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Article *in* The Indian journal of animal sciences · April 2010

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Indian Journal of Animal Sciences **80** (4) (Suppl. 1): 109–124, April 2010

Cryopreservation of fish gametes and embryos

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ABSTRACT

Cryopreservation of gametes is one of the important *ex situ* methods of conservation of germplasm and has wide ranging applications in aquaculture and fisheries management. Though sperm cryopreservation has been a success in fishes, yet developing successful protocols for eggs and embryo cryopreservation still remains elusive. Despite successful sperm cryopreservation protocol known for more than 200 fish species, the adoption of the technique at commercial level for fish seed production has been limited. High degree of variability in the procedural requirements and success not only between the species but at times within the species, is considered one of the limiting factors in its utilization. The present paper records the status of gamete cryopreservation in fish species. The paper provides a comprehensive review of various aspects of milt cryopreservation such as milt collection, sperm quality and viability assessment, extender compositions, cryoprotectants equilibration periods, freezing rates, thawing of cryopreserved milt and fertilization of eggs and ultrastructural studies on damages in cryopreserved spermatozoa. The paper also provides a brief review of cryopreservation of fish embryos and embryonic stem cells.

Key words: Cryopreservation, Egg, Embryos, Fish, Sperm

The conservation measures are broadly classified into *in-situ* programmes which protect and manage animal populations within their natural habitats and *ex-situ* conservation programmes which remove individual animals, their gametes or embryos from wild populations for captive breeding as in the case of cryopreservation of gametes (Rall, 1985). Maintenance of a species in live gene banks has two major drawbacks, viz. they offer no long-term guarantee of the genetic stability of the population or species over time and it is very expensive to maintain the desired representative genetic information of a population or a species (Rana, 1995). Cryopreservation of gametes is one of the important *ex situ* methods of conservation of germplasm, and FAO has endorsed it as a major strategy for conservation of fish resources (Khoshoo, 1997). Cryopreservation of gametes aims to increase the longevity of gametes for several years without any drastic change in the fertilizing capacity of the gametes by lowering the temperature and thereby reducing their metabolic rate. Even though the need for cryopreservation of fish eggs and embryos assumes a lot of significance in the light of the role played by the mitochondrial DNA, so far the attempts have met with no or limited success (Hagedorn *et al.*1997; Ahmmad *et al.* 1998; 2002, 2003(a,b), 2004). However, studies on the cryopreservation of invertebrate eggs, embryos and larvae have met with some success (McAndrew *et al.* 1993; Diwan and Kandasami, 1997)

The cryopreservation of fish spermatozoa has been a success story so far. Sperm cryopreservation protocols are available now for over 200 species of finfish and shellfish (Horton and Ott, 1976; Legendre and Billard, 1980, Kerby, 1983, Leung and Jamieson, 1991, Holtz, 1993 McAndrew *et al.* 1993; Lakra, 1993; Billard *et al.* 1995; Diwan and Nandakumar, 1998; Lerveroni and Maisee, 1998, 1999; Horvath, *et al.* 2000, Chao and Liao, 2001; Huang and Tiersch, 2004). A few sperm banks for fin fishes have been created, notably for groupers, salmonids and a few commercial and endangered fish species (Chao *et al.* 2002, Rana, 1995; Ponniah, 1998a). The cryopreservation of fish spermatozoa has many potential applications, like conservation of endangered fish species by establishing genetic material reserves for selective breeding, evolving desired genotype through cross-breeding, easy transportation and time-independent distribution of genetic material from one area to another. to produce fish seed in species with differential maturity with respect to sexes, seed production in fish species which are sequential hermaphrodites and to help in reducing the cost by eliminating the need for maintenance of the male broodstock in hatcheries (Rao, 1989).

Cryopreservation of fish sperm

Cryopreservation is a process where cells or whole tissues are preserved by cooling to low sub-zero temperatures,

typically to –196°C (the boiling point of liquid nitrogen). At these low temperatures, any biological activity, including the biochemical reactions that would lead to cell death, is effectively stopped. However, in the absence of vitrification solutions, the cells being preserved are often damaged during freezing to low temperatures or while thawing to room temperature. Phenomena which can cause damage to cells during cryopreservation are solution effects, extracellular ice formation, dehydration and intracellular ice formation. Solution effects are caused by concentration of solutes in non-frozen solution during freezing, as solutes are excluded from the crystal structure of the ice. When tissues are cooled slowly, water migrates out of cells and ice forms in the extracellular space. Too much extracellular ice can cause mechanical damage due to crushing, and the stresses associated with cellular dehydration can cause the damage directly. Nevertheless, while some organisms and tissues can tolerate some extracellular ice, any appreciable intracellular ice is almost always fatal to cells. Vitrification provides benefits of cryopreservation without the damage due to ice crystal formation (Stoss, 1983).

Due to several anthropogenic alterations and consequent habitat and environmental degradation, several animal species are under threat. The scenario is not only limited to terrestrial ecosystems but similar conditions also prevail in aquatic ecosystems. There is a need for conservation of fish population and sustenance of aquatic biodiversity. Gamete cryopreservation is a powerful *ex situ* conservation tool besides its wide ranging applications in aquaculture. Under cooled conditions, gametes can be preserved up to several weeks (short term preservation) and in frozen form, for years together (Long-term preservation). To maintain their viability for longer periods, gametes need to be cryopreserved. It is reported that under optimum cryopreservation and storage protocols, the viability of gametes can be preserved up to 32,000 years (Ashwood-Smith 1980). Polge (1980), for the first time, reported the cryoprotective action of glycerol and observed that fowl spermatozoa retained full motility after freezing and thawing in the presence of glycerol. Blaxter (1953) reported the first successful fertilization of herring *Clupea herrengus* eggs with cryopreserved spermatozoa. Sherman (1954) protected the spermatozoa using glycerol and emphasized the importance of rate of freezing and suggested a method of slower freezing using dry ice. Successful cryopreservation of semen of higher animals like cattle, led to similar attempts to cryopreserve fish spermatozoa. Since then, considerable work has been carried out with special emphasis on temperate fish species like salmonids (Horton and Ott, 1976; Holtz *et al.* 1977; Stoss and Holtz, 1981, Lahnsteiner *et al*. 1996a,b, 1997, Lahnsteiner, 2000).

Regarding tropical and sub-tropical fish species, a significant progress has been made in the last two decades (Lakra and Krishna, 1997). Several researchers have worked on the cryopreservation of spermatozoa of fish species, like silver carp (*Hypophthalmichthys molitrix*), bighead carp (*Aristichthys nobilis*), common carp (*Cyprinus carpio*), grass carp (*Ctenopharyngodon idella*), *Puntius gonionotus*, rohu (*Labeo rohita*), grey mullet (*Mugil cephalus*); *Pangasius sutchi* and in several marine fishes (Sin, 1974; Chao *et al*. 1975; Moczarski, 1977; Withler, 1982; Billard *et al.* 1995; Legendre *et al.* 1996; Lahnsteiner; 1996, a, b; Suquet *et al;* 2000 and Diwan and Nandakumar, 2000).

Early attempts on short-term storage of spermatozoa of Indian Major Carps (IMC) were made by Jhingran (1982) who observed that milt maintained in Ringer-glycerin solution at 28ºC retained the fertilizing ability for 4 hours. At present, sperm cryopreservation has been successful for several finfish species. However, the fish sperm cryopreservation needs development of species-specific protocols. Such protocols are developed through experimental standardization of various parameters, after the captive breeding protocol is developed. But there is a bottleneck due to protracted breeding season and low domestication in most fish species. In all such cases, the time available in a year for conducting the experiments is small and determined by breeding cycle of the species in question. Therefore, it is essential that candidate species for sperm cryopreservation be prioritised.

Success of induced breeding mainly depends on availability of ripe males and females. Sometimes we do not get mature male fishes or get less quantity of sperm even after hormonal stimulation. Thus, the milt in these cases, is gained by extracting it from testis cut into small pieces and extended in a saline solution (Legendre *et al.* 1996). The use of intratesticular sperm causes further difficulties. Males have to be sacrificed for the collection of testis by surgical operation. Sperm obtained from testis may still not be adequate because of reduced volume or poor quality.

