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Cryopreservation of the Endangered Mahseer (*Tor khudree*) Spermatozoa: Effect of Dimethyl Sulfoxide, Freezing, Activating Media and Cryostorage on Post-Thaw Spermatozoa Motility and Fertility

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ABSTRACT

The present study was conducted with the goal of evaluating the viability of spermatozoa of the Deccan mahseer (*Tor khudree*, Cyprinidae) cryopreserved using different strategies. Immotile spermatozoa pooled from 2–4 males were diluted with modified fish Ringer's solution (pH: 7.48) and protected with dimethyl sulfoxide (Me_2SO) at 5%, 10%, and 15% and subjected to different equilibration periods. Diluted samples (1:10) were drawn into 500 μL plastic straws and frozen at a distance of 5 cm from the level of liquid nitrogen (LN_2) and preserved for 385 days in LN_2. Me_2SO at 10% resulted in higher post-thaw spermatozoa motility rate (46.7%) than 5% or 15% (up to 33.3%) after 385 days of cryopreservation. Of the different equilibration periods, 20–40 min generally produced higher motility (%) rates than 0, 10, 50, 60, 70, 80, or 90 min. The highest post-thaw motility of spermatozoa was obtained when they were frozen at 2 cm (−120.3°C) above the level of LN_2 and the optimum freezing rate was found to be 14.5 ± 0.1°C /min. Freezing spermatozoa at 1 cm (−160.4°C) or 3 cm (−103.0°C) or 4 cm (−77.9°C) from the level of LN_2 resulted in a significant decrease in post-thaw motility. Among the different activating media tested in the presence of different extenders and cryoprotectants, NaCl (0.3%) was found to be the best, followed by glucose (1%), and tap water. In the presence of different concentrations of Me_2SO, NaCl (0.3%), glucose (1%), and NaCl plus urea (0.3% + 0.4%) generally produced comparable post-thaw motility percentage and duration at all the concentrations of the cryoprotectant. When the spermatozoa were diluted in the Ringer's solution having pH 7.5 and 7.9, cryoprotected with Me_2SO at 10% (equilibration period: 30 min) and frozen at 2 cm from LN_2, the motility rates were 43% and 40%, respectively, after 810 days of cryostorage. High fertilization rate (98%) was obtained for spermatozoa cryopreserved up to 780 days and was close to that of fresh spermatozoa (98.5%). However, the hatching rate and fry survival were marginally lower in the treatment than that of control. After 1 year of rearing in ponds, the growth and survival of fish obtained from cryopreserved and fresh spermatozoa were comparable. The fish produced from cryopreserved spermatozoa were as normal as normally produced fish. The importance of cryopreservation of mahseer spermatozoa vis-à-vis establishment of a frozen gene bank is discussed.

INTRODUCTION

CRYOPRESERVATION is important in the production of new strains/breeds of economically important species and conservation of wild stocks of threatened/endangered aquatic species in terms of long-term maintenance of their gene banks.[1] In addition, cryopreservation offers several other benefits. In this context, a few sperm banks have been created for grouper, salmonids, and Indian cultivated and endangered fish species and other cyprinids. At the International Fisheries Gene Bank, Canada, more than 3000 accessions of salmonid

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germplasm from 6 species and 29 stocks, representing both wild populations and privately held broodstock, are available.[8]

Mahseers belonging to the genus *Tor* are a group of freshwater cyprinid fish inhabiting fast-flowing streams and rivers of hilly regions as well as the plains of India. They are popular game and food fish constituting India's national heritage. Over the years, natural stocks of mahseer have been depleted because of anthropogenic activities, therefore, mahseer has been declared as an endangered species in India.

To conserve and rehabilitate *Tor khudree* (*T. khudree*, Sykes), an important food and game fish in Peninsular India, several *in situ* and *ex situ* conservation strategies were suggested.[9] Studies conducted in the southern part of India have led to the development of a hatchery technology for *T. khudree* for *in situ* conservation,[10] while cryopreservation of mahseer spermatozoa has received greater attention as an *ex situ* conservation method since the early 1990s.[11–16] For the first time, the spermatozoa of *T. khudree* and *Tor* hybrid spermatozoa were cryopreserved and 80% and 70% post-thaw motility rates were obtained, respectively, after 11 months of storage in liquid nitrogen (LN_2).[11] Later, fry of *T. khudree* and *Tor putitora* (*T. putitora*) were produced from 1-year-old cryopreserved milt.[12,13] Recent studies have revealed the development of protocols for the cryopreservation of spermatozoa of *T. khudree* wherein high post-thaw motility, fertility, and fry survival rates were obtained for spermatozoa cryostored for varying periods.[14–16] Several cryoprotectants were tested to ensure maximum protection to spermatozoa during freezing and thawing; dimethyl sulfoxide (Me_2SO) provided the best protection to *T. khudree* spermatozoa.[16] The cryoprotected spermatozoa were subsequently equilibrated for varying periods, with the minimum interval between two equilibration periods being 30 min. This paved the way for attempting narrower equilibration periods to optimize the same results. Studies show that the pH of an extender is an important factor in determining the success of cryopreservation, and for common carp the best motility rates were found at pH 7–8.[17] While some investigators have reported no difference in viability of cryopreserved spermatozoa held in LN_2 for varying periods,[18,19] others have observed decreased viability with the storage period.[20,21]

Studies on the viability of *T. khudree* spermatozoa preserved over long periods (more than 1 year) in terms of motility, fertilization, hatching, and fry survival rates have not previously been accomplished. Information on the growth and survival of mahseer fingerlings produced from cryopreserved spermatozoa vis-à-vis that produced from fresh spermatozoa is also lacking. Also, data on the optimum freezing rate for cryopreservation of mahseer spermatozoa are lacking. Against this background, the present study was undertaken as part of a larger study, to examine the effects of long-term cryopreserved *T. khudree* spermatozoa on spermatozoa motility and fertility and the fitness of fish thus produced.

