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# Studies on fresh milt parameters and cellular changes during cryopreservation of spermatozoa of Deccan mahseer *Tor khudree* (Sykes, 1839)

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## ABSTRACT

Studies on the physico-chemical characteristics of seminal plasma, along with ultrastructure and mitochondrial activity of fresh spermatozoa of the endangered Deccan mahseer *Tor khudree* were undertaken. The ultrastructure and mitochondrial activity of fresh spermatozoa were compared with those of cryopreserved-thawed spermatozoa to understand the nature and extent of cryo-damage. Physico-chemical analyses of fresh milt revealed sperm density of 3.93±0.11 x $10^7$ spermatozoa $ml^{-1}$, spermatocrit value of 67.08±1.22%, higher $K^+$ concentration of 13.16±0.121 mg $l^{-1}$, total reducing sugar and total protein concentration of 47.31±0.82 and 19.60±0.66 mg 100 $ml^{-1}$ respectively. Ultrastructure of the fresh spermatozoa by both scanning (SEM) and transmission electron microscopy (TEM) revealed the spherical head without any acrosomal complex, small mid piece with mitochondria and a long tail. Cross section of tail by TEM revealed typical 9+2 doublet arrangement of the axoneme. Head measured 1.86±0.006 µ in dia with a mid-piece length of 0.53±0.012 µ and tail length of 33.53±0.220 µ. Ultrastructural damages to the spermatozoa following cryopreservation included, loosening of chromatin and disruption of the cytoplasmic membrane as compared to that of fresh spermatozoa. Nitroblue tetrazolium (NBT) assay revealed low levels of activity of the enzymes of the mitochondrial complex in cryopreserved-thawed spermatozoa when compared to fresh spermatozoa indicating damage to the functional integrity of the enzymes of the mitochondrial enzyme complex.

Keywords: Cryopreservation, NBT assay, Physico-chemical characteristics, Spermatozoa, *Tor khudree*, Ultrastructure

## Introduction

Physico-chemical characteristics of fish milt determine the fertilisation ability of spermatozoa (Rurangwa *et al*., 2004). Fish seminal plasma contains various organic and inorganic components which support the viability of spermatozoa (Hajirezaee *et al*., 2010). Hence, biochemical analysis of seminal plasma plays a significant role in the assessment of milt quality (Billard *et al*., 1995), which has applications related to artificial fertilisation and sperm preservation (Billard, 1978). Also, studies on the ultrastructure of fish spermatozoa are very much useful in understanding the phylogenetic relationships among different species (Jamieson, 1991) and they also help in comprehending damages following cryopreservation (Calvi *et al*., 1994; Lahnsteiner *et al*., 1996). Deccan mahseer, *Tor khudree* belonging to the family Cyprinidae, once formed a major fishery from the rivers and streams of Western Ghats and today, it is enlisted as an endangered species (Ogale, 1994; Basavaraja and Keshavanath, 2000; Dahanukar *et al*., 2004). Attempts for its *ex situ* conservation using cryopreservation of spermatozoa have been made (Basavaraja *et al*., 2002; Patil and Lakra, 2005). For efficient mass production of *T. khudree* fingerlings for ranching purposes and also for development of efficient cryopreservation methodology, it is necessary to study the physico-chemical characteristics of seminal plasma. The present study was undertaken with the objectives of investigating the physico-chemical characteristics of seminal plasma as well as for studying the ultrastructure and mitochondrial activity of fresh spermatozoa in comparison with that of cryopreserved-thawed spermatozoa to understand the nature and extent of cryo-damage.

## Materials and methods

The present study was undertaken in the mahseer farm and hatchery complex of the Tata Power Company, Lonavala, Maharashtra State, bordering Sahyadri range of the Western Ghats.