As per protocol, fish sperm cryopreservation needs a diluent with cryoprotectant and commonly stored in 0.5 cc French medium straws. At the time of fertilization, the straws are thawed rapidly and poured directly over the eggs to fertilize. The fertilized eggs are raised in the appropriate hatchery. However, there are species-to-species variations that are overcome through optimization. Successful methods for Cryopreservation of catfish sperm has been reported for several species, *Silurus glanis* (L) (Linhart *et al*.1993), Chanel catfish *Ictalurus punctatus* (Guest, 1973), *Pangasius sutchi* (Withler, 1982); *P. hypophthalmusthus* (Samorn and Bart, 2003), *P. larnauduei* (Samoran and Bart 2006). Horvath and Urbanyi (2000) reported successful cryopreservation of *C. garipinus*, *P. gigas* sperm has been cryopreserved and thawed sperm were used to fertilize eggs of different species, viz. *P. hypophthalmusthus* (Mongkonpunya *et al*.1992), and *Clarias macrocephalus* (Mongkonpunya, *et al.* 1995). Ritar (1999) carried out artificial insemination studies with cryopreserved semen from stripped trumpeter *Latirs lineata*

and reported that fertilization and larval hatch rates were mostly lower for frozen thawed than for fresh semen, higher for semen frozen with a diluent containing DMSO than containing glycerol and similar for semen cryopreserved for 1 year to recently frozen semen. Yao *et al.* (2000) carried out long-term storage of cryopreservation of sperm of ocean pout, *Macrozoarces americanus* indicating changes in motility, fertility and ultrastructural changes. In their findings, they have reported 33% success in fertilization rate with post-thawed semen and the loss of sperm motility during freeze and thaw due to ultrastrusctural changes of sperm, e.g. severe swelling of the mitochondria or dehydration of cytoplasm at the mid piece. Ohta *et al.* (2001), while studying cryopreservation of the sperm of Japanese bitterling *Tanakia limbata,* reported that 10% methanol plus 90% foetal bovine serum is a suitable diluent for cryopreservation of bitterling spermatozoa and that samples should be cooled to –40ºC at a low freezing rate for effective storage. Lakra *et al.* (2006) established a protocol of cryopreservation of *Clarias batrachus* spermatozoa based on chemical and biochemical parameters.

Milt collection and quality assessment

The quality of milt used for cryopreservation is crucial for optimizing post-thaw viability. Milt contamination with urine can be avoided by gentle squeezing to empty the urinary bladder prior to stripping (Harvey, 1983) as fish milt gets often contaiminated with urine, blood or faeces during stripping, which alter the composition of the seminal fluid and induce motility of the spermatozoa. This can have detrimental effects on post-thaw viability (Billard *et al.* 1995). A number of workers collected milt from mature male fishes by catheterization to avoid contamination with urine and faecal matter (Alderson and MacNeil, 1984; Rao, 1989; Cabrita *et al.* 1988). The use of intratesticular spermatozoa obtained after sacrificing the animal are also in practice when satisfactory milt quantities cannot be obtained by stripping. Inserting a catheter into the sperm duct is not always recommended as it might result in irritation of the epithelium, bleeding and infection if frequent sampling is done. Moreover, it might not be possible due to the anatomy of these ducts or size of the gonopore as in the case of turbot, european catfish (Labbe and Maisse, 1996).

Anaesthetizing the donors (if necessary), wiping the anal and caudal fins with a damp towel to remove excess water, rinsing the genital area with sterile 0.85% saline or any other suitable extender, collection of milt in clean, dry and sterile vials for immediate storage of collected milt on ice are found to be advantageous (Rao, 1989; Lakra, 1993; Kurokura and Hirano, 1980). To avoid the deterioration in milt quality, many workers suggested that milt be kept on ice soon after its collection (Kurokura and Hirano,1980; Chao and Liao, 2001). Collection of milt using several anaesthetizing agents like Tricaine methane sulphonate (MS-222) (Coser *et al.* 1984; Piironen, 1993), has also been reported.

Evaluation of fish semen quality is essential to judge the condition of spermatozoa prior to cryopreservation. The quality of milt decides the success of a cryopreservation protocol and there are many important semen quality parameters to be observed, viz. volume, pH, density of the spermatozoa and percentage of motile spermatozoa (Rao, 1989).

Estimation of density of spermatozoa

Several workers have used different types of cell counting chambers for the estimation of density of the spermatozoa in the fish milt samples *viz.,* Neubauer Haemocytometer (Gopalakrishan *et al.* 1999) and Thoma Cell (Linhart *et al.* 1993) and Burker Chamber (Piironen, 1993). Kruger *et al.* (1984) estimated the sperm density of milt of common carp (*Cyprinus carpio*) and tilapia (*Oreochromis mossambicus*) by Sysmex Microcell counter CC-120.

Spectrophotometric method for estimation of sperm cell density has been employed by Suquet *et al.*(2000), Conget *et al.* (1996), Lin *et al.* (1996) and Lahsteiner *et al.* (1997). Lin *et al.* (1996) during the cryopreservation studies of muskeunge spermatozoa, analysed the sperm density by spectrophotometric method at 610 nm after 1:1000 dilution of milt and used a formula $(58.3\ x).D + 0.305) \times 10^9$ sperm cells/ml to calculate the density of sperm cells.

Spermatocrit value has been estimated for a number of fish species (Piironen and Hyvarinen, 1983; Ohta *et al.* 2001; Basavaraja and Hegde; 2004). During a study on correlation and variation of spermatocrit value and sperm density in Altantic cod (*Gadus morhua*), Rakitin *et al.* (1999) found a positive correlation between spermatocrit value and spermatozoa density.

Hara *et al.* (1982) reported the sperm density of milk fish to be 3.6×10^{12} cell/ml. Gupta and Rath (1993) during cryopreservation of milt of carps, observed that the spermatocrit values ranged from 65 to 75, 75 to 85 and 65 to 75 and the sperm cell counts ranged between 2.1 to 2.5×10^7, 3.0 to 3.25×10^7 and 2.0 to 2.5×10^7 cells/ml for catla, rohu and mrigal respectively. Tiersch *et al.* (1994) during cryopreservation of channel catfish spermatozoa estimated the density to be $2.5–2.8 \times 10^9$ sperms/g of testis. Lahnsteiner *et al.* (1997) reported the sperm density of various species of salmonid fishes to be $4.9 \pm 0.6 \times 10^9$ sperm cells/ml for rainbow trout, $2.3 \pm 0.5 \times 10^{10}$ sperm cells /ml for brown trout (*Salmo trutta* var. *fario*) and $0.9 \pm 0.2 \times 10^9$ sperm cells/ml for *Salvelinus alpinus.* Ritar (1999) estimated the sperm density of striped trumpeter to be ranging from 5×10^9 to 15×10^9 sperm cells/ml. Gopalkrishnan *et al.* (1999) during cryopreservation of brown trout spermatozoa reported that the density varied from 9.83 to 18.41×10^9 cells/ml. Basavaraja and Hegde (2004) during cryopreservation of spermatozoa of *Tor khudree,* estimated the sperm density to be 7.45×10^6/ml of milt.

Determination of sperm viability

Determination of sperm viability is necessary when developing a semen cryopreservation method. Fertilisation assays evaluate the fertilising capacity of spermatozoa and are therefore the most reliable quality markers. However, eggs are sometimes limited and hatching eggs, especially in the Salmonidae, is very time consuming. Alternative methods for determination of semen viability are (1) motility investigations in the form of subjective estimations (Billard and Cosson 1992), computer-assisted cell motility analysis (Lahnsteiner *et al.* 1996a) and analysis of flagellar beat frequency (Cosson *et al*. 1997); (2) measurement of biochemical parameters, such as ATP level of spermatozoa and leakage of enzymes (Ciereszko and Dabrowski 1996) and parameters of seminal plasma and of sperm metabolism (Fig. 1, Lahnsteiner *et al*. 1996b); and (3) assays on spermatozoal membrane integrity (McNiven *et al.* 1992).

Unlike sperm of higher vertebrates, the ejaculated fish milt has spermatozoa in inactive state and post activation motility is of short duration varying from 30 to 300 sec in different fishes and these become activated the moment they come into contact with water (Stoss, 1983). Ability of the diluent used for cryopreservation to maintain the spermatozoa in quiescent state is a critical requirement as activation prior to cryofreezing can result in loss of capacity to fertilize (Leung and Jamieson,1991). In brackishwater and marine fishes the spermatozoa remain motile for a longer duration as compared to freshwater fishes. In majority of the freshwater fishes, spermatozoa remain motile for 2–3 minutes and in carps it is only for a short duration 30–60 seconds (Rao, 1989). Basavaraja and Hegde (2004) during cryopreservation of spermatozoa of deccan mahseer, *Tor khudree* found that 95–100% sperm cells were motile for 1–2 minutes after activation with tap water.

