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EFFECT OF CRYOPROTECTANTS ON THE MORPHOLOGY OF FISH SPERMATOOZOA

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JULY 2002

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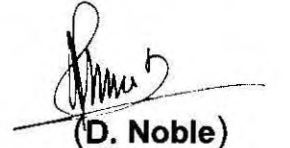
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CERTIFICATE

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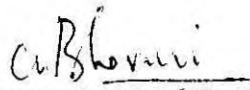
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DECLARATION

I hereby declare that the thesis entitled "EFFECT OF CRYOPROTECTANTS ON THE MORPHOLOGY OF FISH SPERMATOZOA" is an authentic record of the work done by me and that no part thereof has been presented for the award of any degree, diploma, associateship, fellowship or any other similar title.

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सारांश

उच्च संवर्धन शक्यता रहने वाली खारा जल/समुद्री मछली लिज़ा पारिसा के शुक्राणुओं के शीतपरिरक्षण (क्रयोप्रिसरवेशन) के लिए बेहतर जियोप्रोटक्टन्ट को पहचान करने के लिए एक अध्ययन चलाया. विभिन्न शीतपरिरक्षकों (क्रयोप्रोटक्टन्टों) को अकेले और विभिन्न सघनता में मिश्रित रूप में प्रयोग करके परीक्षण चलाया. शीत परिरक्षण के पहले और बाद में शुक्राणुओं के आकृति का अध्ययन ट्रान्समिशन इलेक्ट्रॉन माइक्रोस्कोप के ज़रिए किया. V₂E आयतनवर्धी में 5% डी एम एस ओ और 5% ग्लिसरोल का संयोग, मात्र 10% डी एम एस ओ की अपेक्षा उच्च सुरक्षा प्रदान की. ग्लिसरोल साथ जोड़ने पर डी एम एस ओ की उच्च सघनता से होनेवाला विषैला प्रभाव कम किया जा सकता है.

ABSTRACT

A study was carried out to identify the best cryoprotectant for the cryopreservation of spermatozoa of *Liza parsia*, a brackish / marine fish of great culture potential. Various cryoprotectants alone and in combinations, at different concentrations have been tried. Morphology of spermatozoa before and after cryopreservation studied by transmission electron microscopy (TEM). A combination of 5% DMSO and 5% glycerol in V2E extender accorded better protection than 10% DMSO alone. The toxic effects of high concentrations of DMSO can be minimised by combining with glycerol.

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INTRODUCTION

1. INTRODUCTION

Mariculture sector not only caters to the increasing demands of seafood products, but also leads to sociological, economic and environmental changes in many rural and coastal areas of the world. Enormous opportunities for continued development of mariculture still exist. The key issues required to promote the farming of marine species, as a modern food production industry need to be addressed. Technologies for sustainable mariculture, for example, restoration of over exploited fish stocks requires restocking with cultured juveniles have to be developed. For continuous fish seed production, one of the major constraints is the nonavailability of sufficient spawners at the desired time. In order to solve this problem, efforts are being continuously made world over to evolve suitable methodologies to preserve gametes so that seeds can be made available at any time of the year.

For more than a century, scientists have investigated methods to preserve viable gametes. Early studies were directed to prolong the life of the gametes in the non-frozen state. With advances in the field of cryobiology, the focus has shifted to develop cryoprotective techniques for frozen storage of spermatozoa. Early attempts to cryopreserve fish sperm were patterned after those proven successful with fowl and bull sperm. The first success in preserving the fish sperm at low temperature was reported by Blaxter (1953), who preserved the milt of Atlantic herring *Clupea harengus* for six months at -79°C and obtained 80% fertility. Freezing at any temperature below -130°C will generally maintain the viability of the cells for a longer period. For long term storage, liquid nitrogen (-196°C) is the most suitable medium. Most of the work in literature relates to salmonid fishes and to those from fresh waters; studies on marine fishes are comparatively few.

Cryoinjuries during cryopreservation procedure are often bound to occur. Morphological alteration in the spermatozoa of fish following dilution and freezing has been reported. The success of cryopreservation depends on minimizing the extent of cryoinjuries during freezing and thawing. Cryoinjuries can be reduced in the presence of some protective chemicals called cryoprotectants in a diluting medium called extender.

The use of diluents for the storage of fish spermatozoa provides better control of the physico-chemical conditions during storage than would be possible in undiluted milt. Glycerol was first reported as effective in protecting sperm from freeze-thaw damage. Later on dimethyl sulphoxide (DMSO) was observed to be highly successful in protecting spermatozoa of some species. Methanol and ethylene glycol/propylene glycol also accorded varying degrees of protection. Diluent is a balanced salt solution (extender) containing the correct proportion of protective agent (cryoprotectant) is the basic requirement for cryopreservation of spermatozoa of fish.

The most commonly used technique for cryopreservation comprises of the collection of milt, dilution with a balanced salt solution containing either 7-10% DMSO, glycerol or methanol filled in straws, and stored in liquid nitrogen (LN) at -196°C . The frozen milt is thawed in $20-40^{\circ}\text{C}$ water bath prior to fertilization. The feasibility of cryopreservation of spermatozoa demonstrated in over hundred fish species, notably salmonids, carps, and tilapias. Spermatozoa of many marine fishes have recently been cryopreserved successfully. Compared with fresh water species, high percentage of marine spermatozoa, survives cryopreservation and the process is relatively simple. Hence, the cryopreservation of marine fish sperm is suited for application in mariculture in a large scale.

In the process of cryopreservation, pre-treatment of biological material in cryoprotectants is very important in preventing cell damage during freezing and thawing. Cryoprotectants help in preventing the crystallization of water within the even at very low temperatures and thereby indirectly protect the cell membranes. The cryopreserved samples are thawed to the room

temperature before use. The rates of cooling and thawing are highly critical and can cause damage to the cells if not properly controlled. The optimum rates of cooling and thawing differ from one cell type to another.

Post-thaw motility rate and fertilizing capacity of fish cryopreserved sperm depends greatly on the type of cryoprotectant, its concentration, the extender used and quality of sperms employed. The effectiveness of cryoprotectants in suppressing cryoinjuries varies with different concentration and along with different extenders. Toxicity of using high concentrations of cryoprotectants can be minimized by using combinations of compatible cryoprotectants. Synergistic effects may also happen when combinations of cryoprotectants are used.

Although the ultimate success of cryopreservation depends on the fertilizing ability of cryopreserved sperms, the assessment of efficiency of cryopreservation based on fertilization experiments may be undependable and impracticable. Ultrastructural study of the extent of injuries on milt suspension can be a suitable alternative to evaluate the effectiveness of cryopreservation.

It is very imperative to study the structural damage of the spermatozoa which occurs during cryopreservation process in order to arrive at most efficient diluent-cryoprotectant and freezing and thawing rates. The structural damage to spermatozoa will invariably reduce the fertility rate. The causes of such impairments can be arrived at by the study of the ultra structure of sperm by Transmission Electron Microscopy (TEM).

The candidate species used for the present study is Gold spot mullet *Liza parsia* (Hamilton-Buchnan, 1822) which is a brackish water fish. It has got immense culture potential and commands a high price in market.

The aim of the present study is to compare the effects of different cryoprotectants at different concentrations and combinations along with a suitable extender on preventing injuries during preservation; also to find out the optimal concentration of cryoprotectant/combination of cryoprotectants for the spermatozoa of fish *Liza parsia*.

REVIEW OF LITERATURE

2. Review of Literature

In recent years there have been growing demands for the establishment of gene banks of threatened wild species as well as culturable marine animals. As a result, the cryopreservation of fish semen has been the subject of many investigations (Scott and Baynes, 1980; Billard, 1988). Most of the work pertained to salmonid fishes and to those from fresh water; studies of marine fishes are comparatively few. Even for the ones reports are available some times a variety of protocols are advocated. Since the parameters of cryopreservation such as milt quality, freezing, storage conditions, packing, thawing, fertility protocol evaluation vary considerably between and within studies, evaluation and development of a standardised protocol for a particular species assumes significance.

2.1. Assessment of Sperm Fitness and Milt Quality

2.1.1. Motility

Billard and Cosson (1992) had improved earlier techniques used to assess fish sperm motility. The visual spermatozoan motility is said to be a good indicator of sperm viability. The sperm concentration in milt is high and hence the assessment of sperm motility requires a high dilution rate (Billard and Cosson, 1992). Sperm motility is an important criterion for sperm quality and is usually expressed as percentage. A 0-5 scale motility score can be given to assess the sperm motility (Billard, 1984). Duration of motility of sperm after activation is also taken into account to assess sperm fitness (Lahnsteiner *et al.*, 1996). The percentages of motile cells significantly correlate with the fertilization of seabass and turbot spermatozoa (Suquet *et al.*, 2000). A relation between motility rate and fertilisation capacity of frozen thawed semen was determined by Lahnsteiner *et al.* (1995a). Apart from visual motility analysis another alternate method for determination of sperm viability are computer assisted cell motility analysis (Lahnsteiner *et al.*, 1996a) and analysis of flagellar beat frequency (Cosson *et al.*, 1997).

A computer assisted sperm analysis (CASA) system allows an analysis of video tapes using software, the set-up parameters of which are adjusted for the studied species sperm characteristics (Lahnsteiner *et al.*, 1996a). CASA provides a simple but rapid quantitative assessment of the quality of fish sperm and may predict its ability to fertilize eggs. In combination with CASA, morphological measurements can be used to determine the causes of reduced sperm motility (Kime *et al.*, 2001). Using stroboscopic illumination, the flagellar beat frequency can be measured with reference to the calibrated frequency of the flash illuminator (Suquet *et al.*, 2000).

2.1.2. Biochemical and Biometrical Analysis

The study of biochemical and biometrical analysis of semen is necessary to assess the quality of semen before it is used for gene banking (Chutia *et al.*, 1998). The motility and fertility of spermatozoa are subjected to wide fluctuations due to various physio-chemical and biological parameters such as, ionic concentration of seminal plasma, pH, salinity, temperature of the medium and spermatozoan concentration. The effect of pH on motility was reported in many fishes. Baynes *et al.* (1981) and Billard and Cosson (1998) showed that in the at pH 7.5 trout spermatozoan motility is totally inhibited and added calcium and magnesium ions can overcome this problem. Chao *et al.* (1992) observed high motility at pH 7.5 and 8.0 than at other levels in grouper (*Ephinephelus malabaricus*), and the motile period of spermatozoa in seawater was prolonged for more than 60 minutes and 40% to 60% spermatozoa were motile.

The duration of spermatozoan motility also depends on temperature of the medium. Prolonged duration of spermatozoan motility was obtained in most fishes when the spermatozoa were brought to below room temperature (Paul and Jayaprakas, 1996).

The physical and chemical composition of the semen is highly species specific. This can also be well correlated with the spawning behaviour of the fish (Chutia *et al.*, 1998). The percentage of motile spermatozoa also depends on

osmotic pressure and nature of the ions (Perchec *et al.*, 1995). The concentration of K^+ and Na^+ in the seminal plasma also varies from species to species. A much higher concentration of Na^+ and K^+ (72.5 and 49.5mM) was observed than Mg^{++} (0.41mM) in *Cyprinus carpio* milt (Chutia *et al.*, 1998). Some energy yielding substances such as glucose and fructose are found in the seminal plasma and sperm but in small amounts, which are generally 10 times lower than in mammals (Ford and Rees, 1990). The measurement of intracellular ATP content at various times after activation and the assessment of oxygen consumption were carried out to study the degree of cell integrity before and after cryopreservation (Dreanno *et al.*, 1997). The oxaloacetic transaminase activity of the spermatozoa was also considered as a quality test (Malejac *et al.*, 1990).