MATERIALS AND METHODS

The mahseer broodstock maintained at the fish farm of the College of Fisheries, Mangalore, was used for milt sampling, whereas the stocks reared at Harangi fish farm of Department of Fisheries (Government of Karnataka), Kodagu District, southern India were used for fertility studies. The broodstock was managed in the same manner as described earlier.[14]

Collection of spermatozoa

Spermatozoa were collected from healthy, mature *T. khudree* (200–500 g) males after sedating them in 20 ppm quinaldine (Sigma, St. Louis, MO) for 1–2 min. Prior to collection of spermatozoa, the region around the genital opening was cleaned with a filter paper/ small cotton gauze to remove water, mucus, urine, and faecal material. While applying gentle abdominal pressure, the spermatozoa were stripped into either chilled, sterile cryovials (1.8 mL) or first drawn into chilled disposable syringe (2.0 mL) and then transferred to vials. The collected milt was held on ice and immediately brought to the laboratory for observation. The

quality of fresh spermatozoa was determined by following the method described earlier.[15]

Cryopreservation procedure

Modified fish Ringer's solution (extender E_1) was chosen as an extender for this study because it yielded the best post-thaw spermatozoa motility rates in this species.[16] Similarly, Me_2SO was used as a cryoprotectant in this study because it was found to be superior to other cryoprotectants.[16] Prior to mixing with spermatozoa, the extender was freshly prepared using cold double-distilled water mixed with freshly collected spermatozoa at a dilution ratio of 1:10, and the required quantity of cryoprotectant (Me_2SO cooled to 4°C) was immediately added to yield the required final concentration of the diluent (V/V). The milt–diluent mixture was pre-cooled to 4°C then equilibrated for varying periods of 0, 10, 20, 30, 40, 50, 60, 70, 80, and 90 min in the presence of three levels of Me_2SO (5%, 10%, and 15% of the diluent) . The interval between collection of milt and addition of milt to extender ranged between 1 and 2 h. The diluted aliquots of 400–450 μL were drawn into labeled 500 μL transparent plastic straws (French medium design Indian straws), sealed with a polyvinyl chloride (PVC) gel (pink) and, after the completion of each equilibration period, frozen in a styrofoam box at a distance of 5 cm from the surface of LN_2 for 10 min. Then, the frozen straws (the inner temperature was -73.5 ± 0.26°C at 5 cm, after 10 min) were immediately transferred to a LN_2 can (20 liters) and stored for 385 days. The cooling rate was measured using a temperature probe (Salvin, India).

Effect of freezing on post-thaw motility

In this trial, extenders E_1 and E_2 (composition of E_2: NaCl [0.75%], $CaCl_2$ [0.02%], KCl [0.05%] and $NaHCO_3$ [0.02%] were used. After the specified equilibration period, the labeled plastic straws containing diluted milt were frozen by placing them horizontally at different heights such as 1 cm (-159.27 ± 0.6°C), 2 cm (-120.2 ± 0.12°C), 3 cm (-101.57 ± 0.26°C), and 4 cm (-76.33 ± 0.38°C) from the level of LN_2 in a styrofoam box (18 × 11 × 5 cm). The freezing period was 10 min. The frozen straws were immediately immersed in LN_2 for 1–2 min and then quickly transferred to a canister and stored under LN_2 for varying periods. The motility of spermatozoa frozen at different heights was periodically recorded in triplicate.

Influence of spermatozoa activating media on motility

To evaluate the effect of spermatozoa activating solution on motility of fresh and thawed spermatozoa, tap water (unchlorinated, drawn from a well), NaCl (0.3%), glucose (1%), and NaCl plus urea (0.3% +0.4%) were selected as sperm-activating solutions. The spermatozoa (fresh as well as cryopreserved) were activated on a prefocused slide by placing a small drop of fresh (5 μL) or thawed (50 μL) milt and mixing it quickly with an activating solution (200 μL). Motility rates were recorded in triplicate.

The required pH level of the extender was adjusted by adding either Na_2HPO_4 or KH_2PO_4 solution and was determined using a digital pH meter (Systronics, India).

To accomplish the effect of storage duration, spermatozoa samples were diluted in the extender (at pH 7.5 and 7.9), protected with Me_2SO at a concentration of 10% and equilibrated for 30 min. Following this, the samples (450 μL) were drawn into plastic straws (500 μL), frozen in LN_2 vapor at 2 cm (−120°C) above the level of LN_2 for 10 min and subsequently stored under LN_2. At periodic intervals, the post-thaw motility percentage was determined.

Estimation of post-thaw motility

As in the case of freshly collected spermatozoa, the motility estimates for thawed samples were made as described earlier.[16] Percentage motile sperm was used as the criterion for judging the quality of post-thaw spermatozoa. The straws containing spermatozoa were thawed at 37 ± 1°C for 5–10 sec.

Fertilization studies

Because *T. khudree* did not attain full maturity at Mangalore (a coastal region where temperature ranges between 24 and 31°C), fertil-

ization studies were conducted during the mahseer breeding season at Harangi fish farm, approximately 200 km southeast of Mangalore, to determine fertilization rate using spermatozoa cryopreserved for 780 days.

Fertilization of eggs

For fertilization studies, the spermatozoa samples diluted in the Ringer's solution, protected with 10% Me_2SO (V/V), equilibrated for 30 min, frozen, and long-term cryopreserved in LN_2 for 780 days, were transported to Harangi, by road. The female broodstock of *T. khudree* (1.5–2.5 kg body weight, 2+ years) were injected intramuscularly with a ready-to-use fish-spawning agent, containing a synthetic gonadotropin releasing hormone (sGnRH) and domperidone (WOVA-FH, India), at 0.8 mL/kg body weight, while males received a reduced dose of 0.2 mL/kg body weight. The breeders were anesthetized for 1–2 min with 10 ppm quinaldine solution before injection. Eggs from two females were stripped approximately 17 h after injection at a water temperature of 23 ± 1°C, pooled and divided into six groups of 700–900 eggs each. Two batches of eggs, taken in clean plastic bowls were fertilized with 400 μL of cryopreserved (dilution 1:10) spermatozoa (extender pH 7.5), and another two batches of eggs with 400 μL of cryopreserved spermatozoa (extender pH 7.9). The remaining two batches were fertilized with fresh spermatozoa. NaCl (0.3%) was used as a motility activator. After removing excess milt, mucus, and blood clots using a chicken feather, the eggs were kept for water hardening.

Hatching of eggs and fry rearing

After estimating fertilization rate, the developing eggs at early morula (3–4 h after fertilization) were transported by road from Harangi to Mangalore (6-h journey) in 18-liter capacity polythene bags under oxygen (1/3 water and 2/3 oxygen). During transport, approximately 10%–25% of eggs suffered mortality. Eggs took approximately 3 days to hatch and 6 days for yolk-sac absorption at a water temperature of 26 ± 1°C. A few deformed (caudal bent) fry observed in all the groups perished during the course of rearing.