Hara *et al.* (1982) estimated the percentage of motility in milk fish by picking up fresh milt with a pointed glass rod and by mixing the tiny drop of milt with 3 drops of seawater on the slide and observing under 40× with a microscope. Withler (1982) during the assessment of motility of spermatozoa, used cover slip and sperm activated by flooding the sample with distilled water applied to the edge of the cover slip. Ritar (1999), during assessment of motility percentage of fresh milt, used 18G needle to pick up approximately 0.5–1 µl of milt and used a dilution of 1 : 50 to 1 : 100 (v/v). Many workers assessed the motility percentage of fresh milt by two-step dilution, the final dilution

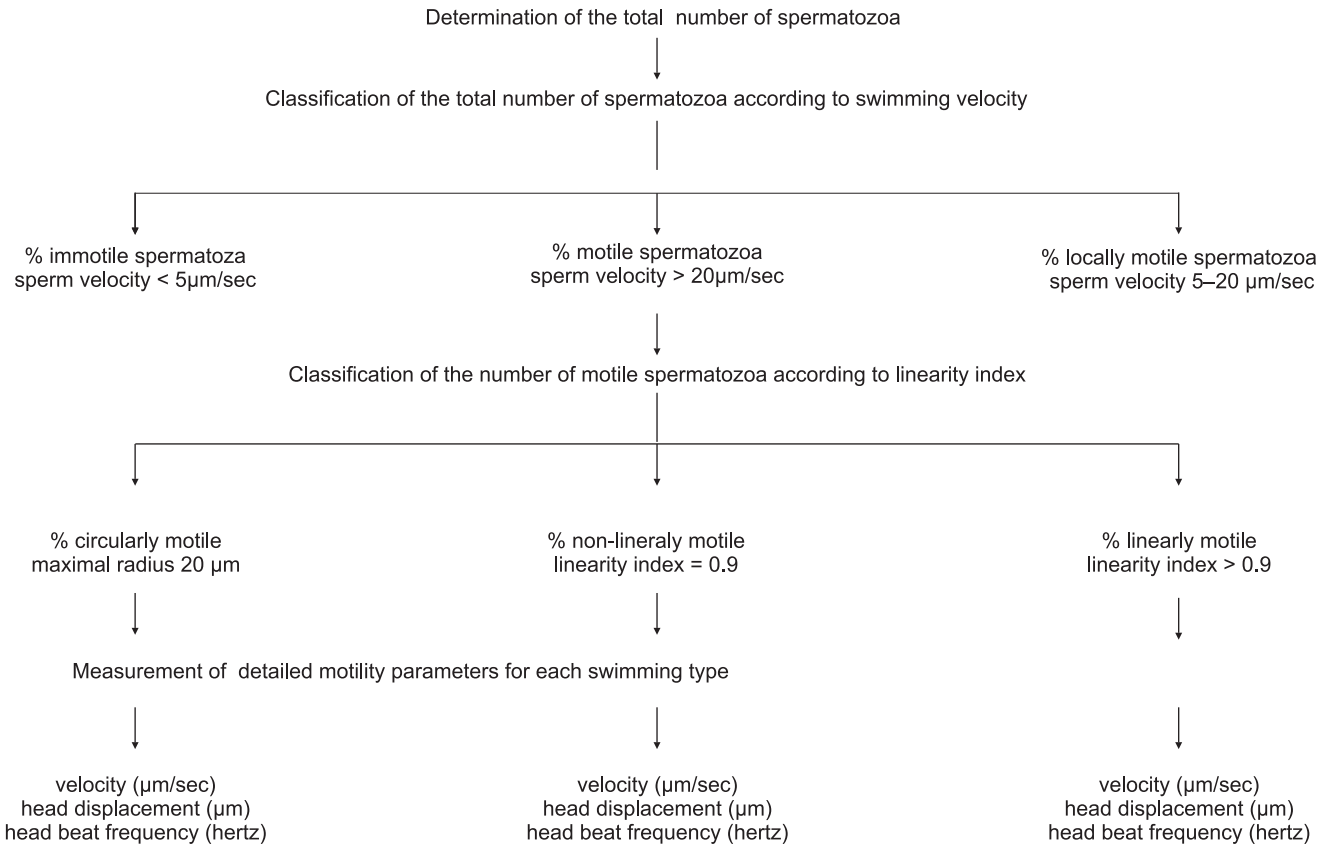


Fig. 1. Steps in the computer-assisted analysis of sperm motility (Source : Lahnsteiner, 2000)

being 1 : 1000 (v/v) (Linhart *et al.* 1993; Cabrita *et al.*1988). Thakur *et al.* (1997) and Ponniah *et al.* (1999) used a pin head to pick up about 1 : 1 of fresh milt (equated with a micropipette) and mixed with 1, 2, 3 : 1 of activating media or dechlorinated tap water to estimate the percentage of motility and duration of motility.

A six point qualitative scale of 0–5 based on mass motililty of spermatozoa was developed to assess the quality of the semen by Sanchez-Rodriguez and Billard (1977) and has been widely used by many workers (Kumar, 1988; Fabbrocini *et al.* 2000). Ponniah *et al.* (1999) scored the motility percentage on a 10-point scale at 10% intervals from 0 to 100%. Many workers conducted motility assessment studies with the help of video camera by frame analysis/computer-assisted analysis of motility parameters (Lahnsteiner *et al.* 1997; Toth *et al.* 1997; Ravinder *et al.* 1997; Linhart *et al.* 1993; Ohta *et al.* 2001). Lahnsteiner (2000) used fertilisation assay method while working on salmonids because they directly reflect sperm fertilisation capacity, as also computer assisted cell motility analysis method since this provides different motility parameters in a large member of individual spermatozoa.

In fertilisation method, eggs are mixed with fresh or frozen-thawed semen by gentle stirring and the viability of sperm is assayed by the number of eggs developed to an eye-stage embryos. In computer assisted cell motility analysis, sperm motility is recorded in videotapes and video sections are analysed in a cell motility analysis programme originally developed for mammalian sperm cells.

Rana and McAndrew (1989) used deactivator solution (0.7% NaCl and 0.6%, KCl, pH 8.2) to arrest sperm motility and only samples with more than 99% deactivated spermatozoa were taken for cryopreservation. Many workers accepted only the milt samples with more than 70% motile spermatozoa with forward motility and used them for cryopreservation (Coser *et al.* 1984; Piironen, 1993; Lahnsteiner *et al.* 1997). The milt samples with a minimum of 40% sperm motility were used for cryopreservation by Linhart *et al.* (1993) where as Cabrita *et al.* (1988) used the milt samples with more than 60% forward motility for cryopreservation.

Cabrita *et al.* (1988) used propidium iodide for estimating the percentage of viable spermatozoa by using flow cytometry. Yao *et al.* (1987) conducted the sperm viability studies with salmon milt using 1% trypan blue. They used milt, phosphate buffer saline (PBS) and 1% trypan blue in the raio of 0.1 : 1 : 0.1 (v/v). Kruger *et al.* (1984) estimated the percentage of live spermatozoa in the millt of common carp (*Cyprinus carpio*) and tilapia (*Oreochromis mossambicus*) by using eosin-nigrosin stain.

Optimization of milt dilution

In a study on cryopreseration of milt of tilapia, *Orechromis* spp., Rana and McAndrew (1989) observed the optimum sperm to egg ratio to be 1.40×10^5 sperm cells/egg. Linhart et *al.* (1993) observed that in case of European catfish, the optimal sperm to egg raio was $2.4–3.0 \times 10^6$ sperm cells/egg. Conget *et al.* (1996), during the cryopreservation of rainbow trout milt, maintained an optimum sperm to egg ratio of 3×10^6 spermatozoa/egg. Gopalakrishnan *et al.* (1999), during cryopreservation of brown trout milt, reported that the sperm to egg ratio was 3.38×10^7 sperm cells/egg. Linhart *et al.* (1993) maintained a sperm to egg ratio of $1.8–2.4 \times 10^5$ spermatozoa/egg during the fertilisation trials of common carp. Basavaraja and Hegde (2004) reported the optimum spermatozoa number per egg to be 10^3 sperm cells.