Sperm quality has often been estimated by the concentration of spermatozoa and their motility observed after activation of the initially motionless spermatozoa. Milt concentration has been assessed by three techniques: Counting in a haemocytometer chamber (Buyukhatipoglu and Holtz, 1984), spermatocrit (Bouck and Jacobson, 1976; Munkittrick and Moccia, 1987) and spectrophotometric evaluation (Billard *et al.*, 1971). As in rainbow trout (Billard *et al.*, 1971) a Spectrophotometric evaluation allows an accurate estimation of milt concentration. Suquet *et al.* (1992) counted the sperms of turbot *Scophthalmus maximus* in a malassezs cell. The technique was an accurate but time consuming one. Trypan blue exclusion tests and application of supravital fluochromes to discriminate live and dead sperms in both diluted and undiluted milt suspensions have been developed for 'acrosomal' sperms of higher vertebrates. Lack of acrosome (anacrosomal) and the small size make teleosts sperms unsuitable for above test (Billard, 1983). Nigrosine-Eosin dye exclusion method was used for evaluating the histochemical viability of spermatozoa of grey mullet, *Mugil cephalus* (Chao *et al.*, 1975). Fluorometry provided a rapid method of detection of the percentage of dead sperm in each treatment, even when samples were autolytic in *Oncorhynchus mykiss* (Mc Niven *et al.*, 1993). Spermatozoal membrane integrity of cryopreserved semen can be assessed fluorometrically using ethidium bromide as a stain that binds to double stranded, intact DNA and fluoresces (Mc Niven *et al.*, 1992). The ratio of two fluorescent readings, (damaged/total spermatozoa) signifies the percentage of damaged spermatozoa.

2.2. Milt Extender

Undiluted milt, with or without a cryoprotectant added directly to it, is not suitable for freezing. It appears that semen must be diluted with a suitable extender (Scott and Baynes, 1980; Gopalakrishanan *et al.*, 1999). An extender has been defined as a solution of salts, sometimes including organic compounds, which helps maintain the viability of cells during preservation. One of the functions of salts or organic compounds is to inhibit osmotically the activation of ova and motility of sperm besides providing isotonicity. Although, a range of chemicals are used in extender formulation, it should be noted that the simplest formulations have generally been the most successful ones (Scott and Baynes, 1980). The use of diluents for the storage of fish spermatozoa provides better control of the physio-chemical conditions than would be possible in undiluted milt (Stoss, 1983).

The diluents in which sperms remain immotile would always be preferable for cryopreservation (Jameison, 1991; Gopalakrishanan *et al.*, 2000). Mounib's diluent was used successfully for spermatozoa of fresh water fish (Mounib, 1978) but was not appropriate in freezing Atlantic croaker, *Micropogonias undulatus* L spermatozoa (Gwo *et al.*, 1991). Sperms suspended in extender solution retained motility significantly longer than did sperm in intact testis.

V2E extender was found to be the best cryodiluent for *Liza parsia* after storage in liquid nitrogen with almost 60% of the sperms exhibiting near normal structure (Sandhya Sukumaran, 2001). Organic materials, including bovine serum albumin, lecithin, and protein and egg yolk are thought to protect spermatozoa from cooling damage, and are widely used for deep-freezing salmonid spermatozoa (Horton and Ott, 1976; Stoss and Holtz, 1983; Baynes and Scott, 1987). The cryodiluent consisting fish ringer with 5% DMSO as cryoprotectant was successfully used to cryopreserve the milt of *Liza macrolepis* (Santiago *et al.*, 1996).

Stoss and Donaldson (1982) emphasized that extender composition was not very important given the brief exposure of spermatozoa to extender before freezing. 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 the loss of capacity to fertilise (Jamieson, 1991). *Mugil cephalus* milt diluted (1:1) and stored at 5°C in Marine teleosts Ringers showed motile spermatozoa following activation after 23 days. (Chao *et al.*, 1975). Babiak *et al.* (1998) observed that the presence of egg yolk in extenders decreased fertilization success of the cryopreserved milt of a cyprinid, *Aspius aspius*. Single agents, such as 0.5% honey or 0.5% glucose in the Ringers solution, were found to be effective as a diluent for milkfish and black porgy sperm (Chao and liao, 1987) The extenders used for cryopreservation of spermatozoa of various cyprinid and salmonid fishes were found to be unsuitable for *Tenualosa ilisha* as they activated the sperm on dilution (Kuldeep Lal *et al.*, 1999). Ohta and Izawa (1996) found that addition of sucrose/glucose to an extender system may improve the preservability of the milt. The motility and fertilizing ability of sperm in salt solutions remains longer than in freshwater probably because they are not killed by osmotic shock (Scott and Baynes, 1980). As motility depends on internally stored ATP, which can be resynthesized only at very low rates; the extender must inhibit sperm motility before freezing (Suquet *et al.*, 2000).

Dilution rates of sperm in extender range from 1:1 to 1:20(volume of semen: volume of diluent) (Suquet *et al.*, 2000). Chao *et al.* (1975) found that mullet spermatozoan motility was reduced at dilution ratios above 20, and Rana and Mc Andrew (1989) showed that dilution of tilapia semen at ratios between 2 and 20 did not affect fertilization capacity. However, Legendre and Billard (1980) reported better post-thaw fertility at a dilution ratio of 3 compared to 1 or 9 in rainbow trout spermatozoa. Lower survival of frozen-thawed spermatozoa was recorded for dilution ratios greater than 1:20 in Atlantic croaker (Gwo *et al.*, 1991) and greater than 1:50 in sea bream (Chambeyron and Zohar, 1990). Increasing dilution rate from 1:1 to 1:9 did not modify the percentage of motile frozen thawed turbot spermatozoa (Dreanno *et al.*, 1997). It is suggested that seminal plasma proteins protect sperm viability, and dilution ratios higher than 1:10 may reduce this effect.

Various extenders and cryoprotectants have been reported to be species specific for freezing fish spermatozoa (Stoss and Refstie, 1983). Erdahl *et al.* (1984) prepared an artificial seminal plasma (ASP) extender and

reported a mean fertility of 54% for brown trout (*Salmo trutta*). Due to the complexity of the interaction of these compounds with spermatozoa, and with freezing and thawing technique, their precise role in protecting spermatozoa is still far from being completely known (Gwo *et al.*, 1991).

2.3. Cryoprotectants

The multiple roles of cryoprotectants during the cooling process were reviewed by Jamieson (1991). Cryoprotectants bind to water molecules and reduce pure ice crystal formations and also help to stabilize hydrated protein molecules. They also lower the freezing point of the intracellular fluids to -45°C (Lebio *et al.*, 1978). However to be effective, a cryoprotectant must permeate cell, otherwise it would not prevent a rise in intracellular electrolytes during freezing (Scott and Baynes, 1980). There are two types of cryoprotectants: permeating and non-permeating. Glycerol, methanol, DMSO and ethylene glycol can permeate into the cell. Glycerol and Dimethyl sulphoxide bind electrolytes there by preventing these substances from concentrating in the residual unfrozen solution in and around the cell during freezing (Scott and Baynes, 1980).

The non-permeating cryoprotectant like polyvinylpyrrolidone, glucose, sucrose, egg yolk, serum and skimmed milk form a coating externally around the cell and prevents ice formation in its vicinity. The non-permeating cryoprotectants in conjugation with a permeating cryoprotectant, helps to depress the freezing point and prevents ice crystal formation. The adjuvant like egg yolk provides additional strength to membrane stability (Padhi and Mandal, 2000). In case of *Liza parsia* sperm motility could be preserved successfully for more than 240 days using a suitable cryoprotectant (Diwan and Nandakumar, 1998). Cryoprotectant agents are needed at the ultra low temperature of preservation in liquid nitrogen. Penetrating, internal cryoprotectants are essential for the cryopreservation of fish semen (Maisse, 1996). Non-permeating cryoprotectants (mainly sugars) are often used in the freezing medium too. Although large selections of cryoadditives are available, glycerol, DMSO, and methanol are most widely used for fish gametes preservation studies (Mc Andrew *et al.*, 1993)

The choice of cryoprotectant and its concentration are of much importance. For the commonly used cryoprotectants, final concentrations of between 7 and 15% have been successfully used. The optimum concentration may, however, vary between cryoprotectants, species and equilibration time used. For *Oreochromis niloticus* 10% methanol was demonstrated to be optimal (Rana and Mc Andrew, 1989) whereas 5% methanol was optimal for *O. mossambicus* (Harvey, 1983). Recent studies have shown that dimethylacetamide (10%) can confer good cryopreservation to rainbow trout spermatozoa (Gallant and McNiven, 1991). Dimethyl sulphoxide (DMSO) provided best protection of salmonidae spermatozoa from damage by freezing (Horton and Ott, 1976; Gopalakrishnan *et al.*, 1999). Withler (1982) used dimethyl sulphoxide at concentrations ranging from 5% to 20% to cryopreserved spermatozoa of shark fin catfish. Because of rapid penetration into cells and relatively low and controllable toxicity at low temperature, DMSO has been recommended as a preferred choice of cryoprotectant (Leung and Jamieson, 1991).

DMSO is rated as better cryoprotectant for most cells than glycerol; probably because it enters and leaves the cell much faster than glycerol. However, DMSO is toxic at high concentration. Their toxicity may cause gamete mortality in pre-treatment and post-thawing. It is necessary to determine the point of equilibrium between cryoprotective efficiency of cryoprotectants and the toxicity of the cell types to be cryopreserved (Chao *et al.*, 2001). The motility duration of frozen thawed barramundi *Lates calcarifer* (Bloch) spermatozoa was reduced when DMSO concentrations was higher than 5% (Leung, 1987) and in the black grouper, sperm motility was also decreased at a concentration of 30% (Gwo, 1993). Studies with *Oreochromis niloticus* spermatozoa suggest that DMSO is more toxic than methanol. (Rana and Mc Andrew, 1989). The mechanism of cryoprotection of fish sperm by DMSO lies in its fast penetration into the sperm and increase of osmotic pressure of the sperm (Zhang *et al.*, 1994). Studies indicate that a gradual addition of the cryoprotectant can minimize osmotic stress and thus increase sperm survival (Stoss and Holtz, 1983).

Glycerol as a cryoprotectant was suited in fishes like *Mugil cephalus*, *Gadus morhua* and *siluris glanis* (European catfish) but it was toxic to salmonids and

to grouper *Epinephelus tauvina* where DMSO could be used (Chao *et al.*, 1975; Withler and Lim, 1982). In tilapia spermatozoa the optimum concentration of glycerol was found to be between 10-20%, while maximum post-thaw motility of spermatozoa was observed in 20% concentration (Rexaline Sumathi and Sukumaran, 1998). Methanol has low (Barramundi, Leung 1987) or no (Atlantic croaker, Gwo *et al.*, 1991; Black Grouper, Gwo, 1993) cryoprotective efficiency in frozen spermatozoa of marine fish.