*Sperm density, spermatocrit value and motility percentage*

Mature males of *T. khudree* caught by gillnetting and in oozing condition were hand stripped. Milt samples not contaminated with water, faecal matter or blood were collected from individual males in separate clean, dry, sterile vials and stored in ice soon after collection. Density of spermatozoa in fresh milt of individual males was estimated, with improved Neubauer Haemocytometer following the method of Ax *et al*. (2000). Method of Rakitin *et al*. (1999) was followed for the estimation of Spermatocrit value. The spermatocrit value of fresh milt of individual males was estimated. For estimation of motility percentage of spermatozoa of individual males, method of Billard *et al.* (1995) was followed. Modified BWW (Ravinder *et al*., 1997) extender was used for milt dilution.

*Elemental composition of seminal plasma*

$Na^+$, $K^+$, $Ca^{2+}$, $Mg^{2+}$ and $Zn^{2+}$ in the seminal plasma were estimated as per Gopalakrishnan *et al*. (1998) using Atomic Absorption Spectrophotometer (AAS) (Electronics Corporation of India Ltd. Model No. AAS 4129). Absorbance was measured at 589.0 nm for Na, 766.50 nm for $K^+$, 422.70 nm for $Ca^{2+}$, 285.20 nm for $Mg^{2+}$ and 213.90 nm for $Zn^{2+}$. Samples were run in triplicates and 10% TCA was used as blank.

*Total reducing sugars and total proteins in seminal plasma*

Total reducing sugars in the pooled, fresh seminal plasma was estimated as per the modified method of Nelson and Somogyi (1945) and total proteins were estimated following Gopalakrishnan *et al*. (1998). The samples were run in triplicates and physiological saline was used as blank.

*Cryopreservation of spermatozoa*

Modified BWW extender and a combination of 9% dimethyl sulfoxide (DMSO) and 11% glycerol as cryoprotectant were used (Patil and Lakra, 2005). Only milt samples with more than 70% motile spermatozoa were subjected to cryopreservation (Lahnsteiner *et al*., 1997). The cryopreserved milt was subjected to fertilisation trials after a storage period of 30 days in $LN_2$.

*Ultrastructure studies*

Scanning electron microscopic (SEM) studies were performed following the protocol of Ghadially (1986) using a Philips SEM Model Number L30 at ICAR-Central Institute for Research on Cotton Technology, Matunga, Mumbai. Morphometric parameters of fresh spermatozoa *viz*., head diameter, length and width of mid-piece and length and width of the tail were measured randomly for 100 spermatozoa. Transmission electron microscopy (TEM) studies were undertaken as per Ghadially (1986) in a Jeol-TEM Model no. Jem-1010 at Jaslok Hospital and Research Center, Pedder Road, Mumbai. Percentage of damaged spermatozoa was estimated randomly for 100 spermatozoa and fresh milt was used as control.

*Sperm mitochondrial activity Iidex (SMAI)*

Physiological changes in the cryopreserved-thawed spermatozoa were evaluated using sperm mitochindrial activity index (SMAI) by nitroblue tetrazolium (NBT) assay following the modified method of Gopalakrishnan *et al*. (1991) and Stasiack and Baumann (1996). The activity of the mitochondrial enzyme system was measured in terms of NBT reduction. The absorbance was measured at 620 nm using ELISA reader (Lab Systems Multiskan MS) with the help of "Genesis" software version 3.03.

*Statistical analysis*

Normality of the data was tested by Box-Plot method. Wherever needed, arcsine and logarithmic (to the base 10) transformations were carried out. Analysis of variance was performed between the mean values of different treatments at 5% level. All the statistical analyses were performed using SAS Analyst Package (Version 8.2).

