopamine

Dopamine used in oliguric patients with severe OHSS results in significant improvement in renal function

[69, 70]. Dopamine produces its renal effect by increasing renal blood flow and glomerular filtration. Dopamine therapy should be given cautiously and under strict observation. In one report, intravenous dopamine 4.32 mg/kg per 24 hours, was administered to seven patients hospitalized with severe OHSS following gonadotropin stimulation for IVF or GIFT, beginning within 10 hours of admission [69]. Additional treatment consisted of: bed rest, restriction of fluid intake to 500 ml/day, and daily monitoring of urine output, abdominal girth, and weight. Biochemical and hematological clotting factors were measured daily in the pregnant women. Serum hCG was measured every 2 days and the patients were given a protein- and salt-rich diet in order to increase the oncotic and osmotic blood pressure. Dopamine treatment was continued until there was complete resolution of ascites. In the five patients who were pregnant, dopamine treatment was required for 9 to 22 days. The duration of treatment was related to the magnitude of the increase of hCG and was the longest (18–22 days) in patients with triplets, shortest (9–10 days) in patients with a singleton pregnancy, and intermediate (14 days) for patients with twins. In the two non-pregnant women, dopamine was only required for 7 days. In another report, a 750 mg tablet of docarpamine (Dostinex) was taken orally every 8 hours by 27 patients, hospitalized because of OHSS and refractory to the initial therapy with intravenous albumin [70]. Clinical symptoms associated with ascites were gradually improved; there were no major adverse effects.

Aspiration of ascitic fluid and pleural effusion in severe OHSS

The presence of ascites is the hallmark of OHSS. Symptoms resulting from ascites are the most common reason for hospitalization. Aspiration is not indicated in every patient. Paracentesis by the transabdominal or transvaginal route is indicated for severe abdominal pain, pulmonary compromise as demonstrated by pulse oximetry, and tachypnea and renal compromise as demonstrated by oliguria and increased creatinine concentration [71–74].

Abdominal paracentesis

Paracentesis is followed by increased urinary output shortly after the procedure, with a concomitant decrease in the patient's weight, leg edema, and abdominal circumference. Also the creatinine clearance rate is increased following the procedure. Paracentesis offers temporary relief of respiratory and abdominal distress, but, since the fluid tends to recur, some patients needed repeated paracentesis and drainage of effusions before spontaneous improvement occurs. The amount of fluid aspirated can range between 200 and 4000 ml. The risk of injury to ovaries is minimized by ultrasonographic guidance. Monitoring of plasma proteins is essential and human albumin should be infused whenever necessary. Percutaneous placement of a pigtail catheter is a safe and effective treatment modality for severe OHSS that may represent an alternative to multiple vaginal or abdominal paracentesis [71, 72].

Transvaginal ultrasound-guided aspiration

Transvaginal ultrasound-guided aspiration is an effective and safe procedure. Injury to the ovary is easily avoided by puncture under ultrasonic visualization [73]. No anesthesia is required for the procedure, and better drainage of the ascitic fluid is accomplished because the pouch of Douglas is the most dependent part [73].

Autotransfusion of ascitic fluid

Transvaginal aspiration of ascitic fluid and autotransfusion of the aspirated fluid has been used in treatment of severe OHSS [74]. The procedure is simple, safe, and straightforward, and shows a striking physiological success in correcting the maldistribution of fluid and proteins without the use of heterogenous biological material. However, the author does not recommend autotransfusion of ascitic fluid because of the possible reinjection of cytokines into the circulation [10].

Pleurocentesis and treatment of pulmonary complications

Evaluation and treatment of patients with severe OHSS complaining of dyspnea includes physical examination, chest ultrasound and X-ray, and arterial blood gases. It is essential to evaluate accurately any pulmonary complications that may result in hypoxia. If a pulmonary embolus is suspected, a CT scan or ventilation perfusion scan should be performed. Pulmonary comprise should be treated with oxygen supplementation. Thoracocentesis may be necessary for patients with significant hydrothorax. However, a dramatic improvement in the clinical status may occur after paracentesis [10].

Adult respiratory distress syndrome (ARDS)

ARDS is encountered after fluid overload. The importance of a strict fluid input/output balance in patients with moderate complications of OHSS is stressed. Optimum management may require admission to an intensive care unit. ARDS subsides after 3 to 6 days with fluid restriction, forced diuresis, and dopamine therapy [10].

Pericardiocentesis

Pericardial effusion rarely occurs but if it does, drainage by specialists may be necessary [75].

Surgical treatment

Anesthesia considerations in OHSS patients

Surgery is infrequently needed, but if required there are several aspects important for the anesthesiologist (Table 13.20). Careful positioning of patients during surgery is important as the Trendelenberg position may further compromise the residual pulmonary functional capacity. Establishment of access lines may be necessary in patients with contracted vascular volume. Drainage of pleural effusions may assist in improving pulmonary status [76].

Surgery for ruptured cysts

Laparotomy, in general, should be avoided in OHSS. If deemed necessary, in cases of hemorrhage or ruptured ovarian cysts, it should be performed by an experienced gynecologist and only hemostatic measures undertaken so as to preserve the ovaries.

Table 13.20. Challenges to the anesthesiologist in OHSS

• Pulmonary compromise
• Severe hemoconcentration
• Pleural effusions
• Restricted IV access
• Infections and febrile morbidity
• Positioning in surgery
• Thrombophlebitis
• Pelvic masses
• Ascites
• Electrolytic disturbances

Ovarian torsion

Ovarian torsion is an infrequent complication of ovulation induction which if unrecognized and untreated results in the loss of one or both ovaries [59]. Presenting symptoms are severe unilateral adnexal pain, in a patient with enlarged ovaries due to ovulation stimulation, or with multiple pregnancy. Sonography with Doppler flow analysis can be diagnostic but a finding of apparently normal blood flow does not rule out ovarian torsion [59].

In a review of 77 cases of ovarian torsion at a single hospital, Doppler flow sonography was performed preoperatively in 90% of cases but demonstrated compromised blood flow in only 29% of those scanned [60]. Of these, 39 (51%) required oophorectomy. Ovarian torsion followed ovulation induction in 21 of the 77 cases, and of these only one required oophorectomy, possibly because of earlier diagnosis. Twenty-four patients were pregnant, two-thirds as a result of ovulation induction. The mean gestational age was 10.4 weeks (range 4–28) at the time of ovarian torsion. Although the adnexa usually appear dark, hemorrhagic and ischemic, they can be saved, if the diagnosis is made soon enough, by simply unwinding often as a laparoscopy procedure.

Surgery for ectopic pregnancy associated with OHSS

The association between OHSS and ectopic pregnancy is not commonly encountered. Diagnosis of tubal pregnancy by vaginal ultrasound examination at this stage is not always possible. The presence of large ovaries filling the pelvis makes ultrasound scanning of other structures difficult. Fluid in the pouch of Douglas is of limited diagnostic importance in the presence of ascites.

Other surgery

Mesenteric resection after massive arterial infarction has been reported. Rarely vascular surgery is required to treat thromboses that are complicated by recurrent emboli or resistant to medical intervention. Posterolateral thoracotomy and subclavian arteriotomy and thromboarterectomy by the Fogarthy technique have been reported. Inferior vena cava interruption to prevent massive thromboembolism has also been used [10].

Key points

- Ovarian hyperstimulation syndrome is characterized by bilateral cystic ovarian enlargement and third-space fluid shift resulting in ascites and pleural effusion.
- Severe OHSS might be complicated by thromboembolism, adult respiratory distress syndrome, and kidney failure.
- Human chorionic gonadotropin increases VEGF production by granulosa cells and endothelial cells, which results in increased vascular permeability.
- The cornerstone of successful prevention of OHSS is accurate prediction.
- Previous history of OHSS or polycystic ovary syndrome is highly predictive of the development of OHSS during ovarian stimulation.
- Ultrasound is essential for the prediction of OHSS before, during, and after the treatment cycle.
- High basal antral follicle count and ovarian volume are strongly associated with OHSS.
- The presence of a large number of follicles (> 20 per ovary) is associated with an increased risk for the development of severe OHSS.
- Increased intraovarian blood flow and low intravascular ovarian resistance are correlated with the severity of OHSS in patients who develop the syndrome.
- The presence of multiple pregnancy increases the risk of the severity and duration of OHSS.
- The primary prevention of OHSS can be achieved by the use of low-dose gonadotropins and, in some cases, ovarian drilling prior to IVF.
- The secondary prevention of OHSS involves delaying the hCG, known as "coasting," and, in some cases, cancelation of the hCG.
- The medical treatment of OHSS consists of correction of circulatory volume and electrolyte imbalance.
- Ultrasonographic guidance of transvaginal or transabdominal aspiration of ascites improves the symptoms of patients with OHSS.

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Chapter 14

The evidence-based practice of assisted reproduction

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Despite numerous developments in the field of assisted reproduction, the pregnancy and delivery rates in couples undergoing IVF and ICSI remain low. In the latest report by the Society of Assisted Reproductive Technology (SART), the pregnancy rate per initiated cycle was 32.8% and the delivery rate was 27.2% [1]. In order to improve the results, various modifications have been suggested and practiced during the various stages of the treatment, but not all of these have been substantiated by randomized controlled trials (RCTs). This chapter will review these modifications in the light of clinical evidence.

Stimulation protocols

The first successful IVF was achieved in a natural non-stimulated cycle [2]. However, it soon became clear that controlled ovarian hyperstimulation leads to the recruitment of more oocytes and better pregnancy rates. Controlled ovarian hyperstimulation was first achieved with clomiphene citrate, but this was later superseded with the use of human menopausal gonadotropins (hMGs), urinary FSH preparations, and more recently by recombinant FSH (r-FSH) preparations [3]. Nevertheless, the results were hampered by the possibility of premature luteinization leading to cancelation of many treatment cycles. The introduction of GnRH agonists (GnRHa) permitted the down-regulation of the pituitary gland in order to eliminate any premature LH surge and various protocols have been suggested and used successfully [4, 5]. In the short protocol of GnRHa administration, the agonist is started on day 1 or 2 of the menstrual cycle. The injection of hMG (or FSH) is commenced a few days after the start of GnRHa, and the agonist is continued until the day of hCG administration. In the ultrashort protocol, the agonist is administered for 2 or 3 days only at the beginning of the cycle in order to take advantage of its flare-up effect and the hMG (or FSH) is started afterwards. In the long protocol, hMG (or FSH) administration is delayed until pituitary desensitization has been achieved, usually after 2 to 3 weeks of starting GnRHa. In the long protocol, GnRHa is started either in the midluteal phase or in the early follicular phase. More recently, GnRH antagonists were introduced with various claims of success. These compounds do not have a flare-up effect and down-regulation is achieved immediately. Two protocols for GnRH antagonists have been described: the single-dose protocol and the multiple-dose protocol. In the single-dose protocol, one single dose of 3 mg is administered in the late follicular phase, while in the multiple-dose protocol the effect is achieved by daily administration of 0.25 mg of the antagonists, starting on day 5 or 6 of the cycle [6].

GnRH agonists versus HMG

It has now been established that the routine use of GnRHa prior to IVF and gamete intrafallopian transfer (GIFT) increases the clinical pregnancy rate compared to the use of hMG alone. In a meta-analysis of RCTs, Hughes *et al.* found that the clinical pregnancy rate per cycle commenced was significantly improved after GnRHa use for IVF (odds ratio [OR] 1.80; 95% CI 1.33 to 2.44) and GIFT (OR 2.37; 95% CI 1.24 to 4.51) [5]. Cycle cancelation was decreased (OR 0.33; 95% CI 0.25 to 0.44), whereas spontaneous abortion was similar with and without GnRHa use. However, GnRHa was associated with a slight, but insignificant, increase in the incidence of ovarian hyperstimulation syndrome (OHSS) and multiple pregnancies (OR 2.56; 95% CI 0.95 to 6.91).

Human Assisted Reproductive Technology: Future Trends in Laboratory and Clinical Practice, ed. David K. Gardner, Botros R. M. B. Rizk, and Tommaso Falcone. Published by Cambridge University Press. © Cambridge University Press 2011.

Long versus short protocols

Randomized controlled trials have shown that long stimulation protocols are superior to short and ultrashort protocols in terms of clinical pregnancies. In a Cochrane review conducted by Daya, the long protocols were compared to the short and ultrashort protocols [4]. The common OR for clinical pregnancy per cycle started was 1.32 (95% CI 1.10 to 1.57) in favor of the long GnRHa protocols. When in the long protocol, the GnRHa was commenced in the follicular phase, the respective ORs were 1.54 (95% CI 1.11 to 2.13) and when the GnRHa was started in luteal phase, the respective ORs were 1.21 (95% CI 0.98 to 1.51). When the long protocols were compared to the short protocols, the ORs were 1.27 (95% CI 1.04 to 1.56) in favor of the long protocols. Similarly, when the long protocols were compared to the ultrashort protocols, the ORs were 1.47 (95% CI 1.02 to 2.12), in favor of the long protocol.

GNRH antagonists versus agonists

The value of the GnRH antagonist protocols compared to the long stimulation protocols is still controversial. In a Cochrane review conducted by Al-Inany *et al.* 27 trials fulfilled the inclusion criteria. The reviewers found that, in comparison to the long protocol of GnRHa, clinical pregnancy rate was significantly lower in the antagonist group (OR 0.84, 95% CI 0.72 to 0.97). The ongoing pregnancy/live birth rate was also significantly lower in the antagonist group (OR 0.82, 95% CI 0.69 to 0.98). On the other hand, there was a statistically significant reduction in incidence of severe OHSS with antagonist protocol (RR 0.61, 95% CI 0.42 to 0.89) [6]. In contrast, another meta-analysis conducted by Kolibianakis *et al.* showed no significant difference in the probability of live birth between the two GnRH analogs (OR 0.86; 95% CI 0.72 to 1.02). However, in the latter study, some of the live birth rates were calculated by extrapolating the clinical pregnancy rates assuming that 84 % of clinical pregnancies reaching 7 weeks and 92% of those reaching 12 weeks result in live births [7].

Urinary FSH versus hMG

hMG preparations contain FSH and LH in the ratio of 1:1, while urinary FSH preparations contain very small amounts of LH. This suggests that the follicles may be exposed to high levels of LH during their early stages of development when stimulated with hMG compared to the urinary FSH preparations. Many studies have demonstrated that too much LH during the time of follicle development and in the peri-ovulatory phase is associated with reduced rates of fertilization and pregnancy and an increase in the probability of spontaneous abortion. In a Cochrane review, Daya compared the use of urinary FSH to hMG in assisted reproduction. He found that the CPR was higher when urinary FSH was used compared to hMG. The overall ORs for clinical pregnancies in favor of FSH per cycle started, per oocyte retrieval, and per embryo transfer were 1.70 (95% CI 1.11 to 2.60), 1.68 (95% CI 1.10 to 2.56), and 1.69 (95% CI 1.10 to 2.59), respectively [4].

Recombinant FSH versus hMG

The main source of hMG and FSH preparations is the urine of postmenopausal women. New developments in drug manufacturing have resulted in the production of pure FSH in vitro by recombinant DNA technology. These preparations have an extremely high purity and batch-to-batch consistency which make them attractive alternatives to hMG and urinary FSH preparations.

In a Cochrane review including four studies published in 2003, there were no significant differences between hMG and r-FSH in ongoing pregnancy/live birth per woman (OR 1.27; 95% CI 0.98 to 1.64) [8]. However, in a recent meta-analysis conducted by Al-Inany *et al.* published in 2008, the live birth rate was significantly higher with hMG versus r-FSH (OR 1.20, 95% CI 1.01 to 1.42), while OHSS rates were not significantly different (OR 1.21, 95% CI 0.78 to 1.86) [9]. These findings were confirmed in another meta-analysis by Coomarasamy *et al.* [10].

Natural cycle IVF versus stimulated IVF cycles

Despite the fact that the first successful IVF resulted from a natural (non-stimulated) cycle, many subsequent non-randomized studies showed that the clinical pregnancy rate was much higher in stimulated cycles. These results were later confirmed in a RCT comparing the outcome of IVF performed during natural cycles to clomiphene-stimulated cycles [11]. It has also been suggested that performing IVF during natural cycles may improve the outcome in poor responders, endometriosis and in cases of unexplained infertility, but these claims have not been supported by RCTs. In a review of the literature, Pelinck *et al.* found that in 20

selected studies involving 1800 natural (non-stimulated) cycles, the ongoing pregnancy rate was 7.2% per cycle and 15.8% per embryo transfer [12]. However, the real advantages of assisted reproduction performed during natural cycles nowadays are its lower cost and the absence of hyperstimulation. In 2001, Nargund *et al.* performed an economic analysis and calculated that the cost of a natural cycle IVF was approximately 23% of the cost of a stimulated cycle [13].

Patients with polycystic ovaries and PCOS

In a RCT, MacDougall *et al.* found that patients with ultrasound evidence of polycystic ovaries undergoing assisted reproduction had significantly higher serum estradiol levels, developed more follicles and produced more oocytes compared to patients with normal-looking ovaries [14]. The fertilization rate was significantly reduced in these patients, but the pregnancy rate was not affected. More importantly, they had a significantly higher incidence of OHSS. Various modifications to the stimulation protocol have been suggested, mainly to reduce the incidence of OHSS in patients with polycystic ovaries (PCO) and those with the polycystic ovary syndrome (PCOS). These regimens include the step-up protocol, the step-down protocol, the alternate-day protocol, and the sequential protocol [15–19] and although some of these regimens resulted in significant reduction in the risk of OHSS, none of them resulted in improving the pregnancy rates significantly. The addition of metformin has also been suggested in patients with PCOS undergoing IVF or ICSI. A recent Cochrane review reported that the risk of OHSS was reduced significantly (OR 0.27, 95% CI 0.16 to 0.47). However, there was no evidence that metformin treatment before or during assisted reproduction improved live birth or clinical pregnancy rates (OR 0.77, 95% CI 0.27 to 2.18 and OR 0.71, 95% CI 0.39 to 1.28, respectively) [20].

Stimulation protocols for poor responders

There is no consensus on the definition of poor responders in assisted reproduction and Surrey and Schoolcraft reported that 27 different definitions were used by various clinicians [21]. However, some patients are known to repeatedly respond poorly to stimulation protocols with fewer follicles developing, fewer oocytes retrieved, and lower fertilization and pregnancy rates. Various treatment protocols have been suggested and used in these patients. These include increasing the dose of hMG/FSH, using purified hMG or r-FSH instead of hMG, starting FSH in late luteal phase, diminishing the dose and/or duration of GnRHa administration (flare up protocols), or using GnRH antagonists. Other suggestions include supplementing hMG/FSH therapy with growth hormone, clomiphene citrate, DHEA, trans-dermal testosterone, low-dose aspirin, letrozole, or L-arginine [21]. However, none of these suggestions has been confirmed by RCTs, except for a small study favoring the use of r-FSH over hMG [22]. Table 14.1 summarizes the clinical outcome associated with some of the modifications in the stimulation protocols used in assisted reproduction.

Table 14.1. Odds ratios and 95% confidence intervals for the clinical pregnancy (CPR) and live-birth (LBR) rates with various stimulation protocols for patients treated with IVF and ICSI

Stimulation protocol	Odds ratio (95% CI)
Urinary FSH versus HMG stimulation (CPR) [3]	1.70 (1.11–2.60)[a]
Long versus short and ultrashort protocols (CPR) [4]	1.32 (1.10–1.57)[a]
Long versus short protocols (CPR) [4]	1.27 (1.04–1.56)[a]
Long versus ultrashort protocols (CPR) [4]	1.47 (1.02–2.12)[a]
GnRH agonists versus HMG stimulation (CPR) [5]	1.80 (1.33–2.44)[a]
GnRH antagonists versus agonists (CPR) [6]	0.84 (0.72–0.97)[a]
GnRH antagonists versus agonists (LBR) [6]	0.82 (0.69–0.98)[a]
HMG versus reccombinant FSH (CPR) [9]	1.20 (1.01–1.42)[a]
Metformin versus no metformin in PCOS (CPR) [20]	0.77 (0.27–2.18)
Metformin versus no metformin in PCOS (LBR) [20]	0.71 (0.39–1.28)

[a] Statistically significant.

Laboratory issues

In order to optimize the results of assisted reproduction, various laboratory modifications have been suggested. These include performing ICSI rather than IVF for all oocytes, even in cases of non-male factor infertility, using co-culturing techniques, assisted hatching techniques, as well as selecting the embryos with the best potential for implantation based on their morphology, their metabolism, or by prolonging their culture in vitro to the blastocyst stage.

ICSI versus IVF in non-male factor infertility

It has been suggested that performing IVF and ICSI on sibling oocytes for patients with non-male factor infertility could improve the outcome of assisted reproduction [23]. A clinical review of four RCTs found that this approach improved the fertilization rate significantly and prevented total fertilization failure in these patients [24]. However, a subsequent Cochrane review found that performing ICSI for non-male factor infertility did not improve the clinical pregnancy rates (OR 1.4; 95% CI 0.95 to 2.2) [25].

Co-cultures and group culture

Culturing human embryos in the presence of other cells was tried as a method to improve the cleavage rate and hence the pregnancy and implantation rates. Various co-culture systems have been used including the Vero cell line, granulosa cells as well as autologous cryopreserved endometrial cells [26]. Many RCTs have reported improvement in the cleavage rate, embryo morphology, and blastocyst formation rate and a meta-analysis showed that co-cultures resulted in an improvement in the clinical pregnancy rate and the ongoing pregnancy rate. However, the studies were not homogeneous and the results should be interpreted with caution [27]. In addition, there have been concerns regarding the transmission of disease from non-human cells lines to the developing embryo or the intended parent and the US Food and Drug Administration (FDA) formally addressed these concerns in April 2002 by recommending that non-human co-culture cell lines not be used in human IVF [27].

Embryo selection

Different methods of embryo selection have been suggested in order to maximize the implantation rate, while diminishing the incidence of multiple pregnancies. Embryos can be selected at the pronuclear stage based on the polarity of the nucleoli inside the two pronuclei [28]. At the 2 or 4 cell stage, embryo selection is based on the size and regularity of the blastomeres and the presence of fragments [29] or on the zona pellucida thickness variation [30]. Scoring of the blastocyst stage embryo has also been described [31]. However, the clinical value of embryo selection based on these scoring systems has not been established by RCTs. We have attempted to establish an objective method for embryo selection based on computer-aided pattern recognition, but this has not also been tested in a RCT [32].

Other methods of embryo selection include preimplantation genetic screening (PGS) to exclude the abnormal embryos [33], the measurement of embryo respiration rate [34], of amino acid turnover [35], of embryo metabolomics [36], and of embryo proteomics [37]. However, the clinical value of these techniques has not been evaluated by large RCTs.

Assisted hatching and fragment removal

It has been suggested that assisted hatching can improve the implantation capacity of the embryos. Assisted hatching can be performed mechanically, chemically (using a microjet of acid Tyrode), or using the Erbium-YAG laser.The technique is usually reserved for older patients (> 40 years), patients with thick or abnormal zona pellucida, and patients with repeated implantation failures [38, 39].

A recently updated Cochrane review found that assisted hatching had no effect on the odds of live births (OR 1.13, 95%; CI 0.83 to 1.55). However, the clinical pregnancy rate was increased significantly (OR 1.29; 95% CI 1.12 to 1.49) as well as the multiple-pregnancy rate (OR 1.67; 95% CI 1.24 to 2.26), while the miscarriage rates were similar in both groups (OR 1.13; 95% CI 0.74 to 1.73) [40].

Removing cytoplasmic fragments from fragmented embryos has also been claimed to improve clinical pregnancy and implantation rates, but this has not been substantiated by RCTs [41].

Blastocyst culture

Prolonged culture of the embryos has been suggested as a method for selecting the embryos with the best potential for survival and hence for implantation [42]. A recently updated Cochrane review reported an increase in live birth rate with blastocyst transfer on days 5/6 compared to cleavage stage transfer on days 2/3 (OR 1.35; 95% CI 1.05 to 1.74). However, failure to transfer any embryos per couple was significantly higher in the day 5/6 group (OR 2.85; 95% CI 1.97 to 4.11) [43]. It must be added that the real advantage of blastocyst-stage transfer is the reduction of multiple pregnancies, as one or two embryos only can be replaced with the best potential for implantation [44].

In vitro maturation

In vitro maturation (IVM) of human oocytes was suggested in order to achieve fertilization of immature

oocytes occasionally retrieved during stimulated cycles, oocytes retrieved from PCOS patients in natural as well as stimulated cycles, and also after freezing-thawing of immature oocytes. The technique has been shown to lead to normal fertilization, embryo development, pregnancies, and the delivery of healthy children. However, the overall efficiency is still low, indicating that embryo viability is compromised.

In a prospective non-randomized trial, comparing IVM to IVF, the clinical pregnancy rate (44.7% vs. 20.0%) and live birth rate per cycle started (39.5% vs. 20.0%) were lower in the IVM group, but not statistically significant [45]. Large RCT are needed to further evaluate the technique.

Other laboratory issues

It has been suggested that a high oxygen concentration is detrimental to embryo culture in vitro. In a recent RCT, embryos cultured in a 5% O_2 environment resulted in higher rates of implantation (OR 12.2; 95% CI 3.9 to 20.3) and live births (OR 4.8; 95% CI 1.9 to 27.0) compared to the natural 20% concentration present in the atmosphere [46].

Similarly, RCTs have shown that culturing human embryos in antibiotic-free media improves the cleavage rates and that prolonged exposure of the oocytes to the sperm during IVF is associated with lower fertilization rates [47]. Table 14.2 summarizes the clinical outcome associated with the various modifications in some of the laboratory techniques used in assisted reproduction.

Table 14.2. Odds ratio and 95% confidence intervals for the clinical (CPR), ongoing pregnancy rate (OPR), miscarriage rate (MCR), multiple-birth rate (MBR), or live birth rate (LBR) with various laboratory techniques used in assisted reproduction

Technique	Odds ratio (95% CI)
ICSI for non-male factor infertility (CPR) [25]	1.40 (0.95 – 2.20)
Assisted hatching versus no hatching (LBR) [40]	1.13 (0.83 – 1.55)
Assisted hatching versus no hatching (CPR) [40]	1.29 (1.12 – 1.49)[a]
Assisted hatching versus no hatching (MBR) [40]	1.67 (1.24 – 2.26)[a]
Assisted hatching versus no hatching (MCR) [40]	1.13 (0.74 – 1.73)

[a] Statistically significant.

Embryo transfer

Embryo transfer (ET) is arguably the most critical step in assisted reproduction and the least successful. In 1995, Edwards observed that 85% of the embryos replaced in the uterine cavity fail to implant [48].

A consensus of opinion has always existed that a gentle and atraumatic ET technique is necessary for maximizing the chances of pregnancy. Obstacles during transfer include difficulty in negotiating the cervical canal, the necessity of using a volsellum (tenaculum), and the presence of blood after transfer. In 2003, we conducted a meta-analysis of published studies and found that difficult ETs were indeed associated with diminished clinical pregnancy rates (OR 0.73; 95% CI 0.63–0.85) [49].

Various techniques have therefore been suggested in order to optimize this apparently simple step, including performing a trial ET prior to the actual procedure, performing the transfer under ultrasound guidance, using soft catheters rather than the rigid ones, and asking the patients to rest in bed following the transfer.

Dummy (trial) embryo transfers

It has been suggested that performing a trial (mock or dummy) ET before the actual transfer diminishes the incidence of difficult transfers and increases the pregnancy and implantation rates. This can be performed during the luteal phase of the preceding cycle, at the time of oocyte retrieval or immediately before the real transfer [49]. In 1990, Mansour *et al.* conducted a small RCT and found that the clinical pregnancy and implantation rates increased significantly in patients who had a trial ET compared to those who did not (22.8% vs. 13.1%, $P < 0.05$, and 7.2% vs. 4.2%, $P < 0.05$, respectively) [50].

A subsequent study by Henne and Milki challenged the value of performing a mock ET and showed that a retroverted uterus at mock transfer will often change position during the actual procedure [51]. The authors suggested that ultrasound guidance during the real transfer is a better method of judging the direction of the uterine axis, as shown in a study by our group [52].

Ultrasound-guided embryo transfer

Many studies have reported that ultrasound-guided ET is associated with an increase in the clinical pregnancy and implantation rates. In 2003, we conducted

a meta-analysis of RCTs and found that ultrasound-guided ET increased the clinical pregnancy rate significantly (OR 1.42; 95% CI 1.17. to 1.73) as well as the ongoing pregnancy rate (OR 1.49; 95% CI 1.22 to 1.82) [51]. These results were subsequently confirmed in a more recent meta-analysis which found that the technique significantly improved the clinical pregnancy rate (OR 1.50; 95% CI 1.34 to 1.67), the ongoing pregnancy rate (OR 1.51, 95% CI 1.31 to 1.74), as well as the live birth rate (OR 1.78; 95% CI 1.19 to 2.67) [54].

Ultrasound-guided ET is mainly used to confirm that the embryos are properly deposited in the uterine fundus and to follow the embryo-associated air bubble afterwards. We have also suggested that ultrasound can be used to measure the uterocervical angle (Figure 14.1) and mould the ET catheter according to this angle. In a prospective controlled trial, we have found that this technique increased the clinical pregnancy (OR 1.57; 95% CI 1.08 to 2.27) and implantation rates (OR 1.47; 95% CI 1.10 to 1.96) significantly compared to the clinical touch method. The incidences of difficult transfers (OR 0.25; 95% CI, 0.16 to 0.40) and blood (OR 0.71; 95% CI 0.50 to 0.99) during transfers were also significantly reduced [52] (Figure 14.1).

Soft catheters versus rigid catheters

Different types of ET catheters have been used in assisted reproduction with various claims of success. In particular, it has been suggested that soft catheters do not indent the uterine fundus and result in higher clinical pregnancy rates [55]. In a meta-analysis by Abou-Setta *et al.* soft catheters were found to be associated with a significantly higher clinical pregnancy rate compared to firm catheters (OR 1.49, 95% CI 1.26 to 1.77) as well as a higher ongoing/take home baby rate (OR 1.25, 95% CI 1.02 to 1.53) [56]. These results were confirmed by another meta-analysis by Buckett [57].

Site of embryo deposition

It has been suggested that depositing the embryos too near to the uterine fundus could risk injuring the endometrium and diminish the outcome of assisted reproduction and this hypothesis has been supported by a RCT. In this study, Coroleu *et al.* reported a significantly higher implantation rate when the embryos were deposited 2 cm below the uterine fundus compared to when deposited 1 cm below the fundus ($P < 0.05$) [58]. A subsequent meta-analysis advised the

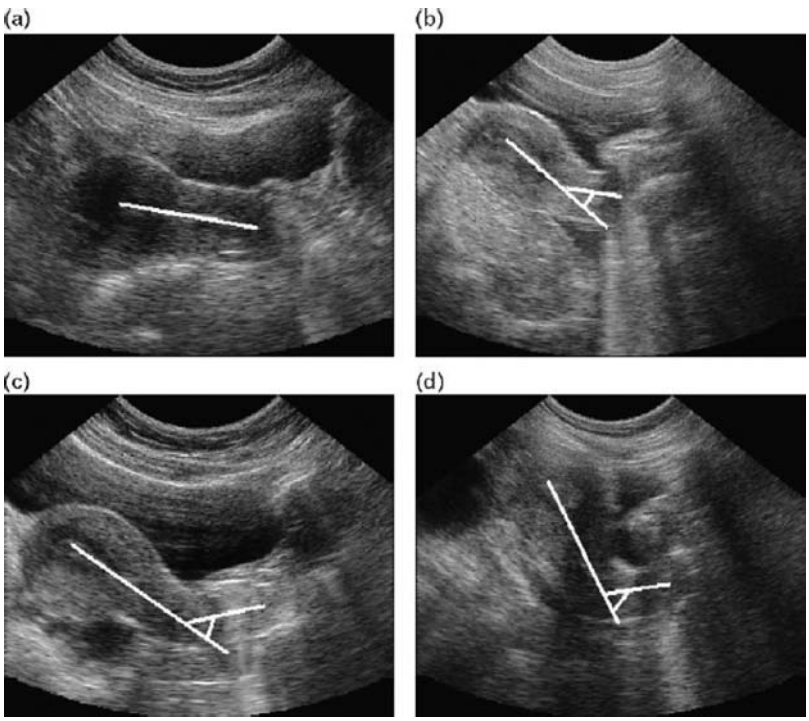


Figure 14.1. Measuring the uterocervical angle prior to embryo transfer. (a) No angle. (b) Small angle (< 308). (c) Moderate angle (30–608). (d) Large angle (> 608). Reproduced with permission from Sallam *et al. Hum Reprod* 2002;17:1767 [52].

performance of more well-designed and powered RCTs to confirm this conclusion [59].

Bed rest after embryo transfer

Prolonged bed rest after ET does not improve the outcome of assisted reproduction. In 1997, Botta *et al.* conducted a RCT of 182 patients undergoing ET and found no statistically significant difference in clinical pregnancy rates between patients who rested in bed for 24 h after transfer compared to those who rested for 20 min only [60]. In a recent Cochrane review, we have found that 30 minutes of bed rest did not affect the ongoing pregnancy rate (OR 1.00; 95% CI 0.54 to 1.85). In addition, there was no significant difference in the clinical pregnancy rate between less bed rest and more rest (OR 1.13; 95% CI 0.77 to 1.67) [61]

Other factors affecting embryo transfer

It has also been suggested that cervical infection is detrimental to assisted reproduction. In an observational study, Egbase *et al.* cultured the tip of the ET catheter and found that the clinical pregnancy rate was significantly lower for patients with positive cultures compared to those with negative cultures [62]. When antibiotics were routinely administered to patients with positive cultures, the clinical pregnancy and implantation rates improved significantly (from 17.8% to 41.3%, $P < 0.01$, and from 9.3% to 21.6%, $P < 0.001$, respectively). The routine administration of antibiotics at ET has also been tested in a RCT. Catheter contamination was significantly diminished but the clinical pregnancy rate was not improved [63].

The effect of the time interval from loading the ET catheter to depositing the embryos in the uterine cavity has also been studied. In a RCT, Matorras *et al.* found that the longer this time interval, the lower the pregnancy and implantation rates and that an interval of more than 120 seconds carries a poor prognosis. They recommended speeding up the transfer process, whenever possible [64].

On the contrary, RCTs have shown that there was no significant difference in the clinical pregnancy rate (RR 1.23; 95% CI 0.96 to 1.58) or live birth rate (RR 1.34; 95% CI 0.85 to 2.11) when ET was performed with acupuncture [65] and that waiting for 30 s before withdrawing the ET catheter does not improve the clinical pregnancy rate [66, 67]. Another RCT reported no differences in pregnancy or implantation rates when the embryo-containing droplet of medium was bracketed by air compared with when the transfer catheter was completely filled with medium [68].

Table 14.3. Odds ratio and 95% confidence intervals for the clinical (CPR), ongoing pregnancy rate (OPR), or live birth rate (LBR) with various techniques used in embryo transfer

Technique	Odds ratio (95% CI)
Gentle versus difficult transfers (CPR) [49]	0.73 (0.63 – 0.85)[a]
U/S guided ET versus no U/S guidance (CPR) [53]	1.42 (1.17 – 1.73)[a]
U/S guided ET versus no U/S guidance (OPR) [53]	1.49 (1.22 – 1.82)[a]
U/S guided ET versus no U/S guidance (CPR) [54]	1.50 (1.34 –1.67)[a]
U/S guided ET versus no U/S guidance (OPR) [54]	1.51 (1.31 – 1.74)[a]
U/S guided ET versus no U/S guidance (LBR) [54]	1.78 (1.19 – 2.67)[a]
Soft catheters versus rigid catheters (CPR) [56]	1.49 (1.26 – 1.77)[a]
Soft catheters versus rigid catheters (OPR) [56]	1.25 (1.02 – 1.53)[a]
Bed rest for 30 minutes versus no rest (OPR) [61]	1.00 (0.54 – 1.85)
Bed rest 30 min versus 24 hours (CPR) [61]	1.13 (0.77 – 1.67)
ET with acupuncture (CPR) [65]	1.23 (0.96 – 1.58)
ET with acupuncture (LBR) [65]	1.34 (0.85 – 2.11)

U/S, ultrasound.
[a] Statistically significant.

In addition, a recent Cochrane review concluded that, at present, there is no evidence of benefit with the following interventions: performing ET with a full bladder, removal of cervical mucus, or flushing the endocervical canal or the endometrial cavity prior to ET. In addition, the authors did not identify any eligible studies for changing patient position (from supine to knee–chest), the use of a tenaculum (volsellum), or embryo afterloading [69]. Table 14.3 summarizes the clinical outcome associated with some of the modifications in the ET technique used in assisted reproduction.

Implantation and endometrial receptivity

Various attempts have been made to improve endometrial receptivity in order to increase the clinical

outcome of IVF and ICSI. These include various regimens of luteal support, the use of corticosteroids, the removal of hydrosalpinges, diminishing uterine contractions as well as enhancing the endometrial blood flow.

Progesterone versus hCG supplementation

It has been shown that the use of GnRHa down-regulation protocols results in luteal-phase deficiency during assisted reproduction [70, 71]. In order to counteract this effect, luteal-phase support has been provided by both progesterone supplementation and/or hCG administration. In a meta-analysis of RCTs, Daya and Gunby found that hCG administration improved the clinical pregnancy rate significantly in patients receiving GnRHa stimulation protocols (OR 2.38, 95% CI 1.32 to 4.29) and decreased miscarriage rates (OR 0.12, 95% CI 0.03 to 0.50) [72]. Similarly, progesterone use resulted in a small but significant increase in pregnancy rates (OR 1.34, 95% CI 1.01 to 1.79), but no effect on the miscarriage rate was observed. On the other hand, no significant difference was found between progesterone and hCG or between progesterone and progesterone plus hCG in terms of pregnancy or miscarriage rates, but the odds of OHSS were more than two-fold higher with treatments involving hCG than with progesterone alone (OR 3.06, 95% CI 1.59 to 5.86). Consequently, progesterone has become the agent of choice for luteal supplementation [73].

Timing and duration of progesterone administration

The time of starting progesterone supplementation has also been a point of debate. In a RCT, Mochtar *et al.* randomized 385 women treated with IVF and ICSI. They found no significant difference in ongoing pregnancy rate between patients who started the progesterone on the day of hCG administration (20.8%), on the day of oocyte retrieval (22.7%), or the day of embryo transfer (23.6%) [74]. The time of stopping the luteal-phase support has also been disputed. In a RCT, Aboulghar *et al.* found no significant difference in the rate of miscarriage when the progesterone supplementation was stopped after the observation of a pulsating fetal heart by ultrasound and when this was continued for 3 more weeks (OR 0.94; 95% CI 0.3 to 3.1) [75].

Route of administration of progesterone for luteal support

The route of administration of luteal-phase progesterone has also been debated. In a RCT, Licciardi *et al.* found no significant difference in clinical pregnancy rate and live birth rate between oral administration of 600 mg of micronized progesterone (45.8%) and i.m. administration of 50 mg progesterone (57.9%). However, the implantation rate was significantly lower with oral progesterone supplementation [76]. Similarly, in another RCT by Chakravarty *et al.* there was no significant difference in LBR between oral administration of 20 mg dydrogesterone (24.1%) and intravaginal administration of 600 mg of micronized progesterone (22.8%) [77]. On the other hand, three RCTs have shown that i.m. administration of progesterone results in higher implantation, ongoing, and live birth rates over vaginal administration [78–80]. However, vaginal administration is sometimes preferred as it is less painful and is associated with a lower incidence of allergic reaction [81].

Estradiol for luteal-phase support

Estradiol supplementation has also been suggested as a method for luteal-phase support with various claims of success. In a meta-analysis of 10 RCTs, Gelbaya *et al.* found no significant difference between luteal-phase support by progesterone alone as compared to estrogen plus progesterone in the clinical pregnancy rate (OR 1.19; 95% CI 0.74 to 1.90) or the ongoing pregnancy rate (OR 1.37; 95% CI 0.81 to 2.30) [82]. A more recent meta-analysis reached the same conclusions [83].

Corticosteroids

Clinical studies have suggested that the administration of corticosteroids during IVF/ICSI therapy might improve the clinical pregnancy rate [84]. However, a recent meta-analysis of RCTs showed that the preimplantation administration of corticosteroids did not improve the CPR (OR 1.16; 95% CI 0.94 to 1.44). However, a subgroup analysis of women undergoing IVF (not ICSI) revealed a significantly higher pregnancy rate for women using corticosteroids (OR 1.50, 95%; CI 1.05 to 2.13) but the significance of this finding is unclear [85]. Similarly, another RCT showed that low-dose glucocorticoids after in vitro fertilization and embryo transfer have no significant effect on the clinical pregnancy rate [86].

Removal of hydrosalpinges

Various studies have shown that the presence of a hydrosalpinx has a detrimental effect on the outcome of assisted reproduction. In 1999, Camus *et al.* conducted a meta-analysis and showed that the clinical pregnancy rate was significantly lower in patients with hydrosalpinges undergoing IVF or ICSI compared to those with no hydrosalpinges (OR 0.64; 95% CI 0.56 to 0.74). The implantation rate was also diminished (OR 0.63; 95% CI 0.55 to 0.72) as was the delivery rate (OR 0.58; 95% CI 0.49 to 0.69). Pregnancy loss was also significantly higher in patients with hydrosalpinges (OR 1.78; 95% CI 1.31 to 2.48) [87].

Consequently, the removal of hydrosalpinges prior to assisted reproduction has been suggested as a measure to improve the outcome. A RCT conducted by Strandell *et al.* in 1999 showed that the removal of the fallopian tubes in patients with bilateral hydrosalpinges increased their implantation, clinical pregnancy, and delivery rates significantly [88] The removal of a unilateral hydrosalpinx also increased the implantation and pregnancy rates, but this did not reach statistical significance, probably because the study lacked enough power. A recent Cochrane review conducted by Johnson *et al.* confirmed these results and showed that laparoscopic salpingectomy increased the ongoing pregnancy rate (OR 2.14, 95% CI 1.23 to 3.73) significantly as well as the clinical pregnancy rate (OR 2.31, 95% CI 1.48 to 3.62). In addition, the review showed that laparoscopic occlusion of the fallopian tube (without its removal) improved the clinical pregnancy rate significantly (OR 4.66, 95% CI 2.47 to 10.01) but not the ongoing pregnancy rate (OR 7.24, 95% CI 0.87 to 59.57) [89].

Ultrasound-guided hydrosalpinx aspiration during oocyte collection was also suggested. In a RCT, Hammadieh *et al.* found that aspiration resulted in a greater biochemical pregnancy rate (RR 2.1; 95% CI 1.02 to 4.60). The clinical pregnancy rate was also improved but this did not reach statistical significance (RR 1.8; 95% CI 0.8, 4.3) [90].

Endometriosis and assisted reproduction

A meta-analysis conducted by Barnhart *et al.* in 2002 showed that patients with endometriosis-associated infertility undergoing assisted reproduction have significantly lower pregnancy rates compared to patients with tubal factor infertility (OR 0.56; 95% CI 0.44–0.70). Moreover, patients with stages III and IV endometriosis have a lower pregnancy rate compared to those with stages I and II (OR 0.60; 95% CI 0.42 to 0.87) [91].

In an attempt to improve these results, various approaches have been proposed to treat the endometriosis prior to assisted conception. Retrospective studies have shown that surgical removal of endometriomas (endometriosis cysts) prior to IVF or ICSI diminishes the pregnancy rate [92], while laparoscopic cystectomy of endometriomas with or without laser vaporization of their internal wall had no effect on the results [93, 94]. Ultrasound-directed endometriotic cyst aspiration prior to IVF was associated with mixed results and an increased incidence of infection and tubo-ovarian abscess formation [95].

Various medical approaches have also been proposed and two small RCTs have shown that immunotherapy with corticosteroids [96] and treatment with danazol prior to IVF or ICSI [97] improved the clinical pregnancy rate in those women. More importantly, in a Cochrane review, we have found that the administration of GnRH agonists for a period of 3 to 6 months prior to IVF or ICSI in women with endometriosis increases the odds of clinical pregnancy by four-fold (OR 4.28; 95% CI 2.00 to 9.15) [98].

Fibromyomata and assisted reproduction

The effect of fibromyomata on the outcome of assisted reproduction is still a matter of controversy. In a retrospective study conducted in 1995, Farhi *et al.* found that the implantation rate and pregnancy outcome were impaired in women with uterine leiomyomata only when they cause deformation of the uterine cavity [99]. However, in a recent meta-analysis by Sunkara *et al.* the clinical and live birth rates were found to be significantly diminished even in women with non-cavity-distorting intramural fibroids treated with IVF compared with those without fibroids (RR 0.85; 95% CI 0.77 to 0.94 and RR 0.79; 95% CI 0.70 to 0.88, respectively) [100].

Whether to remove the fibroids before IVF/ICSI is another question and is still a matter of debate. Most authorities agree that the removal of submucous fibroids prior to IVF/ICSI is beneficial. In a retrospective study by Narayan and Goswamy, hysteroscopic resection of the submucous fibroids resulted in a higher pregnancy rate compared to patients with no fibroids (48.2 % vs. 26 %; $P < 0.02$), while the miscarriage rate was not significantly different (23.1 % vs. 15.8 %) [101]. However, these findings are also disputed. In a

retrospective case-controlled study, Surrey *et al.* found that neither the ongoing pregnancy rate nor the implantation rate was significantly different among patients undergoing IVF-ET after hysteroscopic polypectomy (61% and 24%, respectively) or patients with previous myomectomy (52% and 26%, respectively) compared to controls (53% and 23%, respectively) [102]. Prospective RCTs are needed in order to settle the issue.

Other factors affecting implantation

Uterine contractions during embryo transfer have been blamed for diminishing the outcome of assisted reproduction. Fanchin *et al.* recorded uterine contractions during ET and found that fewer uterine contractions were associated with a higher clinical pregnancy rate. They also found that plasma progesterone concentrations and the frequency of uterine contractions were negatively correlated (r 0.34; $P < 0.001$) [103]. In a subsequent prospective non-randomized study, the same group found that vaginal progesterone administration starting on the day of oocyte retrieval induced a decrease in uterine contraction frequency on the day of ET [104]. The effect of these observations on the outcome of IVF and ICSI remains to be studied.

Sexual intercourse around the time of ET has also been suggested as a cause of low implantation rates after assisted reproduction. However, when a RCT was conducted by Tremellen *et al.*, the clinical pregnancy rate was not affected by sexual intercourse and, contrary to expectations, the implantation rate was significantly increased for patients who had sexual intercourse around the time of ET [105].

Low-dose aspirin has also been suggested as an adjuvant during the luteal phase. However, various RCTs and meta-analysis have shown that its routine administration does not improve the outcome in those patients [106, 107].

In an attempt to increase endometrial receptivity, Sher and Fisch used sildenafil (Viagra) in four patients

Table 14.4. Odds ratio and 95% confidence intervals for the biochemical (BPR), clinical (CPR), or ongoing pregnancy rate (OPR) or miscarriage rate (MCR), with various factors affecting implantation for patients treated with IVF

Stimulation protocol	Odds ratio (95% CI)
HCG versus no HCG in GnRH agonist protocols (OPR) [72]	2.38 (1.32–4.29)[a]
Progesterone versus no progesterone in all protocols (CPR) [72]	1.34 (1.01–1.79)[a]
OHSS with HCG versus OHSS with progesterone [72]	3.06 (1.59–5.86)[a]
Stopping progesterone at U/S versus 3 weeks later (MCR) [75]	0.94 (0.3–3.1)
Estradiol + progesterone versus progesterone only (CPR) [82]	1.19 (0.74–1.90)
Estradiol + progesterone versus progesterone only (OPR) [82]	1.37 (0.81–2.30)
Corticosteroids versus no corticosteroids (CPR) [84]	1.16 (0.94–1.44)
Hydrosalpinx versus no hydrosalpinx in IVF patients (CPR) [87]	0.64 (0.56–0.74)[a]
Removal of hydrosalpinx versus no removal (CPR) [88]	2.31 (1.48–3.62)[a]
Removal of hydrosalpinx versus no removal (OPR) [88]	2.14 (1.23–3.73)[a]
Occlusion of hydrosalpinx versus no occlusion (CPR) [89]	4.66 (2.47–10.01)[a]
Occlusion of hydrosalpinx versus no occlusion (OPR) [89]	7.24 (0.87–59.57)
U/S aspiration of hydrosalpinx versus no aspiration (BPR) [90]	2.1 (1.02–4.60)[a]
U/S aspiration of hydrosalpinx versus no aspiration (CPR) [90]	1.8 (0.80–4.30)
Endometriosis versus tubal factor infertility (CPR) [91]	0.56 (0.44–0.70)[a]
Stages III and IV versus stages I and II endometriosis (CPR) [91]	0.60 (0.42–0.87)[a]
GnRHa prior to IVF versus no GnRHa (CPR) [98]	4.28 (2.00–9.15)[a]
Fibroid uterus versus no fibroid (CPR) [100]	0.79 (0.70–0.88)[a]

U/S, ultrasound; OHSS, ovarian hyperstimulation syndrome.
[a] Statistically significant.

with previously failed IVF attempts and three of them conceived [108]. However, to date, no RCTs have been published to confirm this interesting observation. Table 14.4 summarizes the clinical outcome associated with some of the modifications in the luteal-phase support used in assisted reproduction.

Conclusions

The multifaceted nature of assisted reproduction requires a meticulous evidence-based approach to the various steps involved in this treatment modality. In this chapter, the current status of assisted reproduction techniques has been presented in the light of the best evidence available. The field is dynamic and new evidence is emerging all the time. With the ever-increasing numbers of patients in need of assisted reproduction, the clinical outcomes of these techniques can only be improved by relying on properly conducted RCTs and continuously updated meta-analyses.

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Chapter 15

Three-dimensional in vitro ovarian follicle culture

Eugene Galdones, Lonnie D. Shea, and Teresa K. Woodruff

Introduction

The mechanisms underlying ovarian follicle development, or folliculogenesis, are poorly understood. Revealing these processes will provide researchers and clinicians with the information necessary to develop therapeutic strategies aimed at preventing and treating premature ovarian failure and female infertility. In addition, knowledge about follicle biology may provide new options for preserving fertility in women who must undergo potentially fertility-threatening treatments, such as radiation therapy or chemotherapy. To date, cryopreservation of embryos generated by in vitro fertilization (IVF) is the most successful method to preserve the fertility of young female cancer patients. Oocyte and ovarian tissue retrieval and cryopreservation are also emerging as promising methods for "banking" immature or mature oocytes prior to undergoing fertility-threatening treatment. Indeed, for cancer patients who are unable to undergo IVF prior to starting a potentially gonadotoxic therapeutic regimen, oocyte or ovarian tissue cryopreservation may be the only options for preserving their future ability to start a family.

However, methods for utilizing the oocytes in banked ovarian tissue to achieve a successful pregnancy are still being developed and refined. Autotransplantation of thawed ovarian tissue has led to resumption of reproductive function and subsequent follicle development in vivo, and has resulted in several live births [1], but this option also carries a risk of reintroducing cancer cells to the patient. The development of in vitro ovarian follicle culture systems may circumvent this problem and open up new fertility preservation strategies by allowing for the ex vivo production of an ovulatory-stage follicle and the harvest of a fertilizable egg (Figure 15.1).

From the first two-dimensional follicle (2D) cultures performed in 1977 [2] to the recent advancements in three-dimensional (3D) follicle culture [3], in vitro follicle culture methodologies lie at the cusp of biomedical research and hold great promise, not only for answering fundamental questions about follicle biology, but for providing new options in fertility preservation. Given the complexity of the molecular, cellular, and physical mechanisms essential for proper follicle and oocyte growth, in vitro follicle culture strategies must be carefully designed to closely mimic the in vivo ovarian environment. A multidisciplinary approach has been taken to tackle the challenges of growing immature follicles in vitro, and has resulted in the development of various biomaterials able to sustain follicle growth and development as well as oocyte health. The purpose of this review is to describe the progress that has been made in the development of in vitro ovarian follicle culture systems and to highlight the importance of maintaining follicular architecture to facilitate follicle growth and oocyte development.

Follicle architecture during folliculogenesis

The follicle is the main reproductive unit of the ovary, and is composed of a single oocyte and its surrounding somatic support cells. The process of folliculogenesis encompasses the cyclical recruitment, selection, and maturation of follicles followed by the ovulation of a metaphase II oocyte that is competent for fertilization. At birth, the ovary has a relatively greater proportion of primordial follicles, characterized by the presence of a dormant oocyte surrounded by one layer of

Human Assisted Reproductive Technology: Future Trends in Laboratory and Clinical Practice, ed. David K. Gardner, Botros R. M. B. Rizk, and Tommaso Falcone. Published by Cambridge University Press. © Cambridge University Press 2011.

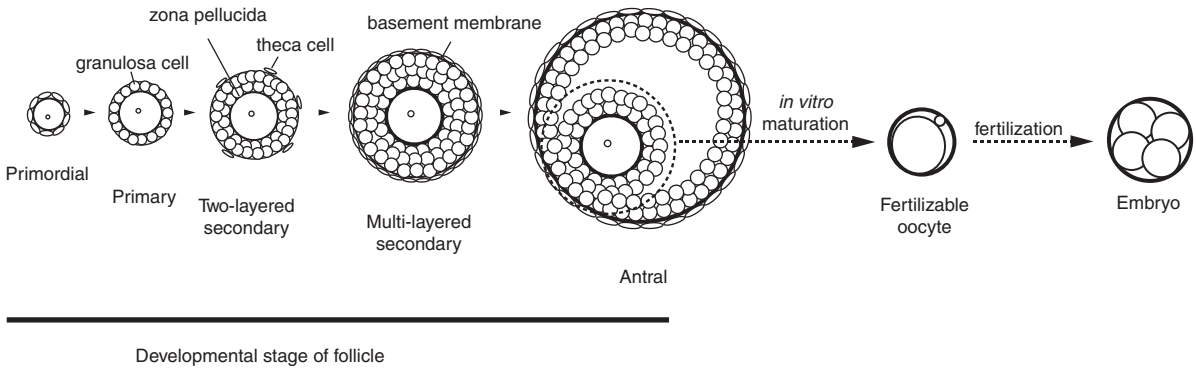


Figure 15.1. Schematic representation highlighting follicle architecture and morphological changes that occur during follicle development.

squamous granulosa cells. At the onset of folliculogenesis, as yet unknown mechanisms initiate the primordial to primary follicle transition, during which the surrounding layer of granulosa cells become cuboidal and oocyte growth is resumed (Figure 15.1). Additionally, a basement membrane forms around the granulosa cell layer, and a glycoprotein layer referred to as the zona pellucida appears, delineating the border between the granulosa cells and the oocyte. Proliferation of granulosa cells leads to the formation of multiple cell layers surrounding the oocyte. The presence of multiple granulosa cell layers, along with the differentiation of theca cells outside the basement membrane, is a morphological hallmark of secondary follicle development. Only after puberty, when follicle-stimulating hormone (FSH) is produced by the pituitary, will the granulosa cells in secondary follicles continue proliferation and differentiation into either cumulus or mural cells. Whereas cumulus cells directly surround the developing oocyte and provide key signaling and structural support, proliferating mural cells remain associated with the outer basement membrane and are involved in antral cavity formation. Antrum formation is the key feature that defines Graafian (or antral) follicle development. At this point, with the right hormonal cues, the oocyte undergoes meiosis, and rupture of the follicle results in the release of a mature, fertilizable egg (ovulation).

Control over the morphological and functional changes that occur during folliculogenesis is attributed to complex endocrine, paracrine, and autocrine signaling loops. However, recent work has revealed the added importance of the physical environment in dictating follicle outcome. Throughout folliculogenesis, the oocyte is highly dependent on proper granulosa cell and theca cell function. The different cell types within a single follicle are in constant communication via gap junctions that facilitate the transport of signaling molecules in homologous (i.e. granulosa-granulosa) or heterologous (i.e. granulosa-oocyte) cellular associations. In addition, the interaction between follicle cells and the surrounding extracellular matrix (ECM) is essential for facilitating intercellular communication within an individual follicle. The diverse set of proteins that make up the ECM not only support and maintain follicle architecture, but also appear to contribute to follicle fate [4]. Thus, the ultimate goal in ovarian follicle culture, particularly with follicles from larger mammalian species, is to mimic the complex molecular, functional, and structural changes that occur in vivo in order to produce a fully mature, fertilizable egg in vitro (Figure 15.1).

Follicle development in vitro

In 1977, seminal work by Eppig provided the first indication that late-stage (antral) follicles could be isolated and cultured in vitro [2]. This work also illustrated the fact that oocyte development is reliant on its association with the adjacent granulosa cells: only oocytes cultured while in physical contact with GCs exhibited growth, whereas denuded oocytes were unable to survive [2]. Since then, a significant effort has been made to identify the culture conditions and media components that support consistent follicle growth in vitro. Various culture media formulations have been used with a number of experimental successes. Examining the contribution of the culture media to follicle growth and development in vitro is beyond the scope of this review, and readers are directed to several comprehensive papers on the subject [5–8].

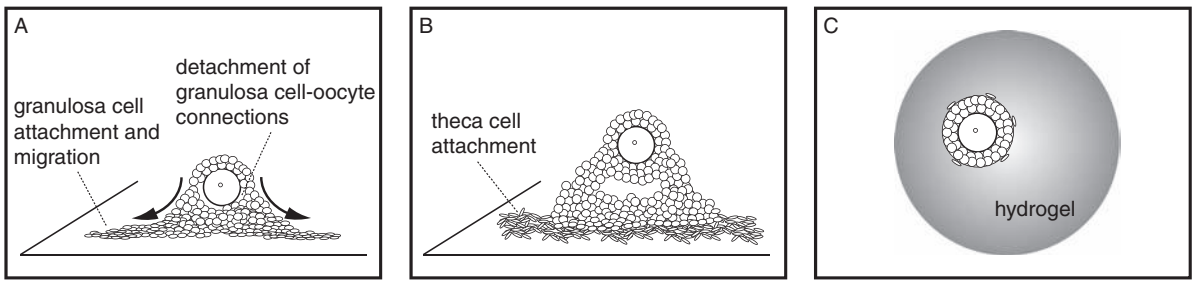


Figure 15.2. Examples of in vitro follicle culture systems. (A) Culture of follicles on a planar surface results in the migration of the granulosa cells through the basement membrane onto the culture surface. Granulosa cell adhesion and splaying results in compromised follicle architecture with specific disruption of the granulosa cell-oocyte connections required for proper oocyte development. (B) Culture of follicles within a droplet on top of a hydrophobic surface results in the migration and attachment of theca cells onto the culture surface. This prevents granulosa cell adhesion and migration, resulting in the maintenance of the requisite cell-cell interactions. (C) Preantral follicles encapsulated within inert or bioactive matrix materials retain their 3D architecture, more closely mimicking the in vivo ovarian environment.

Various experimental approaches have been taken to culture preantral (secondary) follicles in vitro. Initial attempts included adhesion of isolated follicles on 2D culture surfaces; however, this approach resulted in compromised follicle ultrastructure [9]. Others have successfully limited follicle adhesion, thus preserving (at least in part) the association between the granulosa cells and the oocyte [10]. More recent work had involved the complete encapsulation of secondary follicles within biomaterial scaffolds that support the follicular ultrastructure [3, 11], which can preserve the paracrine and autocrine signaling necessary for follicle development. To date, the culture of individual very early-stage (primordial/primary) follicles to the antral stage has yet to be accomplished, and research efforts in this area will be addressed in the latter part of this review. The following sections will review the various systems that have been developed for the in vitro culture of preantral follicles.

Two-dimensional (planar) follicle culture

Initial work focused on achieving oocyte growth in vitro relied on adherent 2D culture systems. Following follicle isolation by enzymatic and/or mechanical means, these 2D culture systems involve attachment of the follicle to a planar surface. Such surfaces have included uncoated tissue culture plastic or porous membranes and plates coated with cellular adhesion-inducing agents such as collagen or poly-L-lysine [9, 12]. As the cultures proceed, however, overall 3D follicle morphology becomes compromised as granulosa cells migrate through the basement membrane, attach to the culture surface and simultaneously detach from the oocyte (Figure 15.2A). Nonetheless, successful fertilization and production of healthy live offspring in mice have been achieved using oocytes recovered from 2D culture systems [13]. Unfortunately, the success of this technology appears to be limited to the rodent. Follicles isolated from larger animal species do not survive the rigors of 2D culture because their terminal sizes are much larger relative to that of rodent follicles. Moreover, oocytes from larger mammals require more time to reach a fully mature stage. Sustaining follicles in vitro for prolonged periods of time and to larger terminal sizes requires the preservation of cell-cell and cell-matrix integrity to ensure proper oocyte maturation. Without the maintenance of follicle architecture, the essential association between the oocyte and granulosa cells is lost.

Maintaining follicle architecture in vitro

Other follicle culture approaches have been devised to better preserve follicle architecture and cell-cell communication in vitro. The first attempt, albeit unsuccessful, to culture follicles in a non-adherent system was made by Roy and Greenwald [14]. Using enzymatically isolated preantral hamster follicles, tissue culture plates were coated with agar to prevent attachment. While adhesion was prevented, a lack of antral cavity formation was also observed; however, this was attributed to the detrimental effect of enzymatic isolation of the follicle rather than to the culture conditions.

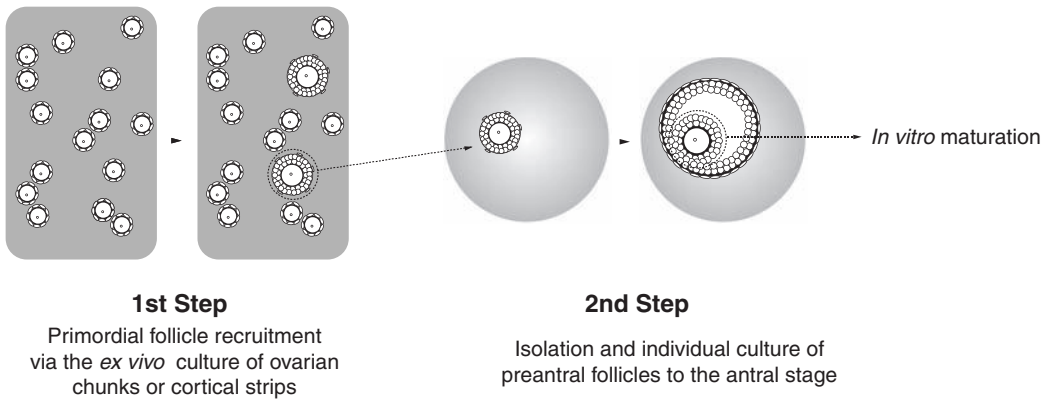


Figure 15.3. Diagram illustrating the two-step culture system that allows for the complete development of primordial follicles in vitro. Ovarian cortical strips allow *in situ* culture of primordial follicles and growth to the secondary stage; secondary follicles are isolated and encapsulated in an alginate hydrogel to support growth to the antral stage.