Ethylene glycol and propylene glycol gave intermediate results in Atlantic croaker (Gwo *et al.*, 1991). Glycerol gave good protection in yellow fin sea bream (Gwo, 1994), but it provided no protection in black grouper (Gwo, 1993). Ethylene glycol is rarely used in fish sperm cryopreservation protocols and never for freezing African catfish sperm (Horvath and Urbanyi, 2000). It had a damaging effect on the spermatozoa after 2 minutes of equilibrium in African catfish. This corresponds with results reported for European catfish by Linhart *et al.* (1993) who also found ethylene glycol highly toxic before freezing. DMSO 15% and ethylene 15% (Marine and Krasznai, 1987) have been successfully used for European cat fish *Silurus glanis*.

DMSO at a concentration of 5% with either 15% milk powder or 20% egg yolk gave optimal results in cryopreservation of *Lates calcarifer* (Post-thaw motility of spermatozoa: 70-100% for 7 min) compared with glycerol and very inferior results for methanol (qv) (Leung, 1987). DMSO was found inferior to methanol with skim milk in cryopreservation of spermatozoa of Tilapia, *Sarotherodon mossambicus* (Harvey, 1983). Tilapia (*Oreochromis. sp*) spermatozoan protected with 12.5% methanol in fish ringers remained viable for at least 13 months (Rana and Mc Andrew, 1989).

Methanol and DMSO have proven most satisfactory as Cryoprotective agents, often yielding post thaw motility value of greater than 40% in many species (Tiersch, 1995). 40% post thaw motility was achieved using a cryodiluent of 5% glucose as and extender and 5% glycerol as a cryoprotectant in African catfish sperm cryopreservation (Urbanyi *et al.*, 1999). In the cryopreservation of Sea bream (*Sparus auratus*) spermatozoa, five cryoprotectants, dimethyl sulphoxide, ethylene

glycol, 1-2- propylene glycol, glycerol and methanol were tested at concentrations between 5 and 15% by volume to evaluate their effect on the motility of semen exposed for upto 30 minutes at 26^o C. On thawing, the best motility was observed with 5% dimethyl sulphoxide although both 10% propylene glycol and ethylene glycol showed good results (Fabbrocini *et al.*, 2000).

(Tiersch *et al.*, 1994) successfully used 5% methanol and fish ringer solution to cryopreserve channel catfish spermatozoa. The post thawed spermatozoa of two Asian fresh water catfishes *Heteropneustes fossilis* and *Clarius batrachus* cryopreserved using 10% glycerol as the cryoprotectant yielded upto 87% fertilization with respect to control (Padhi and Mandal, 1995). Piironen and Hyvarinen (1983) recommended glycerol as one suitable cryoprotectant for white fish, a salmonid even though glycerol has been reported to be toxic to salmon and trout spermatozoa (Scott and Baynes, 1980). Cloud *et al.* (1990) obtained 23% hatchability in the spermatozoa of rainbow trout frozen in liquid nitrogen for 11 months using 6.25% DMSO. Rurangwa *et al.* (2001) has recently described a method in which CASA can be used to improve the cryopreservation of fish sperm. A range of cryoprotectants which has least harmful effect on sperm is assessed simply by testing motility after storage of sperm in protectant at 4^oC for 24 hrs. The most promising protectant is then modified in composition and retested.

2.4. Cryopreservation Protocol

The protocols for cryopreservation of spermatozoa of marine fish species vary considerably between and within studies and have led to confusing results. A variety of protocols even for the same species are advocated. The components of cryopreservation protocols viz., equilibrium time, freezing, storage and thawing need to be standardized for a particular species to achieve success.

2.4.1. Semen equilibration

After dilution in the extender, the semen is immediately frozen or equilibrated before loading the straws. Only a few studies have indicated that

equilibration improves the post thaw quality of spermatozoa (Baynes and Scott, 1987). Because of small cell size and cryoprotectant toxicity, the immediate freezing of semen without equilibration is recommended (Legendre and Billard, 1980; Babiak *et al.*, 1995; Piironen and Hyvarinum, 1983; Stoss and Holtz, 1983b). However, equilibration time is usually recommended for slow penetrating cryoprotectants like glycerol.

Fish spermatozoa usually become motile upon mixing with diluents containing the cryoprotectant. Equilibration time should be kept to a minimum (few minutes to less than one hour) to avoid exhaustion. (Billard, 1978; Chao *et al.*, 1975). With the exception of glycerol, the permeability of most cryoprotectants is not markedly reduced by low temperatures and, therefore, equilibration is usually performed at 0°C to reduce cryoprotectant toxicity (Leung and Jamieson, 1991). Prolonged equilibration is clearly detrimental in tilapia spermatozoa and allowing no equilibration resulted in best fertility (Harvey, 1983). The best result was obtained when 5 min equilibration was used together with 20% DMSO in Atlantic croaker. (Gwo *et al.*, 1991). The effect of equilibration time seemed to play a major role in the success of the freezing protocol when using DMSO as cryoprotectant in cryopreservation of African catfish *clarius gariepinus* (Horvath and Urbanyi, 2000).

2.4.2. Freezing and Thawing

Optimal cooling rate is -30°C per minute (Harvey, 1982). The rate of cooling is a critical variable during cryopreservation (Scott and Baynes, 1980). During freezing, intracellular water should be allowed to come out of the cell. Rapid cooling fails to provide sufficient time for the cells to dehydrate and avoid the detrimental effects associated with intra-cellular ice crystal formation. In contrast, slow cooling results in excessive dehydration and reduction of cellular volume to the extent that is not reversible. (Padhi and Mandal, 2000). Chao (1982) reported that freezing rates between -21°C and -119°C per minute were favourable for grey mullet spermatozoa.

Commonly used freezing vessels for fish sperm are vials, pellets and straws. However, use of straws is superior to pellets because recrystallisation occurs when pellets are thawed (Erdahl *et al.*, 1984). French straw method was designed to speed up the collection, freezing and distribution of sperm (Scott and Baynes, 1980). Straws with a volume of 0.5ml and 1.2ml were suited to cryopreservation of semen of salmonidae (Lahnsteiner *et al.*, 1997). Freezing protocol varies with species. For grey mullet, black porgy and grouper, two steps freezing by exposing straws with sperm-cryoprotectant mixture to liquid nitrogen vapour at -100°C , and then quickly quenching to -196°C is optimal (Chao and Liao, 1987; Chao, 1991).

The most critical step is the selection of the optimum depth for freezing positions in a container. This may be because the temperature in the container varies at different freezing positions. The freezing rate is also possibly dependent on the size and the material of the freezing tube and holder. Thus, it is highly important for assuring the reproducibility to use a fixed type of freezing tube and a holder in a series of experiments (Aokil *et al.*, 1997). Storage temperature is usually -196°C (liquid nitrogen) and maintenance of the storage temperature is very essential.

Generally, thawing rates should be high to avoid recrystallization. Straws are thawed in warm water (Baynes and Scott, 1987) and pellets directly in the fertilization solution (Holtz, 1993). Thawing was the most sensitive parameter during cryopreservation and slight deviations from the optimal conditions, significantly reduced the fertilization success in semen of salmonidae and northern pike (Lahnsteiner *et al.*, 1995, 1996a, 1998a). The delay between thawing of semen and its activation may decrease sperm quality. Thawed spermatozoa must be used rapidly. Sixty minutes after thawing, the percentage of motile turbot spermatozoa stored on crushed ice decreased by 35 % (Dreanno *et al.*, 1997).

2.5. Morphological Alternations in Spermatozoa

It is well known that damage occurs in spermatozoa during cryopreservation. Motility of frozen thawed semen is lowered due to morphological

alternations and the number of spermatozoa necessary for fertilization must be increased (Lahnstneiner, 1996). Cryoinjuries have been reported for thawed spermatozoa of many fresh water fish species (Rana, 1995a). In marine fish, the fine structure of the head region of 90% of the thawed black grouper spermatozoa was similar to untreated ones (Gwo, 1993). Damages or disfunctioning have been observed in thawed spermatozoa at the level of the plasma or the mitochondrial membrane, suggesting alternations in the energetics or ionic metabolism (Ogier de Baulny *et al.*, 1996).

Cell death during the process of freezing and thawing may be the primary factor that accounts for the differences between fresh and frozen spermatozoa fertility. The pH fluctuations caused by freezing or addition of cryoprotectants may result in temporary or permanent cryoinjuries (Chao *et al.*, 2001). Shrinkage of the plasma membrane of the mid piece was reported for frozen thawed spermatozoa of the ocean pout (Yao *et al.*, 1995). In frozen-thawed Atlantic croaker spermatozoa the cristae of mitochondria were disrupted, the plasma membrane was swollen or disrupted and the axoneme was coiled (Gwo and Arnold, 1992). In rainbow trout non-damaged cryopreserved spermatozoa presented the same characteristics of fresh spermatozoa, except for chromatin, which was extensively clumped. Spermatozoa that were damaged by cryopreservation exhibited a swelled and non-continuous membrane with a clumped chromatin, showing high electron density zones. The flagella of fresh spermatozoa showed a continuous and well organized membrane which surrounds the typical 9+2 structure of the axoneme, while the same flagella structure was observed in non-damaged cryopreserved spermatozoa. In spermatozoa damaged by cryopreservation, a break and a displacement of axoneme, along with changes in flagella membrane were observed. The shape of mitochondria was altered by cryopreservation (Conget *et al.*, 1996).

During freezing several physio-chemical changes take place within the cell and its surrounding area, leading to morphological alterations. The plasma membrane that surrounds the cell gets affected by cold shock. The plasma membrane consists of lipid protein bilayer and controls the transport of metabolites and ions. Lipid remains in a liquid state at normal temperature. At low temperature

lipid gets frozen which affects the permeability and structural integrity of the membrane. It is noted that the temperature range between 0 to -40°C is most critical during freezing since most of the cryoinjuries take place in the extra cellular solution and the dehydrated cytoplasm vitrify and further cellular damage does not take place (Rall *et al.*, 1984; Leung and Jameison, 1991).

In thawed spermatozoa of rainbow trout and brown trout after cryopreservation considerable ultra structural alternations was observed in chromatic structure (Billard, 1983). Within the spermatozoa, plasma membrane is probably the site most susceptible to damage due to water flux during freezing and thawing (Baynes and Scott, 1987). A low membrane cholesterol or phospholipids ratio was strongly correlated with better freezing resistance of the corresponding sperm (Labbe *et al.*, 1996). The morphological alternations observed in frozen thawed semen of rainbow trout following cryopreservation were mainly the swelling of the plasma lemma of the head, mid piece and the tail regions as well as of the mitochondria (Lahnsteiner, 1996a). Most of the damage occurred within 10 minutes of adding DMSO to the sperm suspension in carps (Lubzens *et al.*, 1997). Sperm damage caused by freezing and thawing is generally believed to be due to the formation of intra cellular ice crystals and the osmotic effects (Horton and Ott, 1976; Scott and Baynes, 1980).

Electron microscopy indicated obvious structural damage, especially in the trout spermatozoa (Gwo *et al.*, 1993). Knowledge of morphology of sperm has progressed considerably since the development of the technique of Electron Microscopy and these were exhaustively reviewed by Jamieson (1991). Electron micrographs of trout spermatozoa that had been cryopreserved showed particles grouped in rounded clusters on the protoplasmic surface of both head and tail. In some spermatozoa, folding of protoplasmic membrane, with the particle free sites were found (Drokin *et al.*, 1998).