Rearing of fingerlings up to maturity

The rearing of fingerlings (produced from spermatozoa cryopreserved up to 70 days as described earlier[14]) up to maturity was also conducted at Mangalore. After estimating the survival, the fingerlings from all the treated groups were pooled and transferred to a prepared concrete pond (10 × 5 × 1 m; 15–20 cm soil base), while the fingerlings of control replicates were stocked in another pond at similar stocking densities. The fingerlings of both the experimental and control groups were fed once daily, on a supplementary feed (as per details given under broodstock rearing) at 5%–10% of their body weight. Every 6 months, the fish were sampled to assess their growth rate and readjust feed quantity. Survival was recorded after one year of rearing in ponds. At the end of 1½ years, milt was collected from mature males of both the treatment and control groups and spermatozoa motility recorded.

Statistical analysis

Spermatozoa motility, fertilization, hatching, and fry survival rates expressed in percentages were first angular transformed to induce homogeneity of percentage data. Also, the data were statistically analyzed using a Minitab Package (V 3.0, Minitab Inc., State College, PA) at a probability level of $p < 0.05$.

RESULTS

Influence of Me_2SO and equilibration period on post-thaw motility of spermatozoa

When different equilibration periods were employed with three levels of Me_2SO (5%, 10%, and 15%), spermatozoa equilibrated up to 40 min at 5% and 10% Me_2SO exhibited higher motility percentage (after 385 days of cryopreservation) than those treated for higher (50, 60, 70, 80 or 90 min) equilibration periods (Table 1). Spermatozoa protected with Me_2SO at 5% and equilibrated for 70, 80, and 90 min showed no motility, while the shorter periods yielded poor motility rates. Cryoprotection of spermatozoa with 15% Me_2SO and equilibra-

Table 1. Effect of Me_2 Dimethyl Sulfoxide and Equilibrium Time on Post-Thaw Motility (%) of Spermatozoa Cryopreserved Up to 385 Days (Mean ± SE, $n = 3$)

Eq. time (min) / *Me_2SO (%)*	*0*	*10*	*20*	*30*	*40*	*50*	*60*	*70*	*80*	*90*
5	$15.0^{a12} \pm 2.87$	$20.0^{a12} \pm 0.0$	$21.67^{a12} \pm 1.6$	$23.33^{a1} \pm 1.66$	$13.33^{a2} \pm 1.66$	$8.33^{a23} \pm 1.66$	$5.0^{a3} \pm 0.0$	0.0	0.0	0.0
10	$36.67^{b1} \pm 3.3$	$45.0^{b1} \pm 2.89$	$41.67^{b1} \pm 1.66$	$46.67^{b1} \pm 3.33$	$40.0^{b1} \pm 2.89$	$21.67^{b2} \pm 1.7$	$13.33^{b23} \pm 1.66$	$15.0^{a23} \pm 0.0$	$10.0^{a23} \pm 2.87$	$10.0^{a23} \pm 2.89$
15	$21.67^{a1} \pm 1.7$	$18.33^{a1} \pm 1.7$	$20.0^{a1} \pm 0.0$	$23.33^{a1} \pm 1.66$	$33.33^{b2} \pm 1.66$	$30.0^{b12} \pm 0.0$	$33.33^{c2} \pm 1.66$	$23.33^{b1} \pm 1.66$	$21.67^{b1} \pm 1.66$	$18.33^{b1} \pm 1.66$

Extender: E_1, dilution ratio: 1:10, freezing: 5 cm from the level of LN_2, activating solution: tap water. Means with different letters within columns and numbers within rows are significantly ($p < 0.05$) different.

LN_2, liquid nitrogen; SE, standard error.

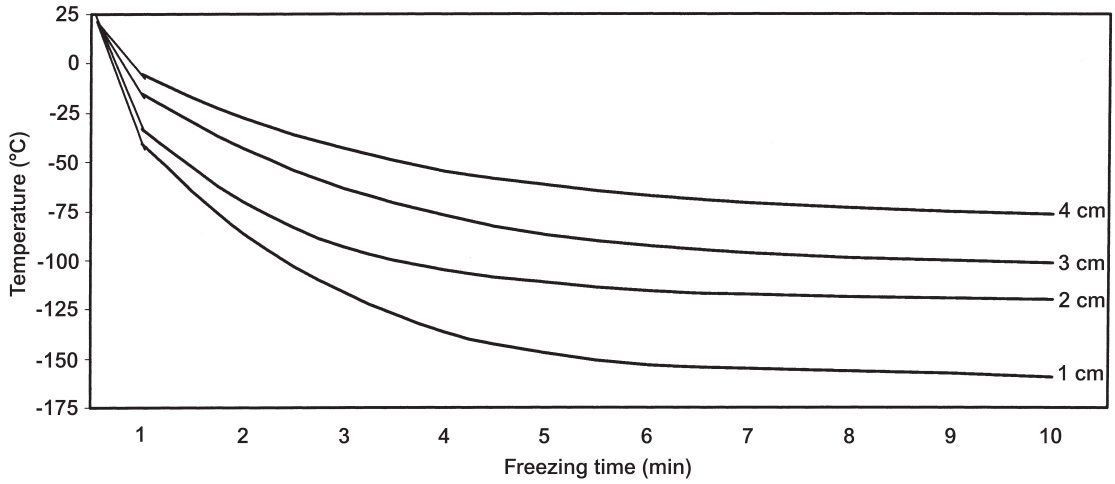

FIG. 1. Freezing curves for straws frozen at 1, 2, 3, and 45 cm from the surface of liquid nitrogen.

tion between 40–60 min produced superior post-thaw motility rates, compared to other equilibration periods. Of the three concentrations of the cryoprotectant, 10% was found to be better than 5% and 15%.

Influence of freezing on post-thaw motility

Freezing curves for straws frozen at 1, 2, 3, and 4 cm above LN_2 are shown in Figure 1. Expectedly, the temperature at 1, 2, 3, and 4 cm height from the surface of LN_2 declined significantly ($p < 0.05$) with an increase in cooling time up to 6–7 min, thereafter the decline was insignificant ($p > 0.05$). The cooling rates, averaged for 10 min, showed a decline as the freezing height increased (Table 2). At all four freezing heights tested, cooling rates that were high during the first 5 min, were significantly ($p < 0.05$) low during the next 5 min (6th–10th min).

Freezing spermatozoa (extender E_1) at 2 cm (-120.20 ± 0.12°C) from the surface of LN_2 resulted in slightly higher motility rates after 7 and 35 days of cryopreservation than freezing spermatozoa at a distance of 1 cm (-159.27 ± 0.61°C), 3 cm (-101.57 ± 0.26°C) or 4 cm (-76.33 ± 0.38°C) from the level of LN_2 (Table 3). Post-thaw motility rates (percentage as well as duration) were better after spermatozoa were activated with tap water than those found before spermatozoa activation. Prefreezing motility percentage and duration were the same in all the groups. However, post-thaw motility percentages obtained were relatively low in all the groups.