## Results and discussion

Average sperm density of fresh milt from twelve males was estimated as $3.93\pm0.11 \times 10^7$ spermatozoa $ml^{-1}$ (Table 1). However, a lower sperm density of $7.45 \times 10^6$ spermatozoa $ml^{-1}$ for *T. khudree* was reported by Basavaraja *et al.* (2002). Gupta and Rath (1993) reported sperm densities of 2.0 to 2.5 x $10^7$, 3.0 to 3.25 x $10^7$ and 2.0 to 2.5 x $10^7$ cells $ml^{-1}$ for other cyprinid fish like catla (*Catla catla*), rohu (*Labeo rohita*) and mrigal (*Cirrhina mrigala*) respectively. Average spermatocrit value of fresh milt from twelve males was estimated to be 67.08±1.22 (Table 1). Gupta and Rath (1993) recorded spermatocrit values ranging from 65-75, 75-85 and 65-75% for catla, rohu and mrigal respectively. On the contrary, a very low spermatocrit value of 11.5% was reported by Gasco *et al.* (1999) in rainbow trout (*Oncorhynchus mykiss*), which is also a cold water species. The differences in the findings by different researchers with respect to the sperm density and spermatocrit values may be due to differences in feeding conditions, husbandry procedures, age, environmental factors, spawning time or dilution ratio (Izquierdo *et al*., 2001; Aliniya *et. al*., 2013). It was observed in the present study that only nine males exhibited more than 70% motility and the average motility percentage of spermatozoa from the nine males was 95.01±0.85 (Table 1). The present findings are in agreement with the observations of Basavaraja *et al.* (2002) who reported 95-100% motility of the spermatozoa from fresh milt of *T. khudree*.

Table 1. Density, spermatocrit value and motility% of spermatozoa in fresh milt of *T. khudree*

| Sperm density (x10$^7$ spermatozoa ml$^{-1}$) (Mean ± SE) | % Spermatocrit (Mean ±SE) | % Motile spermatozoa (Mean ±SE) |
|---|---|---|
| 3.93 ± 0.11 | 67.08 ± 1.22 | 95.01±0.85 (only from 9 males with ≥ 70% motility) |

In the present study, it was found that the concentration of $K^+$ in the serminal plasma was higher (13.16±0.121 mg l$^{-1}$) when compared to that of $Na^+$, $Ca^{2+}$, $Mg^{2+}$ and $Zn^{2+}$ (Table 2). This explains the fact that the spermatozoa remain non-motile in the seminal plasma as high levels of potassium are found to inhibit sperm motility (Baynes *et al*., 1981). Similar observations were made by Plouidy and Billard (1982) that the concentration of $K^+$ was higher than the other elements in the seminal plasma of common carp.

Higher concentrations of total reducing sugars at levels of 47.31±0.82 mg 100 ml$^{-1}$ in the seminal plasma were observed in the present study (Table 2). However, Lahnsteiner *et al*. (1994) reported lower concentration of total reducing sugars (glucose, fructose, galactose and xylose) in the seminal plasma of cyprinid fishes varying from 11.06 to 24.75 mg 100 ml$^{-1}$. In some fish species, total reducing sugars in the seminal plasma do not play any role in providing energy for sperm locomotion and the ATP required for motility seems to be pre-accumulated in the spermatozoa (Billard *et al*., 1995). However, Gardiner (1978) reported that in some fish species, sperm motility remains longer, when extracellular source of glucose was provided and this indicates the ability of the spermatozoa to utilise energy-substrates in the seminal plasma. In the present investigation, it was found that the concentration of total proteins in seminal plasma was 19.60±0.66 mg 100 ml$^{-1}$ (Table 3). Kruger *et al*. (1984) reported very low concentration of total proteins in the seminal plasma of common carp (0.04 to 0.38 mg 100 ml$^{-1}$) and tilapia (0.00 to 0.06 mg 100 ml$^{-1}$). However, the findings of the present study are similar to that of Lahnsteiner *et al*. (1994) who observed that the total protein content in the seminal plasma of cyprinid fishes to be ranging from 9.38 to 12.67 mg 100 ml$^{-1}$. It has been reported that the protein content in the seminal plasma varies from species to species (Lahnsteiner *et al*., 1994). Proteins play important role by protecting the spermatozoa by acting as buffers in the fish seminal plasma and help in maintaining the osmotic pressure (Kruger *et al*., 1984).