Another non-adherent system developed by Nayudu and Osborn involved the culture of isolated follicles in droplets of media atop a hydrophobic membrane that restricted cell attachment, allowing for the structure to remain intact [15]. Follicle culture in media under a layer of mineral oil resulted in the migration and adhesion of theca cells to the plate surface [16, 17]. The monolayer of theca cells acted as an anchor to the culture dish and prevented granulosa cells from rupturing through the basement membrane and adhering to the dish. In this culture system, a clear reorganization of the follicle structure was observed, with the oocyte oriented near the top of the droplet, while granulosa cells proliferated between the oocyte above and the theca cells below to produce an antral cavity (Figure 15.2B). Even though this culture was performed on a planar surface, this culture approach allowed the developing follicles to retain the physical association between the proliferating granulosa cells and developing oocyte. Follicles isolated from fresh or cryopreserved tissues have been successfully cultured in vitro using this method, which supports oocyte growth and produces oocytes with the capacity to re-initiate meiosis, undergo fertilization, and form viable embryos [18, 19].

Since then, various other systems have been developed to maintain the cellular and structural integrity of the growing follicle. In several cases, simply limiting the exposure of the follicle to an adhesive substrate has been shown to result in successful follicle development. Methods include the daily transfer of follicles to a new culture dish [16], suspension of follicles within inverted drops of media [20], mechanical agitation of cultured follicles by rotating the culture vessel [21], and encapsulation of follicles in various 3D matrices (Figures 15.2C, 15.3). While all of these procedures have the ability to produce mature follicles in vitro, complete follicle encapsulation holds the most promise as the method to sustain follicle ultrastructure, particularly in cultures of follicles from larger mammalian species.

Both bioactive (collagen, Matrigel) [11, 22] and inert (alginate, PEG, agarose) biomaterials have been used as 3D culture matrices. While collagen and Matrigel appear to be excellent candidates, and provide bioactivity that mimics aspects of the ovarian ECM, retaining the cellular connections within the follicles at the termination of culture is difficult. The bioactive matrices can lead to cell migration from the follicle. Additionally, the digestion of these hydrogels for follicle retrieval actually damages the follicles. Additionally, given the heterogeneity of these bioactive matrices, the physical and biochemical interactions between the growing follicle and the bioactive biomaterial are poorly characterized and difficult to control.

Conversely, the use of an inert 3D culture matrix, such as alginate, for follicle encapsulation has proven to be a truly customizable tool for in vitro follicle culture. Alginate is a naturally derived polysaccharide extracted from brown algae and is composed of homogeneous and heterogeneous blocks of α-L-glucuronic acid (G) and β-D-mannuronic acid (M) monomers. Alginate is water soluble and can exist in a liquid state; hydrogel formation is achieved through crosslinking of G blocks, which is induced by the addition of

calcium. The gelation of alginate is a relatively gentle process and thus ideal for follicle encapsulation. In addition, cultured follicles can be retrieved from the alginate using an alginate-specific lyase that selectively degrades the biomaterial but not the follicle. Alginate is an inert, non-degradable substance that does not interact with mammalian tissues and exhibits no endogenous bioactivity. This key characteristic of alginate is of particular interest in follicle culture because the biomaterial can be modified to include key signaling or ECM components that promote follicle growth and oocyte health in vitro. Customization of the alginate matrix in this way retains specific extracellular signals that would otherwise be lost following follicle removal from the ovary (see below) [23]. Preantral murine follicles encapsulated in an alginate matrix have produced mature, meiotically competent oocytes in vitro that were fertilized and produced live, healthy pups [24].

Dynamics of ovarian rigidity

The physical and mechanical properties of any hydrogel can affect follicle outcome by dictating the extracellular mechanical signals that impact biological endpoints such as cellular proliferation and differentiation [25]. While a great deal of research has focused on understanding the role that the physical environment plays in several pathologies (e.g. breast cancer), the involvement of mechanotransduction in ovarian follicle development has only recently been investigated [26]. Preantral murine follicles grown in alginate have attained sizes similar to follicles grown in vivo, and it was subsequently found that growth was dependent on the solids content, and the resulting relative "stiffness" or rigidity, of the alginate [3, 27]. As shown by Xu and colleagues, matrices containing a lower solids concentration provided the encapsulated follicle with a less rigid, growth-permissive environment that maintained the follicular architecture and allowed for substantial follicle expansion [28]. In contrast, follicles cultured in a more rigid, non-permissive environment (higher solids content) displayed stunted growth, the absence of antral cavity formation, deficiencies in steroidogenesis, and aberrant terminal oocyte quality [28]. Independent from the solids concentration of alginate, chemical modifications to alginate caused by oxidation or gamma irradiation can lead to the truncation of alginate polymers, thus creating weaker hydrogels with decreased rigidity. These chemical modifications have resulted in improved follicle growth relative to follicles cultured in unmodified solids and solids content-matched controls [27, 28].

Primordial and primary follicles are mostly localized to the rigid ovarian cortex, whereas growing follicles appear to migrate into the less rigid medulla. It has been hypothesized that the developing follicle is exposed to a less rigid physical environment within the ovarian medulla, which facilitates growth into more advanced developmental stages by offsetting the outward mechanical stresses associated with increasing follicle volume [27]. Thus, the development of dynamic biomaterials provides the opportunity to mimic the heterogeneous in vivo physical environment of the ovary, and tailoring the stiffness of the culture matrix will have to be taken into account to ensure proper follicle growth and development in vitro.

When follicles are encapsulated in alginate, follicle growth leads to increased compressive forces due to the non-degradable characteristics of the biomaterial. To address this issue, recent advances in biomaterial engineering led to the development of fibrin-alginate interpenetrating networks (IPN) for in vitro follicle culture [29]. Within the IPNs, the alginate component provides structural support and does not degrade, while the fibrin component provides matrix bioactivity, promotes follicle-matrix interactions, and provides additional mechanical support. Furthermore, the fibrin component is degradable, allowing plasmin (and other proteases) produced by follicle cells to progressively break down the local matrix. In effect, as the follicle increases in size, the stiffness of the IPN matrix decreases and facilitates follicle expansion. Thus, the dynamic mechanical properties of the alginate-fibrin IPN mimic the changing microenvironment of developing follicles within the ovary. Importantly, fibrin alone degrades and is unable to support the matrix after a couple of days in culture, and thus the other matrix component is essential for providing this support. Growth, survival, and steroid production of follicles cultured within the biodynamic IPN matrix were comparable to those of follicles encapsulated in alginate alone [29]. However, a greater number of meiotically competent oocytes were recovered from the IPN-cultured follicles compared with the alginate-cultured follicles, highlighting the significance of the physical microenvironment for follicle and oocyte development.

Importance of the ovarian extracellular matrix

The ovary consists of the follicle pool as well as a heterogeneous and complex stromal cell population and ECM. Apart from providing structural support for follicle maintenance and growth, the extra-follicular tissue and ECM of the ovary interact closely with both quiescent and developing follicles. It is unclear whether the stroma and ECM produce soluble signals that influence follicle growth, development and survival, or whether they serve as physical points for migration and adhesion of follicles. In vivo, cells attach to the ECM through integrin receptors; the heterogeneity of the ECM dictates the involvement of particular integrin receptors, and thus impacts cellular function [30]. In vitro studies have linked ECM composition with granulosa cell function [31], confirming the importance of the ovarian stromal microenvironment during folliculogenesis. Thus, the potential role of the stroma and ECM in driving aspects of folliculogenesis cannot be overlooked. Finding ways to recapitulate the specific interactions and functions of the extra-follicular ovarian tissues using engineered biomaterials will further enhance 3D in vitro follicle culture systems.

Natural bioactive substances such as collagen and Matrigel have been shown to encourage cell adhesion and support follicle development [32]; however, how their structural components influence follicle outcomes has been difficult to characterize. To elucidate the role of the ovarian ECM in vitro, otherwise inert biomaterials can be modified with ECM-like components, or novel matrices could be created that consist of both inert and bioactive substances (see below) [23, 29]. The covalent attachment of ECM components and binding motifs to inert materials has been shown to support numerous cellular processes, including adhesion, migration, and proliferation [30]. The conjugation of ECM components (e.g. laminin, fibronectin) or a highly conserved integrin-binding arginine-glycine-aspartic acid (RGD) sequence (present on many ECM components) to inert hydrogels, such as alginate, has provided researchers with a means to manipulate the cellular microenvironment. Encapsulation and culture of follicles with RGD-modified alginate significantly increased growth of secondary follicles and improved the meiotic competency rates of oocytes compared with follicles cultured in alginate alone [23]. Recreating the external cues and specific integrin-dependent associations between follicles and the ovarian stroma and ECM will be crucial for the optimization of 3D in vitro follicle culture systems.

In vitro follicle culture in larger mammalian species

Although successful rodent follicle in vitro culture has been well documented, the in vitro culture of preantral follicles originating from larger animals (i.e., domestic livestock, non-human primates, and humans) has proven difficult. Unlike mouse ovaries, the ovaries from larger animals contain more fibrous stroma relative to follicle content, making follicle isolation challenging and reducing oocyte yields. Nevertheless, following enzymatic isolation, the preservation of follicle architecture and integrity during isolation from ovarian tissue has been accomplished successfully. In one study, granulosa cell-oocyte complexes enzymatically isolated from ovine ovarian tissue were successfully grown to the antral stage following a planar, non-encapsulated 3D culture for 12 to 30 days [33]. Unlike what occurs during the culture of murine follicles in this system, granulosa cells from larger mammals maintained three-dimensionality and exhibited very limited cellular migration and attachment to the culture surface. In other studies, mechanically isolated preantral follicles from buffalo [34] and non-human primate [35] ovaries have been embedded in either collagen or alginate hydrogels, respectively. In both instances, the preservation of the follicle ultrastructure resulted in successful follicle growth and development.

With respect to human tissues, Abir and colleagues have embedded individual ovarian follicles in collagen and cultured them for 24 hours [36]. Despite the fact that the follicles did not survive longer culture periods, increased granulosa cell proliferation and oocyte diameters were observed. More recently, the in vitro growth of human secondary follicles embedded in Matrigel or encapsulated in alginate for an extended culture period (30 days) has been achieved [37]. Both culture environments supported the significant growth of the follicle and the oocyte, and antrum formation was readily visible in 75% of follicles at the midway point of culture. The secretion of steroid and peptide hormones was also demonstrated in the in vitro grown human follicles. Encapsulated follicles were able to preserve transzonal projections between the oocyte and granulosa cells.

Culture of early-stage follicles in vitro

Currently, much of the work assessing the feasibility of in vitro follicle culture has been performed using granulosa cell-oocyte complexes or isolated secondary or preantral follicles. By comparison, the in vitro culture of individually isolated primordial and primary follicles has proven quite challenging. The extended culture of primary follicles in collagen ultimately results in the loss of follicular architecture and degeneration, whereas follicles encapsulated within inert biomaterials survive and avoid degeneration, but follicle growth does not progress [36].

The ability to culture immature follicles will be invaluable for the development of more robust fertility-preserving strategies. Primordial follicles represent the most abundant follicle stage in the ovary and are present at all ages [38]. Conversely, secondary and preantral follicles are sparse, especially in tissues from larger mammalian species. Gaining the ability to activate and maintain the in vitro growth of early-stage follicles would provide researchers, clinicians, and patients with a viable untapped source of potentially fertilizable oocytes from cryopreserved ovarian tissue. Indeed, early-stage follicles appear to be less susceptible to the damaging effects associated with cryogenic freezing and cancer therapeutics, making them an ideal resource for expanding fertility preservation options.

The mechanisms underlying the successful in vitro growth of primordial and primary follicles have yet to be fully elucidated, yet the present roadblocks to follicle growth may be, at least in part, the result of the absence of the stroma and ECM. One approach to this problem is the use of a two-step culture system involving ex vivo ovarian tissue culture followed by secondary follicle isolation and growth in vitro (Figure 15.3). Eppig and O'Brien attempted the first two-step culture system in which whole murine ovaries were explanted and cultured in vitro, allowing for the activation and progression of primordial follicle development within the native physical and biochemical environment [13]. Specifically, the first step entailed the extraction of whole ovaries from newborn mice (containing only primordial follicles), which were then isolated and cultured for 8 days on polycarbonate membranes floating atop culture media. Following organ culture, the ovaries were enzymatically treated to facilitate the collection of cumulus cell-oocyte complexes, which were then cultured on collagen-treated membranes for 14 days. Following this two-step process, the oocytes were successfully matured in vitro, fertilized, and transferred to recipient females, and one pup was born [13]. In 2003, this culture protocol was optimized and led to an increase in the number of live births, illustrating the significance of the two-step approach [39]. Since the original studies, fetal ovaries (embryonic day 12.5) and cryopreserved ovaries have been cultured successfully using a two-step strategy [40].

Recently, the combination of organ culture followed by the encapsulation and culture of individual murine follicles was performed [41]. Briefly, whole postnatal day 8 mouse ovaries containing primordial follicles were cultured for 4 days to allow for the *in situ* development of early-stage follicles to the secondary stage. Results indicated that whole-organ culture resulted in a comparable transition of early-stage follicles to secondary follicles relative to proportions observed in vivo in 12-day-old ovaries. After organ culture, secondary follicles were mechanically isolated and encapsulated within fibrin-alginate IPN and grown for an additional 12 days. Comparable to cultured preantral follicles isolated from fresh (uncultured) ovaries, IPN-encapsulated follicles from the two-step culture method exhibited exceptional follicle survival (74%) and antral cavity formation (72%). These rates surpassed not only follicles grown in 2D culture, but follicles encapsulated in alginate alone. Following in vitro maturation, the majority of oocytes resumed meiosis. Importantly, in vitro fertilization of meiotically competent oocytes resulted in the formation of two-cell embryos [41].

In vitro culture of human primordial follicles

Using a modified two-step method, in vitro culture of fresh and cryopreserved early-stage human follicles has been achieved [22]. Rather than culturing whole ovaries, human ovarian cortical strips are isolated and cultured. This not only provides early-stage follicles with an *in situ* environment that promotes follicle growth but also circumvents the follicle damage that occurs with enzymatic and/or mechanical follicle isolation from the fibrous human ovarian stroma. Moreover, the smaller size and larger surface area of ovarian strips promotes efficient gas and nutrient exchange, in contrast to whole organ cultures in

which necrosis and apoptosis can occur. Thus far, in vitro culture of ovarian fragments or cortical strips originating from the cow [42], baboon [43], and human [44] has led to successful primordial follicle growth.

The ability to produce antral-stage follicles from early-stage follicles in the two-step culture system has not been as easy for larger mammalian species as it has been for rodents [13]. Recently, Telfer and colleagues mechanically dissected preantral follicles from cultured human cortical strips and cultured them for 6 days [45]. The isolated follicles were then maintained in a non-spherical 3D culture system for up to 4 days. In the presence of activin, individually cultured secondary follicles developed antral cavities and achieved significant oocyte growth, providing the first demonstration that early-stage human follicles could be cultured to the antral stage.

Future directions

It is clear that follicle development is a very intricate process involving both biomechanical and hormonal signals, and recapitulating folliculogenesis in vitro is proving to be quite challenging. However, the optimization of in vitro follicle culture approaches holds great potential for biomedical research and clinical application to treating or preventing infertility. The developing follicle is a particularly demanding tissue that requires a plethora of nutrients, culture conditions (temperature, pH, and oxygen tension), and signals (biochemical, hormonal and physical) that work in harmony to ensure proper oocyte development. To add to the complexity, follicles at different stages are dependent on diverse conditions that favor growth; therefore, designing dynamic follicle culture systems will be the key to the success of these experimental approaches. In addition, the requirements for coordinated follicle development in vitro differ according to species. From the recapitulation of requisite signaling pathways to determining the duration of follicle growth in vivo to accommodating the sheer size of the fully developed oocyte, work performed in rodent species may not necessarily translate to the culture of primate and human tissues. Follicles from larger mammals not only exhibit much larger follicle volumes relative to their rodent counterparts, but require more time to develop in vivo (reviewed in Ref. 46).

The development of novel 3D follicle culture systems has provided new insights into follicle biology and the means for successful maintenance of intact follicles in vitro. Follicles encapsulated within alginate hydrogels are provided with a physical scaffold that preserves the follicular architecture and can be tailored to the particular needs of the follicle. Modifications to the composition of the hydrogel better mimic the in vivo interactions between the follicle and the ECM, provide the physical support needed to ensure proper cell-cell contacts during follicle growth and provide a bioactive dynamic physical environment that can change as needed to accommodate the growing follicle. Ultimately, this customizable culture environment will provide an excellent tool for the in vitro culture of human follicles [47].

In addition, *in situ* culture of ovarian cortical strips prior to follicle encapsulation appears to be a promising model that will facilitate the growth of primordial and primary follicles to the preantral stage. With respect to fertility-sparing strategies, further work will be needed to establish the most favorable methods for the culture of cryopreserved tissues [33, 37, 48].

All in all, while much progress has been made with 3D culture of follicles *in situ* and in vitro, future work must continue to focus on methods to preserve follicle architecture and on optimizing the ever-changing culture conditions and technical requirements, which is imperative for the translation of these technologies to clinical use.

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Chapter 16

Artificial gametes

Jan Tesarik and Raquel Mendoza Tesarik

Introduction

The progress in the assisted reproduction technologies during the last two decades has made it possible to treat with success most types of infertility. This progress was accelerated by the development of intracytoplasmic sperm injection (ICSI) in the early nineties [1]. Further modifications of ICSI enabled fertilization, pregnancies, and births by elongated spermatid injection (ELSI) [2] and round spermatid injection (ROSI) [3]. Subsequently, in vitro maturation of testicular germ cells made it possible to achieve pregnancies and births in cases of male germ cell maturation arrest at the primary spermatocyte stage [4]. Transfer of a small amount of cytoplasm from donor oocytes into the patients' oocytes at the time of ICSI [5] helped alleviate the consequences of some oocyte cytoplasmic deficiencies.

However, there still remain serious limitations to the successful application of assisted reproduction in the treatment of infertility. This mainly applies to cases in which the germline is completely absent or the gametes produced in the gonads bear serious defects incompatible with embryo creation.

Gamete donation is an efficient therapeutic tool in these situations. However, this solution is not always easily accepted by the infertile couple, and the availability of donated gametes is limited, especially as far as the female gamete is concerned.

The idea of using non-germline cells to produce gametes matured in the last three decades and was encouraged by the recent achievements of nuclear transfer technology and embryo cloning. However, the production of a gamete by nuclear transfer from somatic cells is a more complicated issue than embryo cloning because of the obvious problems of meiotic-like reduction of the chromosomal complement from the diploid to the haploid status (haploidization).

This chapter reviews the relevant studies with human and animal cells aimed at the development of artificial gametes, outlines the main methodological problems, and suggests possible ways of their solution. In this chapter the term 'artificial gamete' is used to denote a cell or a cell component which is able to transmit the individual's genome to a biparentally gendered embryo. The biparental origin of the embryos makes the difference between the use of artificial gametes for assisted reproduction, on the one hand, and reproductive cloning, on the other hand, since the latter would result in monoparentally derived embryos whose genome will be identical with that of the parent of origin.

Artificial female gamete

During female gametogenesis (oogenesis), oogonia start the first meiotic division in the fetal period of life, but the process becomes arrested at a late prophase until puberty. The cell resulting from this incomplete meiotic division is called the primordial oocyte. After birth, oocytes grow and accumulate stock materials to be used after fertilization, but 2 days before ovulation the oocytes still contain a prophase nucleus, called the germinal vesicle. It is not until shortly before ovulation that meiotic division is reactivated. The oocytes complete the first meiotic division, extrude the first polar body, and start the second meiotic division. However, the process is interrupted again at metaphase (metaphase II). Such oocytes are called mature oocytes, but strictly speaking the maturation process is not achieved until shortly after fertilization, when the oocytes complete the second meiotic division and extrude the second polar body.

If a somatic cell nucleus, which is diploid by nature, is to be used as a source of artificial gamete

Human Assisted Reproductive Technology: Future Trends in Laboratory and Clinical Practice, ed. David K. Gardner, Botros R. M. B. Rizk, and Tommaso Falcone. Published by Cambridge University Press. © Cambridge University Press 2011.

genome (haploid), it must undergo a similar reduction of genetic material as that occurring in oocytes during the two meiotic divisions. A number of experimental studies have examined whether this process, called haploidization, can be induced by transfer of somatic cell nuclei to previously enucleated oocytes.

Several studies have addressed the question whether somatic cells can be haploidized after transfer to oocytes, making use of the oocyte's ploidy-reduction machinery including the first and the second meiotic spindle. It has been shown in the mouse that metaphase II oocytes can reduce nuclear DNA from secondary spermatocytes [6], primary spermatocytes [7], and even diploid somatic cells fibroblasts [8]. After the transfer of embryos resulting from oocytes injected with spermatocyte nuclei to foster mothers the live birth rate was 24% for the secondary spermatocytes [6] but only 4% for the primary spermatocytes [7].

These data were at the origin of an experiment in which six enucleated human metaphase II oocytes (ooplasts) from an oocyte donor were injected with cumulus cell nuclei from a patient in whom oocytes failed to be recovered after ovarian stimulation for an assisted reproduction attempt [9]. After 14 h of incubation the reconstructed oocytes were injected with spermatozoa from the patient's husband. A structure similar to the second polar body (pseudopolar body) was extruded in three oocytes by 5 h after sperm injection, followed by the formation of two pronuclei and cleavage in two of them (Figure 16.1). The three pseudopolar bodies were analyzed by fluorescent *in situ* hybridization, and a single fluorescent signal for the four chromosomes studied (13, 18, 21 and X) was found in two of them. The cleaved embryos were cryopreserved at the 2-cell stage to be stored until further research confirms the safety of their eventual transfer to the patient [9]. A schematic representation of the manipulations used in that study for somatic cell nucleus haploidization, and of the main observations, is shown in Figure 16.1.

In the same year Takeuchi *et al.* [10] published a study in which they tried to generate human oocytes with a haploid maternal genome by injecting human cumulus cells to enucleated metaphase human II oocytes obtained after 24 hours of in vitro maturation from the germinal vesicle stage. Of a total of 85 in vitro matured and successfully enucleated oocytes, 59 survived after cumulus cell injection. None of those survivors extruded a polar body following activation, but 16 among them displayed one pronucleus, and 12 had

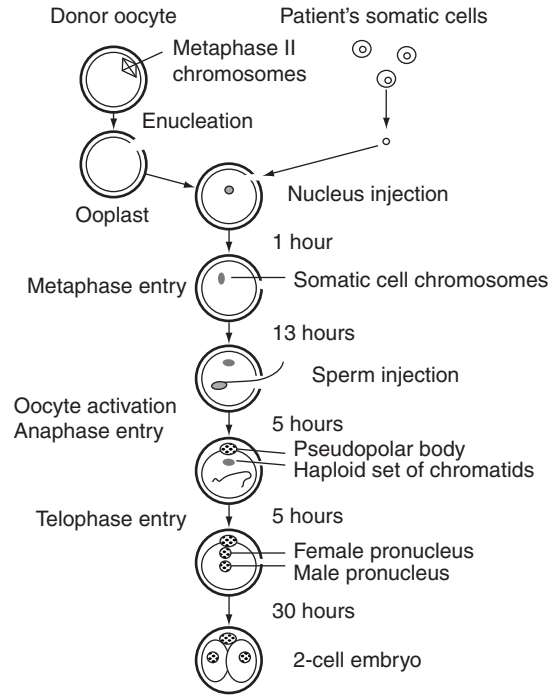


Figure 16.1. Schematic representation of the manipulations for somatic cell nucleus haploidization using enucleated metaphase oocytes. Reprinted with permission from Tesarik *et al. Reprod Biomed Online* 2001;2:160–4.

two pronuclei. Fluorescent *in situ* hybridization analysis of the pronuclei, performed with specific probes for chromosomes 16, 18, and X, confirmed their haploid state [10].

Lacham-Kaplan *et al.* [11] injected a total of 725 mature mouse oocytes with mouse cumulus cells, resulting in 429 oocytes having survived the manipulation, 102 of which could be successfully fertilized, but only 13 embryos developed to the blastocyst stage.

Artificial male gamete

Unlike oogenesis, male gametogenesis (spermatogenesis) is a continuous process in which spermatogonia enter meiosis and form primary spermatocytes.

A diploid spermatogonium which resides in the basal compartment of seminiferous tubules divides mitotically to produce two diploid intermediate cells called primary spermatocytes. Each primary spermatocyte then moves into the adluminal compartment of the seminiferous tubules and duplicates its DNA and subsequently undergoes meiosis I to produce two haploid secondary spermatocytes which still possess 2N

chromatid content. Secondary spermatocytes then rapidly enter meiosis II and divide to produce haploid spermatids (1N chromatid content).

Unlike the construction of artificial oocytes, where the manipulation aims at haploidization of a somatic cell nucleus within the cytoplasm of an enucleated host oocyte, followed by fertilization of the resulting construct, somatic cell nuclei intended to substitute for the male gamete are introduced into complete (non-enucleated) oocytes. The oocyte is then expected to carry out haploidization of the somatic cell nucleus in parallel with its own meiotic progression.

In 2001 Lacham-Kaplan *et al.* [11] reported an experiment in which 57 mature mouse oocytes were injected with adult male fibroblast cells as sperm substitutes. Nine of them extruded two second polar bodies and formed two pronuclei. Only two embryos reached the blastocyst stage [11].

Common problems in artificial female and male gamete production

In spite of the initial enthusiasm, further research revealed serious problems with the use of mature and immature oocytes as machines for somatic nucleus haploidization.

In 2003 Tateno *et al.* [12] tested the efficiency of somatic cell haploidization in the mouse model. Mature mouse oocytes were enucleated and injected with single nuclei from mouse or hamster cumulus cells. After artificial activation the resulting constructs were cultured for an additional 13–14 hours in the presence of vinblastin and processed for chromosome examination. The number of mouse cumulus chromosomes that remained in activated oocytes after pseudopolar body extrusion was less than or greater than the expected haploid number of 20 chromosomes in 153 of 168 cases [12]. Other oocytes were injected, under the same conditions, with hamster nuclei, and it was shown that even those oocytes that presented the expected haploid number of chromosomes (18 out of 128 oocytes; 14.1%) did not develop a normal haploid chromosome complement [12]. Similar findings were published a year later by Heindryckx *et al.* [13] and by Chen *et al.* [14], who found the expected number of chromosomes only in 15% and in 6% of artificial oocytes, respectively. In addition to chromosome analysis, Chen *et al.* [14] also transferred 324 two-cell semi-cloned embryos to foster ICR mothers, but no implantations or live births were achieved.

In 2005 Galat *et al.* [15] reported results of a cytogenetic analysis of 41 human artificial oocytes reconstructed by injecting cumulus cell nuclei (presumably in G0 phase) to 129 enucleated metaphase II human oocytes obtained by in vitro maturation of immature (germinal vesicle) oocytes donated by consenting patients. Only four of the 41 oocytes analyzed by 5-colour fluorescent *in situ* hybridization or DNA analysis for copy number of chromosomes 13, 16, 18, 21, 22, and X showed a normal distribution of chromosomes; the remaining 37 (90.2%) were abnormal, with one, two, or three errors [15]. These data were confirmed by Takeuchi *et al.* [16] using a similar experimental design.

In order to find out whether immature (germinal vesicle) oocytes would perform better than mature (metaphase II) oocytes as tools for somatic nucleus haploidization, Fulka *et al.* [17] evaluated the behavior of somatic and embryonic diploid cell nuclei after their transfer to germinal vesicle oocytes. Enucleated mouse oocytes were fused with mouse cumulus cell nuclei or nuclei of two-cell mouse embryos. Polar bodies were extruded in about 75% of oocytes reconstructed with embryonic cell nuclei, but only in 1.5% (2/132) reconstructed with somatic nuclei from cumulus cells; moreover, the metaphase plates were abnormal in almost all cases and chromosomes were often arranged in abortive metaphase plates [17].

The above data show that neither mature nor immature oocytes ensure sufficient fidelity of chromatid partition between the reconstructed oocyte and the polar body to be usable for artificial gamete production in clinical practice.

Mechanisms of somatic cell haploidization failure

Fidelity of chromosome and chromatid separation

All studies showing the low efficiency of mature and immature oocytes as a tool for haploidization of somatic cell nuclei coincide in a high incidence of chromosomal abnormalities in the resulting constructs (see the previous section). Hence the fidelity of homologous chromosome and chromatid separation seems to be a major problem. The idea underlying the use of mature oocytes for somatic cell nuclei haploidization was based on the observation that the cytoplasm of the metaphase II oocyte

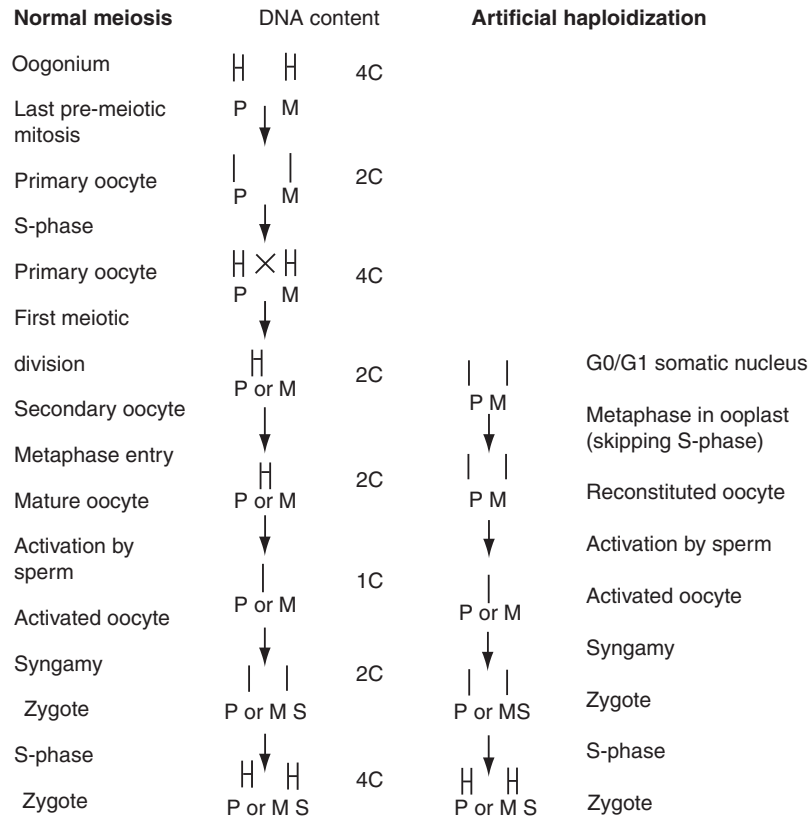


Figure 16.2. Schematic representation of cell cycle events occurring during artificial somatic cell haploidization for oocyte reconstruction, as compared with normal meiosis. Only one chromosome is represented. Vertical bars represent individual chromatids. The letter above each represented chromosome indicates its paternal (P) or maternal (M) origin. Sperm-derived chromosome set (S) complementing the oocyte derived set in the zygote is also represented. Attachment of sister chromatids at centromeres is represented by horizontal bars between individual chromatids. The cross between homologous chromosomes symbolizes the presence of exchange/chiasma sites. In different stages of normal meiosis and of artificial haploidization the represented chromosome occurs in two versions or only in one version, each version consisting of one or two chromatids. Reprinted with permission from Tesarik J. *Hum Reprod* 2002;17:1933–7.

is capable of forcing somatic cell nuclei at G0/G1 phase of the cell cycle to a premature M-phase without a previous S-phase, resulting in segregation of one set of single-chromatid chromosomes to a pseudo-second polar body [18]. This process is schematically represented in Figure 16.2, showing the successive events during meiosis and somatic cell haploidization occurring at the level of a single chromosome. In different stages of normal meiosis and of artificial haploidization the represented chromosome occurs in two versions or in only one version, each version consisting of one or two chromatids. In both normal meiosis and artificial haploidization the reduction division is achieved after oocyte activation by the fertilizing spermatozoon so that the set of single chromatids of maternal origin can be complemented by a sperm-derived chromatid set to restore the normal chromosome number after the subsequent first zygotic S-phase (Figure 16.2). In normal meiosis reductional division occurs at meiosis I and results in the elimination of either the paternally derived or the maternally derived chromosome set with the exception of chromosome parts exchanged at chiasmata during crossing over. On the other hand, a complete (no crossing over) elimination of either paternally or maternally derived chromosomes occurs only at anaphase II, in activated oocytes, during artificial haploidization (Figure 16.2).

In order that chromosomes become aligned correctly on the meiotic spindle, a temporary cohesion between homologous chromosomes appears to be needed [19]. Chromosome cohesion and its timely release are just as important as kinetochore arrangement for correct reduction of chromosome number [20]. If cohesion is absent, chromosomes segregate at random, so cohesion must be present before anaphase. As an example, a relationship between recombination and meiotic chromosome attachment to the spindle has been demonstrated in mouse oocytes from animals homozygous for a targeted disruption of the DNA mismatch repair gene *Mlh1*; in these oocytes the absence of MLH1 protein dramatically reduces the meiotic recombination, and the meiotic chromosomes are present as univalents, unable to establish the correct spindle attachment [21]. Similarly, the absence of Spo11p results in the defects of chromosome synapsis

and a random segregation at meiosis I [22], and the mouse meiotic mutation *mei1* disrupts chromosome synapsis and compromises the proper organization of chromosomes on the spindle [23]. Cohesion must be released at anaphase to allow chromosomes to move to opposite poles. Thus, problems of cohesion between chromosomes in meiosis appears to be related to a failure of meiotic recombination and the absence of chromosome synapsis [24]. However, somatic cell nuclei, by nature, lack chiasmata, where during meiosis I homologous recombination of chromosomes occurs [25].

As compared with mature oocytes, the use of germinal vesicle oocytes for somatic cell nucleus haploidization represents an even greater challenge because two steps of reduction are necessary. In meiosis I, two chromatids move to each spindle pole and then, in meiosis II, the two are distributed, one to each future gamete (in the case of sperm) or a polar body (in the case of oocytes). This requires that meiosis I chromosomes attach to the spindle differently than meiosis II chromosomes and that they regulate chromosome cohesion differently [26]. In mitosis, and also in the second meiosis, sister kinetochores lie back-to-back and capture microtubules from opposite poles; as a result, sister chromatids move to opposite poles in anaphase [20]. In the first meiotic division, however, sister chromatid kinetochores lie side-by-side, and they capture microtubules from the same spindle pole; as a result, sister kinetochores move to the same pole in anaphase I [20]. Also, in mitosis chromosomes are held together along chromosome arms and between sister centromeres until anaphase, when cohesion lapses along the entire length of the chromosome. Similarly, the linkage of homologous chromosomes (bivalents) in the first meiotic division is a result of cohesion along chromatid arms related to recombination between the two homologous chromosomes, and at centromeres. The chromatid arm cohesion is released in anaphase I, but cohesion between the centromeres of sister chromatids is maintained, which allows homologous chromosomes to separate from one another while the chromatids which make up each homolog remain united at the centromere [27]. It is not until anaphase II that centromere cohesion is released and sister chromatids separate from one another [26]. In order that chromosomes of somatic cells behave in the same way as meiotic chromosomes a sequential change in both the orientation of chromatid kinetochores and the cohesion and cohesion release between chromatid arms and centromeres, similar to those occurring in germ cells, would be required. This is unlikely to occur in chromosomes of somatic cells even if transferred to maturing oocyte cytoplasm because this behavior is determined by properties built into the meiotic chromosomes [20] and can thus hardly be conferred to the mitotic chromosomes by the oocyte cytoplasm.

Genomic imprinting

Genomic imprinting, first discovered in the mid-eighties [28, 29], is a process whereby the genomes of the male and the female gametes are differentially conditioned during gametogenesis, resulting in gender-specific repression of certain (imprinted) genes. In other words, genomic imprinting is an allele-specific modification of DNA which allows the recognition and differential expression of maternal and paternal alleles of certain genes [30]. The parentally specific marker is present on imprinted genes in somatic cells during the fetal and postnatal life and is closely related to differential methylation at the 5 position of cytosine in specific CpG sites of DNA [31]. The parental imprint is stable during somatic cell division, but it is erased in primordial germ cells and re-established during gametogenesis [32].

Problems of genomic imprinting are suspected to be one of the causes of the currently low efficacy of mammalian cloning. In fact, embryos resulting from transfer of somatic cell nuclei have been shown to have widespread epigenetic defects in imprinted genes [33], in CpG island methylation [34, 35], in DNA methyltransferase expression [36], and in DNA methylation and histone acetylation of the entire genome [37]. It remains to be determined whether the human immature and mature oocytes might be able to erase and re-establish imprinting information in somatic cell nuclei. If this does occur, somatic cell nuclei might be epigenetically reprogrammed to the oocyte-like pattern. On the other hand, it is more difficult to imagine how the oocyte could reprogram male-derived somatic cell nuclei to act as sperm nucleus precursors.

New prospects in artificial gamete production – the use of stem cells

Stem cells, by definition, are able to differentiate into a diverse range of specialized cell types. Basically, two

types of stem cells, embryonic stem (ES) cells and adult stem (AS) cells, can be distinguished. The former are isolated from the inner cell mass of blastocysts and can differentiate into all of the specialized embryonic tissues, while the latter are found in different organs, serve as a repair system for the body and, as compared with the ES cells, are more limited in their differentiation potential. These properties of stem cells suggest that they may be manipulated in vitro to differentiate into the male and the female germ cells. Although the ES cells, owing to their differentiation totipotency, can be expected to be better adapted for this use as compared with the AS cells, they cannot be obtained from adult infertile patients and have thus no direct clinical application in reproductive medicine. However, experiments with the ES cells can help understand the mechanisms guiding the differentiation of stem cells towards the germline and thus prepare the route for the work with the AS cells.

Germ cells from ES cells

The potential of ES cells as a source of germ cells was first demonstrated, independently, by Toyooka *et al.* [38] and Geijsen *et al.* [39]. The authors of these experiments used a three-dimensional culture system, supporting the development of embryoid bodies, and added bone morphogenetic protein-4 (BMP-4) [38] or retinoic acid [39] to derive male germ cells from mouse ES cells. The ES cell-derived male germ cells obtained by Geijsen *et al.* [39] were injected into mouse oocytes, resulting in fertilization and embryonic development to the blastocyst stage. Later, Nayernia *et al.* [40] have shown that embryos developing from intracytoplasmic injection of mouse ES cell-derived germ cells can develop into adult animals. Mouse ES cells have also been shown to be able to develop into oogonia that enter meiosis, recruit adjacent cells to form follicle-like structures, and later develop into blastocyst-like entities [41]. Human ES cells behave in a similar way during in vitro culture; they are recruited spontaneously into embryoid bodies and there begin to express meiosis-specific proteins, such as VASA, BOL, SCP1, SCP3, GDF9, and TEKT1 [42]. Tilgner *et al.* [43] have described a robust protocol to differentiate human embryonic stem cell lines from primordial germ cells using a simple fluorescence-activated cell sorting strategy for their isolation. These authors have also demonstrated removal of parental imprints and chromatin modification changes in the ES cell-derived cells that support their primordial germ cell identity [43]. Moreover, primordial germ cells arising from human ES cells have been shown to form post-meiotic spermatids in vitro [44].

However, thorough analysis of the meiotic process in mouse germ cell-like cells derived from ES cells, using a panel of meiosis-specific markers, showed that many meiosis-specific proteins, including those involved in meiotic chromosome cohesion, were lacking in these cells and those present were abnormally distributed [45]. Moreover, the germ cell-like cells did not contain synapsed homologous chromosomes but instead displayed a chromosomal organization normally found in somatic cells [45]. This is the same kind of problem as that observed in studies trying to haploidize adult somatic cell nuclei by injecting them into immature or mature oocytes (see section 5.1), and this finding suggests that the germ cell-like cells derived from ES cells might suffer a risk of chromosomal abnormalities.

Germ cells from AS cells

In 2004 Johnson *et al.* [46] reported a surprising and somewhat controversial finding that juvenile and adult mouse ovaries possess mitotically active germ cells that, based on estimated rates of oocyte degeneration (atresia) and clearance, are needed to continuously replenish the follicle pool. These data challenge the basic doctrine of reproductive biology saying that most mammalian females lose the capacity for germ-cell renewal during fetal life. The germline stem cells capable of generating oocytes in the adult mouse ovaries were suggested to be of bone marrow origin, based on the finding of germline markers in bone marrow and on the observation that bone marrow transplantation restores oocyte production in sterile female mice [47]. Moreover, donor-derived oocytes were also observed in the sterile mice following peripheral blood transplantation [47]. Spermatogonia-like cells could also be derived in vitro from mouse bone-marrow stem cells [48].

Nevertheless, subsequent investigations demonstrated that putative bone marrow- and blood-derived germ cells fail to generate developmentally competent oocytes after induced or spontaneous ovulations [49]. Even so, bone marrow transplants reportedly sustain or restore the function of mouse ovaries that are failing due to chemotherapy exposure [50, 51] or advancing age [52], with all offspring derived from the host

females. Moreover, a recent study has demonstrated that oocytes derived from transplanted neonatal mouse female germline stem cells are capable of producing offspring [53].

Oocyte-like cells have also been produced in vitro from stem cells derived from porcine fetal skin [54] and from a clonal pancreatic stem cell line [55]. Moreover, the imprinted gene *H19* undergoes demethylation during the differentiation in vitro of porcine fetal skin cells to primordial germ-like cells in a similar way to that of natural primordial germ cells in the pig ovary [56], suggesting an ability of the in vitro generated primordial germ cell-like cells to undergo imprint erasure required for correct epigenetic reprogramming of a functionally competent gamete.

Following the finding of germline stem cells in adult mouse ovaries, several studies tried to determine whether a comparable population of cells exists in adult human ovaries. The first study attempting to identify mitotic germ cells or meiotic entry in adult human ovaries was unsuccessful [57], but two subsequent studies reported the presence of rare stem-like cells with germline characteristics in the ovarian surface epithelium of post-menopausal women [58, 59]. It was shown that these cells, isolated by biopsy of the ovarian surface, have the ability to spontaneously form oocyte-like cells in vitro [58] and the oocyte-like cells derived from these putative stem cells can undergo parthenogenetic activation to form blastocyst-like structures [59]. However, when a similar experiment was performed with putative germline stem cells recovered from follicular aspirates of ovulating women, no development of oocyte-like cells was observed [60]. It is possible that the germline stem cells, present as contaminants in follicular fluid aspirates, are not captured in the puncture needle in large enough amounts or in the right ratio with respect to other cell types as compared with ovarian biopsy. However, it is also possible that the differentiation of germline stem cells to oocytes is silenced by some components of the functionally active ovary, and can only be reactivated after the ovarian follicle pool depletion with the onset of menopause.

A synthetic view

As shown in the previous sections, methods are now available to carry out crucial steps on the way from diploid somatic cells towards haploid cells which might act as artificial gametes in the near future. What is now needed is the elaboration of a robust and reliable method with which artificial gametes could be produced from easily available adult cells with acceptable efficiency and with the minimal risk of abnormalities in the progeny resulting from the use of these cells in assisted reproduction.

The experimental data reviewed in this chapter illustrate two major directions in which the efforts at the production of artificial gametes progressed in the last decade. The first direction was marked by the use of immature or mature oocytes as tools for somatic cell nucleus haploidization. These works demonstrated the haploidizing capacity of the oocyte cytoplasm, but problems arose from the inability of the somatic cell nuclei to reduce their chromosomes with the requisite fidelity, resulting in a high incidence of non-disjunction leading to chromosomal abnormalities. The second direction was characterized by the recourse to stem cells, making use of their ability to spontaneously differentiate into primordial germ cell-like cells. The main obstacle of this approach was the difficulty of defining an optimal somatic cell support, similar to that provided by granulosa cells and Sertoli cells for oocytes and spermatozoa in the female and the male gonad, respectively.

More research is needed to find out whether the two approaches can be successfully combined. In fact, stem cell nuclei might be better suited to properly react to the oocyte haploidizing machinery as compared to the initially used non-stem somatic cells. Since the first step – the conversion of stem cells to primordial germ cell-like cells – can apparently be obtained in vitro without the need for somatic cell support, nuclei of these stem cell-derived cells may subsequently be introduced to the oocytes to complete the process. Further research is needed to determine the optimal source of the adult stem cells to be used and to find out the optimal protocol for their transformation.

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Chapter 17

Current status and future trends of the clinical practice of human oocyte in vitro maturation

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Introduction

The treatment of infertility is now widely based on in vitro fertilization (IVF) as the technology of choice. Conventional IVF (which, for the purposes of this review, includes IVF by intracytoplasmic sperm injection, i.e. ICSI) requires substantial levels of gonadotropin hormone stimulation of the ovary to induce the growth of multiple follicles, which then receive an ovulatory stimulus during a single cycle. Harvesting of the mature oocytes that have developed within the follicle then occurs. Such ovarian hyperstimulation comes at a cost, as there can be significant immediate side effects and potential health risks. Oocyte in vitro maturation (IVM) is an established technique in many animal species, but has yet to make a significant impact as a treatment option for infertile couples. IVM of human oocytes provides options in the treatment of infertility, especially for women with polycystic ovarian syndrome, but also for women who respond poorly or wish to reduce/avoid ovarian hyperstimulation regimes used in conventional IVF, for couples who cannot afford ovarian hyperstimulation, and for couples where male factor infertility is the only identified cause of infertility.

Widespread uptake of IVM has been limited in particular by reduced embryo development and approximately 5–15% implantation rates following embryo transfer, with high levels of early miscarriage (25% vs. 15% for IVF). There are also fears regarding long-term health consequences for children derived from the IVM procedure. However, despite low numbers of children born to date (probably around 1000–2000 worldwide), there is little evidence that IVM is associated with perturbations in fetal and neonatal growth, chromosomal abnormalities, congenital malformations, or impaired cognitive development, over and above that seen within conventional IVF (approximately 5% of births). However, there is a clear reduction in the risk to women of ovarian hyperstimulation and related complications when undertaking IVM treatment as compared to conventional IVF. The burden of treatment is reduced with IVM compared to IVF as there is substantially less cycle monitoring and patient injections. There are also notably reduced costs per cycle to couples and healthcare providers.

This chapter examines the process of IVM, its efficacy and safety, and explores why it is not more routinely used to treat infertility and what is required to make it a more viable clinical option.

Infertility treatment and current limitations

In vitro fertilization (IVF, including ICSI) is a routine technology that relieves the distress for couples caused by infertility which cannot be resolved by other less invasive assisted reproductive technologies, such as artificial insemination. During the early development and growth phases of clinical IVF in the 1980s and 1990s, academic reproductive medicine units promoted advances in the science of IVF, whereas today IVF is a mature technology that operates largely within risk-averse private IVF clinics. IVF requires both clinical and psychological management of patients as well as highly developed laboratory systems for the collection of gametes and production and, if necessary, storage of embryos. The success of current IVF procedures is heavily reliant on the use of gonadotropins and/or GnRH analogs to achieve controlled ovarian hyperstimulation. Despite the wide success of IVF, there are still patient groups that lack ready access to the technology, either for financial reasons or because

Human Assisted Reproductive Technology: Future Trends in Laboratory and Clinical Practice, ed. David K. Gardner, Botros R. M. B. Rizk, and Tommaso Falcone. Published by Cambridge University Press. © Cambridge University Press 2011.

they remain unsuitable for IVF treatment. For example, treatment of women with the condition of polycystic ovarian syndrome is problematic using conventional IVF, due to the need to avoid ovarian hyperstimulation syndrome in these patients. IVM is an alternative approach for generating mature oocytes that eliminates or significantly reduces the need for hormonal stimulation of the ovary.

Procedures for IVM

IVM of oocytes refers to the practice of removing immature cumulus-oocyte complexes from antral or pre-ovulatory ovarian follicles, which are then matured for 1–2 days within the laboratory environment, before standard IVF or ICSI procedures are used to generate a viable embryo. The procedure differs from conventional IVF in particular, in that immature oocytes can be collected from small antral follicles in unstimulated ovaries, negating the need for ovarian hyperstimulation regimes (Figure 17.1). Many other aspects of laboratory procedures and patient management are the same in IVM and in IVF treatment cycles.

For IVM, oocytes and their surrounding "nurse cells," the cumulus cells – the collective entity known as a cumulus-oocyte complex (Figure 17.2A) – are aspirated from antral follicles using techniques that differ only subtly from that used in conventional IVF, and still involve the insertion of an ultrasonography-guided needle through the vaginal wall and into the follicles of an ovary. Nevertheless, most follicles aspirated are smaller (3–12 mm) than aspirated during conventional IVF and more vigorous techniques are required to collect the oocyte, as cumulus expansion should not yet have occurred, especially if exogenous hCG has not been administered 36 to 38 h before collection.

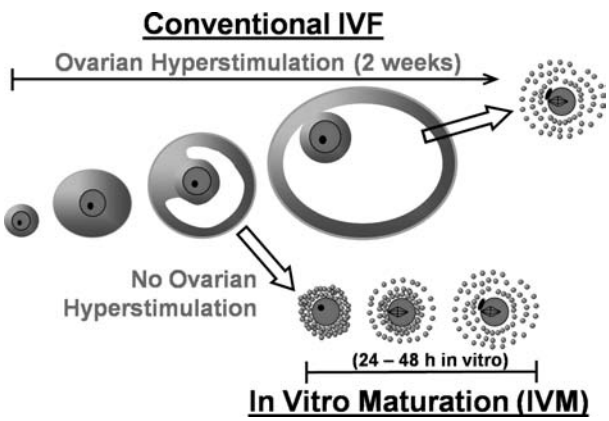


Figure 17.1. Schematic of the major differences between IVM and conventional IVF. First, for IVM, patients receive no or minimal ovarian hormonal stimulation, whereas in IVF patients' ovaries are hyperstimulated with large doses of gonadotropins. Second, in IVF, oocyte maturation occurs in vivo, whereas in IVM oocyte maturation occurs in vitro for 1–2 days, prior to standard fertilization procedures.

There are two relevant clinical IVM protocols for the preparation of pre-ovulatory follicles for oocyte collection. The different protocols affect the time required for IVM of human oocytes. In an unstimulated IVM cycle, oocyte pick-up is performed in a natural cycle when the lead follicle is ~10–12 mm in diameter or in an anovulatory cycle when the endometrial thickness is at least 5 mm [1]. In this scenario, oocyte maturation occurs in vitro over a 30–36 h period and is referred to as spontaneous IVM, which is modeled on a significant body of work conducted in animal-based studies. During spontaneous IVM, the act of artificially removing the cumulus-oocyte complex from the meiotically inhibiting environment of the follicle and placing it in vitro allows the immature germinal vesicle-stage oocyte to spontaneously mature to the metaphase II stage [2] (Figure 17.2). In some cases short follicular-phase FSH priming is used to increase oocyte numbers [3]. The second protocol involves administration of a large bolus of hCG (10 000 IU) 36 to 38 h prior to oocyte retrieval. In this case the time for IVM is significantly reduced (usually to 24 h) as many oocytes have initiated meiotic resumption, but not matured, at the time of collection [4]. The bolus of hCG appears to initiate a cascade of molecular events which possibly mimics an ovulatory cascade although this remains controversial. Oocyte meiotic resumption in this scenario may be a reflection of a blunted ovulatory EGF-like cascade in these small follicles. Currently there is ongoing debate in the literature as to the most appropriate work-up protocol for IVM patients.

Oocyte IVM requires a chemical formulation to support the maturation process in vitro [5]. The maturation medium should comprise a mixture of inorganic salts, a pH buffering system, and various additives including antioxidants. Energy substrates (for example, glucose and pyruvic acid) and amino acids are also necessary to achieve substantive results [5]. The IVM chemical solution is further supplemented with agents that are required for biological activity known to influence oocyte developmental competence. In particular, recombinant human FSH

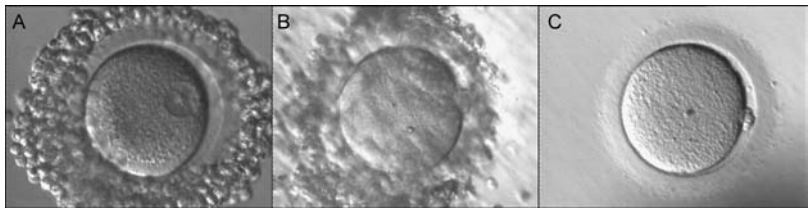


Figure 17.2. (A) An immature cumulus-oocyte complex soon after collection with a compact cumulus mass and an intact germinal vesicle. (B) A cumulus-oocyte complex after 29 hours in a serum-free IVM system. Note that there is only moderate cumulus cell expansion in serum-free IVM. (C) A mature, metaphase II oocyte following IVM which has had all cumulus cells removed. Note the presence of the first polar body.

is known to stimulate (amongst other biochemical events) the process of cumulus expansion (Figure 17.2B). A source of protein is also required and autologous patient serum is usually added to human IVM. In some countries, animal sera is used instead [6, 7] although this practice is likely to be rejected by these countries in the longer term. Future human IVM media are likely to replace serum with recombinant human serum albumin as a more defined component, in the presence of FSH and/or epidermal growth factor [8] (Figure 17.2).

Prevalence of IVM and regulatory aspects

Even though the first description of IVM using human oocytes was reported in 1965 by Edwards [9], IVM is still not widely practiced today, although it has increased slowly in popularity over the past decade in a small number of countries. This slow uptake of the technology is generally attributed to the poor efficiency of pregnancy establishment and lower "live birth" rate following transfer of IVM-derived embryos, when compared to embryos from conventional IVF. Indeed, this is not unique to humans, as for most species implantation and pregnancy rates are generally lower following IVM than from in vivo matured oocytes [10, 11]. Nonetheless, IVM is widely practiced in a wide range of animal species, most specifically in the mouse as a research tool and in sheep, cattle, pig, deer, and horses for both research purposes and for producing multiple embryos from selected dams for the purposes of genetic selection and breeding. Furthermore, IVM is also practiced for some applications of species conservation.

The first child born from IVM was in 1983 [12], but there were no subsequent IVM offspring reported in the 1980s. With advances in animal IVM in the 1990s, significant resources were put into attempts to implement clinical IVM. Approximately 3500 IVM cycles were studied throughout the 1990s, yielding just 50 successful births. Despite these disappointing early results, success rates have increased since the turn of the century. It is difficult to report the prevalence of IVM with any degree of accuracy. There is no central world-wide registry of IVM pregnancies/offspring, and a large proportion of units practicing IVM do not report or publish results or pregnancies. Approximately 500 offspring are reported in the peer-reviewed literature although an estimate of >1000 is probably more realistic [13].

As IVM is regarded as an adjunct procedure to IVF, IVM is usually not separately regulated. As such, to the best of our investigations, IVM has not been approved or banned in any country, with the exception of the UK, which has approved the practice. IVM is currently under evaluation by government health agencies within New Zealand. The practice of IVM is quite geographically specific, with Asia and Scandinavia the largest users. South Korea has recorded more pregnancies than any other country, while other prominent locations include Japan, China, Canada, Finland, Denmark, Italy, and France.

Benefits of IVM

The major benefit of IVM is that the procedure removes the need to administer large multiple doses of FSH normally used in conventional IVF treatment to women (Table 17.1). Women undergoing IVM receive either no or substantially reduced levels of gonadotropins (less than 600 IU/treatment). Exogenous FSH can lead to ovarian hyperstimulation syndrome (OHSS), which occurs in approximately 5–10% of women undertaking IVF cycles. The mild form of OHSS is common (20–33% of all women undergoing IVF) and is usually self-limiting; however, in some cases, urgent medical attention is required [14]. When severe (0.5–5%), the syndrome can be

Table 17.1. Incidence of OHSS using IVM vs. IVF in women with PCOS (adapted from Child *et al.* [27])

	IVM ($n = 107$)	IVF ($n = 107$)	*P*
Total units FSH injected	0	2355 ± 833	<0.01
Moderate or severe OHSS	0%	11.2%	<0.01

potentially life-threatening, requiring hospitalization, intravenous fluids, pain relief, and other medication. Thrombosis at different sites of the venous network and pulmonary embolism or complications of severe dehydration may occur in rare cases. As women with the condition of polycystic ovarian syndrome (PCOS) are at least double the risk of developing OHSS in response to FSH, these patients require IVM in preference to conventional IVF.

IVM can also be applied to women whose preference is to minimize ovarian hyperstimulation in order to receive a less invasive form of infertility treatment, or a simplified protocol requiring less monitoring. There is currently a resurgence in interest in "natural cycle IVF" or minimal stimulation IVF, and IVM represents an important adjunct therapy in this area [15–17]. There are data suggesting that the use of gonadotropins for ovarian hyperstimulation is associated with increased health risks including increased risk of ovarian, breast, and endometrial cancers [18, 19] and increased risk of stroke [20], although these claims are also disputed [21]. Hence, the reduced use of gonadotropins in IVM may be associated with a reduced risk of long-term adverse health outcomes, relative to conventional IVF. IVM is also associated with less discomfort to the patient as it requires less drug administration, which is usually performed by the patients themselves. Importantly, there is also accumulating evidence demonstrating that ovarian hyperstimulation has adverse effects on oocyte and embryo quality [22] and aneuploidies [23], which at the very least impact negatively on pregnancy and live birth rates [24], but may also cause long-term health problems in offspring. Significantly, recent data in a mouse model revealed that gonadotropin administration was associated with a loss of maternal and paternal imprinted methylation in a dose-dependent manner [25].

Due to the reduced use of gonadotropins and need for monitoring, the other major advantage of IVM over conventional IVF is a substantial reduction in cost. Hence, in lower-income families and in countries where infertility treatment is not financially subsidized or covered by health insurance providers, IVM is likely to provide treatment opportunities to couples who would otherwise have limited access. In this sense, IVM could be viewed as being more socially equitable than conventional IVF. These factors are likely contributors to the rapidly increasing rate of IVM use in Asia.

Finally, an intriguing and unique situation in Italy caused a substantial increase in IVM usage in that country. In 2004, the Italian government introduced a new law that prevents the use of more than three oocytes per IVF cycle and the freezing of embryos, such that there has been a large increase in low-stimulation IVF and natural cycle IVM/IVF in Italy [26].

Current practice and perceived problems associated with IVM

Efficiency

Current IVM conditions compromise subsequent embryo development rates following fertilization [1]. There is ample and consistent evidence of this phenomenon in both human and animal studies. Interestingly, fertilization rates themselves are generally not compromised, with between 70 and 80% of IVM oocytes fertilized following ICSI.

Embryo development rates per se of human oocytes post-IVM are rarely reported as embryos are usually transferred back to the uterus on day 2 or day 3. Furthermore, there is still very limited blastocyst culture of embryos derived from IVM oocytes. As a consequence, the best measure of embryo development rate in human IVM oocytes is "implantation rate," which is defined as the number of gestational sacs at 6 weeks divided by the number of embryos transferred. Implantation rates from IVM oocytes are usually half that of IVF oocytes as typified in Table 17.2, from Child *et al.* [27].

A summary of some of the more recent major IVM studies (Table 17.3) demonstrates implantation rates consistently above 10% (for example, ~13% in non-PCOS patients and 11.6% in PCOS patients [28, 29]). The reasons for the impaired developmental potential of IVM oocytes, compared to IVF oocytes, are complex and not yet fully understood. This topic is the subject of intense animal research and embryo developmental rates post-IVM are slowly, consistently improving with time.

Table 17.2. Reproductive efficiencies of IVM vs. IVF in women with PCOS

	IVM	IVF	*P*	Reference
Implantation rate	9.5%	17.1%	<0.01	[27]
Pregnancy rate	21.5% (107)	33.7% (107)	n.s.	[27]
Miscarriage rate	25.3% (99)	15.7% (705)	<0.05	[65]
Live birth rate	15.9% (107)	26.2% (107)	n.s.	[27]

Table 17.3. Embryo development and implantation rates after IVM

Cycles (*n*)	Cleavage rate (%)	Embryos transferred (mean)	Implantation rate (%)	Reference
94	88	4.9	6.9	[66]
24	95	2.7	15.7	[4]
36	60	1.8	15.0	[3]
121	93	3.2	9.3	[67]
68	88	3.8	10.5	[68]
203	N/A	5.0	5.5	[69]
45	96	2.5	10.9	[70]
48	75	1.6	18.5	[1]
140	90	3.2	15.4	[7]

Pregnancy rate is usually defined as the percentage of cycles commenced producing a positive hCG result, confirmed at 6–8 weeks by the presence of a fetal heartbeat by ultrasound. Pregnancy rates are confounded by the number of embryos transferred per cycle, as many clinics compensate for poor embryo developmental potential by transferring more embryos. As IVM oocytes have a lower developmental potential, it has been common practice to transfer more embryos than would be transferred in an IVF cycle. Indeed, in Child *et al.* [27], significantly more embryos in IVM cycles were transferred (3.2) than in IVF cycles (2.7), yet IVF embryos yielded a higher pregnancy rate (non-significant; Table 17.2).

Major recent studies of IVM reveal that pregnancy rates are generally greater than 20% in both PCOS patients and non-PCOS patients [28, 29] (Table 17.4). Furthermore, it is perhaps noteworthy that two recent studies [6, 7], both from China, report IVM pregnancy rates of around 40% when transferring between 3.0 to 3.5 embryos.

Miscarriage rates after IVM range from 20 to 57% (Table 17.4). With very few adequately controlled studies, it is difficult to determine whether miscarriage rates are significantly higher than in conventional IVF. However, this seems likely and possibly reflects a combination of poorer oocyte/embryo developmental potential and suboptimal endometrial preparation [30].

Live birth rate is defined as the percentage of cycles commenced resulting in at least one live offspring. As with pregnancy rates, live birth rates are affected by the number of embryos transferred and the inherent developmental potential (implantation rate and miscarriage rate) of the embryo. As can be seen from the data in Table 17.2 [27], IVM generates approximately half the live birth rate of IVF. Although in this case this is not significantly different, it is reasonable to conclude that currently IVM has a lower live birth rate than IVF.

Clinical issues

The collection procedure for immature oocytes differs slightly from collecting mature oocytes in IVF. While both procedures involve ultrasonography-guided trans-vaginal ovarian follicle aspiration, oocytes are easier to aspirate in conventional IVF. In IVM, when

Table 17.4. Pregnancy and miscarriage rates in PCO and PCOS undergoing IVM

Cycles (*n*)	Pregnancy rate (%)	Miscarriage rate (%)	Reference
94	27	20	[66]
24	33	25	[4]
36	23	57	[3]
121	27	40	[67]
68	34	22	[68]
203	22	37	[69]
45	23	33	[70]
48	42	36	[1]
89	41	21	[6]
140	40	9	[7]

an ovulatory dose of hCG is not injected, the cumulus-oocyte complex is embedded in the mural granulosa cell layer and hence is strongly attached to the follicle wall. To effect collection, the cumulus-oocyte complex is usually scraped from the follicle wall using the bevel of the aspiration needle. Hence, an IVM oocyte collection is usually associated with more ovarian bleeding than occurs in conventional IVF. However, there is no report outlining an adverse event arising from oocyte pick-up during an IVM cycle and there appears no additional patient discomfort during the procedure [31], even though it may take longer.

