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CRYOGENIC FREEZING OF SILVER BARB (*Barbonymus gonionotus)* SPERMATOZOA FOR GENE POOL CONSERVATION

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ABSTRACT

Experiments were conducted to develop and standardize the protocols of cryopreservation of sperm of *Barbonymus gonionotus.* Three extenders Alsever's solution, urea-egg-yolk and egg-yolk citrate and four cryoprotectants methanol, ethanol, DMSO and DMA were used. Cryodiluents were prepared by adding 10% cryoprotectant to 90% extender (% v/v). Milt and cryodiluents were mixed at a ratio of 1 : 9 for Alsever's solution and 1 : 4 for urea egg-yolk and egg-yolk citrate solutions. Alsever's solution with 10% DMSO showed the best performance and produced 78 ± 2.55% sperm motility at the post-thaw period. Urea egg-yolk and egg-yolk citrate with 10% DMSO produced 76 ± 1.87% and 74 ± 1.87% post-thaw motility respectively. When cryopreserved sperm was stored in liquid nitrogen for 130 days, a gradual reduction in motility ranging from 31.25 to 37.50% was observed. This could be happened due to frequent opening of the nitrogen dewar that could cause thawing of the sample. In breeding trials, sperm preserved with Alsever's solution and urea egg-yolk plus 10% DMSO produced 14.28 ± 7.06% and 15.46 ± 5.50% hatching respectively. In contrast, sperm preserved with egg-yolk citrate and 10% DMSO produced poor hatching as 8.01 ± 2.15%.

Key words : Silver barb, Cryopreservation, Sperm, Fertilization

INTRODUCTION

Bangladesh is blessed with enormous endemic fish resources consisting of 260 freshwater fishes and 24 prawn species; 475 marine fishes and 36 shrimp species (DoF, 2009). Besides, a considerable number of exotic fish species have been introduced since 1960 (Rahman, 1985). Silver barb (*Barbonymus gonionotus*), locally known as Thai sarpunti is an herbivorous species native to South East Asia and is cultivated in freshwater ponds in Thailand, Indonesia and Vietnam (Tantong *et al*., 1980). It is introduced in Bangladesh from Thailand in 1977 (Rahman, 1989). Due to its bright silvery appearance, good taste, faster growth and good response to comparatively low cost and simple management

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practices (Akhteruzzaman, 1991), silver barb becomes a popular aquaculture candidate among the marginal fish farmers.

Fish production through aquaculture has been expanded many folds over the last two decades and artificial production of fry in the hatcheries has made it possible. As many as 900 private and government fish hatcheries are involved in producing seed of Indian major carps and exotic carps including silver barb (DoF, 2009) but the quality of seed has been deteriorated due to inbreeding, genetic drift, hybridization and unplanned broodstock management. Inbreeding could be happened in hatcheries specially in small hatcheries where the founder stocks are very limited (Hussain and Islam, 1999). Genetic deterioration of existing stocks of silver barb has been reported (Hussain and Mazid, 2001). It is, therefore, urgent need to take necessary steps to minimize these problems specially for silver barb as it is an exotic fish and could not possible to replenish the founder stock whenever necessary. Cryopreservation of sperm could be considered as an important technique to conserve the gene pool of the species. However, no work on cryopreservation of silver barb has been reported in Bangladesh and elsewhere. In the present study, attempts were taken to develop the cryopreservation protocols by optimizing cryodiluents and by assessing storage effect on post-thaw motility of sperm and fertilization capability of cryopreserved sperm.

MATERIALS AND METHODS

Experimental fish

The broods of silver barb were collected from Bangladesh Fisheries Research Institute (BFRI) and from some other known hatcheries and stocked in the ponds at the vicinity of Fisheries Faculty premises, Bangladesh Agricultural University, Mymensingh. Fish were reared with supplementary feed comprising of mustard oil cake, rice bran and wheat bran at the rate of 45%, 30% and 25%, respectively. Vitamin E was provided with the feed to enhance the gonadal development. The feed was supplied twice a day at the rate of 4-5% of the total body weight of the fish.

Experimental design

Three experiments were conducted in which determination of the suitable extender, cryoprotectant and their combination was the first one. Assessment of storage effect on post-thaw motility of sperm and fertilization capability of cryopreserved sperm were the second and third experiments.

Though the three experiments were conducted separately the procedures for collection of milt and its preparation for preservation were same in all the experiments. In the first experiment, three extenders and four cryoprotectants were used. The extenders were Alsever's solution, urea egg-yolk and egg-yolk citrate while methanol, ethanol, DMSO and DMA were the cryoprotectants (Table 1). Cryodiluents were prepared by mixing 90% extender with 10% cryoprotectant (v/v). Milt samples were diluted with the cryodiluents at the ratio of 1 : 4 (milt : cryodiluent) for egg-yolk citrate and urea egg-yolk and 1 : 9 for Alsever's solution.

