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CRYOPRESERVATION OF FISH SEMEN

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Cryopreservation is a process where biological materials such as cells and tissues are preserved by cooling to very low temperatures, usually at -196°C (the temperature of liquid nitrogen), yet remain viable after later warming to temperatures above 0°C. It makes possible almost indefinite storage of the desirable gene pools and ensures the availability of cryopreserved semen for artificial insemination/breeding.

Nearly 60 years have passed since the first demonstration of effective cryopreservation of sperm was made (Polge *et al*., 1949). It leads a major breakthrough in animal husbandry with successful cryopreservation attempt on bull spermatozoa (Smith and Polge, 1950). Soon after, first report on fish sperm cryopreservation was published by Blaxter (1953) and since then flurry of publications demonstrating the feasibility of cryopreserving the fish semen from a large number of marine and freshwater species have appeared. To date milt of over 200 species of freshwater and marine fish have been cryopreserved (Lakra, 1993; Rana, 1995; Blesbois and Labbe, 2003; Hiemstra *et al.,* 2005).

Advantage of cryopreserving the fish semen is well established. It is not only a useful management tool, it offers several benefits such as stock protection from being totally eliminated due to sudden outbreak of disease, natural disaster, over exploitation etc. Other application of cryopreservation include stable supply of sperm for optimal utilization in hatchery production and laboratory experiments, easy stock transportation among hatcheries, improvement in selective breeding whereby stock can be maintained more economically and effectively, experimental material for advanced studies such as gene transfer.

A number of different protocols are advocated in literature for the preservation of fish semen but most of them are concerned with the salmonids, tilapia, and carp (Ott, 1975; Scott and Baynes, 1980; Chao *et al.*, 1987; Baynes and Scott, 1987; Koldras and Bienarz, 1987; Harvey and Kelley, 1988; Leung and Jamieson, 1991; Gwo, *et al*., 1993; Rana 1995; Babiak *et al*., 1997; Akcay *et al.*, 2004). Extenders and cryoprotectants are important and play a vital role in cryopreservation. Irrespective of the species, fish semen requires dilution before

it has to be cryopreserved. Extenders used for diluting the fish semen are generally designed to be compatible with the physico-chemical composition of seminal fluid of the candidate species. The chemical constituents of extenders vary enormously (Scott and Baynes, 1980; Stoss, 1983). A range of cryoprotective agents of permeating and non-permeating categories are available for the use to minimize cryoinjuries during cooling and thawing process. The DMSO and glycerol are widely used cryoprotective agents. Suitability of extenders and cryoprotectants differs from one fish to another (Muchlisin, 2005). Semen is commonly packaged in cryovials (Ott and Horton, 1971), plastic straws (Erdhal, 1986; Chao *et al.*, 1987) or visotubes (Mounib, 1978; Stein and Bayrle, 1978) cooled over liquid nitrogen vapor or in programmable freezer and stored in liquid nitrogen (Cognet, *et al*., 1996). Fish semen can also be cryopreserved as pellets on dry-ice blocks and then stored in caped cryovials in liquid nitrogen (Leung and Jamieson, 1991). Various cooling methods have been successfully used to cryopreserve the fish sperm. Careful manipulation of temperature excursion is required to control the size, configuration and location of ice crystals. Thus choice and concentration of cryoprotectants and rate of cooling is needed to be optimized for each species as the basis for any protocol development.

From the current state of art of fish spermatozoa cryopreservation and species differences, one universal protocol cannot be suggested since response to cryoprotectant and freezing vary with the different biology. Thus, optimization of the protocol is needed for each individual species though some general rules are applied for each fish species. In the present communication, basic principles and essential steps of cryopreservation techniques for the sperm of fresh water fish species are explained with the example from a snowtrout species (*S.richardsonii*) as a model. For the development of any reliable protocols for fish semen cryopreservation, emphasis should always be placed on the standardization.

CRYOBIOLOGIC PRINCIPLES

Nature dictates that biological material will decay and die. The structure and function of organisms are changed and lost with the time. An attempt to stop the biological clock, experiments with temperature and water contents of the cell is the basic theme of cryopreservation research. The use of much lower temperatures has proved a means of storing living organisms in a state of suspended animation for extended periods. The removal of water from biological material in the frozen state (freeze-drying) provides another means of arresting the biological clock by withholding water, and commencing again by its addition. This is all attempted after realization that water is the major component of all living cells and essential for the chemical processes of life to occur. Cellular metabolism stops when all water in the system is converted to ice during cooling and freezing. Further, reduced temperature lowers the motion of molecules. At absolute zero (-273^{o}C) all molecular motions are inhibited and all biological and biochemical events cease. It is those events at ultra-low temperatures that provide basic mechanisms for long term preservation of biological material in genetically stable form. In practice, no significant change of biological importance occurs below -150 ^{o}C and therefore material can be conveniently stored in liquid nitrogen vapour or liquid nitrogen at -196^{o}C.

Most of the events associated with freezing are a result of the osmotic properties of cells. The cell is made up of water, organelles, dissolved salts, sugars, proteins and lipids, all surrounded by a semi-permeable membrane. This membrane permits water to flow relatively freely across it, while the membrane acts as a barrier to the larger solutes. Intracellular and extracellular environments associated with water transport and membrane permeability form the basis behind cryopreservation of cells/sperm. By nature, the water tends to move across the cell membrane to maintain an equal concentration of solutes inside and outside the cells (concentration of solutes in a solution is measured as osmolality). Hypertonic solutions cause water to be released from the cell, hypotonic solutions cause an uptake of water into the cell and isotonic solutions maintain equilibrium.

The cellular damage during the freezing process is all due to the osmotic shock, intracellular ice formation, increased intracellular concentration of solutes and solution effects. The addition of cryoprotectants interacts with the membranes to make them more flexible and thus reduces damage due to solution effects (Mazure, 1970; Leung, 1991). Thus the basic principle of cryopreservation is to cause cell dehydration and eventually concentrate the cytosol with minimum injury so that ice crystallization in the cytosol is minimized during quenching in liquid nitrogen. However ice formation and changes in osmotic pressure are the major causes of spermatozoa damage during cryopreservation, and the ability of sperm plasma membrane to resist structural damage during cryopreservation may be related to the type of fatty acids in the spermatozoa plasma membrane and the strength of the bonds between membrane components. It is assumed that fatty acids protect the cell from osmotic pressure of extenders and cryoprotectants solution and cold or hot shock during freezing and thawing further prevent cell dehydration and damages.

A Partial List of Fish Species whose Semen has been Cryopreserved

The semen of more than 200 fish species with external fertilization have been cryopreserved with varying success (Mounib, 1978; Blesbois and Labbe, 2003; Hiemstra *et al.,* 2005). Cryopreservation of salmonids sperm (rainbow trout- *Salmo gairdneri*, brown trout -*Salmo trutta fario,* atlantic salmon- *Salmon salar*) are most extensively worked out (Ott 1975; Holtz *et al.*, 1976; Buykhatipoglu and Holtz, 1978; Legendre and Billard, 1980; Billard, 1992; Stoss and Holtz, 1981; Alderson and Macneil, 1984; Erdahl *et al*., 1984; Baynes and Scott, 1987; Schmidt and Holtz, 1989; Cloud *et al*., 1990; Gwo *et al*., 1993 Conget *et al.*, 1996; Lahnsteiner, *et al.* 2000; Cabrita *et al.*, 2001). Tilapias (mostly *Oreochromis mossambicus*) are also well represented (Harvey, 1983; Chao *et al*., 1987; Harvey and Kelly, 1988; Rana and McAndrew, 1989). Some economically important marine species for which spermatozoa have been frozen include Asian sea bas (Leung, 1987), Atlantic halibut (Bolla *et al*., 1987), milk fish (Chao and Liao,1987), black progy (Chao *et al*., 1986) and bluefin tuna (Doi *et al*., 1982); various cat fishes (Van Vuren and Steyn, 1987), walleye (Moore, 1987), whitefish (Piironen, 1987) and striped bass (Kerby, 1983) have also been studied. Some tropical and sub-tropical freshwater species having potential for aquaculture, have also received attention of fishery scientists. These species are *Hypophthalmicthys molitrix* (Sin, 1974); *Mugil cephalus* (Chao *et al.*, 1975); *Cyprinus carpio* (Moczarski,

1977; Kurokura *et al.*, 1984, Babiak *et al.,* 1997; Akcay *et al*., 2004); *Pangassius sutchi* (Withler, 1980); *Ctenopharyngodon idellus* (Withler, 1980; Durbin *et al*., 1982); *Puntius gonionotus* and *Labeo rohita* (Withler, 1982); *Epinephalus tauvina* (Withler and Lim, 1982); *Esox masquinongy* (Lin *et al.*, 1996; Glogowski *et al*., 1999); *Oryzias latipes* (Aoki *et al.*, 1997) and *Pleuronectes ferrugineus* (Richardson *et al*., 1999).