2.6. Post-Thaw Evaluation

The motility of frozen thawed semen is a reliable parameter for quality determination since there is a statistically greater correlation with post-thaw fertilization rate (Lahnsteiner *et al.*, 1995a). Only an empirical quantification of actual fertilization is a reliable indicator of success of the cryopreservation procedure (Leung and Jameison, 1991). The duration of motility in post-thawed sperm markedly reduces, from half an hour in fresh sperm to 30 sec in frozen thawed sperm for the grouper (Withler and Lim, 1982). For salmonid sperm, a delay of 30 sec between thawing and addition of sperm to eggs reduced the fertilization rate from 72-56% (Stoss and Holtz, 1981). Artificial insemination is therefore performed immediately after thawing.

It is often proposed that marine species have a better ability to withstand the rigors of cryopreservation than most of the freshwater species (Scott and Baynes, 1980). The factors involved in cryoresistance are complex to identify. The electron microscopy has got great potential in identifying the injuries. Spermatozoa with a damaged nucleus would still retain fertilization ability, but the embryonic development would be impaired (Labbe *et al.*, 1998). In a series of fertility test in carps, the chemical constituency of the medium in which fertilization was attempted influenced the fertility of cryopreserved sperm (Kurokura *et al.*, 1984). Cryopreserved sperm showed motility percentages between 20 and 30% (Oteme *et al.*, 1996). A strong positive correlation was found between the post-thaw motility and fertility of frozen sperm securing high fertilization in common carp (Magyary *et al.*, 1996). In *Liza macrolepis* spermatozoa, which displayed a post-thaw motility of 30-40% were selected for inseminating the ova (Sulthana *et al.*, 1998). In studies in Muskellunge spermatozoa, although spermatozoa cryopreserved with extender containing 10% methanol had only traces of motility or no motility at all after thawing, fertilization trials indicated that their fertilizing ability was preserved (Ciereszko *et al.*, 1999). Other morphological criterion could be investigated such as the number of sperm mitochondria, or the flagella attachment to the sperm head however, attachment of the flagella to the nuclear fossa is similar in turbot

(Suquet *et al.*, 1993) and rainbow trout (Billard, 1983) although turbot retain a good motility after cryopreservation contrary to rainbow trout.

The ATP level of sperm also affects the fitness of spermatozoa before and after cryopreservation. ATP losses after cryopreservation can reach 70% of the initial level in rainbow trout measured using flow cytometry technique (Ogier de Baulny *et al.*, 1997b).

A low cholesterol/phospholipids ratio in rainbow trout sperm plasma membrane was significantly correlated to a best ability to fertilise after cryopreservation (Labbe *et al.*, 1996). A decrease in the activity of mitochondria and a loss of ATP during freeze-thawing could have dramatic consequences for sperm motility (Ogier de Baulny *et al.*, 1997a).

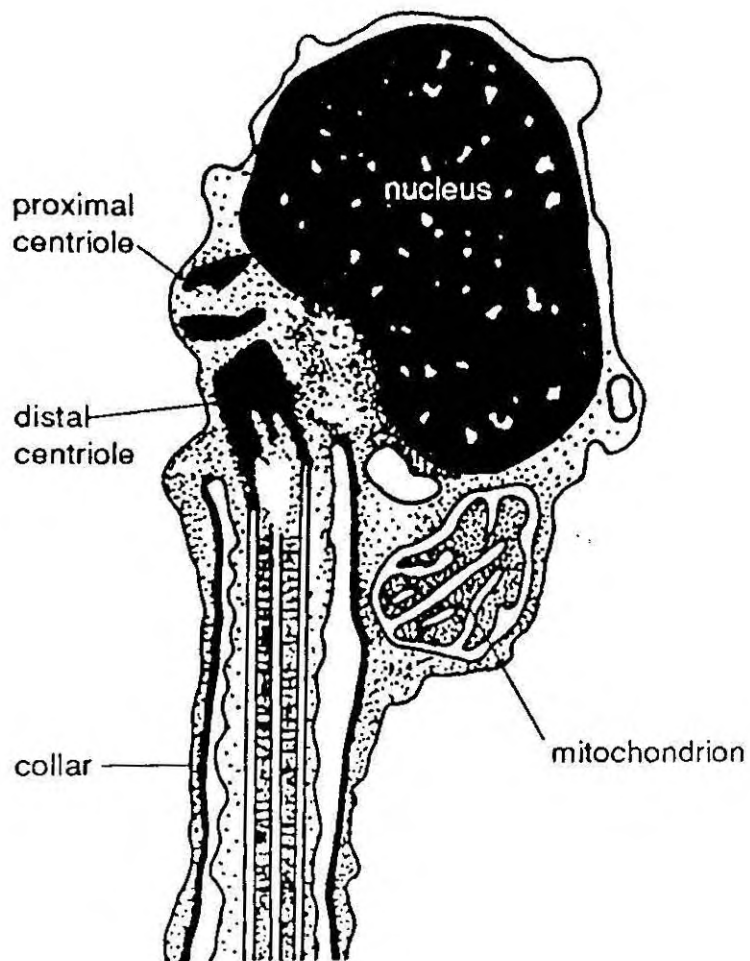


Figure 1. Diagrammatic representation of spermatozoa of *Liza Spp.* (Jameison, 1991)

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Specimen Collection and Maintenance

The Gold spot mullet *Liza parsia* was the candidate species selected for the cryopreservation studies. The fishes were collected from the wild with the help of Chinese dip nets located in Vypeen bar mouth. The male fishes of size (TL) 10-12cm were selected and transported to the laboratory in big plastic jerry cans with proper aeration and maintained in fibre glass tanks. They were maintained at salinity 20-22 ppt.

3.1.2. Collection of Milt

The specimens were given an intramuscular injection of Ovaprim (Syndel Laboratories, Canada, marketed by Agrivet Farm Care Division of Glaxo) at the rate of 0.3ml per Kg body weight and were stripped manually 8 hours after injection by applying gentle pressure on the abdomen. The milt obtained was collected in dry and sterile eppendorf tubes and kept over crushed ice. Care was taken to avoid contamination with blood, urine, scales and faeces.

3.2. Motility Assessment

The quality of milt collected was tested by taking one drop of milt from sample and mixed with seawater and observed under binocular microscope (10x x 10x). A convenient scale based on type of motility was adopted (Billard, 1984) and motility scores were given from 0-5. The method was standardised by repeating the process with at least four samples. The milt samples which exhibited rapid progressive movement (score 5) when mixed with seawater were selected for further studies. The maximum duration of motility obtained from *Liza parsia* sperms when induced with seawater was 5 minutes.

3.3. Selection of Extender

The undiluted sperms are not suitable for freezing and they must be diluted with a suitable extender-cryoprotectant mixture, which extends the viability of spermatozoa by rendering it immotile. The extender selected for the present study was V2E (Scott and Baynes, 1980), since in this extender sperms were not activated on dilution in *Liza parsia*. V2E is also found to be the best one for *Liza parsia* (Sandhya Sukumaran, 2001). Chemical Composition of Extender V2E (Scott and Baynes, 1980) used in this study is shown in Table 1. As seawater was the spawning medium for *Liza parsia*, extender was prepared with seawater as the base solution.

3.4. Cryoprotectants Used

The permeating cryoprotectants, dimethyl sulphoxide (DMSO), Glycerol, Methanol, Propylene glycol and their combinations were used in various concentrations. The final concentrations of cryoprotectants used are given in the Table 2. The milt is mixed with the cryodiluent in the ratio of 1:3 (milt: extender: cryoprotectant)

3.5. Experimental Protocol

- One part of semen was mixed with three parts of cryodiluent (Zero seconds after mixing with cryodiluent) and all solutions were kept at +20°C.
- Equilibration time (time required for the cryoprotectant to diffuse through the cell membrane) of 10 minutes over ice was given including time to fill the diluted milt into 0.5ml French straws. Filled straws are sealed with polyvinyl alcohol powder.
- Exposing to liquid nitrogen vapours (-120°C) for 5min.

- Plunging the straws into liquid nitrogen after vapour phase.

The duration of preservation is 3 days in liquid nitrogen. After step 1 and 4, samples were fixed for electron microscopic studies using gluteraldehyde as a fixative, following the method of Elizabeth (1987), Diwan and Nandakumar (1998) and Gopalakrishanan *et al.* (2000)

3.6. Percentage of Live Sperm

Nigrosine-eosin stain was used to assess the percentage of live and dead spermatozoa in semen sample (Chao *et al.*, 1975).

One drop of milt was mixed with two drops of Eosin (2%) and Nigrosine (10%) and a thin smear was prepared on clean, grease free glass slides. Two slides from each semen sample were prepared. The slides were air dried and observed under oil immersion microscope (1000X).

The dead sperms took pink stain while live spermatozoa appeared clear and transparent. The numbers of live sperms were counted and expressed in relation to total number of sperms. Randomly about 3 to 4 fields were counted for live sperms.

% of live sperms = (No. of live sperms ÷ Total number of sperms counted) x 100.

3.7. Post-Thaw Motility Assessment

The cryopreserved samples were thawed by rapidly plunging the straws into water bath at 37 °C for 20 sec. After thorough wiping, the sealed ends of straws were cut to expel thawed milt. Post-thaw motility of the spermatozoa was assessed using binocular microscope (10X x 10X) immediately after thawing. A small drop of cryopreserved milt was taken on the glass slide and mixed with seawater (salinity 35‰) and immediately observed under the microscope. The post-thaw motility was judged by two variables:

(a) Percentage of motile frozen spermatozoa was estimated on 5 point Scale.

(b) Duration of post-thaw motility.

Duration of sperm motility was taken approximately 5 sec after mixing the frozen milt with seawater which is the natural sperm activator, using a count up timer.

Table 1. Chemical composition of V2E (Scott and Baynes, 1980).

Chemical	Composition (in mg)
NaCl	750
KCl	38
NaHCO ₃	200
Glucose	100
Egg yolk	20
Seawater	100
pH	7.0

Table 2. Concentrations of different cryoprotectants used.

Sl. No.	Cryoprotectants	Concentration (in per cent)
1	DMSO	7.5, 10, 12.5
2	Glycerol	5, 10
3	Methanol	5, 10
4	Propylene glycol	8, 12
	Combinations:	
5	DMSO and Glycerol	5+5
6	Methanol and Glycerol	5+5
7	DMSO and Propylene glycol	5+5
8	Methanol and Propylene glycol	5+5

3.8. Processing For Electron Microscope

The samples were fixed in 3% gluteraldehyde buffered to pH 7.3. The samples after each treatment, ie. zero seconds after dilution and after plunging into liquid nitrogen were taken for ultrastructural studies. They were fixed for 2 hours at 4⁰C following immersion fixation. Then the supernatant was decanted and the pellets were washed with 0.1M sodium cacodylate buffer thrice for 15 minutes each. The samples were then centrifuged for 10 minutes at 8000 rpm. The pellets were post-fixed in 1% osmium tetroxide for 1 hour at 4⁰C. Then the samples were centrifuged at 5000 rpm for 10 minutes and decant the solution. The pellets are then washed thrice for 15 minutes each using 0.1M buffer. The pellets obtained were then transferred to 2% agar. The agar blocks were trimmed to 1mm³ size. The agar embedded samples were transferred stepwise through a concentration series of acetone (30%, 50%, 70%, 80%, 90%, 100% v/v) following standard time schedule.

Infiltration was carried out in standard Spurr medium with acetone in 3 steps at 4⁰C, each one hour.