In the case of extender E_1, freezing spermatozoa at 2 cm from the surface of LN_2 yielded maximum motility percentage both before and after activation of spermatozoa in 7- and 35-day–cryopreserved spermatozoa (Table 4).

TABLE 2. FREEZING RATES (°C) (AVERAGED BASED ON RESULTS OF FIGURE 1) OF STRAWS FROZEN OVER LN_2 (MEAN ± SE, $N = 3$)

Freezing time distance from LN_2	*0–5 min*	*6–10 min*	*0–10 min*
1	$34.48^{a1} \pm 0.14$	$2.37^{a2} \pm 0.03$	$18.43^{a} \pm 0.06$
2	$27.30^{b1} \pm 0.14$	$1.74^{b2} \pm 0.12$	$14.52^{b} \pm 0.01$
3	$22.40^{c1} \pm 0.09$	$2.91^{c2} \pm 0.04$	$12.66^{c} \pm 0.03$
4	$17.31^{d1} \pm 0.07$	$2.95^{c2} \pm 0.06$	$10.13^{c} \pm 0.04$

Means bearing different letters and numbers within columns and within rows, respectively, are significantly ($p < 0.05$) different.

LN_2, liquid nitrogen; SE, standard error.

TABLE 3. INFLUENCE OF FREEZING SPERMATOZOA ON POST-THAW MOTILITY (MEAN ± SE, $N = 3$) (EXTENDER E_1)

Distance from LN_2 (cm)	*Prefreezing motility*		*7 days after cryopreservation*				*35 days after cryopreservation*			
			Before activation		*After activation*[a]		*Before activation*		*After activation*[a]	
	Motility (%)	*Duration (sec)*	*Motility (%)*	*Duration (sec)*	*Motility (%)*	*Duration (sec)*	*Motility (%)*	*Duration (sec)*	*Motility (%)*	*Duration (sec)*
1	$90.0^{a} \pm 0.0$	$125.0^{a} \pm 2.89$	$5.0^{a1} \pm 0.0$	$23.33^{a3} \pm 3.33$	$18.33^{ab2} \pm 1.67$	$33.33^{a4} \pm 3.33$	$5.0^{a5} \pm 0.0$	$20.00^{ab} \pm 0.0$	$10.0^{a5} \pm 2.89$	$30.0^{a7} \pm 0.0$
2	$90.0^{a} \pm 0.0$	$125.0^{a} \pm 2.89$	$6.67^{a1} \pm 1.67$	$25.0^{a3} \pm 2.89$	$28.33^{b2} \pm 1.67$	$70.0^{b4} \pm 5.78$	$6.67^{a5} \pm 1.67$	$25.0^{a7} \pm 2.89$	$20.0^{b6} \pm 0.0$	$41.67^{bc8} \pm 1.67$
3	$90.0^{a} \pm 0.0$	$125.0^{a} \pm 2.89$	$5.0^{a1} \pm 0.00$	$21.67^{a2} \pm 1.67$	$13.33^{a1} \pm 1.67$	$41.67^{ac3} \pm 1.67$	$5.0^{a4} \pm 0.0$	$21.67^{a7} \pm 1.67$	$16.67^{ab6} \pm 1.67$	$33.33^{ab7} \pm 3.33$
4	$90.0^{a} \pm 0.0$	$125.0^{a} \pm 2.89$	$5.0^{a1} \pm 0.00$	$23.33^{a2} \pm 1.67$	$11.67^{a1} \pm 1.67$	$48.33^{c3} \pm 1.67$	$5.0^{a4} \pm 0.0$	$23.33^{a5} \pm 1.67$	$13.33^{a4} \pm 1.67$	$46.67^{c5} \pm 2.89$

Cryoprotectant: Me_2SO (10%); equilibrium time, 30 min; dilution ratio, 1:20; activating solution, tap water; means with different letters within columns and numbers within rows under motility percentage and motility duration are significantly ($p < 0.05$) different.

Me_2SO, dimethyl sulfoxide; LN_2, liquid nitrogen; SE, standard error.

TABLE 4. INFLUENCE OF FREEZING SPERMATOZOA ON POST-THAW MOTILITY (MEAN ± SE, $N = 3$) (EXTENDER E_2)

Height from LN_2 (cm)	*Prefreezing motility*		*7 days after cryopreservation*				*35 days after cryopreservation*			
			Before activation		*After activation*[a]		*Before activation*		*After activation*[a]	
	Motility (%)	*Duration (sec)*	*Motility (%)*	*Duration (sec)*	*Motility (%)*	*Duration (sec)*	*Motility (%)*	*Duration (sec)*	*Motility (%)*	*Duration (sec)*
1	$90.0^{a} \pm 0.0$	$46.67^{a} \pm 3.33$	$5.0^{a1} \pm 0.0$	$25.0^{a2} \pm 2.89$	$5.0^{a1} \pm 0.00$	$25.0^{a2} \pm 2.80$	$5.0^{a1} \pm 0.0$	$28.33^{a} \pm 4.41$	0.0	0.0
2	$90.0^{a} \pm 0.0$	$46.67^{a} \pm 3.33$	$33.33^{b1} \pm 3.33$	$393.33^{b3} \pm 17.66$	$76.67^{b2} \pm 1.67$	$40.0^{b4} \pm 0.0$	$33.33^{b5} \pm 3.33$	$380.0^{b7} \pm 11.56$	$63.33^{a6} \pm 3.33$	$40.0^{a8} \pm 0.0$
3	$90.0^{a} \pm 0.0$	$46.67^{a} \pm 3.33$	$26.67^{b1} \pm 1.67$	$470.0^{c3} \pm 26.49$	$63.33^{c3} \pm 3.33$	$45.0^{b4} \pm 2.89$	$26.67^{b5} \pm 1.67$	$460.0^{c7} \pm 20.84$	$53.33^{a6} \pm 3.33$	$46.67^{a8} \pm 3.33$
4	$90.0^{a} \pm 0.0$	$46.67^{a} \pm 3.33$	$5.0^{a1} \pm 0.0$	$118.33^{d2} \pm 4.41$	$11.67^{d1} \pm 1.67$	$35.0^{ab3} \pm 2.89$	0.0	0.0	$26.67^{b} \pm 1.67$	$38.33^{a} \pm 4.41$

Cryoprotectant: Me_2SO (10%); equilibrium time, 30 min; dilution ratio, 1:20; activating solution, tap water. Means with different letters within columns and numbers within rows under motility percentage and duration are significantly ($p < 0.05$) different.