Packaging materials used for cryopreservation of milt

During cryopreservation of fish spermatozoa, most of the workers made use of French straws of 0.25 ml and/or 0.5 ml capacity, sealable with polyvinyl alcohol (PVA) powder (Hara *et al.* 1982; Alderson and MacNeil, 1984; Scheerer and Thorgaard, 1989; Gupta and Rath, 1993; Tiersch *et al.*1994 Thakur *et al.* 1997; Ponniah *et al.* 1998a, 1998b, 1999, Gopalakrishnan *et al.* 1999; Basavaraja and Hegde 2004). Alderson and MacNeil (1984), during cryopreservation of milt of Altantic salmon, used both 0.25 ml and 0.5 ml size French straws and observed that good sperm viability was achievable with 0.5 ml straws.

Several workers used screw capped cryo-vials/glass ampoules of different sizes, viz. 1.0 ml or 1.5 ml or 2.0 ml (Durbin *et al.* 1982; Withler, 1982; Coser *et al.* 1984; Rana and McAndrew, 1989; Diwan and Nandakumar, 2000; Linhart *et al.* 1993). Rana and McAndrew (1989) observed that with 1.5 ml cryo-tubes, the percentages of fertilisation showed unacceptable levels of variability and hence they used 0.5 ml straws for further studies on cryopreservation of spermatozoa of tilapia, *Oreochromis* spp. Kurokura *et al.* (1984) used 10 ml aluminum foil bags (20 mm × 100 mm) in addition to 0.5 ml straws. Lahnsteiner *et al.* (1997), during cryopreservation of salmonid fish species, studied the performance of 0.5 ml, 1.2 ml and 5 ml straws with respect to fertilisation percentage and observed that with 0.5 ml and 1.2 ml straws the fertilisation rates were similar and 5 ml straws resulted in a fertilisation success of only about 40% of fresh semen (control). Cabrita *et al.* (1988) used 1.8 ml flat plastic straws and 5.0 ml macro-tubes in additon to 0.5 ml straws for a comparative study on their performance for application in large scale fertilisation and observed that the percentage of fertilisation was low in large volume straws (73.2% and 61.9% for 1.8 ml straws and 5.0 ml macro-tubes) as compared to 0.5 ml straws (77.4%) during cryopreservation of rainbow trout milt. Ohta *et al.* (2001) used long, acrylic capillary tubes (110 mm length × 2 mm diameter, volume 80µl) sealed with PVA powder at both the ends after filling.

Extenders used for the cryopreservation of fish spermatozoa

An extender is essentially a solution of balanced salts and

sometimes, organic compounds. Spermatozoa must be diluted before freezing with a suitable extender. One of the functions of the extender is to inhibit the osmotic activation of the spermatozoa (Leung, 1991). The extender is based on a buffered physiological saline solutions, originally described by Borchand and Schmidt (1979). This is because such buffered solution resembles the inorganic composition of seminal plasma of a spermatozoa. Therefore, the composition of extender differs from species to species. A large number of extenders with varying chemical compositlion and complexity, including those proven successful for cattle semen cryoprservation and some animal tissue culture media like, Ringer's solution, Cortland's solution, Alserver's solution etc., have been tried for the cryopreservation of spermatozoa of fish. Several simple extenders, isotonic to fish milt, with inorganic salts like Nacl, KCl, $CaCl_2$, $NaHCO_3$, $NaHPO_4$, $MgSO_4$, $MgCl_2$, KH_2PO_4 and others with organic compounds like fructose, mannitol, glucose, lecithin, glycine, egg yolk, Bovine Serum Albumin (BSA) have been used with varying levels of success (Rao, 1989).

Withler (1982) observed that during cryopreservation of spermatozoa of *L.rohita, Puntius gonionotus, Pangasius sutchi, Ctenopharyngodon idella, Aristichthys nobilis* and *Cyprinus carpio,* medium 189M gave good results for *L. rohita* and for *C. carpio*, medium 251 gave better results but in the case of *Pangasius sutchi,* the performance of both the medium amd 189M was poor. Durbin *et al.* (1982) observed that post-thaw motililty in the NaCl-$NaHCO_3$ extender was better during cryopreservation of spermatozoa of *C. idella.* Kurokura *et al.* (1984), during the cryopreservation of milt of *C. carpio*, used two extenders namely extender-1 and extender-2 and observed that extender-1 gave more eyed eggs (68.6%) as compared to extender-2 (11.0%). Kumar (1988) studied the suitability of seven extenders for cryopreservation of spermatozoa of *Catla catla, L. rohita, Cirrhinus mrigala* and *Hypophthalmichthys molitrix* and reported that egg yolk citrate and extender-M_a gave better results in terms of percentage of post-thaw motility as well as fertilization percentage. Gupta and Rath (1993) used an extender with NaCl, KCl, $CaCl_2$ and $NaHCO_3$ during the cryopreservation of spermatozoa of *C. catla, L. rohita* and *C. mrigala.* During a study on the short-term preservation of milt of *C. carpio* at low temperature, Ravinder *et al.* (1997) used eleven different extenders namely, KCL, TLP, Cytomix, Mannitol, FPS, Corland's Fish Ringer (FR), FR+Tris, NAS, TSM and BWW and found that BWW and TLP were the most suitable storage buffers as the milt stored in these buffers showed no significant decrease in percentage of motile spermatozoa up to 24 h upon activation. Lakra and Krishna (1997) during cryopreservation of spermatozoa of *Cyprinus carpio* and *Labeo rohita,* tested seven extenders for their suitability and found that Tris-egg yolk was the most effective of all and gave higher post-thaw motility percentage (50%) as compared to other extenders. Ponniah *et al.* (1998b) used CC-1 extender during cryopreservation of milt of *C. carpio.* Ponniah *et al.* (1999) use the same extender for cryopreservation of *Tor khudree* milt. They also tested an egg yolk-citrate extender. Ponniah *et al.* (1999) used five different extenders composed of various levels of NaCl, KCL, $CaCl_2.2H_2$), $NaHCO_3$, sodium citrate, $MgCl_2.6H_2O$. $MgSO_4.7H_2O$, $NaH_2PO_4.2H_2O$, glucose, egg yolk, streptomycin and penicillin for the cryopreservation of spermatozoa of *Tor putitora* and observed that extender NBFGR-2 gave higher post-thaw motility of 90% and a hatching rate of 12.1% when activated with diluter-532. Diwan and Nandakumar, (2000), while carrying out studies on cryopreservation of sperms of certain cultivable marine species, used nine extenders with different cryoprotectants. They found out of these nine, extenders, use with Marine Ringer and physiological saline solution are the best extenders for long-term cryopreservation studies. Basavaraja and Hegde (2004) used modified Fish Ringer's solution for cryopreservation of deccan mahseer. *Tor khudree* spermatozoa. Lal *et al.* (1999) observed that the potassium concentration (KCl @ 1500 mg /100 ml or 201 mM) required in *T. ilisha* to maintain sperm in inactive state is ralatively high as compared to most of the fishes studied.

Cryoprotectants used for the cryopreservation of fish spermatozoa

Durbin *et al.* (1982) used 10% DMSO as the cryoprotectant for cryopreservation of grass carp spermatozoa and achieved mean fertilisation percentages of 32, 51 and 57%. Hara *et al.* (1982), during cryopreservation of milt of milt of milk fish, used 15% DMSO as the cryoprotectant and obtained fertilisation percentage of 67.5% with milkfish serum as an extender. Withler (1982) used two cryoprotectants, 8% DMSO and 8% glycerol individually with extenders 189M and 251 and found that for rohu, 8% DMSO gave better percentage of feritilisation (58% with 189M) and for *Puntius gonionotus*, 8% DMSO with either 189M or 251 gave poor percentage of fertilisation (5%). Alderson and MacNeil (1984) protected the milt of Atlantic salmon with 9% DMSO. Coser *et al.* (1984), during cryopreservation of two freshwater south American fishes, *Prochilodus* sp. and *Salminus* sp. used 10% DMSO (v/v) and found that the post-thaw motility percentage varied widely from 1 to 4%. Kurokura *et al.* (1984) used 15% DMSO as the cryoprotectant during cryopreservation of common carp. Kumar (1988) used 8% glycerol individually during the cryopreservation of spermatozoa of India Major Carps (IMC) and silver carp. Rana and McAndrew (1989) tested the suitability of two cryoprotectants, methanol and DMSO in modified Fish Ringer's solution for cryopreservation of spermatozoa of tilapia at six concentrations from 5 to 40% (v/v) and reported that 10% methanol was better. They obtained widely variable fertilization rates ranging from 38.7% to 93.4% on subsequent cryopreservation with 12.5%