- Spurr/ acetone ratio 1:3
- Spurr/ acetone ratio 1:1
- Spurr/ acetone ratio 3:1

Embedding was carried out in Spurr's medium in plastic mould and kept in incubator for 12 hours at 70⁰C, for polymerisation. The polymerised blocks were cut into ultra-thin sections in the LKB ultra tome Nova. Ultra thin sections were then stained with 2% uranyl acetate followed by 0.4% lead citrate and were mounted on grid and observed in Transmission Electron Microscope (TEM mode of Hitachi, H600). The record of normal and abnormal spermatozoa was made. A photograph as far as possible representing the overall changes of each sample was taken.

3.9. Post-Thaw Intact Spermatozoa

In all experiments, spermatozoa from each samples were examined utilizing TEM images. The ratios of intact and damaged spermatozoa for each treatment were calculated by counting sperms under low magnification (4000X in TEM). Randomly selected portions of at least three grids were taken for each treatment and observed. TEM images of untreated raw milt served as control.

RESULTS

4. RESULTS

4.1. Ultrastructure of untreated spermatozoa

The sperm of *Liza parsia* is a typical anacroosomal aqua sperm. The nucleus is bilobed or kidney shaped and is also tilted relative to axoneme. Chromatin is very coarsely granular and not condensed. Matrix spaces are clearly visible (Plate 2). A cytoplasmic collar, which extends around the base of the flagellum by a periaxonemal space, the cytoplasmic canal. The small cristae mitochondria are present within the midpiece. The flagellum is parallel to the base of the nucleus and a depression is present at the base of nuclear fossa. The plasma membrane surrounding the whole structure is present (Plate 3).

4.1.1. Percentage of live spermatozoa

The percentage of live spermatozoa in raw milt of *Liza parsia* was 90%. The live sperms were clear with out any stain whereas dead sperms were pink in colour (Plate 1).

4.2. Ultrastructural Changes due to Cryoprotectants

4.2.1. DMSO 7.5% in V2E.

4.2.1.1. Zero seconds after dilution (Plate 4)

The sperms were uniformly shaped and plasma membrane was intact in almost 75% of the sperms. The nucleus is condensed and chromatin appeared coarsely granular.

4.2.1.2. Frozen thawed spermatozoa (Plate 5)

The nucleus appeared almost intact but the chromatin showed more granulation compared to raw milt. The nuclear membrane was disintegrated and the mitochondria in the mid piece appear to be ruptured. About 56.4% of the spermatozoa exhibited structural changes.

4.2.2. DMSO 10% in V2E.

4.2.2.1. Zero seconds after dilution (Plate 6)

Almost all the spermatozoa appeared similar to untreated ones. The plasma membrane was intact. There was no disintegration of chromatin layer. Nuclear fossa was clear and nearly 80% of the spermatozoa exhibited near normal structure.

4.2.2.2. Frozen thawed spermatozoa (Plate 7)

Structural features of spermatozoa did not differ much after exposing to liquid nitrogen and thawing. However, condensation of chromatin and slight disorientation of mitochondria was observed in many cases. About 53% of the spermatozoa exhibited rupture of plasma membrane.

4.2.3. DMSO 12.5% in V2E.

4.2.3.1. Zero seconds after dilution (Plate 8)

The structure of spermatozoa appeared similar to raw milt. The clear nuclear fossa was visible and the flagellum was almost intact. Almost 67% of the sperms appeared normal as in the case of raw milt in this treatment.

4.2.3.2. Frozen thawed spermatozoa (Plate 9)

There is clear indication of rupture of plasma membrane. The mitochondria appeared less conspicuous. Several vacuoles were present in the nucleus and the whole structure showed signs of disintegration. 62% of spermatozoa exhibited structural changes.

4.2.4. Glycerol 5% in V2E.

4.2.4.1. Zero seconds after dilution (Plate 10)

Maximum changes were observed in plasma membrane. The clear disintegration and rounding of nuclear material were observed. The flagellum is found to be intact. Almost 64.8% sperms became abnormal.

4.2.4.2. Frozen thawed spermatozoa (Plate 11)

The midpiece and mitochondria were totally disrupted. Plasma membrane showed severe undulations. Considerable damage occurred in chromatin structure. Almost 90% sperms were deformed.

4.2.5. Glycerol 10% in V2E.

4.2.5.1. Zero seconds after dilution (Plate 12)

The nucleus remained intact with clear nuclear fossa. The mitochondria were deformed. Clear disintegration of chromatin structure was observed in almost 37.6% of the sperms.

4.2.5.2. Frozen thawed spermatozoa (Plate 13)

Almost 66.2% of the spermatozoa exhibited structural changes and became altogether ruptured. The plasma membrane exhibited clear undulations.

4.2. 6. Methanol 5% in V2E.

4.2.6.1. Zero seconds after dilution (Plate 14)

The plasma membrane was ruptured and midpiece totally damaged. The flagella got separated and nucleus exhibited many vacuoles inside chromatin material. Almost 51% of spermatozoa exhibited abnormal structure.

4.2.6.2. Frozen thawed spermatozoa (Plate 15)

Complete swelling and disintegration of the nucleus and midpiece were observed. The chromatin appeared web like and totally shattered. Such changes were observed in 77% of spermatozoa.

4.2.7. Methanol 10% in V2E.

4.2.7.1. Zero seconds after dilution (Plate 16)

Almost 46.8% of the spermatozoa exhibited near normal structure. Midpiece and mitochondria were intact.

4.2.7.2. Frozen thawed spermatozoa (Plate 17)

Complete destruction of the spermatozoal structure noticed in almost 82% of the spermatozoa. The plasma membrane was severely undulated. Clumping of nuclear material with rounded particles departing out of the head region was observed and flagellum was found to be distorted.

4.2.8. Propylene glycol 8% in V2E.

4.2.8.1. Zero seconds after dilution (Plate 18)

The plasma membrane was more or less intact. About 41% of spermatozoa showed disintegration of nucleus and rupture of midpiece.

4.2.8.2. Frozen thawed spermatozoa (Plate 19)

After plunging into liquid nitrogen the spermatozoan nucleus was found to be dilated and nuclear fossa was less conspicuous. Midpiece and mitochondria were completely ruptured and there is condensation of chromatin material. About 74% of sperms were highly deformed.

4.2.9. Propylene glycol 12% in V2E.

4.2.9.1. Zero seconds after dilution (Plate 20)

Morphology of spermatozoa remained more or less unchanged. Midpiece is found to be intact and the nuclear material became more condensed. 53.8% of spermatozoa remained intact.

4.2.9.2. Frozen thawed spermatozoa (Plate 21)

Swelling in the plasma membrane of head, midpiece and tail region with rupture of mitochondria was observed in almost 73.7% of spermatozoa.

4.2.10. Methanol + propylene glycol (5+5%) in V2E.

4.2.10.1. Zero seconds after dilution (Plate 22)

The same intact nucleus was present but plasma membrane appeared ruptured. The percentage of damaged spermatozoa was 69.7%.

4.2.10.2. Frozen thawed spermatozoa (Plate 23)

Plasma membrane was totally disrupted with scattering of nuclear material noticed. The percentage of abnormal spermatozoa increased to 92%.

4.2.11. DMSO + Glycerol (5+5%) in V2E.

4.2.11.1. Zero seconds after dilution (Plate 24)

Mitochondria and plasma membrane were intact in almost 86% of the spermatozoa. Condensed nucleus with clear nuclear fossa was present. Chromatin was coarsely granular and shape of spermatozoa was uniform.

4.2.11.2. Frozen thawed spermatozoa (Plate 25)

Almost 55.6% of the spermatozoa were normal with intact plasma membrane. Mitochondria appeared near normal without much disorientation. Nucleus was dense and bilobed or kidney shaped.

4.2.12. Glycerol + Propylene glycol (5+5%) in V2E.

4.2.12.1. Zero seconds after dilution (Plate 26)

The head region appears hypertrophied or swollen, but the plasma membrane was intact. The percentage of abnormal spermatozoa was 60%.

4.2.12.2. Frozen thawed spermatozoa (Plate 27)

Almost 86.4% of spermatozoa became damaged and exhibited structural changes. Rupture of plasma membrane and detachment of mid piece were the changes.

4.2.13. DMSO+ Methanol (5+5%) in V2E.

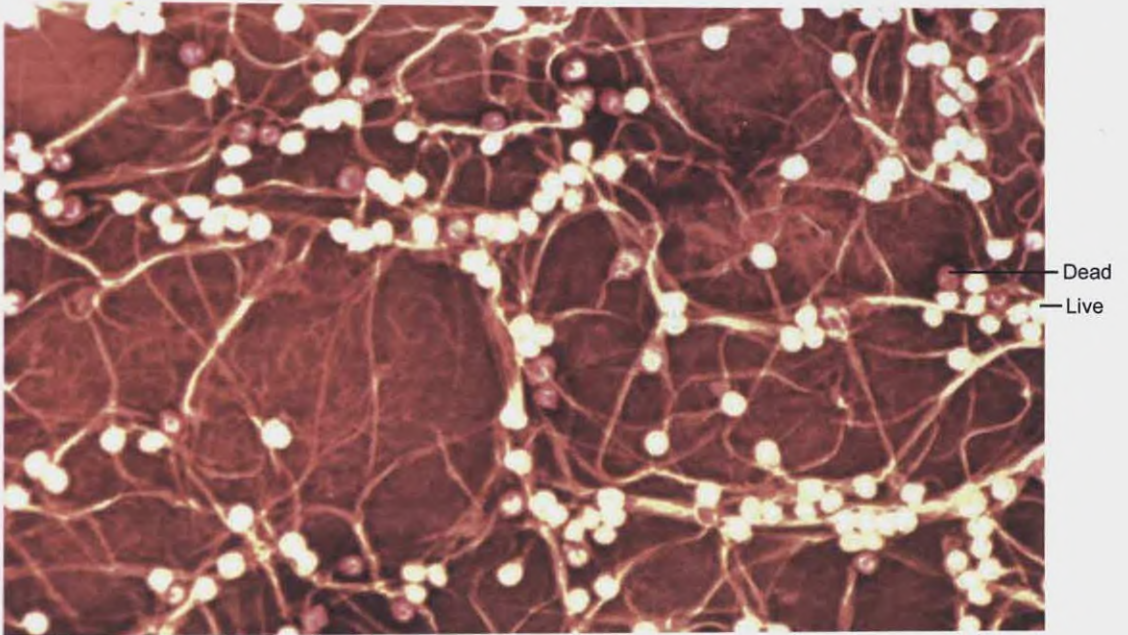
4.2.13.1. Zero seconds after dilution (Plate 28)

About 30% of the spermatozoa maintained near normal structure with clear nuclear fossa and condensed chromatin material.

4.2.13.2. Frozen thawed spermatozoa (Plate 29)

Complete disintegration of the bilobed nucleus, with highly disrupted plasma membrane was observed. Few spermatozoa also exhibited hypertrophy and chromatin material became clumped and rounded particles were found scattered all along. Mitochondria and midpiece were also highly damaged. About 95% of the spermatozoa exhibited abnormal structure.