Me_2SO, dimethyl sulfoxide; LN_2, liquid nitrogen; SE, standard error.

TABLE 5. EFFECT OF ACTIVATING MEDIA ON POST-THAW MOTILITY OF SPERMATOZOA PRESERVED UP TO FIFTY DAYS (MEAN ± SE, $N = 3$)

		Tap water		*Glucose (1%)*		*NaCl (0.3%)*	
Extender	*Cryoprotectant*	*Motility (%)*	*Motility duration (sec)*	*Motility (%)*	*Motility duration (sec)*	*Motility (%)*	*Motility duration (sec)*
E_1	Me_2SO	$46.67^{a1} \pm 1.67$	$78.33^{a3} \pm 1.67$	$56.67^{a1} \pm 3.33$	$125.0^{a4} \pm 2.89$	$60.0^{a2} \pm 0.00$	$83.33^{a3} \pm 1.67$
	Methanol	$5.0^{b} \pm 0.00$	$18.33^{b} \pm 1.67$	0.0	0.0	0.0	0.0
	Propylene glycol	$6.67^{b1} \pm 1.67$	$33.33^{c3} \pm 3.33$	$11.67^{b2} \pm 1.67$	$33.33^{b3} \pm 3.33$	$15.0^{b2} \pm 2.89$	$33.33^{b3} \pm 3.33$
E_2	Me_2SO*	$53.33^{a1} \pm 3.33$	$48.33^{a4} \pm 1.67$	$71.67^{a2} \pm 1.67$	$101.67^{a5} \pm 7.27$	$80.0^{a3} \pm 0.0$	$93.33^{a6} \pm 3.33$
	Methanol	0.0	0.0	0.0	0.0	$5.0^{b} \pm 0.0$	$16.67^{b} \pm 1.67$
	Propylene glycol	$8.33^{b1} \pm 1.67$	$21.67^{b4} \pm 1.67$	$26.67^{b2} \pm 1.67$	$26.67^{b4} \pm 3.33$	$45.0^{c3} \pm 2.89$	$90.0^{a5} \pm 0.0$
E_3	Me_2SO*	0.0	0.0	0.0	0.0	0.0	0.0
	Methanol	0.0	0.0	0.0	0.0	0.0	0.0
	Propylene glycol	$5.0^{1} \pm 0.0$	$28.33^{3} \pm 1.67$	$21.67^{2} \pm 1.67$	$43.33^{4} \pm 3.33$	$8.33^{1} \pm 1.67$	$43.33^{5} \pm 1.67$
E_4	Me_2SO*	$55.0^{a1} \pm 2.89$	$60.0^{a3} \pm 0.0$	$81.67^{a2} \pm 1.67$	$65.0^{a3} \pm 0$	$86.67^{a2} \pm 1.67$	$75.0^{4} \pm 2.89$
	Methanol	0.0	0.0	0.0	0.0	0.0	0.0
	Propylene glycol	$11.67^{b1} \pm 1.67$	$33.33^{b3} \pm 3.33$	$21.67^{b2} \pm 1.67$	$40.0^{b3} \pm 0.0$	$25.0^{b2} \pm 0.0$	$63.33^{4} \pm 3.33$

Cryoprotectant level, 10%; equilibrium time, 30 min; dilution ratio, 1:10, freezing; 2 cm from the level of LN_1. Thawed samples showed slight motilityy (10–30%, 50–70 sec) prior to activation. Means with different letters within columns and numbers within rows under motility percentage and duration are significantly ($p < 0.05$) different.

Me_2SO, dimethyl sulfoxide; LN_2, liquid nitrogen; SE, standard error.

Freezing spermatozoa at 3 cm was the next best. Spermatozoa frozen at 1 cm from the surface of LN_2 showed minimum motility rates. Notwithstanding, maximum motility duration (before as well as after activation) was seen when spermatozoa were frozen at a height of 3 cm, in comparison to 1, 2, or 4 cm. In most cases motility duration declined significantly ($p < 0.05$) after spermatozoa activation as against that found before activation. Paradoxically, spermatozoa frozen at 4 cm from the level of LN_2 exhibited slightly higher post-activation motility percentage after 35 days of cryopreservation, compared to 7-day storage period.

Effect of activating media on post-thaw motility of spermatozoa

Of the three activating media used, 0.3% NaCl yielded higher post-thaw motility percentage than 1% glucose (second best) (Table 5). Tap water produced poor motility. Among the three cryoprotectants tested in the presence of four extenders, Me_2SO yielded consistently better motility percentage, barring extender E_3 wherein Me_2SO did not give any protection to spermatozoa during cryopreservation. While propylene glycol was observed to be the second best cryoprotectant, methanol was found to give least protection to spermatozoa.

In terms of motility duration, glucose (1%) was found to be better in extenders E_1 and E_2, while NaCl (0.3%) showed better motility in the presence of extender E_4. Tap water was found to be a poorer spermatozoa motility activating solution than NaCl (0.3%) and glucose (1%).

The effects of spermatozoa activating solutions and Me_2SO concentrations on post-thaw motility of spermatozoa cryopreserved for 60 days are shown in Table 6. It is observed that among the four activating solutions used, NaCl (0.3%), glucose (1%), and NaCl plus urea (0.3% + 0.4%) generally produced comparable post-thaw motility percentage and duration at all the concentrations of Me_2SO, barring 12.5% and 15%. Among the different levels of the cryoprotectant, 5.0%, 7.5%, and 10.0% resulted in generally higher motility percentage and duration than 2.5%, 12.5%, and 15.0%, when spermatozoa were activated with the four activating solutions.

Influence of spermatozoa storage period and pH on post-thaw motility

Motility of spermatozoa cryopreserved in fish Ringer's solution having pH 7.5 and 7.9 decreased gradually with storage period (Table 7). At pH 7.5, spermatozoa that exhibited 93% motility 2 days after freezing, yielded 43% motility after 810 days of cryostorage. The percent motile spermatozoa was still above 50% even after 415 days of cryopreservation. Between 415 and 810 days of storage also, the decline in motility rate was insignificant ($p > 0.05$).

At pH 7.9, spermatozoa cryopreserved up to 810 days revealed a trend in post-thaw motility that was similar to that observed when spermatozoa were frozen with the same extender but at pH 7.5 (Table 7). Motility declined gradually with increasing storage period, but it was still 50% after 365 days of cryopreservation and 40% at the end of 810 days preservation in LN_2.

Motility rates were not different ($p > 0.05$) between pH 7.5 and 7.9 after a storage period of 810 days and they were significantly different ($p < 0.05$) at 170, 270, and 365 days of storage.