methanol. Scheerer and Thorgaard (1989) used 9.0% DMSO as the cryoprotectant during the cryopreservation of rainbow trout milt. Young *et al.* (1992), during cryopreservation of milt of summer whiting, tested four cryoprotectants, viz. 0.75M glycerol, 0.75M methanol, 0.75M DMSO and 0.75M propylene glycol individually, and observed that glycerol gave better results with respect to post-thaw motility (duration of motility-41 min) and gave better results with egg yolk (duration of motility-50 min). Gupta and Rath (1993), during cryopreservation of spermatozoa of IMC, used 15% DMSO as a cryoprotectant. Lakra and Krishna (1997), during cryopreservation of carp spermatozoa and catfishes used sperm DMSO and Glycerol individually at 5% and 10% levels as cryoprotectants and found that glycerol gave better post-thaw in *C. carpio* and DMSO gave better post-thaw motility in case of *L. rohita* and catfishes. Thakur *et al.* (1997) used 10% DMSO as the cryoprotectant with six extenderes during cryopreservation of spermatozoa of rain trout. Ponniah *et al.* (1998a, 1998b), during the cryopreservatioon of spermatozoa of *C. carpio* used DMSO as the cryoprotectant at a concentration of 8% or 10% with varying levels of success. Ponniah *et al.* (1999) in their study on cryopreservation of spermatozoa of *Tor putitora*, used two cryoprotectants DMSO and glycerol individually and reported highest hatching percentage of 12.1% with glycerol. Basavaraja and Hegde (2004) used DMSO at three levels, 5,10 and 15% (v/v) and observed high post-thaw motility of 92% and 98% with 5 and 10% DMSO respectively, while 15% DMSO significantly reduced the post-thaw motility. Among the various cryoprotectants used for long-term cryopreservation of sperm of marine fishes like *L. parsia*, *S. siham*, *M. cephalus* and *G. oyena*, Diwan and Nandakumar (2000), reported that DMSO in combination with Marine Ringer and Glycerine are found to be most suitable cryoprotecants. Further, they mentioned that preservative media supplemented with addition of oxygen showed high survival rate of cryopreserved sperm.

Oxygen enriched environments

Maintaining sperm cells in an aerobic environments is a prerequisite for *in vitro* preservation (Stoss 1983, Billard 1988). Studies on rainbow trout (Buyukhatipoglu and Holtz 1978), Billard 1981, Stoss, 1983) suggest that the fertility of spermatozoa can be prolonged when preserved under oxygen compared with air. To ensure high oxygen availability and distribution to the cells several different approaches have been reported. Milt has been stored in polythene bags (Stoss, 1983, Billard,1988) or continuously flushed in a moisture-saturated desiccator (Stoss, 1983, McNiven *et al.* 1993). By combining this technique with the use of antibiotics and lowering the storage temperature to 0ºC rainbow trout milt has been successfully stored for 34 days (Stoss, 1983).

The use of perfluorocarbon emulsions (PFC) such as fluosol and FC-77, which were originally used for respiratory gas transport in human medicine and cell culture (King *et al.* 1989, Lowe 1991) has increased the longevity of poultry semen under chilled conditions (Rogoff 1985). The use of such inert organic gas carriers, which have a very high affinity for oxygen, to prolong the viability of fish milt was recently reported for rainbow trout (McNiven *et al.* 1993). In these studies rainbow trout milt held over a non-aqueous layer of PFC (FC-77) in a moisture laden atmosphere at 0ºC remained viable for 37 days. Similar studies on Atlantic salmon using fluosol, however, at 4ºC and –4ºC showed that although milt could be stored for up to 29 and 69 days, respectively, at each temperature there was no significant advantage over storage in air at either temperature.

The depth of milt in the storage container, is also reported to influence the fertility capacity of milt after storage (Stoss, 1983). By sampling rainbow trout milt at various depths in a test tube, it has been demonstrated that there is a pronounced decrease in post-activation motility at depths below 5 mm (Rana, 1995).

Different Equilibration Periods for Cryopreservation of Fish Spermatozoa

Kumar (1988) used a range of equilibration periods from 2 to 30 min during the cryopreservation of spermatozoa of Indian major carps and silver carp. Many workers did not use any equilibration time during cryopreservation of milt of may fish species (Durbin *et al.* 1982; Coser *et al.* 1984; Ciereszko and Dabrowski, 1996). Gupta and Rath (1993), during cryopreservation of milt of IMC, used an equilibration time of 45–60 min. Conget *et al.* (1996), during rainbow trout milt cryopreservation used an equilibration of less than 10 minutes. Lahnsteiner *et al.* (1997), during cryopreservation of salmonid fishes, used an equilibration time of 15 min. Lakra and Krishna (1997), during cryopreservation of milt of carps and catfish, used equilibration times of 4, 120 and 170 min. Thakur *et al.* (1997) equilibrated the milt with diluent on ice for 10 minutes during the cryopreseration of milt of rainbow trout. Ponniah *et al.* (1998a) used an equilibration time of 10 min at 4ºC during cryopreservation of spermatozoa of common carp, *Cyprinus carpio*. Gopalkrishnan *et al.* (1999) used an equilibration time of 15 min during the cryopreservation of brown trout. Equibration periods of 60, 120, 180 min and 70–95 min (in three steps) were used in a study by Ponniah *et al.* (1999) for cryopreservation of spermatozoa of *Tor putitora,* and they observed higher hatching percentages at an equilibration time of 60 min. They also reported that there was no marked difference in percentage of motile spermatozoa between the different equilibration times. Basavaraja and Hegde (2004) used varying equilibration times ranging from 10 to 90 min during cryopreservation of deccan mahseer *Tor khudree* and observed that very high post-thaw motility rates of 92–98% were obtained at 10, 20, 30 min of equilibration and they also observed that fertilisation rates were independent of

equilibration time.

Different freezing rates used during the cryopreservation of fish spermatozoa

The freezing rate is the most critical factor affecting the success of a cryopreservation protocol. If the freezing rate is too high, there will not be much time for the free water to separate from the cytoplasm and hence it results in the formation of small ice crystals within the cell which is undesirable as it punctures the cell membrane and the membranes of the cell organelles. On the other extreme, if the rate of freezing is too low, it results in the exposure of the cell to the concentrated cytoplasm for a long time. It is a sort of pickling effect and due to the high salt concentration and subsequent changes in the pH, the biomolecules in the cell get denatured. Hence, the optimum freezing rate is a moderate rate between the two extremes of the freezing rate (Franks, 1985). The rate of freezing is a very critical factor in freezing experiments and instant immersion in LN_2 has been found to cause significant decrease in post-thaw duration of motility (Young *et al.* 1992).