PLATE 1



Live and Dead count
(Nigrosine - Eosin Staining)

PLATE 2



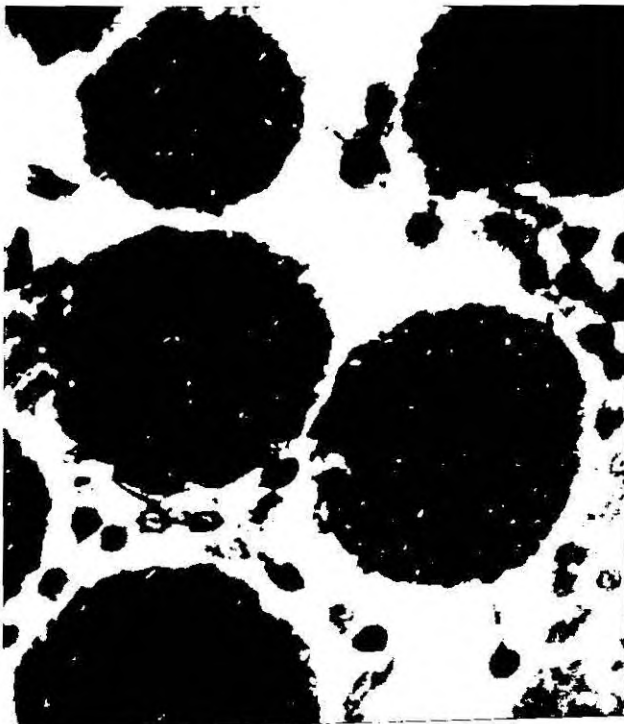
Untreated spermatozoa of *Liza parsia*
(25000 x)

PLATE 3



Untreated spermatozoa of *Liza parsia*
(40000x)

PLATE 4



V2E extender + 7.5%DMSO
Zero seconds after dilution
(25000x)

PLATE 5



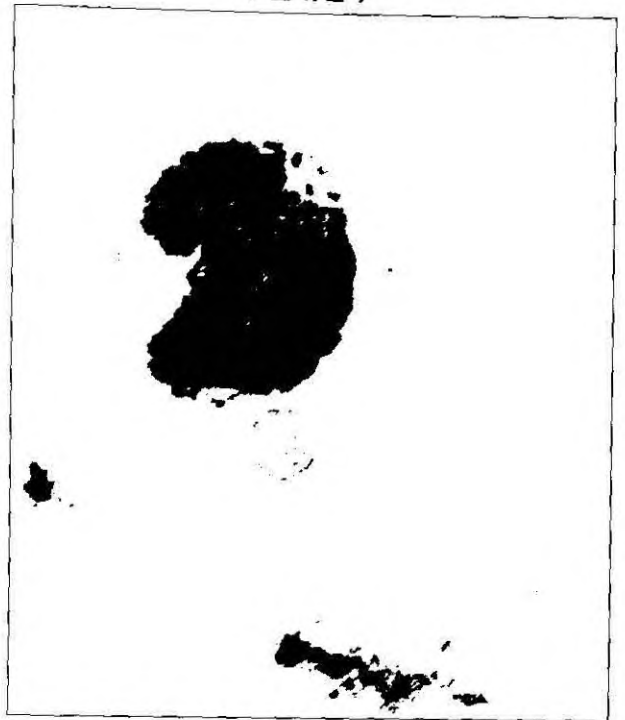
V2E extender + 7.5%DMSO
After plunging into liquid nitrogen
(25000x)

PLATE 6



V2E extender + 10% DMSO
Zero seconds after dilution
(25000x)

PLATE 7



V2E extender + 10% DMSO
After plunging into liquid nitrogen
(25000x)

PLATE 8



V2E extender +12.5% DMSO
Zero seconds after dilution
(25000x)

PLATE 9



V2E extender +12.5% DMSO
After plunging into liquid nitrogen
(25000x)

PLATE 10



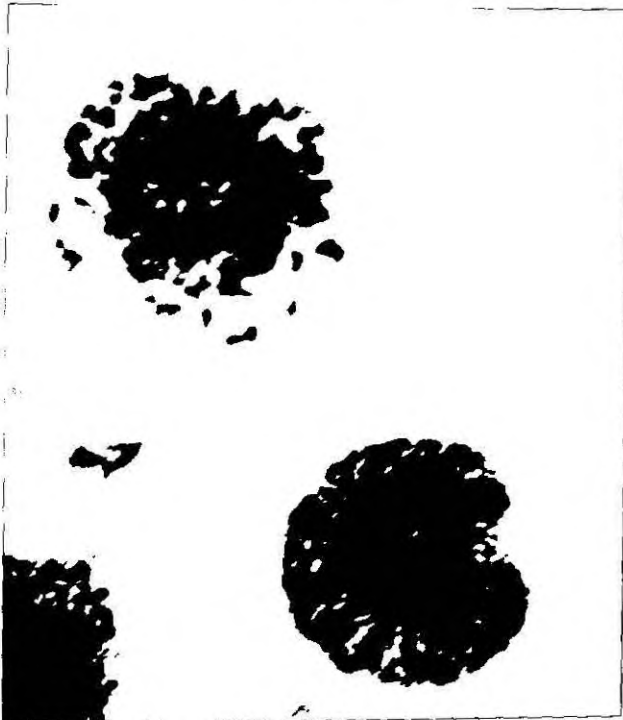
V2E extender + 5% glycerol
Zero seconds after dilution (25000x)

PLATE 11



V2E extender + 5% glycerol
After plunging into liquid nitrogen (25000x)

PLATE 12



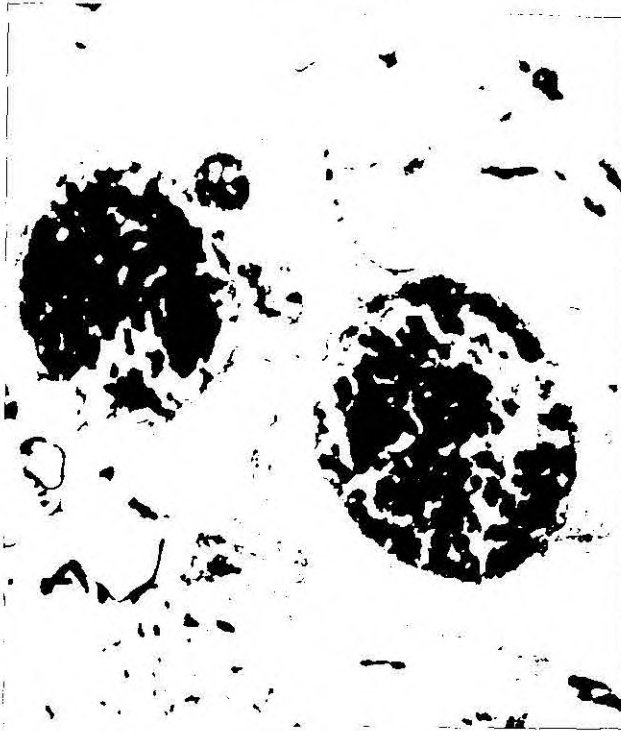
V2E extender + 10% glycerol
Zero seconds after dilution (25000x)

PLATE 13



V2E extender + 10% glycerol
After plunging into liquid nitrogen (25000x)

PLATE 14



V2E extender + 5% methanol
Zero seconds after dilution (25000x)

PLATE 15



V2E extender + 5% methanol
After plunging into liquid nitrogen (25000x)

PLATE 16



V2E extender + 10% methanol
Zero seconds after dilution (25000x)

PLATE 17



V2E extender + 10% methanol
After plunging into liquid nitrogen (25000x)

PLATE 18



V2E extender + 8% propylene glycol
Zero seconds after dilution (25000x)

PLATE 19



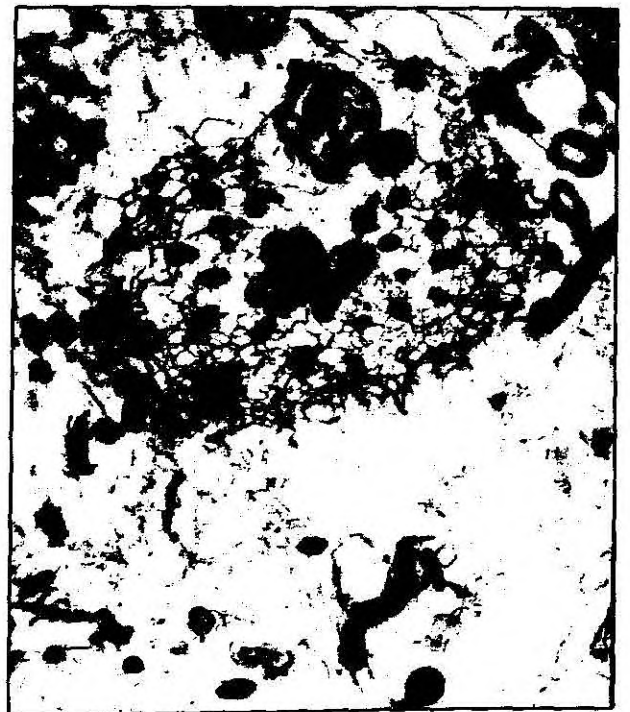
V2E extender + 8% propylene glycol
After plunging into liquid nitrogen (25000x)

PLATE 20



V2E extender + 12% propylene glycol
Zero seconds after dilution (25000x)

PLATE 21



V2E extender + 12% propylene glycol
After plunging into liquid nitrogen (25000x)

PLATE 22



V2E extender + 5% methanol
and 5% propylene glycol
Zero seconds after dilution (25000x)

PLATE 23



V2E extender + 5% methanol and
5% propylene glycol
After plunging into liquid nitrogen (25000x)

PLATE 24



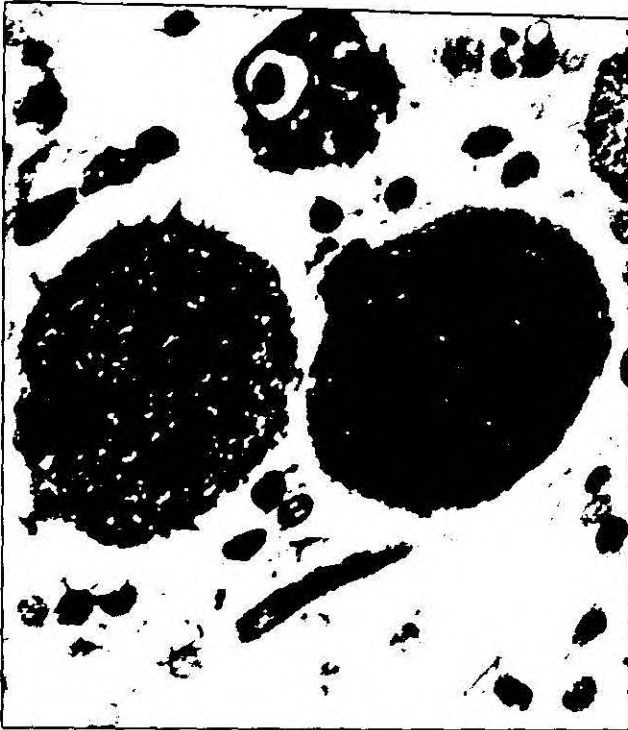
V2E extender + 5% DMSO
and 5% glycerol
Zero seconds after dilution (25000x)

PLATE 25



V2E extender + 5% DMSO
and 5% glycerol After plunging into
liquid nitrogen (25000x)

PLATE 26



V2E extender + 5% glycerol
and 5% propylene glycol
Zero seconds after dilution (25000x)

PLATE 27



V2E extender + 5% glycerol
and 5% propylene glycol
After plunging into liquid nitrogen (25000x)

PLATE 28



V2E extender + 5% DMSO
and 5% methanol
Zero seconds after dilution (25000x)

PLATE 29



V2E extender + 5% DMSO
and 5% methanol
After plunging into liquid nitrogen (25000x)

Table 3. Percentage of intact spermatozoa of *Liza parsia* before and after cryopreservation.