As far as the Indian fishes are concerned, cryopreservation of semen of few freshwater carps (Bhowmick and Bagchi, 1971; Kumar, 1989; Lakra and Krishna, 1997) and catfish- *Ompok malabaricus* (Basheer *et al.*, 2003) is worked out. Cryopreservation protocol for the semen of mahseer sp. *Tor khudree* (Ponniah *et al.*, 1992; Basavaraja *et al.*, 2003, Patil and Lakra, 2003) and coldwater snowtrout- *Schizothorax richardsonii, Schizothorax curvifrons* and *Schizothoraichthys progastus* (Agarwal 2005, Agarwal *et al*., 2007) have also been developed.

MODULE FOR THE DEVELOPMENT OF CRYOPRESERVATION PROTOCOL FOR SEMEN OF FRESH WATER SPECIES

Equipments required for Cryopreservation

- Cryovials or sterile Eppendorf tubes for semen collection
- Haemocytometer
- Micro centrifuge with haematocrit rotor
- 0.5 ml French medium straws of different colors
- PVA (Poly-venyl alcohol) powder or beads
- Laminar flow
- Programmable freezer
- Polysterine/Thermocol box and Freezing rack
- Liquid nitrogen containers (cryocans BA-3, BA-20, BA-35)
- Refrigerant: LN2 or dry ice for alternative cooling
- Water bath
- Safety equipment; cryogloves, goggles, visor etc.
- Flow throw hatchery unit

Basic steps of cryopreservation technique for fish sperm are similar to that of cattle with some exceptions. The process for cryopreservation of semen typically includes a series of steps: 1) Procurement of ripe brooder, 2) semen collection, 3) refrigerated (non-frozen) storage and shipping of samples, 4) semen quality examination, 5) extender formulation, 6) use of cryoprotectants, 7) diluent prepration, 8) semen-diluent ratio and dilution process, 9) packaging of the samples, 10) Cooling/freezing, 11) frozen storage procedures, 12) thawing, 13) Post thaw sperm motility evaluation, and 14) production of early life stages for assessment of cryopreservation success. Protocol establishment involves evaluation and optimization of multiple factors at each step (e.g., the type and concentration for each cryoprotectant), and recognition of the interactions among the steps (e.g., between cryoprotectant and cooling rate).

1. Procurement of Brooders

The ripe male brooders may be procured from the wild stock (river/streams/reservoir) by gill net during the breeding season or from the brood stock maintained in stress free conditions of hatchery. Male brooders should be maintained in the hatchery with females in a spawning environment to stimulate semen production. The identification of male ripe brooder is easy in those species where sexual dimorphism exist. The male brooder of snowtrout species shows prominent white tubercles on the snout on approach of sexual maturity in the breeding season.

2. Semen Collection

Collection of semen should always be performed from easy oozing male ripe brooders by stripping method in ice cold, sterilized, 4.5 ml cryovials (Fig. 1). It is very important that collected semen should be in quiescent form and should be free from contaminants, such as water, mucus, blood, and gut exudates. The receptacle (graduated cryovials) are first cleaned and dried. Donor brooders are sedated before milt collection to facilitate handling and minimizing damage to personnel and fish. Contamination of semen with water, mucus, blood, and fecal matter activate sperm and lowering the semen quality.

To avoid contamination, brooder is first dried from soft cotton cloth from the vent and first 1-2 drops of semen should be discarded. The risk of urine and fecal contamination of semen from male brooders producing small quantity of semen is greater. During stripping if color of semen seems pinkish there is always a chance of contamination with blood due to internal injury during netting/ handling. Such milt should also be discarded.

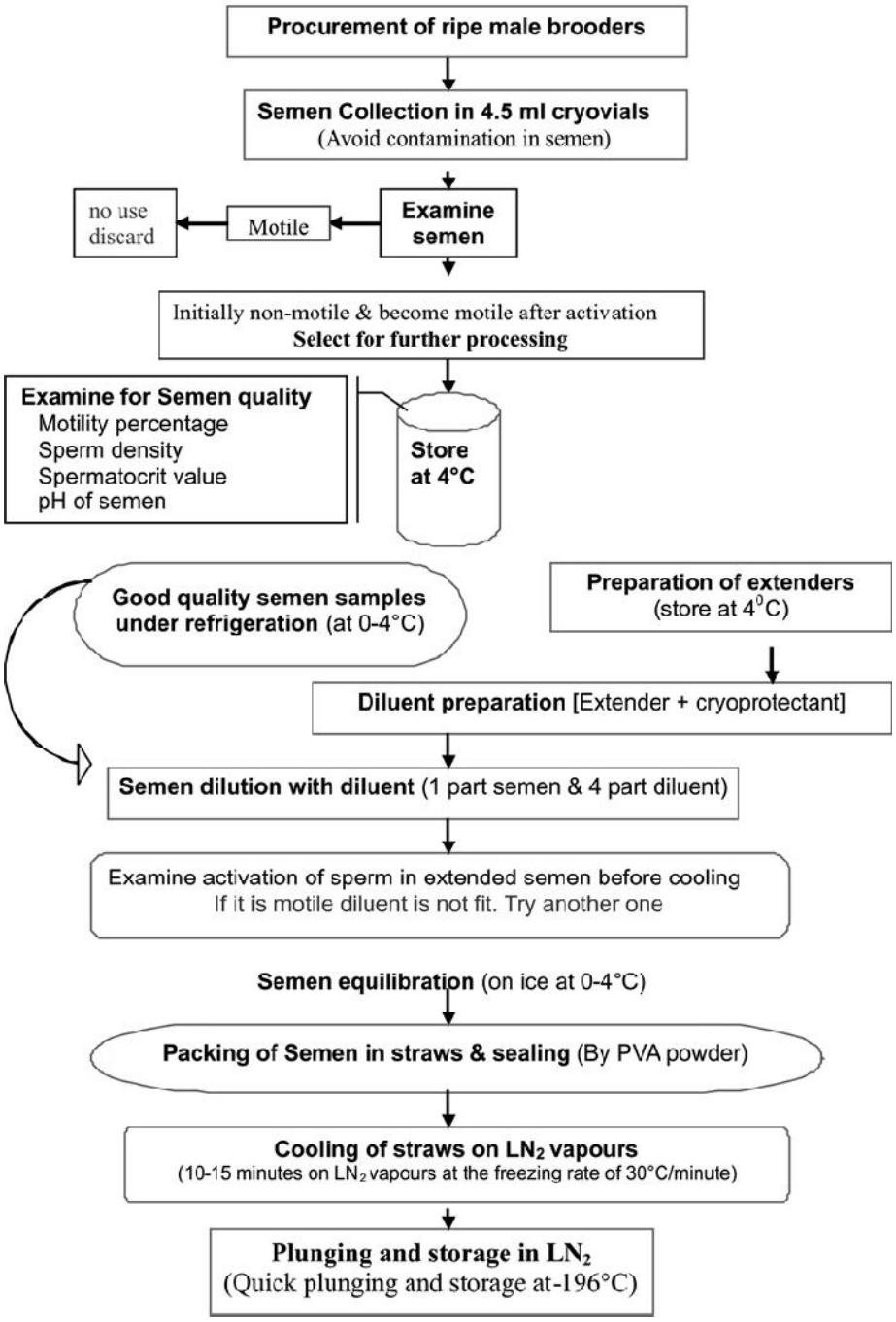
3. Refrigerated (non-frozen) Storage & Shipping of Semen Samples