Fertilization studies

When spermatozoa were stored in LN_2 for more than 2 years, motility percentage declined to 42.5% and 40% at pH 7.5 and 7.9 respectively, as against 97.5% of freshly collected spermatozoa (Table 8).

The eggs exhibited very high fertilization rates (absolute) of 98.1% and 98% at pH 7.5 and 7.9 respectively, compared to 98.5% of control. The fertilization rates as a percentage of control were close to 100% in both treatments, while the hatching rates were lower than that of control. The hatching rates as relative to control were 74.9% and 85.5% for the spermatozoa diluted in the extender having pH 7.5 and 7.9, respectively (Table 8).

Fry survival was marginally better in control than that of the experimental groups; the rates (absolute) were more or less comparable (41.5% and 44.6%, respectively) between the two hydrogen ion concentrations (Table 8). Fry produced from long-term cryopreserved spermatozoa was not different from normally produced *T. khudree* fry.

Table 6. Effect of Activating Solution and Dimethyl Sulfoxide on Post-Thaw Motility of Spermatozoa Preserved up to Sixty Days (Mean ± SE, $N = 3$)

DMSO (%)	*2.5*		*5.0*		*7.5*		*10.0*		*12.5*		*15.0*	
Activating solution	*Percentage*	*Duration (sec)*	*Percentage*	*Duration (sec)*	*Percentage*	*Duration (sec)*	*Percentage*	*Duration (sec)*	*Percentage*	*Duration (sec)*	*Percentage*	*Duration (sec)*
Tap water	$13.33^{a1} \pm 1.66$	$35.0^{a4} \pm 2.89$	$33.33^{a23} \pm 3.33$	$35.0^{a4} \pm 2.89$	$35.0^{a23} \pm 2.87$	$35.0^{a4} \pm 2.89$	$36.67^{a2} \pm 3.33$	$33.33^{a4} \pm 1.67$	$21.67^{a3} \pm 1.66$	$35.0^{a4} \pm 2.89$	$15.0^{a3} \pm 2.87$	$40.0^{a4} \pm 0.0$
NaCl (0.3%)	$25.0^{b1} \pm 0.0$	$66.67^{b5} \pm 3.33$	$73.33^{b2} \pm 1.66$	$50.0^{b6} \pm 2.89$	$83.33^{b3} \pm 1.66$	$55.0^{b6} \pm 2.89$	$75.0^{b2} \pm 2.87$	$46.67^{b6} \pm 3.33$	$55.0^{b47} \pm 2.87$	$43.33^{ab6} \pm 1.67$	$63.33^{b4} \pm 1.66$	$41.67^{a7} \pm 1.67$
Glucose (1%)	$26.67^{b16} \pm 1.66$	$56.67^{bc7} \pm 3.33$	$66.67^{bc2} \pm 3.33$	$45.0^{b8} \pm 2.89$	$75.0^{b3} \pm 2.87$	$55.0^{b7} \pm 2.89$	$48.33^{a4} \pm 4.41$	$41.67^{ab8} \pm 1.67$	$16.67^{c5} \pm 1.66$	$36.67^{a8} \pm 1.67$	$35.0^{c6} \pm 2.87$	$45.0^{ab8} \pm 0.0$
NaCl + Urea (0.3% + 0.4%)	$35.0^{b1} \pm 2.87$	$55.0^{c2} \pm 2.89$	$55.0^{c2} \pm 2.87$	$60.0^{c4} \pm 0.0$	$70.0^{b3} \pm 2.87$	$46.67^{b25} \pm 3.33$	$81.67^{b3} \pm 1.66$	$45.0^{bc5} \pm 2.89$	$50.0^{d2} \pm 2.87$	$50.0^{b45} \pm 0.0$	$50.0^{d2} \pm 5.77$	$51.67^{b45} \pm 1.67$

Extender, E_2; equilibration time, 30 min; dilution ratio, 1;10; freezing, 2 cm from LN_2.
At 0% Me_2SO, there was no motility. Means with different letters within columns and numbers within rows under motility percentage and duration are significantly ($p < 0.05$) different.
DMSO, dimethyl sulfoxide; SE, standard error.

Table 7. Influence of Storage Period (up to 810 Days) on Post-Thaw Motility (%) of Spermatozoa (Mean ± SE, $N = 3$)

	pH	
Storage period (days)	*7.50*	*7.90*
2	$93.33^{a1} \pm 1.67$	$93.33^{a1} \pm 1.67$
25	$91.67^{a1} \pm 1.67$	$90.0^{a1} \pm 0.0$
108	$83.33^{b1} \pm 1.67$	$81.67^{b1} \pm 1.67$
138	$78.33^{bc1} \pm 1.67$	$76.67^{c1} \pm 1.67$
170	$75.0^{c1} \pm 0.0$	$68.33^{c2} \pm 1.67$
270	$58.33^{d1} \pm 1.67$	$51.67^{d2} \pm 1.67$
365	$55.0^{de1} \pm 0.0$	$50.0^{d2} \pm 0.0$
415	$51.67^{def1} \pm 1.67$	$48.33^{df1} \pm 1.67$
685	$48.33^{ef1} \pm 1.67$	$43.33^{ef1} \pm 1.67$
730	$45.0^{ef1} \pm 2.89$	$41.67^{e1} \pm 1.67$
810	$43.33^{f1} \pm 1.67$	$40.0^{e1} \pm 0.0$

Cryoprotectant, Me_2SO (10%); equilibration period, 30 min; freezing, 2 cm from the level of LN_2; motility duration, 40–60 sec; activating solution, tap water. Means with different letters within columns and numbers within rows are significantly ($p < 0.05$) different.

Me_2SO, dimethyl sulfoxide; LN_2, liquid nitrogen; SE, standard error.

Growth and survival of fingerlings

There was no significant difference in weight and length between the control and cryopreserved group at the end of 1 year rearing in ponds (Table 9). The survival of fish produced from cryopreserved spermatozoa was comparable with that of control. At the end of $1\frac{1}{2}$ years, weight and length between the control and cryopreserved groups, approximately 25% of the fish of both the treatment and control groups attained gonadal maturity and produced spermatozoa upon abdominal pressure. Spermatozoa obtained from fish that were produced from cryopreserved spermatozoa were observed to be as normal (in terms of motility percentage and duration) as those stripped from fish that were produced using fresh spermatozoa.