Cryoprotectant	Zero seconds after dilution(Before cryopreservation)	After freeze-thawing (After cryopreservation)
DMSO 7.5%	75.02 ±3.61 (4)	43.52±3.34 (2)
DMSO 10%	80.67±5.33 (4)	47.83 ±6.64 (3)
DMSO12.5%	67.23 ± 2.31 (3)	37.48±6.94 (2)
Glycerol 5%	35.11 ± 2.98 (2)	10.92 ± 5.86 (1)
Glycerol 10%	62.46 ± 2.31 (3)	33.74 ± 5.32 (2)
Methanol 5%	49.71 ± 4.02 (3)	23.12 ± 6.61 (1)
Methanol 10%	46.82 ± 6.87 (2)	18.46 ± 2.64 (1)
Propylene glycol 8%	59.35 ± 4.96 (3)	29.96 ± 4.71 (2)
Propylene glycol 12%	53.84 ± 5.93 (3)	26.24 ± 2.73 (2)
Methanol + Propylene glycol (5%+5%)	30.26 ± 4.98 (2)	7.08 ± 0.39 (0.5)
DMSO + Glycerol (5%+5%)	86.23 ± 6.68 (5)	55.6 ± 1.76 (3)
Glycerol + Propylene glycol (5%+5%)	40.03 ± 2.41 (2)	13.56 ± 3.89 (1)
DMSO+ Methanol (5%+ 5%)	29.91 ± 5.11 (2)	5.01 ± 0.24 (0.5)

Figures in parenthesis () indicate motility score. Simple comparison of means by student's t- test was employed, with a significance level of $P < 0.05$.

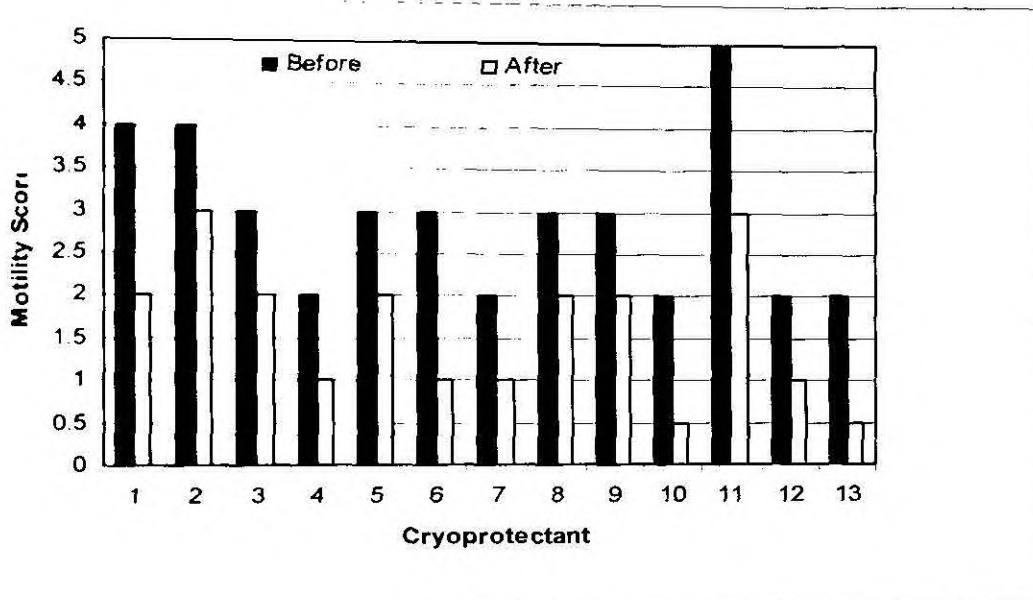


Figure 2. Motility score of *Liza parsia* spermatozoa in different cryoprotectants before and after cryopreservation.

Cryoprotectants 1: DMSO 7.5%; 2: DMSO 10%; 3: DMSO 12.5%; 4: Glycerol 5%;
 5: Glycerol 10%; 6: Methanol 5%; 7: Methanol 10%;
 8: Propylene glycol 8%; 9: Propylene glycol 12%;
 10: Methanol + Propylene glycol (5%+5%); 11: DMSO + Glycerol(5%+5%);
 12: Glycerol + Propylene glycol (5%+5%); 13: DMSO+ Methanol (5%+ 5%).

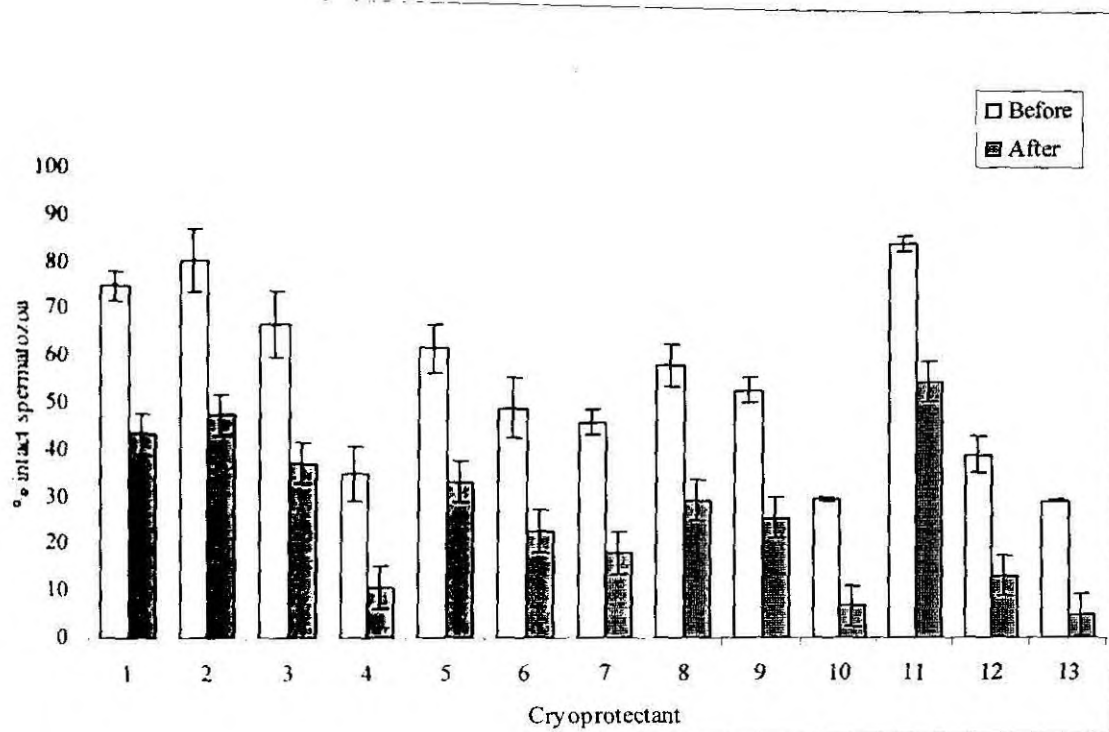


Figure 3. Percentage of intact spermatozoa of *Liza parsia* in different cryoprotectants before and after cryopreservation.

Cryoprotectants 1: DMSO 7.5%; 2: DMSO 10%; 3: DMSO 12.5%; 4: Glycerol 5%;
 5: Glycerol 10%; 6: Methanol 5%; 7: Methanol 10%;
 8: Propylene glycol 8%; 9: Propylene glycol 12%;
 10: Methanol + Propylene glycol (5%+5%); 11: DMSO + Glycerol (5%+5%);
 12: Glycerol + Propylene glycol (5%+5%); 13: DMSO+ Methanol (5%+ 5%).

DISCUSSION

5. DISCUSSION

Cryoprotectants are added to extenders to suppress cryoinjuries, but when used in higher concentrations most of them become toxic to biological material. The cryoprotectant depresses the freezing point and prevents injury due to ice crystal formation. The cryoprotectants may also be toxic to spermatozoa leading to abnormalities. A comparison of the extent of injuries damages on treatment with different concentration of cryoprotectants were made to get an in depth knowledge about the extent of protection accorded during freeze-thaw process with the specific objective to identify the best extender-cryoprotectant combination for the spermatozoa of *Liza parsia*.

The spermatozoa of *Liza parsia* is a typical teleostean anacrosomal aqua sperm. The untreated spermatozoa (raw milt) exhibited clear and intact nucleus and clear nuclear fossa. Two centrioles are present and around the centrioles mitochondria are located. The region containing the mitochondria forms the midpiece. All these structures are clearly visible in the untreated spermatozoa (Plate 2 and Plate 3).

5.1. Dead and Live Count

Dead and live count using Nigrosine-Eosin stains is the commonly used technique for evaluation of mammalian spermatozoa (Chao *et al.*, 1975). The dead sperms take pink colour and live ones are white/clear in appearance (Plate 1). The abnormalities and immature sperms if any can be made out to some extent. However, the morphological changes may not be clearly visible. It was reported that lack of acrosome and the small size of spermatozoa make the teleosts sperms unsuitable for above tests (Billard, 1983), thus limiting the use of this technique to assess the quality of milt in cryopreserved samples unlike in mammals.

With all these limitations, it is opined from the present study that Nigrosine- Eosin staining to estimate the percentage of live and dead spermatozoa is very much useful in preliminary evaluation of the spermatozoa. It gives fairly good idea about the gross surface details and also about intactness of head and tail. Any abnormality in head and tail are also discernible. The percentage of live spermatozoa after each treatment also coincides with the motility test.

Most of the tests now available for assessment of sperm fitness for cryopreservation do not fully satisfy the requirements for which it is done. At least combinations of more than two tests need to be conducted to arrive at a fairly valid conclusion. As in mammalian cases, dead and live count coupled with motility/ mass activity would have been sufficient for fish spermatozoa also. Costly, cumbersome and time consuming tests like electron microscopic imaging, computer assisted sperm analysis could have been avoided. From the present study it appears that motility test coupled with dead and live count would be sufficient enough to evaluate sperm fitness.

5.2. Ultrastructural changes of spermatozoa in different cryoprotectants

Cryoprotectants have multiple roles, mainly bind to water molecules and reduce ice crystal formation and also help to stabilise hydrated protein molecules. Dimethyl sulphoxide (DMSO), methanol, glycerol, propylene glycol are permeating cryoprotectants. DMSO is most widely used one. However, cryoprotectants are toxic in high concentrations and may cause structural damages result in lowered fertility. The ultrastructural observations of spermatozoa are the most reliable method to study the morphological changes to understand freeze-thaw effects. On the basis of the degree of protection accorded from cryoinjuries, a cryoprotectant can be culled or selected for use.

5.2.1. Dimethyl sulphoxide (DMSO)

The sperms were uniformly shaped with intact plasma membrane when DMSO was used as a cryoprotectant at concentration of 7.5% upon zero seconds after dilution (Plate 4). On cryopreservation and subsequent thawing the chromatin showed more aggression and granulations compared the raw milt. The nucleus appeared almost intact but there was clear disintegration of nuclear membrane. (Plate 5). The percentage of injured spermatozoa increased to 56.4%.

About 80% of the spermatozoa exhibited near normal structure on dilution with 10% DMSO (Plate 6). However, slight disorientation of mitochondria and detachment of midpiece was observed in spermatozoa treated with 10% DMSO on freeze thawing (Plate 7). When the spermatozoa were exposed to 12.5 % DMSO at 20°C, the structure of spermatozoa remained intact and the nucleus appeared similar to raw milt (Plate 8). There was a clear indication of rupture of plasma membrane and several vacuoles were present in the nucleus (Plate 9). About 62% of spermatozoa were completely deformed. So, the cryoprotectant DMSO at concentration of 10% was found to be the optimum cryoprotectant than at 7.5% and 12.5 % to prevent injuries.