Table 1. Chemical constituents of different extenders used for cryopreservation of *B. gonionotus* sperm

Extender / Constituent	I	II	III
	Urea egg-yolk	Egg-yolk citrate	Alsever's solution
Sodium chloride	0.3%	0.4%	0.4 %
Urea	0.4%	-	-
Sodium citrate	-	0.3%	0.8 %
	Dissolved in 100 ml of distilled water. Pancromycin (1000 IU/ml) and streptomycin (1000 IU/ml) were added to the buffer solution. Egg yolk was added to the buffer at a ratio of 1:4 (egg yolk: buffer). Therefore, 80 ml of buffer was mixed with 20 ml of egg yolk.		Dissolved in 100 ml of distilled water

For the second experiment, required number of straws were prepared at a time and stored in liquid nitrogen for 130 days. Three extenders, Alsever's solution, urea egg-yolk and egg-yolk citrate solutions and two cryoprotectants, methanol and DMSO were used. Two straws were sampled for each treatment at 10 days interval. Post thaw motility was recorded by eye estimation under the microscope at X10 magnification.

For the third experiment, matured females those were induced with PG extract were stripped immediately after ovulation to collect the eggs. The collected eggs were fertilized with both cryopreserved and fresh sperm. Sperm used for fertilization were preserved with three different extenders such as Alsever's solution, urea egg-yolk, egg-yolk citrate and one cryoprotectyant, DMSO.

Milt collection

Milt was collected following the procedure mentioned by Sarder *et al*. (2007). Mature males were selected by applying gentle pressure on abdomen which extrudes white milt. Before collection of milt, little pressure was applied on abdomen to remove some milt that contains urine and water. When the milt looked concentrated and creamy white it was collected in a 5 ml glass tube. The milt was stored on ice. The quality of sperm in the tube was checked under the microscope at X10 and X40 magnifications, by placing 1-2 µl of milt on a glass slide. Milt sample that contained >80% motile sperm was used for preservation.

Preparation of milt for freezing

According to the experimental design milt sample was diluted with the cryodiluents and drawn into 250 µl plastic (French) straws with the help of a micropipette. The open end of the straw was sealed by heating and placed them into the cryochamber. Computer-controlled freezer (CL-3300) was used to freeze the sample using the two-step freezing protocol, where milt sample was first cooled from the ambient temperature (0^{o}C) to -4^{o}C at a decreasing rate of 4^{o}C per min, and then to -80^{o}C at a decreasing rate of 10^{o}C per min. Frozen samples were removed from the cryochamber and loaded into the canisters and finally placed into the liquid nitrogen (-196^{o}C) for long-term preservation.

Determination of the effect of storage time on the post-thaw motility of sperm

To determine the effect of storage period on post-thaw motility of sperm, a desired number of straws were prepared at a time and stored in liquid nitrogen for 130 days. Three extenders, Alsever's solution, urea egg-yolk and egg-yolk citrate solutions and two cryoprotectants methanol and DMSO were used. Methanol and DMSO was mixed with Alsever's solution, and DMSO was mixed with urea egg-yolk and egg-yolk citrate. Two straws were sampled for each treatment at 10 days interval. The motility of thawed sperm was observed under microscope by adding physiological saline. Post thaw motility of sperm was recorded by eye estimation under the microscope at X10 magnification.

Collection, fertilization and hatching of eggs

Matured females were induced by pituitary gland extract injection. Eggs were collected from female fish by stripping into a plastic bowl immediately after ovulation. Stripped eggs were divided into four batches where each batch contained approximately 1200 eggs. Three batches of eggs were fertilized with cryopreserved sperm (sperm preserved with three different cryodiluents) and one batch was fertilized with fresh sperm as control. The frozen straws, after thawing at room temperature for about 30-60 sec, were cut at both ends. Sperm from 15 straws (each straw contained 230 µl of diluted milt) was used to fertilize each batch of eggs.

After mixing sperm, 5-10 ml of 0.9% NaCl solution was added to the egg mass-milt and mixed with feather for about 1 min. The fertilized eggs were washed carefully 3 to 4 times with tap water and transferred into marked incubation jars that were provided with continuous water flow for egg movement. After 6h of fertilization, some eggs were collected from all jars (eggs fertilized with cryopreserved and fresh sperm) and observed the progress of cell division under microscope. After 18-24 h of fertilization when the eggs were hatched, hatchlings were counted and transferred to the plastic bowls and reared.