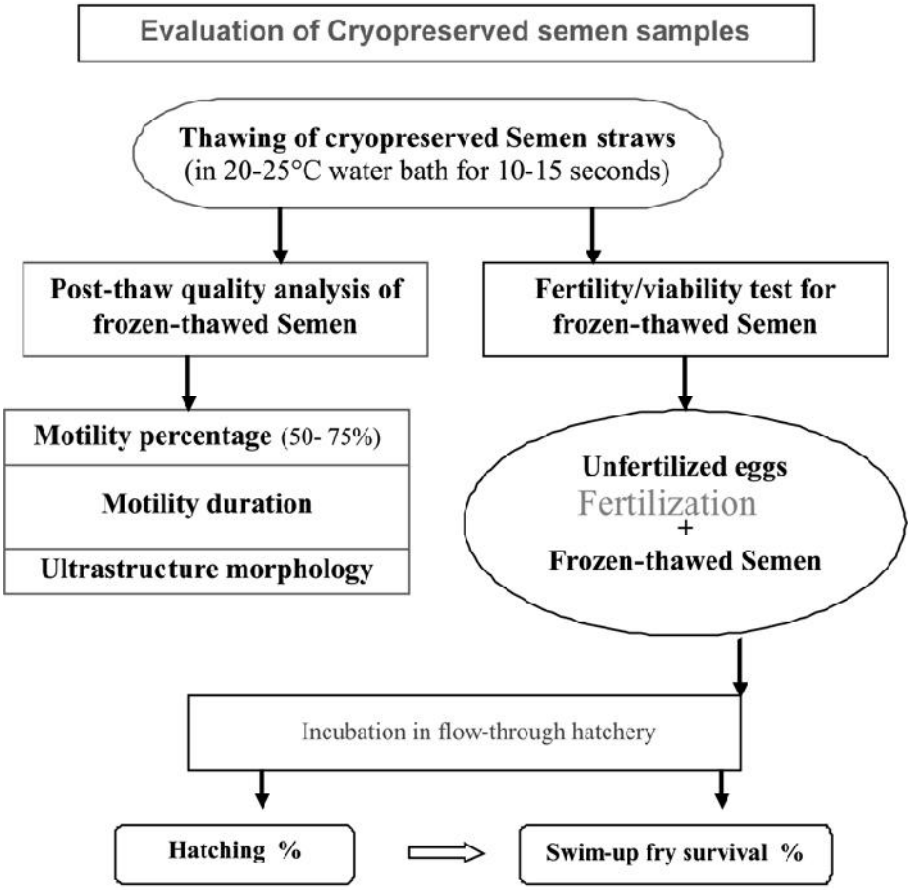
After collection of semen in cryovials/sample containers, they are kept in ice chest (4^0C) and shipped to the laboratory/semen production center as early as possible for follow-up quality analysis and cryopreservation. The semen can be successfully frozen within 4 hours of collection. Condensation within storage vessel should be avoided.

4. Analysis for Semen Quality

Success of cryopreservation is largely depended on the quality of prefrozen semen. As the structure and cryo-resistance of sperm and bio-physicochemical composition of seminal fluid varies species to species thus pre-frezing quality of semen of candidate species should be cautiously studied. Suitability of semen may vary with the prefreezing storage time and condition, breeding season, social hierarchy, diet, and collection techniques. For cryopreservation of snowtrout semen, milt collected during middle of the breeding season has given the good results. Semen from individual fish should be held in isolation until screened for quality, and then pooled if required. Before processing for cryopreservation, the semen quality is assessed on the basis of set criterion (Agarwal *et al*., 2004). The important parameters/test for quality determination are as follows :

Basic sequential steps for Cryopreservation of Semen





(i) **Colour**

The colour of the semen of freshwater fish species is generally white, but sometimes it may be creamy white. The consistency of semen viz. thick/thin/or medium seems to be related with the creamy on milky white colour of fish semen. Sometime collected semen may appears reddish or yellowish, this may be due to its contamination with body fluid viz blood, urine or fecal matter. Contaminated semen slowly looses viability and is of no use in cryopreservation.

(ii) **Volume**

Semen volume can be measured by collecting it directly in to graduated cryovials. Amount of semen from a brooder varies largely among the species and even from the individuals of same species depending more on the state of ripeness than the size.



Fig. 1. Semen collection in 4.5 ml graduated cryovial



Fig. 2. Semen filled microhematocrit capillaries being arranged on haematocrit rotor for measuring spermatocrit value.



Fig. 3. Straws arranged on the freezing rack for frozen in liquid nitrogen vapours.



Fig. 4. Plunging and storing of frozen straws in Liquid nitrogen

(iiii) **pH**

The pH of milt is slightly alkaline in the fresh water fishes. It varies with the species. The pH is an important parameter for consideration during preparation of extender medium for cryopreservation. Therefore it should be carefully measured. The pH of snowtrout of three sp. is ranged between 7.31 ±0.02 to 7.34 ±0.05 (Agarwal, 2005).

(iv) **Sperm Motility**

The sperm are not motile in the testes or seminal fluid. It becomes activated in fertilizing media & performs movement. In external fertilization, activating media is generally the river/pond water. Sperm motility is the most commonly used parameter to evaluate sperm quality, as sperm must be motile to achieve fertilization. The sperm showing progressively forward movement are only capable in fertilizing the ova. Thus these sperms are of main consideration during determination of motility percentage/rating in the semen. The motility is measured on a five point scale from 0 to + 4 as shown here-

Sr. No.	**Motility Percentage**	**Rating**
1.	0 – <1%	0
2.	1 – <25%	1
3.	25 – <50%	2
4.	50 – <75%	3
5.	75 –100%	4

Sperm motility is an important parameter of consideration for good quality of semen. Sample must be observed under a microscope (100x). Only those samples which initially showing no motility but after activation with water/activating media (under microscope) shows motility rating 3 and above considered good for cryopreservation.

After activation, sperm of fresh water species remain motile only for a brief period of seconds or minutes. The duration of motility refers to the total duration of forward motion and survival time of the sperms. The longer sperm-motility duration is advantageous for maximum chances of fertilization and considered good for semen quality viewpoint. In species where sperm motility duration is longer, cryopreservation is generally easier. The mean sperm motility duration for snowtrouts *S. richardsonii*, *S. curvifrons*, *S. progastus* is 58.9, 53.7 and 68.0 sec. respectively (Agarwal, 2005). It is a species specific feature.

(iv) Sperm density (Sperm-cell count):

The number of motile & viable sperm in unit volume of semen has important bearing on the fertilization success. It is an important parameter of quality assessment for cryopreserving the semen. It is a species specific feature. In case of Indian major carps it vary from $2x10^7$ to $3.5x10^7$ sperm ml^{-1}. In snow trout sperm density vary between 2.94×10^8 to 9.76×10^8 sperm ml^{-1} . It is $3.96 \pm 0.49x\ 10^8$ sperm ml^{-1} in *S. richardsonii* and $8.62 \pm 0.56 \times 10^8$ in sperm ml^{-1} *Schizothoraichthys progastus*. The mahseer (*Tor putitora)* has comparatively higher value ($1.70 \pm 0.29 \times 10^9$ sperm ml^{-1}). Sperm density also varied in the different phases of

breeding. It is usually high with the start of breeding season (Agarwal, 2005). Sperm concentration in semen sample should not fall below a number where capacity to fertilize ovum is reduced. The sperm density is estimated in deactivator solution using haemocytometer same as in case of WBCs (Agarwal & Raghuvanshi, 2009).