An important issue in an IVM cycle is adequate endometrial preparation. This is usually achieved by daily oral estrogen and vaginal progesterone from the day of oocyte pick-up. Embryo transfer is typically performed on day 2 or 3.

Because of the reduction in pregnancy rates and live births following IVM, there may be a need to manage a couple's expectations of success of the procedure, especially if they compare against published rates of success for IVF. Although there are no data on anxiety levels in patients undertaking IVM, such management of patient's expectations may reduce any additional anxiety that may occur through IVM treatment, in comparison with conventional IVF treatment.

Alternative methods which are similar to IVM in the treatment continuum are natural cycle IVF or minimal stimulation IVF [32]. Advantages include providing nil (in the case of natural cycle IVF) or a reduced exposure to gonadotropins (therefore minimizing the risk of ovarian hyperstimulation syndrome) and reduced costs. Furthermore, the maturation of the oocyte within the follicle in the absence of exogenous hormone stimulation should provide the optimal environment for the oocyte to achieve developmental competence [22]. However, both these options require more complex monitoring of the patient by ultrasound and through blood plasma hormone analysis, especially for natural cycle IVF compared with conventional IVF. Even with the increased monitoring, there is still a high risk of missing the onset of the endogenous LH surge, especially in natural cycle IVF, which invariably would mean a failed cycle. In order to time the oocyte retrieval in a natural cycle, the LH concentration has to be measured in blood or urine samples every 6 to 8 hours. Furthermore, these alternatives would not apply to patients with anovulatory polycystic ovaries.

Laboratory observations

IVM does not lead to any "observable" or obvious visible damage to the oocyte if collected using appropriate needle gauges and vacuum pressure. Under appropriate culture conditions, oocytes rarely degenerate during IVM, they undergo maturation to metaphase II quite readily. Dependent on medium conditions, especially the use of serum in culture, they undergo comparatively normal cumulus cell expansion, and they generally exhibit comparable fertilization rates to IVF oocytes. However, as has been shown in animal models, IVM undoubtedly compromises the oocyte at the sub-cellular and molecular level. As comparative material is rarely obtained, such studies that mirror the animal studies are unlikely to occur.

Several studies have investigated chromatin stability and aneuploidy following IVM using either immature or partially matured oocytes recovered post-hCG from ovarian-stimulated cycles. Such oocytes should be regarded as having suboptimal developmental competence and therefore it is unsurprising to note that these oocytes appear to have significant levels of spindle and/or chromosomal defects following IVM [33, 34]. However, Li *et al.* [35] utilized immature oocytes from unstimulated PCOS patients and compared spindle and chromosome configurations with those within in vivo matured oocytes from a similar patient group but who underwent conventional ovarian stimulation. They described significantly more abnormal spindles and chromosome configurations in the IVM group

(Table 17.5). Such observations suggest a higher miscarriage rate in IVM-derived embryos. Nevertheless, these results stimulated debate regarding the value of such observations, arguing that such abnormal oocytes could be removed prior to fertilization using suitable microscopic techniques [36, 37].

Recent evidence has demonstrated that epigenetic alterations can occur within animal embryos cultured under suboptimal conditions. Such concerns have also been raised in reference to the impact of IVM; however, there is a lack of information regarding the epigenetic state of oocytes following IVM in both animal and human oocytes. Borghol *et al.* [38], using germinal-vesicle arrested oocytes collected post-hCG from an ovarian stimulated cycle and subsequently in vitro matured, found 15/20 oocytes with normal methylation status for an imprinted differentially methylated region of the gene *H19*, but five oocytes from two patients had disturbed methylation patterns. These oocytes, however, are highly compromised as they have failed to mature in vivo following gonadotropin stimulation. Similarly, oocytes that failed to mature following ovarian stimulation that were subsequently matured in vitro were less methylated in an imprinted region of the *KCNQ1OT1* gene than oocytes that did mature in vivo [39]. Again, there is a need to conduct a genuine examination in epigenetic status of human oocytes matured either in vitro or in vivo collected from unstimulated ovaries. However, the feasibility of gaining experimental material for such work is a major obstacle.

Obstetric and post-natal outcomes

In general, in terms of maternal obstetric outcomes, IVM pregnancies are comparable to conventional IVF and ICSI pregnancies. Multiple-pregnancy rates, mode of delivery, cord pH, pregnancy complications, are all comparable between births resulting from IVM, IVF, or ICSI [29, 30, 40, 41]. ART pregnancies in general are associated with an increased risk of multiple pregnancy (twins: IVM 21%, IVF 20%, ICSI 17%) and cesarean delivery (IVM 39%, IVF 36%, ICSI 36%) compared to non-ART pregnancies (twins, 1.7%; cesarean 26%), but compared to IVF and ICSI, IVM is not associated with any additional risk [40]. Consistent with this, in a separate report of 43 IVM pregnancies, 35% were delivered by cesarean section and 9% were pre-term deliveries (<37 weeks) [42].

It is important to note that there is very limited information available on the obstetric and post-natal outcomes of IVM pregnancies. This is because (1) the technology is relatively new and so the children are still young and (2) there is a relatively low number of reported IVM children born so far. Of the IVM-derived children worldwide, around 300–400 are reported in the literature and this is not an adequate number to calculate absolute risks for specific health abnormalities.

Despite this, reports so far indicate little additional obstetric or perinatal complications as a consequence of the IVM procedure. From the preliminary data published so far, in general, there appears to be no major neonatal or infant health complications from IVM, compared to conventional IVF or ICSI [29, 30, 40, 41]. Gestational age, growth restriction, Apgar scores, birth weights, and sex ratios are all comparable between births resulting from IVM, IVF, or ICSI (Table 17.6) [29, 30, 40, 41]. In a recent review of the topic Suikkari and Soderstrom-Anttila [30] conclude: "More than 300 children have been born and follow-up studies have reported no major concerns about the pregnancies, deliveries or health of the babies."

Of the approximately 300–400 IVM children reported in the literature, approximately half of these have been examined for congenital abnormalities at birth. Approximately 5% of children born from IVM

Table 17.5 Normality of spindle and chromosomal configurations in IVM and in vivo maturated oocytes from PCOS patients (adapted from Li *et al.* [35])

Oocyte group	No. (%) of oocytes with spindle configuration		No. (%) of oocytes with chromosome configuration	
	Normal	Abnormal	Normal	Abnormal
In vitro matured ($n = 48$)	27 (56.3)	21 (43.7)[a]	32 (66.7)	16 (33.3)[a]
In vivo matured ($n = 22$)	19 (86.4)	3 (13.6)	20 (90.9)	2 (9.1)

[a] $P < 0.05$ oocytes matured in vivo.

Table 17.6. Perinatal clinical characteristics of IVM births (adapted from Shu-Chi *et al.* [41])

Characteristic	IVM[a] (*n* = 21)	Control[b] (*n* = 21)	*P*
Gestational age at birth (weeks)	38.1 ± 1.5	38.1 ± 1.5	n.s.
Birth weight (g)	3075 ± 489	3134 ± 287	n.s.
Birth height (cm)	49.6 ± 1.4	49.9 ± 0.9	
Head circumference at birth (cm)	33.5 ± 0.8	34.1 ± 0.9	
Sex (*n*)			
Male	11	13	n.s.
Female	10	8	n.s.
Size for gestational age (*n*)			
Appropriate	20	21	
Small	1	0	
Mode of delivery (*n*)			
Cesarean	14	17	
Vaginal	7	4	
Apgar score (*n* > 6 at 5 min)	21	21	n.s.

[a] Children from PCOS mothers undergoing gonadotropin priming, IVM, and ICSI-ET.
[b] Children from spontaneous pregnancies.
Values are means ± standard deviations.

Table 17.7. Summary of congenital defects from major IVM studies from 1995–2006

Period of study (reference)	IVM children born (*n*)	Congenital defects
1995–1998 [66]	20	0
1995–2001 [69]	28	1 hydrops fetalis (terminated)
		1 omphalocele (45X/46XY mosaicism)
		1 cleft palate
1999–2004 [71]	47	1 soft palate
		1 still birth at 42.3 weeks
1998–2006 [40]	48	1 VSD
		1 hip dislocation
2002–2003 [70]	5	0
Totals	148	7/148 (4.7%)

Table 17.8. Psychomotor and cognitive development of IVM children aged 6–24 months (adapted from Shu-Chi *et al.* [41])

Index	IVM[a] (*n* = 21)	Control[b] (*n* = 21)	*P*
Bayley Mental Development Index	92.7 ± 10.5	97.2 ± 8.9	*P* = 0.07
Bayley Psychomotor Development Index	96.7 ± 8.9	96.2 ± 7.1	n.s.

[a] Children from PCOS mothers undergoing gonadotropin priming, IVM, and ICSI-ET.
[b] Children from spontaneous pregnancies.
Values are means ± standard deviations.

have some form of congenital defect (Table 17.7) [29]), which is similar to rates reported within the general population (≈5%). Due to the very low numbers of IVM infants, it is impossible at present to determine accurately whether IVM is associated with an increased risk of chromosomal abnormalities or congenital birth defects. However, a major malformation rate of 5% associated with IVM (Table 17.7) is comparable with rates reported for ICSI of 7.4% [43], 4.9% [44] and 8.6% [45]. In support of this, Buckett *et al.* [40] recently examined 432 ART children compared to age- and parity-matched controls, and concluded that all ART pregnancies are associated with an increased risk of congenital abnormalities, and that IVM is not associated with any additional risk.

Long-term health outcomes

The first reports on follow-up data on IVM children appeared in 2006, and to date there still remain few in the literature [41, 42]. Based on these very preliminary studies, IVM children appear to have normal physical and neurological development rates from 0 to 2 years of age. In a study that tracked growth parameters including, body weight, body height, and head circumference from 0 to 2 years of age, IVM children were within normal limits [41]. In the same study, all IVM children had normal neurological development (Table 17.8) [41].

In a separate follow-up study of 46 IVM children [42], obstetrics and perinatal outcomes including birth weight were within the normal range. At 6 months of age, no child was assessed as having Considerable Development Disorder and three (7%) had Minor Development Disorder. By 12 months, 19% of children had

Minor Development Disorder and one child had Considerable Development Disorder (further investigation revealed this child had a glioblastoma of the optic nerve). By 2 years of age, neuropsychological development of the cohort was within the normal range; 97% of children with a Bayley Mental Development Index >85 and one child (3%) with an index between 70 and 84 [42].

There are few longitudinal studies on the development and health of ART children and none on children derived from IVM. In fact, there are very few such studies on animals, even though there are many hundreds of thousands of cattle embryos produced and transferred worldwide that are generated using IVM. In an important recent mouse study, Eppig *et al.* [46] compared the long-term health status and lifespans of offspring generated from oocytes matured in vitro versus matured in vivo. There were no differences between the two groups in lifespan or in a wide range of physiological and behavioral analyses, except the pulse rate and cardiac output were slightly and significantly lower in IVM-derived offspring.

Future directions and adoption of clinical IVM

IVM is an established technology in livestock animal breeding. In particular, embryo production in vitro using IVM-derived oocytes has become widely utilized in some parts of the world as an economically viable method of cattle breeding. Many initial problems have been overcome, such as the so-called "fetal oversize" syndrome, which is attributed to deficiencies in the embryo culture medium composition, rather than the IVM systems used [11]. Indeed, even though IVM in animals is generally associated with poor pregnancy rates similar to those found in clinical IVM, there are no major safety concerns in animals in terms of long-term health of adult female oocyte donors or the offspring generated.

A major deficiency in human IVM is the failure of laboratories to exploit what is known and what is emerging in knowledge in other species, especially in the area of laboratory technology (such as culture conditions and media formulations). For human IVM to become acceptable the efficiency rates in embryo yield and viability require improvement and this necessitates translating basic discoveries made in animal models to human IVM. Animal studies (e.g. [10]) have shown that oocyte developmental competence is the "rate-limiting" factor in the production of embryos using in vitro techniques. There is a vast body of literature concerning the mechanisms governing oocyte competence and more recent studies have clearly demonstrated that altering laboratory conditions during IVM can significantly enhance not only blastocyst production yields, but also fetal survival following transfer (see Refs. 47–50 for reviews). Some recent advances that improve the competence of IVM oocytes include the use of FF-MAS [51, 52], oocyte-secreted factors such as GDF-9 and BMP-15 [53, 54], cAMP modulating agents such as phosphodiesterase inhibitors [55–58], and factors that activate protein kinase C including FSH and phorbol myristate acetate [59]. We have recently developed a new approach to oocyte IVM called "simulated physiological oocyte maturation" (SPOM [60]). SPOM is an integrated IVM system that differs from other IVM systems with a short pre-IVM phase (1–2 h) and an extended IVM phase that synergize to generate high embryo and fetal yields following embryo transfer. The pre-IVM phase is specifically designed with the human clinical scenario in mind, whereby at pick-up, COCs are removed from the follicular environment and typically placed in a simple buffered collection medium that leads to a rapid and precipitous drop in oocyte cAMP levels. These various advances in IVM are based around significant new insights into the regulation of oocyte biology and the mechanisms mediating oocyte maturation in vivo [61]. Furthermore, researchers have only begun to appreciate the importance of the underlying requirement for appropriate metabolic conditions and the importance of gap-junctional and paracrine communication within the cumulus-oocyte complex during IVM that is fundamental for the attainment of oocyte developmental competence [5, 48, 62].

In contrast, in human IVM, there is still debate whether hCG-induced IVM or spontaneous IVM is the preferred method of oocyte maturation, although the literature suggests there is, in reality, little difference. Furthermore, there has been little commercial development of specific IVM media formulations as there has been for IVF and embryo culture media and most clinics still use a high concentration of serum during IVM culture. What is required are IVM systems that are specifically designed to meet the nutritional needs of the cumulus-oocyte complex and that mimic the natural cascade of signals that are induced by the ovulatory LH surge, although these remain significant challenges. Whether signaling occurs within follicles and oocytes following the large bolus in hCG-induced IVM has yet to be determined and is most likely not easily manipulated. On the other

hand, removing COCs from the unstimulated follicle and initiating the appropriate signaling cascade is more likely to lead to developmentally competent oocytes. Can such a system be developed? There is no doubt that improving our understanding of oocyte biology coupled with improving culture systems in the laboratory will enable the development of IVM systems that give rise to mature oocytes that are comparable to fully matured in vivo derived oocytes.

There is an increased emphasis in modern human IVF to minimize the administration of high levels of gonadotropins for ovarian stimulation [24, 63]. In part this has been as a consequence of the remarkable improvement in implantation rates and "take-home baby" rates achieved by many IVF units over the past decade, and is accompanied by a reduction in the numbers of embryos transferred, in many cases, down to single embryo transfer [64]. This, as well as acknowledging that high levels of exogenous gonadotropins depress the overall quality of oocytes and embryos obtained [22, 23], suggests that greatly reducing or more judicious gonadotropin stimulation in modern ART programs should be the preferred treatment choice. Hence IVM represents an important technology that is likely to have an increasing impact on the treatment of human infertility and its financial cost to society in the near future [15, 16].

Nevertheless, the uptake of IVM is not likely to be rapid, even if pregnancy rates do increase. Conventional IVF is now a mature technology that is widely accepted in society. Many of the pioneers who oversaw the rapid developments in conventional IVF did so within academically driven units. However, with generational change in personnel within the reproductive medicine field, an increase in more economic forces involved in the business of practicing IVF and a more litigious and regulatory environment, in many countries there is a shift away from adoption of new technology. As a consequence, IVF units are increasingly hesitant about offering "new" intervention technologies to patients until satisfied by a significant weight of evidence and even authority approval. As an emerging technique, IVM is likely to be subjected to much more scrutiny than, say for example, ICSI, blastomere biopsy for PGD, or cryopreservation of oocytes. Perhaps this may lead to the development of IVM occurring more rapidly in countries where there is less regulatory scrutiny.

Another change that has occurred over the past decade is the establishment of culture medium manufacturers that provide the vast number of IVF units with ready-prepared formulations for the production of embryos in the laboratory. This has been a significant and overall beneficial advancement, as the quality of manufacture is monitored far more so than what can be achieved by most IVF units. As it is likely that increasingly efficient IVM systems will require sponsorship and even development by these companies, then their preparedness for research investment into IVM will be another rate-limiting factor in the growth of IVM. Again, this will be driven by economic and litigious risk/benefit analyses.

We conclude that IVM, despite the reduced efficiencies of implantation potential and increased early miscarriage, should be viewed as having no more risk of an adverse outcome to mother and child compared with conventional IVF. This conclusion is reached when IVM treatment risks are balanced against the benefits of reduced risk of ovarian hyperstimulation syndrome and greater access to treatment for patient groups such as women with PCOS or as a new treatment option that is financially less restrictive. However, each IVF unit should undertake its own risk/benefit analysis, which will be the most important step in determining whether clinical IVM is a justifiable technique in their clinic. We further conclude that incorporation of exciting new developments, such as the provision of oocyte secreted factors and regulating cAMP production, already shown to improve oocyte developmental competence in animal models, will eventually cross over into human clinical use, causing embryo production and pregnancy establishment efficiencies to approach levels attained by current conventional IVF practices.

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Chapter 18

Oocyte and zona imaging

David L. Keefe

"Embryogenesis begins during oogenesis"
E. B. Wilson, 1918

Introduction

As women approach their late thirties they experience a sharp decline in fertility [1]. Age, in fact, provides the single best predictor of IVF outcome, yet women age reproductively at strikingly different rates. The oocyte is the locus of reproductive aging in the female, so assessment of oocyte health should help determine the biological age of women undergoing IVF. Morphological assessment of oocytes, however, is limited by the fact that form does not always reflect function. Imaging the oocyte is further constrained by the sensitivity of the human oocyte to all manipulation, including imaging, and by the brief access the clinical embryologist has to the oocyte before it must be fertilized. Also, the morphology of the oocyte reflects not only its developmental potential, but also its stage of meiotic maturation. Not surprisingly embryo morphology remains the most widely used method to predict embryo viability. Recently, however, a number of studies have employed novel methods to image the structure of important organelles within the oocyte. Time-lapse imaging also promises to reveal the dynamic architecture of oocyte development, providing a convergence of anatomy and physiology [2].

Imaging methods

A number of imaging methods are available to the clinical embryologist. Magnification is a necessary but not sufficient requirement for optimal imaging. Resolution, the ability to distinguish between two small structures within the image, is of equal or greater importance than magnification [3]. Resolution is affected by the microscope's optics, illumination, and method of introducing contrast. High-quality objectives and condensers which are properly aligned are necessary to achieve maximal resolution [3]. The lenses in the microscope's objective and condenser are characterized not only by their magnification, but also by their numerical aperture (NA), a reflection of the ability to capture waves of light diffracted by the specimen [2, 3]. The larger the NA, the more light is captured by the lens, and the greater the resolution. At the same time, increasing NA requires shorter working distances and more intense illumination, both problematic in the clinical IVF laboratory, because passing the objective lens too close to the oocyte risks contaminating it and prolonged exposure to high-intensity light perturbs cells, including oocytes.

The approach a microscope uses to introduce contrast affects what structures can be imaged by that microscope. Immunofluorescence microscopy enhances resolution by labeling cellular structures with antibodies and then decorating the antibodies with fluorescent molecules, which emit coloured light. Immunofluorescence disrupts cellular function and, therefore, cannot be applied to clinical IVF. Non-invasive imaging methods introduce contrast by taking advantage of the ability of certain molecules to alter the phase relationships of light waves as they pass through the specimen. Polarization light microscopy, discussed below, images structures based on anisotropy, so it can image structures with high degrees of molecular order, such as the zona pellucida, meiotic spindle, and membranes. Differential interference contrast (DIC) microscopy images structures based on changes in refractive index encountered by light as it passes through the specimen. DIC images

Human Assisted Reproductive Technology: Future Trends in Laboratory and Clinical Practice, ed. David K. Gardner, Botros R. M. B. Rizk, and Tommaso Falcone. Published by Cambridge University Press. © Cambridge University Press 2011.

cytoplasmic structures, nuclei, and metaphase chromosomes [3]. DIC is most efficient when imaging through glass coverslips, an impractical requirement for clinical IVF, so most IVF laboratories employ variations of the DIC method, such as Hoffman modulation (HM). HM allows imaging through plastic dishes.

When imaging the oocyte for IVF it is important to maintain tight temperature control, because both reduced and elevated temperature disrupt the integrity of the meiotic spindle (see below). Quality control in the laboratory should monitor not only the temperature on the microscope stage, but also in the dish itself, since some heated stages have cold or hot spots. Alterations in pH also can perturb microtubule dynamics, so embryologists should minimize the time oocytes remain outside the CO_2 incubator. Finally, light itself can promote reactive oxygen formation, so imaging should be carried out with the lowest light intensity for the briefest period possible.

Oocyte imaging with Hoffman modulation and DIC

Size and shape

Giant oocytes, at times with two polar bodies, are rare, but when they occur are associated with markedly decreased viability (Figure 18.1). Most giant oocytes are aneuploid [4]. Elongated oocytes do not exhibit altered fertilization, cleavage or, pregnancy rates [5].

Cytoplasm

The oocyte cytoplasm contains endoplasmic reticulum, Golgi complex, mitochondria, vacuoles, peroxisomes, and other organelles. Under DIC or HM healthy oocytes contain translucent cytoplasm with fine granularity. Diffuse or localized dark and granular cytoplasm (Figure 18.2) has been associated with decreased pregnancy rate, presumably related to atresia [6, 7].

Vacuoles

Circular cytoplasmic inclusions, known as vacuoles, may appear alone or in groups. Their cellular basis is unknown, but they may be associated with oocyte atresia and decreased pregnancy rates [8].

Perivitelline space

The perivitelline space is the space between the zona pellucida and oolemma. Normally the oolemma nearly touches the ZP. Widening of the perivitelline space has unclear effects on fertilization, preimplantation embryo development, and pregnancy rates. Granules may appear within this space, but they also do not appear to influence oocyte or embryo development [9].

Polar body

hCG or the LH surge resumes meiosis, resulting in extrusion of the first polar body. The second polar body separates only after fertilization or activation. The timing of the first polar body extrusion varies among aspirated oocytes, presumably because of variations in the timing of meiotic maturation [10]. Oocytes with incomplete cytokinesis, which leave the polar body attached to the oocyte, exhibit low

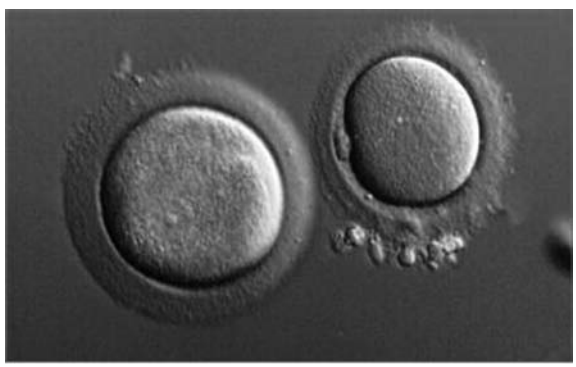


Figure 18.1. Giant oocytes (left) are rare and less viable.

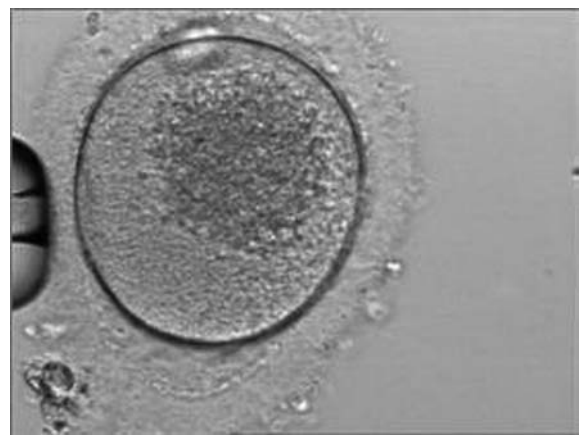


Figure 18.2. Granular structures and vacuoles in the cytoplasm have been linked to decreased oocyte quality.

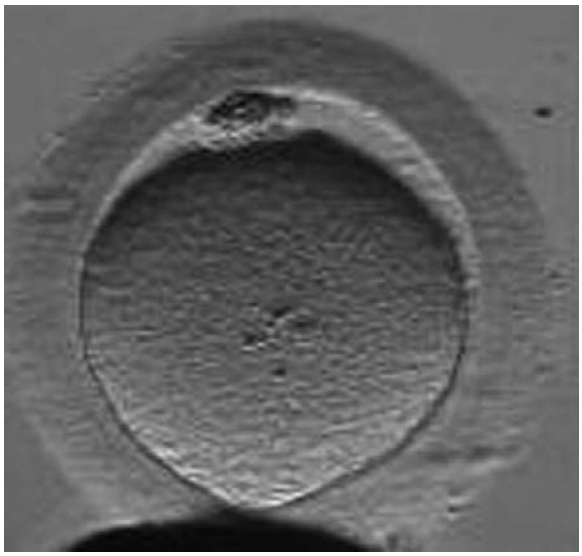


Figure 18.3. The first polar body in this oocyte is degenerating, suggestive of oocyte post-maturity.

fertilization and poor embryo cleavage rates, presumably because of their impaired meiotic competence. In post-mature oocytes the polar body already may be degenerating (Figure 18.3) [10]. More generally, degenerated or excessively large first polar bodies have been associated with poor embryo quality and low pregnancy rate [11–13].

Zona pellucida

The ZP is composed of a matrix of three intermediate filament proteins, ZP1, ZP2, and ZP3, laid down during folliculogenesis, which envelope and protect the oocyte and preimplantation embryo during its trip through the Fallopian tube following ovulation [14]. ZPs 2 and 3 also serve as receptors for sperm binding and help activate the sperm acrosome reaction. The ZP normally averages 15–20 μm in width. Reported alterations of ZP morphology include irregular and excessive thickness, which impairs sperm penetration and hatching [15–17]. Rare cases of missing ZP have been reported [18]. Fractured ZPs presumably result from excess pressure during oocyte aspiration.

Meiotic spindle

Meiosis consists of two reduction steps, meiosis I (MI), which separates homologous chromosomes, follows the LH surge or hCG administration, and meiosis II (MII), which separates chromatids, takes place after fertilization or activation. Mature oocytes (eggs) are arrested in metaphase II (MII), with the metaphase II spindle tethering chromatids, and a single polar body is present. MI oocytes also may be retrieved during IVF, though the oocyte does not arrest in the first meiotic metaphase, so this stage is brief and transient in humans [19]. The MI spindle, which tethers homologous chromosome pairs, is larger and more barrel-shaped than the MII spindle.

Immunofluorescence studies have demonstrated marked alterations in the structure of the meiotic spindle and chromosome congression abnormalities in nearly 80% of eggs from older women [20]. In mouse experiments we found that genetically or pharmacologically induced telomere shortening produced dysmorphic spindles remarkably similar to those from older women, suggesting that spindle abnormalities may arise from altered chromosome structure [21]. Disruption of meiotic spindles from telomere shortening also has been confirmed in yeast [22]. We have proposed that telomere shortening provides a parsimonious and unifying explanation of the diverse and seemingly disparate abnormalities observed in egg aging, including spindle dysmorphologies [23]. Immunofluorescence provides exquisite detail of the spindle and metaphase chromosomes, but requires fixation and exposure to high energy light, and, therefore, cannot be used during the IVF procedure. We have applied polarization light microscopy to non-invasively image spindles to provide a clinical method for oocyte assessment.

Polarized light microscopy

Meiotic spindles cannot be imaged with the contrast enhancement methods commonly used in the IVF laboratory, such as Hoffman modulation and DIC (Figure 18.4). Spindles can, however, be imaged non-invasively by polarized light microscopy. Because spindles are composed of highly ordered macromolecular complexes, they exhibit a property called anisotropy, which means their macromolecular structure influences the state of polarized light as it passes through them [24, 25]. Anisotropic structures, such as microtubules and intermediate filaments (e.g. ZPs), have two orthogonal axes, that is they themselves are polarized. Light polarized parallel to one of the specimen's axes travels at a different speed than light traveling along its orthogonal axis, so these two components of polarized light, in phase when they entered

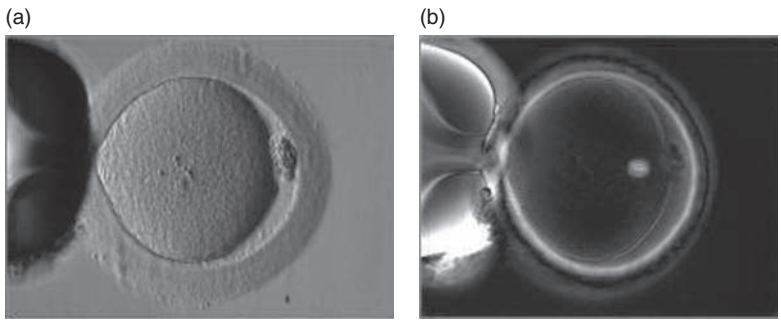


Figure 18.4. Microscopes used for IVF (Hoffman modulation, image of human egg (a), or DIC) do not image spindles. The polscope (image of human egg (b)) measures birefringence on a pixel-by-pixel basis, providing quantitative and reproducible measurements that correlate with spindle structure and function.

the specimen, exit the specimen out of phase. This phase change between the two beams of polarized light provides contrast. The magnitude of this phase shift is directly related to the orientation and retardance of the spindle. Therefore, the retardance or birefringence of an anisotropic structure, such as a meiotic spindle or ZP, represents a direct measure of the orderliness of its macromolecular structure.

Polarization light microscopy first was applied to image spindles non-invasively in non-vertebrate living cells in the early 1950s [24]. However, human meiotic spindles are small and exhibit only low levels of retardance, levels typically below the detection limit of conventional polarized light microscopes. Moreover, the clinical application of polarization light microscopy requires precise quantification of retardance, whose level reflects not only inherent birefringence, but also the orientation of the specimen relative to the plane of the illuminating polarized light. The compensator used by conventional polarized light microscopes was designed to adjust for changes in the relationship between the birefringent specimen and the polarizer, but is too tedious and impractical for quantitative imaging in the IVF laboratory, where speed and ease of use are critical for safety and economy [25].

A quantitative polarized light microscope, the polscope, which uses circularly rather than plane polarized light, provides orientation-independent and, therefore, quantitative measurement of retardance in living specimens. The polscope employs a liquid crystal variable compensator controlled by electronics, a computer programmed with imaging algorithms, and a video camera to provide rapid and accurate measurement of retardance [25]. The embryologist starts with an automated calibration step, which includes subtraction of the background. The spindle can be viewed either in real time through the microscope's eyepieces or in a processed image on the computer screen. The processed image results from the generation of four images created by four different polarization states generated by changing the computer-controlled orientation of the liquid crystals. A computer algorithm processes these four states to create a virtual map of retardance and azimuth (the direction of the molecules), pixel by pixel within the specimen. The spatial resolution of this imaging system approaches 0.2 μm, near the resolution limit of light microscopy and sufficient to image the human meiotic spindle. The use of polarized light combined with the fast speed of the image processing limit the amount of light exposure to the oocyte.

Applications of the polscope to IVF

The polscope (SpindleView, CRI, Woburn, Massachusetts) has been used in human IVF settings to image the retardance, azimuth, and shape of meiotic spindles [26] and the architecture and structure of the zona pellucida [27, 28]. In research settings the polscope also has been used to image chromosomes [29] and to study architectural dynamics, through time-lapse imaging [30]. The accuracy of spindle images generated with the polscope has been validated by immunofluorescence [31]. Indeed, since spindle imaging is non-invasive and is, therefore, not susceptible to fixation and staining artifacts like immunofluorescence, it should provide a more accurate depiction of actual spindle structure.

Presence or absence of meiotic spindle

Early applications of the polscope to IVF discovered that some oocytes imaged in the IVF laboratory exhibited no spindle retardance at all [26, 31] (Figure 18.5). In our first study we found 44% of 533

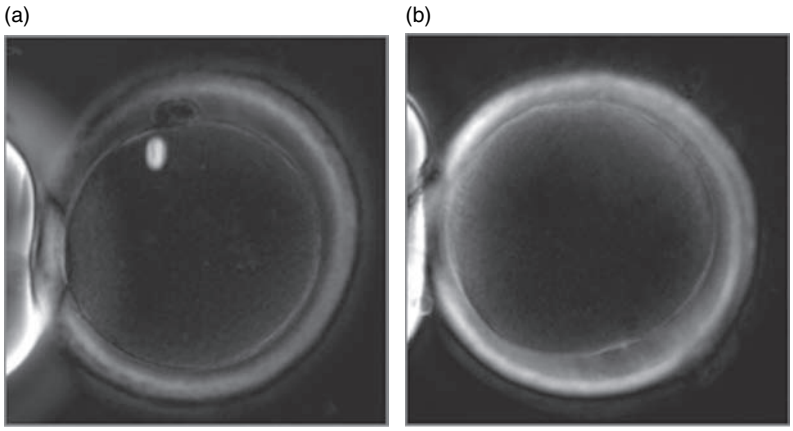


Figure 18.5. Some oocytes lack spindle refringence (b). Oocytes with spindle birefringence (a) are more viable.

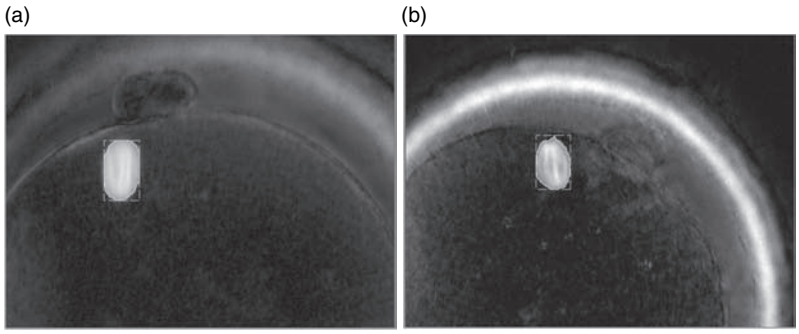


Figure 18.6. Mean retardance of the spindle in (a) is 2.95 nm and that in (b) is 1.45 nm. Oocytes exhibiting higher spindle retardance are more viable.

oocytes lacked spindle retardance altogether. Oocytes with spindle retardance produced significantly higher rates of fertilization and blastocyst development compared to those that did not. This high prevalence of absent spindles, coupled with observations that some spindles seemed to depolymerize before our eyes, led us to wonder whether thermal control on the conventional heating stage was optimal. Careful investigation in fact showed cold and hot spots on the stage heaters widely used at the time. Moreover, we discovered a large temperature gradient between the heating stage and the specimen itself. Spindles are dynamic structures, which are sensitive not only to temperature, but also to pH, gravity, and heavy water. When we undertook systematic studies of the effects of temperature on spindles, we found early depolymerization with only a few degrees drop in temperature and complete depolymerization when the temperature dropped to 33°C for 10 minutes [32]. Only two of five oocytes reformed their spindles upon re-warming after cooling to 28°C for 10 minutes, and no spindles reformed after cooling to 25°C. Spindles that did reform exhibited decreased retardance. We did not evaluate the normalcy of kinetochore attachments to reformed spindles, but presumably depolymerization and repolymerization cycles would disrupt chromosome-spindle attachments as well. Overheating was even more detrimental to oocyte and embryo development than cooling [33]. Rigorous control of temperature during the ICSI procedure led to marked improvements in fertilization and pregnancy rates. Spindle retardance, therefore, provides a natural intracellular thermister to gauge the tightness of thermal control and more generally oocyte handling by the IVF laboratory.

Spindle retardance

Spindle retardance reflects the spindle's molecular order and therefore would be expected to be reduced in abnormal spindles from poor quality eggs (Figure 18.6). A number of studies have found spindle retardance predictive of fertilization and embryo development [35]. Spindles from oocytes in pregnancy cycles were more likely to be structurally normal

(100% vs. 33%) and have higher retardance compared to those from non-pregnancy cycles, though age and fertilization rate did not differ between the groups [36]. Other studies have used spindle retardance to evaluate the efficiency of egg freezing and thawing protocols [37, 38].

Spindle azimuth

Normally, the spindle presents a barrel-shaped structure with its long axis oriented radially to the oolemma. Tripolar spindles, presumably resulting from aneuploid oocytes, have been observed in human eggs aspirated for IVF. Other azimuth abnormalities can result from incomplete meiotic maturation.

Oocyte maturation

Though clinical IVF laboratories employ a discontinuous classification scheme to oocyte maturation (i.e. GV vs. MI vs. MII), meiotic maturation actually is a continuous process and some oocytes aspirated from follicles of various sizes would be expected to be at intermediate stages of maturation. Indeed, oocytes diagnosed as MII based on normal-appearing polar bodies and the absence of GV were obviously in anaphase when imaged with the polscope (Figure 18.7). The polscope, therefore, may optimize the timing of ICSI for each oocyte, and therefore improve egg utilization [39].

Spindle size and shape

The size and shape of the meiotic spindle can be evaluated with simple image analysis software after imaging with the polscope. Small, short spindles are associated with reduced fertilization, embryo cleavage, and pregnancy rate [34–36].

Polarity and relationship of spindle to polar body

Eggs from most species, including human and mouse, exhibit polarity [40]. The meiotic spindle, ruffle-free oolemma and mitochondria all are polarized to a region of the oocyte called the animal pole, in contrast with the vegetal pole on the other side of the oocyte. Extensive polarity also has been reported in the localization of a number of developmentally important molecules, though these are impossible to measure non-invasively. Recent studies in mice suggest that the polarity of the egg establishes polarity in the blastocyst [40]. The entry site of the sperm, itself influenced by polarized changes in the oolemma, also helps establish the axes of the embryo [41]. The polscope provides the ability to non-invasively image the most obvious and important marker of the animal pole, the meiotic spindle.

In our early studies with the polscope we discovered that the spindle does not always lie beneath the polar body [42], a reflection not only of the tendency of the polar body to move freely in the perivitellane space,

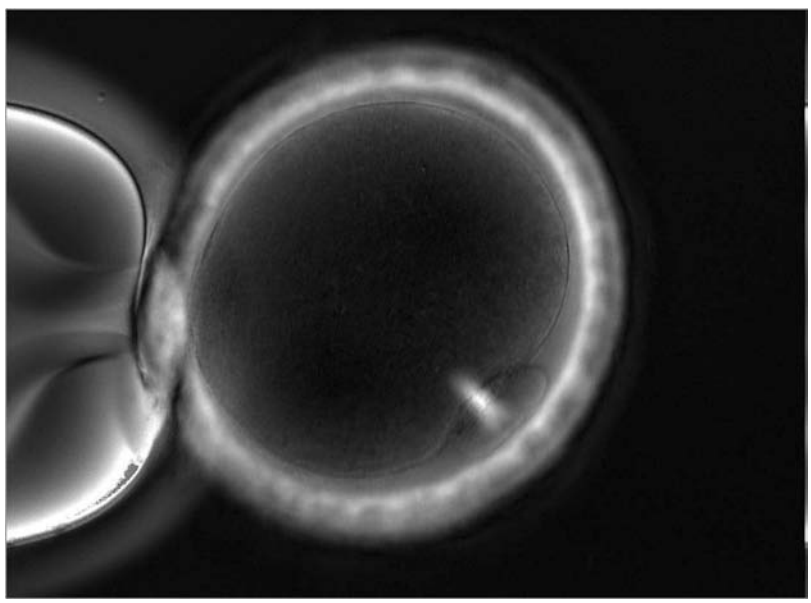


Figure 18.7. This oocyte has not completed anaphase of meiosis 1, though the absence of a GV and presence of the first polar body suggest an MII egg ready to undergo ICSI.

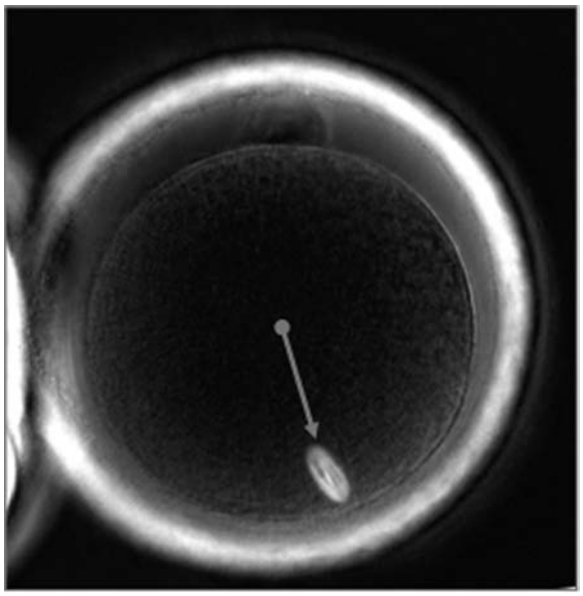


Figure 18.8. The polar body at 12 o'clock does not predict the location of the spindle at 5 o'clock.

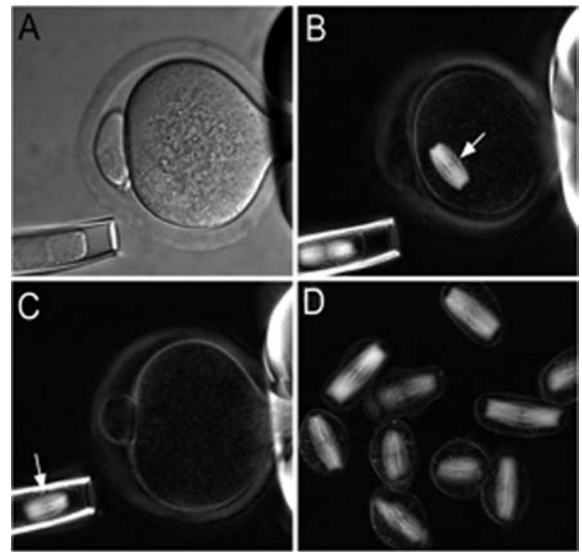


Figure 18.9. Differential interference contrast microscopy does not image the meiotic spindle (A). The spindle can be clearly seen (B) and removed (enucleated) (C) with the polscope. Isolated spindles are shown in (D).

especially with in vitro manipulation, but also of altered polarity of the oocyte (Figure 18.8). Conventionally the polar body has been used to orient the egg for ICSI. The observation that the polar body does not always predict the location of the spindle has important clinical implications. The narrow ICSI needle would be unlikely to skewer the tiny spindle when passed through the vast egg cytoplasm. However, since the polar body does not accurately mark the animal-vegetal poles of the oocyte, the sperm may be inadvertently injected into a suboptimal location within the cytoplasm. Indeed, injecting sperm based on the location of the spindle, as determined by the polscope, as opposed to the polar body, improved the quality of the resulting embryos [43].

Imaging chromosomes

Chromosomes also exhibit anisotropy, though in a different axis compared to spindles. When we imaged chromosomes non-invasively in the MII oocytes of senescence accelerated mice (SAM) we found congression abnormalities [29].

Polscope guided enucleation and spindle transfer

The polscope enables direct, real-time imaging of the meiotic spindle during the enucleation step of nuclear transfer. Conventionally, enucleation for nuclear transfer was performed by incubating the oocyte with fluorescent dye, which intercalates DNA, and then imaging the fluorescing chromosomes under UV light before removing the spindle-chromosome complex with a pipette. We hypothesized that the polscope could be used to enucleate the spindle and chromosomes for SCNT without exposing oocytes to toxic DNA dyes or high intensity light. Using the polscope (Figure 18.9), we enucleated oocytes from mice, cattle, and women without exposure to DNA dyes or UV light [44]. We also hypothesized that spindle enucleation with the polscope would improve the efficiency of nuclear transfer for somatic cell nuclear transfer. Polscope-directed spindle extraction contributed to the first successful cloning of a non-human primate, which had defied previous attempts using conventional enucleation methods.

Zona pellucida

The ZP is laid down during oogenesis and plays an important role in protecting the embryo during preimplantation embryo development. Implantation can occur only after hatching of the blastocyst-stage embryo from the ZP. Intriguingly, the ZP has a trilaminar structure when imaged by scanning electron microscopy. ZP fibers of the inner layer course radially, those from the outer layer course tangentially,

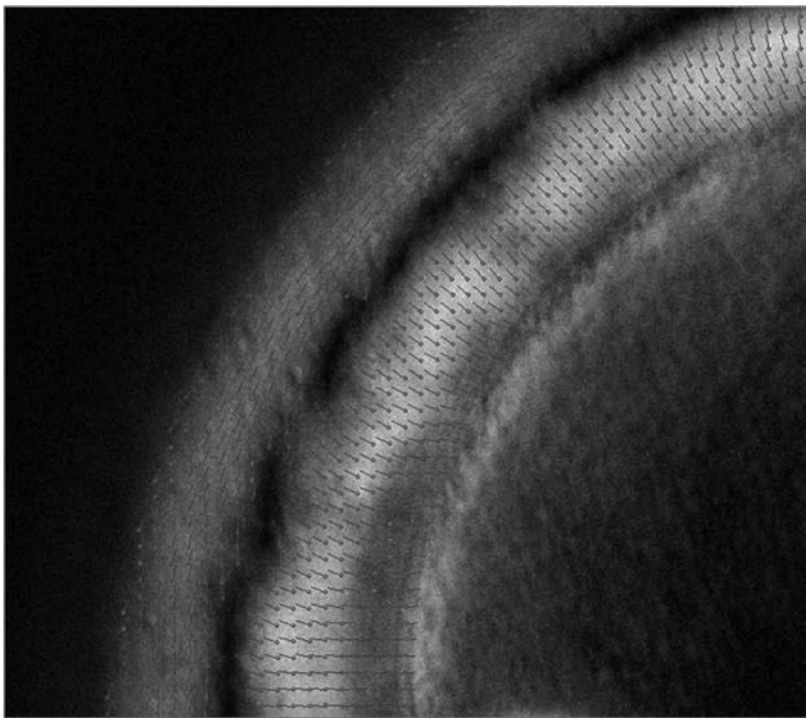


Figure 18.10. The ZP is composed of a lattice of intermediate filament zona pellucida (ZP) proteins organized into three layers. The small red arrows demonstrate the azimuth. The image's gray scale reflects the level of retardance, or molecular order. Filaments of the inner layer orient radially and are highly ordered, as shown by high retardance. Outer layer filaments orient tangentially and have intermediate levels of retardance. Middle layer filaments orient randomly and have minimal retardance. Alterations in the zona are linked to failed or abnormal fertilization, poor embryo development, and implantation. See colour plate section.

and fibers of the middle layer course randomly. The polscope clearly images each of these layers and distinguishes them by retardance level and azimuth (Figure 18.10). Polscope imaging of mouse embryos developed in vitro demonstrated marked structural alterations in the zonae compared to in vivo developed embryos. Future studies should explore whether the polscope can predict which embryos would benefit from assisted hatching. Since the ZP is laid down early during oogensis, we also hypothesized that features of the ZP architecture and structure might reflect these critical stages of early development, just as the rings on a tree stump inform about climate changes from years before. ZP thickness and retardance have both been associated with subsequent embryo development [31–33].

Summary

Polscope imaging provides valuable information on the structure and architecture of the meiotic spindle and the ZP, which helps predict fertilization, embryo development, and pregnancy. Further studies are needed to determine cut-off values to optimize the sensitivity and specificity of these new assays. Additional studies are needed to determine whether spindle abnormalities predict aneuploidy as revealed by the more invasive preimplantation genetic screening (PGS). Theoretically, polscope imaging and PGS could complement each other, much as Pap smears and colposcopy are used to manage cervical dysplasia.

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Chapter 19

Granulosa cells and their impact on human ART

Laurie J. McKenzie

Introduction

The follicle is the basic functional unit of the mammalian ovary, with each follicle containing a single oocyte surrounded by inner layers of granulosa cells and outer layers of thecal cells (Figure 19.1). Granulosa cells nurse the oocyte and differentiate and proliferate in response to gonadotropins, which are crucial for successful follicular growth and ovulation. In the early stages of follicular development, undefined factors, independent of gonadotropins, recruit primordial follicles and induce their growth.

Folliculogenesis

Compared with the initial stages of follicular recruitment, substantially more is known about the regulation of subsequent follicle differentiation and growth. Hormones control the development of individual follicles by triggering sequential, dynamic changes in granulosa cell proliferation and gene expression. Following the initial recruitment of primordial follicles, a primary follicle is formed (Figure 19.2). The primary follicle begins a growth phase and the surrounding follicular cells (now called granulosa) become cuboidal and proliferate. When the growing oocyte is surrounded by more than one layer of granulosa cells, the follicle is deemed a secondary follicle. Cavities then form inside the follicles and fill with follicular fluid (referred to as a follicular antrum). The antrum separates the population of granulosa cells into two subtypes; cumulus cells surrounding the oocyte and mural granulosa cells that line the follicular wall. (For a detailed review of folliculogenesis please see McGee and Hsueh [1]).

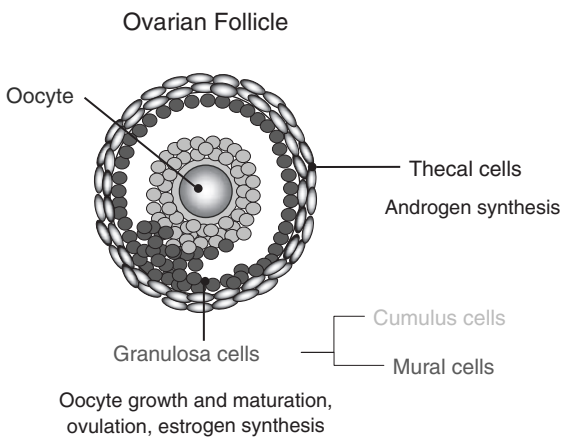


Figure 19.1. Schematic of human ovarian follicle. See colour plate section.

Bidirectional communication between oocytes and somatic cells

It has long been realized that the granulosa cells support oocyte development. These follicular somatic cells direct many specific oocyte functions including maintenance of the oocyte in meiotic arrest, subsequently promoting reinitiation of meiosis and nuclear maturation, and promoting oocyte competence to undergo fertilization. Recently, it has become apparent that the oocyte itself communicates directly with the granulosa cells.

There is bidirectional communication between the oocytes and the surrounding somatic cells via gap junctions and paracrine signaling, with this feedback loop facilitating the growth and development of both cell types during folliculogenesis (Figure 19.3). The oocyte actively directs a favorable microenvironment for the acquisition of its own developmental competence [2–6].

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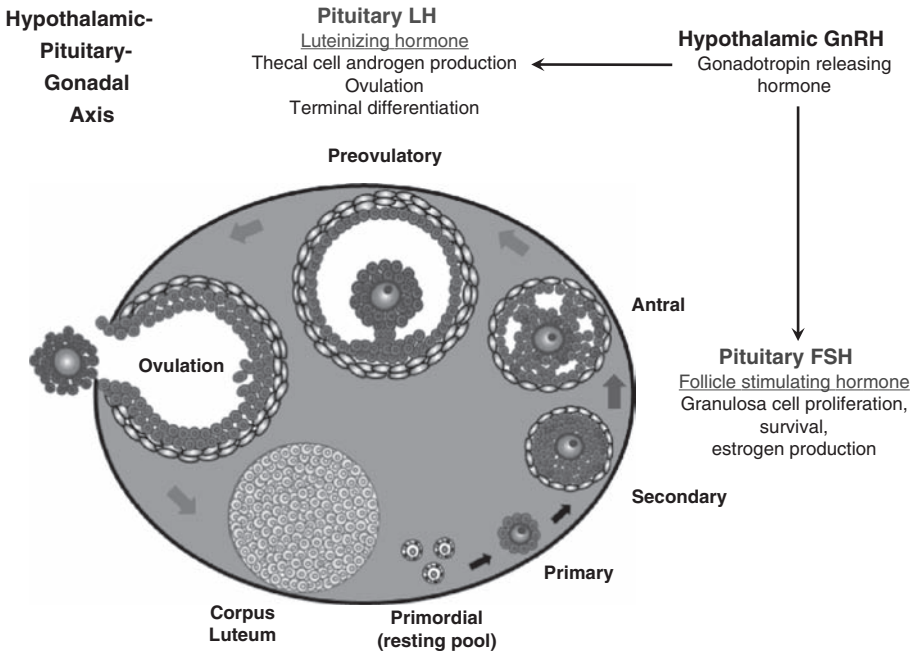


Figure 19.2. Schematic of folliculogenesis. See colour plate section.

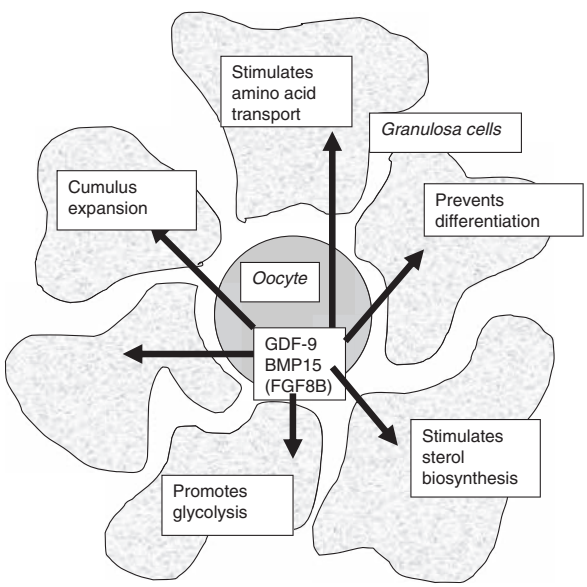


Figure 19.3. Bidirectional communication between the oocyte and cumulus cells.

Cumulus expansion

Cumulus cell expansion is a good example of this cumulus cell/oocyte interaction (for review see Refs. 2 and 4). In the peri-ovulatory period, the cumulus cells expand to form a radiating layer in response to the midcycle surge of luteinizing hormone or exogenous human chorionic gonadotropin (hCG) (Figure 19.4). Cumulus expansion denotes a grade III oocyte, the optimal stage for fertilization [7]. During expansion, cumulus cells secrete a hyaluronic acid-rich matrix that binds the oocyte and cumulus together, facilitates follicular extrusion and oviductal fimbrial capture, and allows sperm penetration and fertilization [8]. Cumulus expansion is imperative for normal oocyte development in vivo, and oocytes not associated with cumulus expansion have limited potential for implantation [7]. Expansion of the cumulus layer appears to be regulated by growth differentiation factor 9 (GDF9), an oocyte-secreted member of the transforming growth factor beta (TGFβ) superfamily, and other oocyte-secreted factors such as bone morphogenic protein 15 (BMP 15) [9–12].

GDF9 induces hyaluronan synthase 2 (*HAS2*) in cumulus cells [9] stimulating the production of hyaluronic acid, which facilitates cumulus expansion [12, 13]. GDF9 also facilitates cumulus expansion via the induction of cyclooxygenase 2 (*PTGS2*) and the resultant prostaglandin E_2 (PGE2) production [14] both of which are required for cumulus expansion [15].

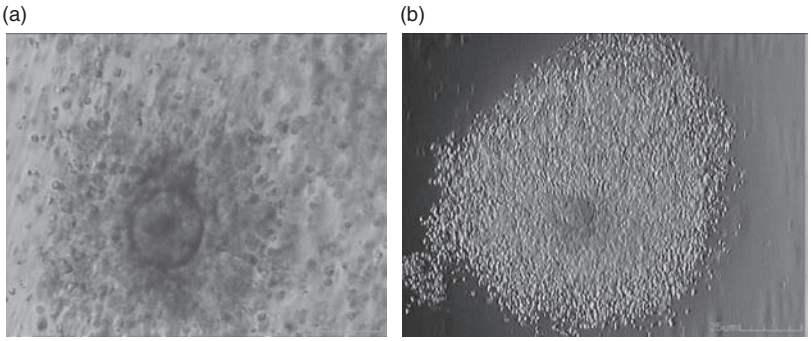


Figure 19.4. Photomicrograph of mature human oocyte with radiant dispersal of cumulus cells. See colour plate section.

Mice lacking functional *PTGS2* have defects in ovulation, fertilization, decidualization, and implantation [16]. Systemic treatment with PGE2 restores the ability of *PTGS2* knock-out mice to ovulate [17]. Calder *et al.* [15] examined expression of *PTGS2* and prostaglandin receptor mRNA in bovine cumulus-oocyte complexes and found that expression of PTGS2 and EP2 mRNA is dependent on time of maturation and oocyte quality. Complexes in which the oocyte is microsurgically removed and cultured with recombinant GDF9 can be induced to undergo cumulus expansion [14].

Proposed oocyte regulators of folliculogenesis

Two oocyte-secreted factors, GDF9 and BMP15, have been proposed as oocyte regulators of follicular development and therefore fertility. GDF9 is required for normal folliculogenesis in female mice and regulates human folliculogenesis [3, 9, 18]. Elvin *et al.* [9] demonstrated that GDF9 protein is expressed in all mouse oocytes beginning at the 3a follicle stage including antral follicles. GDF9 gene mutations lead to infertility due to an arrest at the type 3b (primary) follicle stage, absence of thecal layer formation, and defects in oocyte meiotic competence. Additional data have shown that GDF9 is required for later-stage follicular development as well [3, 18, 19].

Another potential candidate for an oocyte-derived regulator of folliculogenesis is bone morphogenic protein 15 (BMP15), which acts on murine granulosa cells to regulate their function [20–22]. Targeted disruption of mouse BMP15 results in subfertile animals with a defective cumulus phenotype [19]. Transgenic mice with oocyte overexpression of BMP15 reveal a growth promoting role of BMP15 in ovarian follicles [23], and genetic studies clearly illustrate the interaction between BMP15 and GDF9 in the mouse ovary [19].

Cooperativity between BMP15 and another oocyte-produced factor, fibroblast growth factor (FGF) 8b, in promoting glycolysis has been described. Oocytes from *Bmp15*$^{-/-}$ and *Gdf9; Bmp15*$^{-/-}$ double mutant mice are deficient in promoting glycolysis and inducing gene expressions of the glycolytic enzymes, platelet phosphofructokinase (Pfkp), and lactate dehydrogenase A (Ldha). Furthermore, the combination of recombinant BMP15 and FGF8b is capable of promoting glycolysis and gene expression of glycolysis enzymes in cumulus cell cultures [24]. Mammalian oocytes are deficient in the glycolysis pathway and are dependent on the glycolytic products from cumulus cells. Regulation of glycolysis by oocyte factors BMP15 and FGF8b are therefore of particular significance for oocyte development.

Gremlin, another downstream gene upregulated by GDF9, selectively inhibits bone morphogenic protein signaling (*BMP*) and not *GDF9* signaling. It is hypothesized that the differential signaling inhibition may facilitate the luteinization of mural granulosa cells, while allowing cumulus cell expansion [25]. The role of gremlin in human reproduction has not been well elucidated and merits further investigation.

GDF9 signals through SMAD2/3 pathway [26, 27] and induces the expression of expansion-related transcripts (pentraxin 3 [*Ptx3*], hyaluronan synthase 2 [*Has2*], tumor necrosis factor alpha-induced protein 6 [*Tnfaip6*], and prostaglandin-endoperoxide synthase 2 [*Ptgs2*]) in mouse granulosa cells [14, 28]. Furthermore, the SMAD2/3 pathway, one of the pathways downstream of TGFβ superfamily members in mammals, mediates oocyte signals that determine cumulus cell lineage and expansion. Studies on *Smad2/3* conditional knockout mice indicate that ovarian SMAD2 and SMAD3 are indispensable for normal cumulus expansion in mice (Matzuk, unpublished data). The requirement of SMAD2/3 signaling for initiation of cumulus expansion and induction of cumulus

expansion-related genes [29, 30] as well as a defective cumulus phenotype are manifested in a *Smad4* conditional knockout model [31].

Clinical utility

With the current understanding of granulosa cell/oocyte physiology, it is apparent that the granulosa cell pathways are regulated by the oocyte, and the functional properties of the granulosa cells (particularly cumulus cells) are reflective of oocyte quality. Based on this concept, gene expression profiling of granulosa cells may indirectly provide novel and reliable parameters to assess oocyte competence. Regulated expression of downstream GDF9 targets in the cumulus cells may reflect GDF9 activity, and could ultimately predict oocyte health. Knowledge of these downstream targets can be utilized to augment culture media and facilitate in vitro maturation. Additionally, this relationship has been proposed to shed more light on human reproductive conditions such as polycystic ovarian syndrome and premature ovarian failure, and will be discussed below.

Biochemical markers of follicles

Traditional methods for oocyte and embryo selection for human assisted reproductive technologies (ART) rely on subjective morphological assessments. Biochemical assessment of follicles would generate additional objective information to better understand successful fertilization and embryo development. For example, the expression of specific genes (e.g. *PTGS2*, *HAS2*, *GREM1*) expressed in the cumulus during expansion, and discarded at time of IVF, may give a direct assessment of the fertility potential of an individual oocyte without compromising the oocyte integrity. Cumulus cells are abundant and are typically discarded at the time of IVF, making them an ideal substrate for study. Biochemical grading of the follicle could be utilized to select the "optimal" embryos that are produced and transferred. Acceptable pregnancy rates could be maintained while replacing fewer embryos.

Markers of apoptosis and oxidative stress

Fewer than 500 oocytes will ovulate over a woman's lifespan, despite the presence of approximately one million oocytes at birth. Greater than 99% of her follicles will disappear, primarily due to apoptosis of granulosa cells. Acceleration of this normal process of atresia can negatively affect fertility and lead irreversibly to premature ovarian failure. Other processes, such as autophagy [32], may be involved with follicular atresia, but are not considered the primary mechanism for follicular atresia. Therefore, for the purposes of this discussion, we will only describe the impact of granulosa cell apoptosis on human fertility.

Many apoptosis-related factors have been implicated in follicular atresia, including death ligands and receptors, caspases, pro- and anti-apoptotic Bcl-2 family members, gonadotropins, calcium, and gap junctional intercellular communication. Other mediators probably involved in regulation of granulosa cell apoptosis include follicle-stimulating hormone (FSH), GDF-9, nodal, prohibin, TNF, IGF-I, and p53. Differences in apoptosis exists between granulosa cell types and follicular stage. For example, mural GCs have a higher incidence of apoptotic bodies than cumulus cells, and primordial follicles are independent of gonadoptropin support. (For an excellent detailed review of apoptosis and female fertility, please see Krysko *et al.* [33].)

