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Ultrastructural changes in the spermatozoa of the goldspot mullet *Liza parsia* (Hamilton-Buchanan) in different diluents during cryopreservation

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Abstract

The milt of goldspot mullet, *Liza parsia* (Hamilton-Buchanan) was diluted with four extenders containing 10% DMSO. Samples were collected at four critical steps of cryopreservation procedure. Percentage of intact spermatozoa and cryoinjuries at each step was recorded. Extender V2E in seawater base +10% DMSO appeared to be the best cryodiluent as the sperms exhibited least structural changes during the cryopreservation protocol. Chao's extender+10% DMSO was the next preferred cryodiluent. The suitability of various extenders that accorded protection to injuries was evaluated on the basis of electron microscopic images.

Introduction

Studies on the cryopreservation of milt of marine and brackish water fishes are limited (Chao *et al.*, 1975). Marine fish sperm seems to withstand the rigours of cryotreatment better (Scott and Baynes, 1980) compared to freshwater fishes (Suquet *et al.*, 2000). Drokin (1993) proposed that the cryoresistance of marine fish spermatozoa could result from the lipid composition of sperm membranes, mainly due to the molar ratio of cholesterol to phospholipids, which is two or three times higher than that of freshwater fish. Its protective role against osmotic and cold stress also has been reported by Simpson *et al.* (1986).

The success of cryopreservation depends on many factors, like selection of a suitable cryodiluent (cryoprotectant + extender), using the optimum concentration of cryoprotectant, standardisation of equilibration time, optimization of freezing rate, etc. Cryoinjuries can occur at any stage of cryopreservation protocol. A study of the structural changes of the spermatozoa will enable us to identify the right extender-cryoprotectant combination, which can minimise injuries and retain maximum post-thaw sperm fitness and fertility. Some of the cryoinjuries reported to occur in the spermatozoa during cryopreservation are alterations in chromatin structure, coiling up of flagellum, appearance of discontinuities along the plasma membrane, loss and rupture of mitochondria, etc. (Gwo,1995). Genomic

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changes due to alterations in chromatin structure could also affect the embryonic development and survival of larvae. So the structural changes of spermatozoa should invariably be studied before selecting a cryodiluent for preserving sperms of a particular species. The present work was taken up to study the morphological changes in spermatozoa during various stages of cryopreservation protocol with the specific objective to arrive at the most suited cryodiluent for the brackish water fish *Liza parsia* using Transmission Electron Microscopy (TEM).

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Material and methods

Ripe males of the mullet, *L. parsia* collected from Chinese dip nets operating at the Cochin backwater bar-mouth during the peak breeding season were dry stripped manually and the milt collected in vials, avoiding contamination of blood, urine and faeces. Preliminary motility test was done on the collected milt. The sperms were actively motile in seawater of salinity 35ppt. for a duration of about 6 minutes. For cryopreservation studies, only good quality milt was selected, based on the motility of spermatozoa in seawater. A convenient scale based on the type of motility was adopted and motility scores were given from 0 – 5. Only samples with a motility score of 4 and above (showing rapid progressive movement) were chosen for cryopreservation.

The following extenders immobilised spermatozoa on dilution and mobilised them on activation. They are (1) V2E (Kurokura *et al.*,1984), (2) Mixture C (Elizabeth,1987) and (3) Rana and McAndrew extender (Rana and McAndrew, 1989). Apart from these three extenders Chao's extender was also selected for the present investigation since it is one of the most widely used extenders in other mullets.

Experimental protocol: The ratio of milt : cryodiluent for this experiment was fixed as 1:3.The samples were collected at the following steps for assessing the extender damages in the spermatozoa at each stage : (a) zero seconds after mixing with cryodiluents; (b) after an equilibration time of 10 minutes with cryodiluents including the time taken for filling diluted milt into 0.5ml french straws; (c) exposing to liquid nitrogen vapour (-100^{0}C) after step 2 and (d) plunging straws in liquid nitrogen and storing overnight(-196^{0}C).

After each step, the diluted milt was thawed for 20 seconds in a water bath maintained at 37^{0}C and the percentage of intact spermatozoa recorded. The samples

were also processed for Transmission Electron Microscopy (TEM). The stained ultra thin sections were mounted on the grid and observed in the TEM model Hitachi (H 600) electron microscope and recorded the images. The ratio of intact and damaged spermatozoa for each treatment was calculated by counting sperms under low magnification of 4000 x in TEM. TEM images of untreated raw milt served as control.