(v) Spermatocrit Value

Determination of spermatocrit is an easy and quick method of assessing sperm concentration in semen sample. For spermatocrit value estimation of fish semen, micro-haematocrit capillary tubes are used. These capillaries are filled with raw semen by sucking and their both ends are sealed with haemoseal wax. Semen filled volume in capillaries is measured by meter scale in mm. Thereafter, these capillaries are placed in a haematocrit rotor and centrifuged (Fig.2). After centrifugation, the volume of packed cells is again measured by meter scale in mm. Thus the spermatocrit value of semen is calculated as packed cell volume percentage. Centrifugation time for the stable reading of percentage of packed cell volume (sharp interface between packed sperm cell and clear seminal fluid) is needed to standardize for each species separately. It may be done by conducting at least six initial experiments for time duration (10 min. intervals up to 1 h of centrifugation) and centrifugation speed (rpm). For Snowtrout *S.richardsonii* 30 min. centrifugation time (at 7500 rpm, 6315 ×g) is appropriate (Agarwal & Raghuvanshi 2009).

5. Solutions: Extender & Cryoprotectants

For developing cryopreservation protocol for semen of any fish species, prime requirement is to develop extender medium for dilution of semen, as undiluted semen is not suitable for preservation. The extender inhibits the activation of spermatozoa and thereby preserves the vitality & viability of sperm by inhibiting the motility & conserving the energy. The extender also functions as a medium for cryoprotectant - the chemicals that minimize the cryo-injuries to cell due to formation of intracellular ice crystals during cryopreservation at the time of freezing and thawing of semen. Determining the cryoprotectant type and its right concentration in extender medium is an important step of developing cryopreservation protocol for a species.

5.1. Extender Medium

Basically, an extender is the solution of balanced salts. It inhibits the activation of sperm. The sperm require energy for basic cell metabolism and for motility. The endogenous source of energy for external fertilization in fishes is in the form of pre-accumulated ATP, glycogen/glycolipids or glucose present in the middle piece. This energy is utilized for sperm motility when milt is activated during fertilization. To conserve the finite endogenous reserve of fish spermatozoa, extenders should be tailored to maintain the spermatozoa completely quiescent. Therefore a good extender for preservation of semen of a species matches with the properties of the seminal fluid of that particular species and inhibits the activation of sperm. With this concept a long range of extenders media have been used in different fish species such as Cortland's medium (Truscott and Idler, 1969; Randall and Hoar, 1971; Billard

and Jalabert, 1974; Buyukhatipoglu and Holtz, 1978); Alsever's solution (Moczarski, 1977); Mounib's medium (Mounib,1978); NaCl solution, NaCl + urea solution, Fish Ringer's solution (Scott and Baynes, 1980; Stoss, 1983) and KCl medium (Sumathi and Sukumaran, 1998). An ideal extender is isotonic, has a good buffering capacity, contains nutrients, stabilizing colloids and antioxidants; antibacterial and generally has a good maintenance of quality. The different salts, organic and inorganic compound are used for formulating the extender medium for a species. The functions of some of these are listed as follows:

Function of different salts used in extenders

NaHCO3, KHCO3, TRIS	- As buffer
NaCl	- For tonicity
KCl	- To prevent the sperm activation
Mg^{++}, Ca^{++} ions	- Normal components of seminal fluid
Sugars	- for Source of Energy
Glycine	- for improved survival of sperm
Mannitol	- to protect against toxic effect of DMSO
Lecithin, Egg yolk, Promine D, Bovine Serum Albumen	- to protect the membranes

After preparation of extender, it should be tested for its competency of inhibiting motility and extending life span of sperm. For this minute quantity of semen is mixed with extender on glass slide under microscope. After mixing of semen with extender if motility occurred, then extender is not fit for sperm preservation. Such extender should be discarded. The prepared extenders should be stored at 4-5^{0}C and taken in use within one week.

For snowtrout sp. (*S. richardsonii*, *S. curvifrons*, *S. progastus*) three extenders namely, Mounib's medium, Extender modified from Buyukhatipoglu & Holtz, and KCl medium has been evaluated. Mounib's and KCl extender is considered good. (Agarwal *et al.* 2009). The chemical composition and ingredients of all these extenders are summarized in table 1

5.2 Cryoprotectant (CPAs) Preparation

Cryoprotectants are of 2 categories (1) permeating Cryoprotectants and (2) non - permeating cryoprotectants.

Cryoprotectants that are permeable to cell membrane are called permeating cryoprotectants. These chemicals function by reducing the rate of diffusion of water from cell to extra-cellular ice crystal, reducing the cell volume change/salt concentration colligatively, lowering the homogenous nucleation temperature, and reducing the rate of ice crystal growth. The common permeating cryoprotectants are DMSO, Methanol, propylene glycol and glycerol.

Non-permeating cryoprotectants are not permeable to cell membrane. Sucrose, glucose, dextran, egg yolk serum, skim milk and anti freeze protein are of this category. These

Table 1. Extenders, their chemical composition and pH.

Ingredients (mg/100 ml of Distilled Water)	Extender mediums		
	Modified from Buyukhatipoglu & Holtz	KCl medium	Mounib's medium
NaCl	592	-	-
KCl	172	1147	-
$CaCl_2$	68	-	-
$NaHCO_3$	-	-	-
$MgSO_4.7H_2O$	15	-	-
TRIS	2420	-	-
KHCO	-	-	1000
Reduced glutathione	-	-	200
Sucrose	-	-	4270
Fructose	-	-	-
Bovine serum albumen	400	-	-
Promine-D	100-1000	-	-
Vegetable lecithin	-	-	-
Mannitol	-	-	-
pH	7.3	7.3 (with citric acid)	7.3

function by depressing the freezing point and raising the glass transformation temperature of extra-cellular solution.

Dimethyl sulphoxide (DMSO) and glycerol are most widely used permeating type cryoprotectant to depress the freezing point of the extra cellular medium, ameliorate the damaging effects of ice crystals and regulate the rate of cellular dehydration in the freshwater fishes. These cryoprotectants are also toxic to gametes when use in high concentration and prolong exposure to cell during equilibration. Thus it may be advantageous to examine the sensitivity of the sperm to increasing concentrations of the cryoprotective agent (CPA) to determine the optimum. The DMSO/glycerol at concentration of 5-20% has been used successfully for cryopreservation of fish sperm. However, Stoss and Holtz (1983) and Leung (1987) observed toxic effect of DMSO, when used at high concentration. During cryopreservation of snowtrout semen DMSO and glycerol in 5 & 10 % concentration gives better results (Raghuvanshi , 2007; Agarwal *et al.,* 2009).

6. Diluent Preparation

The mixture prepared by mixing of extender and cryoprotectant is termed as diluent. The concentration of cryoprotective agents (CPAs) in the diluent is subjective to the semen of the fish species for which diluent is required. The diluent should be prepared fresh just

prior to their use and checked with a semen sample to ensure that spermatozoa are not activated by the diluent. The diluent is kept in refrigerator as it exhibits exothermic reaction.

7. Dilution of Semen: Semen-Diluent Ratio and Dilution Process

Semen is diluted with the different diluents in various semen-diluent ratios, to test the effective and suitable ratio for fertility trials. The dilution is important because it increases the volume of semen, so that it can be used for multiple inseminations. For salmonid, carp and tilapia, semen is generally diluted 3-20 folds (Rana, 1995). But this variable must be standardized for the efficacy of any protocol. Since sperm density shows high variability between species, within and between individuals of same species, and during breeding seasons; the use of dilution ratio must be complimented with cell density. The number of spermatozoa needed to fertilize one ovum is also species dependent. Therefore optimal sperm to egg ratio should be determined first while calculating the semen-dilution ratio for cryopreserving the semen. As post thaw viability and motility is usually lowered then the pre-freeze motility, therefore this should also be compensated in calculating the viable sperm available to an ovum for fertilization from cryopreserved semen.

For preparation of semen: diluent ratio, the diluent is slowly added in the semen to avoid any shock (due to change in pH & osmolality) to the sperm. After preparation, each sample is tested for motility. If semen sample shows motility after mixing with diluent, such diluent should be discarded and possibility of another diluent by using new extender should be explored. The snowtrout milt is diluted 4-10 fold. Ten-fold dilution is adequate to fertilize about 300 eggs with one 0.5 ml cryopreserved semen straw (optimal sperm to egg ratio is 27,000 sperm egg^{-1} for *S.richardsonii* and 60,000 sperm egg^{-1} for *S. progastus*).

