Indian J. Fish., 47(1) : 21-27, Jan.-Mar., 2000

Fine structural changes of Rohu (*Labeo rohita*) sperm after dilution with cryoprotectants

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ABSTRACT

Spermatozoa of rohu (*Labeo rohita*) were diluted in two cryodiluents and fine structural changes were investigated. Damages were observed in head, mid-piece and tail of about 51% spermatozoa immediately after dilution in one of the cryodiluents. After 10 minutes of equilibration period, the frequency of damaged sperms increased to 85%. The other diluent did not exhibit significant structural changes unlike the former one and nearly 60% sperms remained visibly intact even after 10 minutes of equilibration time. The present investigation points out (1) diluents inhibiting sperm motility alone need not always be ideal for a species and (2) usefulness of ultrastructural images of fish sperms in determining the fertilizing ability of milt and in preliminary screening and selection of cryodiluents for deep-freezing programmes.

Introduction

Efficient methods for long-term storage of fish spermatozoa play an important role in aquaculture and conservation. Therefore cryopreservation of fish milt has been the subject of many investigations (Scott and Baynes, 1980; Stoss, 1983; Billard, 1988). However, results are often variable and satisfactory fertility rates in most cases can be obtained only by using large amounts of milt (Billard, 1988). In spite of many communications on how to successfully cryopreserve fish milt of more than 30 families of fishes, (see reviews by Stoss, 1983; Leung and Jamieson, 1991), there has been little study of changes in morphology which might occur during dilution, freezing and thawing (Billiard, 1983; Gwo and Arnold, 1992; Lahnsteiner *et al.*, 1992, Drokin *et al.*, 1998. Recently it has been shown that the dilution of milt with cryodiluents, freezing and subsequent thawing cause structural changes such as deformed nucleus and acrosome and rupture of membranes in the spermatozoa of various teleosts including rainbow trout, Atlantic croaker and grayling (Billiard, 1983; Gwo and Arnold, 1992; Lahnsteiner *et al.*, 1992, Drokin *et al.*, 1998). These damages ultimately result in reduced spermatozoan motility and fertility (Gwo and Arnold, 1992).

Indian major carps (catla, rohu and mrigal) have been identified as one of the target groups for milt cryopreservation and gamete banking by NBFGR and among these, rohu formed the species for in-depth investigation.

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Fertilization experiments carried out previously using frozen-thawed milt of rohu revealed (1) variations in hatching rates between different extenders but using the same cryoprotectant and dimethyl sulphoxide (DMSO - final concentration 10% v/v) and (2) reduced motility of sperms on dilution and post-freezing. These findings prompted us to initiate a series of detailed examination of the possible fine structural changes in the spermatozoa during pre-freezing and post-freezing. The present work in rohu – forming the first part of the series – was designed to (1) investigate the possible fine structural changes in spermatozoa during dilution of milt using extender-cryoprotectant mixture prior to freezing; and (2) to infer the possible causes of low motility and reduced fertility after dilution.

Material and methods

Specimens of *Labeo rohita* were caught from the stock of Triveni hatchery and Tendua fish farm, Meja approximately 25 km away from Allahabad, U.P. Sperm production was induced by the administration of "Ovaprim" at the rate of 0.2 - 0.3 ml/ kg body weight of the fish during peak breeding season. Milt from each individual was stripped into separate plastic boxes held over ice, taking care to avoid contamination of water, blood, urine and faeces. Milt samples exhibiting more than 80% motile sperms upon microscopic examination were pooled and used for experiment.

Dilution of rohu milt in cryo-diluents : From our preliminary sperm motility analysis, fertilization and cryopreservation experiments, it was found that the extender CC_1 of Kurokura *et al.* (1984) containing NaCl 750 mg; KCl 20 mg; NaHCO3 20 mg and $CaCl_2$ $2H_2O$ 20 mg per 100 ml of distilled water (pH 7.3) and DMSO (final concentration 10%) was a suitale cryodiluent for long term cryo-preservation of rohu spermatozoa in liquid nitrogen (LN_2) producing hatching rates equal to that of control ones whereas the extender developed by Rana and Mc Andrew (1989) containing NaCl 650 mg; KCl 300 mg, $CaCl_2$ 30 mg and $NaHCO_3$ 20 mg per 100 ml (pH 7.3) and DMSO (final volume of 10%) was not an ideal one for this species. The final ratio of milt : extender : DMSO used was 1.0 ml : 2.6 ml : 0.4 ml in both the cases. In pre-freezing toxicity tests also, milt diluted in the former extender and DMSO exhibited better motility on activation and produced higher hatching percentages compared to the latter extender – DMSO milt combination.