DISCUSSION

The results of this study indicate that the modified fish Ringer's solution is suitable for the long-term cryopreservation (810 days) of spermatozoa of *T. khudree*. Earlier, the same extender was successfully used for this species.[14–16] The findings of the present work also indicate that among the three levels of Me_2SO, 10% was found to be marginally better than 5% and 15% (Table 1), while concentrations of 5–20% (v:v) were used for cryopreservation of sperm of several fish species.[22] Similarly, 10% Me_2SO was found to be the best for the cryopreservation of mahseer spermatozoa.[12–16] The optimum cryoprotectant concentration may vary depending on cryoprotectants, species, equilibration time and criteria used for evaluation of post-thaw motility; for most cryoprotectants, final concentrations of 7% and 15% have been used successfully.[22]

To ensure adequate protection to sperm during cooling, sufficient time must be allowed to facilitate the penetration of cryoprotectants into cells and periods from 0 up to 3 h have been used.[22] It is recommended that equilibration time be kept to a minimum (<1 h) to avoid exhaustion of sperm.[23,24] In this study, when different equilibration periods were tested at 5%, 10%, and 15% DMSO, spermatozoa equilibrated for 20–40 min exhibited higher motility percentage (after 385 days of cryopreservation), compared to 0, 10, 50, 60, 70, 80, or 90 min equilibration time, barring 15%. Longer equilibration time (45, 120, and 170 min) showed no difference in the motility of spermatozoa of *T. khudree* protected with glycerol and Me_2SO.[11]

Freezing temperature is one of the most critical variables that affect the success of cryo-

Table 8. Influence of Long Storage (780 Days) of Frozen Spermatozoa in LN_2 on Post-Thaw Fertility Rates (Mean ± SE, $N = 2$)

pH	Motility (%)	Fertilization rate (%) (absolute)	Fertilization rate (% of control)	Hatch rate (%) (absolute)	Hatch rate (% of control)	Fry survival (%) (absolute)	Fry survival (% of control)
7.50	42.5 ± 2.50	98.13 ± 0.25	99.60 ± 0.04	37.26 ± 1.14	74.88 ± 0.94	41.48 ± 1.09	78.97 ± 0.04
7.90	40.0 ± 0.0	98.01 ± 0.65	99.48 ± 0.45	42.01 ± 1.96	85.48 ± 3.49	44.57 ± 1.17	84.85 ± 0.03
Control	97.5 ± 2.50	98.52 ± 0.21	—	49.14 ± 0.29	—	52.53 ± 1.36	—

Cryoprotectant, Me_2SO (10%); equilibration period, 30 min; freezing 2 cm from the level of LN_2.
LN_2, liquid nitrogen; SE, standard error; Me_2SO, dimethyl sulfoxide.

preservation. In *Hucho hucho* (*H. hucho*) and *Thymallus thymallus (T. thymallus)*, freezing semen at 1.5 cm (−110 ± 2°C) above the level of LN_2 was optimal.[25] Likewise, in *Oncorhynchus mykiss* (*O. mykiss)*, *Salmo trutta lacustris* (*S. trutta lacustris*), and *Salmo trutta fario* (*S. trutta fario*), the optimal freezing height was 1.5 cm (−110 ± 2°C) above the level of LN_2 and in *Salvelinus fontinalis (S. fontinalis)* it was 2.5 cm (−92 ± 2°C).[18] They also demonstrated that changes in the freezing height of 0.5 cm (approximately 10°C) resulted in a significant decrease in post-thaw fertility. In this study, a decrease or increase of freezing height by 1 cm from the optimum (2 cm) led to generally lower motility rates. The results of the present study show that when spermatozoa were diluted in extenders E_1 and E_2, cryoprotected with Me_2SO (10%) and equilibrated for 30 min, higher post-thaw motility percentage and duration were recorded at 2 cm (−120.2 ± 0.12°C) than at 1, 3, or 4 cm above the level of LN_2, following 7–35 days cryopreservation (Tables 3 and 4). Effect of different freezing temperatures/heights on the post-thaw motility/fertility of mahseer spermatozoa has not been investigated earlier. In a cyprinid (Nishikigoi), either no significant differences or a marginal effect on percentage of fertilized eggs was found when spermatozoa were cooled in LN_2 vapor at different rates such as 2.5, 5.0, and 10.0°C/min; the lowest value was obtained with spermatozoa cooled at 10.0°C/min.[26] Earlier research carried out on mahseer spermatozoa cryopreservation was limited to manual freezing of straws at a height of 4 cm from the surface of LN_2 in an igloo box.[27] Further research may be needed to confirm obtaining optimum cooling rates using a programmable cooling chamber for cryopreservation of *T. khudree* spermatozoa albeit such attempts are expensive.

In order to prolong the duration of motility, disperse the spermatozoa around the ova and to minimize osmotic shock during fertilization, activating/insemination solutions are recommended.[28] The composition of such solutions is comparable to ovarian fluid[29,30] or fish saline.[31] In this study, maximum motility was

Table 9. Growth and Survival of Mahseer Produced from Cryopreserved (up to 70 Days) and Fresh Spermatozoa (Mean ± SE)

S1. no.	Particulars	Treatment (cryopreserved spermatozoa)	Control (fresh spermatozoa)
1	Size of fry at stocking (cm)	2.50	2.50
2	Size of fingerlings at the end of 6 months (g)	3.50^a ± 0.16	3.45^a ± 0.15 (10)
3	Weight of fish at the end of 1 year (g)	38.24^a ± 0.40 (33)	39.15^a ± 0.35 (33)
4	Length of fish at the end of 1 year (cm)	15.28^a ± 2.11 (33)	15.32^a ± 2.06 (33)
5	Survival after 1 year (%)	85.8	87.4

Numbers in parentheses indicate sample size. Cryoprotectant, Me_2SO; equilibration time, 30 min; freezing 5 cm from the level of LN_2. Means with same letters within rows are not significantly ($p < 0.05$) different.
Me_2SO, dimethyl sulfoxide; LN_2, liquid nitrogen; SE, standard error.

obtained when spermatozoa were activated with 0.3% NaCl, compared to NaCl plus urea (0.3%+0.4%), tap water, or 1% glucose solution, although 1% glucose yielded better post-thaw motility duration than the other activating solutions (Table 5). Motility rates were generally higher at 5.0%, 7.5%, and 10.0% Me_2SO, with all the abovementioned activating solutions (Table 6). In *T. khudree*, Me_2SO and methanol resulted in better hatch rate when thawed milt was not prediluted with an extender (same as the one used for extending spermatozoa prior to freezing) prior to fertilization, while glycerol gave better hatching percentage when thawed milt was prediluted with the extender.[12] Diluer 532 (a sperm activator) (Sanofi Sante Animale, Libourne, France) yielded a significantly higher percentage of motility than hatchery water and Tris-glycine-NaCl.[13] The thawed spermatozoa could be activated with 1.0%–2.0% NaCl to achieve higher survival rate.[31] Higher post-thaw motility rates of spermatozoa recorded in this study may be because of the higher sperm activating potential of NaCl, with Me_2SO treatment groups showing maximum motility.