8. Equilibration Time

For effective protection during cooling, sufficient time must be allowed to facilitate the penetration of cryoprotectants into cells (Grout and Morris, 1987). This time is termed as equilibration time. It varied with the species and even for the same species depending on the use of cryoprotectants / diluents. It may be up to two hours but it should be kept to a minimum to avoid exhaustion (Chao *et al*., 1975). The CPA may be toxic to the cells if the equilibration time is too long. Therefore, equilibration should occur for at least 15 minutes, but no longer than 45-60 minutes. The optimal equilibration time should be determined empirically for the semen being cryopreserved to maximize later recovery. Rana (1995) has commonly suggested 20-30 minutes of equilibration at 4-5°C.

For equilibration, semen diluent mixture is kept on refrigeration temperature (0-4°C). Several sets of experiment should be conducted on time scales (to standardize the equilibration time for diluent medium of each CPA - DMSO / glycerol) for the semen samples. The time taken in gradual addition of diluent (CPA) to semen, filling and sealing of straws should also be taken into account while calculating the total equilibration time. The most appropriate equilibration time for *S. richardsonii* and *S. curvifrons* is as 45 minutes for DMSO and 60 minutes for glycerol. While in case of *S. progastus* it is slightly higher and standardized as 50 minutes for DMSO and 75 minutes for glycerol (Saini, 2008).

9. Packaging of Semen

The equilibrated diluted semen is frozen at very low temperatures. There are three ways of freezing viz. 1) Pellet method, 2) Vial method, 3) Straw method

i. *Pellet method* : Pellet method is extensively used for salmonids sperms. In this method diluted mixture is formed in to pellets in small hole drilled into solid dry ice (-79°C). Pellets are removed to liquid nitrogen for storage.

ii. *Ampoules/vials /visotube method* : In this method ampoules/vials /visotube are used for storing the diluted milt for freezing and cryopreservation (Mounib, 1978)

iii. *Straw method* : This method is more popular now a day, as it has been found to yield better post thaw quality of semen as chance for recrystallization during thawing is less in straw method (Erdahl *et al.*, 1984). This method is also applied for snowtrout semen. The diluted semen is loaded in 0.5 ml French medium straws and sealed manually by PVA (Poly-venyl alcohol) powder. While filling the straws different colour codes should be allotted to the samples for cryoprotectant type, their concentration and semen dilution for ease in identification of semen sample during post thaw motility assessment.

10. Cooling and Freezing

Freezing results as an outcome of cooling. Cooling removes the heat from a solution or biological system resulting in a lowered temperature. Freezing occurs when there is a change in the state of water from liquid to solid phase, *i.e*., the inception of ice crystallization and accumulation. During cooling heat is dissipated from the sperm cytoplasm/seminal fluid by conduction to the surrounding heat sink, and at a point below its freezing point, the semen under-cools. The extent of under-cooling will depend on the probability of random events of ice-nucleation in the cytoplasm/seminal fluid. Once the ice-nucleation begins, the semen starts to freeze. As the water freezes out from the solution, the residual solutes are freeze concentrated. When freezing of the water is complete, the heat continues to be conducted from the system, the temperature will fall. The cooling beyond the eutectic point (the temperature at which solutes solidify) results in a complete transition of the medium from the liquid to the solid phase. At this event all molecular motion is inhibited and all biological and biochemical events cease.

The rate of cooling is important since it affects the rate of formation and size of ice crystals, as well as solution effects that occur during freezing. The slow cooling provide sufficient time for osmotic equilibrium to be maintained by cellular dehydration, but prolonged exposure of the unfrozen sperm to the hypertonic residual freezing solution may effect membrane integrity.

Freezing of diluted semen is accomplished on dry ice (pellet method) or in straws in liquid nitrogen vapour or using programmable freezers. It is variously done at a rate of 10 to 45 ^{o}C min^{-1} until the temperature reaches -70 ^{o}C. Control rate cooling (CRC) process is ideal as it provide uniform cooling rates and is particularly useful for establishing optimal cooling rates for semen of different fish species. But it requires sophisticated and costly system of programmable freezers. The dry ice cooling method offers only one freezing rate of 30-35 ^{o}C min^{-1} and is used for making pallets of trout semen. This method is of limited use as

semen is frozen as pellets that are prone to crumbling and cannot be hermetically sealed. Liquid nitrogen vapor cooling method is widely used and easy for field application. In this method liquid nitrogen is poured in a thermocol/ polystyrene box up to fix height. Thereafter cooling profiles are monitored by placing several sealed straws on a freezing rack at various heights above the liquid nitrogen to achieve various cooling rates (Fig. 3). After empirically establishing the optimal height (cooling rate) for cryopreservation of candidate fish species, semen filled straws are placed on freezing rack at a predetermined height above liquid nitrogen for freezing. Straws are allowed to cool for 10-15 minutes and then rapidly transferred in cryocan for long term storage. During this period, the straws are super-cooled slowly from 0°C to -120°C.

11. Frozen Storage Procedure

After 10-15 minutes on liquid nitrogen vapors, all the straws filled with diluted semen of one extender medium of different CPA combinations and dilution ratios (separate colour coding should be given for type of CPA, its concentration and semen dilution ratio at the time of semen packaging) are kept together in a labeled goblet to avoid confusion with straws of other extender mediums. Thereafter goblets are immediately placed in canisters and plunged directly into liquid nitrogen (-196°C) in cryocans for long period of storage (Fig 4). While storing the straws, sample codes for fishes were also given to the canisters for distinguishing the preserved samples. Comprehensive records of cryopreserved materials should be maintained as shown in table 2.

Table 2. Format for cryopreservation record of fish spermatozoa

Fish	Preservation details	Location
Species/ strain	Date	No. of samples
Origin	Pre-frezing quality	Color coding of straws
Identification no.	Extender, cryoprotectant/ conc./dilution rates/cell density	Canister position
Collection method/date	Freezing method/cooling rate	
Sperm density	Post thaw quality	
Milt volume		

12. Thawing Procedure (Reconstitution)

The effects of thawing (warming) on sperm viability depend on cooling history. Sperm that are cooled slowly may be sufficiently dehydrated before entering into frozen state and thus intracellular freezing injury during warming may be insignificant. Upon warming sperm re-hydrate and any damage may be associated with osmotic stress related to sperm plasma membrane coalesee (re- crystallize). The intracellular stress associated with these events of recrystallization is thought to be the main cause of cryoinjury. This damage can be minimized

by rapid warming from the frozen state. In rapid warming, less time is available for re-crystalization to occur before complete thawing of frozen semen.

Thawing is the process of the revival of cryopreserved spermatozoa. By this technique spermatozoa brought to the fertilizing temperature from the ultra low temperature (-196^{0}c). During thawing, the same physiological processes take place in reverse order as in freezing. Theoretically the thawing process should be the same as the corresponding cooling rate.

Thawing temperature has profound effects on sperm viability after freezing. In several fresh water fish species thawing is done at 30-80^{0}C in a water bath. But for individual group of fishes it should be standardized due to large variability in sperm physiology (Rana, 1995). In practice, carp sperm samples are generally thawed in a 30-35°C water bath prior to insemination or testing for retaining their viability. For coldwater fishes like snowtrouts, thawing at the temperature of natural breeding environment (20-25°C) is advantageous. The cryopreserved semen samples are thawed in water bath by quickly removing from liquid nitrogen. As soon as the semen filled straws are thawed, they should be removed from water bath. To minimize the risk of contamination during reconstitution, disinfect the external surface of straws by wiping with alcohol-soaked gauze prior to cut open.

13. Post-thaw Sperm Motility Evaluation

After thawing, straws are cut open and percentage motility of thawed semen samples is estimated without delay because just after thawing, sperm become activated and remain motile only for a brief period. The percentage of post thaw motility usually decreased after cryopreservation (Linhart *et al*., 1993) and not affected with the time period at cryogenic temperature.