Results

Ultrastructure of untreated spermatozoa

The sperm of *L. parsia* is a typical anacrosomal aquasperm. The nucleus is bilobed or kidney shaped in longitudinal section. It is also tilted relative to the axoneme. Chromatin is very coarsely granular and not condensed. Matrix spaces are clearly visible. A cytoplasmic collar, which extends around the base of the flagellum, is present and it is separated from the flagellum by a periaxonemal space, the cytoplasmic canal. Small cristate mitochondria are present and they are situated in the cytoplasmic collar. The flagellum is parallel to the base of the nucleus and a depression is present at this point, the nuclear fossa. A plasma membrane surrounding the whole structure is also present (Fig.1).

Ultrastructural changes of sperm in freshwater

The most conspicuous changes in freshwater, probably due to osmotic shock, were the bursting of plasma membrane; the nucleus rounded off in 80% of the sperms; the nuclear fossa became less conspicuous and flagellum got separated from the nucleus and coiled up within its membrane (Fig.2).

Ultrastructural changes due to treatments

***Zero seconds after dilution with extenders*:** Maximum damages occurred to spermatozoa in the milt diluted with Rana and McAndrew extender+DMSO. Almost 70% of the spermatozoa became abnormal at this stage. The plasma membrane exhibited undulations; the chromatin material got condensed and vacuoles present; the mid-piece and mitochondria were deformed but nucleus remained intact. In mixture C, 30% of the sperms exhibited slight disruption of mid-piece. In Chao's extender, a condensed nucleus with clear nuclear fossa was present and the mitochondria and plasma membrane were intact in 80% of the sperms (Fig.3). In V2E extender, almost all the spermatozoa appeared intact as in raw milt without much damages. Appearance of flagellum, mitochondria, nuclear fossa, nucleus, etc. were more or less similar to the raw milt in 90% of the sperms. So V2E extender was found to be the best cryodiluent among the 4 studied. Chao's extender was the second best cryodiluent. Maximum damages were observed in sperms diluted with Rana and McAndrew extender among the 4 cryodiluents studied.

***10 minutes after dilution*:** More damages to the spermatozoa occurred in Rana and Mc Andrew extender, the extent of damage was observed to be 85%. The plasma membrane exhibited undulations; the chromatin material got condensed and vacuoles were present in it. The mid-piece and