Can markers of apoptosis be utilized to predict fertility outcomes? Alisch *et al.* [34] collected cumulus-oocyte complexes from women undergoing in vitro fertilization and intracytoplasmic sperm injection (IVF/ICSI) and correlated the proportion of apoptotic bodies in the cumulus to fertilization, oocyte quality, and pregnancy outcome. High-quality embryos demonstrated lower rates of cumulus apoptosis, although the difference in pregnancy outcomes was not significant. The preponderance of data indicates that the degree of apoptosis in cumulus cells negatively correlates with oocyte competence [35–37]. Bcl-2 protein can prevent apoptosis induced by a variety of stimuli [38]. Interestingly, Bax can counter the effects of Bcl-2 on cellular survival by heterodimerizing with Bcl-2, and it has been proposed that the ratio of Bcl-2 to Bax (Bcl-2/Bax) could be used to predict oocyte quality [38, 39].

Apoptotic markers can be used to estimate a function of ovarian reserve in women undergoing in vitro fertilization [40, 41]. Patients from whom a large number of oocytes were retrieved had a lower incidence of apoptosis in granulosa cells compared to those from whom fewer oocytes were retrieved [42]. Endometriosis patients are associated with higher incidence of apoptotic granulosa as compared to normal women [43] and the incidence of granulosa cell apoptotic bodies increased as the severity of the

endometriosis increased [44]. Accelerated granulosa apoptosis has been proposed to play a pivotal role in the etiology of unexplained infertility [45].

Oocytes and somatic cells that constitute the ovary are exposed to reactive oxygen species during the normal aging process as well as during ovulation [46]. Reactive oxygen species are known to induce zona hardening, tanning, and DNA damage. Recently, investigators have attempted to correlate oocyte competence with follicular biomarkers of oxidative stress (8-hydroxy-2′-deoxyguanosine [8-OHdG] and lipid peroxidases in granulosa cells, total anti-oxidant capacity, and reactive oxygen species [ROS]). An increase in the levels of 8-OHdG in granulosa cells during the ovulatory process led to a lower fertilization rate and a decrease in good-quality embryos [47]. ROS generated in culture media by day 1 post-insemination may be an important biochemical marker for early embryonic growth [48]. Others, however, found no correlation between lipid peroxide levels and the total anti-oxidant activity and oocyte maturity, fertilization, or embryo cleavage, although they were positively associated with pregnancy rate [49].

Glutathione *S*-transferases (GSTs) are one mechanism nature has developed to protect cells from oxidative stressors and GST mutations have been linked to cancer predisposition [50–52]. GST theta 1 in human granulosa cells is upregulated by aging and inversely correlated with cumulus-oocyte complex maturity [53]. Survivin, an inhibitor of apoptosis, correlates with pregnancy rates in women undergoing IVF [54]. These results suggest that the incidence of apoptosis in cumulus cells can be used to predict oocyte quality, outcomes of in vitro fertilization, and age-related decline in fertility.

Oocyte competence and embryo development

The developmental potential of human oocytes and embryos is reflected in cumulus cell gene expression. Specifically, expression of three genes (*HAS2*, *PTGS2*, gremlin) downstream of *GDF9* (discussed earlier) correlates with oocyte and embryo development. Cumulus cells from oocytes that develop into higher-quality embryos (grades 3, 4, 5) have 6–15-fold increased median expression of these *GDF9* targets, and may be of clinical use in human assisted reproduction [55]. A subsequent study suggested that *HAS2* and *GREM1* are cumulus markers predictive of oocyte competence [56]. There is some controversy, however, regarding the association of cumulus *PTX3* abundance with oocyte quality [55–57]. Oocyte nuclear maturation is associated with increased expression of *STAR*, *COX2*, *AREG*, *SCD1*, and *SCD5* [58].

Microarray technology is now being applied to define the gene profiles of human ovarian somatic cells and correlate these profiles with oocyte and embryo developmental competence. The variation in cumulus cell gene expression during oocyte nuclear maturation has recently been elucidated via microarray. A global analysis of cumulus genes showed a progressive decrease in the number of genes expressed as the oocyte matured from a GV to MII oocyte, and these genes may serve as additional genetic markers for oocyte competence [59].

Differentially expressed genes between cumulus cells derived from fertilized oocytes developing to early cleavage (EC) embryos and those from oocytes that fail to develop into EC (NEC) embryos were identified. The genes increased in NEC samples (glutathione peroxidase 3 [*GPX3*], chemokine receptor 4 [*CXCR4*], stress-induced apoptosis inhibitor [*HSPB1*], cyclin D2 [*CCND2*], 7-dehydrocholesterol reductase [*DHCR7*], etc.] are reflective of a potentially hypoxic state of the cumulus cell microenvironment or delayed maturation of the oocytes [60]. Surprisingly, none of the GDF9 target genes (*HAS2*, *PTGS2, PTX3*, and *GREM1*) identified by other studies [55, 57] were overrepresented in the EC samples.

Expression of BCL2L11 (involved with apoptotic pathways), PCK1 (important in gluconeogenesis) and NFIB (regulation of tissue-specific gene expression) in cumulus cells significantly correlated with embryo potential and successful pregnancy [61]. Five potential markers in pooled (predominantly mural) granulosa cells were associated with embryos leading to successful pregnancy [62]. The markers were FDX1, CYP19A1, cdc42, SERPINE2, and 3βHSD, of which FDX1, 3βHSD and CYP19A1 are involved with steroidogenesis. SERPEINE2 is a member of the family of protease inhibitors involved in a multitude of functions including apoptosis. CdC42 expression is particularly relevant, as it has been demonstrated to delay the rate of the apoptotic process [63].

Clinical outcomes

Alterations in cumulus physiology occur as women age and may be responsible for their reproductive

disadvantage, either as a direct cause or reflecting a decline in the functional and structural qualities of the oocyte [64]. Cumulus cell gene expression was prospectively evaluated in women undergoing in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) with a maternal age less than 25 years or above 40 years. Gene expression profiling was performed using the illumina human V-2 genechip, and several genes were differentially regulated as related to maternal age. Specifically, carbonic anhydrase 9, zinc finger protein 582, and hyaluronan synthase-2 were upregulated in older patients (nine-fold change, six-fold change and two-fold change respectively). Bone morphogenic protein 1 and syndecan 3 were downregulated in older patients (four-fold change and two-fold change respectively) [65]. Mural granulosa cell expression is differentially expressed as a function of maternal age. Three genes were upregulated and 117 were downregulated (including interleukin [IL]-1beta, IL-1R2, and IL-6R) in GCs of older versus younger patients [66]. Given previously recognized roles for the interleukin gene family in folliculogenesis and ovulation, these findings may partly explain ovulatory and luteal dysfunctions associated with reproductive aging. Diminished ovarian reserve (DOR) has been associated with poor response to ovarian stimulation, lower number of oocytes recovered during IVF, lower pregnancy rates following IVF treatment cycles, and higher miscarriage rates. An inverse correlation with follicle-stimulating hormone (FSH) levels and mural (but interestingly not cumulus) granulosa cell viability has been documented [67].

Differential cumulus gene expression has also been noted with various pathological conditions. In women with polycystic ovarian syndrome (PCOS) lower apoptotic rates were found in the granulosa cells from women with PCOS, compared to those women with regular ovulatory cycles [68]. Interestingly, the high lutenizing hormone (LH) levels in women with PCOS may be responsible for their decreased apoptosis in granulosa cells. LH interferes with Fas-induced apoptosis in human ovarian surface epithelial cancer lines [69]. Using microarray analysis of whole ovarian samples, Diao *et al.* [70] also noted that anti-apoptotic factors were overexpressed in PCOS. This is in contrast to the study by Jansen *et al.* [71], who reported an increase in the expression of apoptotic factors in PCOS ovaries. However, it is worth noting that both studies used archival or whole ovarian tissue. Using quantitative real-time PCR, anti-Mullerian hormone (AMH), AMH receptor II, FSH receptor, and androgen receptor genes were shown to be overexpressed by granulosa cells from stimulated follicles of women with PCOS undergoing controlled ovarian hyperstimulation. This could be the sign of a maturation defect or may be reflective of hyperandrogenism [72].

Interestingly, expression profiles appear to change as a function of the medications employed for ovarian stimulation. A recent study compared the expression profile of mural granulosa cells from IVF/ICSI patients receiving two different exogenous FSH preparations for controlled ovarian hyperstimulation (recombinant FSH, Gonal F®, versus human menopausal gonadotropin (hMG), Menopur®) [73]. Eighty-five genes showed statistically significant differences in granulosa cell expression levels between the two medication groups. Luteinizing hormone/human chorionic gonadotropin (LH/hCG) receptor gene and genes involved in biosynthesis of cholesterol and steroids were expressed at lower levels in the hMG-treated cells; inositol 1,4,5-triphosphate-3-kinase-A and S100-calcium-binding-protein-P (anti-apoptosis protein) were expressed at higher levels in hMG than in recombinant FSH. Controlled ovarian stimulation with a rFSH-only protocol was compared to a rFSH and rLH protocol [74]. Cumulus cells were evaluated for apoptosis via TUNEL assay and anti-caspase 3 immunoassay. The rFSH/LH group exhibited lower cumulus apoptosis rates and lower numbers of immature oocytes. No statistically significant difference was noted with pregnancy and implantation rates, albeit there was a small sample size (40 patients). Whether these differences translate into differential clinical outcomes remains to be elucidated.

Could oocyte-secreted factors that direct the cumulus be responsible for human infertility? Several groups have screened women with premature ovarian failure (POF) for GDF9 or BMP15 mutations. Of 127 Indian women with POF, five women were found to have the missense mutation A199C and two women had a G646A mutation [75]. Sixty-one American women with POF were screened, with a single woman showing a heterozygous perturbation [76] in GDF9. Among 100 Chinese POF cases, one novel missense mutation and a novel single-nucleotide polymorphism in BMP15 were found, and one novel SNP and two novel silent mutations in GDF9 were discovered [77]. Screening of 300 unrelated women with premature ovarian failure led to the identification of six heterozygous BMP15 variations in 29 of the 300 women [78]. In most cases of premature

ovarian failure or other reproductive disorders, specific genetic mutations have yet to be elucidated. Clearly, this is an area that warrants further investigation.

Limitations

Limitations with the use of mural granulosa or cumulus cells for embryo selection include the requirement of single embryo culture and the significant processing/analysis time required for microarray results. Additionally, to truly correlate cumulus gene expression to implantation would require increased utilization of elective single embryo transfer, which still constitute less than 5% of IVF cycles in the United States [79].

Granulosa cell co-culture

In vitro maturation

In vitro maturation (IVM) of the oocyte is the act of culturing and maturing oocytes that have been harvested at an immature phase prior to the LH surge. A major advantage of IVM versus traditional IVF is to bypass the ovarian stimulation procedure that may cause ovarian hyperstimulation syndrome (OHSS) (for review see Rao and Tan [80]). During a typical IVF cycle approximately 15% of the oocytes will be found to be immature and they are typically discarded. The ability to successfully mature these oocytes in culture would result in a greater number of potentially usable embryos. A significant limitation to IVM, however, is that IVM often produces low-quality oocytes in contrast to in vivo matured oocytes [81] and the factors contributing to this difference remain to be elucidated. Only 40–80% of fertilized IVM oocytes progress through early cleavage and of those that do cleave and that are transferred, <15% implant to form a viable fetus [82–84].

The majority of human IVM studies have utilized immature oocytes denuded of surrounding cumulus, yet removal of the cumulus oophorus before IVM appears detrimental to oocyte quality [85–88]. Denuded oocytes exhibit accelerated meiotic resumption in vitro which results in a lack of coordination between nuclear and cytoplasmic maturity. Removal of the cumulus before IVM alters the timing of nuclear maturation, disturbs spindle assembly as well as redistribution and function of mitochondria, and increases maturation-promoting factor activity. Cumulus removal also causes precocious exocytosis of cumulus cells leading to zona pellucida hardening and reduced penetrability of oocytes by sperm [89]. Stripping the cumulus from mouse oocytes prior to IVM impairs mouse oocyte developmental potency and, importantly, co-culture on a cumulus monolayer improves the maturation and development of immature murine oocytes [90]. Given the interdependence of oocytes and cumulus cells, perhaps a shortcoming of traditional IVM culture systems is the absence of somatic cell support. Could granulosa cell co-culture facilitate IVM?

Cumulus co-culture with IVM is not a new concept, having been proposed as early as 1991 when immature oocytes co-cultured with granulosa cells improved fertilization rates from 20% to 54% [91] (see Table 19.1 for a listing of relevant co-culture studies). More recently, Johnson and colleagues [92] stripped the oocytes of their cumulus masses and subsequently "reunited" the immature oocytes with their original cumulus mass. Maturation rates were then determined at 18 hours and although there did not appear to be an improvement in metaphase I maturation, increased maturation rates were noted with germinal vesicle-stage oocytes. Despite an overall 62% fertilization rate, this process was not efficient as it yielded only a 13% "usable" embryo rate.

Further attempts to improve cumulus co-culture have been to create a three-dimensional (3D) culture system with use of an extracellular matrix (ECM). The ECM influences a multitude of cell functions, including morphogenesis, survival, migration, proliferation, communication, metabolism, and response to external stimuli. Utilizing cumulus cells embedded in an extracellular matrix would create a system that permits tight contact between the oocyte and the disassociated cumulus cells, better mimicking physiological structure. A 3D co-culture system for IVM of denuded oocytes was first proposed by Combelles *et al.* [93]. Denuded human oocytes were cultured in 3D collagen gel embedded with cumulus cells. Maturation rates were compared. No difference was observed in the incidence of MII oocytes after IVM in microdrop versus 3D co-culture. Contrary to the above findings, a 3D granulosa cell co-culture system enclosed in a barium alginate membrane gave an eight-fold higher maturation rate than those oocytes that underwent IVM in microdrop [94]. It has been speculated that the encapsulation of GCs may delay the process of lutenization, thus maintaining a more favorable milieu for oocyte maturation.

Further refinements of co-culture for IVM involve directly delaying nuclear maturation while cytoplasmic maturation occurs. In vitro matured oocytes undergo

Table 19.1. Granulosa cell co-culture studies with human IVF; impact on embryo development and pregnancy rate (if applicable)

Author	Cell type	Number of patients	Findings	Pregnancy rate
Plachot *et al.*, 1993 [106]	Predominantly mural granulosa	17	83% blastocyst formation rate	No difference
Mansour, *et al.*, 1994 [107]	Cumulus	95	Decreased embryo fragmentation	Not assessed
Freeman *et al.*, 1995 [108]	Predominantly mural granulosa	21 (randomized) 83 (sequential)	Decreased embryo fragmentation rate (0.7 vs. 1.3, $P < 0.05$) in randomized group	54% clinical pregnacy rate in sequential group. No comparison data available.
Quinn *et al.*, 1995 [101]	Cumulus	39	45% vs. 31% blastocyst rate ($P < 0.05$)	Not assessed
Dirnfeld *et al.*, 1997 [100]	Predominantly mural granulosa	40	51 vs. 14% formation of high quality embryos ($P < 0.01$)	No difference
Carrell *et al.*, 1999 [103]	Cumulus	247	Improved embryo quality	Improved (39% vs. 49%)
Khamsi *et al.*, 1999 [104]	Predominantly mural granulosa	15	No improvement in embryo development	No difference
Fabbri *et al.*, 2000 [102]	Predominantly mural granulosa	18	50% vs. 35% blastocyst formation rate	No difference

maturation 'precociously' while they are still in the process of acquiring the cytoplasmic machinery needed to fully support preimplantation embryonic development [95–98]. By temporarily inhibiting spontaneous meiotic maturation, the immature oocytes would be arrested at the germinal vesicle (GV) stage, enabling cytoplasmic maturation to take place. In this way, a two-step culture has been created consisting of a prematuration culture (PMC) using a PDE3-I phosphodiesterase 3-inhibitor (PDE3-I) followed by IVM [99]. Three-dimensional "prematuration culture" (3D PMC) using a PDE3-I followed by IVM enhanced cytoplasmic maturation but did not yield a significantly higher fertilization rate compared with oocytes that underwent conventional IVM. Although 3D-PMC did not augment the fertilization capacity when compared with oocytes prematured in microdroplets, embryos on day 3 post-ICSI from the 3D-PMC groups were of better quality and had fewer nuclear abnormalities compared with the other groups. These results suggest that the combination of a co-culture in the presence of an ECM may serve as a physiological support during the latter stages of oocyte development.

Granulosa cell co-culture improving embryo quality

Cumulus cell co-culture has also been proposed to optimize embryo quality with in vitro fertilization aside from the adjunct of IVM. Dirnfled *et al.* [100] cultured two- pronuclear embryos on a granulosa cell monolayer for 24–28 hours and found an improvement in the number of good quality embryos as compared to the patient's prior failed IVF treatment cycle without co-culture. Pregnancy rates were higher in the co-culture group compared to other patients with failed IVF during the same time period. Quinn and Margalit [101] found improved fully expanded blastocyst formation rates with cumulus co-culture, as have others [102], and other groups have found improved embryo morphology and clinical pregnancy rates [103].

Not all studies demonstrate improved pregnancy rates. Khamsi *et al.* [104] did not improve either fertilization rate or embryo formation rates. Fabbri and associates failed to demonstrate an improvement in live birth rates; however, the sample size was modest at 18 patients [105] (see Table 19.1 for further description of co-culture studies).

Most studies that evaluate the effect of co-culture on fertilization rate, blastocyst formation rates and IVF success rates utilize conventional IVF. Recent data certainly suggest improved fertilization and blastocyst formation rates when fertilization is accomplished via ICSI; however, this traditionally has necessitated denuding the oocyte prior to the ICSI procedure. Perhaps keeping the majority of the cumulus cells intact during and after the ICSI procedure

could confer a benefit to the oocyte/embryo. In ICSI, however, evidence of any effect of attached CCs on further competence of the ovum is scarce, and only one human study has been performed to date [109]. Sibling cumulus-oocyte complexes for 57 ICSI patients were allocated to a study group (incomplete denudation prior to ICSI, $n = 314$) and a control group (complete denudation prior to ICSI, $n = 336$). With the immature oocytes, the presence of adhered cumulus led to a significant increase in resumption of meiosis. Fertilization rate and ability to cleave, however, were impaired in the study group, potentially due to technical challenges associated with performing ICSI on an incompletely denuded oocyte. Interestingly, once the oocytes were fertilized and cleaved, the above effects were reversed. The embryo quality at the 4- and 8-cell stage was significantly superior in oocytes that were incompletely denuded (irrespective of the cumulus pattern).

Granulosa cell culture is a simple, time-efficient, and inexpensive co-culture method. This method has advantages over other co-culture systems: screening of the granulosa cells for infections is unnecessary in an autologous system and the cells are easily collected during oocyte retrieval and are ready for co-culture 1 day later. The monolayer remains epitheloid and metabolically active and synthesizing protein for a maximum of seven to 10 days. There is no need to collect the sample in previous cycles such as with endometrial co-culture.

Media optimization

Augmentation of culture media to facilitate IVM

Can we exploit our knowledge of cumulus-oocyte physiology to improve culture conditions in human IVF? Can we augment culture media with oocyte secreted factors to improve the microenvironment surrounding the oocyte and thus enhance oocyte development and embryo development? Obviously these studies are difficult to perform in humans, in light of concerns for imprinting and epigenetic modifications, to mention a few. Hussein and colleagues [110] treated bovine cumulus-oocyte complexes (COC) with BMP15 and/or GDF9 and observed an increase in the oocyte developmental potential to blastocyst stage. Application of BMP15 anatagonists (follistatin) or GDF9 (ALK4/5/7 inhibitor; SB431542) reduced the oocyte developmental competence [110]. The same group also demonstrated that the addition of GDF9 in the IVM medium can promote mouse embryo development and increase fetal viability without affecting embryo implantation rates [111].

In the limited human data that exist, immature cumulus-oocyte complexes were cultured with FSH, leukemia inhibiting factor (LIF), or without media augmentation. LIF and FSH were able to induce cumulus expansion [112]. Future studies need to evaluate the effectiveness of combinations of oocyte secreted factors in promoting oocyte and embryo competence.

Facilitation of embryo transfer

In most mammalian species, including humans, the cumulus which surrounds the oocyte is still present at the time of fertilization and remains until embryonic implantation. The cumulus matrix is rich in hyaluronan, as well as in other proteins including inter-alpha-trypsin inhibitor, a dermatan sulfate proteoglycan, and pentraxin-3 [89]. Proteins and hyaluronan are linked together to form a meshwork composed of granules and filament and exhibit adhesive properties. In an effort to improve implantation, the search has been ongoing for "glue" that would allow improved contact between the embryo and the uterus. A novel study was recently performed in which embryo transfer was performed using the women's own cumulus cells. A cluster of 30 µl of cumulus cells that were growing in close proximity to the embryo was mechanically lifted with denuding pipettes and deposited around the embryo. Embryos and cumulus cells were then loaded in the transfer catheter simultaneously. Catheter contents were released into the uterus over a duration of 8–10 seconds, and the catheter was held in position in vivo for approximately 15 seconds. Higher pregnancy and implantation rates were reported in the study group. In light and electron microscopy analysis, cumulus cells showed dendritic-like processes extending toward the cleaving embryo (Figure 19.5) [113].

Other findings

Autoimmunity to human granulosa cells has been reported in infertile women. Prevalence of anti-granulosa cell antibodies was measured in the sera of women attending an IVF clinics, and positive antibodies were found in 28.7% of infertile women as compared

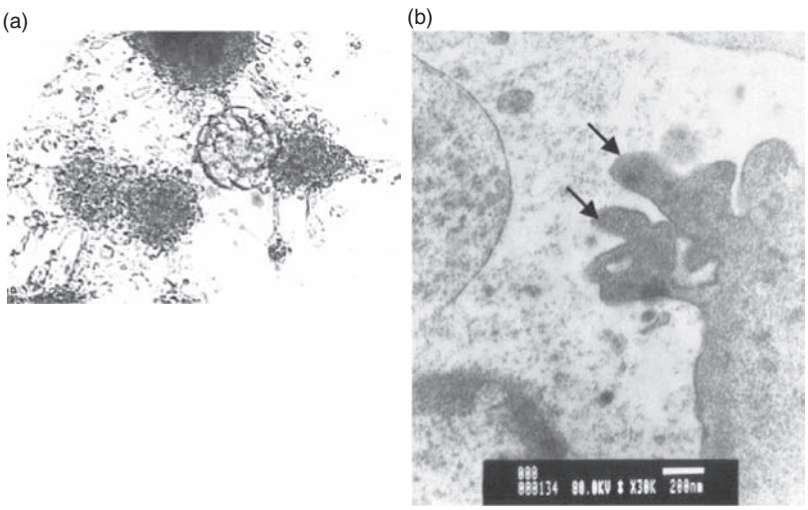


Figure 19.5. (a) Day 3 embryo completely immobilized by the encroaching expanding cumulus cell colonies. Interlacing dendritic processes are pictured. See colour plate section. (b) Electron microscope photomicrograph of the expanded cumulus cells displaying enhanced cellular activity. The arrows indicate cytoplasmic projections. Reprinted from *Fertility and Sterility* 86, Firuza R. Parikh, Suparna G. Nadkarni, Nandkishor J. Naik, Dattatray J. Naik, Shonali A. Uttamchandani. Cumulus coculture and cumulus-aided embryo transfer increases pregnancy rates in patients undergoing in vitro fertilization, 2006, with permission from Elsevier.

to 9.1% of fertile controls [114]. Confirmation of these findings and clinical utility remain to be elucidated.

Conclusion

The body of knowledge regarding granulosa cell physiology offers us an opportunity to improve human assisted reproduction. Investigation of these typically discarded cells can provide additional insight for oocyte and somatic cell interaction and reflect the oocyte microenvironment. Knowledge of the physiology and gene expression of mural and cumulus granulosa can be exploited for in vitro maturation, optimization of embryo culture, and refinement in embryo selection. Abnormal granulosa cell physiology may also further elucidate human conditions such as premature ovarian failure, endometriosis and age-related decline in fertility, and offer opportunity for treatment of these disorders.

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Chapter 20

Sperm diagnosis: selecting the best sperm for IVF/ICSI treatment

Denny Sakkas

Introduction

Infertility has been commonly defined as the inability to conceive after 12 months of regular intercourse, in the absence of contraceptives. A multi-center study conducted by the World Health Organization (WHO) concluded that in 20% of infertile couples, the predominant cause of infertility is the male, while in another 27% of couples both partners contribute. In the 2008 national US statistics, 35% of the IVF-ET cycles reported were diagnosed with male factor infertility, either as a single (17%) or combined (18%) diagnosis (https://www.sartcorsonline.com/). While these statistics underlie the importance of male factor in reproduction, the clinical and analytical methodology used to diagnose male infertility is recognized to have pitfalls [1].

Semen analysis is routinely used to evaluate the male partner of the infertile couple. Although the thresholds for normal semen measurements are standard, the current normal range for sperm concentration, motility, and morphology fail to meet rigorous clinical, technical, and statistical standards. In recognition of these limitations, the terminology in the WHO manual for semen evaluation was changed from "normal" to "reference" values [2]. With the advent of intracytoplasmic sperm injection (ICSI) [3] the onus to understand the biology of which sperm fertilized the egg has taken greater meaning, as the type of male factor patient being treated is much more likely to be problematic.

Beyond routine sperm analysis

In 1980 Evenson *et al.* [4] published an innovative paper in *Science* entitled "Relation of mammalian sperm chromatin heterogeneity to fertility" in which they used flow cytometry measurements of heated sperm nuclei to reveal a significant decrease in resistance to *in situ* denaturation of spermatozoal DNA in samples from bulls, mice, and humans of low or questionable fertility when compared with others of high fertility. They postulated that there were assumed changes in sperm chromatin conformation that may be related to the diminished fertility. They then went on to suggest that flow cytometry of heated sperm nuclei could provide a new and independent determinant of male fertility.

It was not realized by Evenson and colleagues that 12 years later the humans of low or questionable fertility would have the ideal treatment option in ICSI [3]. We have now become more adept at measuring the abnormalities in the sperm nucleus and the use of ICSI to treat humans with severe fertility problems is routine. There are, however, valid concerns about the use of ICSI in these low fertility patients [5], even though large follow-up studies do not show any major differences between ICSI, IVF, and normal conceptions [6]. The testing of sperm DNA fragmentation and concerns about ICSI have therefore become further associated, as it is ICSI patients who are more likely to possess spermatozoa containing faulty nuclear DNA [7]. This chapter will therefore examine the current and new methods available to diagnose and select the best sperm to fertilize the egg.

Sperm testing

Over the past two decades, a number of tests have been introduced for the analysis of sperm and in particular sperm nuclear DNA fragmentation (see review [8]). These tests include TdT-mediated-dUTP nick-end labeling (TUNEL) [9], COMET [10], chromomycin A3 (CMA_3) [11], *in situ* nick translation (ISNT) [12, 13], DNA breakage detection fluorescence *in situ*

Human Assisted Reproductive Technology: Future Trends in Laboratory and Clinical Practice, ed. David K. Gardner, Botros R. M. B. Rizk, and Tommaso Falcone. Published by Cambridge University Press. © Cambridge University Press 2011.

hybridization (DBD-FISH) [14], sperm chromatin dispersion test (SCD) [15], and the sperm chromatin structure assay (SCSA) [16, 17].

One of the open questions about sperm DNA fragmentation analysis is whether the breaks are single- or double-stranded and what is needed to detect them. The different tests therefore sometimes require an initial step of denaturation in order to detect DNA breaks, such as the SCSA, SCD, or COMET at acid or alkaline pH [18]. When DNA damage is observed under acid or alkaline conditions and not under neutral pH conditions they are examining acid/alkali labile DNA sites [19]. Other tests such as TUNEL [9], ISNT [12], and COMET at neutral pH do not require an initial denaturation step and, therefore, measure single-stranded (ISNT, TUNEL and COMET) or double-stranded (TUNEL and COMET) DNA breaks directly. In relation to fertility treatment single-stranded DNA damage is of better prognosis and easier to repair than double-stranded DNA damage. Single-stranded DNA fragmentation may be caused by unrepaired DNA nicks generated during the process of chromatin remodeling. It might also be caused by oxygen radical-induced damage. Double-stranded DNA damage is more likely to lead to failure of an individual sperm to pass on its paternal DNA [8].

Therefore, two type of tests currently exist: (i) tests that measure DNA damage directly, such as TUNEL, ISNT, or COMET at neutral pH; and (ii) tests that measure DNA damage after denaturation such as the SCSA, SCD, and COMET at acid or alkaline pH [18]. Controversy definitely exists as to which test is best. A recent study reported by Borini *et al.* [20] showed that sperm DNA fragmentation values in aliquots of the same spermatozoa used for IVF, measured by TUNEL, were significantly correlated with pregnancy outcome. This is in sharp contrast to the results reported by Bungum *et al.* [21], where no correlation was found between sperm DNA fragmentation values in the samples used for IVF, as measured by the SCSA test, and pregnancy outcome.

The test that has been more extensively studied to date from the clinical point of view is the SCSA test [22–25]. The SCSA test measures DNA susceptibility to DNA denaturation after exposure to a mild acid. DNA of sperm with a normal chromatin structure do not denature, while if the DNA is somewhat damaged and contains breaks in the DNA strands it can reach different degrees of denaturation. In order to determine the degree of sperm DNA denaturation, after mild acid treatment, the sperm cells are stained with acridine orange (AO). The degree of DNA damage is measured by flow cytometry and is expressed as a DNA fragmentation index or DFI. Previous studies indicate that a DFI value >27% is associated with pregnancy failure in ART [16, 26]. However, as indicated above, recent reports challenge the predictive value of the SCSA test [27, 28].

Some misconception has arisen about TUNEL as the test is frequently used for the determination of cell apoptosis. In spermatozoa TUNEL positivity is not synonymous with apoptosis, for a number of reasons including the presence of protamines and lack of nucleosome-like structures and, more importantly, since DNA damage induced by the hydroxyl radical or ionizing radiation also causes DNA fragmentation and can be detected as a positive test result by the TUNEL test [29]. The concept reported in many sperm DNA assessment papers stating that the TUNEL test is associated with apoptosis is an overestimation of this test's capability.

In general the clinical results point to a greater utility of sperm DNA tests in relation to natural conception and intrauterine insemination rather than assisted reproduction fertility treatments such as normal IVF and ICSI [23–25, 30]. It is important to note that the only final determinant is the quality of the sperm DNA in the fertilizing sperm. In techniques such as IVF or ICSI, where a lot of the natural selection and sperm competition is bypassed, it becomes more important to remove or isolate DNA-damaged sperm for treatment.

To date no sperm test can gauge the degree of sperm DNA damage in a single sperm. In animal studies a correlation has been shown between increasing amounts of DNA damage inducers, heat and radiation, and reproductive outcome [31, 32]. For example, the studies by Ahmadi and Ng [32] showed that fertilization rates of around 60% were achieved when sperm were subjected to between 0 and 100 Gy of radiation. Blastocyst development decreased from 49.8% in the control group to 2.3% with sperm exposed to doses of 100 Gy, respectively. Of the transferred blastocyst in the control group, 33.9% developed into live fetuses, while the rates were 20 and 0% when sperm were exposed to doses of 5 and 10 Gy. The authors concluded that embryonic and fetal development are very much related to the degree of DNA damage.

Selecting the best sperm

Sperm preparation

In 1988 a paper by Aitken and Clarkson [33] demonstrated that centrifugal pelleting of unselected sperm populations from human ejaculates caused the production of reactive oxygen species (superoxide and hydroxyl radicals) within the pellet which induced irreversible damage to the spermatozoa, and impairment of their fertilizing ability. Mortimer [34] therefore proposed that certain iatrogenic failures of IVF could be associated with sperm preparation techniques.

It is clear that sperm quality is improved by density gradient centrifugation techniques. Gandini and co-workers [35] measured DFI and HDS on both the raw and prepared semen aliquots. They found that enriched cell suspensions contained sperm with better motility, morphology, HDS, and DFI. It has also previously been shown that when sperm samples from different men were prepared using density gradient techniques for ART and then stained using the chromomycin A3 fluorochrome, which indirectly demonstrates a decreased presence of protamine, and *in situ* nick translation which examines for the presence of endogenous DNA nicks, a significant ($P < 0.001$) decrease in both CMA3 positivity and DNA strand breakage was observed [13, 36].

Preparation of sperm with media containing antioxidants has shown improvement in the overall functional parameters of the spermatozoa and reduction in the ROS level (37). This may improve the quality of gametes used by protecting the spermatozoa from high oxidative stress.

Sperm source

It could be argued, however, that whatever the preparation technique used or the protection offered to the spermatozoa it may still be too late. Post-testicular sperm DNA damage induced by ROS or after exposure to ionizing radiation is associated with nucleotide damage of the 8-OHdG type. The implications of combined nucleotide damage and DNA strand fragmentation are that, in addition to its diagnostic value (oxygen-radical-induced damage is amenable to antioxidant treatment), it may be of worse prognosis, since the great majority of the sperm cells will be affected by one type of damage or the other.

An approach to circumvent this type of damage occurring in the epididymis is using testicular sperm. Interestingly, a number of reports have now shown that sperm DNA damage is significantly lower in the seminiferous tubules compared to the cauda epididymis [38] or ejaculated sperm [39]. These reports indicate that the use of testicular sperm in couples with repeated pregnancy failure in ART and high sperm DNA fragmentation in semen results in a significant increase in pregnancy rates in these couples [39, 40]. Moreover, a recent report shows that pregnancy rates in the first cycles of TESA-ICSI in these couples are relatively high [8]. These results are consistent with the notion that in couples with long-standing infertility or repeated pregnancy failure in ART without an apparent cause, sperm DNA fragmentation could be the limiting factor responsible for their pregnancy failure. However, the use of testicular sperm may not always solve the problem since sperm DNA damage may also occur in the seminiferous tubule epithelium by apoptosis or be due to defects in chromatin remodeling during the process of spermiogenesis.

Selection of apoptotic spermatozoa

The presence of apoptotic-like sperm in the ejaculate was shown in the late 1990s [41] and has since been verified by a number of other studies [42–45]. The expression of apoptotic marker proteins on ejaculated spermatozoa, for example *Fas*, *phosphatidylserine*, *Bcl-X_L*, *p53* etc., indicates that their presence could be utilized to select non-apoptotic spermatozoa from semen samples [46]. A novel and promising technique of annexin V-conjugated microbeads (ANMB)–magnetic-activated cell sorting (MACS) has been shown to remove spermatozoa with phosphatidylserine externalization (marker of apoptosis) and produce a higher-quality non-apoptotic sperm fraction [47]. Furthermore these "negative-apoptotic marker" cells display higher fertilization rates when used for animal model IVF and ICSI. MACS is considered as a flexible, fast, and simple cell sorting system for separation of large numbers of cells according to specific cell surface markers [48].

Aitken's group recently reported a novel electrophoretic sperm isolation device utilizing a sperm separation strategy based on sperm size and electronegative charge. The suspensions generated by the electrophoretic separation technique contained motile, viable, morphologically normal spermatozoa and exhibited low levels of DNA damage. This method of sperm isolation is potentially simple and may allow

separation from testicular or epididymal biopsies in addition to semen. Reportedly, the electrophoretic sperm isolation procedure is both time- and cost-effective [49] and recently the first pregnancy using this method for a couple suffering from extensive DNA damage in spermatozoa was reported [50].

Another technology that could aid in the preparation of sperm populations is microfluidics [51]. It has been reported that the use of a microfluidic device, designed with two parallel laminar flow channels which preferentially separate motile spermatozoa into a separate outlet, increased sperm motility in a sample from 44% to 98% and morphology from 10% to 22% [52]. As semen samples are prepared without centrifugation it may limit some of the iatrogenic preparation problems described earlier. This technology may also prove useful in isolating motile spermatozoa from oligozoospermic samples, even with high amounts of non-motile gamete and/or non-gamete cell contamination.

Selection of single spermatozoon for ICSI

Intracytoplasmic morphologically selected sperm injection (IMSI)

In 2001 Bartoov and colleagues [53] reported the selection of spermatozoa with normal nuclei to improve the pregnancy rate with ICSI. They went on to verify this technique by performing ICSI using morphologically normal sperm, strictly defined by high-power light microscopy ($\times > 6000$), in couples with repeated ICSI failures. Sixty-two couples, with at least two previous consequent pregnancy failures after ICSI, underwent a single ICSI trial preceded by morphological selection of spermatozoa with normal nuclei. Fifty of these couples were matched with couples who underwent a routine ICSI procedure at the same IVF center and exhibited the same number of previous ICSI failures. The matching study revealed that the pregnancy rate after modified ICSI was significantly higher than that of the routine ICSI procedure (66% vs. 30%). They concluded that microinjection into retrieved oocytes of selected spermatozoa with strictly defined morphologically normal nuclei improves significantly the incidence of pregnancy in couples with previous ICSI failures.

More recently, Antinori *et al.* [54] conducted a prospective randomized study to assess the advantages of IMSI over the conventional ICSI procedure in the treatment of patients with severe oligoasthenoteratozoospermia. As reported above IMSI was based on a preliminary motile sperm organellar morphology examination under $\times 6600$ high magnification. A total of 446 couples with at least two previous diagnoses of severe oligoasthenoteratozoospermia, 3 years of primary infertility, the woman aged 35 years or younger, and an undetected female factor were randomized to IVF micro-insemination treatments: ICSI ($n = 219$; group 1) and IMSI ($n = 227$; group 2). A comparison between the two different techniques was made in terms of pregnancy, miscarriage, and implantation rates. The data showed that IMSI resulted in a higher clinical pregnancy rate (39.2% vs. 26.5%; $P = 0.004$) than ICSI when applied to severe male infertility cases. Despite their initial poor reproductive prognosis, patients with two or more previous failed attempts benefited the most from IMSI in terms of pregnancy (29.8% vs. 12.9%; $P = 0.017$) and miscarriage rates (17.4% vs. 37.5%).

In a recent paper Hazout *et al.* [55] also reported a positive association between high-magnification selection of sperm cells with normal nuclear shape and pregnancy outcome in patients with repeated conventional ICSI failures; in a subgroup of patients ($n = 72$) involved in the study, the amount of sperm DNA integrity (by TUNEL assay) was assessed, and the outcomes of IMSI could be compared in patients with several degrees of sperm DNA damage. A noticeable improvement in clinical outcomes (implantation and birth rates) was observed both in patients with an elevated (> 40%) and moderate (30–40%) degree of sperm DNA fragmentation and in those with normal sperm DNA status (< 30%). The use of IMSI appears promising (see review by Nadalini *et al.* [56]). Some drawbacks are, however, present, in particular the belief that it is a complicated technique that cannot be routinely performed [55].

Another microscope-based technology that is showing promise is one that analyzes birefringence in the sperm head. Gianaroli and colleagues [57] recently performed a prospective randomization including 71 couples with severe male factor infertility and performed ICSI using polarized light for sperm selection which permitted analysis of the pattern of birefringence in the sperm head. Twenty-three patients had their oocytes injected with acrosome-reacted spermatozoa, 26 patient's oocytes were injected with non-acrosome-reacted spermatozoa,

and in 22 patients both reacted and non-reacted spermatozoa. They found no effect on the fertilizing capacity and embryo development of either type of sperm, whereas the implantation rate was higher in oocytes injected with reacted spermatozoa (39.0%) versus those injected with non-reacted spermatozoa (8.6%). The implantation rate was 24.4% in the group injected with both reacted and non-reacted spermatozoa. They concluded that spermatozoa that have undergone the acrosome reaction seem to be more prone to supporting the development of viable ICSI embryos.

Sperm binding to hyaluronic acid (HA)

During human spermiogenesis, the elongated spermatids undergo a plasma membrane remodeling step which facilitates formation of the zona pellucida and HA-binding sites. Various biochemical sperm markers indicate that human sperm bound to HA exhibit attributes similar to that of zona pellucida-bound sperm, including minimal DNA fragmentation, normal shape, and low frequency of chromosomal aneuploidies [58]. In 2005 Jakab *et al.* [59] reported that selection of HA-bound spermatozoa significantly decreased the percentage of sperm showing both apoptotic marker proteins and aneuploidies. It was suggested that clinical use of HA-mediated sperm selection could ultimately solve the pertinent problem of aneuploidies and DNA fragmentation when ICSI is performed with immature sperm samples.

Subsequently a number of studies have now indicated that HA-bound sperm used in the ICSI procedure may lead to increased implantation rates. In one such study, Parmegiani *et al.* [60] showed in 293 couples treated with HA-ICSI versus 86 couples treated with conventional ICSI (historical control group) that all outcome measures of fertilization, embryo quality, implantation, and pregnancy were the same or improved in the HA-bound sperm group. The implantation rate was increased from 10.3% in conventional ICSI to 17.1% in the HA group. The authors concluded that if wider multi-center randomized studies confirm the beneficial effects on ICSI outcome, HA could be considered as a routine choice for "physiological" sperm selection prior to ICSI. A smaller clinical trial assessing the same technology by Worillow *et al.* [61] has also shown that clinical pregnancy rates are improved when using HA-selected sperm compared to conventional ICSI. Furthermore, HA binding can be used as a diagnostic semen analysis platform prior to treatment. Worillow *et al.* [61] showed that if patients with less than 65% binding efficiency are prescreened and selected prior to ICSI their success rates are improved by using this technology.

Future technologies

A number of novel new technologies are being developed which may also serve the purpose of allowing better sperm selection prior to classic IVF or ICSI. These include the use of Raman spectroscopy to non-invasively distinguish the DNA packaging and protamine content between normal and abnormal cells [62]. In this study they showed that Raman spectra of individual sperm cells contain vibrational marker modes that can be used to assess the efficiency of DNA-packaging for each cell. Raman spectra obtained from sperm cells with normal shape provide evidence that DNA in these sperm is very efficiently packaged. They found that the relative protein content per cell and DNA packaging efficiencies are distributed over a relatively wide range for sperm cells with both normal and abnormal shape. Their findings indicate that single cell Raman spectroscopy could be a valuable tool in assessing the quality of sperm cells.

Other molecular techniques may allow the analysis of sperm populations in the future. For example, DNA methylation patterns of key developmental genes have been shown to differ in spermatozoa and this may impact embryo development [63]. In addition the fundamental information being generated from proteomic [64] and RNA [65] analysis of sperm will also create a basis for identifying key points in spermatozoa that might be implicated in the defective sperm function observed in a significant proportion of infertile males and aid in both diagnosis and treatment.

Conclusion

A battery of procedures are now being developed (Figure 20.1) to provide us a better methodology for both understanding and sorting out spermatozoa, which will limit the negative paternal influence on reproductive outcome. The possibility of implementing these tests routinely may be a question of filtering patients based on their combined diagnostic history related to levels of DNA damage, membrane properties, and previous treatment history (Figure 20.2). In

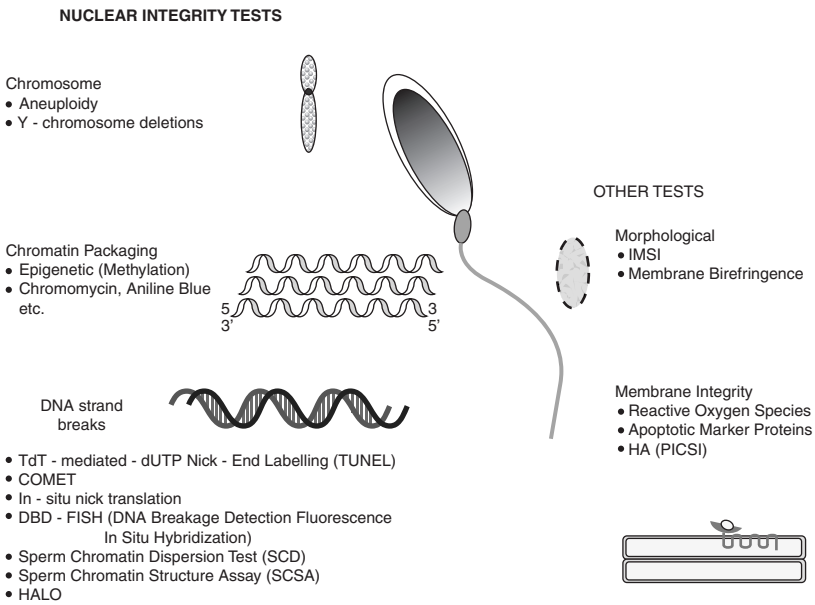


Figure 20.1. Sperm integrity tests and their mode of action. Tests available to measure the integrity of the sperm nucleus at the chromosomal, chromatin, and DNA level. Other tests also include those which examine the proteins present and integrity of the spermatozoal membrane. References to each test are contained within the chapter.

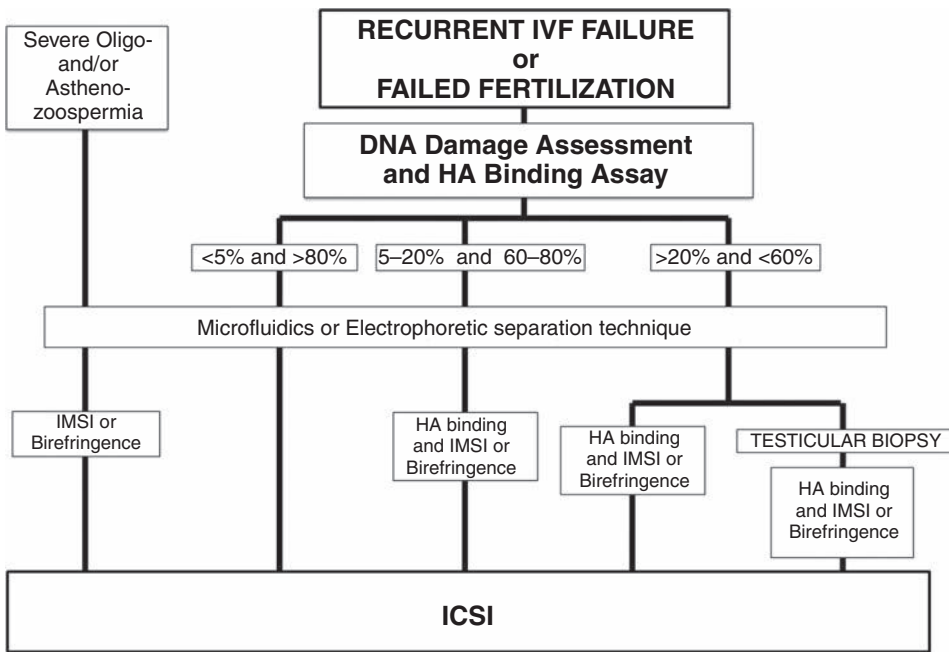


Figure 20.2. Treatment options for couples in which the male has oligo- and/or asthenozoospermia or have failed more than two previous treatment cycles. Males with high levels of DNA damage and poor binding efficiency using hyaluronic acid (HA) would be treated by techniques which would first clean the sperm population by limiting iatrogenic effects and then by techniques which would maximize selection of the best individual spermatozoon for ICSI.

this scenario a couple in which the male partner displays high levels of DNA-damaged sperm and poor membrane integrity who has undergone a number of failed IVF cycles may benefit from both testicular sperm retrieval and sperm selection prior to ICSI. Improving our knowledge of the molecular basis of male infertility will allow us to treat couples with greater confidence and efficiency.

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Chapter 21

Embryo culture in the twenty-first century

Mark T. Johnson and David K. Gardner

Introduction

"Change is the law of life. And those who look only to the past or present are certain to miss the future."
John F. Kennedy

It is remarkable that the prevailing method of culturing preimplantation human embryos remains largely unchanged from techniques that were developed ~50 years ago [1, 2]. In almost all IVF laboratories throughout the world, embryos are cultured singly or in groups in small drops of culture media that are placed on polystyrene dishes and covered with paraffin oil. These plates are then stored in incubators to maintain a constant temperature and gaseous environment throughout the culture period. Entering the second decade of the twenty-first century and the fifth decade of culturing human embryos in this fashion, we are now approaching a revolution in culture techniques, and it is reasonable to propose that the methods and devices that will be used for embryo culture at the close of this decade will little resemble those used today.

Over the past decade, there have been numerous improvements in the laboratory conditions used to culture embryos. Some of the notable advances include more physiological media (containing amino acids and accommodating nutrient gradients), reduced oxygen concentration from 20% to ~5%, the implementation of more rigorous testing of consumables by manufacturers and more suitable lab design and air handling (see Figure 21.1). Such cumulative improvements have led to an overall increase in pregnancy rates in recent times (Figure 21.2). In spite of this progress, however, it is generally accepted that pregnancy rates for embryos conceived in vitro are too low. Out of 80 313 embryo transfers in the USA in 2006, only 35.4% resulted in live-birth deliveries [3]. Until there is solid evidence that pregnancy rates are not adversely affected by the culture of embryos in vitro, there will continue to be a collective drive amongst embryologists to improve culture conditions.

The impetus to improve culture conditions, combined with advances in our understanding of the biology of the preimplantation embryo and the introduction of nanotechnologies, will drive many of the changes in culture technology. A more thorough appreciation of the cellular physiology of the embryo throughout the preimplantation period will enable embryologists to monitor embryonic physiology and modulate culture conditions to ensure that embryos maintain their homeostasis with minimal energetic expenditure. A detailed understanding of embryonic physiology will also be used to develop tests to assess the health of the embryo. In turn, the introduction of new tests for embryos will impact how embryos are cultured, since these evaluations will have requirements for optimal sample acquisition. The rapidly advancing field of nanotechnology will also provide many new tools for manipulating, culturing, and evaluating embryos.

These are thrilling times in embryology: it is clear that change is imminent, but anticipating when changes will emerge and which will prevail is quite challenging. To guide us as we consider the future of embryo culture methods, we shall first consider the parameters important for embryo culture and analysis, then assess how well the current standard culture system addresses these issues and finally look toward the future of embryo culture systems.

Human Assisted Reproductive Technology: Future Trends in Laboratory and Clinical Practice, ed. David K. Gardner, Botros R. M. B. Rizk, and Tommaso Falcone. Published by Cambridge University Press. © Cambridge University Press 2011.

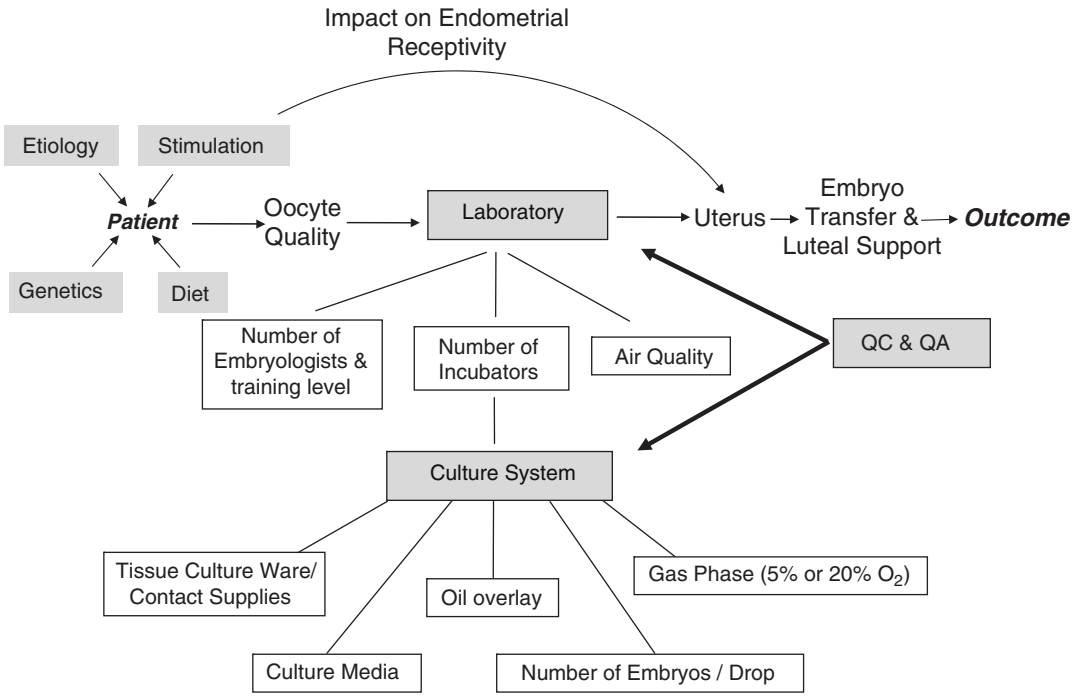


Figure 21.1. A holistic analysis of human IVF. This figure serves to illustrate the complex and interdependent nature of human IVF treatment. For example, the stimulation regimen not only impacts oocyte quality (hence embryo physiology and viability) [97], but can also affect subsequent endometrial receptivity [98–100]. It is also apparent that a patient's etiology and genetics will impact their cycle outcome. Furthermore, the health and dietary status of the patient can have a profound effect on the subsequent developmental capacity of the oocyte and embryo. The dietary status of patients attending IVF is typically not considered as a compounding variable, but growing data would indicate otherwise. In this schematic, the laboratory has been broken down into its core components, only one of which is the culture system. The culture system has in turn been broken down into its components, only one of which is the culture media. Therefore, it would appear rather simplistic to assume that the results of a given laboratory or clinic can be mimicked by changing only one part of the culture system (i.e. culture media). A major determinant of the success of a laboratory and culture system is the level of quality control and quality assurance in place. For example, one should never assume that anything coming into the laboratory that has not been pretested with a relevant bioassay (e.g. mouse embryo assay) is safe merely because a previous lot has performed satisfactorily. Only a small percentage of the contact supplies and tissue culture ware used in IVF comes suitably tested. Therefore it is essential to assume that everything entering the IVF laboratory without a suitable pretest is embryotoxic until proven otherwise. In reality many programs cannot allocate the resources required for this level of QC; and when embryo quality is compromised in the laboratory, it is the media that are held responsible, when in fact the tissue culture ware is more often the culprit. Adapted from Gardner DK, Lane M. [101]

What an embryo needs

In contemplating the future of embryo culture systems, a very valuable initial exercise is to consider the constraints that are likely to shape future developments. One of the fundamental guiding principles for culture systems is to provide conditions that promote both optimal development to the blastocyst stage and maximal pregnancy rates. The advantages of culturing to the blastocyst stage are well recognized [4]. Readers should be reminded, however, that assessing preimplantation development by morphological criteria is a poor predictor of developmental potential, and progression to a normal-appearing blastocyst should not be equated with optimal developmental potential. A tremendous number of studies over the last several decades have defined many conditions that influence developmental potential. We will only briefly outline these conditions in this chapter and will refer readers to other works for elaboration on these conditions [5–8].

Much of the attention regarding embryo culture conditions over the last few decades has focused on the culture medium [9]. This liquid serves the vital functions of providing hydration, ions, and nutrients and diluting waste products. At a bare minimum, embryos require a mix of electrolytes, energetic substrates, amino acids, vitamins, and oxygen for proper development in vitro. A macromolecule such as albumin is also

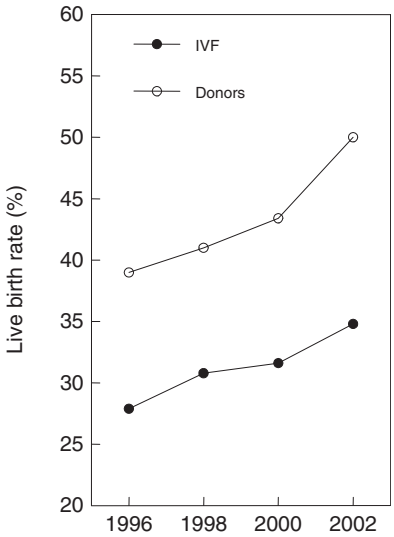


Figure 21.2. IVF success rates (1996–2002). These data are taken from Centers for Disease Control [3]. Overall, success rates for IVF have continued to improve. The higher rates for donors reveal the impact of maternal factors such as age on outcome.

Table 21.1. Conditions for enhancing the developmental potential of the preimplantation embryo

Embryo's needs – physiological ranges of: Electrolytes[a]: Na, K, Ca, Mg, Cl, PO_4 Energetic substrates[a]: glucose, lactate, pyruvate, amino acids, fatty acids[b] Macromolecules: serum albumin, hyaluronan, α and β globulins[b] Gases[a]: O_2, CO_2 Osmolarity pH Temperature Gravity Growth factors[a,b] Mechanical movement/compression[a,b]
Stressful/toxic conditions to be avoided: Accumulation of toxic levels of waste products – ammonia, lactate Exposure to embryo toxins – volatile organics, unincorporated polystyrene
Shear stress
Light

[a] Requirements likely to change during the course of preimplantation development.
[b] Factors that are likely to be required, but have fewer supporting data.

routinely added to the culture medium, principally for the dual purposes of reducing adherence of the embryo to handling equipment and binding toxins. More recently, another macromolecule, the glycosaminoglycan hyaluronan, has also been introduced due to its benefit at transfer and in improving cryopreservation outcome. A generalized list of common culture medium components is provided in Table 21.1. Given the complexity of culture media and the inherent adaptability of biological systems, it is not likely that there is only one specific formulation that is optimal, but rather a range of modified compositions that will work well [10].

A very important aspect of any culture system is that it is highly dynamic. Over the course of the culture period, the needs of cells and the composition of the culture medium change. This is particularly the case for preimplantation mammalian embryos. During the 5-day culture period in which the embryo progresses from an undifferentiated single cell to a blastocyst containing upwards of 100 cells, the embryo undergoes dramatic changes in its metabolism. Two of the most marked changes that occur during the preimplantation period in vitro are shifts in the needs for energetic substrates and amino acids. The developing preimplantation embryo reduces its uptake of pyruvate while increasing its uptake of glucose and changes its needs from a subset to all amino acids [11, 12]. Of note, the composition of pyruvate and glucose differ in fluid from different regions of the female reproductive tract so that embryos are supplied with higher concentrations of pyruvate during the early stages of preimplantation development and then are exposed to higher concentrations of glucose during the latter stages [13]. In response to these changing embryonic needs and to mimic the environment of the female reproductive tract, a number of different two-stage culture systems have been developed that use different medium compositions for pre- and post-compaction embryos [14, 15]. Another dynamic feature of culture medium is that many of the components have increased lability at 37°C and when exposed to light. Furthermore, it should not be forgotten that the embryo makes significant changes to the culture environment itself [8]. Changes include the depletion of nutrients, thus altering effective concentrations, and the release of metabolites from various metabolic reactions. The latter includes lactate production from glucose, which can alter the redox state of the embryo and acidify the medium if the volume is too small, and the formation of ammonium from amino acids (see below). In the case of the human embryo, ammonium

production is problematic as development proceeds, as the blastocyst has been shown to generate significant levels, on top of that created by spontaneous amino acid breakdown [16].

Within the immediate environment of the embryo there are a number of conditions in addition to culture medium components that are required for enhanced developmental potential of the embryo. Embryos require defined ranges of temperature, pH, osmolarity, and concentrations of O_2 and CO_2. The ranges for almost all of these parameters are close to those found in the female reproductive tract [15]. These environmental conditions are highly interrelated. For example, pH is dependent on temperature and is maintained in culture medium through a CO_2–HCO_3 buffering system. In most culture systems, roughly 25 mM HCO_3 and 5–6% CO_2 within the incubator are used to maintain a physiological pH. Exposure of incubator-equilibrated culture medium to ambient air, which contains only 0.038% CO_2, results in off-gassing of CO_2 and a rise in pH due to the commensurate loss of free hydrogen ions. Another environmental factor that is often not considered is gravity. Exposure of mouse zygotes to approximately 0.1% of the standard gravitational force resulted in significantly increased rates of developmental arrest at three time points within the preimplantation period [17]. Although the effects of microgravity may seem irrelevant for the IVF laboratory now, this issue could become important in the future should space exploration or habitation become more common. In general, it appears that the embryo is sensitive even to small or transient deviations in almost all of these environmental parameters. Exposure of mouse embryos to room temperature for even 5 minutes can alter expression of developmentally important genes such as *Glut3*, *IGF2*, and *IGF2R* and a reduction in CO_2 concentration by only 20% can impair preimplantation development [18]. It is thought that fluctuations out of the normal ranges within the female reproductive tract cause the embryo to expend additional energy to maintain its homeostasis [19]. This diversion of energy may have dramatic impact on the maternal to embryonic transition and early developmental processes. An increasing body of literature suggests that increased energy expenditure by preimplantation embryos is associated with lower development potential [20].

We fully expect that the list of requirements for the embryo outlined in Table 21.1 will grow over the coming years. Although there are still insufficient data to consider a number of conditions that putatively improve developmental potential, two additional conditions are worthy of consideration: growth factors and movement. Preimplantation embryos are endowed with receptors for TGF-α, HB-EGF, EGF, CSF, GM-CSF PAF, PDGF, IGF-1, IGF-2, LIF, and activin, and ligands for all of these receptors are present within the fluids of the female reproductive tract [21, 22]. A considerable number of studies using animal models have demonstrated that supplementation of culture medium with embryotrophic factors is beneficial, but studies to define the optimal mix of factors and evaluate their safety are required before the addition of growth factors to human embryo culture medium can be recommended. Even in the absence of supplemented growth factors, embryos will still be exposed to many trophic factors since the embryo itself secretes TGF-α, TGF-β, IGF-1, IGF-2, VEGF, PDGF, PAF, LIF, GH, HLA-G, and PGE2 [21, 22]. The presence of these autocrine trophic factors has been proposed to explain the observation that mammalian embryos often develop better in smaller volumes/embryo [23, 24]. In vivo, the preimplantation embryo is transported into and through the oviduct by a combination of ciliary movement and peristalsis. During the course of this transit the embryo is exposed to shear stress from the movement of surrounding fluid and to compression from the rhythmic contractions of the smooth muscle of the tract. Given the exposure to these mechanical forces, it would not be surprising if preimplantation embryos have adapted to thrive in this environment.

What an embryo doesn't need

Another consideration for current and future culture systems is to avoid or minimize the exposure of embryos to conditions that are toxic or stressful. Attention must be paid to more than avoiding situations in which any of the above conditions deviate from acceptable ranges. There are a number of stressors or toxins that can be introduced or accumulate within culture systems. One of the biggest challenges of embryo culture is to avoid the introduction of substances that are toxic to the embryo. Embryos appear to be more sensitive than most other cell types and cell lines to toxic agents. A good example of this sensitivity comes from the observation that roughly one-quarter of standard tissue culture grade polystyrene and polypropylene plasticware

impair embryonic development when tested with a combination of two different one-cell mouse embryo assays [25]. One of the biggest challenges to avoiding embryo toxins is that our understanding of embryonic toxicology is very poor; we know very little about what concentrations of chemicals are toxic and even less about their mechanisms of toxicity. In general, the current approach is to take every precaution to minimize exposure to embryotoxins through rigorous environmental control that includes specially constructed laboratories, with appropriate environmental regulation, coupled with rigorous quality control testing [25]. An additional precautionary step is to include agents, that can absorb or detoxify toxins in the culture media such as EDTA to chelate heavy metals, reducing agents such as glutathione to protect against oxidizing agents, and serum albumin to non-specifically absorb other toxins [5]. The issues of embryotoxicity will be an even greater challenge in developing new culture systems that will probably increase the number of materials that are in contact with the culture medium.