14. Production of Early Life Stages for Assessment of Cryopreservation Success (protocol evaluation)

Fertilization success of gametes and subsequent development of early life stages are the reliable measures of cryopreservation success (Gupta *et al*., 1995). Therefore fertility/ viability test of cryopreserved semen should be performed by artificial fertilization experiments and incubation and hatching of fertilized eggs in hatchery system to evaluate the success of cryopreservation technique. The fertilization rate, hatching percentage and swim-up fry survival rate should be calculated and compared with the control treatments for evaluation of any cryopreservation protocol.

Cryopreservation Protocol for the Semen of Coldwater Fish Species (*Schizothorax richardsonii*)

Cryopreservation protocol for the semen of a commercially important coldwater food fish species (*Schizothorax richardsonii*) is developed first time in order to make all time availability of frozen semen (viable & fertile) for healthy seed production in hatchery, for conservation and propagation strategies and for selective/cross breeding programmes. Though semen cryopreservation technique is well established in mammal live stock species but for fish semen this has gain importance very recently and this is the first time when Author's

group have succeeded in developing the protocol for live preservation of semen of commercially important snowtrout species of Uttarakhand. As mentioned earlier in this chapter success of semen cryopreservation technique lie in proper collection of semen from healthy donor fish, development & use of suitable extender to prevent depletion of sperm energy reserve and to maintain sperm in quiescent condition but alive, using of appropriate cryoprotectants in right concentration to reduce cryoinjury from cold and thermal shock, and freezing & thawing procedure to minimize sperm damage.

The salient features and step-to-step technical details of the developed protocol is as follows :

i. Procurement of male brooders

- Procure live ripe brooders by gill net from the wild by taking utmost care to avoid any injury.
- Prefer procurement of brooders during the middle phase of breeding/spawning season.

ii. Collection of semen

- Anesthetize the brooder and wipe with soft cotton cloth/ tissue paper.
- Collect the semen directly in clean, sterilized and dry 4.5 ml cryovials by stripping the brooder.
- Avoid any contamination to semen from water, mucus, urine, blood, fecal matter etc. by discarding the first 1 or 2 drops of milt.
- Keep individual semen samples in isolation until screened for quality, and then pooled, if required.
- Maintain semen samples at 0-4 ^{0}C in ice chest until analyzed for semen quality and used for cryopreservation.

iii. Semen - Quality analysis

- Observe the semen for milky white in colour, otherwise it is contaminated and should be discarded.
- Inspect the semen samples under microscope. Samples showing no initial motility and motility rating 4 (75-100% motility) after activation with water should be selected for cryopreservation.
- Estimate the sperm density using haemocytometer. Sperm density in the range of 3.77 $\pm 0.78 \times 10^{8}$ sperm ml $^{-1}$ of semen is adequate.
- Calculate the spermatocrit value (63.13 ±10.27%) of each sample by micro-haematocrit centrifuge for quick estimation of sperm concentration.
- Measure the pH of individual semen samples by pocket-pH meter. It should be 7.31 ±0.07.

iv. Extender preparation

- Prepare the Mounib's medium by dissolving $KHCO_3$ 1000 mg, reduced glutathione 200 mg and Sucrose 4270 mg in 100 ml of Distilled water.

– Prepare the extender in advance from the day of cryopreservation and keep in refrigerator at 4^0C. Extender may be prepared 1-2 days in advance.

v. Diluent preparation

– Prepare diluent by mixing 5% DMSO as cryoprotectant with the Mounib's extender for giving cryoprotection to the sperm.
– Diluent should be prepared prior to use and keep it at 0-4^0C .

vi. Semen dilution

– Dilute the semen sample with the above diluent in 1:4 dilution by mixing one part of semen and 4 parts of diluent.
– In diluted/extended semen final sperm concentration should be $7.19 \pm 0.25 \times 10^7$ sperm/ml. This concentration is adequate to fertilize about 1200 eggs of *S. richardsonii* with 1 ml frozen-thawed diluted semen (2 straws with capacity of 0.5 ml each).
– The semen should be added very slowly in the diluent to avoid osmolarity and pH shock.
– Check the extended semen sample to ensure that spermatozoa are not activated by the diluent. They should only be motile by activating with water. Check pre-freeze motility percentage at this point. It should not be less than the motility rating 3.

vii. Equilibration time and temperature

– Allow the extended semen to be equilibrated at 4^0C for 45 minutes as DMSO is used as cryoprotectant.
– The contact time between spermatozoa and diluents before cooling should not be exceed from this period. Therefore filling and sealing of straws with diluted semen should be completed within equilibration period.

viii. Filling and sealing of straws

– Load diluted semen manually into 0.5 ml French medium straws (IMV). For straws allow an air space of 1 cm in straws to accommodate expansion of diluent during freezing.
– Seal straws with PVA powder.
– If semen of more than one species has to be cryopreserved simultaneously, give the colour codes of straws to the species.

ix. Liquid nitrogen vapour cooling (cooling/freezing of semen filled straws)

– Pour liquid nitrogen in a rectangle, wide and covered polystyrene / thermocoal box.
– Transfer semen filled straws on a freezing rack (already placed in the thermocoal box having LN) at a 2 cm height above the level of liquid nitrogen (at this height the temperature of ^{2}LN vapour is -100 $\pm 2^0$C).
– Allow straws to^2 cool for 10-15 minutes (cooling rate 30^0C /minute).

x. Storage in liquid nitrogen

– Rapidly transfer the cooled straws in the goblet placed in the canister and plunged into LN_2 for long-term storage. The plug of the straw should be in down position and the sealing of the straw in up position.
– The semen straws of individual fish should be retrievable with minimal disturbances to other straws. Therefore separate goblet / canisters should be used for each species and marked accordingly.

xi. Thawing to recover frozen spermatozoa

– From each batch of cryopreserved semen, 2-3 straws should be checked for post-thaw motility after thawing them to ensure quality of cryopreserved semen. If the cryopreserved semen quality is not found satisfactory, the whole batch should be discarded.
– Remove the cryopreserved semen straws from LN_2 and transfer rapidly to 20-25^{0}C water bath for 10-15 seconds.

xii. Post-thaw quality analysis

– Cut open the frozen-thawed semen straw from one end.
– Immediately assess the percentage of motile sperm and motility duration under microscope. As thawed spermatozoa of *S. richardsonii* do not require activation with water and have very short motility duration, therefore they need quick assessment.
– Percentage of motile sperm in frozen-thawed semen should not be less than 50%. However, this is less than the pre-freeze phase, but still the cryopreserved semen will be able to give high fertilization success. Motility duration may also decrease up to 23% and is acceptable.

xiii. Insemination

– Perform stripping of female brooder in the dry enamel tray to obtain fresh and ripe eggs for artificial fertilization by dry method.
– Cut and empty the two frozen-thawed semen straws over 1200 fresh eggs. Mix the semen and eggs gently with bird's feather.
– Add river water after 2-3 minutes and leave the eggs for one hour for complete fertilization and water hardening (Agarwal *et al*., 2007).
– Wash the fertilized water hardened eggs with river water and transfer to the hatching tray in flow-through hatchery for hatching and rearing.
– Evaluate fertilization percentage that will come to nearly 98%.
– The hatching percentage and swim-up fry survival rate in flow-through hatchery may be obtained up to 36% and 21% respectively with the cryopreserved semen by adopting this protocol. This percentage is very close to the hatching and swim up fry survival rate obtained from the eggs fertilized with fresh semen in artificial fertilization in flow through hatchery.

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REFERENCES

Agarwal N. K. and Raghuvanshi S. K. (2009). Spermatocrit and sperm density in snowtrout (*Schizothorax richardsonii*): Correlation and variation during the breeding season. *Aquaculture*, 291: 61-64. DOI 10.1016 / j. aquaculture.2009.03.002

Agarwal N. K., Raghuvanshi S. K. and Saini V. (2009). Cryopreservation of snowtrout (*S.richardsonii*) milt as a means for propagation and ex-situ conservation of species. In: *Fish Genetic Resources*, (eds. W.S. Lakra, A.K., Singh & P.C. Mahanta), 273-284. Narendra Publishing House, New Delhi.

Agarwal N. K., Raghuvanshi S. K., Vandana Saini, and Rawat U. S. (2004). Milt Quality and short term storage of snowtrout (*Schizothorax richardsonii*) sperm. *J. Inland Fish. Soc. India*, 36(2):13-18.