Many workers have used manual freezing method which makes use of freezing the filled straws at different heights over liquid nitrogen vapours depending on the freezing rates required using Styrofoam containers and racks for placing the straws. Witheler (1982) froze the glass ampoules filled with diluted milt by keeping them at a height of 2 cm above LN_2 surface for 5–10 min. During a study by Alderson and MacNeil (1984), the straws were frozen over LN_2 vapours for a period of 5 min for 0.25 ml straws and for 10 minutes in the case of 0.5 ml straws and observed that freezing rates had no effect on post-thaw fertility over a range of 20–140°C/min. Coser *et al.* (1984) used a method in which the straws were frozen at a height of 13 cm above LN_2 surface for 2 minutes. In a study conducted by Kumar (1988), the straws were frozen by placing them at a height of 2 cm over LN_2 surface for a period of 2–5 min. Gopalkrishnan *et al.* (1999) used a method in which straws were frozen over LN_2 surface at a height of 6–8 cm for 10 min during cryopreservation of brown trout milt. Tiersch *et al.* (1994) employed a protocol in which straws were frozen on a stainless steel tray suspended over LN_2 and a temperature of –80°C was maintained at the tray and the straws were frozen for 4 min (after a temperature of –70°C was reached) before immersion into LN_2. In an experiment conducted by Lahnsteiner *et al.* (1997) during cryopreservation of salmonid fishes, the straws were frozen on a horizontally mounted rack in an insulated box for a period of 10 min and used heights of 1.0 cm (final temperature reached –130°C for 1.2 ml straws), 1.5 cm (final temperature reached –110°C for 1.2 ml straws) and 2.5 cm (final temperature reached –92°C for 0.5 ml straws) over the LN_2 surface. Lakra and Krishna (1997), during cryopreservation of carps and catfishes, froze the straws below –120°C over LN_2 surface for 10 min. In an experiment conducted by Ritar (1999) during cryopreservation of milt of striped trumpeter, the straws were frozen over LN_2 at a height of 4 cm for a period of 270 seconds (final temerature reached in the straw was –120°C). Cabrita *et al.*(1988) used a rack that floated on the LN_2 surface for freezing 0.5 ml straws (at a height of 2 cm above the surface) and in a metallic support in a closed Styrofoam box for 1.8 ml and 5 ml straws for 10 min. Ohta *et al*. (2001), during cryopreservation of milt of Japanese Bitterling, studied the effect of freezing rates on the percentage of post-thaw motility and observed that at a freezing rate of 18°C/min, the post-thaw motility percentage (31.9%) was high at a final temperature of –40°C. Basavaraja and Hegde (2004), during cryopreservation of *Tor khudree* spermatozoa, froze the straws at a height of 5 cm over LN_2 for 10 minutes.

Several workers have made use of methanol-dry ice bath (Kurokura *et al.* 1984) for freezing before immersion into LN_2 for storage. Several others froze the extended milt with cryoprotectant over crushed dry ice (Scheerer and Thorgaard, 1989). A technique of pelletization, by dropping specific volumes of diluted milt over dry ice (solid CO_2) which serves to eliminate the need for individual straws as well as serves to freeze the milt was used by many workers (Piironen, 1993; Clereszko and Dabrowski, 1994; Ritar, 1999). Linhart *et al.* (1993) pellet-froze (pellet volume 40:1) the diluted milt of European catfish over small aluminium discs placed at a height of 4 mm above LN_2 surface. Ritar (1999) observed that fertilization rates were lower for milt frozen as 0.25 and 2.0 ml pellets (67 and 69% respectively) and higher for 0.25 ml straws (75%).

Some other workers have used programmable freezers for freezing the diluted milt samples of several fish species with different programmes and different final temperatures were attained before immersion into LN_2, viz. (Linhart *et al.* 1993; Rana and McAndrew, 1989; Ponniah *et al.* 1998a; Conget *et al.* 1996). Ponniah *et al.* (1998a) observed that maximum hatching percentage could be achieved (56.3% of control) with an optimal freezing termperature of 20ºC without ice seeding and with ice seeding, the hatching percentage was low (51.9% of control) with an optimal freezing temperature of –50ºC.

Thawing of cryopreserved milt

The rate of thawing is also a very important step, which determines the success of a cryopreservation procedure. It is the reverse of freezing but rapid thawing is preferred. However too high and too low rates of thawing are detrimental for the cryopreserved spermatozoa.

Durbin *et al.* (1982) during thawing, transferred the frozen vials over dry ice for 1 hour and then thawed at 20ºC quickly in a water bath and obtained the highest mean fertilization percentage of 57%. Whithler (1982) thawed the cryopreserved milt by swirling the frozen ampoules in tap water at 29ºC. During the fertility trials for testing the

viability of cryopreserved milt of Atlantic salmon, Alderson and MacNeil (1984) thawed the cryopreserved milt at 37–40ºC for 5 sec in a water bath. Kurokura *et al.* (1984) thawed two batches of cryopreserved milt at 23ºC in a water bath. Kumar (1988) thawed the frozen milt by swirling the straws in tap water at 30ºC in case of IMC and silver carp. During fertility trails, thawed milt was applied immediately to fresh ova and mixed by stirring with a feather and tap water was added immediately after the semen was mixed. Rana and McAndrew (1989) thawed the cryopreserved milt at 40ºC in water bath for 8 sec and the spermatozoa were activated with equal volume of pre-warmed hatchery water (at 28ºC) and the contents of single straw/cryotube were mixed with 50 eggs, in a petri plate for 3 min before rinsing eggs with excess water. Scheerer and Thorgaard (1989) thawed the milt at 10ºC for 30 sec in water bath during the fertilisation trials for the cryopreserved spermatozoa of rainbow trout. Young *et al.* (1992), during cryopreservation of milt of summer whiting, observed that post-thaw duration was not affected by different temperatures of thawing (0ºC, 23–25ºC and 40ºC). Gupta and Rath (1993) thawed the cryopreserved IMC spermatozoa at 38±2ºC in a water bath and obtained higher hatching percentages of 30–40%. Linhart *et al.* (1993) during the fertilisation trials, thawed the cryopreserved milt at 36ºC for 20 secondes and reported a hatching percentage of 45.2 (hatching percentage for control-70.6%). In the fertility trials conducted by Piironen (1993) the cryopreserved milt pellets of brown trout and arctic charr were thawed by immersing the pellets in 20 ml (10 pellets/20 ml) of 0.12M $NaHCO_3$ at 25–30ºC for 10–15 sec. Tiersch *et al.* (1994) thawed the cryopreserved spermatozoa of channel catfish at 40ºC for 7 seconds in a water bath and estimated the percentage of motility as well as percentage of fertilisation. During fetility trials of the cryopreserved milt of rainbow trout, Ciereszko and Dabrowski (1996) thawed the cryopreserved milt pellets in 0.7% NaCl at 23–25ºC for 5–7 sec. Lahnsteiner *et al.* (1996a,b), during fetility trials, tested different thawing rates and observed that the optimal thawing was at 25ºC for 30 seconds in a water bath. During the fertilisation trails by lahnsteiner *et al.*(1997) for the cryopreserved milt of salmonid fishes, the milt was thawed at 25ºC for 30 seconds for 0.5 ml straws and at 30ºC for 30 sec for 1.2 ml and 5.0 ml straws in a water bath. Thakur *et al.* (1997) thawed the straws at 1, 4, 5, 20, 32 and 37ºC for 30 sec and at 32ºC and at 37ºC for 30 sec and at 32ºC for 60 sec and at 37ºC for 5 sec and reported that thawing at 37ºC for 5 sec gave highest post-thaw motility percentages of 70 to 80%. Ponniah *et al.* (1998b) thawed the straws by briefly exposing them to air at 24ºC for 5 sec and then by rapidly immersing the straws in a water bath at 35ºC for 20 sec. Gopalakrishnan *et al.* (1999) thawed the cryopreserved milt of brown trout by waving the straws in the air for 2–3 sec and then by immersing in a water bath at 37ºC for 5 sec and the post-thaw percentage of motility was assessed.

Ponniah *et al.*(1999), during the fertilisation trials for cryopreserved milt of *T. khudree* milt, thawed the cryopreserved milt at 37ºC for 40 seconds in a water bath. Linhart *et al.* (1993) conducted fertility trials by thawing the straws at 35ºC in a water bath for 110 seconds. Cabrita *et al.* (2001) thawed the cryopreserved milt at 25ºC in water bath for 30 seconds for 0.5, 1.8 ml straws and at 60ºC for 30 seconds/80ºC for 20 seconds for 5 ml straws. Ohta *et al.* (2001) thawed the cryopreserved milt at 20ºC for 7 seconds in a water bath. Basavaraja *and* Hegde (2004) thawed the cryopreserved milt of *Tor khudree* by quickly plunging the straws into a cooler box with water maintained at 37±1ºC for 5–10 seconds and obtained high post-thaw percentage of motility of 92–98% and obtained a high hatching percentage of 25.7%.

Use of activation / fertilisation solution

There have been some differences of opinion, among different researchers about the effect of the ovarian fluid as an activation solution. But it was proven beyond doubt by that normal ovarian fluid in fact induces sperm motility but the presence of broken ova progressively suppresses this ability and reduces the success of fertilisation.