More insidious stresses to the embryo arise from changes in culture medium that occur during culture and embryo handling or assessment. A well recognized culprit is the presence of elevated concentrations of ammonium in the culture medium. Ammonium is released as a waste product from embryos as part of the catalysis or interconversion of amino acids, and can also arise from the spontaneous deamination of amino acids that are present in the culture medium [26]. Studies of mouse embryos have shown that elevated levels of ammonium adversely impact preimplantation development and subsequent prenatal development [27]. At the end of the preimplantation period, the embryo converts much of the consumed glucose to lactate, which can also lower the pH of the culture medium. Handling and examining embryos, two essential parts of current embryo culture protocols, also subject the embryo to a variety of stresses. Beyond all of the previously discussed environmental ramifications of removing embryos from the incubator, handling of embryos can expose the embryo to shear stress and light. Transferring embryos between culture dishes at present requires the embryo to be drawn into a pipette with a bore that is often not much larger than the embryonic diameter. Studies have shown that manipulation of embryos with pipettes with internal diameters that are 1.5× the diameter of the embryo can result in the induction of a number of intracellular markers of stress [28]. In almost all embryology laboratories, embryos are routinely assessed for developmental progression by microscopy using broad-spectrum white light. There is mounting evidence that exposure of embryos to visible light in the blue and red spectra can adversely impact development of the preimplantation embryo [29, 30].

Gamete and embryo analysis

From the advent of the culture of preimplantation mammalian embryos analysis has been an integral part of the process. For much of the first several decades of culture, analyses of embryos have been limited to morphological assessment. Identification of certain morphological characteristics such as the presence of multiple nuclei or extensive fragmentation has been shown to be a reliable indicator of a poor outcome for these embryos. Unfortunately, it has also been shown that many normal-appearing embryos have chromosomal abnormalities that preclude the possibility of producing a healthy liveborn child [31]. A wide variety of other approaches to analyze the health and function of gametes and embryos are being developed. Given the availability of gametes for analysis and their importance for the health and developmental potential of the embryo, it is almost certain that both gametes and embryos will be analyzed and selected in order to improve the outcomes of assisted reproductive technologies [32]. Methods for gamete and embryo diagnostics are often divided into two categories: invasive and non-invasive. Invasive approaches encompass all procedures that disrupt the integrity of the gamete or embryo by either removing a part of or inserting a foreign object into the cell(s). The most common invasive approach is the removal of one or more cells of the embryo for performing genetic analysis, commonly referred to as preimplantation genetic diagnostics (PGD) [33]. Recently, a large number of studies have shown that the most common means of assessing the chromosomal complement of human preimplantation embryos, which involves removing one or two cells of the 8-cell embryo and performing fluorescent *in situ* hybridization on the biopsied cell(s), has not been shown to improve pregnancy rates [34]. Current efforts to assess the chromosomal complement of embryos are focused on performing more comprehensive genetic analyses on polar bodies or embryonic cells, but limited data have been produced to date using these approaches. PGD for assessment of inheritance of a small number of specific alleles to avoid

transmission of genetic disease or for HLA matching, in contrast, is very effective with diagnostic accuracy of 99% [35]. There are many technical and logistical challenges associated with PGD. First, specialized equipment and extensive technical expertise are required to perform the biopsy procedures. Second, there are only a few reference laboratories in the world that perform the genetic analyses on these samples as a service. Given that these diagnoses are based on the analysis of one or two copies of a given genomic sequence, rigorous precautions must be taken to avoid the introduction of contaminating DNA. Although the field of PGD, particularly for single gene disorders, continues to grow, the utilization of PGD is still quite low with fewer than 4000 babies being born in Europe over the two decades since its introduction [35]. It is anticipated that the demand for PGD will grow tremendously as whole-genome screening methods begin to be applied to preconceptional screening.

Non-invasive approaches do not impact the integrity of the gamete or embryo. Most non-invasive approaches to date have focused on either imaging of the embryo or evaluation of culture media following exposure to the embryo. Imaging approaches have principally focused on morphology of the embryo at specified developmental time-points [36]. Evaluations of culture media in the presence of embryos have been used to assess oxygen consumption [37, 38]. A wide range of analyses have been performed on spent culture media aimed at assessing consumption/production of small molecules or protein secretion [39, 40]. The more common approaches have been to assess utilization/production of energetic substrates using microfluorometric analyses, amino acids using HPLC, proteins using mass spectrometry, or global changes in media composition using various spectroscopic methods. The fact that there are a number of studies that support the use of each of these approaches in assessing embryonic viability and developmental potential indicates that embryos develop distinctly different metabolic activities in the preimplantation period and these metabolic differences portend the developmental success of the embryo [39, 40] (see Chapter 27 by Gardner). With advances in culture media analysis, there will be a move toward culturing all embryos individually. Much like invasive diagnostics, the interfaces between embryo culture system and the various platforms are cumbersome at best, and much of the analytical equipment required is far beyond the financial reach and technical expertise of the standard embryology laboratory. As technologies become more effective and less costly, it is certain that analytical systems will be integral parts of culture devices in the future.

Practical issues to consider

Although biomedical research may drive technological advances, practical issues such as cost, reliability and ease of use will play large roles in determining whether these advances become part of clinical practice. Assisted reproduction is costly throughout the world. In 2002, average costs for an IVF cycle were $9547 in the USA and $3518 in 25 other countries [41]. Laboratory costs account for between 12 and 20% of the total costs [42]. These high costs make assisted reproduction unavailable for a large segment of the infertile population, which comprises roughly 9% of the global population [43]. Thus, an important goal for future culture systems is that they help reduce costs of assisted reproduction. Per cycle costs for assisted reproduction could be lowered by reducing laboratory costs and/or improving live birth rates. The simplicity and robustness of the current culture system set high standards for future systems. It will also be imperative that future culture systems are simple to set up, highly reliable and easy to run.

How well does the prevailing current culture system meet these requirements?

Embryos are currently cultured in small drops of culture medium placed on culture dishes that are then overlaid with oil, a system that will be referred to as the droplet-oil-dish culture system. These culture plates are then stored in incubators that maintain a constant temperature and gaseous environment. The fact that this method of culture has remained unchanged for so long attests to its many advantages. The fact that many small drops can be placed on standard tissue culture plates makes it easy to culture many embryos in a single dish, and embryos can be easily evaluated and accessed. The relatively large volumes of culture medium used provide both a vast supply of nutrients at relatively steady concentrations and a large reservoir for diluting out waste products. The overlay of oil protects against evaporative losses and also serves as a thermal and gaseous sink, temporarily protecting the embryos from fluctuations in these important

parameters when the incubator door is opened or the dish is removed from the incubator.

Droplet-oil-dish culture has some deficiencies as well. First of all, preparation of culture plates consumes labor since the plates need to be prepared by embryology laboratory staff shortly before use. Exchange of culture medium also is performed manually. Not only does this procedure require skilled personnel time, but it also can potentially expose the embryo to suboptimal culture conditions due to handling of the embryo, loss of autocrine factors, and potential shock to the embryo as it is shifted between media with different compositions. The standard volumes used in droplet-oil-dish systems also have some disadvantages. A 10 microliter drop has almost 1800 times the volume of a 110 μm-diameter embryo, which substantially dilutes autocrine factors and proteins secreted by the embryo. Furthermore, this large volume provides such a large pool of nutrients that it may not be possible to assess consumption or production of certain substrates due to the very small changes in concentration. It is possible to reduce volumes in this system to some extent, but limitations of volume accuracy of air displacement pipettes and the need to exchange media more frequently pose substantial practical barriers. Another drawback of current culture systems is that all manipulations, such as washes, media exchanges and analyses, are performed outside the incubator, which requires opening the incubator door and removing the dish of interest. Opening the incubator door of an incubator, with a thermocouple CO_2 sensor inside, only 11 times per day was shown to impair development of mouse preimplantation embryos [44]. Incubators with integrated imaging systems have recently been developed to allow for image capture without breaching the incubator chamber [45], but such systems are costly and do not help for situations in which media exchange or manipulations are required.

Embryo culture systems of the future

Up until very recently, a major impediment to the development of novel embryo culture systems has been the lack of technological tools that are capable of handling the small number of cells and volumes required for single embryo culture. With the recent emergence of the field of microfluidics, the discipline that deals with the handling of volumes in the nanoliter range or less, the small sizes are no longer an issue. The incredible variety of materials, channels, chambers, valves, and pumps developed for microfluidic systems provides an overwhelming number of options for device design, and this is still in the very early stages of this field [46, 47]. Similarly exciting advances in the development of microanalytic devices that can detect millimolar and lower concentrations of molecules in submicroliter samples, and can be integrated in line into microfluidic culture devices, will also spur on the introduction of microfluidics into embryology. The era of complete microdevices for culture and analysis of single or small numbers of cells is dawning, and there is hardly a more ideal biological system to apply these technologies to than free-living mammalian gametes, and preimplantation embryos. To guide us in looking toward the future, we will consider some of the major issues that will need to be addressed as these new systems are being developed.

Smaller culture volumes

One major constraint for future culture systems will be the optimal volume per embryo. At present, very little work has been done to address this issue, and the answer will be dependent upon a number of factors associated with the specific culture system such as: (1) whether future culture media can supplant the need for autocrine factors, (2) rates of consumption of nutrients and production of waste products, (3) optimal rate of turnover of culture medium and (4) what types of analyses are performed on culture medium. It is anticipated that with any culture system, there will be a range of volumes that will be optimal; volumes above a certain point will dilute out autocrine factors (if not supplied exogenously) and volumes below a certain point will deplete nutrients or concentrate waste products. Smaller volumes will also improve many analyses of culture medium by increasing the magnitude of changes effected by the embryo. Considering these factors, it is likely that, at least for the near future, the volumes used in culture systems will be less than those that are currently used, although medium renewal may need to be built into the device design in order to accommodate these lower volumes. Of note, pairs of 2-cell mouse embryos cultured in 100 nl of culture media within a microfluidic device developed to the blastocyst stage at similar rates to embryos cultured in standard droplet-oil-dish culture [48]. One drawback of lowering the volume of the

culture medium is that the surface area-to-volume ratio will increase, which raises the risks of evaporative losses and introduction of toxins.

Biphasic vs. monophasic

Another question for the future is whether embryos will continue to be cultured in a biphasic system such as droplet-oil-dish culture. Biphasic microfluidic systems have been devised in which cells are maintained in small bubbles of culture medium that can be shuttled around channels, split into smaller bubbles, or merged into larger bubbles [49, 50]. One of the great advantages of such a system is that an embryo and a defined volume of culture medium can be moved to specific points within a device without loss or cross-contamination of culture medium from surface wetting. Drawbacks to a biphasic system that we have found are that (1) the larger bubbles required for embryos are prone to breaking up or sticking to channel walls, (2) movement of fluid requires higher pressures, (3) the negative buoyancy of embryos can cause the embryos to contact the oil and (4) oil can foul bioanalytical probes and sensors. Such technical issues are not uncommon when moving to submicroliter volumes and will require further work to resolve.

Device materials

Materials used in culture microdevices should share many of the properties of paraffin oil including permeability to $CO_2/O_2/N_2$ (but not water vapor), optical transparency, minimal absorption or adsorption of media components and lack of embryo toxicity. Other desirable properties of materials for microfluidic devices are that they are inexpensive and are amenable to low-cost manufacturing processes. The material that predominates in microfluidic devices at present is poly(dimethylsiloxane) (PDMS). This optically transparent elastomer is highly permeable to $CO_2/O_2/N_2$ and water vapor. Permeability to water vapor of PDMS can lead to dramatic evaporative losses if the culture medium is in contact with a thin (0.2 mm or less) layer of PDMS [51]. To prevent water loss, thicker PDMS is usually equilibrated with water or culture medium and thinner layers of PDMS can be layered with impermeable polymers such as parylene [51]. It is also becoming well appreciated that PDMS is not inert. PDMS has been shown to absorb small hydrophobic molecules and release unpolymerized monomers into contacting aqueous liquids [52, 53]. There are likely to be effective extraction methods or modifications to the surface or bulk PDMS that can avoid these properties, but standardized, embryo-friendly protocols have yet to be developed.

Four microfluidic devices fabricated from PDMS have achieved comparable or better preimplantation and subsequent development of mouse embryos [48, 54–56]. The most extensively studied device is the microfluidic embryo culture chip, which consists of a funnel for loading connected to a 250 μm (h) × 1000 μm (w) channel that has a constriction at the midpoint that is too small for embryos to pass [57]. Embryos are loaded into the channel and moved until they are stopped at the constriction. When embryos are stationary at the constriction, it is estimated that the effective volume that the embryo is exposed to is around 250 nl [58]. Studies have demonstrated that mouse embryos in this system undergo faster rates of cleavage and produce more blastocysts relative to standard droplet-oil-dish culture [56]. Embryo culture in this device and others suggest that this polymer may be embryo compatible, but additional studies assessing embryonic physiology and long-term effects are warranted before this material is deemed to be safe. There are a number of other polymers with similar physical properties that are likely to be evaluated for embryo culture devices in the future.

One great advantage of PDMS is that this polymer is amenable to rapid prototyping using soft lithography [59]. For this process, masks are designed using computer-aided design software and then printed using high-resolution printers. Molds are then fabricated by photolithography, much the way materials are applied to silicon wafers for the production of semiconductors. Photolithography requires technical expertise and very costly equipment [46]. PDMS casting and assembly, in contrast, are relatively straightforward and can be done in virtually any laboratory setting. In the future, barriers to microdevice production will be lessened by the development of affordable chip design and production services and the availability of pre-made PDMS building blocks that can be easily assembled on site to make chips with desired functions [60].

Dynamic culture

If embryo culture systems of the future are designed with the goal of emulating conditions in vivo, then

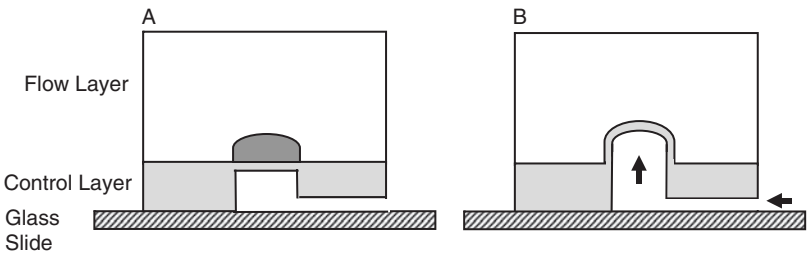


Figure 21.3. Monolithic microfluidic valve. One of the most common designs for valving channels is to have an underlying valve line that is separated from the flow channel by a thin membrane of PDMS. (A) Unpressurized valve line. (B) When pressure is applied to the valve line, the membrane balloons out and occludes the flow channel.

these systems will certainly be dynamic, continuously moving the embryo and exchanging culture media. Prior to development of microfluidic technology, perfusion systems were limited by the inability to deliver physiologically low rates of flow in small volumes [61]. Microfluidics, by definition, deals with the handling of fluids in the nanoliter-picoliter range. Amongst the numerous approaches to move fluids, the most common methods used in microfluidics are either to apply positive or negative pressure to one end of a channel, or to use a pliable material in the device that allows channels to be compressed in a sequential manner that produces peristaltic flow. Channels can be compressed by making one wall of the channel very thin and then applying pneumatic, hydraulic, or mechanical pressure to the thin membrane (Figure 21.3) [62, 63]. It is likely that devices of the future will have integrated valves as this not only allows for pumping, but also can mix and deliver very precise volumes [47]. Of the currently available options for integrated valves, those that are actuated by pneumatic or hydraulic pressure have the advantages that (1) the valves are optically clear, (2) the valves take up very little space, (3) the valves can be placed at almost any location on the chip as they are not constrained by location of mechanical mechanisms and (4) single pressure lines can be used to operate multiple valves. On the other hand, mechanical valves can deliver higher pressures, which have some advantages in certain designs.

The precision and automation of fluid handling in microfluidic systems open up many possibilities for dynamic culture. Media may be completely or partially exchanged or recirculated through a dialysis system to adjust the composition of culture medium while preserving autocrine factors. Much work over the coming years will address many important questions regarding dynamic embryo culture such as whether flow should be continuous or intermittent, what flow rates are optimal, how should media be exchanged, and whether flow-induced movement of embryos is beneficial. A recent study using a device in which mouse embryos were cultured in a funnel with small inlet and outlet channels at the base showed that delivery of a pulsatile flow resulted in preimplantation development and pregnancy rates that were comparable to those from embryos that developed in vivo, and were better than rates of embryos cultured in wells without flow or in droplet-oil-dish culture [54]. This study suggests that a dynamic culture system may enhance developmental potential in these conditions, but it did not address whether the beneficial effect was due to the media exchange of almost 10 volume exchanges over the course of the culture period and/or to the observed rocking movement. In contrast, embryos cultured in the previously described channel device with flowing media resulted in poorer blastocyst rates than embryos cultured in static channels or standard drop-oil-dish culture [64]. It is also possible to expose embryos to gentle fluctuations in pressure that are probably experienced by embryos as they are moved through the oviduct by peristalsis. Exposing bovine zygotes to intermittent gentle pressure by drawing them through a constricted channel resulted in a significantly better development to the 8-cell stage as compared to a straight channel (57 vs. 24%) [65]. Another dynamic aspect of embryo culture relates to the composition of culture media. As discussed previously, the embryo is exposed to different compositions of fluid as it migrates through the reproductive tract. By coupling a mixing system with flow, it should be possible to mimic the kinetics of changes in small molecules that occur in vivo. In the future, when more is understood about embryonic physiology and methods have been developed to follow important physiological parameters in real time, it is likely that flow and medium composition will not be preset, but rather will be dictated by the embryo's physiological responses, much like how renal dialysis or artificial heart-lung machines operate.

Co-culture

Another possibility for future culture systems is to line the culture chamber with endothelial or endometrial cells, making a cellular lining similar to that found in vivo. Much progress has been made in growing adherent cells on specific surfaces of microfluidic devices [66]. Recently, a device was developed that allows mouse embryos to be co-cultured in wells containing polarized endometrial cells that are grown on a microporous membrane overlying a flow channel that perfuses the basolateral side of the cells [55]. Co-culture systems would more closely approximate conditions in vivo since the endothelial cells are likely to interact with the embryo and produce trophic factors, but complexities of producing these devices and concerns that co-culture may expose embryos to infectious agents and may complicate or prevent analyses of culture media are formidable challenges to the implementation of such systems. It is also important to keep in mind that the embryo for almost all of the preimplantation period is surrounded by the zona pellucida (ZP), a porous glycoprotein structure that sterically prevents direct contact with objects of greater than 20–30 nm in diameter and is selectively permeable to smaller molecules [67]. Therefore, the embryo's only interaction with the surrounding environment is via molecules that transverse the ZP. It is likely that these molecules will ultimately be identified so that they may be provided exogenously without the need for cells during the preimplantation period. Endometrial cells would, however, be essential for the development of in vitro systems that could support postimplantational embryonic development.

Adaptability

A key feature of any embryo culture system is that it is sufficiently flexible to accommodate differing protocols used in different laboratories as well as differing protocols that are applied to individual clinical cases. Microfluidic devices are well suited for such demands even today. The options for design are practically limitless and it is no exaggeration to state that devices already exist or could readily be developed to execute any task that is performed in the IVF lab. For example, microfluidic devices have been developed to sort human sperm, remove cumulus cells from bovine oocytes, remove the zona pellucida from bovine embryos, perform in vitro fertilization in mice and pigs, inject zebrafish zygotes, culture mouse embryos, move mouse embryos to specific locations, and orient an embryo at a fixed location [68–77]. Integrated valves such as the monolithic, pressure-activated valves previously described are ideal for making a flexible platform. The abilities to vary the number and location of valves and the possibility of using the same pressure line to actuate numerous valves are great advantages. The power of such a system was demonstrated by Thorsen *et al.* through the development of a device with 1000 individually addressable chambers using only 22 pressure lines [78]. Inputs may be even further simplified by the incorporation of pneumatic logic circuits on-chip to control valving [79].

Microfluidic devices should be capable of culturing from one to thousands of embryos simultaneously. In the future, the laboratory should never be the limiting factor for IVF clinics. The ability to culture large numbers of embryos will become essential if methods are developed to obtain larger numbers of MII oocytes from women or if it becomes possible to support oogenesis in vitro, possibly from germline or embryonic stem cells [80]. The ability to generate large numbers of embryos from couples will be particularly important for situations in which embryos with specific genotypes at multiple loci are needed, such as when there is a desire to reduce the risk of having a child with a complex genetic disease. The ability to culture large numbers of embryos in isolated chambers will also be a boon for embryo research and will most certainly be used for projects such as optimizing culture media and screening for embryotoxins.

Microanalysis

One of the most exciting prospects for microfluidic culture platforms is that many mature analytical technologies are available that can be readily incorporated into chips. Microdevices have been developed to analyze nucleic acids, proteins and peptides, amino acids, and small metabolites using the full complement of biochemical analytical processes that are performed routinely on the macro scale [81–83]. Microdevices are or will soon be available for performing any type of assessment that is useful for evaluating gametes or embryos. On-chip imaging is a developing technology, but the recent construction of a lensless aperture array system for producing images with resolution below 1 μm shows promise [84]. A variety of technologies are applicable for integrated non-invasive analysis of the metabolism of embryos [85]. To give readers an

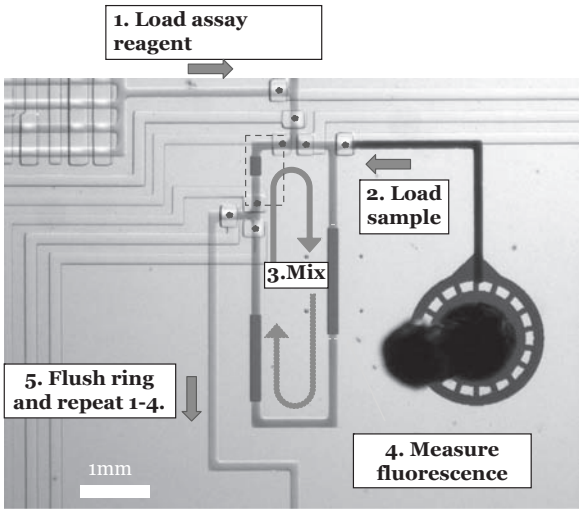


Figure 21.4. Microanalytical device for determining the concentrations of metabolites in culture media. This photomicrograph shows a section of a microfluidic device that we developed in collaboration with the laboratory of Dr. Todd Thorsen [86]. Above the image is a multiplexor with 10 input channels for automated loading of assay cocktail and standards. To the right is a sample loading port where approximately 100 nl of culture medium is loaded manually. By automatically activating pneumatic valves (marked with red dots) in defined sequences, 9/10 of the mixing circuit with assay cocktail and 1/10 of the ring with sample (section of the mixing ring outlined with the red dashed line) is filled. Sequential firing of the valving within the mixing circuit then mixed the samples and then NAD(P)H fluorescence of the fluid within the measurement chamber (highlighted by dashed yellow lines) was determined using an automated fluorescence microscope equipped with a photomultiplier tube. Nine measurements (glucose, lactate, and pyruvate) were determined automatically using this sequence. The sample port and mixing circuit were then flushed clean, another culture medium sample was loaded and the sequence was repeated. To see this procedure in real time, we refer readers to the supplementary video file included with Urbanski *et al* [86]. See colour plate section.

appreciation of the capabilities of microanalytical devices, we have included an image of a micro metabolite analyzer that we developed in collaboration with Dr. Todd Thorsen at the Massachusetts Institute of Technology (Figure 21.4) [86]. This automated device can determine the concentration of glucose, lactate, or pyruvate in only 4 nl of culture medium in 20 seconds per assay. Particularly attractive devices for in-line monitoring of metabolites are biosensors that convert binding or a chemical reaction of a substrate on the surface of a sensor to an electrical signal [87]. These sensors can be fabricated very inexpensively using thick film printing and can be developed to detect a wide variety of analytes. In collaboration with Dr. C. C. Liu at Case Western Reserve University, we have recently tested biosensors that can determine the concentrations of glucose, lactate, and pyruvate using 5 µl of culture medium in 5 minutes, and efforts are under way to reduce the volume and time requirements (data not shown) [88]. With the tremendous amount of ongoing research in microanalytics, we expect that even better technologies will emerge in the near future. Real-time monitoring of embryonic physiology is soon to come and will revolutionize how embryos are cultured. Culture conditions will no longer be static or invariably dynamic, but instead will be constantly adjusted in response to the embryo's changing needs. Real-time read out of embryonic physiology will be tremendously valuable both for optimizing and monitoring culture conditions and for assessing the health and developmental potential of the embryo.

Automation

Another appealing attribute of microfluidic devices is that their operation can be automated. Automation has the advantages of removing human error and reducing personnel costs. A less obvious advantage is that an automated system would permit assessments and manipulations of gametes and embryos to be based on optimal developmental stage of the embryo rather than the work schedule of personnel. It is well appreciated that embryos within a cohort often show differences in developmental rates, even when they have the same parents and are fertilized at the same time. It is likely that some of the biological variability in analyses could be removed if embryos were analyzed according to precise developmental staging rather than based on the time post-fertilization. It is also likely that embryos will tolerate certain procedures better if they were performed at certain developmental stages. One additional word about automation: the replacement of personnel will probably be a slow process, and it is unlikely that the IVF laboratory will ever be run without human supervision. Embryologists will still play important roles in interpreting qualitative data and communicating embryo culture and testing results to clinicians and patients. The good news for the future is that embryology staff will be relieved of many of the mundane duties that are part of current job responsibilities.

Self-contained

In light of the benefits to the embryo of maintaining a very stable, physiologically appropriate environment, it is likely that future microdevices will be entirely self-contained, including systems to monitor and maintain

an appropriate thermal and gaseous environment. Many different on-chip heating devices and systems for regulating temperature have been developed [89]. For the near future, it is likely that the gaseous environment will be maintained either by filling the system with the desired gas such as 6% CO_2/5% O_2/89% N_2 and sealing it or connecting it to a premixed gas cylinder to perfuse a low amount of gas continuously through the system. The low rates of oxygen consumption (around 0.3 nl/h) and CO_2 production would require quite small volumes to maintain a stable gaseous environment throughout the culture period [90]. Further into the future, it is likely that devices will have integrated gas generators combined with on-chip regulators. Microdevices have already been developed that can produce O_2, CO_2 and N_2 [91–93]. Additional benefits of self-contained systems will be that they will be portable and will dramatically reduce the potential for the introduction of contaminating molecules, which is particularly important for many exquisitely sensitive technologies that are used for embryonic analysis.

Practical issues

Another benefit of soft lithography is that once the moulds are made, PDMS devices can be cast for pennies. Fabrication costs could rise significantly as imaging and other analytic components are integrated, but they are likely to still be far less than the current costs of culturing embryos. In our and others' experiences, PDMS appears to be very durable, capable of handling thousands of pneumatic actuations without failure. The flexibility in design of devices also allows for larger ports to be incorporated for loading and retrieval of embryos. Since these microfluidic systems will be run by computers, software will need to be improved and simplified. The work by Thies *et al*. to develop portable java-based software for designing and operating devices provides a framework for such software [94]. Given the dynamic nature of embryo culture and the variations in procedures that are performed with embryos, it will also be important that the operator has control over parameters such as media exchange, rates of flow, media sampling, and embryo location. Although there will be a lot of sophisticated technology incorporated into these devices, the goal will be to hide all of this technology and present the user with an intuitive interface much as has been done for portable electronic devices such as the iPod.

Current state of the field

These are still the very early stages of applying advances in microfluidics and nanotechnology to assisted reproduction. To date, the four devices that have been presented in previous discussions in this chapter are really the only ones that have been documented in sufficient detail in peer-reviewed publications to warrant comment. These culture systems have all provided proof of principle that embryos can be cultured in microdevices, and most have demonstrated comparable or improved development over standard culture conditions. These devices have also demonstrated that they can be used to culture embryos in new conditions such as flowing culture medium and sub-microliter volumes, and preliminary results suggest that these new culture conditions are likely to be beneficial. This body of work provides a solid, encouraging foundation for much future work. The small number of published papers on microfluidic culture systems could provide readers with the mistaken perception that reproductive nanotechnology is still a very small field. This is far from the case. To our knowledge, we are aware of more than 10 academic laboratories that are working on microfluidic-based culture or analytic platforms for embryos or gametes. We are also aware of five companies that are currently advertising microfluidic devices for embryo culture. In this period of tremendous excitement and opportunity, it is critical that we keep in mind that much groundwork needs to be performed before these devices are ready for clinical application.

The dream system

With the coming advances in our understanding of embryonic biology and in technologies to culture and analyze embryos, it will be possible to build the dream system (hopefully arriving before the end of our careers!). A dream culture system in essence is quite simple. Such a system would input gametes and would output blastocysts with quantitative assessments of developmental potential. A blastocyst with an acceptably high developmental potential, if available, could then be loaded into a cathether for transfer and any other embryos of suitable potential could be prepared for cryopreservation. The challenges in realizing this dream system are two-fold: (1) optimizing culture conditions so that there is no undue harm sustained to the embryo during the time in culture and (2) developing highly accurate methods of

assessing the developmental potential of each cultured embryo. We are well on our way to tackling the first, but the second will pose formidable challenges, particularly in light of our growing appreciation of the degree of genetic mosaicism in human preimplantation embryos [95, 96].

Closing comments

These are exhilarating times to be in the field of embryology. Looking back over our brief history, it is stunning to consider the tremendous scientific accomplishments and their clinical impact. It is hard to believe that a generation ago, many of our patients had little or no chance of having children of their own. And turning toward the future, we are in equally as much awe. Extraordinary developments in embryology, genetics, nanotechnology, and microfluidics are occurring. We will be able to watch as a process that was largely manual, and therefore was built on a human scale, finally becomes reduced to a scale more appropriate for embryos. The insight into the biology of the embryo that will follow will be fascinating. We look forward to understanding which traits of the embryo represent vestiges of the mother and what are the first signs of expression of a new genome, as well as understanding what signs embryos give that they have serious problems with their genome, or epigenome. We also eagerly await the time when these advances will make single embryo transfer the standard of care. And most of all, we look forward to seeing how all of these advances will impact our patients. Improving the rates of healthy children born through ART and reducing barriers to accessing ART through the use of portable, low-cost culture/analytic devices will be the greatest rewards.

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Chapter 22

Morphometric analyses of embryos

Jennifer Kahn, Thomas Elliott, and Zsolt Peter Nagy

Introduction

In the United States and Europe more than 1% of children born are a result of some form of assisted reproductive technology. High success rates of IVF clinics are partly due to the transfer of more than one embryo – in most clinics in most countries of the world. A mean number of 2.8 embryos were transferred in the United States in 2004 resulting in a 27.7% delivery rate per initiated IVF cycle and a 33.5% multiple birth rate [1].

One of the major concerns to the IVF patient and to the practitioners of assisted reproductive technology, in general, is the high incidence of multiple pregnancy. High-order pregnancies pose a risk to both the mother and infant such as low birth weight, preterm delivery, and an increased risk for cerebral palsy [2]. As a result, the infant is troubled with a higher incidence of perinatal, medical, and neonatal complications when compared to a singleton delivery [3]. In order to limit multiple pregnancies the embryologist attempts to identify the most viable embryo(s) for transfer by using non-invasive microscopical observation. This procedure allows the embryologist to assess the morphological appearance of the transformations that occur in the developing embryo.

Morphological assessment is evaluated on days 0, 1, 2, or 3. Assesment on days 5 and 6 (or occasionally on day 4) are associated with extended culture to the blastocyst stage. Day 0 evaluation is based on oocyte morphology and only used when intracytoplasmic sperm injection (ICSI) is performed when cumulus and corona cells are removed (prior to insemination). Day 1 embryos are evaluated on pronuclear morphology, cytoplasmic appearance, early cleavage and polar bodies. The characteristics of a zygote with two pronuclei (PN) have been proposed to be associated with viability [4, 5]. Tesarik and Greco showed that the presence of an even number of nucleolar precursor bodies (NPB) in each pronuclei and NPBs in each pronucleus that were either polarized or non-polarized showed a higher implantation rate than the opposite [4]. PN orientation in relation to the polar bodies has also shown increased embryo viability. On day 2 or 3 the embryos are scored based on the extent of fragmentation, blastomere symmetry, presence or absence of multinucleated blastomeres, and cleavage speed [6]. Commercially available culture media have allowed the extended culture of embryos to the blastocyst stage in recent years. Gardner and Schoolcraft developed a scoring system for the blastocyst based on the structure and evenness of the trophectoderm cells and inner cell mass and the state of expansion [7].

Day 0 morphological evaluation

Oocyte meiotic maturation is the most cardinal parameter to assess when grading the retrieved oocytes from patients submitted to controlled ovarian hyperstimulation (when ICSI insemination is planned). The use of ovarian stimulation is thought to decrease the quality of oocytes because abnormal oocytes are matured rather than becoming atretic [8]. Immature oocytes, those that are arrested at prophase I and show a germinal vesicle, cannot be used for ICSI insemination (without further maturation in vitro) because the oocyte has a diploid set of chromosomes (2n, 4C) and has not expelled the first polar body [9, 10]. Oocytes that are at the MII phase of meiosis are at the stage when insemination can be performed and thus should be used for fertilization. A mature oocyte should show an unfragmented first polar body, a clear and somewhat granulated cytoplasm, a colourless zona pellucida, and a small perivitelline space [11–13]. Ebner *et al.* showed that an intact first polar body with a smooth surface showed positive values in relation to embryo quality and successful fertilization [13, 14].

Human Assisted Reproductive Technology: Future Trends in Laboratory and Clinical Practice, ed. David K. Gardner, Botros R. M. B. Rizk, and Tommaso Falcone. Published by Cambridge University Press. © Cambridge University Press 2011.

The end of meiotic maturation is seen with the extrusion of the first polar body. Oocytes with a polar body that is indented or fragmented have shown spindle abnormalities, non-disjunction events and aged oocytes [15–20].

Day 1 morphological evaluation

A normal zygote forms on day 1 and should have two PN that are of equal size, closed, and in the center of the cytoplasm; possess at least three NPBs in each PN; and NPB that are polarized (preferentially) or non-polarized in both PN. PN morphology should be graded between 16 and 18 hours post-insemination (relative to the type of insemination, IVF or ICSI) [4]. PN morphology is a very important step when evaluating morphology because it is the first point to truly grade the embryo. This evaluation can help separate those embryos with little chance of implantation from those with normal implantation potential. Figure 22.1.1a shows an optimal day 1 zygote with equally sized PN and aligned nucleoli.

Zygote/embryo evaluation

The presence of two PN and two polar bodies is the typical proof that fertilization has occurred. The timing of pronuclear formation and breakdown, DNA

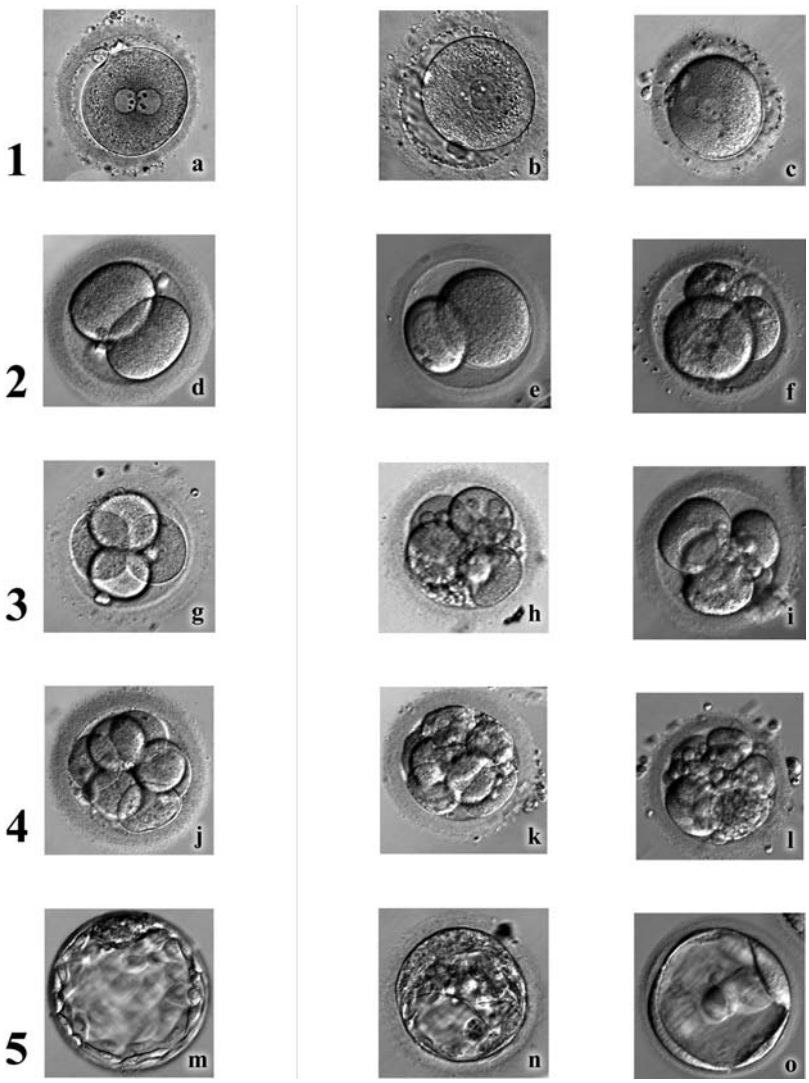


Figure 22.1. Image (1a) shows an optimal day 1 zygote with equally sized PN and aligned nucleoli; (1b) shows a day 1 zygote with asymmetrical PN and scattered nucleoli; (1c) shows a day 1 zygote with unequal sized nucleoli. The two nucleoli in the right PN are much larger than the nucleoli in the left PN. Image (2d) shows an optimal 2-cell embryo due to the equal size of each blastomere; (2e) shows a 2-cell embryo with asymmetrical blastomeres; a mild degree of granularity can be seen in the blastomere on the right; (2f) shows a 3-cell embryo with a slight amount of fragmentation. Image (3g) shows an optimal day 2 embryo with four equally sized blastomeres; (3h) shows a suboptimal day 2 embryo due to the fragmentation; (3i) shows a day 2 embryo with asymmetrical blastomeres and a vacuole in one blastomere. Image (4j) shows an optimal day 3 embryo with eight similar sized blastomeres; (4k) and (4l) show day 3 embryos with some fragmentation which would be considered suboptimal (embryo in (4k) also shows preliminary signs of early compaction). Image (5m) shows an optimal day 5 blastocyst; (5n) shows a suboptimal day 5 blastocyst due to the smaller size of the blastocoel and fragmentation within the trophectoderm cells; (5o) also shows a suboptimal-quality blastocyst due to the lower number of cells.

synthesis and cleaving during the first cell cycle of human embryogenesis following conventional IVF has been investigated in detail [21, 22]. PN form between 8 and 10 h post-insemination, the S-phase commences between 8 and 14 h post-insemination until it is completed between 10 and 18 h post-insemination, M-phase is observed between 22 and 31 h and cleavage to the 2-cell stage takes place between 25, and 33 h post-insemination. Studies describing the dynamics of pronuclear formation following ICSI have found that PN were first observed 6 h post-injection, and 91% of embryos had developed PN by 8 h post-ICSI [23].

Delayed appearance of PN after IVF is associated with poor embryo development [24]. Giorgetti *et al.* observed no pregnancy from a single transfer of oocytes with delayed PN visualization, indicating their poor developmental capacities [25]. One of the most concerning aspects of delayed pronuclear formation after insemination is the very substantial increase in chromosomal abnormalities [26]. Furthermore, the absence of PN after insemination does not indicate that sperm has not penetrated the oocyte. Failures of PN formation arise from specific defects within either gamete after sperm penetration, particularly in the microtubule organization ability of the sperm centrosome, growth of the sperm astral microtubules responsible for positioning the genome, and in progression through the first cell cycle [27]. Similar types of fertilization dysfunction have also been observed in ICSI failures [37]. Payne *et al.* used time-lapse video cinema photography to find that normal fertilization follows a definite course of events. Waves of granulation were seen in the ooplasm after ICSI [28]. At this time the sperm head begins to decondense which is followed by the formation of the male pronucleus. The female pronucleus forms in the cytoplasmic region, close to the second polar body (PB). Both PN are formed at about the same time. The female PN migrates toward the male PN until the two abut. NPBs are abundant, small, and distributed randomly within the PN. NPB function as the center of what will ultimately become the nucleoli [20, 29]. rRNA are produced in the nucleoli and also function as the protein powerhouse of any cell [30].

A study completed by Gamiz *et al.* has shown that PN score is correlated with age and normal chromosome outcome, with patients < 37 years of age [31]. Women over 37 years have a higher incidence of chromosomal abnormalities which is due to non-disjunction of bivalent chromosomes during oogenesis [32]. Pronuclear scoring evaluates the number and distribution of NPB and assesses the pronuclear zygote morphology. Embryos that had different-sized PN showed a higher incidence of chromosomal abnormalities.

Gianaroli *et al.* found that embryos with PN that were centralized/separate, unequal in size, and fragmented showed a very high proportion of aneuploidy and poor embryo development [33]. A majority of embryos that showed small scattered nucleoli and PN with nucleoli of different size and number were mostly abnormal, particularly showing complex abnormalities. Morphological changes may be related to the activity of protein synthesis and DNA transcription. These processes are very active in immature oocytes. In this stage the nucleoli synthesize RNA and proteins; however, this activity decreases with maturation. When the embryonic genome takes over, the nucleoli are fully competent due to formation over the first mitotic cycle. Figure 22.1.1b shows a day 1 zygote with asymmetrical PN and scattered nucleoli. Figure 22.1.1c shows a day 1 zygote with unequal-sized nucleoli. The two nucleoli in the right PN are much larger than the nucleoli in the left PN.

PN evaluation is very time-dependent due to the coalescence of NPB to nucleoli. A transition phase can be observed [28]. Two pronuclei can be seen as early as 6 h post-ICSI and at 16 h post-ICSI almost all normally fertilized oocytes show two pronuclei [34]. PN evaluation in conventional IVF oocytes is slightly different with relation to PN appearance. Nagy *et al.* found that sibling oocytes showing two PN were visible at 18 h post-insemination. This delay could be due to the time the spermatozoa need to pass through the cumulus and corona cells and the zona pellucida of the oocyte [34].

A decreased amount of ATP that is available to the oocyte could be the cause of delayed or abnormal PN formation. Oocytes that have a decreased amount of energy storage due to the switch from oxidative phosphorylation to glycolysis may not have enough ATP to sustain PN formation. Energy availability is also important for the correct alignment of the chromatin onto the mitotic plate and incorrect alignment could result in mosaic or aneuploid embryos [35].

Nucleoli evaluation and nucleolar precursor bodies (NPB)

The nucleoli separate into different parts during mitosis and in the final stage of maturation of the

oocyte. As the chromatin is condensing on the meiotic spindle, the NPB are also condensing. Nucleoli provide the fascinating possibility of linking morphologically distinct structures such as those seen in the electron microscope with biochemical features of the formation and stepwise maturation of ribosomes [36, 37, 38, 39]. The molecular components and the function of NPB have been investigated by several authors who found that these electrondense, homogeneous, and finely filamentous intranuclear structures occurring in mammalian zygotes during the early postfertilization develop after an early pronuclear RNA synthesis [21, 40–42]. Uneven distribution or unequal numbers of NPB can result in an irregular cell cycle, karyo- and cytokinesis which can ultimately lead to a decrease in the development potential of the embryo [43]. Poor embryonic development can also be attributed to the lack of organization or synchrony between nucleoli. PN score in combination with day 3 embryo morphological evaluation has given rise to an increase in implantation rates from 19% to 31% [5].

Another study completed by Nagy *et al.* found that polarization of NPB in both pronuclei is also a reliable marker for implantation when combined with day 3 embryo morphology evaluation. Unequal size of blastomeres and distance between both pronuclei were also noted in this study. There was a significant difference in the quality and rate of cleavage in embryos where the NPB were not polarized and blasotmere size was unequal. A 21.1% implantation rate was seen in embryos that were checked for NPB polarization and evaluated on day 3 for embryo morphology [44].

Pronuclear orientation

Pronuclear orientation in relation to the polar bodies can also be used to evaluate embryo morphology on day 1. The position of the pronuclei and the polar bodies arrange to become parallel to each other before cleavage, therefore a correlation between early cleavage and PN orientation may be true [45–47]. A study completed by Kattera and Chen found that the embryos with both PN and polar bodies parallel to each other had a higher percentage of early cleavage when compared to those that did not show a parallel relationship [48]. However, this observation alone is not responsible for early cleavage.

Cytoplasmic appearance

Gamiz *et al.* evaluated the appearance of a cytoplasmic halo [31]. Contraction of organelles from the cortex to the center of the oocyte leaving a clear cortical zone is known as a cytoplasmic halo [28]. Gamiz *et al.* found that zygotes with halo-positive showed a significantly higher blastocyst rate as compared to zygotes with halo-negative [31].

Early cleavage

Preimplantation embryos cultured in vitro grow and differentiate at constant rates. The earliest time the human concepti can reach the 2-cell stage ranges between 20 and 27 h post-insemination [21, 22, 49]. By days 2 and 3, normally developing embryos have reached the 2- to 4-cell and 8-cell stages, respectively. The mean time for the first three cleavages has been reported to be 34–36, 46, and 54–56 h after insemination [49, 50]. Arrested or disordered development is an overt sign of abnormal growth, and care must be taken to ensure that these abnormalities do not arise through suboptimal methods of culture. Figure 22.1.2d shows an optimal 2-cell embryo due to the equal size of each blastomere. Figure 22.1.2e shows a 2-cell embryo with asymmetrical blastomeres. A mild degree of granularity can be seen in the blastomere on the right. Figure 22.1.2f shows a 3-cell embryo with a slight amount of fragmentation.

Several studies have shown that transferring early-stage cleavage embryos results in a higher pregnancy and implantation rate when compared to transferring non-early-stage cleavage embryos. Sakkas *et al.* found that ICSI patients with embryos that cleaved early had a pregnancy rate of 25.9% as compared to those patients whose embryos did not cleave early resulting in a pregnancy rate of 3.2% [51]. (This observation is particularly interesting, because it is somewhat against "nature's wisdom" – as most physiological changes are considered optimal when they happen in a "timely manner" – meaning that not only too slow (or delayed) cleavage but also too early (too fast) cleavage may be suboptimal. Time-lapse video recording (such as Embryoscope) may help in the future to further clarify this point.)

Day 2 and 3 morphological evaluation

Embryos on day 2 should have four blastomeres that are equal in size and show no multinucleation, the cytoplasm of each blastomere should be pale and

clear with some granulation, and fragmentation should be ≤ 20%. Figure 22.1.3g shows an optimal day 2 embryo with four equally sized blastomeres. Figure 22.1.3h shows a suboptimal day 2 embryo due to the fragmentation. Figure 22.1.3i shows a day 2 embryo with asymmetrical blastomeres and a vacuole in one blastomere. Day 3 embryos that are developing normally should have eight blastomeres; however, it is possible for embryos with five, six, or seven blastomeres to have unequal sized cells. Figure 22.1.4j shows an optimal day 3 embryo with eight similar-sized blastomeres. Table 22.1 shows embryo evaluation criteria for day 2 and day 3 embryos, as well as the significance of each evaluation

Blastomere number / cleavage speed

It has been reported by several authors that too slow or too rapid embryo growth has a negative impact on implantation rate [25, 50, 52–55, 94]. In all these studies, the transfer of 4-cell-stage embryos on day 2 resulted in a significantly higher implantation rate as compared with other cell stage embryos. Furthermore, when the above optimal cleavage pattern was used for single embryo selection at day 3, very high implantation rates have been reported [56–58].

A correlation between embryo timely cell division and chromosomal constitution has also been reported [59–63]. Slow cleavage and cell division arrest, as well as fast cleavage, has been related to embryo chromosomal abnormalities (such as mosaicism, polyspermia, and aneuploidies).

Fragmentation

The presence of anuclear small cellular fragments (fragmentation) is the norm in in vitro cultured human embryos. The degree of fragmentation varies

Table 22.1. Evaluation of embryo quality on day 2 (42 h to 48 h after IVF/ICSI) and on day 3 (68 h to 74 h after IVF/ICSI).

Embryo evaluation criteria	Relative importance	
1. Number of blastomeres		Strongly important
	Day 2	Day 3
Optimal	4(–6) cells (>6 cells is negative sign)	7–8 cells or more (not more than 10 cells)
Medium	2–3 cells	5–6 cells
Poor	1 cell	2–4 cells
2. Dimension/symmetry of blastomeres – day 2 and day 3		Strongly important
Optimal	similar or equal size blastomeres	
Suboptimal	different size blastomeres	
3. Proportion of anucleate fragments – day 2 and day 3		Strongly important
Optimal	between 0–10%	
Good	between 10–20%	
Medium	between 20–50%	
Poor	more than 50%	
4. Quality of the cytoplasm		Moderately important
Optimal	normal appearance	
Suboptimal	presence of cytoplasm abnormalities (granulated, vacuoles, refractile bodies)	
5. Multinucleation of blastomeres (Multinucleated blastomere may be discounted from the number of blastomeres present) The evaluation of multi-nucleated blastomeres better performed at the 2–4-cell stage Number of cells with visible and non-visible nucleus should be noted		Moderately important
Optimal	Every blastomere with a single nucleus	
Suboptimal	<20% of cells with multinucleation – cells with two equal-sized nuclei	
Poor	>20% of cells with multinucleation – cells with two unequal sized nulei or more than two nuclei	
6. Early cell compaction Cell compaction should start after 8–10-cell-stage embryo (end of day 3 or day 4)		Moderately important
Optimal	Cell compaction is not observed (or not strong) until the end of day 3	
Suboptimal	Cell compaction is strongly present late day 2 or early day 3	

from 5% or 10% to 100%, the fragments may be either localized or scattered and initially appear from the first mitotic division on. However, when the degree of fragmentation exceeds 10% of the embryonic volume it can have a negative effect on development. Large fragments that are found in the cells of a 2- or 4-cell embryo are due to the electrondensity of the mitochondria in each fragment. Mitochondria are more electrondense in later stages of development than in earlier stages. When large fragments are released in the early stages of development the embryo loses important organelles such as the mitochondria. When fragmentation occurs the section of the cell that is left with the nucleus can arrest due to the loss of important organelles [64]. Fragmentation has also been attributed to spermatozoa [65]. Spermatozoa were implicated as a cause of fragmentation through DNA damage occurring before fertilization [66, 67], whereas others have reported that metabolic disturbances in the oocyte may play a role [68]. Fragmentation percentage has also been known to be associated with chromosome abnormalities [62, 69, 70]. Both apoptotic and necrotic processes have been suggested as causes of blastomere fragmentation in human embryos [71]. Figure 22.1.4k,l shows day 3 embryos with some fragmentation which would be considered suboptimal.

According to Van Royen *et al.*, a top quality embryo will have no multinucleated blastomeres; four or five blastomeres on day 2; and < 20% fragments. The presence of large fragments was found to be harmful to the developing embryo [57]. On the other hand small and scattered or localized fragments did not significantly impact implantation potential [72,73]. Fragments can be removed by microsurgical techniques, which improves the developmental potential because the spatial relationship between the blastomeres is restored. Frozen embryos also show fragmentation and detriment to the embryo. Elliott *et al.* showed that by removing lysed cells in mouse embryos the developmental potential was restored to that of the control [74]. Lysed cells in frozen/thawed embryos are thought to interfere with cell to cell communication.

Uneven cleavage / blastomere symmetry

Uneven cellular cleavage is an indicator of poor quality and slow development; this can lead to an uneven distribution of genetic material. Embryos with unevenly sized blastomeres displayed a much higher multinucleation and aneuploidy rate resulting in a lower implantation rate [75].

Multinucleation

Multinucleation is the presence of two or more nuclei inside a single blastomere. Multinucleated blastomeres can be observed more easily on day 2. However, multinucleation is only visible for those blastomeres that are at the interphase stage [76]. Multinucleation results from a failure in cytokinesis during early cleavage and also has a detrimental effect on the developing embryo [77]. Observing multinulceation in day 2 embryos is a simple, non-invasive detection and therefore should be added to the cumulative embryo grading system. Day 2 embryos have a larger dimension and less overlap than day 3 embryos, therefore day 2 is the best day to assess multinucleation [76]. Van Royen *et al.* also found that multinucleation is correlated with early cleavage. As mentioned earlier, early cleavage is associated with embryo quality. Day 2 embryos that had an optimal cleavage pattern exhibited a lower number of multinucleated blastomeres. Embryos not only with a lower number but also with a higher number of blastomeres on day 2 showed a significant increase in multinucleation rate. Normal day 3 embryos, those with eight blastomeres, showed the lowest frequency of mulitnucleation. Van Royen *et al.* also found that a shorter than average stimulation, higher than average number of oocytes retrieved, and a higher than average FSH dose for stimulation was related to a higher incidence of multinucleation [76].

Cytoplasmic appearance

When the cytoplasm of a developing embryo is pale and clear or finely granular in appearance it is considered normal. Unusual colour or granular texture is considered abnormal, as well as the presence of vacuoles or dense bodies [78]. There is no correlation established between the cytoplasm of blastomeres and embryo morphology in terms of regularity and fragmentation rate and blastomere number [79].

Early compaction

Compaction is when the embryo cleaves to the point where one cannot distinguish the number of cells and cell membranes. The embryo now is a solid mass of cells. Tight junctions are formed that cause the

blastomeres to come together and form a morula. During compaction the blastomeres lose their totipotency due to their interactions and the embryonic genome takes over and this usually occurs on day 4. Early compaction occurs on day 3 and it is debated whether it has a negative association with embryo viability, but some take it as a sign of increased developmental potential. Some studies suggest that early compaction will improve implantation potential when compared to embryos with some or no compaction. Skiadas *et al.* concluded that compaction grading is another assessment tool to use when evaluating embryos and choosing the most viable embryo for transfer on day 3. One drawback to this grading system was the degree of fragmentation. Embryos with < 10% fragmentation showed implantation rates that increased with the degree of compaction. For embryos with ≥ 10% fragmentation, the implantation rate decreased [80].

Days 5 or 6 (blastocyst stage) morphological evaluation

At approximately 112–114 hours post-insemination, a normal developing embryo should be at the blastocyst stage. At this developmental stage the embryo should exhibit a distinct inner cell mass protruding into the cavity, a defined and large blastocoel that covers at least half of the embryo, and a ring of evenly sized and spaced trophectoderm cells [81]. The blastocyst has two distinct cell types, the inner cell mass and the differentiated trophectoderm. The inner cell mass gives rise to the embryo proper and extraembryonic membranes. The trophectoderm cells form a fluid-supporting epithelium that is responsible for the accumulation of fluid in the blastocoel and blastocyst expansion. Figure 22.1.5m shows an optimal day 5 blastocyst. Figure 22.1.5n shows a suboptimal day 5 blastocyst due to the smaller size of the blastocoel and fragmentation within the trophectoderm cells. Figure 22.1.5o also shows a suboptimal quality blastocyst due to the lower number of cells.

The quality of the blastocyst can be determined by the formation of blastocoele and trophectoderm cells, as well as the size of the inner cell mass (ICM). All of these factors were found to be the most important in relation to increased implantation rates [7, 82–84]. The ICM can be scored with respect to the cell number. Blastocysts with a tightly packed ICM with multiple cells represents grade A, which is the best-quality grade. A grade B embryo exhibits a loosely grouped cell mass with several cells, and a grade C embryo shows very few cells. In addition to scoring the ICM, the trophectoderm can also be graded. The trophectoderm was thought to be of good quality if the cells formed an organized epithelium [83]. A study completed by Racowsky *et al.* found that a key determinant for selecting embryos for blastocyst transfer was the number of 8-cell embryos on day 3 [85].

Transferring embryos at the blastocyst stage has become an option for most assisted reproductive clinics due to the development of sequential culture media. This type of media was designed due to the many physiological changes that occur in the female reproductive tract; therefore culture media provide different metabolic nutrients for the development of cleavage and blastocyst stage embryos [86]. In order to meet the increased energy demands of the developing embryo, the nutrients provided in the culture media need to switch from pyruvate to glucose. The inner cell mass of the blastocyst also requires the essential group of amino acids and the trophectoderm cells require glutamine and the non-essential amino acids [87].

Blastocyst transfer has been shown to provide the possibility for improved embryo selection, thus reducing the number of embryos transferred and consequently reducing multiple pregnancies while increasing implantation rates [88, 89]. Patients that cannot medically sustain multiple pregnancies (due to medical conditions), those with recurrent implantation failure, and patients who are undergoing preimplantation genetic diagnosis will benefit from blastocyst transfer. Those patients with poor prognosis, such as poor-quality embryos on day 2 or 3, may also benefit from blastocyst transfer – though in these situations it is a common "fear" that no embryos will develop to the blastocyst stage by day 5. Synchronization between the endometriosis and embryo at the time of transfer is another advantage of blastocyst transfer [87]. Contractions in the uterus on day 5 are reduced by 1.5 contractions per minute when compared to early-stage transfers on day 2, therefore day 5 blastocyst transfers appear to be more beneficial for successful implantation [47, 90]. Another advantage of extended culture is the activation of the embryonic genome. A 4–8-cell embryo begins to rely on the embryonic genome and if cultured up to the blastocyst stage it is possible to identify those embryos with a developmental block on day 2 or 3 [91, 92].

In the early days of IVF the transfer of multiple embryos was common practice due to the low implantation and pregnancy rates that were obtained [87]. Blastocyst transfer has the ability to decrease the risk of multiples while increasing implantation and pregnancy rates. Multiple pregnancies pose serious risks to mother and fetus, therefore reducing the number of embryos transferred minimizes the incidence of multiples. In order to reduce the frequency of multiple births, IVF clinics have opted for single embryo transfer in young women. Papanikolaou *et al.* reported that women less than 36 years of age who were undergoing their first or second IVF cycle had an increased chance of pregnancy from a single blastocyst embryo transfer when compared to the transfer of a single cleavage-stage embryo [93]. However, optimal laboratory conditions and a concrete selection process is necessary when culturing embryos to the blastocyst stage, as well as a proven blastocyst-freezing program [87].

Paternal effect on blastocyst development

Several studies have shown that ICSI embryos produce a lower number of viable embryos when compared to IVF cycles. Studies published by Balaban *et al.* and Miller and Smith confirmed that poor-quality sperm can decrease embryo development [95, 96]. Abnormal sperm, such as those with low motility, can affect the function of the centrosome of the sperm. The centrosome is a very important structure in spermatozoa that is responsible for proper fertilization and cell division in the egg. The functional proximal sperm centriole is brought into the oocyte and arranges a sperm aster. It then replicates at the PN stage and migrates to one pole of the first mitotic spindle during prophase. It will ultimately occupy both poles of the bipolar spindle at metaphase. In humans the inheritance of the centrosomes is completely paternal; therefore if its structure is altered in any way it can have a negative affect on fertilization and cleavage [97].

Cumulative embryo score

Cumulative embryo scoring was proposed by Steer *et al.* This scoring system allowed IVF clinics to select the most viable embryo for transfer and to decrease the incidence of multiple pregnancies. This scoring system encompassed embryo morphology, cleavage rate, and number of embryos transferred into a single figure [98]. Since then many other morphological scoring systems have been developed and differ from clinic to clinic based on the experience/familiarity of the embryologists. Cumulative embryo scoring is usually performed on day 3 and takes into account the extent of fragmentation, blastomere symmetry, presence or absence of multinucleated blastomeres, and cleavage speed [6] and it also requires a single embryo culture – which is not favored by some other embryologists.

Video monitoring is a tool that is used to follow embryo development. Different monitoring systems allow embryologist to visualize the changes that occur while in culture, such as cleavage, fragmentation, and blastulation.

Future developments in embryo morphology evaluation

Time-lapse video monitoring is a tool that is used to follow embryo development and can supply embryologists with more accurate information about embryo morphology than the routine microscope observations. Different monitoring systems allow embryologists to visualize the changes that occur while in culture, such as cleavage, fragmentation, and blastulation. Lemmen *et al.* used time-lapse recording to find that embryos which implanted had a more synchronous appearance of nuclei in each blastomere after the first cleavage. From this observation the number of noticeable nuclei in the evaluation of early cleavage can be used as a parameter for embryo scoring. Lemmen *et al.* also found that embryos that had four or more blastomeres had earlier disappearance of PN and first cleavage than those embryos with three or two blastomeres [99].

Video monitoring can also be used as a quality control tool because it supplies real-time visualizations of embryo development. For instance, it can detect toxic damage or infection from the culture media or dish before regular quality control measures.

When culture dishes are removed from the incubator embryo(s) they are exposed to non-physiological environment (which may impact their further development) and there is a chance of disturbing paracrine factors that are released by each embryo. With the use of time-lapse video monitoring the embryos can stay in a controlled and optimal environment for several days until transfer.

Automatic analysis of different patterns of cleavage, symmetry of blastomeres, and degree of fragmentation may be a future improvement when scoring embryos. This procedure in conjunction with time-lapse video monitoring may have the potential to increase the

embryologist's ability to choose the best embryo for transfer and subsequently improve implantation potential. It is expected that these new time-lapse video monitoring systems alone or in combination with other non-invasive assessment technologies (proteomics and metabolomics) will revolutionize how embryology is performed and help to achieve the highest pregnancy rates with single embryo transfer to establish healthy singleton pregnancies.

Summary

In an in vitro fertilization program, embryo viability should be estimated before transfer, in order to replace only viable concepti, aiming to optimize the efficiency of the technique. An important issue in IVF is how to evaluate the viability of the embryo and how to select the right embryo(s) for transfer. A non-invasive morphological assessment of developing embryos can be completed on days 1 through 4, and day 5 for blastocysts. Extended culture allows the embryologist to follow the development of several blastocysts and select the embryo that has the highest potential for implantation. A combined evaluation of pronuclear and embryo parameters can further improve embryo selection procedure that can result in (1) lower number of embryos transferred, (2) higher implantation rates, (3) higher pregnancy rates, and (4) lower multiple-pregnancy rates.

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Chapter 23

Embryo biopsy: towards trophectoderm isolation and blastocyst analysis

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Introduction

There are primarily three stages at which biopsy is performed to obtain cellular material for genetic testing. These are the polar body stage (day 0 and/or day 1); the cleavage stage, typically on day 3; and the blastocyst stage (day 5 or 6). All require breaching the zona pellucida in order to facilitate the removal of cellular material. This chapter will mainly focus on biopsy procedures at subsequent stages of embryo development after fertilization with particular emphasis on blastocyst biopsy.