Table 2. Biochemical composition of the seminal plasma of fresh milt of *T. khudree*

| Chemical constituent | Concentration (Mean±SE) |
|---|---|
| Elements | |
| Sodium (mg l$^{-1}$) | 6.82±0.016 |
| Potassium (mg l$^{-1}$) | 13.16±0.121 |
| Calcium (mg l$^{-1}$) | 0.950±0.010 |
| Magnesium (mg l$^{-1}$) | 0.275±0.004 |
| Zinc (mg l$^{-1}$) | 0.018±0.004 |
| Total reducing sugars (mg 100 ml$^{-1}$) | 47.31±0.82 |
| Total proteins (mg 100 ml$^{-1}$) | 19.60±0.66 |

Table 3. Morphometric characteristics (Mean±SE) of fresh spermatozoa of *T. khudree* as revealed by SEM

| Head diameter (µm) | Mid-piece | | Tail | |
|---|---|---|---|---|
| | Length (µm) | Width (µm) | Length (µm) | Width (µm) |
| 1.86±0.006$^a$ | 0.53±0.012$^a$ | 0.60±0.004$^a$ | 33.53±0.220$^a$ | 0.31±0.005$^a$ |

*Values with different superscripts are significantly different

Scanning electron microscopy (SEM) investigations in cryopreserved-thawed spermatozoa, revealed several morphological deformities which included, winding of the tail, loss of tail, shrunken mid-piece, appearance of verrucosities on the surface of sperm head and severe roughening of the surface of the head (Fig. 1) when compared to that of fresh spermatozoa which revealed spherical head, small mid-piece, a long tail (Fig. 2) with a head diameter of 1.86±0.04 µ and a tail length of 33.53±1.56 µ (Table 4). Similar ultrastructural changes which included, disruption of plasma membrane, loss or winding of tail and appearance of verrucosities were reported by several researchers in SEM studies of cryopreserved-thawed/frozen-thawed spermatozoa of carps (Saad *et al*., 1988; Wei-xin and Ren-Liang, 1991).

Transmission electron microscopy (TEM) studies on the cryopreserved-thawed spermatozoa revealed amoeboid, triangular or irregular shapes, loosening or

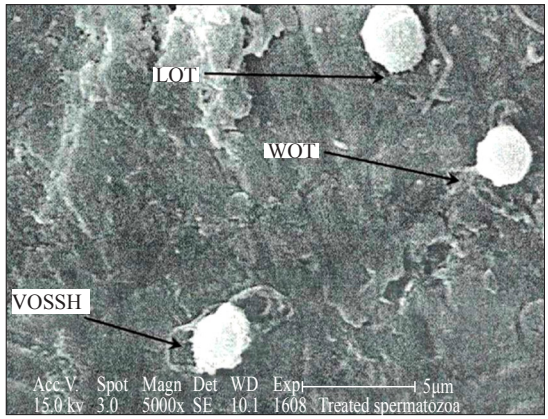


Fig. 1. Scanning electron micrograph of cryopreserved-thawed spermatozoa of *T. khudree* (x 2,500). LOT - loss of tail; WOT - Winding of tail; VOSSH - Verrucosities on the surface of sperm head

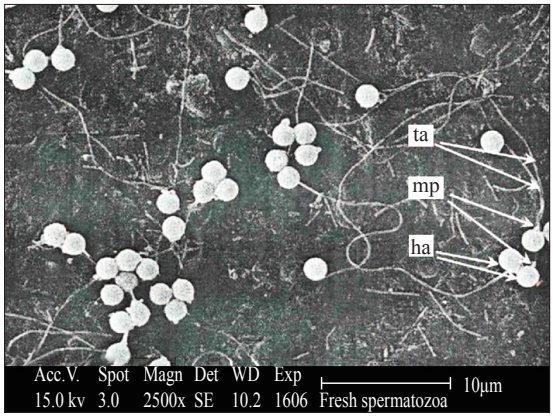


Fig. 2. Scanning electron micrograph of fresh spermatozoa of *Tor khudree* (x 2,500). ha - head; mp - mid piece; ta - tail