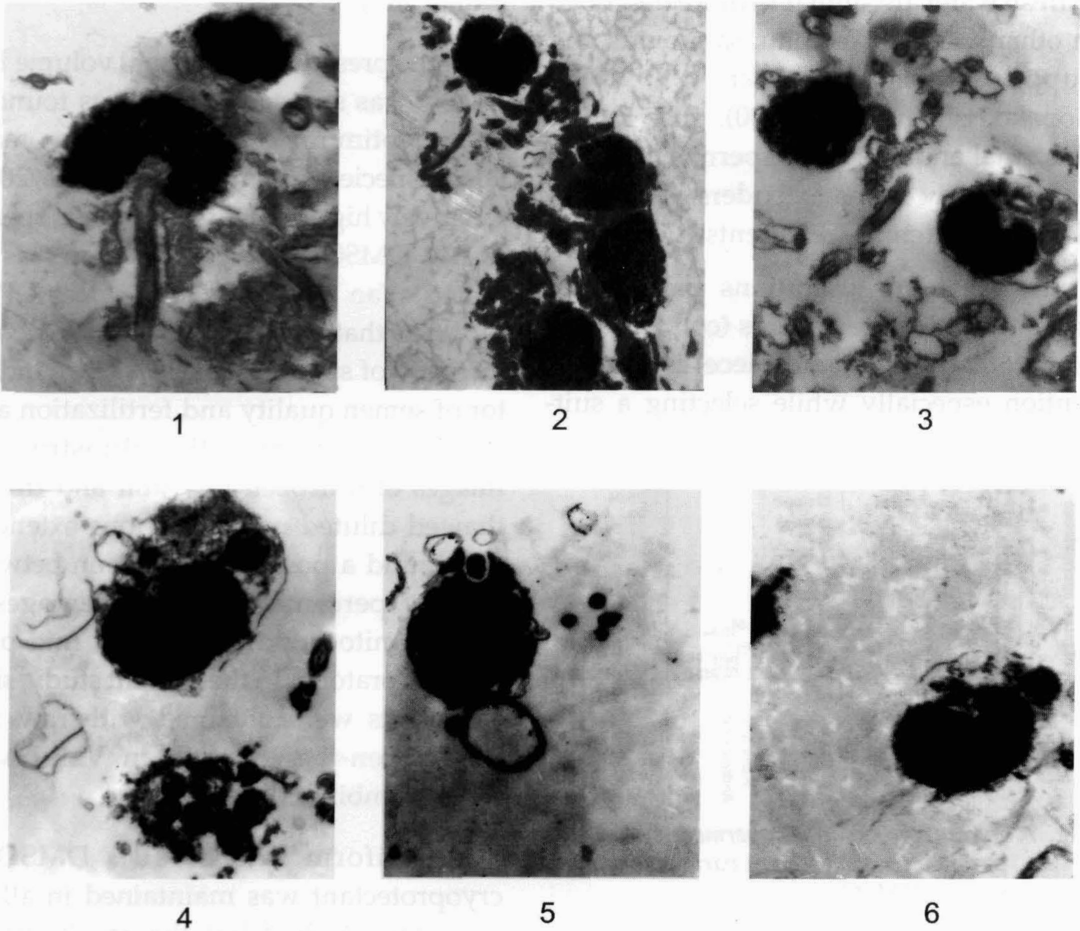
mitochondria were deformed (Fig.4). In mixture C, damages occurred in almost 60% of the sperms. The mitochondria were less conspicuous; the plasma membrane exhibited corrugated appearance and the chromatin got more condensed. In Chao's extender, about 33% of the sperms exhibited abnormalities like dilated nucleus and less conspicuous nuclear fossa. In V2E extender, most of the spermatozoa (76%) appeared similar to sperms in raw milt retaining flagellum, mitochondria, nucleus, nuclear fossa, etc. Twenty four percent of sperms showed relatively minor damages like disintegration of plasma membrane and rupture of mid-piece.

Exposure to liquid nitrogen vapour: In Rana and McAndrew extender, the mitochondria of the spermatozoa were totally damaged and the plasma membrane showed severe undulations. In mixture C, the plasma membrane was totally disrupted, the mid-piece and mitochondria found altogether ruptured (Fig.5). In Chao's extender, the percentage of damaged spermatozoa was 42% and the gross morphology remained more or less similar as compared to second step. Plasma membrane was present, nucleus dilated and nuclear fossa became less visible and chromatin material got condensed. In V2E extender, almost 60% of sperms exhibited near normal structure. Plasma membrane was more or less intact and did not exhibit much invagination. Nucleus was intact, mitochondria also appeared near normal. So V2E was found to be the best cryodiluent after exposure to liquid nitrogen vapour. Severe damages were observed in Rana and McAndrew extender.

Effect of plunging and storage in liquid nitrogen: Very severe damages were observed in Rana and McAndrew extender, wherein almost 95% of the sperms suffered damages in head and mitochondria. The mid-pieces were also found totally damaged. In mixture C, almost 75% of the spermatozoa became abnormal, plasma membrane was totally disrupted and the nucleus exhibited vacuoles inside the chromatin material. In Chao's extender, the percentage of damaged sperms was 52. Plasma membrane appeared to be ruptured but nucleus was intact. In V2E extender, the percentage of damaged sperms was 46. Structural features of spermatozoa did not differ further after freezing and thawing protocol, only the plasma membrane appeared to be slightly more disrupted compared to the previous step. The nucleus and nuclear fossa were intact and appeared almost similar to the normal spermatozoa (Fig.6). V2E extender was again the best cryodiluent after plunging and storing in liquid nitrogen.

