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Cryopreservation of Brown Trout (*Salmo trutta fario*) Sperm: The Influence of Extender Composition and Fertilization Procedure

A. Gopalakrishnan*, K.L. Thakur, A.G. Ponniah, K. Kumar** and R. Dayal**
National Bureau of Fish Genetic Resources, Canal Ring Road
Dilkusha P.O., Telibagh, Lucknow-226 002, India

Seven extenders were tested to check their relative efficiency in cryopreserving the milt of brown trout (*Salmo trutta fario*). Hatching rates equal to that of control ($p > 0.05$) were obtained when a cryodiluent containing egg yolk (2%) and dimethylsulphoxide (10%) was used. Removal of coelomic fluid prior to the addition of thawed milt and addition of 5 mM theophylline to the "Dexter" solution enhanced the hatching percentage. The sperm density varied from 9.83 to 18.41 x 10^9 sperms/ml.

Key words: Cryopreservation, brown trout, activator, cryo diluent, extender, milt

Long-term storage of fish spermatozoa plays an important role in aquaculture. Sperm has been successfully cryopreserved for a number of species and standard methods have been developed for the milt of salmonids (Scott & Baynes, 1980; Stein & Bayrle, 1978; Stoss, 1983; Baynes & Scott, 1987; Cloud *et al.*, 1990; Holtz, 1993). However, in many cases, the results are often highly variable (Piironen, 1993) and satisfactory fertility rates can be obtained only by using large quantity of milt (Lahnsteiner *et al.*, 1992). As observed by many workers, various factors influencing fertility of cryopreserved milt are not fully understood, which makes the generalization of the methods impossible.

Among the various cold water fishes introduced in India by the British settlers, brown trout (*Salmo trutta fario*) forms one of the most important species and constitutes recreational fishery in cold water streams, lakes and reservoirs. The species is being produced on an increasingly large scale in hatcheries using a limited number of parent stock. As a result, the descendants of the founderstock now exhibit retarded growth and loss of general vigour and the status of recreational fishery has become bleak these days (Sehgal, 1989). A project for genetic rejuvenation of brown trout was taken up to correct the negative effects of inbreeding, using the cryopreserved milt of the species to transfer genes among population (Ponniah *et al.*, 1993).

Despite the progress made in milt cryopreservation in many salmonids, especially in rainbow trout during recent years, information on brown trout is scanty (Stein & Bayrle, 1978; Erdahl & Graham, 1980; Stoss & Refstie, 1983; Piironen, 1993). Recent studies indicate that species - specific modification with respect to cryoprotectant, extender and freezing - thawing protocol may be needed. Very little work has been done on this species on the efficiency of different extenders, activators and role of insemination medium during fertilization. The present investigation was thus aimed at developing a long-term cryopreservation procedure that can be carried out under normal field conditions for brown trout sperm.

* Present address: N.B.F.G.R., Cochin Unit, C/o CMFRI, P.B. No. 1603, Cochin-682014, India
** Present address: Fisheries Directorate, Bilaspur-174 001, Himachal Pradesh, India

Materials and Methods

All the experiments were carried out under field conditions during the breeding season (December, 1992 & January, 1994) in the trout farm at Barot, Himachal Pradesh. During the design of the experiment, constraints like relatively short breeding period of brown trout in the Himalayas (from last week of November to the end of December); long incubation period of ova (35-38 days); nonavailability of sufficient hatching troughs for brown trout eggs (due to overlapping of breeding season of commercially more important rainbow trout in December) and extreme remoteness of the hatchery location (Barot) during heavy snowfall etc. had to be taken into account.