Table 4. Percentage of damaged spermatozoa in cryopreserved-thawed milt of *T. khudree* as revealed by TEM

| Milt sample | % of damaged spermatozoa (Mean±SE) |
|---|---|
| Cryopreserved-thawed milt | 62.88±1.53[a] |
| Fresh milt (Control) | 2.17±0.12[b] |

*The values with different superscripts are significantly different

spillage of chromatin material and disruption of the cytoplasmic membrane (Fig. 3, 4) when compared to that of fresh spermatozoa which revealed a spherical head, smaller mid piece, two tiny cytoplasmic extensions on either side of the tail containing mitochondria, posterior end of head with a basal plate and cross section of the tail with typical 9+2 arrangement of tail fibers (Fig. 5, 6, 7) which play an important role in the beating of the tail that leads to the movement of the spermatozoa.

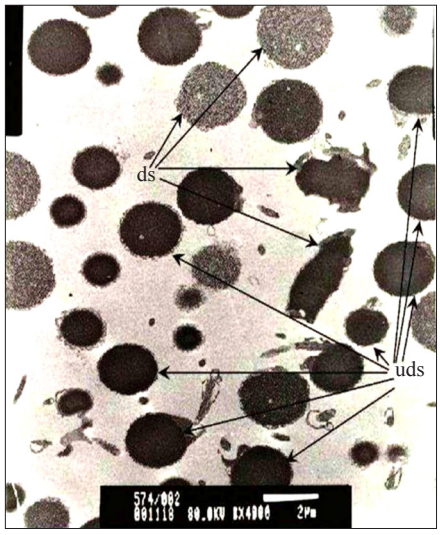


Fig. 3. Transmission electron micrograph showing damaged and intact spermatozoa during thawing (x 4,000). uds - undamaged spermatozoa; ds - damaged spermatozoa

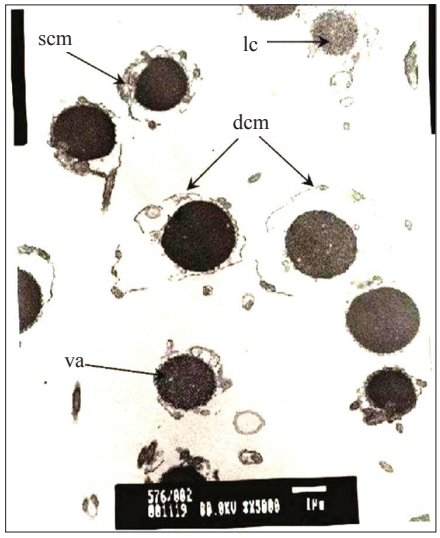


Fig. 4. Transmission electron micrograph showing the damage caused to the spermatozoa of *T. khudree* due to thawing (x 5,000). va - vacuolisation; lc - loosening of chromatin; dcm - disrupted cytoplasmic membrane; scm - spilled chromatin material

Similar ultrastructural damages to the spermatozoa of common carp were recorded by several workers which included detachment of plasma membrane, loss of the central doublet, vacuolisation of nucleus and loosening of the chromatin of spermatozoa of common carp due to cryopreservation-thawing or freezing-thawing cycles (Saad *et al.*, 1988; Drokin *et al*., 2003). Similar findings with a spherical head, smaller mid-piece and the typical 9+2 arrangement of tail fibres in fresh spermatozoa were reported in case of *Morone saxatilis* (He and Woods III, 2004)