5.2.2. Glycerol

More damages occurred than DMSO in 10% Glycerol +V2E with almost 67% of spermatozoa exhibiting structural damage after freeze-thaw (plate 13). The mid piece and mitochondria became altogether dislocated with plasma membrane exhibiting clear undulations. Even zero seconds after dilution in 5% glycerol, almost 64.8 % of the sperms became abnormal, but on dilution with 10% glycerol, the structure of spermatozoa was maintained with clear nuclear fossa, but the mitochondria was detached and dispersed in the media with almost 37.6% of spermatozoa showing abnormality (Plate 12). So, 5% or 10% glycerol is not a suitable cryodiluent for preserving *Liza parsia* spermatozoa as in both cases severe morphological alternations were observed.

5.2.3. Methanol

The concentration of 5% and 10% methanol was studied. In case of 5% methanol, zero seconds after dilution, almost 51% of spermatozoa exhibited severe structural damage with ruptured plasma membrane, separated flagella and coiled up tail, Vacuoles inside chromatin material were observed. (Plate 14). In frozen-thawed spermatozoa there was severe hypertrophy and disintegration of nuclear material with almost 67.8% of spermatozoa exhibiting gross deformity (Plate 15). So, 5% methanol does not serve as a good cryoprotectant for *Liza parsia* spermatozoa. In case of 10% methanol, nearly 46.8% of spermatozoa exhibited near normal structure (Plate 16), but on freeze-thawing, almost similar destruction of spermatozoa structure with severely undulated plasma membrane, clumped nuclear material with rounded particles oozed out of the head region were observed (Plate 17). 10% methanol also with V2E extender will not serve as a good cryoprotectant in preserving spermatozoa.

5.2.4. Propylene glycol

In V2E extender containing 8% propylene glycol, the spermatozoa exhibited comparatively less damage than after freezing as seen in plate 19. There was clear destruction of nuclear material, mitochondria and mid piece completely ruptured and almost 74% sperms were highly deformed. In 12% propylene glycol also, about 73% sperms were abnormal (Plate 21) after freeze thawing.

5.2.5. Methanol and propylene glycol (5+5%)

Zero seconds after dilution the structure of spermatozoa remained intact with condensed nucleus and some cases with ruptured plasma membrane (Plate 22). After thawing the percentage of abnormality increased to 92% (Plate 23). So, it can be concluded that even combination of methanol and propylene glycol (5+5%) also will not serve as a good cryoprotectant.

5.2.6. DMSO + Glycerol (5 + 5%)

Mitochondria and plasma membrane were intact with clear nuclear fossa but slight condensation of nucleus, observed in some spermatozoa zero seconds after dilution in V2E extender treated with DMSO + glycerol (5+5%). Almost 86% of sperms were normal (Plate 24). Freeze thawed spermatozoa also remained as normal with intact nucleus and clear nuclear fossa (Plate 25). DMSO being a fast permeating cryoprotectant and glycerol being less toxic, the combination of both might have worked well in preserving the morphology of spermatozoa. The percentage of abnormal spermatozoa is very less. Hence the combination of DMSO + glycerol appears to protect well and has an edge over 10% DMSO as cryoprotectant.

5.2.7. Glycerol and Propylene glycol (5+5%)

Even zero seconds after dilution, the sperms were found to be damaged. About 86.4% of sperms became damaged and complete destruction was noticed in frozen thawed spermatozoa (Plate 27). So, glycerol + propylene glycol will not act as a good cryoprotectant for *Liza parsia* spermatozoa.

5.2.8. DMSO + methanol (5+5%)

About 30% of sperms exhibited near normal structure with clear nuclear fossa and condensed chromatin material. After freeze thawing, complete destruction of the bilobed nucleus with highly disrupted plasma membrane was observed. Chromatin became clumped and hypertrophied. Almost 95% of spermatozoa exhibited abnormal structure.

5.3. Ultrastructural changes in spermatozoa before cryopreservation

Changes in the ultrastructure of spermatozoa are possible even before freezing due to toxicity difference in osmolality, inadequacy of the concentration and

low permeability of the cryoprotectant. A study of changes will give an insight into the acceptability of cryoprotectant and optimal concentration requirement. Structural changes in spermatozoa following dilution and deep-freezing have been reported in several fish species. (Billard 1978, 1983; Gwo and Arnold, 1992; Diwan and Nandakumar, 1998; Gopalakrishnan *et al.*, 2000). The ultrastructural changes in *Liza parsia* following dilution in different cryoprotectants have been described in other species like rohu, salmonids and guppy (Lahnsteiner *et al.*, 1992; Gopalakrishnan *et al.*, 2000). The sensitiveness of spermatozoa to osmotic pressure may cause morphological alternations including rupture of membrane, swelling and disruption of mid piece (Billard, 1983).

The sperms were uniformly shaped with intact plasma membrane in almost 75% of the sperms on treatment with 7.5% DMSO. Where as in 10% DMSO, the percentage of intact spermatozoa increased to 80% with almost all the spermatozoa appearing similar to untreated ones. More damages occurred in 5% glycerol treated sperms zero seconds after dilution. Maximum changes were observed in plasma membrane and rounding of nuclear material was also observed. In spermatozoa treated with methanol + propylene glycol (5+5%) on dilution 69% of the sperms exhibited structural damage. On dilution with DMSO and methanol (5+5%) with V2E, about 30% of the spermatozoa maintained near normal structure with clear nuclear fossa and condensed chromatin material. But 70% of the spermatozoa showed disintegration of plasma membrane and rupture of mid piece. The motility of the spermatozoa also gradually decreased in these treatments. Fish sperm motility is closely related to the existence of mitochondria in mid piece (Gwo, 1995). About 41% of spermatozoa showed disintegration of nucleus and rupture of mid piece along with mitochondria on dilution with 8% propylene glycol. The destruction of mitochondria results in exhaustion of energy supply thus decreasing the motility of sperms. In addition, the rupture of plasma membrane, condensation of chromatin, hypertrophy of nucleus may also result in reduced viability and structural integrity as seen in plate 22 and 26.

The Morphology of spermatozoa did not differ significantly in undiluted milt and sperms diluted with DMSO + Glycerol (5+5%). In 10% DMSO +V2E the structural damages were to a lesser extent upon dilution.

5.4. Ultrastructural changes during freezing

Maximum cryoinjuries occur during freezing phase. Exposure to liquid nitrogen vapours and subsequent freezing followed by thawing can cause cold shock to biological systems (Jamieson, 1991). Cold shock is mainly caused by the change of membrane lipids from the liquid to the solid phase. Ice crystal formation during the freezing will effectively dehydrate and create phase transition of the lipids in biological membranes. This would increase the membrane tension leading to its rupture. In addition the likelihood of formation of intracellular ice increases with cooling rate and the degree of injury is proportioned to the size of ice crystals. Recrystallisation of small ice crystals during thawing inside and outside the cell also is detrimental. All these reactions will end up with mechanical destruction of membrane structures and cell death (Jamieson, 1991).

DMSO is rated as a better cryoprotectant and for most of the cells due to its fast penetration. On treating the sperms with 7.5% DMSO +V2E and upon freeze thawing the nucleus appeared almost intact but the chromatin showed more granulation compared to the untreated spermatozoa. About 57% of spermatozoa showed structural changes with several vacuoles in the nucleus and the whole structure exhibited signs of total disintegration in freeze thawed spermatozoa treated with 12.5% DMSO. Maximum structural changes were noticed in DMSO+ methanol (5+5%) with V2E. Few spermatozoa also exhibited abnormal structure. Shrinkage of the plasma membrane of the mid piece was reported for frozen thawed spermatozoa of the ocean pout. (Yao *et al.*, 1998). In frozen thawed Atlantic croaker spermatozoa the crystal of mitochondria was disrupted, the plasma membrane was swollen and the axoneme was coiled. (Gwo and Arnold, 1992). Rupture of plasma membrane and the detachment of mid piece were the changes observed in frozen thawed spermatozoa treated with Glycerol + Propylene glycol (5+5%) with V2E. Swelling in the plasma membrane of head, mid piece and tail region with rupture of mitochondria was observed in almost 73.7% of spermatozoa treated with 12% propylene glycol (Plate 21).

Methanol was observed very poor in according protection both in 5 and 10% concentrations. Frozen thawed spermatozoa treated with 10% methanol with V2E exhibited complete destruction of the spermatozoal structure in almost 82% of the spermatozoa. The plasma membrane was severely undulated with clumping of nuclear material with rounded particles departing out of the head region. Even 5% methanol exhibited poor results with 51% abnormal spermatozoa.

Within the spermatozoa, plasma membrane is probably the site most susceptible to damage due to water flux during freezing and thawing (Baynes and Scott, 1987). Almost 66% of the spermatozoa exhibited structural changes and became altogether ruptured. The plasma membrane exhibited clear undulations in frozen thawed spermatozoa treated with 10% glycerol.

In the present investigation, combination of DMSO + Glycerol (5+5%) with V2E exhibited fairly good results with almost 55.6% of intact spermatozoa. 10% DMSO in V2E also accorded significant protection with 53% of the spermatozoa exhibiting normal structure after freeze thawing. The study indicated the superiority of combination of dimethyl sulphoxide (DMSO) and glycerol over other cryoprotectants used for *Liza parsia* spermatozoa. Though DMSO is rated as better cryoprotectant owing to its faster penetrability, toxic effects may appear at higher concentrations. Glycerol is least toxic to biological material but also least permeable to the cell membrane and hence takes longer to equilibrate with glycerol osmolality. Because of the gene activation properties of DMSO (Ashwood-Smith, 1985), possibility of genetic effects in fish cryopreservations cannot be ruled out. Hence any attempt to reduce the concentration of DMSO without sacrificing the viability of spermatozoa will be a desirable one.

The study has succeeded in reducing the concentration of DMSO by combining glycerol with DMSO as cryoprotectant. The concentration of 5% DMSO + 5% glycerol not only maintained the viability of cryopreserved spermatozoa comparable with 10% DMSO but also proved to be better than that. This not only reduced the harmful effects of DMSO but also combined the favourable attributes of both DMSO and glycerol.

SUMMARY

An investigation was carried out to identify the best cryoprotectant for the cryopreservation of the spermatozoa of *Liza parsia* on the basis of morphological changes observed in Transmission Electron Microscopic images.

- ❖ Male specimens of *Liza parsia* 10-12cm were collected from the wild and milt collected by manual stripping.
- ❖ Motility score and percentage intact spermatozoa on TEM images used for evaluation of sperm fitness before and after cryopreservation.
- ❖ Four commonly used permeating cryoprotectants and its combinations at different concentrations in V2E extender were tried.
- ❖ The efficiency of different cryoprotectants to prevent injuries before and after storage in liquid nitrogen had been studied.
- ❖ A combination of 5% DMSO and 5% glycerol in V2E extender appeared to be better than 10% DMSO alone as cryoprotectant.
- ❖ Toxic effects of high concentrations of DMSO can be minimized by a combination of reduced quantity of DMSO and glycerol.
- ❖ Dead and live count of spermatozoa by Nigrosine-eosin staining combined with motility score is sufficient to evaluate sperm fitness and cumbersome tests can be avoided.

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