Milt samples were collected from individual specimens (weighing 900-1500 g; n=20) by abdominal massage and stored in clean, dry, serially numbered plastic containers in a thermocole box containing crushed ice. Faecal contamination of milt was avoided by starving the milters for 24 h prior to milting. Immediately after collection of milt, motility of spermatozoa was determined microscopically following the methods of Chao (1982) and Steyn *et al.* (1985) by activating sperms using an activator solution prepared by dissolving 123 mg of DILUER 532, a product of M/s Sanofi, Sante Animale, Libourne, France, in 10 ml of distilled water (temperature, 10°C pH 8.5) (Dexter solution). Motility was assessed subjectively on a scale of 0 to 5+, depending on the proportion of spermatozoa

Table 1. Extenders used and their chemical composition in the cryopreservation of brown trout sperm

Chemical composition	Extenders*						
	NBFGR 3	NBFGR 3A	NBFGR 3B	NBFGR 4	NBFGR 6	NBFGR 7	NBFGR 9A
Sodium chloride (mg/100 ml)	750	750	750	650	730	750	750
Potassium chloride (mg/'100 ml)	38	38	38	300	38	20	150
Calcium chloride dihydrate (mg/100 ml)	0	0	0	30	23	20	30
Sodium bicarbonate (mg/100 ml)	200	200	200	20	750	20	0
Magnesium sulphate ($MgSO_4$ $7H_2O$) (mg/100 ml)	0	20	20	0	23	0	20
Sodium dihydrogen phosphate (mg/100 ml)	0	50	50	0	41	0	0
Glucose (mg/100 ml)	100	100	100	0	100	0	100
Glycine (mg/100 ml)	0	500	500	0	0	0	500
Mannitol (mg/100 ml)	0	0	0	0	250	0	0
Yolk (hen's egg) (%v/v)	2	0	2	0	0	0	0
pH	7.4	7.4	7.4	7.4	7.1	7.3	7.2

*Extender references: NBFGR 3, 3A & 3B are modifications of V2e of Scott & Baynes (1980); NBFGR 4 – refer Rana & Mac Andrew (1989)' NBFGR 6 – # 164 of Scott & Baynes (1980); NBFGR 7 – extender et al., Kurokura, 1984); NBFGR 9A – V7/B & S Lahnsteiner, et al., 1992).
In all experiments, DMSO (10% v/v final concentration) was used as cryoprotectant.

Table 2. Hatching rates of brown trout eggs fertilized with frozen-thawed milt - effect of different extenders

Fertilization trial	Extender code (NBFGR)	Amount of coelomic fluid removed (ml)	Volume of sodium bicarbonate added (ml)	Hatching rate (Mean± S.D.)*	Hatching rate as % control
I					
December					
1992	3	0	3	$42.4^{a,b}$±5.41	84.80
	4	0	3	0.98^{c}±0.80	1.96
	6	0	3	6.85^{d}±5.72	13.70
	Raw milt (control)	0	3	50.0^{b}±5.2	-
II					
January					
1994	6	0	3	35.76^{e}±2.6	57.75
	7	0	3	49.11^{f}±1.83	79.31
	9A	0	3	45.88^{f}±0.99	74.10
	3A	0	3	41.39^{e}±1.41	66.84
	3B	0	3	53.11^{g}±1.41	85.77
	Raw milt (control)	0	3	61.92	-

*n=3, wherever standard deviation values are given.
Means superscribed with same letter are not significantly different ($p>0.05$); t-test carried out between different treatments of the same fertilization trial only.

activated and the intensity and duration of their movement. The samples with high motility values (5+) alone were frozen. 12-15 samples culled at this step were pooled together and they were processed for cryopreservation within 30 min. The sperm density was determined using a Neubauer haemocytometer, and it varied from 9.83 to 18.41 x 10^9 sperms/ml (13.53 ± 3.75 x 10^9). The values were in conformity with sperm concentration of other salmonids (Stoss, 1983).