Agarwal N. K., Thapliyal B. L. and Raghuvanshi S. K. (2007). Induced Breeding and artificial fertilization of snowtrout, *Schizothorax richardsonii* through the application of ovaprim. *J. Inland Fish. Soc. India* 39(1):12-19.

Agarwal, N.K. (2005). *Developing cryopreservation protocols for the spermatozoa of snowtrout and other endangered coldwater species*. Final report, ICAR Scheme (2002-2005).

Akcay, E.; Bozkurt, Y.; Secer, S. and Tekin, N. (2004). Preservation of fish semen. *Ege Univ. J. Fish. Aquat. Sci.* 12: 367-373.

Alderson, R. and Macneil, A.J. (1984). Preliminary investigations of cryopreservation of milt of Atlantic Salmon *Salmo salar* and its application to commercial farming. *Aquaculture* 43: 351-354.

Aoki, K.; Okamoto, M.; Tatsumi, K. and Ishikawa, Y. (1997). Cryopreservation of Medaka Spermatozoa. *Zool. Sci*. 14 (4): 641-644.

Babiak, I.; Glogowski, J.; Brzuska, E.; Szumiec, J. and Adamek, J. (1997). Cryopreservtion of sperm of common carp, *Cyprinus carpio L. Aquaculture Research* 28: 567-571.

Basavaraja, N.; Hedge, S.N. and Palaksha, K.J. (2003). Cryopreservation and viability of spermatozoa from the endangered mahseer (*Tor khudree*). *Nat. Symp. on Genetics and Gene Banking of Fish and Shellfish* (29-30 March): 53.

Basheer, V.S.; Musammilu, K.K.; Gopalakrishnan, A.; Lal, K.K.; Singh, S.P.; Kapoor, D.; Haniffa, M.A. and Nagarajan, M. (2003). Milt cryopreservation of butter catfish, *Ompok malabaricus* (Val.). *Nat. Symp. Genetics & Gene Banking of Fish and Shellfish* (29-30 March): 62.

Baynes, S.M. and Scott, A.P. (1987). Cryopreservation of rainbow trout spermatozoa: the influence of sperm quality, egg quality and extender composition on post thaw fertility. *Aquaculture* 66: 53-67.

Bhowmick, R.M. and Bagchi, M.M. (1971). A note on the preservation of sperm of carps. *J. Inland Fish. Soc. India* 31: 119-120.

Billard R. and Jalabert, B. (1974). L'insemination artificielle de la truite (*Salmo gairdeneri* Richarson). II.Comparison des effects des different dilueurs sur la conservation de la fertility des gametes avant et apres insemination. *Annales de Biologie Animale,Biochmie,Biophysique* 14: 601-610Das, P. and Pandey, A.K. (1999). Endangered fish species: measures for rehabilitation and conservation. *Fishing Chimes* 19 (6): 31-34.

Billard, R. (1992). Reproduction in rainbow trout: sex differentiation, dynamics of gametogenesis, biology and preservation of gametes. *Aquaculture* 100: 263-298.

Blaxter, J.H.S. (1953). Sperm storage and cross-fertilization of spring and autumn spawning herring. *Nature* 172: 1189-1190.

Blesbois, E. and Labbe, C. (2003). Main improvements in semen and embryo cryopreservation for fish and fowl. In: *Workshop of Cryopreservation of Animal Genetic Resources in Europe* (Ed., D. Planchenault), Paris. ISBN 2-908447-25-8: 55-65.

Bolla, S.; Holmefjord, I. and Refstie, T. (1987). Cryogenic preservation of Atlantic halibut sperm. *Aquaculture* 65: 371-374.

Buyukhatipoglu, S. and Holtz, W. (1978). Preservation of trout sperm in liquid or frozen state. *Aquaculture* 14: 49-56.

Cabrita, E.; Robles, V.; Alvarez, R. and Herraez, M.P. (2001). Cryopreservation of rainbow trout sperm in large volume straws: application to large scale fertilization. *Aquaculture* 201: 301-314.

Chao, N.H. and Liao, I.C. (1987). Application of honey in cryopreservation of sperm of milkfish *Chanos chanos* and black porgy *Acanthopagrus schlegeli*. *Proc. 3rd Int. Symp. Reprod. Physiol. Fish*, St. John's Newfoundland (Aug. 2-7): . 94-96.

Chao, N.H., Chao, W.C., Liu, K.C. and Liao, I.C. (1986). The biological properties of black porgy (*Acanthopagrus schlegeli*) sperm and its cryopreservation. *Proc. Nat. Sci. Council*, Part-B. life Sciences, Taipei, Taiwan: 145-149.

Chao, N.H., Chao, W.C., Liu, K.C. and Liao, I.C. (1987). The properties of tilapia sperm and its cryopreservation. *J. Fish Biol*. 30: 107-118.

Chao, N.H.; Chen, H.P. and Liao, I.C. (1975). Study on cryogenic preservation of grey mullet sperm. *Aquaculture* 5: 389-406.

Cloud, J.G.; Miller, W.H. and Levanduski, M.J. (1990). Cryopreservation of sperm as a means to store salmonid germplasm and to transfer genes from wild fish to hatchery populations. *Prog. Fish Cult.* 52: 51-53.

Conget, P.; Fernandez, M.; Hirrera, G. and Minguell, J.J. (1996). Cryopreservation of rainbow trout (*Oncorhynchus mykiss*) spermatozoa using programmable freezing. *Aquaculture* 143: 319-329.

Doi, M.; Hoshino, T.; Taki, Y. and Ogasawara, Y. (1982). Activity of the sperm of the bluefin tuna *thunnus thynnus* under fresh and preserved conditions. *Bull. Japan Soc. Sci. Fish* 48: 495-498.

Durbin, H.; Durbin, F.J. and Scott, B. (1982). A note on the cryopreservation of grass carp milt. *Fish Manag.* 13: 115-117.

Erdahl, A.W. (1986). *Factors affecting storage and fertilization of fish gametes*. Ph.D. Thesis, University of Minnesota, MN: 128.

Erdahl. A.W., Erdahl, D.A. and Graham, E.F. (1984). Some factors affecting the preservation of salmonid spermatozoa. *Aquaculture* 43: 341-350.

Glogowski, J.; Ciereszko, A. and Dabrowski, K. (1999). Cryopreservation of muskellunge and yellow perch semen. *North Am. J. Aquaculture* 61: 258-262.

Grout, B.W. and Morris, G.J. (1987). *The effect of low temperature on biological systems*. Edward Arnold, London.

Gupta, S.D.; Rath, S.C. and Dasgupta, S. (1995). Fertilization efficiency of cryopreserved spermatozoa over four years at different time intervals after thawing. *Geobios.* 22: 208-211.

Gwo, J.C.; Kurokura, H. and Hirano, R. (1993). Cryopreservation of spermatozoa from rainbow trout, common carp and marine puffer. *Nippon Suisan Gakkaishi* 59: 777-782.

Harvey, B. (1983). Cryopreservation of *Sarotherodon mossambicus* spermatozoa. *Aquaculture* 32: 313-320.

Harvey, B. and Kelley, R.N. (1988). Practical methods for chilled and frozen storage of tilapia spermatozoa, In: *2nd Int. Symp. tilapias in Aquaculture* (Eds., R.S.V. Pullin, T. Bhukaswan, K. Tonguthai and J.L. McLean), ICLARM conference proceedings, Manila: pp. 179-189.

Hiemstra, S.J.; Van der Lende, T. and Woelders, H. (2005). The potential of cryopreservation and reproductive technologies for animal genetic resources conservation strategies. *Proc. Symp. role of biotechnology*, Villa Gualino, Turin, Italy (5-7 March): 25-35.

Holtz, W.; Buyukhatipoglu, S. and Stoss, J. (1976). Preservation of trout spermatozoa for varying periods. FAO Tech. Conf. on Aquaculture. Kyoto, Japan FAO/Conf/76/E.65:3 p.

