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The Fertility of Cryopreserved Deccan Mahseer, *Tor khudree* (Sykes) Spermatozoa

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Abstract

The present study was undertaken with a view to develop a simple practical protocol for the cryopreservation of spermatozoa from the endangered mahseer (*Tor khudree*, Cyprinidae), a well-known sport fish of India.

The study reports on the fertility of cryopreserved *T. khudree* spermatozoa. Immotile spermatozoa samples pooled from several males were diluted with fish Ringer's solution (pH: 7.48) and protected with dimethyl sulfoxide (DMSO) at 5, 10 and 15%, each being subjected to equilibration periods of 10, 20, 30, 60, 70, 80 and 90 min. Diluted samples were stored in 500 µl plastic straws and cooled over liquid nitrogen (LN_2) and preserved in LN_2 up to 70 days. Thawing of samples was accomplished by swirling in water at 37±1°C for 10 sec. Viability of post-thawed spermatozoa was determined based on motility rate, fertilization and hatching rates of eggs and fry survival.

Very high post-thaw motility rates (92 to 98%) were obtained with 5 and 10% DMSO, equilibrated for 10, 20 and 30 min, while 15% DMSO at three equilibration periods significantly reduced ($P < 0.05$) post-thaw motility of spermatozoa stored up to 70 days. Very high fertilization rates of eggs were observed and interestingly, they were independent of cryoprotectant level and equilibration time. Spermatozoa protected with 15% DMSO at equilibration periods of 30, 60 and 90 min showed significantly higher ($P < 0.05$) hatching rates up to 41% as compared with 10 or 20 min. Maximum fry survival was obtained when spermatozoa were equilibrated up to 30 min in all three levels of DMSO. Fry produced from cryopreserved spermatozoa were normal.

The fertility studies indicate that *T. khudree* spermatozoa can be successfully cryopreserved using fish Ringer and 5 to 15% DMSO at an equilibration time of 10 to 90 min and viable fry is produced. Being the first report on the successful production of viable fry of mahseer from cryopreserved spermatozoa, this study will pave the way for the creation of a sperm bank for *T. khudree*.

Introduction

With the rapid global expansion of aquaculture, there is a need for year-round availability of larvae that could, to some extent, be met through cryopreservation of gametes of cultivated species. Sperm cryopreservation protocols are now available for over 200 species of finfish and shell fish (Scott and Baynes 1980, McAndrew et al. 1993 and Billard et al. 1995). Studies on cryopreservation of invertebrate eggs, embryos and larvae have also been met with success (McAndrew et al. 1993, Subramoniam and Arun 1999). However, techniques for the cryopreservation of fish eggs/embryos have not been developed. Cryopreservation plays an important role in the production of new strains/breeds of economically important species and in the conservation of wild stocks of threatened aquatic species. In this context, a few `sperm banks' have been created, notably for grouper, salmonids and Indian cultivated and endangered fish species (Chao et al. 1992, Rana 1995, Ponniah 1998).

Mahseers are a group of freshwater fishes inhabiting fast flowing streams and rivers of hilly regions in India. They are well-known game and food fish and once attracted anglers all over the world. Over the years, natural stocks of mahseer have depleted owing to anthropogenic activities, and the Deccan mahseer (*Tor khudree*) has been declared as a threatened species. To conserve and rehabilitate *T. khudree*, several *in situ* and *ex situ* conservation strategies have been suggested (Basavaraja and Keshavanath 2002). Recent studies conducted in the southern part of India have led to the development of a hatchery technology for *T. khudree* (Nandeesha et al. 1993, Keshavanath 2001), which forms one strategy of *in situ* conservation.

On the other hand, cryopreservation of mahseer spermatozoa is receiving more attention. Ponniah et al. (1992) cryopreserved *T. khudree* and Tor hybrid spermatozoa and obtained 80 and 70% post-thaw motility, respectively after 11 months of storage in liquid nitrogen (LN_2). Basavaraja et al. (1998) successfully cryopreserved *T. khudree* spermatozoa and activated them with a high motility rate of 93.75% after 70 days of preservation in LN_2. Ponniah et al. (1999a) produced viable hatchlings of *T. khudree* from one year-old cryopreserved milt. Similarly, Ponniah et al. (1999b) obtained viable hatchlings of *T. putitora* from spermatozoa cryopreserved for one year. Isswara et al. (1999) have suggested guidelines for establishing commercial sperm banks in India.