Seven extenders were selected from a range of 15 tested on cyprinids at NBFGR and on the basis of their performance in other salmonids, tilapia and carps (Table 1). All the solutions were maintained at 4°C before mixing with milt. Dimethyl sulfoxide (DMSO) with a final concentration of 10% was used as the cryoprotectant. The yolk of hen's egg, a non-permeating cryoprotectant, was added to the extenders 3 and 3B at a relatively low concentration (2% v/v). This was based on the earlier results in Indian cyprinids (Ponniah, A.G., personal communication). The ratio of milt:extender:cryoprotectant was 1:26:0.4. An equilibration period of 15 min was given which included the time for filling and sealing of 0.5 ml French straws. The straws were later kept approximately 6-8 cm over liquid nitrogen (in vapour phase) for 10 min and thereafter rapidly plunged into liquid nitrogen for storage.

The motility of the cryopreserved milt was examined 36 h after freezing. The straws taken out of liquid nitrogen were waved in air for 2-3 seconds and quickly placed in a water bath at 37°C for 5 seconds. They were then wiped rapidly and the milt was poured into clean dry microfuge vials. The motility of cryopreserved spermatozoa was determined immediatley as mentioned earlier for raw milt, using Dexter solution.

Two series of fertilization experiments were carried out using milt cryopreserved for four days. Ripe eggs were stripped (two men method) from spawners weighing 900-1500 g and eggs pooled from 5-6 females were used. For each treatment, subsamples having 100-150 ova (containing approximately 5 ml coelomic fluid) were taken in separate containers. 3 ml of 1% sodium bicarbonate solution was added to this (Scott & Baynes, 1980; Holtz, 1993). The eggs were fertilized immediately with thawed milt from 2 straws (sperm to egg ratio - approximately 3.38 x 10^9 sperms to 100 eggs) and mixed well for 30 seconds. 5 ml of Dexter solution was added and mixing continued for another 2 min. Water was sprinkled over the fertilized eggs, mixing continued and the eggs were washed in running water for two min. Soon after this, they were given a dip in 2% malachite green solution (zinc-free) for 30 sec, again washed thoroughly in water and distributed in horizontal hatchery trays away from light. The hatchery was maintained at a constant water flow of 3-5 l/min and average water temperature of 12°C. A set of eggs, fertilized with freshly stripped, pooled milt (150 eggs/ 200 µl raw milt containing approximately $2.8x10^9$ sperm) was treated as control.

The hatching rate after 38-40 days was determined. The results are expressed as number of alevins (sacfry) as proportion of total number of eggs fertilized. Variations from routine fertilization procedure were made to study the effect of removal of ovarian fluid, the concentration of sodium bicarbonate and the changes in the composition of the activator solution by changing the fertilization protocol in selected treatments (Table 3).

All treatments except the variation in fertilization with extender 3B were carried out in triplicate, and the mean and standard deviation values were calculated. The percentage of hatching was subjected to angular transformation (TP = $\sin^{-1}\sqrt{P}$, where P is the proportion of alevins) to normalize the variance. Statistical analyses were performed by the t-test (wherever replicates were available) between the different treatments of same trial only and not between two trials.

Table 3. Hatching rates of brown trout eggs - effect of variation in fertilization protocols

Fertilization trial no.	Extender code (NBFGR)	Protocol	Volume of coelomic fluid removed (ml)	Volume of 1% sod. bicarbonate added (ml)	Activator (5 ml)	Hatching rate (Mean± S.D)*
I (Dec. 1992)	6	Standard 1 **	0	3	Dexter	6.85^{d}±5.72
		Variation 1.1	1	2	Dexter	36.4^{a}±4.4
		Standard 2 **	0	3	Dexter	53.11±1.41
II (Jan. 1994)	3B	Variation 2.1	0	3	Dexter @	55.75
		Variation 2.2	0	0	Dexter	49.47
		Variation 2.3	1	3	Dexter	55.06

@ 5 ml Dexter solution contained 5 mM theophylline (11 mg/10 ml)
*n=3, wherever standard deviation values are given.
Means superscribed with same letter are not significantly different ($p>0.05$); t-test carried out between different treatments of the same fertilization trial only.
** Hatching rates same as in table 2 as experiments were carried out simulteneously.