Methods of breaching the zona pellucida for biopsy

Since the advent of preimplantation genetic diagnosis (PGD) there have been three methods primarily used to breach the zona pellucida to facilitate the removal of cells for testing. The first approach utilizes a mechanical technique called partial zona dissection or PZD [1]. This procedure requires a holding pipette and a fine needle to penetrate into and under the zona pellucida and then back out. The skewered section of zona is then cut by gentle rubbing against the holding pipette creating a slit-type opening. PZD was first used successfully by Cohen *et al.* [2] to facilitate sperm penetration; however, there were concerns regarding constriction of the hatching blastocyst through the slit-type opening. Subsequently, a modification of PZD termed "3D-PZD" was developed, in which a second intersecting slit is made at a right angle to the first, creating essentially a flap opening in the zona pellucida [3]. Through this flap, biopsy pipettes can be introduced to remove polar bodies or blastomeres. Advantages of this method, as compared to those described below, include no change in pH (i.e. zona drilling) and no change in temperature (i.e. heat ablation using a non-contact infrared laser) for the embryo. It does require a double tool holder for control of the PZD needle and biopsy pipette. It also requires practice to quickly rotate the oocyte or embryo back into position so that the slit opening is in focus and in the right position to perform biopsy and therefore has a longer learning curve when compared to the use of a non-contact laser.

The second method developed to breach the zona pellucida involves chemical digestion with acidified Tyrode's solution (pH 2.2–2.4). This method does not require expensive equipment but requires considerable skill to create the correct hole size while not exposing the embryo to excessive acid. A small micropipette is used to apply acid to the outer surface of the zona pellucida while holding the embryo in a fixed position with a holding pipette. Afterward the embryo is thoroughly rinsed to remove any residual acidic solution. This procedure allows for a rapid creation of a 20 to 30 μm opening in the zona pellucida termed zona drilling and was first applied to enhance fertilization in the presence of oligospermia [4]. However, its use in assisted fertilization was abandoned after it was thought to cause developmental arrest after fertilization from exposure to the acidic pH. Other studies have shown considerable intracellular pH changes to the oocyte before and after fertilization with zona drilling, and have suggested the human oocyte is unable to compensate acidosis [5, 6]. Therefore, zona drilling with acid has never been the method of choice prior to polar body biopsy. This technique, however, has been widely used for assisted hatching at the cleavage stage. Quick and careful application of the acidic solution followed by thorough rinsing does not seem to have a negative affect on embryo development and has been shown to enhance embryonic implantation

Human Assisted Reproductive Technology: Future Trends in Laboratory and Clinical Practice, ed. David K. Gardner, Botros R. M. B. Rizk, and Tommaso Falcone. Published by Cambridge University Press. © Cambridge University Press 2011.

with selective application for poor-prognosis patients [7]. Others have used this technique to breach the zona pellucida prior to embryo biopsy using a third micropipette or the same micropipette to remove one or two blastomeres [8–10]. More recently, there has been an extensive investigation regarding the potential deleterious effects of zona drilling at the cleavage stage, particularly to the cells in close proximity to the opening [11], emphasizing the need for caution when applying this technique or perhaps abandoning it all together.

The third and most recently applied method for creating an opening in the zona pellucida involves the use of a computer-controlled non-contact 1480 nm infrared diode laser beam. Various lasers of different wavelengths as low as 248 nm have all been found to be effective in breaching the zona pellucida but not without concern for possible mutagenic effects at wavelengths close to the absorption peak of DNA at 260 nm [12]. The evolution of this technology has resulted in a non-contact laser, delivered through the microscope objective, with minimal absorption by the culture dish and culture medium, allowing for the quick formation of a hole which corresponds to the laser power and pulse duration. Tinney *et al.* [13] investigated for use of the 1480 nm diode laser for assisted hatching in mouse embryos with regard to beam intensity, the number of pulses and the duration of the pulse to determine the optimal technical settings. From this study it was concluded that the use of lower laser intensity and more than one pulse could safely generate a sufficiently sized hole for complete hatching. Montag *et al.* [14] investigated the use of laser-assisted microdissection on mouse zygotes to facilitate second polar body removal and concluded that it made the procedure more accurate and effective for preimplantation genetic diagnosis. With this in mind the use of the laser has been applied for use on human embryos. There have been several studies regarding the use of the laser at the cleavage stage of embryo development. Comparison of laser drilling to the use of acid Tyrode's (zona drilling) resulted in a greater number of intact blastomeres obtained during biopsy for preimplantation genetic diagnosis when laser drilling was applied [15]. Other studies revealed equal or improved outcomes with laser drilling for preimplantation genetic testing when comparing sibling embryos [16], and when used for assisted hatching [17]. The advantage of using a computer-controlled laser beam is the uniformity of the opening with each embryo, thereby reducing operator variability. Additionally it requires less time in which to create the opening, reducing the time the embryos are outside the incubator. However, despite these aforementioned studies, there are still concerns regarding the proper use of the laser to avoid heat damage, keeping the firing position of the laser away from the nearest blastomere [18] as well as firing in a row, moving across the zona pellucida from the outside to the inside in a location adjacent to the largest perivitelline space [19].

Polar body biopsy

Polar body biopsy involves the removal of both the first and second polar bodies in order to make an accurate diagnosis of the oocyte. The analysis of polar bodies can be used to determine chromosomal aneuploidy related to maternal meiosis and maternally derived unbalanced translocation chromosomes and detect single gene defects in the maternal genome allowing for day 3 embryo transfers.

Polar bodies can be removed together after fertilization for aneuploidy testing or separately for single gene disorders. Concerning the latter, the first polar body is removed shortly after retrieval followed by ICSI with the removal of the second polar body on day 1.

To date the vast majority of polar body biopsies (over 20 000 oocytes, personal communication) for aneuploidy testing have been performed by one PGD laboratory, whose founder, Yury Verlinsky, pioneered polar body removal using the mechanical method of PZD to breach the zona pellucida [20]. Rapid analysis of the genetic material by fluorescent *in situ* hybridization (FISH) for aneuploidy and maternally derived chromosomal rearrangements has been performed successfully focusing on a limited number of chromosomes [21, 22]. FISH, however, can be difficult and technically demanding to perform on polar bodies due to limitations in the number of chromosomes which can be investigated, requiring several rounds of hybridization. Other laboratories, specifically in Europe, have adopted the procedure with some variation. The use of non-contact infrared laser is now used to breach the zona pellucida prior to polar body removal [23]. Due to legislation in some countries, PGD can only be performed prior to syngamy, creating a small window of time in which to complete polar body analysis. Because of these constraints, the removal of only the first polar body, termed "preconception genetic diagnosis," is performed, providing

information on the first meiotic division only. Some have attempted to perform both first and second polar body analysis within this time frame as well. These variations, along with the limitations of FISH, may contribute to the conflicting studies regarding polar body biopsy and decreased subsequent embryonic development and viability [23–25].

Cleavage-stage embryo biopsy

Collectively, since the first report of cleavage-stage embryo biopsy for PGD [26], day 3 embryo biopsy has been the most common method utilized in the past two decades. Investigation of both maternal and paternal genetic contributions can be performed for single gene mutations, HLA compatibility, chromosomal rearrangements, and aneuploidy.

All three of the above-mentioned methods for breaching the zona pellucida have been utilized to facilitate cleavage-stage biopsy. Traditionally blastomeres have been removed using gentle suction from a biopsy pipette with a diameter of 30 μm but more recently by displacement caused by positive pressure generated from the expulsion of medium under the zona pellucida [27]. Studies on cleavage-stage biopsy in the mouse [28] and on human embryos [29] have shown that an optimal number of cells [6–10] are required at the time of biopsy so as not to remove a considerable portion of embryonic cell mass with detriment to further development and implantation. For that same reason, the removal of a single blastomere has been found to be less detrimental to blastocyst formation [30] and implantation [31] when compared to the removal of two cells to ensure a more accurate diagnosis.

Cleavage-stage biopsy can be performed with the use of Ca^{2+}/Mg^{2+}-free medium, which has been found to ease the removal of a single blastomere with less chance of embryo damage since it allows for the dissociation of blastomeres by loosening the membrane adhesions [32]. Prior to breaching the zona pellucida, the embryo should be examined and positioned so that a blastomere with a clearly visible single nucleus can be removed to ensure successful genetic analysis. In order to allow time for genetic testing and embryo transfer within the same cycle, day 3 embryo biopsy requires subsequent culture of the embryo to the blastocyst stage, and therefore an excellent blastocyst culture system.

Several studies have shown day 3 embryo biopsy of a single blastomere to be non-detrimental to further development to the blastocyst stage [8] even when performed following polar body removal in order to obtain more material for testing [24, 33]. However, for as many studies supporting PGD at the cleavage stage there are equal numbers of reports reporting the poor benefit/risk ratio, specifically regarding aneuploidy testing. Multiple randomized trials have compared aneuploidy screening using fluorescent *in situ* hybridization and day 3 embryo biopsy to control embryos which were cultured to the blastocyst stage without biopsy or FISH analysis. To date, none of these randomized trials has shown a benefit from embryo biopsy and genetic screening by FISH. Furthermore, several of these trials showed either a trend toward or a statistical lower rate of implantation and pregnancy rates in the embryo biopsy group [34–36].

There are a number of reasons for this confounding picture. As mentioned above, the manner in which the zona pellucida is breached may play a role in further embryo development. Overexposure to Ca^{2+}/Mg^{2+}-free medium to dissociate compacting embryos may contribute to poorer embryo development following biopsy if exposed for no more than a few minutes. Plausibly the removal of one or two cells negates the beneficial effects of PGD [37, 38]. Goossens *et al.* [30] studied the controversial question of whether to biopsy one or two cells at the cleavage stage and showed that removing two blastomeres on day 3 had a significant influence on day 5 embryo quality, particularly for embryos of slower development. However, that being said, the authors still emphasized the need for two cells to be removed from good-quality embryos when utilizing PCR technology due to the incidence of allele dropout and failed amplification. The analysis of two cells would increase diagnostic efficiency, reducing the number of "no result embryos" in a cycle. This definitely presents a conundrum.

Biopsy at the cleavage stage is based on the premise that the embryo is totipotent at this stage; in other words, each blastomere is capable of developing into an entire organism. Studies on polarity of the embryo, however, have challenged this dogma, suggesting the removal of even a single cell can be detrimental due to the uneven distribution of mitochondria and regulatory proteins within the cells of the preimplantation embryo that are necessary for further embryonic development [39, 40]. Embryo biopsy has been shown to reduce pyruvate and glucose uptake in biopsied embryos compared to controls and results in

fewer cells in the embryo at the blastocyst stage [8]. The fact the biopsied embryos are less likely to survive with conventional slow cooling methods after zona drilling and embryo biopsy demonstrates the detrimental effects of biopsy at cleavage stage, leaving the embryo in a fragile state [41, 42]. In a telling study by Chatzimeletiou *et al.* [18] embryos were biopsied on day 3 after breaching the zona pellucida by zona drilling with acid or by laser and each compared to control embryos which were not biopsied. When all three groups of embryos were re-analyzed on days 5, 6, and 7, there was a marked reduction in cell numbers in both biopsy groups compared to controls. By day 7, the biopsied embryos had less than half the number of cells that control embryos possessed.

Additionally, the controversy over PGD at the cleavage stage can also be attributed to the limitations of the testing being performed. Genetic analysis using FISH with probe cocktails targeting, at best, 7–12 chromosomes may overlook other chromosomal errors which result in implantation failure. Finally, mosaicism of the embryo at the cleavage stage makes the testing of a single blastomere particularly problematic and has been a major cause attributed to misdiagnosis [43–47]. As such cases, normal or viable embryos may be discarded due to a false misdiagnosis of aneuploidy. Taking into account all of these aforementioned reasons, scientists and clinicians have searched for better methods to perform PGD.

Blastocyst biopsy

Trophectoderm biopsy on human embryos for preimplantation genetic testing was explored as early as 1990 by Dokras *et al.* [48]; however, it was not universally applied due to poor pregnancy success rates with few embryos reaching the blastocyst stage [49]. Also, the extremely tight time constraints in which to obtain genetic results and perform embryo transfer within the implantation window do not allow for fresh transfer, requiring cryopreservation and transfer in a future cycle. Fortunately, embryo biopsy at the blastocyst stage is now a reality due to the convergence of several key technologies. First, advancements in embryo culture such as low-oxygen and sequential culture systems using chemically defined media formulated to meet the needs of the embryo's metabolism at particular stages have made culture to the blastocyst stage common practice for many laboratories [50, 51]. Second was the development of a non-contact infrared laser as an effective means of biopsying trophectoderm cells with ease and without negatively impacting the viability of human blastocysts [52]. The third convergent technology was the improved method of cryopreservation using vitrification, which allows for biopsied human embryos to be cryopreserved with a high rate of efficiency. The fourth technology arriving in parallel with these other aforementioned technologies was the appearance of new platforms for comprehensive chromosomal screening. SNP microarrays, BAC microarrays, CGH and real-time PCR have all recently been developed as platforms to assess all 23 pairs of chromosomes simultaneously from trophectoderm biopsies [53–55]. The trophectoderm cells (between 4 and 8) can be removed on day 5 or 6 without disturbing the inner cell mass. The negative impact of embryo biopsy on embryonic cell mass is greatly reduced as a smaller fraction of cells is removed from the developing embryo. Furthermore, the removal of several cells for aneuploidy testing may reduce the risk of missing mosaicism [56]. Blastocyst-stage biopsy can also be utilized for single gene mutation analysis and in some cases analysis can be performed in time for day 6 embryo transfer or in combination with cryopreservation. The removal of several cells at the blastocyst stage for the investigation of single gene disorders may alleviate the problem of failed amplification and allele dropout in PCR-based protocols due to the increased amount of DNA template and therefore may reduce the potential for misdiagnosis and uninformative outcomes seen with single cell analysis [57].

Trophectoderm biopsy as performed at the Colorado Center for Reproductive Medicine

As mentioned above, an excellent blastocyst culture system is required for successful trophectoderm biopsy. Utilization of high-quality sequential culture media in microdrops with an oil overlay and 5% oxygen gas phase is a critical component of success. The use of a modified neonatal isolette is used to maintain temperature and pH when embryos are outside the incubator for micromanipulation procedures. A Ca^{2+}/Mg^{2+}-free medium is not required for this procedure; however, careful efforts to maintain temperature and pH during the biopsy are critical since embryos are biopsied in the same bicarbonate-buffered medium used for culture. This is accomplished with oil overlay

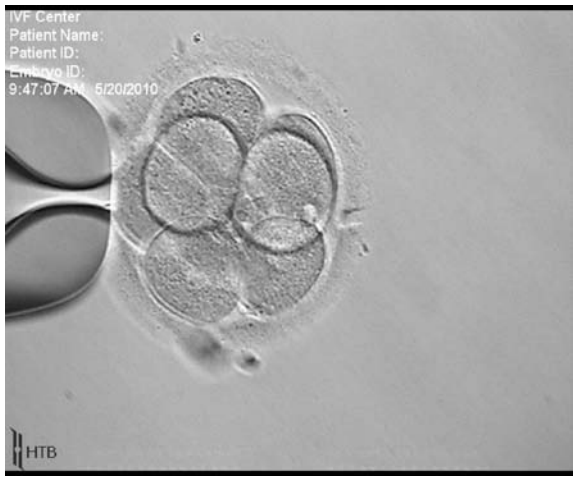


Figure 23.1. Channel opening made on day 3 of embryo development. Opening can be seen at 3 o'clock.

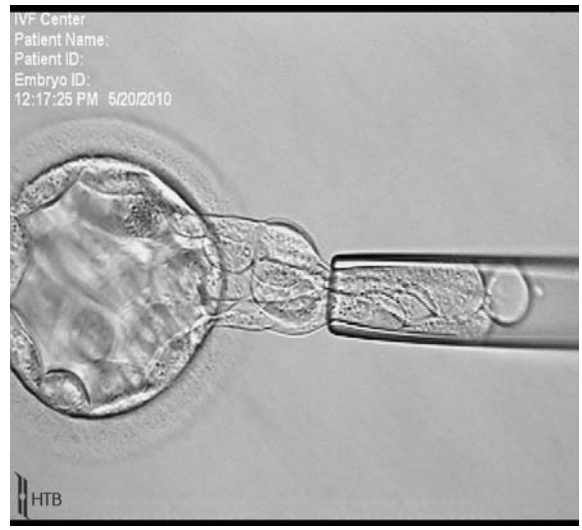


Figure 23.2. Herniated cells protruding through the zona pellucida are aspirated into a micropipette and the laser beam is targeted to the area just beyond the zona pellucida immediately in front of the micropipette opening.

and a microscope stage warmer provided the biopsy procedure time does not exceed 5 minutes, after which detrimental changes in pH and temperature are sure to occur.

On day 3 prior to transferring cleavage-stage embryos to extended culture medium, the zona pellucida is breached using a non-contact laser so that upon further development, formation and expansion of the blastoecoel cavity, trophectoderm cells will herniate through the opening (Figure 23.1). A small breach in the zona pellucida is made in an area of considerable perivitelline space. This is accomplished with 100% power and a 200-µs pulse from a Zilos-tk laser system (Hamilton Thorne Biosciences, Beverly, MA), creating a hole approximately 5 µm in diameter. A channel opening is created by firing 3–5 pulses in a row across the width of the zona pellucida. This size opening allows for sufficient herniation of the trophectoderm so that approximately four to eight cells can safely be removed. This is done in microdrops of culture medium under oil, followed by rinsing through a center well dish containing equilibrated extended culture medium. Afterward, embryos are transferred into new microdrops of fresh blastocyst-stage media for two additional days of culture. This procedure can also be applied to embryos previously frozen at the blastocyst stage; the zona pellucida is breached immediately after thawing, before the embryo has a chance to fully re-expand.

On the morning of day 5, blastocyst development is assessed and the expanding blastocyst undergoes trophectoderm biopsy. At this stage, a small number of trophectoderm cells can be seen herniating through the breach in the zona pellucida created on day 3. A holding tool and biopsy micropipette with a 30 µm diameter are required. It is imperative that all dishes are labeled with the corresponding number of the embryo being biopsied. Under the inverted microscope, with laser objective, the cells protruding through the zona pellucida are aspirated into a micropipette while the blastocyst is held by the gentle suction of the holding pipette (Figure 23.2). The laser beam, set for a 500 µs pulse at 100% power, is aimed and fired in the area of attenuated cells away from the inner cell mass just outside the opening of the biopsy micropipette. From 1 to 3 pulses are generally required to complete the process in conjunction with gentle pulling to separate the group of cells from the rest of the blastocyst.

After biopsy is complete, under supervision of a witness, each blastocyst is individually rinsed in equilibrated blastocyst medium and placed in separate appropriately labeled (name and embryo number) organ culture dishes for further culture until vitrification is performed. The biopsied cells are left behind in the appropriately labeled drop of the biopsy dish. Afterward, the cells are removed from the microdrops, under supervision of a witness, and processed according to the type of testing being performed

(i.e. microarrays, CGH, FISH). The requirement for a witness when transferring embryos and cells into appropriately labeled vessels cannot be stressed enough and is critical to avoiding misdiagnosis. Because chromosomal analysis (i.e. full embryonic karyotype) can take 2 to 4 weeks with the above-cited new platforms, embryo cryopreservation is required. We have adopted a vitrification protocol involving ethylene glycol and DMSO using a cryotop storage device [58, 59]. The entire process is repeated on day 6 of embryo development for embryos that have reached the blastocyst stage and are suitable for biopsy.

Blastocyst transfer following biopsy

Endometrial preparation is accomplished using oral contraceptives for 14 days followed by oral contraceptive and Lupron for an additional 5 days. On the third day of bleeding, transdermal estrogen patches begin. The dose is increased from 0.1 to 0.4 mg over the subsequent 2 weeks. When an adequate endometrial thickness is achieved and serum estradiol levels are appropriate, progesterone is begun either intramuscularly or vaginally. On the sixth day of progesterone administration, the blastocyst-stage embryos, which have been deemed chromosomally normal after genetic testing, are warmed and transferred. Generally, no more than two blastocysts are transferred at any one time due to their high implantation rates.

To date, over 200 patients have undergone blastocyst-stage trophectoderm biopsy and vitrification primarily for comprehensive chromosomal screening with subsequent warming and transfer. Biopsy was successful in 100% of embryos yielding between four and eight cells (mean five) for subsequent genetic analysis. Of these embryos, about half were diagnosed as aneuploid. No diagnosis was obtained on about 10% of the embryos. Embryo survival after biopsy, vitrification, and warming was excellent (97%). To date, 100 live births and many more ongoing pregnancies are currently under way. Currently, a prospective randomized trial for women of advanced maternal age or repetitive IVF failure is active to further assess the benefit of blastocyst-stage embryo biopsy in combination with vitrification and comprehensive chromosomal screening [54].

Conclusions

There are many factors to consider when performing biopsy procedures for preimplantation genetic testing: a culture system which is capable of development to the blastocyst stage, the information that will be provided from the sampled material in relation to the stage of development, the manner in which the zona pellucida is breached and the amount of material to be removed in order to obtain sufficient information without detriment to the embryo, and finally a successful cryopreservation method allowing for embryo transfer in a subsequent non-stimulated cycle. With the evolution and convergence of several technologies, embryo biopsy at the blastocyst stage offers many advantages. Fewer embryos progress to the blastocyst stage, even in the best culture systems, and as a result there are fewer embryos requiring biopsy. The fact that an embryo has developed to the blastocyst stage reflects its quality and hardiness and perhaps a higher tolerance for biopsy when compared to biopsy on days 1 or 3 of embryo development. Unlike polar body biopsy, assessment of the trophectoderm allows for an assessment of both the maternal and paternal contribution to the embryo's chromosomal status. Unlike cleavage-stage biopsy, the presence of several cells in the biopsied material allows for a larger DNA template to work with and therefore a better chance to get an accurate result and fewer embryos with "no result" outcomes. Mosaicism is thought to be less of a problem at the blastocyst stage as compared to the cleavage stage since several cells are analyzed, reducing the chance of missing mosiacism. The averaging of several cells into one sample allows low-level mosaicism to be filtered out. Furthermore, studies have shown excellent concordance in the ploidy of the inner cell mass and trophectoderm compartments, demonstrating that biopsy of trophectoderm, while not disturbing the inner cell mass, is reflective of the entire embryo's genetic constitution [53, 60].

In the future, this work may also allow for the development of non-invasive methods to assess embryo viability. At the blastocyst stage, spent culture media can be examined using metabolomic and proteomic assays, and these can be correlated with the chromosomal status of that same embryo. It is hoped that such correlations may some day allow non-invasive means to predict embryo viability at a similar rate to what is still an invasive method of genetic diagnosis. Further gains in embryonic implantation potential may be seen as a result.

Couples who have had previous IVF failures represent a difficult and challenging group of patients. Blastocyst culture and trophectoderm biopsy for

aneuploidy screening represent an effective way to assess such patients. If these patients fail to achieve blastocyst development in an excellent sequential culture system in addition to previously failed day 3 embryo transfers, their cause of IVF failure is self-evident. If blastocyst formation does occur, aneuploidy screening will determine whether any of the embryos developing are in fact chromosomally normal. If chromosomally normal blastocysts are obtained and implantation continues to fail, a uterine factor must be considered. The diagnostic potential of this platform allows patients to resolve their history of IVF failure and move onto appropriate therapies such as egg donation or the use of a gestational carrier as appropriate. Finally, as the field of IVF worldwide continues to increase the emphasis on single embryo transfers, a greater demand will be placed on clinicians and embryologists to determine which single embryo has the greatest developmental potential. Blastocyst culture, trophectoderm biopsy and comprehensive chromosomal screening represent a way forward toward that goal. Highly efficient cryopreservation systems are also required to reduce the pressure on patients to transfer more than one embryo at a time. These patients will have the comfort of knowing that the transfer of a single blastocyst after vitrification and chromosomal screening will achieve a high pregnancy rate. The couple can continue to thaw and transfer one embryo at a time until their family is complete, while avoiding multiple gestation.

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Chapter 24

Analysis of the embryonic transcriptome

Claude Robert, Andrew J. Watson, and Marc-Andre Sirard

Introduction

The "omics" revolution has had immense impact on our investigation of preimplantation mammalian development [1–11]. Several studies using diverse microarrays have reported the characterization of the embryonic transcriptome [1, 2, 5–11]. To date it has been difficult to fully integrate the large dataset that is provided by these studies into practical advances that assist in our selection of viable embryos for embryo transfer due to numerous technical considerations that mostly arise from the unique nature of oocytes and early embryos. However, virtually every transcript in which the abundance dramatically fluctuates during early development or, better yet, is turned on or turned off during the first week of mammalian development has been cataloged and identified by the "omics" during mouse preimplantation development [1, 2, 5–11]. The approach is almost equally complete for early embryos of other species such as the sheep, cow, pig, and even the human preimplantation embryo [5, 7, 12–17]. However, with our current state of knowledge, the complete description of transcript-level fluctuations during early development is far from being complete nor does it provide sufficient information to reliably sort preimplantation-stage embryos into different development competence classes for embryo transfer. Nonetheless, experiments have quickly advanced beyond comparatively simple embryo stage specific genome characterization to now using the technologies to address questions such as how do specific treatments affect embryonic gene expression [18]? What impact does the embryo's environment have on embryonic gene expression [19, 20]? How do gene expression patterns vary between gametes and early embryos with different developmental competencies [18, 21, 22]? And perhaps the most important questions of all, how can we use (?) or should we use (?) this technology in the fertility clinic to assist in determining embryo fitness for development and initiation of pregnancy before embryo transfer [1, 2, 7, 13]?

The "omics" are important tools for investigating every step of a cellular response to treatment including chromatin remodeling and DNA sequence modifications by either surveying thousands of known polymorphic regions (using for example SNP chips) or by systematically measuring DNA copy number variants or abnormalities in chromosomal structure and number (using for example tiling arrays). This type of analysis is typically also used to survey transcribed elements either coding for proteins and/or for non-coding RNAs which are an increasing focus of investigation. A third critical level of "omics" research includes the survey of protein composition, giving rise to the term proteomics, while the potential to investigate a large spectrum of cellular outputs or metabolites has sparked the field of metabolomics [23–27].

This chapter will focus on the application and methods used to analyze the embryo's transcriptome. Two of the authors (Robert and Sirard) are experts in the application of these technologies to investigate the transcriptome of mammalian gametes (oocytes) and early embryos, while the third (Watson) has investigated the regulation of gene expression in the preimplantation embryo and has become interested in exploring the ethics of how such technologies can be applied in the clinical setting.

Gene expression during preimplantation development

The basic genetic program controlling preimplantation development includes activation of the

Human Assisted Reproductive Technology: Future Trends in Laboratory and Clinical Practice, ed. David K. Gardner, Botros R. M. B. Rizk, and Tommaso Falcone. Published by Cambridge University Press. © Cambridge University Press 2011.

embryonic genome, compaction, and cell lineage specification, and cavitation or blastocyst formation [2, 7, 28, 29]. Activation of the embryonic genome occurs characteristically quite early during mammalian preimplantation development, varying with the species, but certainly by the 2-cell stage in the mouse, 8-cell stage in the human, and 8–16-cell stage in the cow and sheep [12, 21]. This event is complex and represents to this day an intensive area of research, but generally involves genome-wide chromatin remodeling due to histone/DNA demethylation and histone acetylation/deactylation changes [12, 21]. These changes occur either before fertilization in the oocyte or coincident with fertilization. Embryonic genome activation encompasses a shift from a "repressed" state characteristic of oocyte chromatin to a "permissive" state required for transcriptional activation and embryonic gene expression. Activation of the embryonic genome allows the preimplantation embryo to assume control over its developmental program, but despite a large turnover of oogenetic mRNAs and proteins that also occurs during this event, the remaining oogenetic products still have an important contribution to at least the preimplantation developmental program and probably even beyond [2, 28, 30]. Following a series of cleavage divisions, the embryonic blastomeres become tightly associated with one another by the formation of stable cell to cell adherens junctions [2, 28, 30]. This formation of adherens junctions signals the onset of compaction, which is characterized by a loss of individual blastomere outlines, which results in the formation of a morula [2, 28, 30]. Compaction also signals the beginnings of cell differentiation as by the 16-cell stage the embryo has sufficient cells to result in the distribution of both outer (cells with a free apical surface) and inner (cells completely enclosed on all sides) cells [2, 28, 30]. The outer cells will become the epithelial trophectoderm while the enclosed inner cells retain their pluripotency and become the inner cell mass [2, 14, 28, 30]. Recent research has greatly improved our understanding of these events, as it is now known that trophectoderm differentiation is governed by expression of transcription factors such as TEAD4, Cdx2 and GATA 3 while the pluripotency of the inner cell mass is associated with a retention of OCT4 expression [2, 4, 14, 28, 30–33]. Cavitation is orchestrated by the trophectoderm as it matures to a true epithelium by the appearance of functional apical tight junctional complexes, basolaterally localized transporters such as the Na/K-ATPase, and the expression of water channel aquaporins localized to both apical and basolateral membrane domains that collectively allow water to cross the epithelium and become confined to a fluid-filled cavity that defines the blastocyst structure [5, 7, 29, 34]. While these details represent the foundation of our understanding of the genetic mechanisms controlling preimplantation development, the more recent application of transcriptome analysis to stage-specific early embryo pools has clearly illustrated the full complexity of the gene expression patterns governing preimplantation development [7].

The preimplantation embryo transcriptome

The transcriptome: what do we know already?

The transcriptome includes all transcribed elements [1, 7, 29]. This definition involves the inclusion of every type of RNA known so far. Traditionally, the focus has been on the study of transcripts bearing protein-coding sequences [1, 7, 29]. However, mRNAs only represent a fraction (2–5% in somatic cells) of the entire RNA content. The other RNA types include ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), and a group of RNA molecules involved in transcript maturation located in the nucleus and nucleolus respectively called small nuclear RNAs (snRNAs), and small nucleolar RNAs (SNoRNAs) [35–37]. In recent years, deep sequencing studies have identified other types of non-coding RNAs (ncRNAs) that have been categorized according to their length as either small (generally less than 100 nucleotides) (sncRNAs) or long (lncRNAs) [35–37]. The discovery that these sncRNAs are actively regulating gene expression either by targeting the specific destruction of endogenous RNAs (such as small interfering RNAs (siRNAs)) or by modulating the translation of mRNAs (this function is being conducted by microRNAs (miRNAs)) by binding specific sequences in their untranslated regions to block or to activate the translation machinery has sparked tremendous research interest [35–37].

The discovery of sncRNAs is paralleled by the identification of lncRNAs [38]. Currently, the role of these longer RNA molecules is still mostly uncharacterized. However, early embryo development has been

the focus for the characterization of three of the most studied lncRNAs. The first two are the X-inactive specific transcript (Xist) and its complementary counterpart (Tsix) which are major effectors of X inactivation in mammalian females [39]. Furthermore, another lncRNA is strongly suspected to be involved in a pathological outcome from in vitro culture in the bovine as H19, a lncRNA partner of Igf-2 in a switch locus epigenetically controlled, is abnormally expressed in the large offspring syndrome [40]. These examples illustrate that the transcriptome still holds many secrets to be revealed and that even our cursory understanding of their function indicates the significant contribution these ncRNAs play in early embryo development [36, 39, 41].

The unique nature of the oocyte and early embryonic transcriptome

The mammalian oocyte and preimplantation embryo represent unique biological systems where total RNA content, cell size, cell numbers, and transcriptional activity differ dramatically between developmental stages [2, 7, 9, 28, 42]. For example, during oogenesis, the gamete accumulates large amounts of RNA that are stored in ribonucleo-complexes [2, 7, 9, 28, 42]. The stored messenger RNAs are de-adenylated (although we still do not know the extent of the de-adenylation) and are re-adenylated upon recruitment for translation or simply sent off for degradation [2, 7, 9, 28, 42]. This implies that the mRNA population coding for the same gene may be composed of molecules bearing a poly(A) tail of various length [2, 5]. This situation has profound impact on sample-handling strategies as explained below.

A second consideration unique to early embryo development is that the transcriptional activity of the blastomeres shifts dramatically prior to and following maternal to embryonic genome activation (MET) [2, 5]. Upon MET, the genome rapidly becomes transcriptionally active, therefore allowing the embryo to control its own developmental fate and adjust its program to changing environmental conditions [7, 9–11, 15, 43–46]. The transcriptional status of the embryo is a major factor regarding the interpretation of transcriptomic data since variations in RNA abundance during early development do not occur only due to transcription.

A third and most important issue when dealing with samples containing early embryos is data normalization [7, 9–11, 15, 43–46]. This is especially true for across-stage comparisons since cell sizes and numbers differ [43]. In addition, total RNA as well as polyadenylated RNA content fluctuates during development and does not follow a linear pattern [47, 48]. This raises the question of whether we are comparing apples with apples or apples with oranges when we apply this analysis to contrast transcript levels between embryo stages (Figure 24.1). What are the precise criteria that allow datasets to be compared? Some have suggested the use of standard housekeeping gene candidates [49], cell numbers [50]. or fixed amount of total RNA [22, 46, 51]. So far, none of the proposed methods has been fully satisfying since all housekeeping gene candidate standards fluctuate during preimplantation development, resulting in biased variations in normalizing the outcomes, and the other options result in a loss of physiological relevance [10]. Indeed, are two 2-cell embryos comparable to a 4-cell-stage embryo? If RNA abundance differences are observed, should the data be adjusted by cell number and reported on a per cell basis? This cannot be easily done since 4-cell embryos in many species other than the mouse contain less RNA than 2-cell embryos even though 4-cell embryos have twice the number of blastomeres (Figure 24.2).

Furthermore, in a microarray context, relative quantitation of the outcomes can only be applied if the hybridization kinetics are identical for all probes and the output fluorescence is linear, which is not usually the case. Thus the debate regarding how to

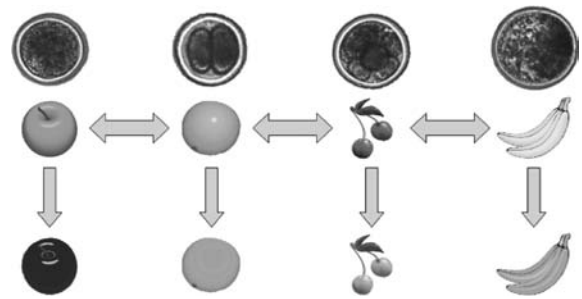


Figure 24.1. Challenges associated with contrasting transcriptomes across embryo stages. Preimplantation development is fraught with dramatic changes in cell number and size as well as very distinct intervals in terms of nuclear activity. As a consequence, the RNA composition fluctuates greatly between developmental stages. Sample processing can thus profoundly impact the physiological relevance of the downstream transcriptomic data. It is expected that within-stage comparisons (green apple vs. red apple) will be less prone to the introduction of methodological biases than inter-stage comparisons (apple vs. orange). See colour plate section.

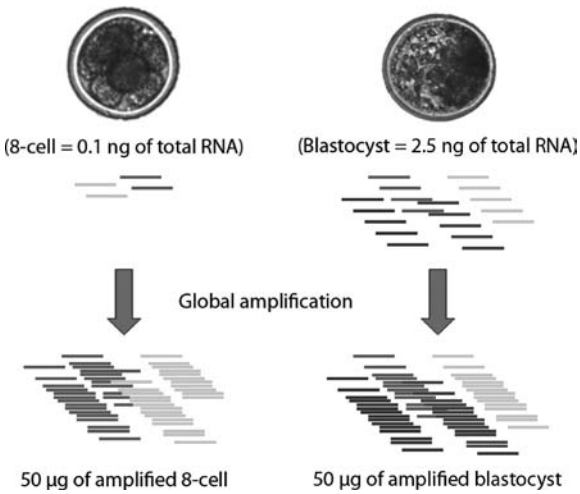


Figure 24.2. Loss of physiological representation following sample amplification. Due to the unique nature of preimplantation development, additional care must be taken to ensure sample processing does not result in a loss of physiological relevance. The global amplification step most often yields a constant output regardless of the RNA content of the input sample. This proportionally skews the downstream gene expression data. See colour plate section.

generate accurate mRNA quantitation results during preimplantation development is currently not resolved. A solution may reside partly in the use of exogenous controls [4] and/or the use of a normalization strategy considering multiple metabolically unrelated housekeeping candidates to normalize according to the geometric mean [6, 7, 9, 10, 18, 22, 43, 46–48, 51–53].

Transcriptomic platforms

Transcriptomic analysis is currently undergoing a renaissance as many are predicting the decline of microarray use, which could soon be replaced by new deep sequencing approaches. Many of the methodological considerations that must be considered regarding mining and interpreting microarray datasets also apply to deep sequencing. The use of deep sequencing to survey the transcriptome is still in its infancy but an important bottleneck is slowly surfacing as the explosion of data it generates requires the expertise of a strong bioinfomatic and biostatistic core which is not accessible to many laboratories. Additionally, in order to truly claim we have reached an acceptable depth in the coverage of a transcriptome would technically include the ability to decipher mRNA splice forms which would require several tens of millions of sequence reads especially for Solexa and ABI Solid platforms. To perform this coverage with the required biological replication remains unaffordable for many laboratories. However, it is clear that with the current and constant rapid strides in the development of sequencing technologies, the opportunity presented by deep sequencing for transcriptome analysis is almost certainly going to grow in the long term.

The past decade has seen the rise and fall of cDNA microarrays, mostly developed using academic infrastructures by independent research teams to be replaced by commercial oligo-based microarrays. The cDNA microarrays are often referred to as "boutique" since they contain a limited set of candidates selected on the basis of their suspected involvement in the phenotype under study. The oligo arrays (25 to 70 bases depending on the synthesis technology) have become the standard for species that have had their genome sequenced. The gene collection has been organized in silico and oligos are designed following specific rules to minimize cross gene overlap. The commercially available microarrays are currently dominating the market and have the benefits of containing larger numbers of probes (sometimes more than 1 million) and their production is subjected to manufacturing quality controls that ensure batch to batch reproducibility.

Over the course of microarray development, we have utilized nearly every type of genomic technology from boutique in-house printed cDNA arrays (the bovine BlueChip) as well as oligo arrays from academic consortiums and commercial oligo arrays (Afymetrix and Agilent). They were all successfully used and have generated valuable information on oocyte maturation, developmental competence, and embryonic development [6, 7, 9, 10, 18, 22, 43, 46–48, 51–53]. However, the generated datasets are hardly comparable to one another due to the numerous fundamental differences in technologies that make comparison across platforms very troublesome. This situation has led to our ongoing effort to develop embryo-specific oligo arrays derived from deep sequencing results where the printing is outsourced to a microarray manufacturer (Agilent). This microarray developed from the EmbryoGENE project will consider attributes specific to the embryo (such as the inclusion of splice variants) as well as specific to the sample processing procedures, namely the global amplification that is required when starting with such small amounts of biological material.

RNA sample collection: single embryo? Pools? Or cell biopsy?

Preimplantation embryos from all mammalian species do not represent abundant sources of mRNA. For example a mouse oocyte or blastocyst contains approximately 1 ng of total RNA. Since the protocols for transcriptome analysis using most "omics" platforms requires μg quantities of mRNA the application of these methods to defining the preimplantation embryo transcriptome requires the inclusion of a global mRNA amplification step that increases the template sufficiently to enable array hybridization [6, 7, 9, 10, 18, 22, 43, 46–48, 51–53]. Thus in all cases, whether embryo pools, single embryos, or even embryonic blastomeres (in the case of applying diagnostic analysis prior to embryo transfer), there is insufficient starting material to proceed directly to transcriptome analysis (see next section for details). This requirement is important as it imposes additional constraints on the interpretation of the outcomes from such experiments [6, 7, 9, 10, 18, 22, 43, 46–48, 51–53]. The mRNA amplification step must be applied with great care to ensure that relative mRNA abundance in the original sample pool is minimally affected. Also the outcomes regarding changes in transcript abundance either across embryo stages or following specific treatment regimes must be validated by subsequent experiments employing q-RT-PCR (see below for details). If these constraints are understood and handled properly then the application of these methods to embryo pools, single embryos, or embryo cell biopsies can be done to generate informative data regarding the global embryonic transcriptome [6, 7, 9, 10, 18, 22, 43, 46–48, 51–53]. The entire process is summarized below.

RNA isolation and amplification

Since the mRNA population found in early embryos, especially prior to the MET, is heterogeneous with regard to poly(A) tail length, the RNA extraction method can profoundly influence the downstream RNA abundance results [6, 7, 9, 10, 18, 22, 43, 46–48, 51–53]. Some RNA extraction procedures are designed to selectively isolate RNA bearing a poly(A) tail (such as magnetic beads-based applications). The minimal poly(A) tail stretch required for binding to the "capture" system is undefined but any type of segregation of the RNA molecules will definitely result in the study of a subpopulation of the entire set of mRNAs [1, 4, 7, 9, 15, 52].

The next sample processing step is to globally amplify the sample to generate sufficient output for microarray hybridization [43]. Regardless of the method used, the first step is to reverse transcribe the RNA to produce cDNA that will be used as template for the amplification. This step is also key as the priming strategy, either using random hexamers or targeting the poly(A) tail with oligo dT, can also select a subpopulation of transcripts leading to divergent downstream results. This is particularly important in oocytes and early embryos where the mRNA poly(A) tail often shortens during storage at low temperatures.

We have demonstrated that the amplification step can be a major source of artifact especially when the targeted comparisons involve embryos of varying stages and treatments [43]. This is caused by the plateau phase, which is reached within the first hour of the amplification reaction [43]. This homogenizes the amplification outputs regardless of the initial RNA content of the input biological samples and, as a consequence of this equalization, the natural differences in RNA content that were present prior to the amplification are lost, eliminating the physiological relevance of the downstream analysis [43]. When comparing the RNA content of germinal-stage oocytes and 8-cell embryos by following standard procedures, the microarray results yielded a false discovery rate of more than 60% (Figure 24.2) [1, 4, 8, 9, 15, 34, 43].

Array hybridization

Once sufficient amounts of cDNA have been produced, the labeling can be performed following diverse procedures [1, 4, 8, 9, 15, 34, 43]. The addition of the fluorescent dye is not a considerable source of variation. By contrast, hybridization of the microarrays using equal amounts of each sample is another process that results in the loss of the naturally occurring differences in RNA abundance. As mentioned, this issue is less problematic with samples of a similar type (i.e. comparing two cells to two cells; Figure 24.1) but can have profound consequences on the value of the data when the samples are naturally divergent [1, 4, 8, 9, 15, 34, 43]. This bias can be effectively controlled by the introduction of known amounts of exogenous control RNAs that are used to standardize the dataset and recover the initial physiological representation [10].

Data analysis

Following the scan, the fluorescence values must be pre-processed to account for background correction, intra-array, and inter-array normalization. The literature contains an ever-growing list of algorithms for microarray data pre-processing [6, 20, 45–48]. The normalization strategy is directly linked with the platform as well as the experimental design. Two-colour arrays are handled differently than single colour and the design influences replication. However, it can be stated that the choice of normalization procedures must fit not only the platform and design but also the biological samples' intrinsic characteristics. For instance, many normalization algorithms are based on assumptions that may not be suitable. The widely employed Loess normalization assumes that only a small fraction of genes are differentially expressed and that the proportion of candidates over-expressed is equal to the number under-expressed. Such conditions may not apply to early embryos. For example, it is expected that the oocyte has larger pools of mRNAs than the 8-cell-stage embryo for most species in addition to the fact that following depletion of the maternal RNA, it is also expected that most mRNAs would be found at a higher level in the oocyte comparatively to the 8-cell embryo in most species. Therefore, the normalization assumptions may not apply in this case.

Validation of array outcomes (qRT-PCR)

This vital step is generally performed by testing a set of candidate gene transcripts, usually a handful, through quantitative RT-PCR [6, 13, 20, 24, 45–48, 54]. The fact that this method is very different from the common microarray procedure is an important benefit when transcript variation between samples is confirmed but it may also be troublesome when the two approaches provide different conclusions. The discrepancies in the conclusions between the methods can arise from methodological considerations as well as being representative of some level of imprecision in the gene list. Due to the more direct assessment of RNA level, qRT-PCR is accepted as the gold standard for validating a microarray dataset. Ideally the samples should be different (i.e. experimental replicates) than the ones used for microarray analysis to ensure that problems in the initial handling of the samples are not masked by the qRT-PCR (such as sample quality degradation).

Transcriptome analysis vs. physiology

In addition to all the precautions mentioned above concerning the way mRNA relative abundance is measured, it must be emphasized that variations in mRNA levels do not necessarily convey a physiological change in gene function. This is especially true in oocytes and early embryos (before the embryonic genome activation) where RNA can be stored and translated later. In some cases the level of RNA may parallel the level of protein in the embryo but in other cases it is the opposite, where RNA levels decrease as the protein level rises from translation followed by degradation. Therefore before any possible conclusion is made regarding function and the physiological effects of variations in mRNA levels, one must investigate the protein level and when possible even protein function as post-translational modifications such as phosphorylation are often required for protein function and thus physiological changes in embryonic development.

Integration into the fertility clinic: what do we need to know?

Given the range of significant concerns regarding the use of this methodology as a diagnostic method you may be surprised to discover that there is great interest from fertility patients and clinicians to apply these technologies in the clinic as tools to improve our ability to measure embryo developmental competency and increase overall pregnancy rates following embryo transfer. The forces propelling their application in the clinic include: the limitations of using morphological criteria and systems to assess embryo developmental competence; the increasing and important trend of employing single embryo transfer each cycle to minimize the production of "multiple" pregnancies; and a legitimate desire to use the global power of transcriptome analysis to minimize the chances of transferring embryos that may be carrying deleterious mutations or have a high susceptibility to the onset of major disease later in life.

Transcriptome analysis will introduce several other major changes to current clinical practices. The clinic must incorporate "state of art" embryo culture methods (other chapters in this issue) to foster development to the blastocyst stage; and incorporate "state of the art" trophectoderm biopsy methods in their program. It is expected that transcriptome analysis

would be developed in commercial diagnostic laboratories and biopsied samples would be sent to these labs for analysis. The time required for this to be conducted and the data analysis would impose a requirement for blastocyst cryopreservation before embryo transfer.

The application of trophectoderm biopsy instead of 8-cell-stage blastomere collection is preferred as the embryo is better able to survive the procedure at the blastocyst stage and no embryo progenitor cells, i.e. inner cell mass cells, are affected. The employment of 8-cell-stage biopsy methods has been severely criticized due to the large variation in chromosomal number that exists between early embryonic blastomeres and thus the poor representation that using a single cell provides for determination of overall embryonic health. Thus the ability to sample from more than one embryonic cell (3–8 trophectoderm cells can be safely removed if the skill required to successfully accomplish this is available) provides the obvious advantage that a more representative sample of the embryo's transcriptome can be made, but great care must still be applied to not over-interpret the outcomes. The most challenging aspect of offering this technology in the clinic is the ability to produce reliable results and interpret the outcomes properly to use that information to decide whether the embryo is to be transferred or not.

Ethical concerns on integrating transcriptome analysis in the clinic

The ethical questions regarding the introduction and application of these technologies into the fertility clinic include asking: how reliable will the data be? How will the data be interpreted? What criteria will be developed to assist clinicians in making appropriate decisions regarding whether to transfer the embryo or not? It is our position that our current ability to interpret the cumulative data from the application of global transcriptome analysis to preimplantation embryos for any species is insufficient to make a fully informed decision regarding which embryo to transfer. We expect this situation to change progressively in the coming years though. Bioinformatics and gene cluster analysis is improving at a rapid pace and our understanding of how gene networks collaborate to affect cellular and embryonic health is progressing rapidly. To apply this technology ethically in the clinic we must increase our comprehension of the variation in gene expression patterns that matter and truly affect the ability of a preimplantation embryo to initiate a pregnancy or not.

Thus in the end and perhaps not surprisingly we conclude that we must continue to support and push research efforts in this area so that the scientists can supply the vital information that clinicians will need to make fully informed choices and assist their patients in the best way possible to resolve their infertility.

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Chapter

25

Analysis of embryo-derived factors as markers of developmental potential and viability

Chris O'Neill

Introduction

Since the inception of IVF it has been an attractive notion that the preimplantation embryo may release into its culture media a molecular "fingerprint" that allows the prediction of its developmental capacity. The need for such a non-destructive diagnostic tool arises from the relatively poor developmental potential of many embryos produced in vitro. It is reasoned that a non-destructive means of identifying the cohort of embryos with greatest developmental potential would allow these to be selected for transfer to the uterus. This would be expected to improve pregnancy rates, reduce the temptation to transfer multiple embryos (and hence the risk of multiple pregnancies) and result in a reduction in both the morbidity and costs associated with these procedures. Several candidate antigens have been identified, yet to date a definitive marker for the embryo's developmental fate has not been rigorously confirmed.

Perhaps the limited success in developing a test for viability is not surprising given the small size of the embryo. The amount of biological material released by a few cells creates a significant technical challenge in achieving a quantitative signal of sufficient strength. Yet efforts to date do provide encouragement for the concept and ongoing investigation of this question. The development of such markers has proceeded along two relatively independent tracks. One track has been the rational approach of using conventional cell biological methodology to investigate the nature of normal embryo development and from this understanding identify likely targets for investigation (for example, immunomodulatory agents, bioactive trophic ligands). The second approach has been to use empirical screening approaches without a-priori assumptions of the likely targets (for example, metabolomic and proteomic screening). Both approaches have their strengths and weaknesses and it is too early to assess which will be the most likely to yield a useful diagnostic tool. This chapter focuses on the embryo's bioactive secretome. It aims to provide a brief overview of the early developments in the field, an analysis of the current state of development of diagnostic markers for embryo development and discuss the limitations in their development. This paper will also provide a personal perspective of the future challenges to development and considers the broader benefits and risks that may accrue from a detailed analysis of the embryo's secretome.

Historical perspective

It is now accepted that there is an active bi-directional dialog between the embryo and the female reproductive tract (and beyond) which commences soon after fertilization. This picture gradually emerged during the 1970s and 80s and became firmly established in the 1990s. By the 1970s there was much experimental interest in the question of how the embryonic "allograft" survived within an immunologically alien female reproductive tract. Of the many hypotheses under consideration at that time, one was the possibility that the embryo released immunosuppressive agents that caused modulation of the maternal immune system allowing the embryo to escape its effects. Testing this hypothesis revealed a soluble embryo-dependent

Human Assisted Reproductive Technology: Future Trends in Laboratory and Clinical Practice, ed. David K. Gardner, Botros R. M. B. Rizk, and Tommaso Falcone. Published by Cambridge University Press. © Cambridge University Press 2011.

activity within serum of pregnant females with putative immunosuppressive effects [1]. This activity was termed "early pregnancy factor" and was detected in many species. The remarkable feature of this activity was that it appeared within hours of fertilization [1]. The molecular characterization of early pregnancy factor has always remained controversial, yet an important feature of the activity was that a component of its activity was released by the newly fertilized zygote (ovum factor) [2].

Based upon this observation, but adopting the hypothesis that the putative immunosuppressive effect was an epi-phenomenon of a more fundamental physiological response to early pregnancy, a new investigation of the phenomenon established that the embryo-derived component was a potent platelet-activating factor (Paf) [3–6]. Embryo-derived Paf was capable of inducing a transient thrombocytopenia in peripheral blood within hours of fertilization in a range of species, and also sometimes caused a rebound thrombocytosis [7]. This factor was identified as the ether phospholipid 1-*o*-hexadecyl/octadecyl-2-acetyl-*sn*-glyceryl-3-phosphocholine [6, 8]. This was the first chemically defined biologically active agent to be identified as released by the preimplantation embryo. As well as inducing a wide range of changes in maternal physiology, it was found that a fundamental role for Paf was its action back on the embryo to create an autocrine loop which promoted the survival of the embryo [9, 10]. After the discovery of Paf, a number of other putative autocrine trophic ligands and their receptors were also discovered to be synthesized by the embryo [11, 12].

These observations were made around the time that human IVF was becoming a mainstream medical therapy for infertility. Some anecdotal observations indicated that communal culture of human embryos may be more beneficial than their individual culture. Animal studies showed that the benefits of communal culture arise from the required actions of autocrine trophic agents released by embryos into their media [9, 13, 14]. Paf is the best characterized of the embryo-released trophic factors and it was shown, first in mice [15, 16] and then in humans [17, 18], that adding Paf to media improved the embryo's metabolic rate, morphology, cell number and implantation and development rate following embryo transfer. Similar findings were subsequently found for other putative autocrine embryotrophins such as the insulin-like growth factors [19], LIF [20, 21] and EGF [20]. It was also shown that reducing the activity of these autocrine embryotrophins reduced the developmental potential of treated embryos. This important role for the released trophic agents argues for their potential role as reliable markers of embryo viability. To date the full trophic effect of communal culture has not been accounted for by the limited range of ligands identified. The use of mass spectrometric analysis of embryo-conditioned media has identified a more diverse secretome [22, 23]. Factors released include lactate dehydrogenase, a range of enzymes implicated in covalent modification of proteins and a number of proteins that govern the cell's redox state [23]. Functional analysis of the embryonic role(s) of these secreted products is yet to be reported.

A number of studies showed that embryo-conditioned media that contained immunomodulatory activities were not obviously accounted for by the then defined secretome [24]. HLA-G is a non-classical major histocompatibility antigen. This antigen is released by a range of cell types and has immune tolerogenic actions [25]. This gene was expressed by the early embryo [26], its expression levels showed an association with embryo viability and the antigen was present in some media conditioned by the fertilized human embryo in vitro [27]. Other surface antigens that modulate the immune response, including immunoglobulin superfamily members, such as ICAM, are also expressed by the oocyte and early embryo [28]. ICAM is also released by the human oocyte and IVF embryo into media [29].

Biological roles for the embryo's secretome

It is now clear that the embryo releases a host of antigens during its culture in vitro. To date there has been very limited analysis of the functions of the embryo's secretome, and much is left to explore in this area. To date the functions of Paf and HLA-G have been studied in most depth.

The mammalian embryo is unusual in that much of the Paf they make is released as a soluble mediator [30]. There is currently a poor understanding of the mechanisms of release of Paf by cells and there is no satisfactory explanation of why some cells release Paf while others primarily retain it. Following its synthesis within the intracellular organelles, Paf is moved to the inner leaf of the plasma membrane, followed by its transbilayer movement to the outer leaf of the

plasma membrane, possibly under the regulation of cellular transglutaminase [31] and/or the actions of P-glycoprotein [32, 33] depending upon the cell type involved. Upon translocation to the outer membrane, Paf is available for release. The ether-linked, long-chain alcohol at C1 and the acetyl group at C2 of the glycerol backbone cause Paf to behave differently than C1 and C2 long-chain esterified membrane lipids [34]. Paf is less hydrophobic than many membrane lipids [35] and is therefore more readily lost from the membrane surface. Paf's release requires the actions of an extracellular protein acceptor, and it seems that this role is primarily fulfilled by serum albumin [30, 36].

The amount of Paf released by the embryo increases as the extracellular albumin concentration increases [10, 30]. In the mouse, the culture of zygotes reduced the amount of Paf released by embryos compared to those collected at the 2-cell stage, and the production of embryos by IVF further exacerbated this. As a consequence IVF embryos on average released around 20-fold less Paf than those developing in the oviduct and seven-fold less than those fertilized in the oviduct and then cultured in vitro [10, 30]. This was not associated with a reduction in the amount of Paf retained within the embryo and a rate-limiting enzyme for Paf synthesis (lyso-Paf acetyltransferase) was acutely upregulated (~ 10-fold) in IVF embryos compared with those fertilized *in situ*. It seems that the primary lesion induced by IVF and culture is to perturb the capacity of the embryo to release Paf in vitro. Expression of the Paf-receptor required new transcription from the embryonic genome and culture of embryos, irrespective of their method of fertilization, caused a marked retardation in this expression [37]. Thus, fertilization and culture of the embryo had the combined effects of reducing Paf release and retarded Paf-receptor expression, leading to a variable breach of this autocrine loop. Culture and IVF have also been shown to decrease or delay the expression of other putative autocrine ligands in the mouse, such as Igf1 and Igf2 [38, 39].

Paf acts on a membrane receptor as early as the zygote stage to induce characteristic transient increases in the embryo's cytoplasmic calcium concentration. These transients occur with a periodicity of about 90–120 min [40]. The transients result in the simultaneous activation of membrane potassium and chloride channels [41, 42], and all these responses are necessary for normal embryo development. The calcium transients cause calmodulin-dependent activation of CREB which then governs transcription from the embryonic genome [43]. The Paf-receptor activates the 1-*o*-phosphatidylinositol-3-kinase signaling pathway. This leads to the improved survival of embryos by inducing the latency of TRP53 expression via the action of AKT and MDM2 [44, 45]. The autocrine loop activated by the release of Paf by the embryo results in the improved survival and growth of the preimplantation embryo. A range of the other putative autocrine and paracrine embryotrophins also seem to act in a similar fashion. The extent that their action overlaps with those of Paf or exerts discrete functions of their own requires detailed investigation.

Genetic analysis of the regulation of embryo mouse development identified a critical role for a HLA-G homolog (*Ped*, Qa-2) [46, 47]. *Ped* gene expression resulted in improved preimplantation embryo growth and development rates. *Ped* codes for the non-classical class 1b MHC protein, Qa-2, which is considered to be a functional homolog of the human HLA-G antigen [48]. HLA-G has few polymorphisms and therefore does not function as a classical histocompatibility antigen. It is a cell surface protein, lacking a cytoplasmic tail but having linkage to the membrane lipid glycophosphatidylinositol on the outer membrane [48]. This form of attachment allows for its shedding into the extracellular milieu. Analysis of the HLA-G gene expression in the early embryo shows a complex pattern of expression of alternatively spliced variants. Yet a different pattern of HLA-G protein forms was detected and it is proposed that the early embryo's protein content is largely carried over from maternal stores within the oocyte [49]. If this is the case then the amount of HLA-G released during the cleavage stages may more accurately reflect oocyte quality. This does not invalidate its use.

An essential tolerogenic role of preimplantation HLA-G is not established. In the mouse model embryos that express Qa-2 grow at a faster rate than those without the antigen [47]. Human IVF embryos that reached the blastocyst stage of development earliest tended to have higher levels of HLA-G [26]. Yet, such embryos grow in entirely defined media where no interaction with the maternal immune system is possible. This argues that the released HLA-G is associated with a function that is largely independent of its putative tolerogenic actions. From this perspective it is of interest that in mouse T-cells the cross-linking of Qa-2 on the cell membrane induces

signaling events via membrane lipid raft-associated intracellular signaling which, under appropriate experimental conditions, can induce cell proliferation [50]. Removal of surface antigen with phospholipase C caused embryos to grow more slowly than controls [47]. Furthermore, in the mouse model, the expression of the *Ped* gene caused a marked negative regulation of miR-125a, a microRNA implicated in growth regulation in a number of settings [51]. These observations suggest that in the early embryo HLA-G/Qa-2 may serve pro-proliferative functions at the membrane independently of immune modulation. Definitive evidence on this point is awaited.

If a pro-proliferative function relates to the membrane-bound HLA-G fraction, as seems to be the case in T-cells, this leaves the role of the secreted component an open question. Does it serve some function in its own right or is it merely a reflection or artifact of the embryo's proliferative activity? Some studies show that HLA-G expression is not strongly associated with the embryo's morphological quality [52] but is higher in faster-growing embryos [53]. Definitive evidence that the release of the HLA-G antigen is biologically relevant for the development of the embryo, rather then an epi-phenomenon of its surface expression and signaling, would add weight to an argument for its utility as a marker of embryo viability.

An interesting link between HLA-G (*Ped*, Qa-2) release and Paf release is the observation that *Ped*-negative mouse embryos released significantly more Paf into media than *Ped*-positive embryos [54]. The molecular basis for this difference has not been defined but the result suggests the possibility of some form of compensatory interaction between the two regulatory systems. If this is the case then it will be worth assessing whether the combined measurement of these two putative markers provides a better measure of developmental potential than either one alone.

Analysis of evidence for the role of biomarkers of embryo viability

The early studies of Paf in media used bioassays which relied upon the detection of platelet activation. In the first such study media conditioned by human IVF embryos cultured for 36 h after fertilization were analyzed [55]. Media from 12 embryos that were known to have resulted in an ongoing pregnancy released more than three times the level of Paf compared with embryos known not to have resulted in an ongoing pregnancy. For a total of 85 embryos where their fate could not be unequivocally identified (because more embryos were transferred than resulted in ongoing pregnancies), 43% of media had Paf activity in the same range as those embryos that caused a definitive pregnancy. Since this was a higher percentage than the incidence of pregnancy it indicated that there was a high false-positive rate, that is, many embryos that produced Paf in the "pregnant" range did not result in pregnancy. This indicated either that the tool has poor discrimination, or that there are factors other than the embryos' viability that determine their capacity for ongoing development. The study showed that there was a strong association between the release of Paf and the morphology and growth of the embryo. There was also some association between aspects of oocyte development and culture conditions.

Using a more quantitative bioassay 228 media in which human embryos had been cultured were found to contain a wide range of Paf concentrations [56]. Around half had a Paf concentration above their corresponding control media. Embryos transferred to women who achieved a pregnancy produced more Paf (295 nM) in vitro than those transferred to women who failed to achieve a pregnancy (75 nM). However, the proportion of Paf-positive embryos transferred to women achieving pregnancy was not different from the proportion transferred to women who did not become pregnant. A significant false-negative rate was identified, that is, around 13% of women achieving pregnancy had embryos transferred that did not produce significant amounts of Paf. By contrast, 26% of women not achieving pregnancy received only Paf-negative embryos. A clear trend for high levels of Paf release by embryos with fastest cleavage rates was observed [56].

The development of immune-based assays for Paf allowed more rigorous approaches to its analysis. Immune assay showed greater sensitivity and reproducibility than bioassays [57]. A pilot study of a total of 46 human IVF media samples showed that embryos that were transferred to women who became pregnant had higher levels of Paf in media than those that failed to produce pregnancies [57]. Receiver operator curve (ROC) analysis [58] showed a positive pregnancy outcome indicator for Paf with predicted cut-off limit of 45 pmol/l per embryo and a diagnostic accuracy of 0.76, with positive predictive value of 0.88 and negative predictive value of 0.46.

In a larger trial [59], the Paf concentration in culture media in which eggs were fertilized was higher than in media of non-fertilized eggs and control media. Partitioning the data showed significant association between the methods of ovarian stimulation and stage of development at the time of embryo transfer, with the fastest-growing embryos showing on average the most Paf release. This study found no association between the size (volume) of the follicle the oocyte was derived from and Paf release by the resulting embryo.