Kerby, J.H. (1983). Cryogenic preservation of sperm from striped bass. *Trans. Am. Fish Soc.* 112: 86-94.

Koldras, M. and Bienarz, K. (1987). Cryopreservation of carp sperm. *Pol. Arch. Hydrobiol.* 34: 125-134.

Kumar, K. (1989). Studies on cryogenic preservation of carp spermatozoa. In: *Fish Genetics in India* (Eds., P. Das and A.G. Jhingran). Today and Tomorrow's Printers and Publishers, New Delhi: 199-206.

Kurokura, H.; Hirano, R.; Tomita, M. and Iwahashi, M. (1984). Cryopreservation of carp sperm. *Aquaculture* 37: 267-273.

Lahnsteiner, F.; Berger, B.; Horvath, A.; Urbanyi, B. and Weismann, T. (2000). Cryopreservation of spermatozoa in Cyprinid fishes. Theriogenology 54: 1477-1498.

Lakra, W.S. (1993). Cryogenic preservation of fish spermatozoa and its application to aquaculture. *Indian Journal of Cryogenics* 18 (1-4): 171-176.

Lakra, W.S. and Krishna, G. (1997). Preliminary trials for cryopreservation of spermatozoa of selected carps and catfishes. *Indian J. Anim. Sci.* 67 (1): 90-92.

Legendre, M. and Billard, R. (1980). Cryopreservation of rainbow trout sperm by deep freezing. *Reprod. Nutr. Dev.* 20: 1859-1868.

Leung, L.K.P. (1987). Cryopreservation of spermatozoa of the barramundi, *Lates Calcarifer* (Teleostei: Cenfropomidae). *Aquaculture* 64: 243-247.

Leung, L.K.P. (1991). Principles of biological cryopreservation. In: *Fish Evolution and Systematics: Evidence from Spermatozoa* (ed., B.G.M. Jamieson). Cambridge University Press, Cambridge, New York: 231-244.

Leung, L.K.P. and Jamieson, B.G.M. (1991). Live preservation of fish gametes. In: *Fish evolution and systematics: evidence from spermatozoa* (ed. B.G.M. Jamieson). Cambridge University Press, Cambridge: 245-269.

Lin, F.; Ciereszko, A. and Dabrowski, K. (1996). Sperm production and cryopreservation in muskellunge after carp pituitary extract and human chorionic gonadotropin injection. *Prog.Fish Cult.*58: 32-37.

Linhart, U.; Billard, R. and Proteau, J.P. (1993). Cryopreservation of European catfish (*Silurus glamis* L.) spermatozoa. *Aquaculture* 115: 347-359.

Mazur, P. (1970). Cryobiology: The freezing of biological systems. *Science* 168: 939-949.

Moczarski, M. (1977). Deep freezing of carp *Cyprinus carpio* L. sperm. *Ser. Sci. Biol.* 15: 187-190.

Moore, A.A. (1987). Short-term storage and cryopreservation of walleye semen. *Prog. Fish Cult*. 49: 40-43.

Mounib, M.S. (1978). Cryogenic preservation of fish and mammalian spermatozoa, *J. Reprod. Fertil.,* 53: 13-18.

Muchlisin, Z.A. (2005). Current Status of Extenders and Cryoprotectants on Fish Spermatozoa Cryopreservation. Biodiversitas 6(1):66-69.

Ott, A.G. (1975). *Cryopreservation of pacific salmon and steelhead trout sperm*. Ph.D.Thesis, Oregon State Univ., Corvallis, Oregon: 145.

Ott, A.G. and Horton, H.F. (1971). Fertilization of steelhead trout (*Salmo gairdneri*) eggs with cryo preserved sperm. *J. Fish Res. Board Can.,* 28: 1915-1918.

Patil, R. and Lakra, W.S. (2003). Cryopreservation of Deccan mahseer, *Tor khudree* (Sykes) spermatozoa and the associated ultrastructural changes. *Nat. Symp. Genetics & Gene banking of fish and shellfish* (29-30 March): 59.

Piironen, J. (1987). Factors affecting fertilization rate with cryopreserved sperm of white fish. *Aquaculture* 66: 347-357.

Polge, C.; Smith, A.U.; and Parkes, A.S. (1949). Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature*, 164, 666

Ponniah, A.G.; Lakra, W.S. and Ogale, S.N. (1992). Study on cryopreservation of mahseer milt. *Punjab Fisheries Bull.* 16 (1): 51-53.

Raghuvanshi, S.K. (2007). Studies on the biology and cryogenic preservation of snowtrout (*Schizothorax richardsonii*) semen. D.Phil. Thesis, HNB Garhwal University, Srinagar Garhwal: 176 p.

Rana, K.J. (1995). Preservation of gamates. In: *Broodstock management and egg and larval quality* (eds. N.R. Bromide and R.J. Roberts). Blackwell Science, Cambridge, U.K.: 53-75.

Rana, K.J. and McAndrew, B.J. (1989). The viability of cryopreserved tilapia spermatozoa. *Aquaculture* 76: 335-345.

Randall, D.J.,and Hoar, W.S.(1971). Special techneqes. In "Fish physiology" (W.S.Hoar, and D.j.Randall, eds.) *Academic Press*, New York.Vol. 6, pp. 511-528.

Richardson, G.F.; Wilson, C.E.; Crim, L.W. and Yao, Z. (1999). Cryopreservation of yellowtail flounder (*Pleuronectes Ferrugineus*) semen in large straws. *Aquaculture* 174: 89-94.

Saini, V. (2008). Studies on the characterization and cryopreservation of milt in *Schizothoraichthys progastus*. D.Phil. Thesis, HNB Garhwal University, Srinagar Garhwal: 155.

Schmidt-Baulain, R. and Holtz, W. (1989). Deep freezing of rainbow trout (*Salmo gairdneri)* sperm at varying intervals after collection. *Theriogenology* 32: 439-443.

Scott, A.P. and Baynes, S.M. (1980). A review of the biology, handling and storage of salmonid spermatozoa. *J. Fish Biol*. 17: 707-739.

Smith, A.U. and polge, C. (1950). Survival of spermatozoa at low temperature. *Nature*, 164: 666.

Stein, H. and Bayrle, H. (1978). Cryopreservation of sperm of some fresh water teleosts. *Ann. Biol. Anim. Biochim. Biophys.*, 18(4): 1073-7076.

Stoss, J. (1983). Fish gamete preservation and spermatozoa physiology. In: *Fish Physiology* (eds. W.S. Hoar, D.J. Randall and E.M. Donaldson). Vol. IX B. Academic Press, New York: 305-350.

Stoss, J. and Holtz, W. (1981). Cryopreservation of rainbow trout (*Salmo gairdneri*) sperm. I. Effect of thawing solution, sperm density and interval between thawing and insemination. *Aquaculture* 22: 97-104.

Stoss, J. and Holtz, W. (1983). Successful storage of chilled rainbow trout (*Salmo gairdneri*) spermatozoa for up to 34 days. *Aquaculture* 31: 269-274.

Sumathi, S.R. and Sukumaran, N. (2000). Role of cryopreservation of gametes in genetic resources conservation and gene bank development in freshwater fishes. *Fishing Chimes* 20 (6): 18-21.

Truscott, B. and Idler, D.R. (1969). An improved extender for freezing Atlantic salmon sperm. *J. Fish Res. Board Can.* 26: 3254-3258.

Van Vuren, J.H.J. and Steyn, G.J. (1987). Cryopreservation of *Clarias gariepinus* sperm and fertilization success. *Proc. 3rd Int. Symp. Reprod. Physiol. Fish*. St. John's, New foundland (August 2-7): . 103.

Withler, F.C. (1980). Chilled and cryogenic storage of gametes of Thai carps and catfishes. *Can. Tech. Rep. Fish Aquat. Sci*. 948: 15.

Withler, F.C. (1982). Cryopreservation of spermatozoa of some fresh water fishes cultured in south and South-East Asia. *Aquaculture* 26: 395-398.

Withler, F.C. and Lim, L.C. (1982). Preliminary observations of chilled and deep frozen storage of grouper (*Epinephelus tauvina*) sperm. *Aquaculture* 27: 389-392.

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