The results of this investigation show that it is possible to cryopreserve *T. khudree* spermatozoa up to 810 days or more which is the longest record for mahseer (Table 7). High motility rates of 80% and 70% were recorded in *T. khudree* and *Tor* hybrids, respectively, after 11 months of cryopreservation.[11] Subsequent studies showed that it is possible to obtain up to 90% motile *T. putitora* sperm one-year after cryopreservation.[13] In rainbow trout (*O. mykiss*, Walbaum), the percentage of immotile spermatozoa in frozen-thawed semen was significantly higher than that of untreated semen and the percentage of motile spermatozoa decreased with time.[20] Nevertheless, it was reported that the motility decreased to 23% in the Siberian sturgeon, *Acipenser baeri* (*A. baeri*) and 15% in the starlet, *Acipenser ruthenus* (*A. ruthenus)* after 4 months of cryopreservation.[21] Fish sperm preserved through cryopreservation can have an almost indefinite storage life. The practical storage period for cryopreserving fish spermatozoa has been estimated to be at least 200 years.[32] In related literature, the longest record of sperm cryopreservation (11 years) was reported in the red sea bream.[33] Decrease in post-thaw spermatozoa motility during cryo-storage (from 93% after 2 days storage to 43% after 810 days storage) may be attributed to periodic removal of canister containing straws for sampling.

While the fertility studies on spermatozoa cryopreserved up to 780 days show that pH of extender has no appreciable influence on the post-thaw motility and fertilization rates, storage slightly decreased the hatching and fry survival rates compared to fresh spermatozoa (Table 8). In cryopreserved sperm of cyprinid, *Cyprinus carpio* (*C. carpio*), a positive correlation ($c = 0.846$) between post-thaw motility and fertilization rate (as high as 99.6% of control) was reported,[34] while the frozen and fresh semen yielded fertilization rates of 54.7% and 82.6% and hatch rates of 50.6% and 59.4%, respectively.[6] Albeit lower motility was observed in frozen thawed sperms than in fresh semen, no difference in hatching rate and larval malformation between fresh and frozen sperm of common carp was found.[34] In the present study, the fertilization rates (absolute and relative to control) did not differ between cryopreserved (780 days) and fresh spermatozoa and also between the pH levels. Similar observations were also made in salmonids.[18,36] Mean fertilization rates ranging from 38.7% to 93.4% of controls were reported after 13 months of storage of tilapia spermatozoa.[37] For the Brazilian curimbata, *Prochilodus scrofa*, (*P. scrofa*), high fertilization rates (101.6% and 102.2% of controls) from 20-day– and 2-year–cryopreserved spermatozoa, respectively, were recorded.[38] After 365 days storage in LN_2, the milt of Indian major carps was able to fertilize 40–50% of eggs.[39] In black porgy (*Acanthopagrus schlegeli* [*A. schlegeli*) 99% and 91.5% fertilization rates were obtained 1 and 342 days after cryopreservation of spermatozoa, respectively.[40] The spermatozoa of Indian major carps cryopreserved over a period of 4 years yielded $56 \pm 7\%$ fertilization rate as against $85 \pm 5\%$ of fresh milt.[41]

Hatching rate is an important criterion used to judge the quality of cryopreserved spermatozoa. In the present research, hatching rates as high as 84.9% of control were recorded for spermatozoa cryopreserved up to 780 days. In contrast, hatching rates of 28.2% and 20.7% of con-

trol were only obtained for *T. khudree* and *T. putitora*.[12,13] Interestingly, hatching rates as high as 155% of control were recorded in *T. khudree*.[14] The hatching rates would have been probably better if the eggs had been aerated during transportation. Even in the normal practices, hatching rates rarely exceed 60%.[10] In gray mullet, the milt cryopreserved for 351 days produced a hatch rate of 92%, compared to 94% of fresh milt.[42] Similarly, fresh and cryopreserved semen of the African catfish, *Heterobranchus longifilis* (*H. longifilis*) yielded hatching rates of 81.1%, 83.4%, and 78.9%, respectively, for the fresh, 1-h– and 8-month–cryopreserved sperms.[14] Lower hatching rates were obtained for frozen-thawed semen than fresh semen.[43] In this study, the fry survival rates obtained from the 780-d–cryopreserved spermatozoa were marginally lower than those of fresh spermatozoa probably because of the lower percentage of motile spermatozoa in the former. Second, the morphology of the fry produced from cryopreserved spermatozoa were as normal as those obtained from fresh spermatozoa.

The results also reveal that the growth and survival of *T. khudree* fingerlings obtained from 70-day–cryopreserved spermatozoa was not different from those of fresh spermatozoa (Table 9). The number and total weight of juveniles of striped bass harvested did not differ significantly between those produced by fertilization with cryopreserved and fresh sperm.[44] Similarly, the growth and survival of 30-day–old tilapia fry produced from milt stored up to 12 months were not significantly different from normal counterparts.[30] No significant difference in growth (22.4–26.3 and 22.5–26.9 g) and survival (74–78.6 and 73.6–79.3%) between cryopreserved milt (up to 3 years) and fresh milt, respectively, was observed in the Indian major carps.[45] The corresponding figures for *T. khudree* in this study were 38.2 and 39.2 g and 85.8% and 87.4%, respectively. In *T. khudree*, the percentage (28.2%) of viable fry produced from 1-year-old–cryopreserved milt was comparable to that of fish produced from 3-day–old cryopreserved milt (26.2%).[12] No significant differences in growth and survival of young turbot, *Scophthalmus maximus (S. maximus)* either obtained from cryopreserved or fresh sperm were found.[46] The fish from both the experimental and control groups attained gonadal maturity after 1 year and produced spermatozoa having normal motility (95–100%) and duration (50–60 sec).

Our study indicates that the spermatozoa of *T. khudree* could be successfully cryopreserved for more than 2 years, using modified fish Ringer's solution and Me_2SO. Freezing spermatozoa at 2 cm from the level of LN_2 has been found to yield best results and Nacl (0.3%) was found to be a superior spermatozoa-activating media. The fertility studies reveal that viable fish could be produced from long-term cryopreserved-spermatozoa that will ultimately help establish a frozen sperm bank for the conservation of genetic material of the endangered *T. khudree*.

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