Detailed studies on the viability/fertility of cryopreserved *T. khudree* spermatozoa in terms of fertilization rate, hatching rate and fry survival are lacking. Major problems associated with mahseer seed production are low fecundity (1,000 to 3,000 eggs per kg body weight), short spawning season (July to October), manual stripping of eggs and high mortality of eggs and larvae (Nandeesha et al. 1993, Basavaraja and Keshavanath 2000) and inconsistency of results of earlier studies on this subject. With this background, the present study was undertaken to develop a viable protocol for the cryopreservation of the endangered *T. khudree* spermatozoa.

Materials and Methods

Collection of spermatozoa

Spermatozoa were collected from *T. khudree* (200 to 500 g) males (1+ years) maintained in 50 m^2 cement cisterns at the fish farm of the College of Fisheries, Mangalore, India. The brood stock was fed daily on a pelleted diet (ingredients: ground nut oil cake 24%, rice bran 20%, rice flour 20%, fish meal 25%, tapioca flour 10% and minerals and vitamins 1%). Sterile cryovials of 1.8 ml were used to collect milt. Prior to collection of milt, the region around the genital opening was cleaned using a filter paper to remove water, mucus, urine and faecal material. While applying gentle abdominal pressure, the spermatozoa were stripped either directly into the vials or drawn first into chilled disposable syringe (2.0 ml) then transferred to the vials. The collected milt was held on ice and immediately transferred to the laboratory for observation.

The quality of fresh spermatozoa was determined by placing a small drop of milt (10 µl) on a glass slide, mixed with 100 to 200 µl unchlorinated tap water and observed under an inverted microscope (x400, Olympus). Spermatozoa that showed no motility before activation, but with 90% or more motility after activation were selected for cryopreservation studies.

Extender, cryoprotectant and equilibration time

Among the different extenders tested by Basavaraja et al. (1998), modified fish Ringer solution containing 5 to 15% DMSO as a cryoprotectant (pH: 7.48) was used as an extender (Table 1). This diluent was chosen for this study because it produced the highest post-thaw motility of cryopreserved spermatozoa of *T. khudree.*

Preparation of spermatozoa samples for cryopreservation

After ascertaining the quality of spermatozoa, the milt pooled from a few males was diluted with the extender at a ratio of 1:10 or 20, afterwards the required quantity of cooled DMSO (< 20^{0}C) was added to produce a final concentration of 5, 10 or 15%. The diluted spermatozoa samples were drawn into labeled 500 µl transparent plastic straws (French medium design Indian straws), sealed using a sealing powder (PVA powder) and equilibrated for 0, 10, 20, 30, 40, 50, 60, 70, 80 or 90 min at 25°C. Each treatment had three replicates. After the specified equilibration period, the straws were cooled at a height of 5 cm over liquid nitrogen (LN_2) vapor in a styrofoam box (18 x 11 x 5 cm) for

Table 1. Chemical composition of modified fish Ringer's solution used for cryopreservation of mahseer spermatozoa

Chemical	g
NaCl	0.75
KCl	0.10
$CaCl_2$	0.016
$MgSO_4$	0.023
$NaHCO_3$	0.04
NaH_2PO_4	0.041
Glucose	0.10
Distilled water (ml)	100
PH	7.48

10 min. The cooled straws were immediately plunged into LN_2 for 1 to 2 min then transferred quickly to canisters and stored under LN_2 for 70 days.

Estimation of post-thaw motility

Frozen spermatozoa were thawed quickly by plunging straws into a cooler box with water maintained at 37 $\pm$ 1^0C for 5 to 10 sec. The cooling and thawing rates were monitored using a temperature probe (Salvin, India). Motility estimates were made visually using an inverted microscope. Percent motile sperm was used as the criterion for judging the quality of spermatozoa. In all cases, the frozen spermatozoa became motile immediately after thawing and hence were not activated by any activating solution prior to fertilization.