Coser *et al.* (1984) thawed the cryopreserved milt of *Prochilodus sp.* and *Salminus sp.* in a solution of 1% $NaHCO_3$ or 0.8% NaCl at 40ºC and observed a post-thaw motility score of 1 (1–5%) for *Prochilodus sp.* and a score of 3 (10–40%) for *Salminus sp.* Rana and McAndrew (1989) used equal volume of pre-warmed hatchery water (at 28ºC) for activation of the spermatozoa. Scheerer and Thorgaard (1989) tested the efficacy of three fertilisation or activation solutions namely, dechlorinated tap water, buffered saline and buffered saline +5mM theophylline during the fertilization trials. In the fertility trials conducted by Piironen (1993) it was observed that highest percentage of fertilization was observed when the fertilization diluent of Billard was used with the thawed milt. Thakur *et al.* (1997) conducated fertilization trials with 1% $NaHCO_3$ and 5ml diluer-532 as activiating solutions. Ponniah *et al.* (1998a) used Diluer-532 (at 123 mg/10 ml) as an activating solution while thawing the straws. Ponniah *et al.* (1998b) used three activating solution vi., tap water (pH 7.5), Diluer-532 at 123 mg/10 ml, pH 8.5) and 1.5% sodium sulphite (pH 8.5). They observed high motility percentage (80%) as well as high hatching percentage (83%–93% as percentage of control) with the activating solution, Diluer-532. Ponniah *et al.* (1999) used three activating solutions namely, hatchery water, Tris-glycine-NaCl in the fertility trials. Ohta *et al.* (2001) thawed the cryopreserved milt and used 0.5% NaCl as an activating solution. Basavaraja and Hegde (2004) did not use any activiation solution during fertility trials in case of *Tor khudree.*

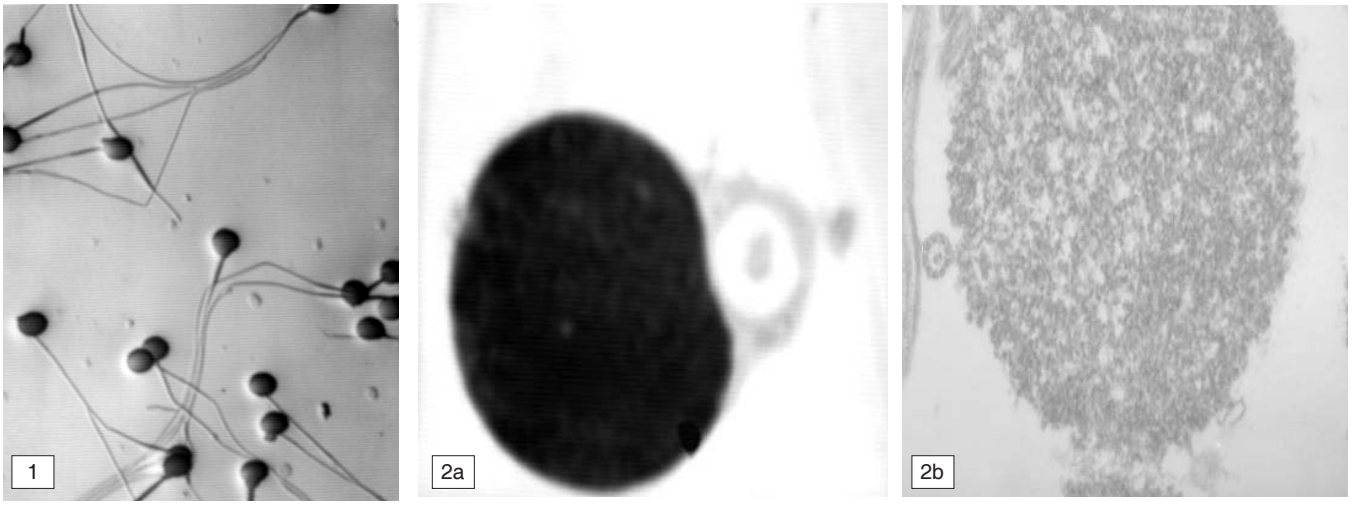
Biochemical analyses of fish milt

Piironen and Hyvarinen (1983) studied the biochemical

composition of milt of six freshwater teleosts namely, landlocked salmon (*Salmo salar* var. sebage), brown trout (*Salmo trutta* var. *Lacustris*), rainbow trout (*Salmo gairdneri*), white fish (*Coregonus lavaretus*), Perch (*Prca fluviatilis*) and Burbot (*Lota lota*) and observed that glucose concentration was five times higher than fructose and fructose level was low when compared to mammalian values. They reported that glycerol concentration was found to be high and could be used as a cryoprotectant in species with high glycerol values with good results. Piironen and Hyvarinen (1983) centrifuged the milt at 5,000 rpm for 30 minutes and the supernatant seminal plasma was stored at –40ºC for 1–5 months till further analysis and was analysed for total lipids, glucose and fructose by kits of Boehringer Mannheim. Kruger *et al.* (1984) analyzed the chemical characteristics of milt of common carp (*Cyprinus carpio*) and tilapia (*Oreochromis mossambicus*) on seasonal basis and collected the supernatant seminal plasma by centrifuging the milt at 7000 rpm for 20 minutes. The milt was analysed for inorganic ions, Na, K, Ca, by flame photometer and organic compounds, fructose, galactose, glucose, lactate, cholesterol, total lipids, proteins, urea, puruvate kinase and ATPase measured with standard biochemical kits from Boehringer Mannhelm. They observed seasonal variations and significant inter-specific variation in the composition except sodium and galactose. Yoa *et al.* (1987) contrifuged the salmon milt at 850 g for 5 minutes at 4ºC and collected the seminal plasma and used for various biochemical analyses. They reported that low temperature preservation of salmon (*Salmo salar*) spermatozoa at –80 ºC led to massive loss of sperm proteins into the seminal fluid which indicated the damage o the cell membranes during freezing, storage and thawing. Lahnsteiner *et al.* (2000) investigated the seminal plasma composition of three cyprinid species, the bleak (*Alburnus*), the chub (Leuciscus cephalus) and the zaehrte (*Vimba vimba*) by qualitative thin-layer chromatography (TLC) and quantitative spectrophotometric assay methods. They analysed the organic compounds, monosaccharides (glucose, fructose, galactose and xylose), lipids (cholesterol, fatty acids, phosphotidylcholine and glycolipids) and proteins and enzyme activities and enzymes like acid phosphatases, a-glucuronidase, proteases and alkaline phosphatase, pH values and osmolarity. Within 15 minutes of collection, the milt samples were centrifuged at 350g for 10 minutes at 4ºC and the supernatant was collected and pooled and the analysis were carried out. Lahnsteiner *et al*. (2000) estimated glucose, fructose, galactose, triglycerides, phosphotidylcholine and proteins. Lin *et al.* (1996) centrifuged the milt of muskellunge at 12,000 rpm for 10 minutes at 4ºC and seminal plasma was collected and kept on dry ice and stored at -80ºC till further use. They analysed the plasma for inorganic ions, Na, K, Mg, Ca, PO_4 and Cl that were estimated by inductively coupled plasma emission spectrophotometer and protein was estimated by Bradford method. Plouidy and Billard (1982) reported that semen was centrifuged at 3600 g for 20 minutes and the supermatant seminal plasma was collected and stored at –20ºC and later analysed for pH, ash, cations, phosphorous and proteins and amino acids. Toth *et al.* (1997) centrifuged the milt of lake sturgeon (*Acipenser fulvescens*), at 4ºC and three aliquots of pooled plasma from nine fishes that was used for all the analyses. They conducted the quantitative elemental analysis of seminal plasma for P, K, Ca, Mg, Na, Zn and Cl using inductively coupled plasma (ICP) emission spectrophotometer.