One study [60] failed to detect Paf in human IVF embryo-conditioned media using one in vitro bioassay, but did detect it using an in vivo bioassay. This led the authors to erroneously conclude that the activity was not Paf. Another study also failed to detect Paf by immunoassay in media conditioned by mouse embryos [61]. The conflicting outcomes from both studies arise from their failure to undertake correct extraction of Paf prior to assay. Being a hydrophobic phospholipid Paf exists in culture media (and all biological fluids) in association with binding proteins. In the case of embryo-derived Paf this has been extensively investigated and it is found that embryo-derived Paf becomes associated with extracellular albumin [30, 36]. This binding requires covalent modification of disulfide bonds within albumin [36] and the resulting high-affinity binding has two important consequences. First it protects Paf from degradation by the ubiquitous Paf-acetylhydrolase (thus markedly extending its biological half-life) and it also makes Paf resistant to extraction by organic solvents. To date the only validated method for its extraction is the Bligh-Dyer method of phospholipid extraction [30, 36]. This extraction procedure has the disadvantage that it is tedious, time-consuming and requires a high level of technical competence. The time-consuming nature of the extraction procedure followed by a lengthy assay has the disadvantage that analysis requires more than a day. Thus, in its current state of development the assay is not ideally suited to prospective screening to allow timely decisions on which embryos are best suited to transfer. A high priority in the field is to devise low-cost, high-throughput quantitative means of Paf extraction that would facilitate rapid screening.

To date, the most determined effort to establish the utility of a released antigen as a non-invasive test of embryo viability has centered on the measurement of released HLA-G into culture media. At least 11, mainly prospective, studies have attempted to assess this utility. Detection of HLA-G has generally used immune-based ELISA, with one study using the high-throughput Luminex system. The most notable feature of these reports is the great heterogeneity in results. Some studies found no HLA-G released by embryos [62] while others found it in a significant proportion of media samples [53]. In one study it was found to have a predictive value for the viability of embryos generated by ICSI but not IVF [63], while others showed a predictive value for IVF [64]. There was no evidence that HLA-G in media provided a tool that allowed genetically abnormal embryos (such as those with defined aneuploidies) to be identified [65]. For studies that detected the antigen, there was considerable disagreement on its predictive capacity. In one multicenter study [66] a poor but statistically significant association ($P = 0.0379$) between HLA-G release and pregnancy was detected for one participating center but no associations were detected for others centers in the study. A meta-analysis of 11 studies concluded that detection of HLA-G in media had a small diagnostic predictive value [64]. Its measurement may provide some clinical benefit if combined as a secondary criterion to other selection criteria such as morphological grading. An interesting feature of HLA-G release is the observation by some groups that it was not highly associated with the subjective morphological quality of embryos. If this is true then it may suggest that HLA-G measurement provides an independent measure of viability which may assist in discriminating between the developmental potential of embryos of similar morphology.

An interesting feature of the meta-analysis [64] is that it did not identify covariates that accounted for the heterogeneity in the results between studies. This might be interpreted as an indication that there are many variables in assaying this antigen that are currently not being adequately controlled. These could include differences in assay sensitivity and precision, sample preparation, handling and storage and media composition (including protein supplementation). A high priority required for progress is the rigorous and systematic investigation of the specifications required for a high-fidelity assay, the nature of media design, storage and preparation required for valid assessment, and identification of the conditions and factors that may confound the assay. Only when these conditions are met will it be prudent to undertake a properly powered, multi-centered prospective trial of the assays.

These studies show that measurement of Paf or HLA-G provides some information about the developmental viability of embryos. It is clear that in their current state of development neither provides a definitive test of viability, since more embryos released these antigens than achieved pregnancy. There were also a number of false-negative results for each analyte. At this time insufficient analysis has been performed to determine whether such false-negatives represent technical failure of the assays or identify embryos that do not release the antigens yet retain viability. Studies have commonly been limited by not being blinded and by the inability to track the fate of individual embryos due to transfer of multiple embryos. There has also not been any analysis of whether the combined analysis of Paf and HLA-G improves diagnostic power. The trend clinically towards increased use of single embryo transfers will facilitate the rigorous reassessment of the value of these tools.

Each of these antigens has their own advantages and disadvantages. HLA-G has the advantage of being a protein that can be readily, rapidly and inexpensively assayed using commercially available kits. It has the disadvantage that the biological case for it being a reliable released marker is not compelling. Paf, by contrast, has a well-developed body of biological evidence for its functions and roles in embryo development. It has the disadvantage of being an ether phospholipid for which little development of high-throughput analysis has been performed.

Alternative uses of the analysis of the embryo's secretome

The conditions for the use of non-destructive testing of embryo viability are yet to be established and validated. Yet, detailed knowledge of the embryo's secretome presents other valuable avenues for development. For instance, the routine measurement of either Paf or HLA-G in embryo-conditioned media retrospectively might be the basis for quality control/assurance of assisted reproductive technologies. A shift in the proportion of antigen-positive media and/or antigen levels might serve as an early indicator of adverse changes in the culture environment. It could also be readily envisaged that such assays would be a suitable early screen for new innovations in embryo culture, such as new media formulations. The value and cost-effectiveness of this strategy have not been assessed.

The observation that the trophic actions of the embryo's released ligands are limited by dilution [9] has led to the consideration of the appropriate volume of media in which embryos should be optimally cultured. Consequentially the culture of embryos in microdrops rather than in large volumes has become more common in clinical practice. The use of microdrops has the advantage of concentrating released mediators and if culture is of groups of embryos it has the further advantage of pooling the released mediators from this group. The communal culture of embryos is likely to allow for high-viability embryos to share their trophic ligands with neighboring embryos. Animal studies provide unequivocal evidence of the benefits of communal culture in small volumes (mouse, cow and pig) [9, 67]. There remains only limited analysis of the benefits in human medicine [68, 69]. There is a growing anecdotal support for such strategies, and they have found adoption in clinical practice, yet the evidentiary basis for their use in humans, and the precise conditions for their optimal use, is currently unsatisfactory. Properly resourced and powered trials to consider the relative optimal media volume, culture duration and the benefits in human embryos are required.

If we accept that autocrine and paracrine mediators are a normal part of the embryo's growth environment, this raises the question – should embryo culture media be supplemented with exogenous ligands routinely? If so, what mediators should be added and at what concentration? Detailed ongoing analysis of the embryo's secretome will help inform this question, yet the solution may not be simple. It is known that the dose-response curve of embryos to some ligands is quadratic, with too high a concentration being detrimental to viability. Thus the addition of a ligand at a concentration that benefits an embryo that releases too little of its own ligand (and thus has reduced viability) may conceivably harm an embryo of high viability (and producing ample autocrine ligand). Such complexity is not trivial and should not be overlooked in the design and analysis of supplementation studies. One solution might be to culture embryos in large volumes of media that effectively dilute endogenous ligands to marginal concentrations and to then supplement media with an empirically derived, optimized concentration of the range of necessary trophic ligands. This strategy would be feasible if we knew the identity and desired concentration of all the critical embryotrophic ligands. Yet it would be a brave

investigator who would make such a claim at this time. If there is extensive redundancy in the action of ligands this will simplify the design of such studies.

A related issue is the extent to which the embryo's trophic milieu is static rather than dynamic. Are different ligands required at different stages of the embryo's development, and are there adverse consequences if this sequencing is incorrectly applied? Conversely we can ask the question: does the use of the various sequential media formulations [70] (increasingly in use in clinical practice) create a disadvantage by the removal of accumulated trophic ligands with each media change? Animal model studies argue that the accumulation of these ligands provides an important survival signal to the embryo [71]. Media changes to optimize the embryo's dynamic metabolic substrate requirements [70] (and toxic waste removal [72]) may create further deprivation of trophic support. This may be exacerbated by the use of more frequent media changes using devices such as the microfluidics approaches currently under development. Thus, media design currently presents a paradoxical trade-off between the benefits of dynamically modifying substrate availability and removal of toxic wastes, and the harm resulting from removal/dilution of released ligands. The challenge for the future is to find ways of accommodating the embryo's dynamic metabolic needs with its requirement for autocrine and paracrine trophic ligands.

There is much complexity governing optimal embryo growth. Continued detailed analysis of the nature and roles of the embryo's secretome will help inform us of the nature of this complexity and how to best exploit it.

Should we be careful of what we wish for?

The identification and selection of embryos with the greatest developmental potential for transfer seems an entirely rational and laudable aim. All processes of artificial selection, however, raise the question – what exactly are we selecting for? To date, the trend in clinical practice is to select embryos that grow at the fastest rate and have the most pleasing morphology. There is little argument that the fertilization and culture of embryos in vitro using current technology imposes a range of exogenous stresses upon embryos which include metabolic imbalances, oxidative and mechanical stresses, genotoxic damage and epigenetic defects. The heterogeneity in the capacity of embryos to grow under these conditions raises the question of whether the capacity to best survive culture is (1) an entirely stochastic responses by the embryo or (2) governed by particular genetic/epigenetic traits that better equip them to survive the rigors of culture. If the response is stochastic then this does not provide a strong basis for selection. If optimal development in vitro is accounted for by definable genetic traits in embryos, then preferential transfer of those embryos creates a selection pressure for those traits.

An example of positive selection for a defined trait has already been considered. The expression of *Ped* (*HLA-G*) caused differential growth and survival of embryos. There is a positive association between this antigen's expression in vitro and pregnancy outcome, and in the mouse model *Ped*-positive embryos have a survival advantage over those lacking the gene within the reproductive tract [73]. *Ped*-positive progeny also have a more favorable growth and homeostatic profile post-natally [74]. Yet there are likely to be many genetic traits that govern the embryo's response to culture. In somatic cells it is well known that many genetic traits foster the survival and rapid growth of cells in culture. Many of these include the gain-of-function mutations to proto-oncogenes or loss-of-function in tumor suppressor genes. Indeed, during the development of modern tissue culture, it was routine to oncogenically transform cells to facilitate their successful survival and propagation in vitro. One mutation that is particularly favorable to the survival and propagation of cells in vitro is the loss-of-function of the tumor suppressor protein, transformation related protein 53 (TRP53). TRP53 plays many important roles as an effector of genotoxic stress and as a regulator of cell-cycle progression and cell death [75]. TRP53 expression is normally held at a very low level during the preimplantation stage of embryo development [44]. This latency of TRP53 expression is essential for the normal development [44, 76]. TRP53 is sometimes elevated in embryos that have been produced by IVF or subjected to culture in a medium lacking amino acids and the presence of 20% oxygen [44]. This increase causes their retarded development, increased incidence of cell death within the embryo and a loss of viability. The genetic deletion of *Trp53* from mouse embryos allows them to grow more rapidly and with better morphology in vitro than their wild-type siblings [44]. This differential growth and survival does

not exist for embryos developing in the reproductive tract. Furthermore, *Trp53*$^{-/-}$ embryos have a higher incidence of pregnancy after their transfer than their sibling embryos expressing *Trp53* (but this was not the case for embryos conceived and grown in vivo). Thus, the culture of mouse zygotes and their transfer to the uterus creates a positive selection pressure for a loss-of-function to *Trp53* [44]. This selection has adverse long-term consequences. Progeny lacking *Trp53* have a profound predisposition to cancer formation [77]. It is noteworthy that one of the mechanisms that maintain the latency of TRP53 expression is the actions of autocrine embryotrophins [44]. These results show that culture in vitro can potentially create positive selection pressures for genetic traits that favor embryo growth and survival in vitro. The inheritance of these traits may either present an unacceptable genetic risk to progeny (e.g. *Trp54)* or be potentially favorable for post-natal development (e.g. *Ped/HLA-G*). It is noteworthy that the spontaneous rate of TRP53 mutation in the population is low so the basis for selection of this mutation is small. But the study illustrates the theoretical point that a seemingly rational basis for artificial selection of embryos for transfer (growth rate and morphology) can result in the selection in favor of entirely undesirable genetic traits. The stricter the selection criteria the more likely it is that such genetic selection might occur. The expression of many different genes is likely to govern the embryo's response to the culture environment and all would be potential targets for selection. To date our understanding of the molecular regulation of the early embryo, particularly the human embryo, is too imperfect to allow an entirely rational assessment of these potential risks. On balance, however, it would seem most unwise to create a set of conditions that creates a selection pressure in favor of embryos best capable of dealing with genotoxic stress.

The challenge to the field is to improve the outcome of these therapies while not creating increased biological risks such as selection for undesirable genetic or epigenetic traits. A low-risk strategy is to use markers of embryo viability as a tool for understanding the aspects of assisted reproductive technologies that compromise the embryo's viability and use this information to reduce the exogenous stressors in the embryo's environment. Optimizing the inherent developmental potential of each embryo created will diminish the potential biological and genetic risks associated with embryo selection.

Conclusions

It is now an accepted biological principle that cells secrete, release or shed a host of biologically active molecules into their surrounding milieu. In this regard the embryo seems no different to other cells. The study of the secretome of all cells is in its infancy and the preimplantation embryo presents particular challenges given the small amount of biological material available for analysis. The limited biological analyses of the embryo's secretome so far indicate that it has important functions and is not simply an artifact or epi-phenomenon of culture in vitro. Much needs to yet be undertaken to establish the diversity and dynamics of the secretome across all preimplantation developmental stages. A high priority is the rigorous development and validation of assays with sufficient sensitivity and precision to meet diagnostic standards. Not until such tools are available will it be cost-effective to undertake extensive trials of their use. These trials need to be appropriately powered, blinded and multicentered. It is essential that any such trials use single embryo transfers so that the fate of each embryo can be unequivocally accounted for. Prior to implementation of any diagnostic methodology into clinical practice a much greater understanding is required of the potential risks of any genetic/epigenetic selection that may inadvertently result.

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Chapter

26 Proteomics analysis of the endometrium and embryo. Can we improve IVF outcome?

Francisco Domínguez, Tamara Garrido, and Carlos Simón

Introduction

A major issue in the field of human reproductive biology is the selection of appropriate embryos for transfer to the uterus. To date, the method used to select the best cleavage embryo or blastocyst has been morphological assessment, but this selection method is highly subjective [1]. Embryo culture with sequential or co-culture systems and the reduction of embryos to be transferred have contributed to reduce the problems related to multiple pregnancies, although implantation rates in ART have not significantly improved in the last decade [2, 3]. The impact of the new technologies on reproductive medicine will enable us to investigate potential new approaches other than morphology to improve embryo viability selection. In this chapter, we will focus on proteomics, first on the endometrial side, and then on the possible non-invasive biomarkers in the human spent embryo medium.

Proteomics of the human endometrium

The implantation process is regulated by the acquisition of endometrial receptivity and the correct development of a viable embryo. Furthermore, implantation needs adequate bi-directional communication between the blastocyst and the endometrium [4].

New technologies allow for a molecular understanding of these events. Transcriptomics, proteomics, and metabolomics may improve our knowledge of the implantation process and enable us to identify biomarkers for endometrial receptivity, embryo development, or associated-pregnancy diseases.

Recently, major advances in the genomics field have been achieved thanks to the microarray and bioinformatics technologies available which provide a vast amount of information on gene expression [5]. However, gene expression is only one aspect of the complex regulatory network that allows cells to respond to intracellular and extracellular signals. Unlike genomics, proteomics represents a true reflection of cellular function. Proteomics is often considered the next step in the study of biological systems (Figure 26.1) [6]. This means that even the basic set of proteins which are produced in a cell needs to be determined. To date, lack of sensitivity is still a stumbling block for the global introduction of proteomics into the field of human reproduction. However, new developments in mass spectrometry using protein profiling and peptide sequencing have been implemented to elucidate the underlying biological processes. In this section, we will review the dynamics of the endometrial transition from the non-receptive stage to the receptive stage at the proteomic level. This fact deserves further attention to understand endometrial receptivity and to identify potential molecules for interception (Figure 26.2).

DeSouza *et al.* [7] employed a quantitative approach to assess the proteomic repertoire using isotope-coded affinity tags (ICAT), affinity purification, and liquid chromatography coupled online to mass spectrometry (LC-MS) between proliferative and secretory endometria. Only five proteins with a significant differential expression were found, of which the glutamate NMDA receptor subunit zeta 1 precursor and FRAT1 were the most interesting. The utility of these proteins as indicators of endometrial receptivity is open to further research.

Our group compared the proteomes of the pre-receptive (day LH+2) versus the receptive (LH+7) human endometrium. Endometrial biopsies were obtained from six fertile woman (n = 6) in the same

Human Assisted Reproductive Technology: Future Trends in Laboratory and Clinical Practice, ed. David K. Gardner, Botros R. M. B. Rizk, and Tommaso Falcone. Published by Cambridge University Press. © Cambridge University Press 2011.

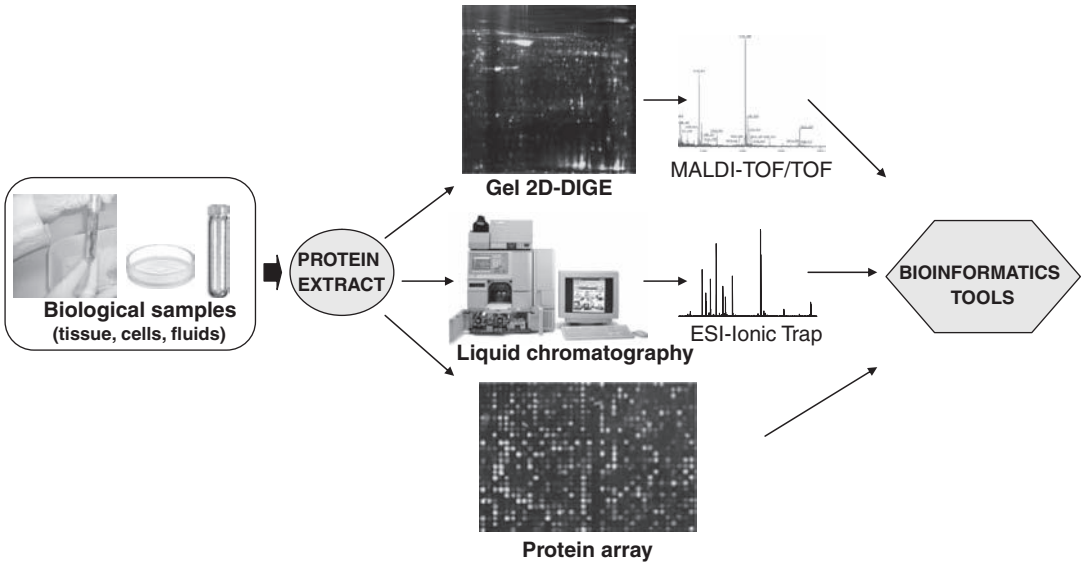


Figure 26.1. Strategies of proteomic analysis. Proteins are extracted from biological samples, fractionated optionally, separated, and analyzed by differential techniques. In the gel-based methods (up), different protein samples are labeled with different fluorescent dyes, and are then mixed together. Next, proteins are separated into two-dimensional difference gel electrophoresis (2D-DIGE) according to their isoelectric point and molecular weight. Gels are scanned by laser scanners, while those spots corresponding to proteins with a differential expression are identified. Finally, these proteins are identified by mass spectrometry (MALDI-TOF/TOF). In the chromatographic separation methods (center), the extracted proteins, protein fraction (SELDI) or one-dimensional gel bands (SDS-PAGE) are digested enzymatically, while the peptidic mix is separated by liquid chromatography (HPLC). Usually, peptides are analyzed and identified by mass spectrometry, typically electronebulization (ESI), and coupled with an ionic trap. Other methods are based on protein arrays (down). These arrays are membranes that contain a certain number of pre-absorbed antibodies that correspond to different proteins. Finally, all the information obtained by different methods is analyzed using bioinformatics tools. See colour plate section.

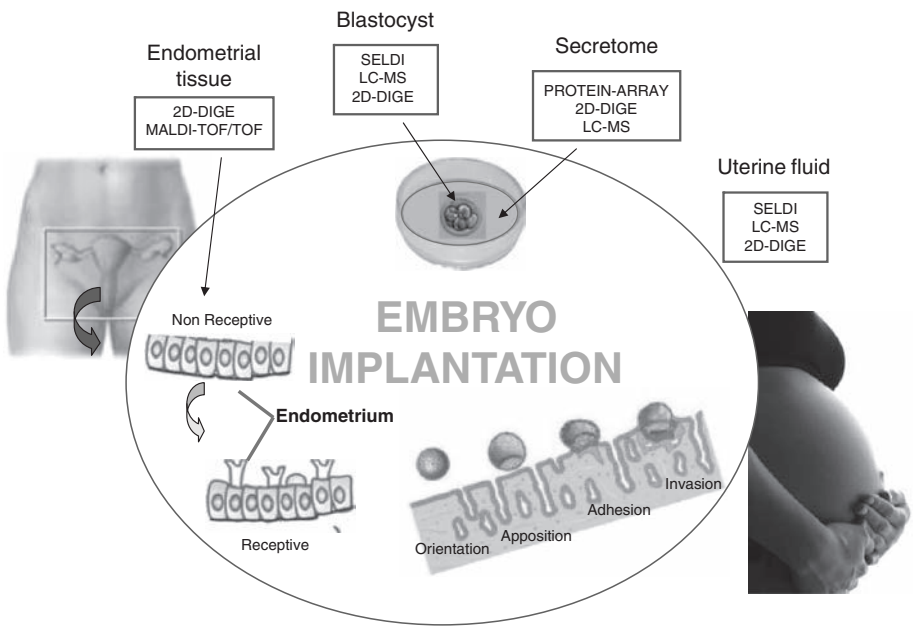


Figure 26.2. Area of use of the proteomics technologies in the human embryo implantation analysis. Different proteomics approaches can be used for the study of the embryo implantation process. The identification of differentially expressed proteins will allow us to understand this complex biological process and to use them as key interceptive markers to prevent embryo implantation, also as markers of endometrial receptivity and embryo viability or to identify causes of diseases. See colour plate section.

menstrual cycle. Proteins were extracted, separated by two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Thirty-two differentially expressed proteins were found in the receptive vs. pre-receptive endometrium, with 23 and 12 up- and down-regulated spots, respectively (Table 26.1). Among these proteins, it is interesting to emphasize that two proteins, annexin A2 and stathmin I, were consistently found to be differentially regulated in two DIGE experiments we performed. These proteins were tested with other techniques such as Western blot and immunohistochemistry, and they followed a similar regulation to DIGE analysis. Finally the endometrial refractoriness model, which was induced by inserting an IUD, was used to test the functional relevance of stathmin I and annexin A2. Our results demonstrate that when an IUD was present, the staining pattern of these proteins was similar to the pre-receptive stage. These results suggest their functional implication in endometrial receptivity and, therefore, annexin A2 and stathmin I may prove important in predicting the receptivity status and could be possible targets for interception [8].

The proteomic approach has also been used in the study of the endometrial receptivity process to analyze endometrial fluid. Endometrial fluid is a complex biological fluid which is in direct contact with the endometrial cavity and contains a multitude of proteins and proteolytic enzymes secreted from the endometrium [9, 10]. It lubricates the endometrium, acts as a line of defense against pathogens, provides nutrients for blastocyst formation, and constitutes a microenvironment where the embryo-endometrial dialog occurs prior to implantation signals. Endometrial fluid can be collected by aspiration in a painless manner using non-invasive methods [11]. Considerable interest has been shown in the protein content of endometrial secretions in recent years and it has been suggested to play a key role in the embryo implantation process [12, 13]. There has been evidence that defects in the expression of these proteins may result in the failure of embryos to implant [14]. Furthermore, uterine secretions are less complex in terms of their protein repertoire, and may serve as a pool of biomarkers for functional endometrial operation.

Endometrial secretion has been shown to contain (i) proteins originating from the transudation of serum, (ii) leakage products of apoptotic epithelial cells and (iii) proteins secreted from the glandular epithelium. This secretion undergoes significant changes in protein content during the transition from the proliferative phase to the secretory phase [15]. Endometrial secretion composition varies during the menstrual cycle as a result of the changes in the ovarian steroid serum concentration [16]. Estradiol (E_2) regulates transudation by blood vessel dilatation and permeability, and progesterone (P) controls the secretory activity of the endometrial glands. Furthermore, endometrial secretion contains cytokines such as leukemia inhibitory factor (LIF) [17], glycodelin (PP14) [18], macrophage colony-stimulating factor (M-CSF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) [19], insulin-like growth factor binding protein 1 (IGFBP-1), interleukins [20–21], as well as steroid hormones (estrogen, progesterone, prolactin, human chorionic gonadotropin, and precursors) [22–23].

In the past, the protein patterns of uterine secretions throughout the menstrual cycle have been analyzed by electrophoresis. These analyses revealed three different protein patterns which are typical of the equivalent phases of the menstrual cycle: the intermediate, proliferative, and secretory phases. The results showed characteristic "families" of proteins bands corresponding to 63 proteins, some of which were identified by their molecular weight [24].

In another work [25], endometrial fluid obtained transcervically by aspiration immediately prior to embryo transfer was analyzed and the protein profile in each sample was determined. Although uterine fluid aspiration is a safe method, the material obtained is sometimes not enough for analysis or may be diluted as a result of uterine washing, which makes the results difficult to consider. These studies also demonstrated that endometrial secretion can be obtained for analysis immediately prior to the embryo transfer in IVF cycles without disrupting implantation [26, 27].

More recently, Van der Gaast *et al.* [28] investigated the effect of ovarian stimulation in IVF on endometrial secretion and markers of receptivity in the mid-luteal phase. The endometrial fluids obtained in this period in the stimulated cycle were compared with the spontaneous cycle. Protein composition was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and gels were stained with Coomassie brilliant blue. The protein pattern was obtained by measuring the relative density of each band by means of a scanning laser densitometer and

Table 26.1. Proteins identified by MALDI-TOF/TOF showing significant changes between human pre-receptive (LH+2) and receptive (LH+7) endometrium

Spot[a]	*P*-value[b]	Fold change[c]	Accession code[d]	Protein description	Mascot score[e]	Mascot expect.[f]	MW / pI theor.[g]	Matched peptides[h]	Sequence cov. (%)[i]	Protein function[j]
1	9.80E-04	−2.5	IBA2_HUMAN	Ionized calcium binding adapter molecule 2 isoform 1	115	2.00E-05	17.1 / 6.6	2	18	Protein binding
2	1.10E-03	−2.6	STMN1_HUMAN	Stathmin (Phosphoprotein p19) (pp19) (Oncoprotein 18)	249	7.90E-19	17.3 / 5.8	7	36	Cytoskeleton, intracellular signaling cascade
3	8.72E-04	−1.6	STMN1_HUMAN	Stathmin (Phosphoprotein p19) (pp19) (Oncoprotein 18)[k]	118	1.10E-05	17.3 / 5.8	3	18	Cytoskeleton, intracellular signaling cascade
4	8.72E-04	−1.4	SYDC_HUMAN	Aspartyl-tRNA synthetase	424	2.50E-36	57.5 / 6.1	19	41	Translation process
5	1.01E-03	1.5	PARK7_HUMAN	DJ-1 Protein	136	5.00E-09	20.1 / 6.3	2	10	Ras protein signal transduction
6	7.33E-04	1.5	PCOC1_HUMAN	Procollagen C-endopeptidase enhancer 1	213	3.10E-15	48.8 / 7.4	12	30	Cytoskeleton
7	9.80E-04	1.5	ANXA2_HUMAN	Annexin A2	117	1.20E-05	38.8 / 7.6	3	9	Skeletal development
8	6.53E-04	1.6	Q59GX5_HUMAN	L-plastin	137	1.20E-07	56.2 / 5.2	1	3	Cytoskeleton
9	7.33E-04	1.7	CATB_HUMAN	Liver Cathepsin B, Chain B	174	2.50E-11	23.0 / 5.2	3	18	Proteolysis, regulation of apoptosis
10	9.80E-04	1.7	TAGL2_HUMAN	Transgelin 2	155	2.00E-09	21.2 / 7.6	8	44	Protein binding, development
11	8.54E-04	1.7	APOH_HUMAN	Apolipoprotein H	103	3.10E-04	36.7 / 8.3	2	7	Cholesterol metabolism
12	6.53E-04	1.8	AL1A3_HUMAN	Aldehyde dehydrogenase 1A3	82	1.20E-02	56.9 / 7.0	1	2	Metabolic process

13	7.33E-04	2.0	TPIS_HUMAN	Chain A, triosephosphate isomerase	238	9.90E-18	26.8 / 6.5	12	42	Metabolism of small molecules
14	9.65E-04	2.1	EI2BL_HUMAN	Translation initiation factor eIF-2B	121	5.00E-06	39.5 / 5.9	2	8	Translation initiation
15	1.06E-03	2.5	ALBU_HUMAN	Albumin, isoform 2, fragment	198	9.90E-14	23.2 / 8.2	4	18	Transport
16	8.72E-04	2.8	ANXA2_HUMAN	Annexin A2	388	9.90E-33	38.8 / 7.6	15	43	Skeletal development
17	9.80E-04	3.4	AOFA_HUMAN	Monoamine oxidase A	97	4.30E-05	60.2 / 7.9	1	2	Electron transport
18	8.72E-04	3.7	APOL2_HUMAN	Apolipoprotein L2	165	2.00E-10	37.1 / 6.3	4	14	Cholesterol metabolism
19	1.74E-02	−2.4	PGRC1_HUMAN	Membrane-associated progesterone receptor component 1	148	4.30E-10	21.8 / 4.6	2	7	Signaling
20	2.53E-02	−2.2	STMN1_HUMAN	Stathmin (Phosphoprotein p19) (pp19) (Oncoprotein 18)	219	3.40E-17	17.3 / 5.8	4	18	Cytoskeleton, intracellular signaling cascade
21	2.55E-03	−1.8	F13A_HUMAN	Coagulation factor XIII A	374	1.10E-32	83.7 / 5.8	17	27	Coagulation
22	1.90E-02	−1.7	SERPH_HUMAN	Serpin H1 precursor	156	6.80E-11	46.5 / 8.8	8	32	Response to unfolded protein
23	3.34E-02	−1.5	VIME_HUMAN	Vimentin	766	6.80E-72	53.7 / 5.1	23	52	Cytoskeleton
24	1.05E-02	−1.4	HNRL1_HUMAN	Heterogeneous nuclear ribonucleoprotein U-like 1	160	2.70E-11	96.3 / 6.5	11	19	RNA processing, response to virus
25	1.51E-02	−1.3	PPIB_HUMAN	Peptidyl-prolyl cis-trans isomerase B	475	8.50E-43	22.8 / 9.3	17	56	Protein synthesis
26	1.23E-02	−1.3	RSSA_HUMAN	40S ribosomal protein SA (p40)	280	2.70E-23	33.0 / 4.8	9	35	Cell adhesion
27	6.87E-04	1.5	TPM3_HUMAN	Tropomyosin-alpha 3	132	1.70E-08	32.9 / 4.7	8	33	Cytoskeleton

Table 26.1. (cont.)

Spot[a]	*P*-value[b]	Fold change[c]	Accession code[d]	Protein description	Mascot score[e]	Mascot expect.[f]	MW / pI theor.[g]	Matched peptides[h]	Sequence cov. (%)[i]	Protein function[j]
28	2.10E-03	1.5	RUVB2_HUMAN	RuvB-like 2	253	1.30E-20	51.3 / 5.5	9	20	Regulation of transcription, cell proliferation
29	2.26E-02	1.6	CO6A1_HUMAN	Collagen alpha-1 (VI)	255	8.50E-21	109.6 / 5.3	15	15	Skeletal development
29	2.26E-02	1.6	HYOU1_HUMAN	Hypoxia up-regulated protein 1	188	4.30E-14	111.5 / 5.2	10	11	Response to stress
30	2.20E-02	1.6	RUXG_HUMAN	Small nuclear ribonucleoprotein G	130	2.70E-08	8.5 / 9.0	2	17	Spliceosome assembly
31	1.52E-03	1.9	ANXA4_HUMAN	Annexin A4	168	4.10E-12	36.1 / 5.8	10	35	Signal transduction
32	1.10E-02	2.1	K1C19_HUMAN	Cytokeratin-19	292	1.70E-24	44.1 / 5	17	43	Response to estrogen stimulus
33	5.10E-03	2.1	ANXA2_HUMAN	Annexin A2	250	2.70E-20	38.8 / 7.6	14	38	Skeletal development
34	2.26E-02	2.2	SSRD_HUMAN	Translocon-associated protein subunit delta	250	2.70E-20	19.2 / 5.8	7	41	Transport
35	2.10E-02	4.8	S10AA_HUMAN	Protein S100-A10	130	2.70E-08	11.3 / 6.8	3	31	Signal transduction

[a] Spot numbering as shown in 2-DE silver gel.
[b] Student *t*-test *P*-value.
[c] Average volume ratio (LH+7 / LH+2) as calculated by the DeCyder BVA analysis.
[d] Protein accession code from SwissProt database.
[e] Mascot score.
[f] Expected value.
[g] Theoretical molecular weight (kDa) and pI.
[h] Number of matched peptides.
[i] Protein sequence coverage for the most probable candidate as provided by Mascot.
[j] Biological function retrieved from SwissProt.
[k] Phosphorylation demonstrated by MALDI-MS/MS.

the GelScan XL software package. In this pilot study, ovarian stimulation did not alter the investigated markers of endometrial maturation in the mid-luteal phase.

In 2009, an integrated work was presented [29] to identify the catalog of proteins present in endometrial fluid aspirate during the secretory phase of the menstrual cycle. To achieve this objective, three different, but complementary, strategies were used: first, in-solution digestion followed by reverse-phase high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS); second, protein separation by denaturing one-dimensional electrophoresis (SDS-PAGE) followed by HPLC-MS/MS analysis; finally, two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) followed by MALDI-TOF/TOF analysis. The combination of these three strategies led to the successful identification of 803 different proteins in the International Protein Index (IPI) human database (v3.48). This catalog of proteins presented here can serve as a valuable reference for the study of embryo implantation and for the future biomarker discovery involved in pathological alterations of the endometrial function.

The current technical limitation of applying proteomics to the study of protein patterns in endometrial fluid is that the majority of identified proteins correspond to serum proteins, thus masking the identification of proteins present at a minor concentration, which is of great interest, such as biomarkers for endometrial receptivity, embryo development, diseases, and/or interception.

In conclusion, the application of proteomics technology to the endometrium can be used to search for new biomarkers to determine endometrial receptivity and possible causes of infertility and to investigate interceptive molecules to prevent embryo implantation.

Proteome and secretome of the human embryo

The proteins translated from RNA transcripts are directly responsible for cellular function. Although gene expression studies of these RNA transcripts are valuable, they have been shown to not often predict protein abundance, with poor clinical translation. Several mechanisms, including mRNA and protein degradation, can be utilized by the cell during transcription/translation regulation, resulting in a lack of association among the gene expression, RNA transcripts, and protein expression. Consequently, an in-depth investigation of the human proteome is vital to understand the cellular function and to comprehend biological processes and/or disease states.

Despite the new advances in proteomic technologies, knowledge of the proteome of the human preimplantation embryo is limited. The combined effect of limited starting material, a low protein expression, and the lack of sensitivity of the new proteomics platforms is the main obstacle. First proteomic studies utilized two-dimensional (2D) gel electrophoresis with computerized analysis of gel images to construct and analyze the protein databases of the preimplantation mouse embryo [30, 31]. Western blotting has also been used to identify the expression of known proteins [32] or to detect post-translational modifications, such as phosphorylation, in relation to embryo development [33].

Technological advances in translational research have enabled the non-invasive determination of the proteomic/secretomic status of an embryo. However, there is still little known about the proteome of the human preimplantation embryo (due to obvious ethical issues), particularly the ability of the blastocyst to secrete/consume different proteins (secretome) from its environment in order to communicate with the maternal endometrium to proceed with the implantation process. More recently, advances in proteomic technologies, with the development of mass spectrometry (MS), have made it possible to identify groups of proteins within limited amounts of complex biological fluids and tissues [34, 35].

Several studies have initiated the analysis of the proteomics of the human and mice embryo/blastocyst using time-of-flight mass spectrometry (TOF/MS) [36–38]. TOF/MS is a mass spectrometry method in which ions are accelerated by an electric field of known strength. This acceleration results in an ion with the same kinetic energy as any other ion with the same charge. The velocity of the ion depends on the mass-to-charge ratio. The time that it subsequently takes for the particle to reach a detector at a known distance is measured. This time will depend on the mass-to-charge ratio of the particle (heavier particles achieve lower speeds). From this time and the known experimental parameters, it is possible to find the mass-to-charge ratio of the ion. Comparing the mass-to-charge ratio with the existing databases, several analytes, and proteins could be unambiguously identified.

Katz-Jaffe and colleagues used surface-enhanced laser desorption and ionization time-of-flight mass spectrometry (SELDITOF/MS) as a highly sensitive and high-throughput method for analyzing the human embryo proteome, including those of low molecular weight and all the isoelectric points [37]. SELDITOF/MS is a variation of matrix-assisted laser desorption/ionization (MALDI) that uses a modified target to achieve biochemical affinity with the analyte compound. In MALDI, a protein or peptide sample is mixed with the matrix molecule in solution, and small amounts of the mixture are deposited onto a surface and allowed to dry. The sample and matrix co-crystallize as the solvent evaporates.

To study the human embryo proteome, a total of 21 blastocyst lysates were used. The blastocysts were divided into three groups: early blastocysts, expanded blastocysts, and degenerating embryos. The comparison of the proteins obtained in the early blastocysts with the expanded blastocysts revealed two negatively charged proteins/biomarkers as being significantly up-regulated in the expanded blastocysts. When developing the blastocysts (early and expanded), a statistical analysis was done to compare them with the degenerating embryos, and a significant number of differences were observed among the negatively charged proteins. In addition, degenerating embryos presented a different protein expression profile to that of developing blastocysts, with a significant up-regulation of numerous proteins/biomarkers [37]. This was the first study in which a system involving TOF/MS was used to successfully analyze the proteome of individual human embryos.

Although this kind of study provides additional information on the physiology and development of the human blastocyst, their relevance for IVF biomarker selection is very poor, mainly due to the invasive properties of the studies. Quick and non-invasive determinations of proteins/analytes to select a good, healthy, or viable blastocyst are still needed.

Katz-Jaffe *et al.* also used the SELDITOF technology to study the secretome of human and mouse embryos [38]. The secretome has been defined as the subset of secreted or consumed proteins that can be found in the media where the embryo/blastocyst grows, and which could be an excellent non-invasive approach to study the proteome of the human blastocyst. The aims of this study were to analyze the human and mouse embryonic secretome at each preimplantation development stage and to correlate these findings with ongoing blastocyst development. The authors divided the conditioned media of the embryo into a 24-hour developmental stage period. Each successive embryonic development stage at 24-hour intervals displayed a distinctive identifiable secretome profile, even though it was evident that several protein biomarkers were observed on consecutive days of development. A detailed statistical analysis of both the mouse and human secretomes across preimplantation development revealed similar patterns and few inter-species differences with distinctive protein profiles characterizing each successive embryonic development stage [38]. The final phase of this work consisted in correlating human day 5 secretome data with ongoing blastocyst development. A statistical analysis was performed to compare the protein profiles generated from spent ART culture media samples of developing blastocysts and spent ART culture media samples of degenerating embryos in the same 24-hour developmental stage. A highly significant difference was observed in the expression of an 8.5-kDa protein biomarker, with developing blastocysts consistently producing larger amounts of this protein biomarker. The near lack of expression of this 8.5-kDa protein biomarker from the degenerating embryos in conjunction with its high expression from the developing blastocysts potentially indicates a direct association between this protein biomarker and ongoing blastocyst development. Protein isolation and identification by tandem MS revealed that the best candidate for this 8.5-kDa protein biomarker was ubiquitin [38]. In conclusion, these distinctive protein profiles characterize the developmental stage of these embryos by their secretome alone, irrespective of morphology.

A recent study by Dominguez and colleagues [39] revealed more proteins that were consumed or secreted by the human embryo by analyzing the secretome of the blastocyst using protein arrays. In this type of array, antibodies are spotted onto the protein chip and are used to capture molecules that detect proteins from cell lysate solutions and/or culture media. The aims of this study were to obtain the human blastocyst secretome using protein arrays and to compare the protein profiles of the blastocysts that implant versus those that do not implant thanks to a single embryo transfer program (SET). Conditioned media from human embryos (implanted and non-implanted) were collected before transfer. Interestingly, the results using the protein arrays, when we compare a control medium to a medium with a human blastocyst, show

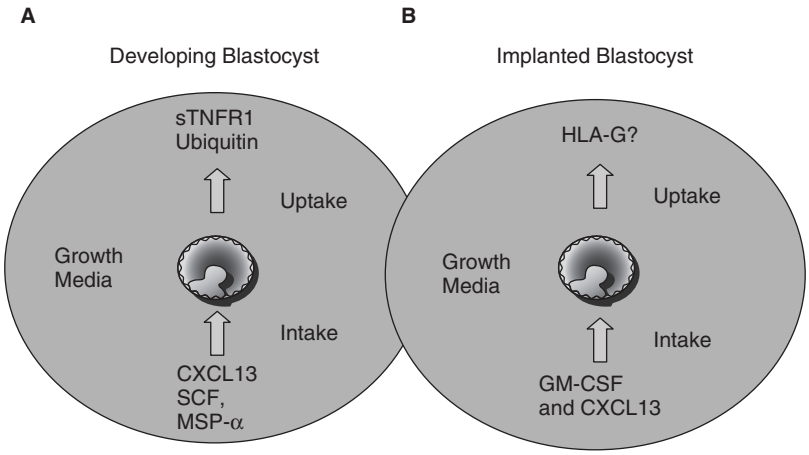


Figure 26.3. Secretome of the human developing and implanted embryo. Human IVF embryos are usually cultured in microdrops of different growth media. By studying the secretome of this medium, we discovered that some proteins, such as sTNFR1 and ubiquitin, are secreted by the developing blastocyst (A), while CXCL13, SCF or MSP-alpha are consumed by this blastocyst. The implanted blastocyst also has some consumed proteins, such as GM-CSF and CXCL13 (B). It is still controversial whether or not HLA-G is secreted uniquely by implanted blastocysts or could be useful as an implantation marker.

that proteins such as CXCL13 (BCL), stem cell factor (SCF), and macrophage-stimulating protein-alpha (MSP-α) significantly decreased ($P < 0.005$) and were, therefore, consumed by the human blastocyst. In contrast, soluble TNF receptor 1 (sTNFR1) significantly increased in the media where the blastocyst was present in comparison with the control media (Figure 26.3).

Additionally, the proteins which significantly reduced or were consumed by the implanted blastocyst versus their non-implanted counterparts in the implantome were GM-CSF and CXCL13 (Figure 26.3). None of the proteins investigated was significantly up-regulated/secreted by the implanted blastocyst. A gene ontology (GO) search with all the proteins found in the protein arrays was used to obtain the predicted functional partners and relationships between the human blastocyst secretome and implantome [39]. However, in this study pooled conditioned media samples in groups of five were employed because of the limited quantity of proteins/analytes produced/secreted by a single blastocyst to the conditioned media. Hence, single embryo-conditioned medium could not be used to analyze the protein changes given the lack of sensitivity of current protein arrays and the limitation of the initial sample (50 µl of conditioned media). With the future arrival of high-throughput and high-sensitivity protein arrays, we may be able to increase the number of proteins found with these techniques in order to analyze the single and complete secretome and implantome of the human blastocyst.

We must not forget that the ability to assess the protein/analyte profile of an individual embryo could lead us to a better understanding of the cellular function at specific embryogenesis stages. Furthermore, such an approach would help us to improve embryo culture media, to identify the interactions between the blastocysts and the maternal uterine epithelium prior to implantation, and help choose which blastocyst to transfer more reliably and not only based on morphological criteria.

Another approach is to study the effect of co-cultures on the secretome and efficiency for obtaining good developing blastocysts [40]. Ten years ago, this group developed a safe, effective clinical program in which embryos were co-cultured with heterologous endometrial epithelial cells (EEC) until the blastocyst stage, and were then transferred back to the patient. The reproductive outcome in IVF patients in terms of pregnancy and implantation rates was statistically higher in EEC co-culture vs. sequential media: 39.1% vs. 27.5% ($P < 0.01$) and 33.3% vs. 20.9% ($P < 0.0001$), respectively. The comparative secretome of the conditioned media from the implanted blastocysts in sequential vs. this EEC co-culture using protein arrays revealed differences in the protein secretion/consumption profile between both embryo culture methods. Interestingly, IL-6 (5.40-fold increase) and PIGF (4.22-fold increase) were the most abundant proteins in the implanted embryo co-culture when compared to the sequential media.

When these data were validated using ELISA on single embryo-conditioned media, it was found that more IL-6 protein in the implanted embryo was consumed from the media when compared to when the blastocysts did not implant. This observation indicates that IL-6 consumption by the blastocyst (mainly

supplemented by EEC) could be necessary for blastocyst development, or even for the preparation of the implantation process.

Whether or not all these proteins are essential or play key roles for blastocyst development and/or implantation merits further research. The truth is, however, that the clinical data derived from this EEC co-culture IVF program indicate that some molecules, such as IL-6 and others still to be identified with high-throughput/sensitivity protein arrays, could prove highly beneficial for blastocyst development and implantation.

Other molecules found to be secreted by the embryo include the soluble human leukocyte antigen G (HLA-G). An analysis of the embryonic secretome has also been done [41, 42]. Subsequent studies revealed higher pregnancy rates when soluble HLA-G was detected in the spent IVF medium of day 3 embryos [43, 44]. However, the results were not absolute as pregnancies were also established from the HLA-G-negative embryos. Furthermore, some studies have raised serious concerns regarding the use of HLA-G production as a marker of further developmental potential [45–47].

Finally, discriminating secretome signatures between individual euploid and aneuploid blastocysts has also been addressed recently. Microdrops of the spent IVF culture medium from individual blastocysts of transferable quality were processed and analyzed by SELDITOF/MS to determine a blastocyst secretome fingerprint. Each individual blastocyst was then subjected to comparative genomic hybridization for a comprehensive chromosomal analysis of all 23 pairs of chromosomes [48]. Of the 14 aneuploid blastocysts analyzed, nine had a single chromosomal aneuploidy and five were chaotic, with more than two chromosomes involved. Secretome fingerprints from the individual blastocysts identified protein signatures which allowed a discrimination between euploid and aneuploid chromosomal constitutions [49].

The proteome's dynamic and sensitive nature to variables during sample collection, storage, handling, and processing needs to be considered, and a consistent protocol for reproducible proteomic data should be followed [50].

All these data shown herein suggest that viable embryos possess a unique proteome and that, potentially, some of these proteins secreted into the surrounding culture medium contribute to the secretome. Consequently, a non-invasive proteomic analysis of the secretome of human embryos throughout preimplantation development may assist in revealing secreted factors that reflect developmental competence and viability, as well as further markers of embryo viability and implantation success.

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Chapter

27 Analysis of embryo metabolism and the metabolome to identify the most viable embryo within a cohort

David K. Gardner

Introduction

The ultimate goal of human IVF is the birth of a healthy singleton child conceived through the transfer of a single embryo [1]. Consequently, over the past decade there has been a move to reduce the number of embryos transferred in a given IVF cycle. In order to successfully attain the routine transfer of single embryos, several important developments have had to take place in the IVF laboratory (Figure 27.1).

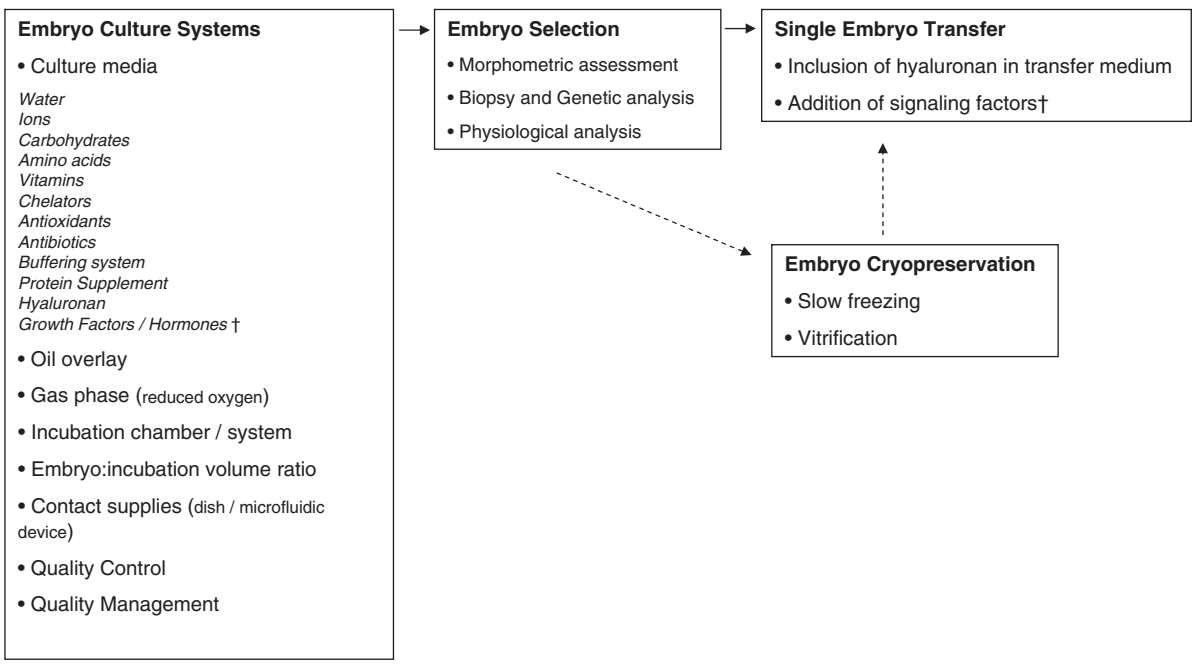


Figure 27.1. Components of an IVF laboratory that have been improved in order to facilitate the move to single embryo transfer. Extensive research on embryo culture systems has been able to identify those components that have a highly significant impact on embryo development [7, 58]. Embryo selection has improved through the development of elegant alpha-numeric grading systems to quantitate morphology, the advent of embryo biopsy for genetic analysis, and the recent ability to quantitate embryo physiology non-invasively through proteomic and metabolomic platforms. Through better catheter design, the use of ultrasound guidance and the inclusion of hyaluronan in the transfer medium [59], the embryo transfer procedure is highly effective and reproducible [60]. For those embryos not selected for transfer, improvements in cryobiology, especially in the area of vitrification, have resulted in further increases in the cumulative pregnancy rate per retrieval. † denotes further research required for validation of efficacy.

Human Assisted Reproductive Technology: Future Trends in Laboratory and Clinical Practice, ed. David K. Gardner, Botros R. M. B. Rizk, and Tommaso Falcone. Published by Cambridge University Press. © Cambridge University Press 2011.

First and foremost has been the need to improve culture conditions for the human embryo. It was not that long ago that it was not possible to routinely culture human embryos beyond the 8-cell stage. Indeed to do so appeared to require co-culture technologies, whereby feeder somatic cells were used to generate a more suitable environment for the embryo to develop [2–4]. However, with a resurgence of interest in embryo physiology and a better understanding of nutrient utilization by the conceptus came more appropriate culture media [5, 6] and laboratory conditions [7]. Consequently there has been an increase in pregnancy rates worldwide, which in no small way reflects improvements in the IVF laboratory. In parallel with improvements in culture systems, morphological grading systems have been developed for the human embryo, with the aim of being able to quantitate specific features of the embryo and relate this to viability, i.e. pregnancy potential. Consequently, there now exist elegant grading systems for the human embryo from the pronucleate oocyte [8, 9], cleavage [10–12], and blastocyst stages [13]. However, although such systems have unquestionably been of great value in selecting embryos for transfer, culminating in improved transfer outcome, it is evident that morphology does not reflect cell function or genetic normality. Indeed, by employing morphology alone for embryo selection one is missing crucial information. With the advent of embryo biopsy [14] and novel chromosomal [15] and molecular screening techniques [16, 17], we are entering a new phase of genetic analysis in assisted human conception, which will increase the probabilities of patients conceiving their pregnancy with a euploid and disease-free embryo. Furthermore, with the phenomenal advances in proteomics and metabolomics in recent years, analysis of single embryo physiology, through non-invasive methods, is rapidly becoming a reality. It is the analysis of embryo physiology, specifically metabolism, that will be discussed in greater detail in this chapter. However, there is one more laboratory component that has greatly assisted in the move to single embryo transfer, and that is cryopreservation of gametes and embryos. It would be a difficult ethical and moral situation to move to reducing the numbers of embryos transferred, if it were not possible to successfully cryopreserve supernumerary embryos that are not to be transferred in the same cycle as the oocyte retrieval. Fortunately the past decade has seen major improvements in our ability to cryopreserve the human oocyte and embryo at all stages. Key developments in cryopreservation have come predominantly in the form of new vitrification procedures [18–20]. Such is the ability to successfully cryopreserve gametes and embryos using this technology, that there is limited loss of cellular function and subsequent viability if vitrification is employed [21]. It would be remiss not to acknowledge the numerous improvements in slow freezing procedures [22, 23]; however, they are now being superseded by vitrification.

Due to improvements in laboratory conditions and embryo culture systems (and hence the ability to sustain the human embryo to the blastocyst stage), the application of morphological criteria for embryo selection, and the introduction of vitrification, we are well positioned to move towards single embryo transfers (SET) for a significant number of patients attending for IVF treatment. Certainly for patients < 37 years old [24] and oocyte donor cases [25], SET should be considered as the primary treatment option. In order for SET to be adopted for further patient groups, and indeed to improve the outcome of SET for all patients, the development of more quantitative means of embryo assessment is highly desirable. The introduction of several "omic" technologies into human IVF should greatly assist in the development of such "viability assays" (Figure 27.2).

Why quantitate the metabolism of the preimplantation embryo?

The production of sufficient energy is fundamental to the survival and proliferation of any cell type, and in somatic cells the pathways used to generate their required energy is tissue specific. Energy production by the preimplantation embryo is highly regulated and, of interest, changes almost daily from fertilization to implantation with regard to the relative activities of the pathways used to generate ATP. Changes in embryo metabolism during the preimplantation period reflect the activation of the embryonic genome, the increased requirement for biosynthesis associated with division and differentiation, and the creation of a blastocoel in preparation for implantation.

Unlike most somatic cells, the mammalian oocyte and cleavage stage embryo do not utilize glucose as their primary energy source, but rather exhibit low levels of oxidation of the carboxylic acids pyruvate and lactate. This type of metabolism reflects the

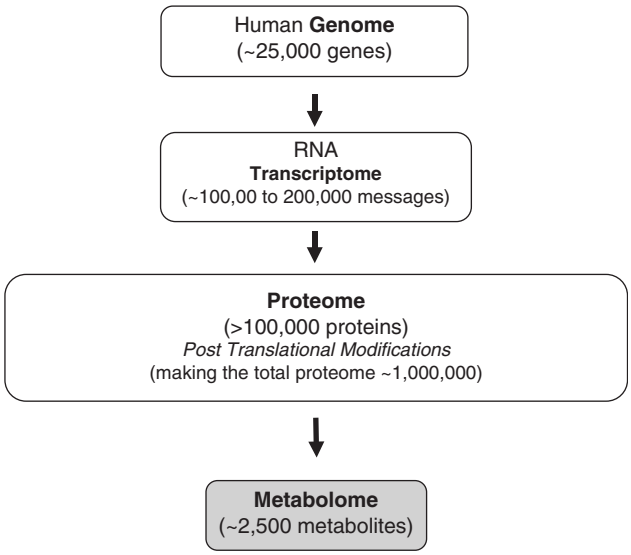


Figure 27.2. The twenty-first century has ushered in the age of the omics. Our understanding of gene expression in the human embryo has been greatly facilitated by the introduction of such technologies as gene array technology and quantitative PCR [61–63]. In contrast to the efforts spent on genomics, the field of proteomics has lagged somewhat, in part due to the limitations in platform technologies. However, this is rapidly changing with the application of novel mass-spectrometry [64, 65] and protein chip platforms [66, 67]. Research into proteomics will serve to elucidate the cellular machinery that determines cell process and fate, and consequently its analysis is of immense value in understanding the biology of reproduction. However, it is the metabolome that provides an insight into the activity of the cellular processes. In terms of its constituents, it is considerably smaller than the genome and proteome, which from a practical perspective makes it perhaps the easiest of the three to analyze. However, due to the size constraints associated with mammalian gametes and embryos, research into metabolomics has been relatively limited. This is now changing with the introduction of novel metabolomic platforms [54, 68].

oocyte's adaptation to a relatively quiescent state while residing in the ovary for several years. Consequently, the oocyte is characterized by a high ATP:ADP ratio as a result of low biosynthesis and no cell division. As embryo development proceeds and energy demands increase, the ATP:ADP ratio falls [26] and the flux through glycolysis increases, hence the demand for glucose goes up. However, the embryo's utilization of glucose is not like that of other tissues. Although at the blastocyst stage the embryo is utilizing glucose as its primary energy source, it only oxidizes around 50%, the remainder being converted to lactate. This so-called "aerobic glycolysis" (not to be confused with adaptive anaerobic glycolysis typically associated with exercise) is observed in rapidly proliferating cell types, such as cancers [27]. This particular type of energy metabolism by the blastocyst can be considered an adaptation to both proliferation and for subsequent implantation, during which the embryos will experience a few hours of relative anoxia [28].

Significantly the relative abundance of nutrients affects the metabolism of the embryo. For example, the ratio of pyruvate:lactate in the surrounding environment can affect the ratio of NADH:NAD^+ in the embryo, which in turn controls the redox state of the cells and hence the flux of nutrients through specific energy-generating pathways. Furthermore, our understanding of embryo energy metabolism has changed recently with the finding that the malate-aspartate shuttle is involved in the control of carbohydrate metabolism [29]. A paradigm held for over 40 years was that the fertilized oocyte had an absolute requirement for pyruvate [30]. However, recent analysis of embryo physiology has revealed that lactate and aspartate can substitute for pyruvate at the 1-cell stage, the two nutrients presumably generating sufficient energy through the malate-aspartate shuttle to support cleavage.

Clearly the regulation of energy metabolism in the embryo is complex and far from understood, although significant developments have occurred in this area in recent years. Ongoing research in this fascinating area will not only assist in the identification of key biomarkers for embryo selection, but will continue to be of value in the optimization of culture media.

Significance of metabolic regulation and function to embryo development

Mammalian embryos can grow in a wide variety of culture conditions. This ability to adapt to different environments has been termed "embryo plasticity." However, we now understand that making the embryo adapt to suboptimal conditions results in impaired cell function and subsequent loss of viability. The significance of maintaining the relative activities of the energy-producing pathways was first observed some 40 years ago by Menke and McLaren [31]. It was determined that when 8-cell mouse embryos flushed from the female tract were placed into culture in a

simple medium (i.e. balanced salts, glucose, lactate, pyruvate, and bovine serum albumin) the resultant blastocysts had an impaired capacity for oxidative metabolism compared to blastocysts developed in vivo. This was a landmark paper as it established that suboptimal culture conditions not only impaired embryo development in culture, but also affected energy metabolism.

Gardner and Leese went on to show that the concentrations of glucose, lactate, and pyruvate in the culture medium had a significant effect on embryo metabolism, and those mouse embryos cultured under physiological levels of nutrients exhibited a metabolism more closely resembling an embryo derived in vivo [32]. Further improvements in culture media formulations, specifically the inclusion of amino acids and vitamins, have also been shown to have profound effects on blastocyst metabolism, maintaining the oxidative capacity and increasing the subsequent viability of the embryo [33, 34].

In summary, loss of metabolic regulation occurs under suboptimal culture conditions and is associated with loss of embryo viability. This has, therefore, provided a marker to assess the effects of nutrients and other media components on the developing embryo, resulting in the formulation of sequential media designed to minimize such metabolic stresses. However, quantitating metabolic activity in the embryo is also of value in assessing the potential viability of an embryo prior to transfer.

Relationship between metabolic activity and embryo viability

Carbohydrates

Renard and colleagues were the first to demonstrate a relationship between the nutrient utilization of an embryo and its subsequent potential to give rise to a viable pregnancy [35]. In a study on day 10 cattle blastocysts, it was determined that blastocysts that had a glucose uptake of > 5 μg/hour developed better in vitro and in utero than embryos with an uptake below this value. This was achieved using spectrophotometric methods. Although suitable for day 10 bovine blastocysts, with a diameter of ~1000 μm, such spectrophotometric technology is not sensitive enough to detect nutrient uptake of earlier stages or smaller blastocysts such as those of the mouse, or indeed the human. Gardner and Leese subsequently used the technology of ultramicrofluoresce to analyze the glucose uptake of individual mouse blastocysts prior to transfer. It was determined that those blastocysts that went on to implant and develop into a fetus had a significantly higher rate of glucose consumption in vitro than those blastocysts which failed to implant [36]. However, both of the above studies were retrospective, and consequently it was not known whether this approach of quantifying metabolism could be used to select viable embryos for transfer. Therefore, a study was designed whereby the metabolism of mouse blastocysts, of the same morphology and diameter, was used to prospectively identify embryos either as viable or non-viable prior to their transfer [37]. As well as measuring glucose consumption by individual blastocysts, lactate was also quantitated in order to obtain an indirect measure of glycolic activity (i.e. one mole of glucose can give rise to two moles of lactate). If an embryo exhibits a 50% glycolytic rate (typical of an in vivo developed blastocyst), then half of the glucose consumed is being shunted to lactate, while the remaining 50% could be used in oxidative metabolism or biosynthetic pathways. A glycolytic rate greater than 50% would indicate loss of metabolic control, while values greater than 100% would reflect the metabolism of endogenous substrates such as glycogen. An initial distribution of glycolytic activity was obtained from 79 blastocysts and the range of glycolytic activity varied from 41 to 280%! It was hypothesized that those embryos with a glycolytic activity close to that of blastocysts developed in vivo would be more viable than those embryos that had clearly lost the ability to regulate their metabolism. When blastocysts were selected prospectively on these criteria it was determined that the viability of blastocysts with a metabolism similar to that of in vivo developed embryos was 80%, while only 6% of those embryos that were metabolically stressed implanted (Figure 27.3a). When compared to the implantation potential of embryos selected solely on the basis of their morphology (20%), it became evident that measuring metabolism could be a valuable and quantitative means of selecting embryos for transfer [37]. A question that remained was whether viable blastocysts were simply more quiescent than those embryos which failed to develop after transfer. In other words, was a high metabolic rate in some way detrimental to the embryo? Subsequent analysis of the glucose consumed by those blastocysts predicted to be viable revealed that they actually had the highest glucose consumption

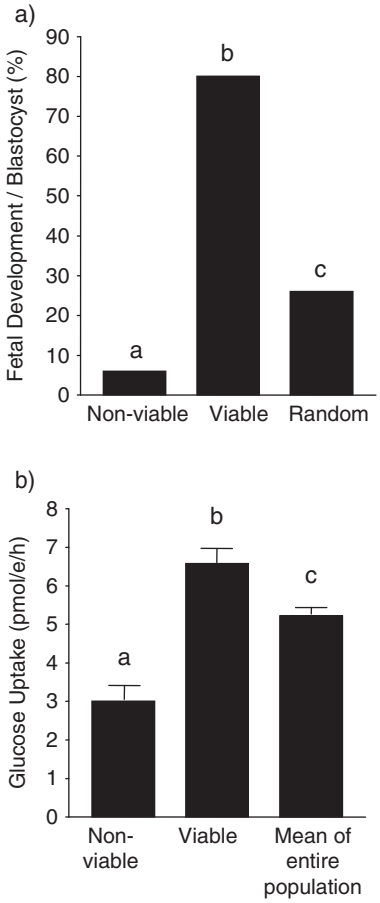


Figure 27.3. (a) Fetal development of mouse blastocysts selected for transfer using glycolytic activity as a biochemical marker. "Viable" blastocysts were classified as those with a glycolytic rate close to in vivo developed blastocysts (< 88%), while "Non-viable" blastocysts had a glycolytic rate in the highest 15% of the distribution (> 160%). On each day of the experiment, a selection of blastocysts were transferred at random, along with those selected as either viable or non-viable. [a,b,c] Different superscripts indicate significantly different populations ($P < 0.01$). (Adapted from Lane and Gardner [37].) (b) Relationship between glucose uptake of mouse blastocysts and subsequent viability. Those blastocysts that went on to term had a higher glucose uptake than those which failed to develop. Signficantly, viable embryos also had the lowest percentage of lactate production, indicating that viable blastocysts had maintained high levels of oxidative metabolism.