Fertilization studies

Field trials were conducted at the Harangi Fish Farm to determine rates of fertilization, hatching and fry survival using spermatozoa cryopreserved up to 70 days. For these trials, the spermatozoa stored in LN_2 were transported to Harangi, 180 km away from Mangalore. The female brood stock of *T. khudree* (1.5 to 2.5 kg) were injected with Ovaprim and eggs were obtained by artificial stripping. The eggs were divided into several groups of 500 to 550. Each group was taken in a clean dry plastic basin, fertilized (dry method) with 450 µl of preserved or fresh spermatozoa with no activating solution. The concentration of spermatozoa of fresh milt was similar to that of cryopreserved milt. The motility of both preserved and fresh spermatozoa (control) was checked prior to fertilization. The egg and spermatozoa ratio was approximately 1:1000 in all treatments, including control. The developing eggs were rinsed with filtered water 15 min after fertilization and kept for water hardening. Fertilization rate was calculated at 2 or 4 cells stage using a dissection microscope.

Hatching and fry rearing

The developing eggs at early morula were transported by road from Harangi to Mangalore in 18 l capacity polythene bags under oxygen. The eggs were incubated in conico-cylindrical fiberglass tanks (60 l) with 1/3 water and aeration (vortex blower). Hatching was completed in 3½ days at 26 to 27°C; hatching rate was calculated based on the number of fertilized eggs and expressed as percentage. The yolk-sac fry took about 4 days for yolk-sac absorption after which they were fed with chicken egg custard (coagulated) and minute plankton. Survival from hatching to 15-day-old fry was also determined.

Analysis of data

Spermatozoa motility, fertilization, hatching and fry survival rates expressed as percentages were first angular transformed and then analyzed

using ANOVA technique. Statistical analyses were carried out using MINITAB PACKAGE.

Results

Spermatozoa count in freshly collected milt was about 7.45 x 10^6 and were motile (95 to 100%) for 1 to 2 min after activation using tap water. The spermatocrit value, an indicator of the concentration of sperm in the milt, was found to be 1:4.6 to 5.0 (fluid: packed sperm cells).

Motility rate

Post-thaw motility of *T. khudree* spermatozoa is presented in table 2. High post-thaw motility rates (92 to 98%) were obtained with 5 and 10% DMSO, equilibrated for 10, 20 and 30 min and were not significantly different ($P > 0.05$), while motility was significantly reduced ($P < 0.05$) at 15% DMSO at all the three equilibration periods, as compared with 5 and 10% after 70 days of storage of spermatozoa in liquid nitrogen. Samples containing 15% DMSO and equilibrated up to 60 and 90 min, led to significantly higher ($P < 0.05$) motility rate when compared to 10, 20 and 30 min. Spermatozoa stored without DMSO showed no motility. Equilibration periods of

Table 2. Effect of cryoprotectant level and equilibration time on post-thaw motility* (%) (absolute)

Cryoprotectant (%)	Equilibration time (min)				
	10	20	30	60	90
5	91.67^{b1}±1.67	95.00^{b1}±0.00	95.00^{b1}±0.00	-	-
10	96.67^{b1}±1.67	96.67^{b1}±1.67	98.33^{b1}±1.67	-	-
15	26.67^{a1}±1.67	33.33^{a1}±1.62	28.33^{a1}±1.67	51.67^{2}±1.67	48.33^{2}±1.67

Values with different superscripts i.e., letters and numbers within columns and rows respec tively, are significantly different ($P < 0.05$).
Control: 98.33±1.67
*Mean±SE

Table 3. Effect of cryoprotectant level and equilibration time on fertilization rate* (%) (absolute)

Cryoprotectant (%)	Equilibration time (min)				
	10	20	30	60	90
5	98.95^{a1}±0.49	98.81^{a1}±0.15	98.80^{a1} ±0.12	-	-
10	98.96^{a1}±0.18	99.01^{a1}±0.18	99.56^{b1} ±0.15	-	-
15	98.15^{a1}±0.14	98.48^{a1}±0.12	99.09^{ab2}±0.14	99.19^{2}±0.15	99.06^{2}±0.11

Values with same superscripts i.e., letters and numbers within columns and rows, respectively, are not significantly different ($P < 0.05$).
Control (fresh spermatozoa): 98.37±0.19
*Mean± SE

0, 40, 50, 70 and 80 were also tested for the three levels of DMSO and motility rates showed a linear trend. This aspect could not be investigated for fertility studies due to shortage of eggs.