Discussion

Structural changes in spermatozoa following dilution and deep freezing have been reported in several fish species (Billard,1978, 1983; Lahnsteiner *et al.*, 1996; Diwan and Nandakumar, 1998; Gopalakrishnan *et al.*, 2000). The spermatozoa are very sensitive to changes in osmotic pressure due to dilution and this would cause morphological alterations including rupture of membrane, swelling and disruption of mid-piece (Billard, 1983). The abnormalities in *L. parsia* sperm were more pronounced when diluted with fresh wa-



Figures

1. *Normal spermatozoa - nucleus, nuclear fossa and flagellum visible*
2. *Osmotic shock - plasma membrane lost completely*
3. *Chao's extender, zero seconds after dilution - nucleus, mid-piece and plasma membrane intact*
4. *Rana and McAndrew extender, 10 minutes equilibration time- mitochondria ruptured*
5. *Mixture C extender, after exposure to liquid nitrogen vapour - plasma membrane and mitochondria disintegrated*
6. *V2E extender, after plunging in liquid nitrogen - nucleus and nuclear fossa intact, similar to normal spermatozoa*

ter, Rana and McAndrew extender and mixture C. The ultrastructural changes in the spermatozoa of *L. parsia* following dilution in different extenders after 10 minutes equilibration time and expossure to fresh water are similar to those described in other species like rohu, salmonids and guppy (Lahnsteiner *et al.*, 1996; Gopalakrishnan *et al.*, 2000). The rate of abnormal and damaged sperms increased progressively in the extenders mentioned above in different treatments (Fig.7).

Considerable alterations particularly those visible in the nucleus (condensation of chromatin) and mid-piece deserve attention especially while selecting a suit-

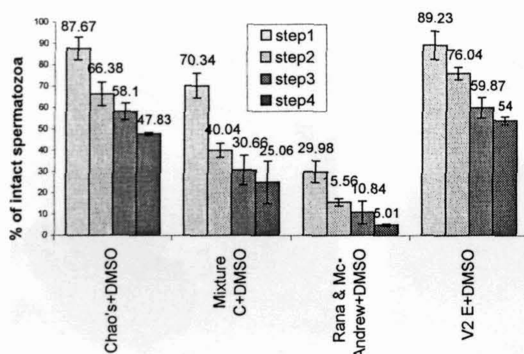


Fig. 7. *Percentage of intact spermatozoa of* Liza parsia *after each step in various extenders + 10% DMSO*

able cryodiluent for mullet sperms. Fish sperm motility is closely related to the existence of mitochondria in mid-piece (Gwo, 1995). The morphology of spermatozoa did not differ significantly in undiluted milt and sperms diluted with V2E and to a lesser extent in Chao's extender. This may be due to exposure of milt to a suitable acceptable extender having proper concentration of salts and DMSO and the ideal dilution ratio. Motility studies carried out also supported the above observation indicating the suitability of V2E and 10% DMSO as an ideal cryodiluent for mullet milt.

In the present study, a final volume 10% DMSO was selected as this was found to be an optimum concentration for many teleost species (Gopalakrishnan *et al.*, 2000). Relatively high percentage of intact sperms in 10% DMSO – V2E combination was obtained. Yao *et al.* (1995) in Ocean Pout reported that the degree of ultrastructural integrity of spermatozoa can be an indicator of semen quality and fertilization ability. They compared the ultrastructural images of unfrozen raw milt and frozen-thawed diluted milt in various extenders and found a positive correlation between fertility percentage and percentages of normal mitochondria, nucleus, mid-piece and perforatoria. In the present study, similar results were obtained with raw milt and frozen-thawed milt in V2E-DMSO (10%) combination.

A uniform rate of 10% DMSO as cryoprotectant was maintained in all the extenders studied but the results varied among different extenders. The cryoprotectant-extender combination is the decisive factor rather than the cryoprotectant alone. DMSO diluted in V2E had provided a better protection to spermatozoa as evidenced by less damages in TEM images and comparatively higher rate of intact spermatozoa (54.0±1.76) after thawing. The Chao's ex-

tender offered cryoprotection to a lesser extent compared to V2E clearly indicating that the ability of a cryoprotectant to prevent injury can vary with different extenders. Similar results were also reported in rohu (Gopalakrishnan *et al.*, 2000).

From the present investigations, V2E with seawater as base and 10% DMSO is selected as the best cryodiluent for *L. parsia* sperms, as in this cryodiluent the spermatozoa exhibited least structural changes at each step of cryopreservation protocol. Chao's extender with 10% DMSO is rated as the second best as this extender maintained structural integrity up to the third stage. The final confirmation of these extenders as best for mullets can be done only after combining with actual fertility tests.

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