Statistical analysis

Data were analyzed by two-factor randomized block design using computer software package (MSTAT) and one way ANOVA followed by Duncan Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

Effect of cryodiluents on spermatozoan motility

Three extenders Alsever's solution, urea egg-yolk and egg-yolk citrate and four cryoprotectants viz. methanol, ethanol, DMSO and DMA were used to preserve the sperm. The effects of these extenders and cryoprotectants on the motility of sperm at both equilibrium and post- thaw periods are presented in Fig. 1 and Table 2. It was found that most of the cryodiluents produced >80% equilibrium motility but it was significantly reduced after thawing.

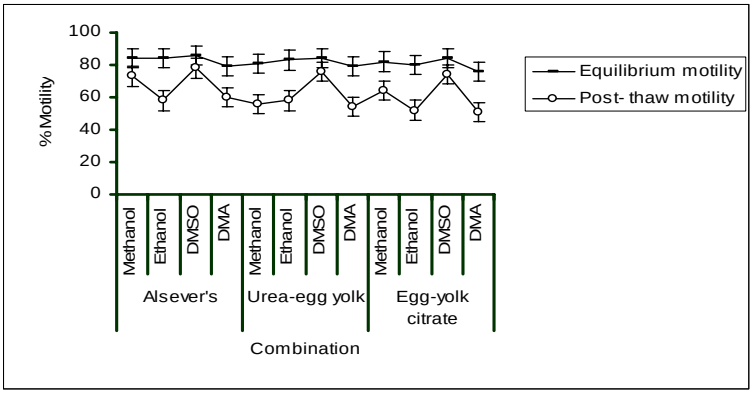

Fig. 1. Comparison between equilibrium and post-thaw motility of *B. gonionotus* spermatozoa at different combinations of extenders and cryoprotectants

Table 2. Effects of different cryodiluents on the equilibrium and post-thaw motility of spermatozoa of *B. gonionotus*

Extender	Cryoprotectant	Equilibrium motility	Post-thaw motility
Alsever's solution	Methanol	84 ± 1.87^{ab}	73 ± 2.55^{a}
	Ethanol	84 ± 1.87^{ab}	58 ± 2.55^{bcd}
	DMSO	86 ± 1.87^{a}	78 ± 2.55^{a}
	DMA	79 ± 1.87^{bc}	60 ± 1.58^{bc}
Urea egg-yolk	Methanol	81 ± 1.87^{abc}	56 ± 2.92^{cd}
	Ethanol	83 ± 1.22^{ab}	58 ± 2.55^{bcd}
	DMSO	84 ± 1.87^{ab}	76 ± 1.87^{a}
	DMA	79 ± 1.87^{bc}	54 ± 1.87^{cd}
Egg-yolk citrate	Methanol	82 ± 1.22^{ab}	64 ± 1.87^{b}
	Ethanol	80 ± 1.58^{bc}	52 ± 2.55^{d}
	DMSO	84 ± 1.87^{ab}	74 ± 1.87^{a}
	DMA	76 ± 1.87^{c}	51 ± 1.87^{d}
Co-efficient of variance		4.68	8.04

Alsever's solution with 10% DMSO produced the best result as 78±2.55% sperm motility at post-thaw period. Egg-yolk citrate and urea egg-yolk with 10% DMSO produced 76 ± 1.87% and 74 ± 1.87% post-thaw motility respectively. All the three extenders produced satisfactory results, closer to each other and they have been selected by observing their performances in the previous studies of Kumar (1988 & 1989) on Indian major carps and Linhart and Rodina (2000); Sarder *et al. (*2007) on common carp. Egg-yolk as extender improves post-thaw motility because the low density lipoprotein fraction (LDL) associates with cell membranes and provides protection against injury during the cryopreservation process (Babiak *et al.,* 2002). The dilution ratio between milt and cryodiluent was selected as 1:4 for egg-yolk citrate, urea egg-yolk, and 1:9 for Alsever's solution as these dilutions were found effective in other studies of Magyary *et al.* (2000); Kumar (1988 and 1989); Shirohara *et al.* (1982); Withler and Lim (1982); Chao *et al*. (1975);

Sarder *et al.* (2007). Cryoprotectant concentration was maintained at 10% (v/v) of the cryodiluent as it was reportedly common and effective for many species (Shirohara *et al.*, 1982; Withler and Lim, 1982; Chao *et al.*, 1975; Sarder *et al.*, 2007). Among the four cryoprotectants used DMSO performed best and its performance in the study is expected as it has been widely used as an excellent cryoprotectant (Stoss, 1983; Rana *et al.*, 1995) due to its easy penetration of the cell (Rao, 1989).