Fertilization rate

Data on the effect of cryoprotectant and equilibration time on fertilization rate are given in table 3. High fertilization rates (98.15 to 99.56%) were obtained in all the treatments and surprisingly, they were independent of cryoprotectant level and equilibration time.

Hatching rate

Results of the effect of cryoprotectant level and equilibration time on hatching rates are shown in table 4. Spermatozoa protected with 15% DMSO at equilibration periods of 30, 60 and 90 min and stored up to 70 days, had significantly higher ($P < 0.05$) hatching rates than 10 or 20 min. Interestingly, 15% DMSO equilibrated for 90 min produced significantly higher ($P < 0.05$) hatching rate of 40.8% against 26.4% of control. The hatching rate, however showed slight variation between other groups, with 10 min equilibration time recording generally higher hatch rates.

Table 4. Effect of cryoprotectant level and equilibration time on hatching rate* (%) (absolute)

Cryoprotectant (%)	Equilibration time (min)				
	10	20	30	60	90
5	$17.70^{a3}\pm0.14$	$12.96^{a1}\pm0.18$	$15.52^{b2}\pm0.36$	-	-
10	$18.83^{a3}\pm0.23$	$15.81^{b2}\pm0.29$	$11.43^{a1}\pm0.44$	-	-
15	$16.92^{a1}\pm0.20$	$14.41^{ab1}\pm0.81$	$25.70^{c2}\pm0.77$	$27.70^{2}\pm0.76$	$40.81^{2}\pm0.82$

Values with different superscripts i.e., letters and numbers within columns and rows, respectively, are significantly ($P < 0.05$) different.
Control: 26.36 ± 0.36
* Mean$\pm$ SE

Table 5. Effect of cryoprotectant level and equilibration time on fry survival* (%) (absolute)

Cryoprotectant (%)	Equilibration time (min)				
	10	20	30	60	90
5	$50.95^{a2}\pm0.87$	$45.97^{a1}\pm0.72$	$68.9^{a3}\pm0.36$	-	-
10	$65.11^{b1}\pm0.87$	$66.32^{c1}\pm1.20$	$69.06^{a1}\pm1.15$	-	-
15	$65.70^{b2}\pm0.65$	$52.56^{b1}\pm0.31$	$74.64^{b3}\pm0.86$	$65.80^{2}\pm0.92$	$64.25^{2}\pm0.64$

Values with different superscripts i.e., letters and numbers within columns and rows, respectively, are significantly ($P < 0.05$) different.
Control: 77.36 ± 0.77
*Mean$\pm$SE

Fry survival

Data on fry survival is presented in table 5. Fry survival was highest at an equilibration period of 30 min for all the three levels of DMSO. Among the different treatments, spermatozoa protected with 15% DMSO and equilibrated for 30 min showed the highest fry survival (74.6%), which was close to that of control. The fry produced from cryopreserved spermatozoa were as normal as those obtained from fresh milt, barring 60 and 90 min of equilibration period where a few deformed yolk-sac larvae were observed.