Ultrastructural studies of cryopreserved spermatozoa of fish after thawing

A number of workers have studied the damages to spermatozoa due to cryopreservation both by Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM) and observed that spermatozoa underwent morphological changes during cryopreservation viz., winding of flagella, loss of flagella, appearance of verrucosities on the sperm head as revealed by SEM. Teleost sperm is primitive type and typically consist of a head with dense chromatin, flagellar tail and a ring of mitochondria. Fig. 1 exhibit scanning electron micrograph of Indian Major Carp, *Labeo rohita*. Structural changes, viz. detachment of nuclear envelope and plasma membrane from the nucleus and loss of the central doublet as elucidated by TEM have also been reported (Yao *et al.* 2000). Lahnsteiner and Patzner (1998) reported a new method for fixation of spermatozoa of freshwater teleosts for electron microscopy consisting of an unbuffered mixture of formaldehyde-glutaraldehyde and osmium tetroxide. The studied the damage caused by freezing to sperm and embryo of carps by SEM and found that freezing damage consisted of expansion of head and neck of the sperm. The plasma membrane of the sperm heads in some cases were also found to be broken. They also observed that the external membrane of the tail appeared disrupted and some sperms were even tail-less. During cryopreservation of grayling, they studied spermatozoa the fine structural changes both by SEM and TEM and noted that morphological damage was observed immediately after dilution of milt with the extenders. After freezing and thawing about 40–50% of the spermatozoa were completely damaged, 30 to 40% changed and only 10–20% showed an intact morphology. Lahnsteiner *et al.*(1996a) studied the ultrastrutural changes caused in spermatozoa of rainbow trout following cryopreservation by TEM. About 20–40% showed intensive signs of swelling of the head and mid-piece regions of the mitochondria. Further, they studied the effect/damage caused to the fine structure of spermatozoa due to cryopreservation of rainbow trout and brown trout by freeze-fracture electron microscopy and observed that changes were induced in the organisation of the plasma membranes of spermatozoa in the form of particles grouped in rounded clusters in a chaotic manner and folding of the plasma membrane as compared to the fresh



Figs 1-2. **1.** Scanning Electron Micrograph of Spermatozoa of *Labeo rohita* (×1800). **2.** Transmission Electron Micrograph of Sperm Head in *Labeo rohita* (×33000). (a) Compact and Dense Chromatin (b) Vacuolation in sperm head and loosely packed chromatin indicating damage during cryopreservation

spermatozoa which showed homogenous distribution of particles in the plasma membrane. Gopalakrishnan *et al.* (2000), during a study on changes in spermatozoa of rohu *Labeo rohita,* observed the damages in head, mid-piece and tail of about 51% spermatozoa immediately after dilution in one of the cryodiluents as revealed by TEM studies. The electron microscopic studies on the spermatozoa of common carp revealed damages during freezing process like vacuolization of nucleus, loosening of the chromatin and morphological damages (Fig. 2) like winding of flagella, loss of flagella, and appearance of verrucosities. Yao *et al.* (2000) studied the ultrastructural changes in spermatozoa of ocean pout (*Macrozoarces americanus*) following cryopreservation and the observed damages inluded, severe swelling of mitochondria and dehydration of cytoplasm at the mid-piece as revealed by SEM.

Cryopreservation Of Fish Embryos And Embryonic Stem Cells

Though cryopreservation of fish sperm has been considerably studied using a number of teleosts as models (Linhart *et al.* 2000), successful cryopreservation of fish eggs and embryos still remains elusive. Several attempts to cryopreserve unfertilised eggs of fish were not successful (Horton and Ott, 1976) due to dehydration problems, because of relatiavely large size of eggs and different water permeability of membranes (Loeffler and Lovtrup, 2000). It is stated that storage of zebrafish embryos using propylene glycol in the liquid nitrogen even or 1 min resulted in damage of mitochondira, disorganisation of ribosomes and plasmamembrane of the yolk syncytial layer (Anchordoguy *et al.* 1987). There was 100% mortaility of some teleost *Cyprinus carpio, Labeo rohia* and *Brachydanio rario*) embryos when stored at liquid nitrogen temperature even for short duration of up to 3 h (Harvey *et al.* 1983). On the other hand, Zhang *et al.* (1989) reported successful cryopreservation of common carp embyros. Nevertheless, these results have not been duplicated. Whittingham and Rosenthal (1978) showed that herring embryos did not survive after cooling below –10ºC when protected with dimethyl sulfoxide (DMSO; Me_2SO).

Considerable studies have been made for the development of suitable cryoprotectant and optimum equilibration time for successful low temperature storage of fish embryos. Zhang *et al.* (1993) found that methanol was more effective cryoprotective agent than either DMSO or ethanediol for zebrafish embryo. Methanol was reported to penetrate the entire embyro within 15 min and other cryoprotectants exhibited little or no permeation into yolk over 2.5 h (Hagedorn *et al.* 1997). In zebrafish embryo, the permeability of the methanol appeared to decrease during embryo development at 22ºC (Zhang and Rawson (1998). Use of ultrasound was reported to enhance the embryo permeability in zebrafish (Bart, 2000). However, methanol was demonstrated to show a limited degree of penetration into prim-6 stage of zebrafish embryos, but it did not penetrate in the later stage embryos (Liu *et al.* 2000).

However, DMSO was also reported to be a good cryoprotectant for medaka (*Oryzias latipes*) embryos (Arii *et al.* 1987). In common carp, the morulae were partially protected against chilling in DMSO and sucrose, half-epiboly in DMSO sucrose and methanol and heart beat stage in methanol and glycerol (Dinnyes *et al.* 1998). Using isotope labelled DMSO and glycerol Harvey *et al.* (1983) found that these solutes permeated into both dechorionated and intact 5 h zebrafish embryos.

Ahammad *et al.* (2003), while working on hatching of common carp, *Cyprinus carpio* embryos stored at 4ºC amd

–2ºC in different concentrations of methanol and sucrose, found that hatching performance wast maximum (41%) in sucrose at 4ºC. No survival was observed at –2ºC with any concentration of sucrose. Further, it was reported that the combination methanol and sucrose produced best results among all concentrtions tested at both temperatures. Same workers (Ahammad *et al.* 2003, *et al.* 2004), while studying hatching responses of rohu embryos at different concentrations of cryoprotectants and temperatures reported that hatching performance of embryos stored at –4ºC temperature in combination of methanol and trehalose showed the highest percentage of hatch out (72%). Cryopreservation of pluripotent blastomeres and grafting of thawed cells into recipient embryos to produce chimeras, open an alternate pathway for production of improved strains of fish and shellfish. Relatively, attempts to cryopreserve inverteberates, especially crustacean larvae, have been more successful than the finfish. In penaeid shrimp, successful survival of thawed larvae has been reported upto freezing temperature of –10ºC (Diwan and Kandasami, 1997).

Research to develop cell lines, embryonic stem cells and germ cells, from Indian fishes, and to develop technology for cloning has been emphasized in the past (Pandian, 2002). There have been some successful studies in developing cell cultures such as ovarian tissue from immature ovary of *Clarius gariepinus* (Kumar *et al.* 2001). Pluripotent cell line from sea bream embryonic stem-like cells (SBESI) has been reported from blastula-stage embryos of the cultured red sea bream, *Chrysophrys major.* In future, development of expertise for other tools, like embryonic stem cells preservation and cloning, need active consideration, to overcome the challenge of long-term storage of finfish eggs and embryos. Embryonic Stem (ES) cells can differentiate to become any tissue in the body (Hong *et al.* 2000). Successful protocols for grafting of embryonic cells to host embryos, for germline transmission of desired genome, can be instrumental in evolving effective programmes for production of transgenics and rehabilitation of endangered species. The tolerance towards cryopreservation procedures and obtaining viable cells after freeze-thaw has been studied in a few species. The grafting of blastomere transplantation to produce chimera has been successful in goldfish, trout, zebra fish and medaka. Expression of primordial germ cells of donor into host system, with successful development of live trout fry has been demonstrated at experimental level. More research in this area may provide simple assays to target germ cells, facilitating pure line *in vitro* culture of primordian germ cells for grafting into host embryos.

CONCLUSION

The science of cryopreservation is quite new to the aquaculture industry. It commenced with an attempt to cryopreserve sperm mainly with the concept of "gene banks". Methods to cryopreserve gametes of aquatic animals are less developed. Although sperm cryopreservation has been done successfully in a number of commercially important aquatic species, particularly teleost fishes and shellfishes, the technology has not yet reached an advanced level suitable for commercial application. Unlike those involving spermatozoa, attempts to cryopreserve fish eggs and embryos have been unsuccessful.

The limited success achieved in the cryopreservation of viable eggs, embryos and larvae of higher animals was attributed to the large size of the eggs and embryos that interferes in the penetration of cryoprotectants and uniform cooling during the cryopreservation process. Sometimes, the large volume of yolk present in the eggs and embryos tends to develop crystals while freezing and if damages the internal parts. In shrimp, though the size of the eggs and embryos is small, the eggs have the tendency to absorb water soon after their release, swell and become activated for fertilisation. After fertilisation, a strong hatching envelop forms around each egg. Therefore, the presence of water and the thick protective envelope surrounding the eggs are some of the disadvantages for successful freezing of viable eggs and embryos. Further research to circumvent these aspects is the need of the hour in paving the way for establishment of Fish Gene Bank.

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