Effects of storage time on the post-thaw motility of sperm

The effects of storage time on the motility of preserved sperm were investigated for a period of 130 days. A gradual reduction in the motility of sperm has been observed with the progress of storage time. Four cryodiluents were used to preserve the sperm and at the end of the storage period 31.25%, 37.50%, 33.33% and 31.25% reduction in the motility of sperm was recorded from Alsever's solution + DMSO, Alsever's solution + methanol, urea egg-yolk + DMSO and egg-yolk citrate + DMSO respectively (Fig. 2). In fact no motility reduction was observed in the first 20 to 30 days of storage from any of the cryodiluents, which agreed with findings of Christensen and Tiersch (2004) who reported no difference in motility reduction of sperm stored in 5% methanol for 1 or 48 h using 0.5 or 0.25 mL straws, or in 5% methanol for 1h or 5-day exposures. The reasons for reduction of motility of sperm in the study are not well understood but lack of storing facility could be one of the most important reasons as only one nitrogen can was used for storing frozen sperm and frequent opening of the lid of the can might be the cause of thawing of sperm.

Effects of cryopreserved spermatozoa on fertilization and hatching of eggs

Eggs were fertilized with cryopreserved and fresh sperm. The eggs fertilized with cryopreserved sperm (preserved with three different cryodiluents) yielded 55.83±1.58% to 64.17 ± 3.0% fertilization and it was significantly ($P<0.001$) lower than that of fresh sperm (average 80 ± 2.45%). The hatching rates of eggs obtained from cryopreserved sperm was between 8.01 ± 2.15% to 15.46 ± 5.50% which was also significantly ($P<0.001$) lower than that of fresh sperm (average 62.91 ± 7.61%) (Table 3).

Table 3. Fertilization and hatching of *B. gonionotus* eggs using cryopreserved sperm

Extender + Cryoprotectant	Eggs No.	Average fertilization rate (%)	Average hatching rate (%)	Average hatching rate (%) compared to controls
Alsever's + DMSO	1200	64.17 ± 3.0^{b} (60-75%)	14.28 ± 7.06^{b} (0.83-40%)	18.92 ± 8.158
Urea egg-yolk + DMSO	1200	62.50 ± 1.22^{b} (40-70%)	15.46 ± 5.50^{b} (0.58-36.6%)	20.79 ± 6.011
Egg-yolk citrate + DMSO	1200	55.83 ± 1.58^{c} (35-65%)	8.01 ± 2.15^{b} (0-13.3%)	10.75 ± 2.633
Control	1200	80 ± 2.45^{a} (75-90%)	$62..91 \pm 7.61^{a}$ (20-87.9%)	-

Values in the same column with different superscripts are significantly ($P<0.001$) different

Linhart *et al*. (2000) observed significant differences between fresh and frozen sperm of common carp, *Cyprinus carpio* on the fertilization rate (68 ± 11% and 56 ± 10%, respectively), but non-significant differences between fresh and frozen sperm on the hatching rate (50 ± 2% and 52 ± 9%, respectively). Lihnsteiner *et al*. (2002) reported that the fertilization rate of frozn sperm was 10% lower than of fresh sperm, but both sperm produced similar hatching rates. Withler and Lim (1982) found the frozen spermatozoa of *Labeo rohita*, after thawing as effective as the fresh control but the fertilization rate has never exceeded 50% versus the controls, although many more cryopreserved spermatozoa were used.

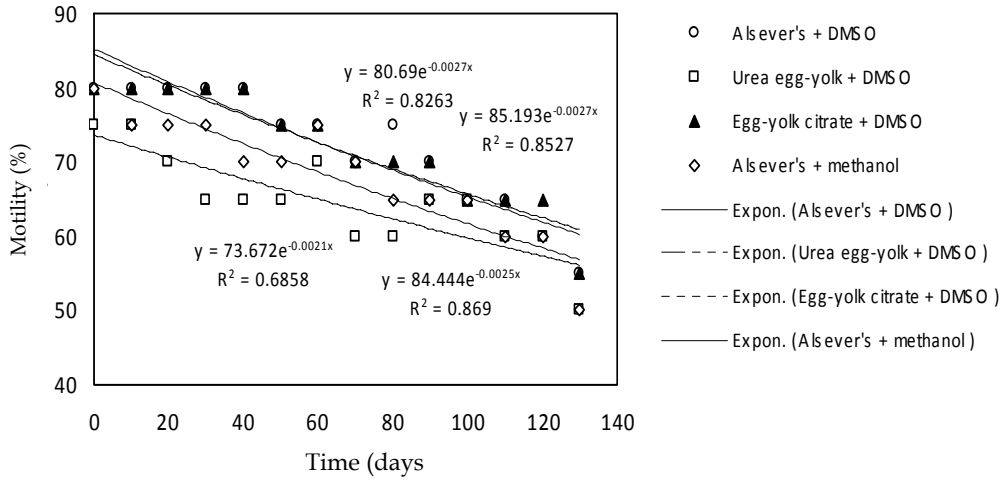

Fig. 2. Effects of storage time on the post-thaw motility of *B. gonionotus* spermatozoa preserved with different cryodiluents

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