Results and Discussion

The results are presented in Tables 2 & 3. In the first fertilization trial, extender 3 gave the highest mean hatching percentage of 42.4 which was not significantly different from control ($p > 0.05$). Similarly, in the second experiment, the extender-3B gave a higher mean percentage of hatching (49.47 to 55.75% in different fertilization protocols) than the other extenders. (Table 3). The common feature in both these was the presence of egg yolk. The difference was more clear in the case of 3B containing 2% egg yolk which gave a significantly higher hatching rate when compared to extender 3A containing no egg yolk ($p<0.01$). The egg yolk lipoproteins firmly bind to bovine spermatozoa and thus play a role of extra-cellular non permeating cryoprotectant along with the penetrating ones (Baynes & Scott, 1987). The present results in brown trout using 2% egg yolk are in accordance with the observations on rainbow trout, Atlantic salmon, summer whiting and some other species (Stein & Bayrle, 1978; Legendre & Billard, 1980; Young *et al.*, 1992) except that the concentration of egg yolk used in this study was much lower. Baynes & Scott (1978) reported that milt of rainbow trout diluted in a sucrose based extender containing 10% egg yolk consistently gave good fertilization rates. Piironen (1993) observed that addition of 20% egg yolk to 0.3 M glucose extender was beneficial for brown trout sperm but not for the milt of Arctic charr. The present experiment yielded equally high hatching rates with as low as 2% egg yolk. This indicates that the optimum amount of yolk required may vary with the species and specific composition of extender used. With extender 6, the hatching rate showed significant increase in the second fertility trial (standard protocol); however it still formed lowest among all the other extenders in this trial (Table 2).

In salmonids, the coelomic fluid extruded together with the ripe eggs at the time of egg-stripping possesses some physiological functions such as activation and prolongation of sperm motility and preservation of unfertilized eggs (Matsubara *et al.*, 1985). Erdahl (1988) recommends the use of coelomic fluid during fertilization procedures. But the volume of coelomic fluid is often not large enough to initiate the motility of all spermatozoa. Also, due to its variable composition, and the tendency of sperms to get agglutinated by contents of broken eggs, the coelomic fluid is often discarded (Leung & Jamieson, 1991; Billard, 1992) and diluents such as 1% sodium bicarbonate are used as the insemination media during artificial fertilization. This would also provide an adequate distribution of milt in relation to ova (Leung & Jamieson, 1991) in addition to activating all the spermatozoa. In the fertilization test I of the present experiment, removal of 1 ml of coelomic fluid from a lot of 150 eggs and addition of 1 ml sodium bicarbonate significantly improved the hatching rate ($p<0.01$; Table 3), when eggs were fertilized with milt cryopreserved in extender-6. However, in the second experiment (using extender-3B), removal of excess coelomic fluid did not yield any significant change in hatching rates (Table 3). Further studies are essential to determine whether such beneficial effects differ between extenders of varying chemical composition. A comparatively low hatching rate of 49.47% was recorded (extender-3B) when coelomic fluid was not removed and no diluent was added.

It had been reported that the addition of methylxanthines (eg. caffeine, theophylline, 3-isobutyl-l-methylxanthine) to fertilization activator solutions prolongs and intensifies sperm motility and fertilizing ability (Scheerer & Thorgaard, 1989). In the present study, the fertilizing capacity of cryopreserved milt (extender-3B) was improved slightly

when eggs were fertilized in activator solution containing 5mM theophylline. However this needs to be confirmed using more replicates.

The highest mean hatching rates of the present experiments using cryopreserved milt are comparable with the hatching rates of the control. The values are comparable with the highest fertilization rates (eyed eggs as % of control) reported for brown trout (Stein & Bayrle, 1978; Stoss & Refstie, 1983 and Piironen, 1993). Thus it can be included that the technique of cryopreservation can be successfully adopted in the genetic improvement programme of brown trout.

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