(Figure 27.3b), consistent with the previous data [36]. Furthermore, as the conversion of glucose to lacate was less in the viable blastocysts, this indicated that these embryos had a much higher oxidative metabolism and more efficient energy production. This observation led to the development of the "Rate and Fate" hypothesis, which predicts that viability is determined not only by the amount (or rate) of nutrient(s) consumed, but also by which metabolic pathway the embryo utilizes to metabolize it (i.e. fate). The Rate and Fate hypothesis predicts that the measurement of more than one parameter will give greater power to the metrics upon which the decision to select an embryo for transfer are based.

In the late 1980s and into the 1990s there were limited reports on the patterns of nutrient utilization by the human embryo conceived through IVF [38–40]. In 2001 Gardner and colleagues demonstrated that the utilization of pyruvate and glucose on day 4 was predictive of blastocyst development in human embryos [41]. However, two other significant observations were also reported in the same paper. First was the finding that the distribution of glucose consumption by human blastocysts of the same score and from the same patient was large, indicating that the morphology and metabolic activity were not as closely related as perhaps envisaged. Such observations are highly consistent with the previous work on the mouse blastocyst [37]. Second, the human embryo produces significant amounts of ammonium at the blastocyst stage, >25 pmol/embryo per hour (Figure 27.4). These data led to the conclusion that the uptake and metabolism of glucose, but not pyruvate, may serve as a suitable marker of human blastocyst viability. Furthermore, with the production of ammonium by the embryo, plausibly reflecting amino acid turnover, it is feasible that either ammonium production and/or amino acid turnover could be used to quantitate embryonic health.

Only a few studies have been performed on nutrient uptake and the subsequent viability of the human embryo. In a retrospective analysis Conaghan *et al.* [42] observed an inverse relationship between pyruvate uptake by 2- to 8-cell embryos and subsequent pregnancy. In a study on human morulae and blastocysts of different degrees of expansion no conclusive data were generated on the ability of nutrient consumption or utilization to predict pregnancy outcome [43]. Unfortunately, in both the above studies the medium used to assess embryo metabolism was a simple one, lacking lactate, amino acids, and vitamins. Under such culture conditions the resultant stress on the embryos would have been detrimental [34], and therefore it is questionable whether any meaningful data could have been obtained. In contrast, Van den Bergh and Devreker [44] showed that in patients who conceived following blastocyst transfer their embryos had an elevated glucose uptake and a higher oxidative

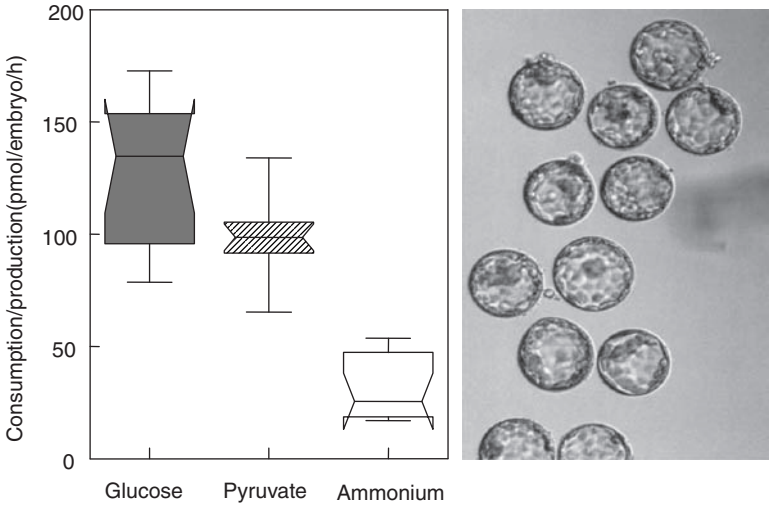


Figure 27.4. Nutrient uptake and ammonium production by individual human blastocysts from the same patient. Thirteen human blastocysts from an oocyte donor were donated to research [41]. These embryos had not undergone cryopreservation. Data are in the form of a notched box plot where the notches represent the interquartile range, therefore including 50% of the data points. Whiskers represent 5 and 95% quartiles. The line across the box represents the median. The spread of glucose uptake is ~100 pmol/embryo per hour, a large spread of nutrient utilization. Pyruvate is not a major nutrient of the blastocyst and does not appear to be different between embryos. The ammonium produced by the blastocysts reflects amino acid turnover. Such is its magnitude that it indicates that the human embryo can modify its immediate environment. This in turn has implications for culture volume and reinforces the need to renew human embryo culture medium every 48 h. The image of the blastocysts indicates how difficult it can be to select them based on their morphology.

rate compared to those blastocysts which failed to establish a pregnancy, supporting the "Rate and Fate" model developed in the mouse. Of note, the study by Van den Bergh and Devreker employed a complete medium (with amino acids) for the metabolic assessment, thereby alleviating the culture-induced metabolic stress. Consequently, this indicates that it is important to assess the embryo under optimal laboratory conditions.

Amino acids

Houghton *et al.* [45] used high-performance liquid chromatography (HPLC) to study amino acid turnover by individual human embryos cultured to the blastocyst stage. Significantly, there was a different pattern of amino acid utilization between embryos that went on to form a blastocyst and those embryos which failed to develop. It was observed that more leucine was taken up from the culture medium by embryos which went on to develop. The profiles of the amino acids alanine, arginine, asparagine, glutamine, and methionine also predicted blastocyst potentiality at > 95%. Subsequently, Brison *et al.* [46] have reported changes in concentration of amino acids in the spent medium of human zygotes cultured for 24 h to the 2-cell stage in an embryo culture medium containing a mixture of amino acids, using HPLC. It was found that aspargine, glycine, and leucine were all significantly associated with clinical pregnancy and live birth. Clinical trials in this area are ongoing. Following on from this, we have recently analyzed the amino acid turnover of individual human blastocysts, together with their glucose consumption, in order to increase the number of parameters assessed [47]. In this analysis, it was determined that distinct patterns of amino acid utilization were evident between viable and non-viable blastocysts, and that glucose utilization by viable blastocysts was significantly greater than by those embryos which did not result in pregnancy (Gardner, Wale, Collins and Lane, unpublished).

An interesting observation from the analysis of amino acid utilization was that overall those embryos that developed exhibited a lower turnover of amino acids than the "non-viable" embryos, leading to the hypothesis that a "quiet metabolism" is optimal [48]. However, given that blastocyst viability is positively correlated to glucose consumption, this hypothesis may be restricted to specific nutrients. Alternatively, the "Rate and Fate" hypothesis can also be applied to amino acid consumption. What the two hypotheses have in common is that embryo viability can be quantitated within a range of uptakes and activities, but that to stray either too far above or below will lead to loss of viability.

Currently, it is possible to quantitate the presence of several potential biomarkers using targeted

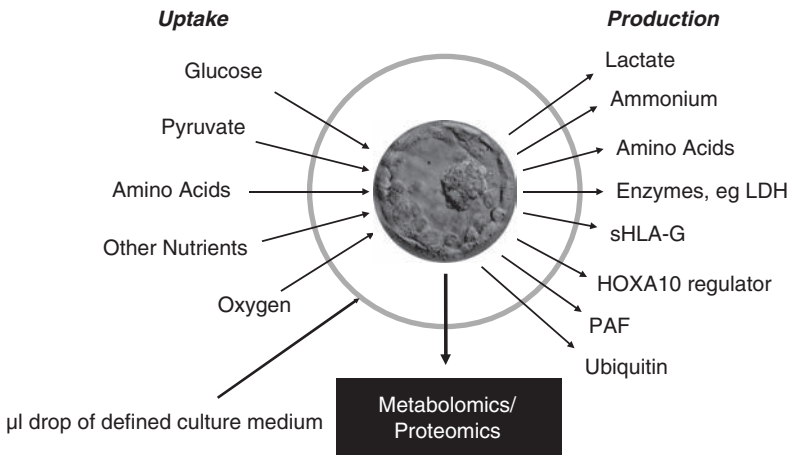


Figure 27.5. Non-invasive analysis of human embryo nutrient consumption and metabolite/factor production. Individual blastocysts are incubated in 1.0 to 10.0 µl volumes of defined medium. Serial or end-point samples of medium can then be removed for analysis. An indirect measurement of metabolic pathways, i.e. glycolysis and transamination, can be obtained by measuring specific nutrients in combination, such as glucose uptake and lactate production, or amino acid turnover with ammonium production.

approaches (Figure 27.5). The potential advantage of measuring a greater number of parameters is the possibility to create weighted algorithms associated with pregnancy outcome.

Enter metabolomics

With relative abundance of data showing the relationship between embryo development and viability and carbohydrate and amino acid metabolism, one may stop to consider why there has not been more clinical application of such work. One of the drawbacks to this type of analysis has been the relative technical complexities involved [49] and the requirement for highly specialized equipment. Furthermore, the work reported to date has for the most part been essential basic research on human embryo physiology. With the advent of emerging technologies, it is feasible that there will be a greater adoption of these kinds of analysis (see below).

An emerging technology in this area is that of metabolomics, in which an overall metabolic fingerprint of the surrounding medium is taken, rather than measuring known nutrients and metabolites [50, 51]. Using such platforms as Raman and near infrared (NIR) spectrometry, it is possible to obtain a spectral profile of the culture medium in which an embryo has developed [52, 53] (Figure 27.6). Although it is not possible to identify specific components that an embryo is using through such spectroscopies, it is possible to attribute specific changes in the spectrum due to the presence of a viable embryo. The potential advantage of such an approach is that one is taking an overall analysis of the culture environment. To interpret the many changes that occur, specific algorithms need to be generated which can then be related to subsequent pregnancy outcome (Figure 27.6). Seli and colleagues [52] established that differences between embryos are detectable in the culture media using both Raman and NIR spectroscopy. A total of 69 day 3 spent embryo culture media, samples from 30 patients with known outcome (0 or 100% sustained implantation rates) were evaluated using Raman and/or NIR spectroscopy. An algorithm was developed to assign a relative "embryo viability score," equating to embryo reproductive potential. It was determined that this score correlated to positive or negative implantation outcomes. Both Raman and NIR spectroscopic analysis of the spent culture media of embryos with proven reproductive potential demonstrated significantly higher viability indices than those that failed to implant [54]. Interestingly when human embryos of similar morphology are examined using the same NIR spectral profile their viability scores vary remarkably in relation to morphology, indicating that the metabolomes of embryos that look similar differ significantly (Figure 27.7). This observation is in agreement with the study on glucose consumption by individual human blastocysts [41], and that of Katz-Jaffe *et al.* [55], who revealed that the proteome of individual human blastocysts of the same grade differed between embryos. Such data reinforce that embryo morphology is not completely linked to its physiology and that there is a precedent for determining which biomarkers best reflect pregnancy potential. What is evident is that as further platforms enter the arena of gamete and embryo analysis, such as LC-MS QQQ [47] and GC-MS, a more detailed understanding of the physiology of human reproduction will emerge.

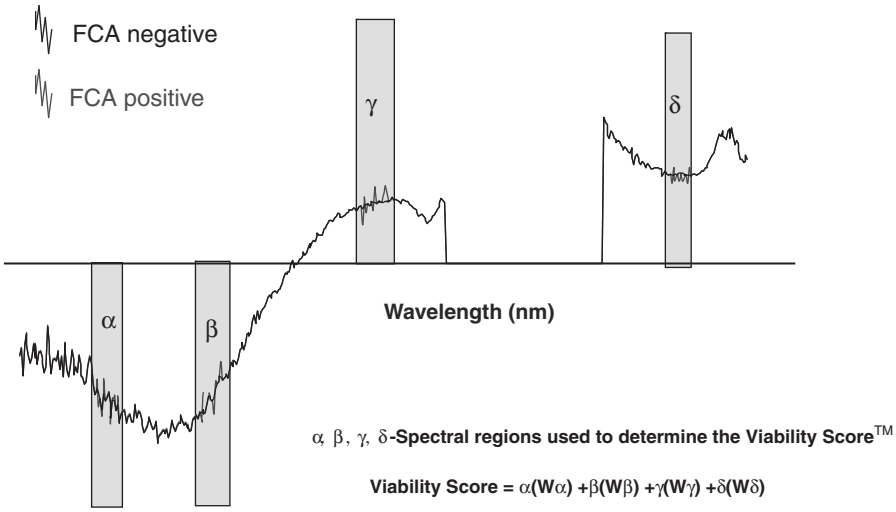


Figure 27.6. Training spectra used to distinguish between embryo culture media giving rise to positive and negative fetal cardiac activity (FCA). Spectra leading to positive and negative FCA outcomes are compared to search for areas of differences in the near infrared spectral range. In this example four wavelet regions discriminate between the positive and negative samples. Once found the wavelet regions are used to create a formula that generates a Viability Score™ whereby the higher the score the closer the spectra resemble that of one that leads to a pregnancy. With permission of Molecular Biometrics. See colour plate section.

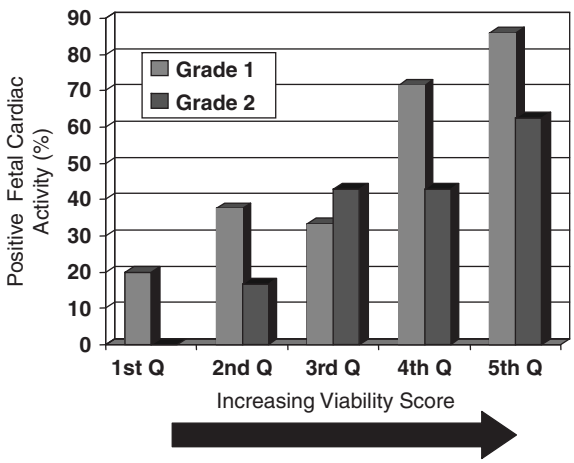


Figure 27.7. Viability scores from 73 day 5 embryos that underwent single embryo transfer. The *x*-axis represents the quintiles of increasing viability scores from day 5 embryos that had an excellent morphology (Grade 1) and good morphology (Grade 2). The *y*-axis represents the % positive fetal cardiac activities from the respective quintiles of Viability Scores™ [69]. With permission of Molecular Biometrics.

Laboratory on a chip

An area of rapid advancement is that of microfluidics, also known as laboratory on a chip. As discussed in Chapter 21 by Johnson and Gardner, this technology holds great promise for both the culture and analysis of gametes and embryos. The ability to work in submicroliter volumes, such as those found within the body, offers unique capabilities to study embryonic function at a level not possible before. Our own endeavors into analysis of embryo physiology led to the development of a microfluidic device capable of analyzing nanoliter volumes of culture medium for three separate nutrients (Figure 27.8) [56]. The potential of such technology in human ART is enormous, with the possibility of being able to perform genetic analysis of cells [57], as well as the types of metabolic determinations described above.

When to assess metabolism?

With the advent and application of several new analysis platforms, a legitimate question would be: when is the optimum time to quantitate embryo physiology? One answer is simply the same day on which one routinely performs embryo transfer. An alternative approach is to consider when the greatest variations in embryo physiology are likely to occur. Given the quiescent nature of the pronucleate oocyte and early cleavage stages, it may be harder to separate embryos due to the smaller variations in activity (Figure 27.9). In comparison, by the blastocyst stage, when the embryo is under predominantly embryonic gene control, there may be most of an opportunity to identify

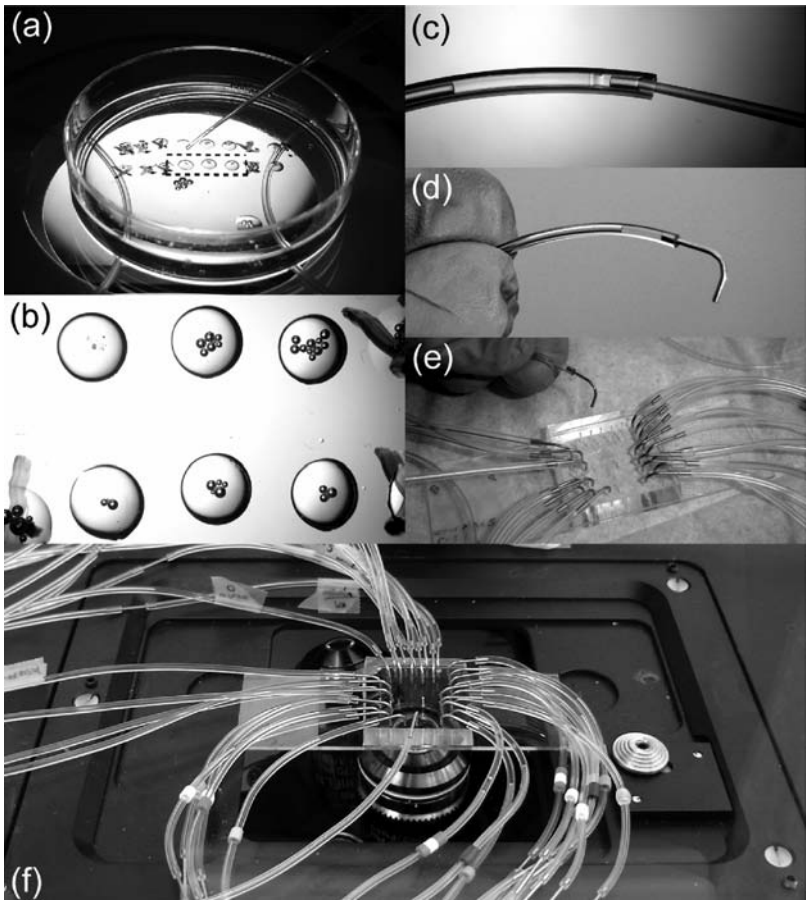


Figure 27.8. An example of a microfluidic chip for the analysis of the culture medium surrounding an individual embryo. Sample loading sequence: (a,b) precollected embryo media samples are stored in a Petri dish (35 mm) under oil to prevent evaporation; (c) a gel loading pipet tip is recommended for transfer of individual media samples (typically 1–5 µl working volume) directly into a length of 500 µm i.d. Tygon tubing; (d) the end of the tubing may be cut to prevent the carryover of any oil prior to adding a sterilized pin; (e) all samples and reagent lines are connected to a detector chip connected to the control manifold, then (f) mounted on an inverted microscope for the experiment. Taken from Urbanski *et al.* [56] with permission.

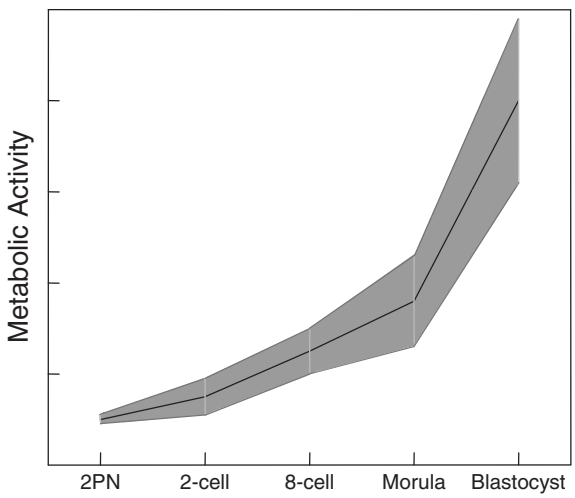


Figure 27.9. Distribution of metabolic actvity with respect to embryo development. As develoment proceeds and metabolic activity increases, there is also an increase in the range of metabolic activities. Consequently, it may be easier to identify a viable embryo within a cohort at later stages of development.

the most viable embryos within a cohort. What is plausible is that the biomarkers identified will be stage specific, and what may be useful for embryo selection on day 1 will be different to those used on day 5.

Concluding thoughts

We are currently entering an extremely exciting period of human assisted conception, one that will surely result in the routine transfer of a single healthy embryo for almost all patients, culminating in the birth of a healthy singleton child. The overall success rates of human IVF have been increasing steadily in parallel with improvements in embryo culture systems and cryopreservation techniques. The development and introduction of novel, non-invasive, quantitative means of selecting embryos for transfer will not only ensure single embryo transfer becomes the standard of care, but the ensuing data on human embryo physiology will help to understand events involved in implantation.

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Chapter

28

Oocyte and embryo cryopreservation

Tetsunori Mukaida and Chikahiro Oka

Introduction

In assisted reproductive technology (ART), cryopreservation of embryos has become important for the best use of supernumerary embryos. During the steps of the cryopreservation of embryos, there is a risk of various types of injury [1, 2]. Among them, the formation of intracellular ice appears to be the most damaging. The first strategy to prevent intracellular ice from forming was to adopt a lower concentration of cryoprotectant and a long slow-cooling stage. This slow freezing method has proven effective for embryos of a wide range of mammalian species. Unlike embryos of laboratory animals and domestic animals, in which dimethylsulfoxide (DMSO), glycerol, or ethylene glycol (EG) are commonly used as the cryoprotectant (cryoprotective agent: CPA), human embryos at early cleavage stages have most often been frozen in a solution of propanediol supplemented with sucrose [3], although those at the blastocyst stage have more frequently been frozen with glycerol and sucrose [4–6]. With slow freezing, however, it is difficult to eliminate injuries occurring from ice formation completely. Furthermore, the slow freezing method requires a relatively long period of time before embryos are stored in liquid nitrogen (LN_2).

In 1985, Rall and Fahy [7] applied the innovative approach of "vitrification," in which injuries related to ice crystal formation are minimized by using very high concentrations of CPA together with rapid temperature change. The definition of vitrification is the solidification of a solution at a low temperature without the formation of ice crystals, by increasing the viscosity using high cooling rates [1, 7]. The rapid cooling process can minimize chilling injury and osmotic shock to the embryos. With recent improvements vitrification has become a reliable strategy, not only because it is technically simple but also because it can lead to high survival rates. To induce vitrification in LN_2 or super-cooled air [8], the solution must contain a high concentration of CPA. This approach simplifies the cooling process, because embryos can be rapidly cooled directly in LN_2. Although embryos subjected to vitrification are potentially liable to be affected by the toxicity of the high concentration of CPA, the method has been refined and proven to be effective for the cryopreservation of embryos at various stages of development in laboratory and domestic species. In 1998, it was demonstrated that vitrification using an EG-based vitrification solution [9] (EFS40) with conventional cryo-straws was relatively effective for human embryos at the 4–8-cell stage [10]. The effectiveness of vitrification was confirmed for human embryos at the 8–16-cell stage [11] and the morula stage [12], also using EG-based solutions.

Over the past decade there have been several advances in vitrification technologies such that it can provide high clinical efficiency along with better clinical outcome. It is proposed that vitrification will become the most suitable method for cryopreservation of any cells and tissues in the near future. This chapter will, therefore, focus on vitrification technologies for cryopreservation in human ART.

Principles of vitrification

The basic procedure for vitrification is simple. Embryos are suspended in a vitrification solution and then plunged in LN_2 or super-cooled air. Embryos are warmed rapidly and diluted quickly

Human Assisted Reproductive Technology: Future Trends in Laboratory and Clinical Practice, ed. David K. Gardner, Botros R. M. B. Rizk, and Tommaso Falcone. Published by Cambridge University Press. © Cambridge University Press 2011.

with a sucrose solution. The most important stage is the exposure of embryos to the vitrification solution before rapid cooling. In order to prevent intracellular ice from forming, a longer period of exposure is desirable. However, if the exposure is too long, embryos suffer from the toxicity of the solution. Therefore, the optimal exposure time for successful vitrification must be a compromise between preventing the formation of intracellular ice and preventing toxic injury. Ironically, embryos may be injured by the toxicity of the cryoprotectant before enough cryoprotectant can permeate inside the embryos. To prevent this, a two-step procedure is commonly adopted, in which embryos are first equilibrated in a dilute (e.g. 10%) CPA solution, followed by a brief (30–60 s) exposure to a vitrification solution before embryos are cooled with LN_2. The optimal exposure time in the vitrification solution depends not only on the CPA solution but also on the temperature, since both the permeability of embryos and the toxicity of the CPA are largely influenced by the temperature [6, 7].

In vitrification, the selection of CPA requires extreme care because their concentration can be as high as 6 M, which can make the toxicity of these compounds a key limiting factor in cryobiology. The most appropriate characteristics of a penetrating CPA are low toxicity and high permeability. For cryopreservation of human embryos, PROH and DMSO have been used as the dominant CPAs, although glycerol is used when embryos are frozen at the blastocyst stage [6]. As a less toxic CPA, ethylene glycol is commonly and widely used [2]. However, few comparative studies have examined the effect of the CPA on the survival of vitrified embryos.

Day 2–3 embryo vitrification

In 1998, an investigation was conducted to find a suitable CPA and suitable conditions for exposing embryos to a vitrification solution using 8-cell mouse embryos [10]. The survival rates of 8-cell mouse embryos vitrified in various solutions after exposure to the solutions for 0.5 and 2 min at 20°C and 25°C were measured. The highest rates of survival were obtained with ethylene glycol-based solutions, regardless of the time and temperature. Although none of the vitrified embryos was morphologically normal when embryos were vitrified after 0.5 min exposure to any mixture of 30% CPA, the survival rate was over 90% when embryos were treated for a longer time (2 min) at a higher temperature (25°C), or when embryos were treated with a higher concentration of ethylene glycol (EFS40) at a higher temperature (25°C).

In addition, a small saccharide (e.g. sucrose) and a macromolecule (e.g. Ficoll 70, BSA or PVP) are frequently included in vitrification solutions. These non-permeating agents are much less toxic, and are known to promote vitrification of the solution [9]. Therefore, their inclusion can reduce the toxicity of the solution by decreasing the concentration of the permeating agent required for vitrification. In addition, inclusion of a saccharide promotes shrinkage of embryos, and thus reduces the amount of intracellular cryoprotectant, which will also reduce the toxic effect of the permeating CPA [9]. At the same time, the osmotic action of saccharide plays an important role in minimizing the swelling of embryos during dilution, since a quick dilution is necessary to prevent the toxic effect of the CPA solution.

Protocols and clinical results of day 2–3 vitrification

There are several protocols that have been introduced for human day 2–3 vitrification. However, in those protocols, the basic concept is similar and the differences between the protocols are related to the type and concentration of CPAs and duration of exposure of CPAs. A summary of those protocols and clinical outcomes are briefly described as follows and appear in Tables 28.1 and 28.2.

Vitrification using conventional cryostraws for day 2–3 embryos

A two-step protocol for vitrification with straw as a container using ethylene glycol-based solutions, EFS20 and EFS40, has been described [1, 10]. The two solutions (EFS20 and EFS40) are used for pretreatment and vitrification, respectively, and contain ethylene glycol diluted to 20% (v/v) or 40% (v/v) with Ficoll-sucrose (FS) solution. This method has been proven suitable for human embryos on day 2–3 (Ref. 10, including Mukaida *et al.*, unpublished data).

In 1998, the effectiveness of this vitrification method for day 2–3 human embryos was reported [10] and its effectiveness was confirmed.

Table 28.1. Summary of each vitrification protocol with respect to the concentration, time, and properties of vitrification solution for day 2–3 human embryo cryopreservation

	Cryostraw	Cryoloop[a]	Cryoloop[b]	Cryoloop[c]	Cryotop
Temp.	Room (25–27°C)	Warm stage (37°C)	Warm stage (37°C)	Warm stage (37°C)	Room (25–27°C)
Equilibration step	EG F S 20:20% EG (2 min)	8% EG (2 min)	7.5% EG + 7.5% DMSO (2 min)	10% EG (5 min)	7.5% EG + 7.5% DMSO (5–10 min[a])
Vitrification step	EG F S 40:40% EG (1min)	15% EG + 15% DMSO + F + S (30 s)	15% EG + 15% DMSO + F + S (35 s)	40% EG + S (30 s)	15% EG + 15 % DMSO + S (1 min[a])
Cooling system	Vapor phase LN2 (3 min), then plunged into LN2	Plunged into LN2 directly (ultra-rapid cooling)	Plunged into LN2 directly (ultra-rapid cooling)	Plunged into LN2 directly (ultra-rapid cooling)	Plunged into LN2 directly (ultra-rapid cooling)
Warming step	One step: 0.5 m S (5 min)	Three steps: 0.6 5 M S (30 s), 0.325 M S (2 min), 0.125 M S (2 min)	Two steps: 0.25 M S (2 min), 0.125 M S (3 min)	Four steps: 1 M S (2.5 min) 0.5 M S, (2.5 min) 0.25 M S, (2.5 min) 0.125 M S, (2.5 min)	Two steps: 1 M S (1 min), 0.5 M S (3 min)

EG, ethylene glycol; F, Ficoll; S, sucrose.
[a] Reported by Balaban in 2008.
[b] Reported by Desai in 2007.
[c] Reported by Raju in 2005.
[d] The duration of equilibration is adjusted according to the time needed for re-expansion of the vitrified cell.

Table 28.2. Summary of the clinical results in each vitrification approach for day 2–3 embryos

	Cryostraw	Cryoloop[a]	Cryoloop[b]	Cryoloop[c]	Cryoloop
Age		31.4 ± 5.1	34.1 ± 4.5	31.3 ± 4.5	35.0 ± 4.5
No. of cycles	127	73	77	40	604 346 patients
Survival rate		222/241 92%	201/236 85%	121/127 95%	1701/1774 95.9%
Cleavage rate[d]	486/661 76%	146/222[e] 66%	184/236 78%		1289/1774 72.7%
Pregnancy rate	34/127 26.8%	33/76 49.3%	34/77 44.2%	14/40 35.0%	164/604 27.2%
Implantation rate		50/168 29.8%	40/121 19.9%	18/121 14.9%	192/1442 13.3%
Delivery rate[f]	22/127 17%	33/73[f] 45%		13/40 32.5%	118/604 19.5%

[a] Reported by Balaban in 2008.
[b] Reported by Desai in 2007.
[c] Reported by Raju in 2005.
[d] Including survival and further cleavage rate.
[e] Number of morula on day 4.
[f] Including on-going pregnancy.
[g] Ongoing pregnancy rates; eight deliveries were confirmed at the time of reporting (two twins, six singletons).

Vitrification using the cryoloop for day 2–3 embryos (Tables 28.1, 28.2)

An improvement to cleavage-stage ultra-rapid vitrification came with the cryoloop [14–17]. This method is effective for embryos on day 2–3, for which conventional vitrification using a straw was found to be less effective. The protocol for vitrification using the cryoloop can be found in the following section on vitrification of blastocysts.

In 2008, Balaban *et al.* [18] reported a two-step protocol of cryoloop vitrification for day 2–3 embryo

cryopreservation. Their protocol (Table 28.1) was originally described by Larman *et al.* [19]. All vitrification and warming procedures are performed at 37°C. The embryos are held in 1 ml of the base holding solution (G-MOPS: Lane and Gardner [20]; supplemented with 12 mg/ml human serum albumin; HSA) for 5–15 min. One or two embryos are placed into the equilibration solution for 2 min. The equilibration solution has the same composition at the holding solution except that it contains 8% (v/v) EG. Once the 2 min have elapsed, the embryos are placed into the vitrification solution for 30 s. The vitrification solution has the same composition as the holding solution except that it contains 16% (v/v) EG, 16% (v/v) PROH, 10 mg/ml Ficoll and 0.65 M sucrose (RapidVit™ Cleave, Vitrolife). The embryos are loaded onto the cryoloop (Hampton Research, Aliso Viejo, CA, USA), transferring as little medium as possible, typically around 50 nl. The cryoloop is then loaded into the cryovial held on a cryocane, which was submerged in liquid nitrogen prior to loading. For warming, the cryoloop is removed from the cryovial and dipped into warming solution 1. Embryos fall off the cryoloop and are moved through 1 ml volumes of a serial sucrose dilution in G-MOPS supplemented with 12 mg/ml HSA (RapidWarm™ Cleave, Vitrolife): warming solution 1 (0.65 M sucrose) for 30 s; warming solution 2 (0.325 M sucrose) for 1 min; warming solution 3 (0.125 M sucrose) for 2 min and warming solution 4 (0 M sucrose) for 5 min. For subsequent embryo development, embryos are then moved into G2.3 (Vitrolife) for 24 (embryo transfer) or 48 h (blastocyst assessment). Clinical outcome was as follows (Table 28.2). A total of 73 women subsequently underwent vitrified-warmed embryo transfers. A mean number of 3.3 embryos were warmed (n = 241). The cryosurvival rate was 92.1%. All blastomeres were intact in 72.1% of the embryos after the warming procedure. The mean number of embryos transferred was 2.3 (n = 168). A clinical pregnancy rate and ongoing pregnancy rate of 49.3 and 45.2% were achieved, respectively. The implantation rate was 29.7% (n = 50), resulting in a multiple-pregnancy rate of 36.1% (n = 13: one triplet, 12 twins). At the time of reporting, eight of the ongoing 33 pregnancies have had successful deliveries of healthy children (two twins, six singletons). Moreover, in this study they revealed that vitrification was a more effective approach to cryopreserving the human embryo than conventional slow freezing.

In 2007, Desai *et al.* [21] reported the post-vitrification development, pregnancy outcomes and live births for cryoloop vitrification of human day 3 cleavage-stage embryos. Tables 28.1 and 28.2 include their protocol and results, which presented consecutive vitrification-warming cycles performed over a 2.5-year interval.

In 2005, Rama Raju *et al.* [22] reported a modified protocol for vitrification of human 8-cell embryos using the cryoloop technique. The protocol including the type of CPA and duration of exposure is different from the one by reported by Desai *et al.* During the cooling steps, embryos are suspended in a 10% EG solution for 5 min at 37°C, and transferred to a 40% EG in 0.6 mol/l sucrose solution for 30 s. For warming, vitrified embryos are passed through four different concentrations of sucrose solution i.e. 1 mol/l, 0.5 mol/l, 0.25 mol/l, and 0.125 mol/l, for 2.5 min at each step at 37°C (Table 28.1). Table 28.2 includes results to show the effectiveness of their protocol.

Vitrification using cryotops

Since the vitrification approach using a cryotop or a cryoloop is similar to that with a minimal volume cooling system, the basic concept of the protocol is the same. The following protocol was originally introduced by Kuwayama *et al.* [23].

For vitrification using a cryotop, initially embryos are exposed to equilibration solution for 5 to 10 min, an equal mixture of 7.5% DMSO and 7.5% ethylene glycol (EG) in HTF supplemented with 20% HSA, and moved to a vitrification solution for 1 min at room temperature (25–27°C), using a mixture of 15% DMSO, 15% EG, and 0.5 M sucrose in HTF/HSA. The duration of the equilibration time is adjusted by assessing morphological changes that indicate shrinkage from dehydration and re-expansion from cryoprotectant (CPA) permeation, and is individually recorded for further analysis. Embryos are then loaded onto a minute nylon sheet (cryotop), and plunged into LN2 immediately. For warming, vitrified embryos on the tip of a cryotop are dipped and kept in 1 M sucrose solution for 1 min and then diluted in 0.5 M sucrose solution for 3 min. Embryos with 70% or more intact blastomeres are considered as indicative of survival and kept in culture until transfer on the following day (Table 28.1).

Table 28.2 includes the results from the use of the cryotops at Nagata Clinic to show the effectiveness of clinical application.

Blastocyst vitrification

Recent advances in culture systems with sequential media have made it possible to develop human IVF embryos to the blastocyst stage quite easily. Because the blastocyst is better suited to the uterine environment and blastocyst formation is a form of selection for more viable embryos, blastocyst transfer has become a promising option to raise the overall pregnancy rate [24, 25]. Accordingly, the need to cryopreserve human blastocysts is increasing. Menezo *et al.* [6] cryopreserved human blastocysts which were developed in a co-culture system using the slow freezing method with glycerol and obtained reasonable clinical results (27% pregnancy rate, 17% implantation rate). However, results reported by other clinics have not been consistent [26–28]. Menezo *et al.* [6] speculated that the cryopreservation outcome might be influenced by the culture conditions, such as a co-culture system.

Recently, human blastocysts were successfully vitrified in straws [29]. However, our own attempts to vitrify human blastocysts using straws resulted in only 45% survival (39/86, unpublished data). Vanderzwalmen *et al.* [30] also reported a low pregnancy rate with human blastocysts vitrified in straws. This is probably because human blastocysts are much less permeable to CPA and water, since it has been observed that they shrink more slowly than mouse and bovine blastocysts in the CPA solution. This suggests that human blastocysts are more likely to be injured by intracellular ice crystal formation.

Increased rates of cooling and warming can help circumvent the problem of intracellular ice formation in less permeable embryos. Faster rates of cooling and warming can be achieved by minimizing the volume of the solution with which embryos are vitrified, i.e. by using minute tools such as electron microscopic (EM) grids [31], open pulled straws [32], cryoloops [2, 33], or cryotops [23]. We showed that the transfer of human blastocysts vitrified with cryoloops can lead to successful births [14]. Since this original report, we have continued to use this vitrification approach for the cryopreservation of blastocysts on day 5 and day 6.

Currently several established blastocyst vitrifications have been reported. As one of the examples, this chapter includes our protocol of blastocyst vitrification and a summary of the clinical outcomes for the last 10 years, which confirms the safety and the effectiveness of the cryoloop technique for the cryopreservation of human blastocysts.

Protocol for blastocysts vitrification (Figure 28.1)

The protocol for the cryoloop vitrification of blastocysts was adopted from the work of Lane *et al.* [17] with slight modifications [14–16]. Procedures of vitrification involve equilibration and vitrification steps carried out at 37°C in 7.5% DMSO and 7.5% EG for 2 min, and 15% DMSO, 15% EG, 1% Ficoll 70, and 0.65 M sucrose for 30–45 s in HTF/HSA. After 30–45 s, the blastocysts are loaded on a small nylon loop (Hamilton Research, CA, USA) and are plunged directly into LN_2. They are warmed by placing the tip of the cryoloop into 0.5 M sucrose in HTF/HSA and keeping it there for 2 min and then in 0.25 M sucrose

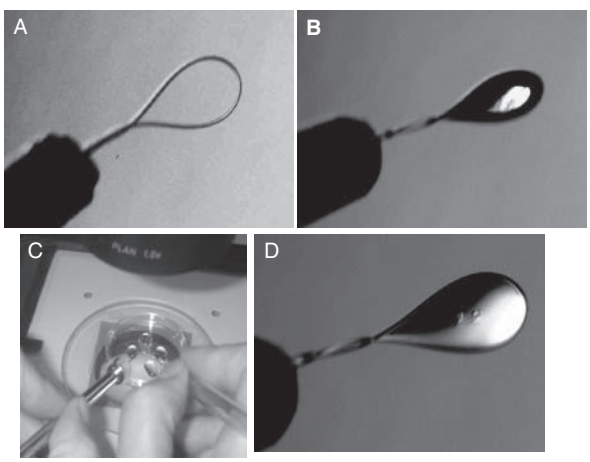


Figure 28.1. (A) A minute nylon loop (20 μm wide, 0.5–0.7 mm in diameter) mounted on a stainless steel pipe under × 100 magnification. (B) A thin layer of the vitrification solution on the nylon loop after dipping the loop into the solution. (C) Under a dissecting microscope, the capping portion of the cryovial with the cryoloop attached with a stainless steel handling rod for manipulation is held by the left hand, and a pulled Pasteur pipette with blastocysts is held by the right hand. Prior to loading, blastocysts are rinsed several times in small drops of final vitrification solution (solution II) on the lid of a culture dish. (D) Blastocysts on the loop with a thin layer of vitrification solution.

in HTF/HSA for 3 min. With the use of a cryoloop as a container, the vitrified blastocyst almost floats in the thin filmy layer of the droplet on the nylon loop, and heat conduction to the blastocyst becomes homogeneous and extremely high. With the extremely high cooling rate, full equilibration of CPA is not necessary to avoid ice crystal formation, and inside the cell is a so-called "meta-stable situation." That is why around 3 minutes of CPA exposure will be enough to reach vitrified status inside the cells. This duration of exposure is shorter than that of other vitrification approaches. Shorter exposure of CPA is more favorable due to avoiding the exposure of the cells to the potentially toxic agent.

Warming of blastocysts, assisted hatching, and assessment of survival

With the cryovial submerged in LN_2, the vial is opened with the aid of the stainless steel rod, and the loop containing blastocysts is removed from the LN_2 and placed directly and quickly into the well containing the 0.5 mol/l sucrose solution. Blastocysts immediately fall from the loop into the solution. Thus blastocysts are warmed and are diluted instantly at around 37°C adjusted by the stage warmer. After 2 min, the blastocysts are transferred to the 0.25 mol/l sucrose solution. After an additional 3 min, blastocysts are washed and are kept in the base medium for 5 min. During this 5 min, assisted zona hatching is always performed on warmed blastocysts with either acidic tyrode as previously described [34, 35] or multiple shots of laser pulse to the zona lesion on warmed blastocysts.

About 2 to 3 h after warming, the appearance of the blastocysts is examined on an inverted microscope at × 400 magnification, and survival is assessed based on the morphological integrity of the blastomeres, inner cell mass, and trophectoderm, and re-expansion of the blastocoel. The surviving blastocysts are scored as to developmental stage and are graded according to quality as described in the section on grading of blastocysts.

Patients, and grading of blastocysts

Almost 4500 cycles have been performed in our clinic for the following three categories of patient groups: Group 1 (1238 cycles), patients who had their fresh embryos transferred on day 2–3, and all the remaining embryos were cultured to allow those which developed into blastocysts to be vitrified;. Group 2 (1864 cycles), patients who received transfers of fresh blastocysts and had all their remaining supernumerary blastocysts vitrified; Group 3 (1413 cycles), patients who had no fresh embryo transfer due to either avoiding OHSS symptoms or attempting only vitrified blastocyst transfer intentionally along with a controlled endometrial cycle (CEC) were supplemented with exogenous female hormones in order to overcome multiple implantation failures, because uterine receptivity under CEC was indicated as better than that under stimulated cycles. After two or three failures of implantation with fresh transfer attempts, fresh blastocyst transfer is intentionally avoided with our clinical concept. One of the reasons is that ovarian hyperstimulation does not always create a suitable uterine receptivity and environment for implantation compared with controlled endometrial preparation using exogenous hormones.

On day 5 after the oocyte pick up, blastocyst development was examined. Only on day 5, each embryo that developed to the blastocyst stage was scored depending on the developmental stage, and graded according to quality criteria [36] with slight modifications [15].

Briefly, blastocysts were first given a numerical score from 1 to 6 on the basis of their degree of development. Second, the blastocysts were graded in three ranks based on morphological appearance. For example, the inner cell mass (ICM) was graded as A (many tightly packed cells), B (several loosely grouped cells) or C (few cells) and the trophectoderm was graded as A (many cells forming a cohesive epithelium), B (fewer cells forming a loose epithelium) or C (very few large cells).

When patients had their fresh embryos transferred on day 2–3, all the remaining embryos were cultured to allow those that developed into blastocysts to be vitrified. Patients who received transfers of fresh blastocysts had all their remaining supernumerary blastocysts vitrified. On day 5, if at least one supernumerary blastocyst was graded as A or B, all the blastocysts of the patient were vitrified regardless of the developmental stage and the grading. In a few cases, compacted morulae forming the cavity were also vitrified with the blastocysts. If all the blastocysts of the patient were graded C, they were not cryopreserved. On day 6, if at least one blastocyst had a large blastocoel (i.e. scored as 3–6) and was graded as A or B, all the developed blastocysts scored as 3–6 were vitrified.

Artificial shrinkage (AS) of expanded blastocysts

In 2003 it was reported that blastocyst survival rates were dependent on the developmental stage and were negatively correlated with the expansion of the blastocoel [15]. The survival rate of early blastocysts with a smaller blastocoel cavity, scored 1 and 2 according to quality criteria [36], were 87% (48/55) and 97% (62/64) respectively. Also, full blastocysts lacking an expanded blastocoel cavity, which were scored 3, had a survival rate of 89% (99/111). The total survival rate of blastocysts scored 1–3 together was 91% (209/230). However, the survival rate of both expanded and hatching blastocysts, scored 4 and 5 respectively, was 85.0% (288/339), which was significantly lower than that of the score 1–3 group ($P < 0.05$). It was therefore postulated that a large blastocoel might lessen cryopreservative potential due to ice crystal formation during the rapid cooling phase of vitrification. In order to overcome this problem, shrinkage of the blastocoel was thought to be the appropriate approach. Several studies reported an increase in the survival rate of blastocysts when the volume of the blastocoel was artificially reduced with a glass microneedle [37], a 29-gauge needle [38] or micropipetting with a hand-drawn Pasteur pipette [39].

Since 2003, we have therefore added artificial shrinkage (AS) after puncturing the blastocoel with a microneedle or laser pulse prior to vitrification to improve the survival rate and clinical outcome of vitrified blastocyst transfer programs. In 2006, we reported the effectiveness of AS prior to vitrification, including the confirmation of the safety of this procedure [40]. Briefly, about 10 minutes before the vitrification, the expanded blastocysts were placed in 50 µl drops of pre-equilibrated medium. The expanded blastocyst was held with a holding pipette connected to the micromanipulator, the ICM was placed at the 6 or 12 o'clock position and a glass microneedle was pushed through the cellular junction of the trophectoderm into the blastocoel cavity until it shrank (Figure 28.2). After removing the microneedle, contraction of the blastocoel was observed within a few minutes. After complete shrinkage of the blastocoel, the blastocyst was vitrified and stored in a LN_2 tank.

Since September 2004, a laser pulse generated by a ZILOS-tk™ laser system (Hamilton Thorn Bioscience Inc., Beverly, MA, USA) has been introduced to perform the artificial shrinkage, instead of microneedle puncture. The inner cell mass should be located away from the targeted point of the laser pulse. A single laser pulse (200 ms) targeted at the cellular junction of the trophectoderm creates a hole to induce collapsing of the blastocoel (Figure 28.3). The blastocoel of the expanded blastocyst shrinks immediately. With the use of this laser system, it is not necessary to hold and locate the expanded blastocyst with a holding pipette connected to a micromanipulator. The laser technique makes the procedure simple and convenient [40].

Clinical results of artificial shrinkage (AS) procedures

In order to show the effectiveness of AS, we summarize the results of 270 cycles in Table 28.3. Results of vitrified expanded and hatching blastocysts in our

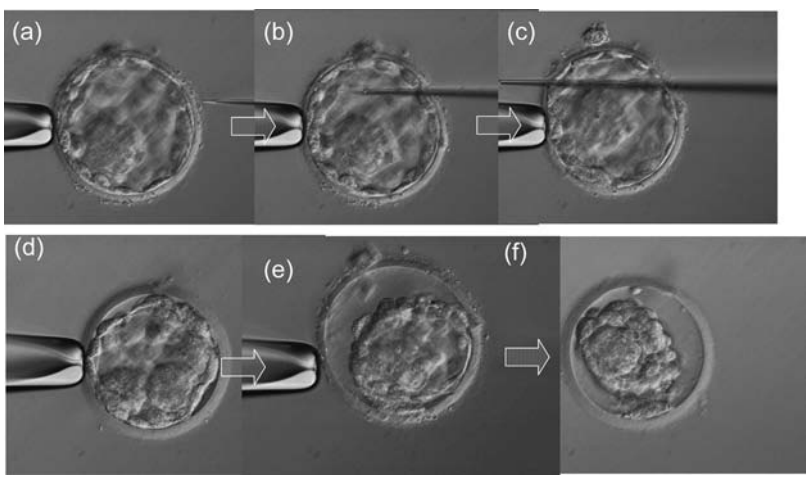


Figure 28.2. Artificial shrinkage of expanded blastocyst with the micro-needle. (a) Holding the expanded blastocyst with the holding micro-pipette connected to micromanipulation; (b) insertion of the micro-needle inside the blastocoel at a point away from the inner cell mass; (c) puncture through the blastocoel and removing the micro-needle gradually; (d) beginning of shrinkage 10 s after puncture; (e) partial shrinkage 30 s after puncture; (f) complete shrinkage 1 min after puncture.

previous study reported in 2003 served as a control group (without AS). The survival rate of both expanded and hatching blastocysts, scored 4 and 5 respectively, was 85.0% (288/339). A statistical difference was noted between the study and the control groups ($P < 0.05$). When the pregnancy rate of the study group was compared with the control group, a statistically significant improvement was noticed in the AS group (60.2% vs. 34.1%; $P < 0.01$).

Also we performed preliminary comparisons between the results achieved using a microneedle or laser pulse for blastocoel shrinkage, to show the difference in AS methodologies. The survival rates achieved with the two methods were similar (microneedle 97.2% vs. laser pulse 97.5%). The mean numbers of surviving blastocysts transferred were also similar. Clinical pregnancy, implantation and miscarriage rates were also similar. No statistical difference was observed in the results achieved with the two methods [40].

Table 28.3. Characteristics of patients and survival of vitrified human blastocysts with (study group) or without (control) artificial shrinkage

	Study group	Control group
No. of patients	245	76
Average age	35.6	34.0
Mean no. of previous IVF/ICSI attempts	2.1	2
No. of initiated vitrified blastocyst cycles	270	
No. of cycles with vitrified blastocyst transfer	266	85
No. of canceled cycles due to no survival of vitrified blastocyst (%)	4(1.5%)	
No. of blastocysts vitrified	502	339
No. of vitrified blastocysts survived	488	288
Survival rate	97.2%	85.0%[a]
No. of vitrified blastocysts transferred	448	
Mean no. of blastocysts transferred	1.7	
Clinical pregnancies	160	29
(%)	60.2%	34.1% [b]

Control: referred to our previous study (Mukaida *et al.* [15]).
[a] $P < 0.05$;
[b] $P < 0.01$.

Clinical results of vitrified blastocyst transfer

Table 28.4 summarizes the clinical results and perinatal outcomes of vitrified blastocyst transfer programs using a cryoloop performed in the 10 years from the beginning of 2000 to the end of 2009. A total of 7754 blastocysts originating from 4515 cycles were vitrified and warmed. The mean age was 36.2 years.

After warming vitrified blastocysts for transfer, 7271 (93.8%) survived. In 82 cycles(1.8%), no blastocysts survived and transfer was not conducted. In 10 cycles, surviving blastocysts were obtained but embryo transfer was canceled, because the number of cells that survived and the quality of the blastocysts were not considered to be suitable for transfer. A total of 6423 blastocysts were transferred in 4433 cycles. The mean number of blastocysts transferred per cycle was 1.45. Of 4433 transfers, 2165 resulted in clinical

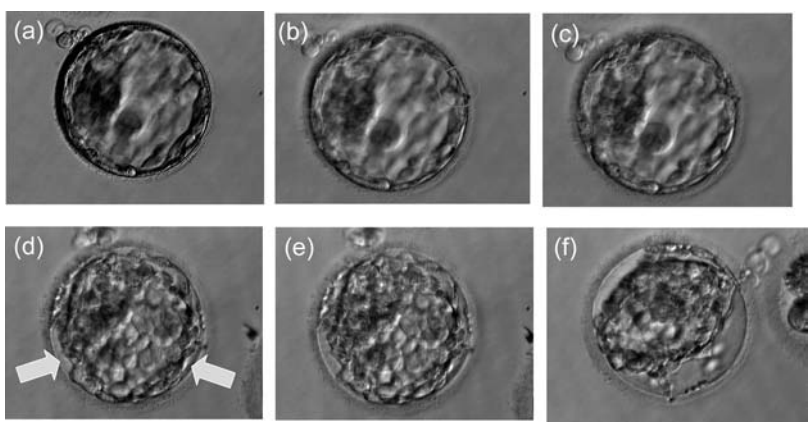


Figure 28.3. Artificial shrinkage of expanded blastocyst with a single laser pulse. (a) Prior to the artificial shrinkage; (b) a single laser pulse at the point of the cellular junction of the trophectoderm cell at a point away from the inner cell mass (circle); (c) beginning of shrinkage 5 s after laser pulse and arrows indicate formation of perivitelline space because of contraction; (d) shrinkage 10 s after laser pulse; (e) shrinkage 20 s after laser pulse; (f) almost complete shrinkage 30 s after laser pulse.

Table 28.4 Clinical outcome of vitrified blastocysts transfer at the Tokyo and Hiroshima HART clinics (2000 to 2009)

Total no. of attempted cycles	4515
Total no. of warmed vitrified blastocysts	7754
Total no. of survived blastocysts	7271
Survival rate	93.8%
No. of transferred cycles	4433
Mean no. of blastocysts transferred	1.45
No. of clinical pregnancies (%/BT)	2165 (4808%)
No. of implantations (%)	2455 (38.2%)
No. of deliveries (babies; boy; girl)	1312 (1517; 779; 738)
No. of miscarriages (%)	608 (28.1%)

pregnancy (confirmed by gestational sac in the uterus); the pregnancy rate was 48.0% per warming cycle, and 48.8% per transfer. The implantation rate was 38.2% (2455/6423).

One thousand five hundred and seventeen babies were born in 1312 deliveries. Since 779 babies were boys and 738 were girls, no bias in the sex ratio was observed. Cesarean sections were performed in 672 deliveries, and the mean gestational age was 38.2 weeks. The mean birth weight of all births through vitrified blastocysts was 2823 g. However, in the singleton delivery outcome (1122 births), the mean gestational age was 39.4 weeks, and mean birth weight was 3071 g. That is not statistically different from national statistics on ART conception reported in 2007 in Japan. One hundred and seventy-eight births were twins (13.6%), and 12 births were triplets (0.9%). Twenty-seven cases had either congenital birth defects or perinatal complications (2.1%), including seven chromosomal abnormalities (two trisomy 18, five trisomy 21), three multiple anomalies, one stillbirth due to hydrops, three stillbirths of unknown causes during delivery (25 weeks, 30 weeks, and 38 weeks), one anencephaly, one spina bifida, four congenital heart malformations, three minor anomalies in hands and/or feet, one congenital esophageal obstruction, one biliary duct obstruction, one Cornelia de Lange syndrome (CdLS), and one Treacher Collins syndrome.

Six hundred and eight pregnant cycles ended in miscarriage (28.1%). A comparison of 1187 pregnancies established from fresh blastocyst transfers in our group of clinics during the same period shows that 249 (21.1%) resulted in miscarriages, and no statistical difference was observed between them. That was also similar to what we reported previously in 2005 [41].

Vitrification of oocytes

Cryopreservation of human oocytes has been significantly improved by refined slow-freezing methods and new vitrification techniques. The establishment of oocyte cryopreservation techniques would provide a number of benefits. First it could prevent ethical and legal issues associated with embryo cryopreservation, particularly in certain countries where embryo cryopreservation is banned or limited by laws and/or regulations. Second, the age at which people marry is rising, resulting in infertility issues, and an egg bank would create an option for older women to have children later in life. Third, it would allow enough time for genetic and infectious screening in donor oocyte programs. It would also provide the ability to synchronize procedures and utilize precious donor oocytes efficiently, which would avoid unnecessary fertilization by the recipient husband's sperm. Finally, oocyte freezing gives the option of fertility preservation for patients who are receiving anticancer treatment or oophorectomy.

Although oocyte cryopreservation has a lot of advantages as described above, clinical outcomes remain unsatisfactory due to lower pregnancy and implantation rates resulting from decreased survival rates and poor embryo development. There are many reasons to be listed why it is difficult to achieve satisfactory results with cryopreservation of oocytes. The oocyte itself is a single cell. Survival can be judged as all or nothing. Multicellular embryos can survive and compensate for the loss of as much as half their cells, as shown by further development after cryosurvival. Membrane permeability of the oocyte is another significant reason. In cryobiology, it is important to achieve acceptable permeation of CPA, dehydration and rehydration. In terms of CPA permeability, the plasma membrane is extremely sensitive and rapidly undergoes a transition from the liquid state to the gel state, an irreversible process that is detrimental for further development. For some reasons, including the releasing of cortical granules, after fertilization the plasma membrane of the fertilized oocyte is much less sensitive to this type of injury. Chilling injury that occurs at relatively high temperatures induces irreversible damage of cytoplasmic lipid droplets, lipid-rich plasma membranes, and microtubules

[42]. The osmotic shock at equilibration may result in shrinking and deformity of oocytes, potentially damaging the cytoskeleton and microfilament. On the other hand, the osmotic swelling shock that can occur during the dilution (rehydration) steps may result in extensive swelling, rupture of the membrane, lysis and immediate death of oocyte. Such damage might be related to the depolymerization of microtubules, misalignment of chromosomes, and the possible increased risk of aneuploidy. However, similar to somatic cell nuclear transfer, in human oocytes spindle reorganization [43] may occur surprisingly efficiently, and the number of chromosomal abnormalities in children born after oocyte vitrification does not seem to be increased.

According to earlier investigations in rabbits [44], the permeability of ethylene glycol is facilitated by DMSO. A possible way to minimize the toxic and osmotic effects of CPA is to decrease the required concentration of CPA while maintaining the ice-free solidification pattern. The only practical way to achieve the above circumstances is to induce an extreme increase in cooling rates. For this purpose, electron microscope (EM) grids [31], cryoloops [33], and cryotops [23, 45] seem to be the most appropriate tools, as well as containers. The problem of zona hardening and subsequent low level of fertilization has been circumvented with the discovery and subsequent widespread application of ICSI.

Recently, there have been many reports related to oocyte vitrification using ultra-rapid vitrification techniques, especially the cryotop [23, 45, 46] method. The cryotop that was originally introduced [23, 45] by Kuwayama is now used in an increasing number of laboratories worldwide for oocyte vitrification as well as embryo cryopreservation [23]. Solutions are based on TCM199 medium supplemented with synthetic serum substitute (SSS), and containing ethylene glycol, DMSO, as permeable CPA, and sucrose as non-permeable CPA. The concept and concentration of these solutions are quite similar to those in cryoloop vitrification described previously. However, no Ficoll is added to the vitrification solution for the cryotop technique.

All media and manipulations should be performed at 25–27°C, except for warming, where the medium should be warmed to 37°C. Oocytes can be vitrified 2–6 hours after the ovum pick-up, immediately after denudation. A stepwise, very mild initial equilibration procedure can be carried out by making 20 µl droplets of washing and equilibration solutions (1 and 2 droplets, respectively) close to each other, and unifying droplets when oocytes seem to have completely recovered from the osmotic effect (a total of approximately 6 min). Finally, oocytes should be placed into an equilibration drop and incubated until they are completely recovered (approximately in an additional 9 min). Subsequently, one oocyte should be placed into a large volume of vitrification solution, mixed well, and after 60 s loaded onto the film strip of the cryotop. All excess media should be removed, leaving only the oocyte covered with a thin layer of vitrification solution. Then the film part should be submerged into liquid nitrogen with a quick and continuous vertical movement to ensure the maximum cooling rate (~23 000°C/min). Finally, under the liquid nitrogen, the cap should be fixed on the cryotop with forceps to protect the film part from mechanical damage during transfer to the container and storage. For warming, the film part of the cryotop should be submerged quickly into the 37°C warming solution to reach an extremely high warming rate (~42 000°C/min). After 10 s, the oocyte can be gently removed from the surface of the cryotop and kept submerged in the warming solution. After 1 min, the dilution should be continued in dilution and washing solutions for 3 and 5 min, respectively. Oocytes should be kept for an additional 2 hours before the ICSI.

The cryotop is now used in an increasing number of laboratories worldwide for oocyte vitrification. Luccena *et al.* [46] reported 89.2% survival rates after cryotop vitrification and a total pregnancy rate of 56.5% (13 of 23 patients) with an average of 4.63 embryos transferred to each patient. In Valencia, Spain, Cobo *et al.* (submitted) have vitrified a total of 225 MII oocytes, of which 217 (96.5%) survived, and 165 (76.0%) were normally fertilized after ICSI, which was not different from the controls. Of zygotes 93.9% underwent cleavage on day 2 and 22.4% of them reached the blastocyst stage. Twenty-one cycles of embryo transfer were performed and resulted in 13 pregnancies (61.9% pregnancy and 37.2% implantation rates).

Conclusions

For embryo cryopreservation, vitrification has many advantages over slow freezing: (1) injuries related to ice are less likely to occur, (2) survival of embryos can be maintained at a higher level if conditions for

embryo treatment are optimized, and (3) embryos can be cryopreserved by a simple method in a short period without a programmable freezer. Therefore, vitrification is suitable for human embryos, where a small number of embryos are cryopreserved frequently. Human embryos at early cleavage stages can be cryopreserved by conventional vitrification using cryostraws or by ultra-rapid vitrification using cryoloops. Human blastocysts are more efficiently cryopreserved by the ultra-rapid approach. Clinical outcomes show that vitrification of blastocysts using the cryoloop technique results in high survival and high pregnancy rates, and confirms the safety of this procedure as seen in our perinatal evaluation.

Since vitrification was introduced as an alternative approach for cryopreservation of human gametes and embryos, vitrification, with recent improvements, has become a more reliable strategy, not only because it is very simple but also because it can lead to high clinical efficiency along with better clinical outcomes. Ultra-rapid vitrification especially opens a new era for oocyte and blastocyst cryopreservation, as described in this chapter. Classically, adequate equilibration of CPA and dehydration are necessary to cryopreserve gametes and embryos. However, the extremely high cooling rate achieved by direct plunging into LN_2 with a minimal volume (≤ 0.5 µl) of final vitrification solution including vitrified cells can produce high survival rates and better viability and help us to avoid ice crystal formation even with the lower concentration of CPA, which could cause devitrification (inducing ice crystal formation) if conventional cooling was applied. Recently this ultra-rapid vitrification approach has been applied for ovarian tissue cryopreservation and stem cell cryopreservation. With the proper preparation of ovarian tissue such as a 1 cm square shape and less than 1 mm thickness with a properly designed container, high survival and better post-warming viability can be expected with this vitrification approach [47]. In future, vitrification will become the most suitable method for cryopreservation of any cells and tissues.

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