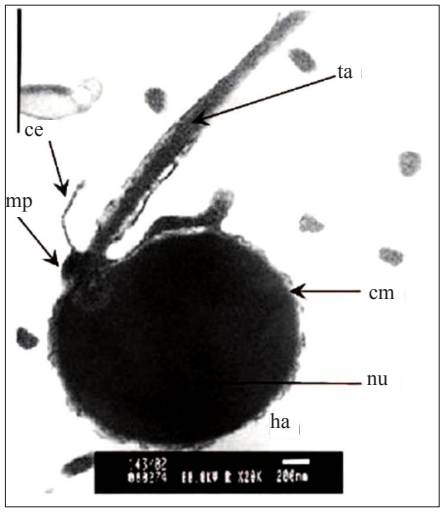


Fig. 5. Transmission electron micrograph of fresh spermatozoa of *T. khudree* (x 20,000). ha - head; mp - mid piece; ta - tail; nu - nucleus; cm - cytoplasmic membrane; ce - cytoplasmic extension

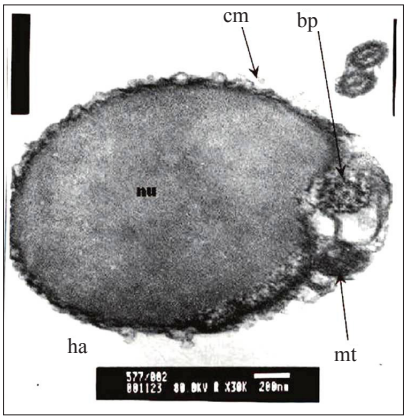


Fig. 6. TEM image of fresh spermatozoa of *T. khudree* (x 30,000). ha - head; nu - nucleus; cm - cytoplasmic membrane; bp - basal plate; mt - mitochondria

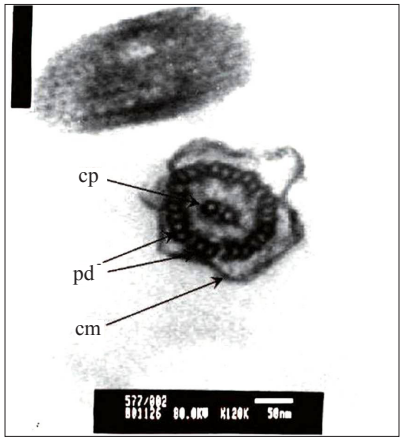


Fig. 7. TEM image of fresh spermatozoa of *T. khudree* showing cross section of the tail (x 1,20, 000). Pd - peripheral doublets; cp - central pair; cm - cytoplasmic membrane

and *Sander lucioperca* (Kristian *et al.*, 2014). In the present TEM studies, percentage of damaged spermatozoa after the cryopreservation-thawing cycle was estimated to be 62.88±1.53 (Table 5). Results of the present study are in agreement with those reported by Lahnsteiner *et al.* (1992) who observed 40 - 50% damaged spermatozoa after cryopreservation-thawing cycle in various fish species.

NBT assay, widely used for assessing SMAI which is considered as the indicator of functional integrity of mitochondria of human spermatozoa (Gopalakrishnan *et al*., 1991; Bergstrom *et al*., 2013), was for the first time successfully applied to fish spermatozoa in the present study. The absorbance values for cryopreserved-thawed spermatozoa were significantly lower than that of fresh spermatozoa in the present study ($p<0.05$) which give clear indication that cryopreservation-thawing cycle causes severe damage to the structural and functional integrity of enzymes of the mitochondrial-enzyme complex, which is responsible for the generation of ATP required for motility of the spermatozoa. Based on the results of the present study, it is suggested that better cryopreservation protocols need to be developed aiming at significant reduction in damages due to cryopreservation-thaw injury in spermatozoa of *T. khudree* and fish spermatozoa in general. Besides results of the physico-chemical parameters of serminal plasma from the present study indicate scope for formulating better extenders for cryopreservation of spermatozoa of *T. khudree*.

Table 5. Absorbance (OD) values of cryopreserved-thawed spermatozoa of *T. khudree* during NBT assay

| Milt sample | Absorbance (Mean±SE) |
|---|---|
| Cryopreserved-thawed milt | 0.087±0.002[b] |
| Fresh milt (Control) | 0.38±0.008[a] |

*Values with different superscripts are significantly different

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