Discussion

The study presents a useful protocol for the cryopreservation of mahseer (*T. khudree*) spermatozoa. Preliminary trials (reported earlier) on cryopreservation of *T. khudree* spermatozoa indicated the suitability of modified fish Ringer's solution for this species (Basavaraja et al. 1998). This extender was found to be suitable for the cryopreservation of spermatozoa of *Oreochromis mossambicus* (Harvey 1983) and *O. niloticus* (Rana et al. 1990). On the other hand, Ponniah et al. (1992) used extenders containing NaCl (0.4%), $NaHCO_3$ (0.8%) and egg yolk (10%) and obtained 80% post-thaw motility (absolute) of *T. khudree* spermatozoa cryopreserved up to 11 months. A number of extenders with varying chemical composition and complexity have been successfully used for the cryopreservation of gametes (Scott and Baynes 1980, McAndrew et al. 1993). Lakra and Krishna (1997) found tris-egg yolk with glycerol as cryoprotectant to be a better extender for the cryopreservation of common carp sperm. Among the seven extenders, egg yolk citrate produced the highest post-thaw motility and fertilizing ability of cryopreserved Indian major carp spermatozoa (Kumar 1986). The freezing and thawing protocol followed in this study was simple and field-oriented. Similar simple freezing and thawing protocols have been successfully used for gene banking of India's cultivated and threatened fish species (Ponniah 1996).

The results demonstrate that DMSO up to 10% has no toxic influence on the motility of spermatozoa cryopreserved up to 70 days, barring 15%, which affected the post-thaw motility of spermatozoa equilibrated for 10 to 90 min. Similarly, Basavaraja et al. (1998) obtained decreased post-thaw motility of spermatozoa at 15% DMSO, equilibrated for 30 to 90 min, but the decrease was not very much pronounced. On the contrary, the same trend was not reflected in terms of fertilization, hatching and fry survival rates, which were equal or higher as compared to those of 5 or 10% DMSO. The differential motility response may be attributed to poorer quality of milt as it belonged to a different batch. Ponniah et al. (1992) obtained 80% motility of *T. khudree* spermatozoa when protected with glycerol

up to 11 months as compared to DMSO, which resulted in 50% motility. While Ponniah et al. (1999a) observed variation in motility and hatching percentage with different cryoprotectants in *T. khudree,* Ponniah et al. (1999b) found no significant difference between different cryoprotectants or equilibration timings (45 to 175 min.) in *T. putitora.* Published literature reveal that there is generally no need for long equilibration of fish spermatozoa because the cells are very small, which facilitate penetration of cryoprotectants (Legendre and Billard 1980; Leung and Jamieson 1991). However Harvey (1983) and Rana and McAndrew (1989) observed methanol to be a better cryoprotectant for cryopreservation of tilapia spermatozoa. Prolonged exposure of spermatozoa to cryoprotectant at higher levels may lead to reduced motility (Stoss and Holtz 1983).

In the present study, higher DMSO level (15%) and equilibration periods (60 to 90 minutes) did not have any adverse effect on the fertilization, hatching and fry survival rates, although 15% DMSO clearly reduced post-thaw motility of spermatozoa equilibrated for 10 to 90 min. Rana et al. (1990) observed that equilibration periods between 30 and 90 min had no significant effect on the post-thaw motility of *O. niloticus* spermatozoa.

In the present study, the absolute fertilization rates obtained were similar and did not differ significantly between different DMSO levels, equilibration periods and control. Similar results were also obtained in salmonids by Lahnsteiner and Patzner (1996). Rana and McAndrew (1989) recorded mean fertilization rates ranging from 38.7 to 93.4% of control after 13 months of storage of tilapia spermatozoa. The spermatozoa having 15% DMSO equilibrated for 30, 60 and 90 min resulted in significantly higher hatching rates than 10 or 20 min, the highest equilibration time leading to a hatching rate higher than that of control in the present study. A maximum hatching rate of only 13.6% was obtained for *T. khudree* spermatozoa cryopreserved up to one year (Ponniah et al. 1999a). The hatching rate could have been higher if the eggs had been aerated during transportation. Even in normal circumstances, hatching rates rarely exceed 60% (Nandeesha et al. 1993). In all the three levels of DMSO, the survival of fry (15 days old) was maximum at an equilibration time of 30 min and the fry produced from cryopreserved sperm were as normal as those obtained from fresh milt in our study.

Conclusion

The results of the present study indicate that the spermatozoa of *T. khudree* could be successfully cryopreserved using modified fish Ringer's solution and DMSO, with high post-thaw motility rates. The fertility studies reveal that high fertilization, hatching and fry survival rates could be obtained and viable fry could be produced from cryopreserved spermatozoa that will ultimately help establish a frozen sperm bank for the conservation of the endangered *T. khudree.*

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