Ultrastructural investigations to study the effect of dilution on rohu milt : To study the effect of dilution, the extended milt was not frozen. The spermatozoa (1) 30 sec. after mixing with extenders and DMSO; (2) after completion of 10 min. equilibration period (i.e., 10 min. following the addition of extender and DMSO) and (3) freshly collected raw milt (control) were processed for TEM and viewed as follows:

Spermatozoa (SPZ) of rohu were fixed in 2% glutaraldehyde buffered to pH 7.3 using 0.1 M cacodylate buffer, over ice for 5 hours. They were then centrifuged (2000 g) at 5°C for 10 minutes. The pellet was then post-fixed in freshly prepared 1% osmium tetroxide in 0.1 M cacodylate buffer, dehydrated in ethanol and embedded

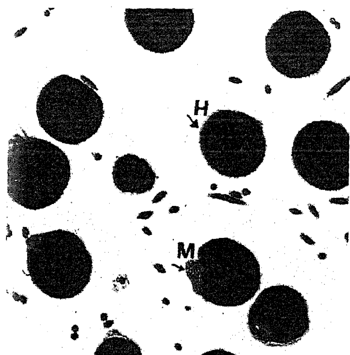

Fig. 1. Transmission Electron Microscope (TEM) image of rohu spermatozoa (SPZ) in raw milt (control). H-Head; M-mitochondria in middle piece. (x 7900).

in firm standard Spurr's resin. Ultrathin sections were stained with 2% uranyl acetate followed by 0.4 % lead citrate and were examined under transmission electron microscope (TEM – Philips CM – 10) and photographed. In all experiments, spermatozoa from each sample were examined utilising TEM photographs. Particular attention was given to damage of the plasma membrane, mitochondria, axial tubules and nuclear material. The ratio of intact and damaged spermatozoa for each treatment was calculated by counting sperms under low magnification (3900 X) in TEM. In this process, randomly selected portions of grids (at least 3 grids for each treatment) were brought under magnification and altogether 150 spermatozoa were screened for each treatment. TEM images of untreated raw milt served as control.

Results

After immersion in cryodiluents (CC_1 & DMSO; Rana and Mc Andrew-DMSO) morphological alterations occu-rred in rohu spermatozoa. The changes were more pronounced in sperms treated with Rana and McAndrew extender - DMSO. The results are summarised in Table 1.

Dilution with Rana & McAndrew extender-DMSO

After an exposure of 30 sec. to this solution, about 50% of spermatozoa exhibited structural changes (Fig. 3) compared to undiluted intact milt (Fig. 1). The middle piece was greatly reduced and sometimes displaced laterally. The head region of many sperms lost their shape and become either rectangular or conical (Fig. 3). The electron dense nucleus exhibited vacuoles in it (Fig. 4). The membrane covering the head region and flagellum appeared detached. After 10 min. of equilibration time, further alterations became evident. Almost 85% of spermatozoa became abnormal and exhibited amoeboid, triangular or hour-glass shaped (Figs. 4, 6, 7). The flagellar axis itself coiled up. The middle piece containing mitochondria was totally deformed.

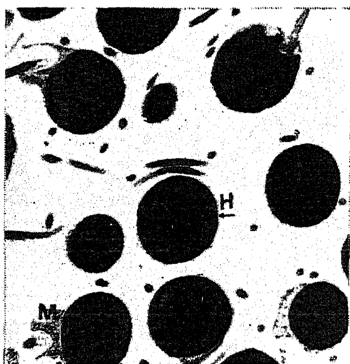

Fig. 2. TEM image of rohu SPZ 30 sec. after dilution in CC_1-DMSO mixture. H-Head; M-mid piece (x 10,500).

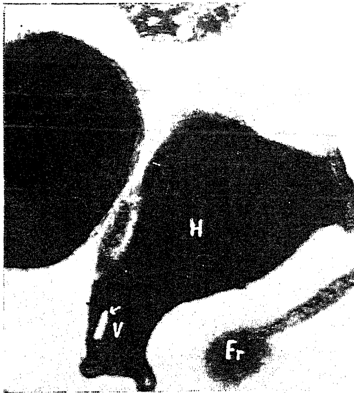

Fig. 7. Enlarged view of rohu SPZ 10 minutes after addition of Rana & McAndrew - DMSO mixture. Fr - ruptured flagellum; H - Head; V - vacuole. (X 25,000)

spermatozoa in inactive condition, but failed to produce comparable results in fertilization experiments using eggs of same lot. This indicated that extenders inhibiting the sperm motility alone need not always be an ideal one for a species. To assess the efficacy of extenders, fertilization experiments may become undependable (due to varying egg quality), expensive (brood-fish maintenance) and impractical (annual breeders with short breeding season). Trypan blue exclusion tests and application of supra vital fluorochromes to discriminate live and dead sperms (in both diluted and undiluted milt suspensions) have been developed for "acrosomal" sperms of higher vertebrates (Ji *et al.*, 1981; Luttmer and Longo, 1986). Lack of acrosome and their small size make teleost sperms, unsuitable for the above tests. Eventhough not as fast as vital staining method, ultra structural observation of milt suspensions can be a suitable and reliable alternative not only to cull

Acknowledgments

The authors sincerely acknowledge Dr. P. Das, former Director, NBFGR for his encouragement and support during the investigation. The services provided by the Regional Electron Microscope Facility, Department of Anatomy, All India Institute of Medical Sciences, New Delhi, for electron microscopy work is greatly acknowledged.

TABLE 1. *Percentage of intact spermatozoa of rohu* (Labeo rohita) *after dilution*

Diluents	Percentage of intact spermatozoa	
	30 sec. after dilution	10 min. after dilution (completion of equilibration time
Extender CC_1 of Kurokura *et al.* (1984) & DMSO (10%)	70.67±8.33	57.29±5.90
Extender of Rana & Mc Andrew (1989) + DMSO (10%)	48.57±4.61	15.04±2.28

n = 150 spermatozoa (3 grids for each treatment and approx. 50 sperms counted from each grid; magnification 3900X)

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