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Development of sperm cryopreservation technique of minor carp, bata (*Labeo bata* Ham. 1822) for *ex-situ* conservation

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Abstract. The study was focused on the development of suitable sperm cryopreservation protocol for minor carp, bata (*Labeo bata*). Sperms were collected from hormone induced males by stripping. Sperm motility activation was assessed in different concentrations of activation solution (NaCl) and observed that sperm motility diminished with increasing concentration of NaCl and fully inhibited at 1.10% NaCl. Toxic effects of cryoprotectants to sperm was assessed using two cryoprotectants- DMSO and methanol along with two extenders (Alsever's solution and egg-yolk citrate) at 5, 10 and 15% concentrations for 5 to 45 min. Alsever's solution along with 10% DMSO resulted in the best equilibration (86.66±3.33%) as well as post-thaw motility (70.0±2.88%) while egg-yolk citrate with DMSO produced similar equilibration motility (68.33±4.41%). In breeding trials, sperm preserved with Alsever's solution and DMSO showed highest fertilization rate (87.5±2.5%) and hatching rate (18.4±6.8%) while sperm preserved with egg-yolk citrate and DMSO produced 82.5±2.5% fertilization and 16.4±5.8% hatching rates. Fresh sperm yielded 92.5±2.5% and 63.6±6.6% fertilization and hatching rate respectively. The protocols that have been standardized can be applied for preservation of sperm of *L. bata*.

Keywords: *Labeo bata*, Cryopreservation, Conservation.

Introduction

Labeo bata is a highly nutritious minor carp species having 66.75% protein, 13.44% lipid, 16.63% ash and 3.51% carbohydrate and also rich in omega-3 fatty acid (Mahfuz *et al*. 2012). It is locally called as ilishbata, bhangonbata or bata, belonging to the family Cyprinidae. Considering the future biodiversity status of *L. bata* in nature, conservation of its gene pool is important to keep the species from being threatened and for genetic improvement through selective breeding. It is, therefore, important to take immediate measures to safeguard the genetic diversity of the species and to do so an integrated approach, for example, live gene banking and cryogenic gene banking can be taken. Cryopreservation of sperm is one the most effective tools of *ex-situ* conservation. Cryopreservation deals with cryobiology that relates to long-term preservation and storage of biological materials at very low temperature, usually at -196°C. At this low

temperature, cellular viability can be stored in a genetically stable form (Ashwood-smith 1980). Cryoprotectants and extenders are used as media where sperm are kept for long or short-term preservation. Because these diluents save sperm from cryoinjuries which occur due to cold shock during freezing and heat shock when thawed. Other reasons of cryoinjuries are pH fluctuation, ice crystal formation, osmotic pressure and cryoprotectant toxicity (Chao and Liao 2001).

Formulation of suitable activation solution is very important for successful cryopreservation. Although immobility is the characteristic of fish sperm in the testis (Morisawa and Suzuki 1980) these become activated on release into the aquatic environment (Billard and Cosson 1992, Alavi and Cosson 2005, Alavi and Cosson 2006, Yang *et al*. 2006). The activation mechanisms of fish sperm vary among species. Sperm of freshwater fish species usually become activated in hypotonic solutions but sperm of marine species are turned on in hypertonic solutions (Morisawa and Suzuki 1980, Yang *et al*. 2006). Development of sperm cryopreservation protocol of *L. bata* would assist to conserve the existing genetic resource of the species and ensure supply of sperm for propagating new generation. Hence, the objectives of the study was to develop a cryopreservation protocol through assessing sperm quality; toxicity level of cryoprotectants to sperm; suitability of diluents; and efficacy of cryopreserved sperm.

Materials and Methods

Experimental site: Brood fishes of *L. bata* were collected from natural sources (*haors* and rivers) in Mymensingh district and stocked in the ponds in the vicinity of Fisheries Faculty premises, Bangladesh Agricultural University, Mymensingh. Fishes were fed twice a day at 4-5% of total body weight of fish with a commercial feed (Mega Feeds) containing 35% protein.

Selection and conditioning of brood fish: Matured male fishes were caught from the pond about 6 h prior to hormone treatment and kept in cistern for conditioning. Additional oxygenation was ensured by supplying water continuously through perforated PVC pipe over the cistern.

Sperm collection, quality assessment and counting: Male broods of 90-120 g body weight were selected and injected once with a single dose of 2 mg PG/kg body weight. The injected fishes were then kept in cistern for 8-12 h with continuous water showering and then the milt was collected by stripping. Milt was stored in eppendorf tubes kept on ice. The sperm quality was evaluated under a light microscope. The count of sperm per unit volume of milt was made using a hemocytometer. Sperm concentration varied between 5.9×10^{9} cells ml^{-1} to 6.5×10^{10} cells/ml.

Estimation of sperm motility: To assess the quality of sperm, motility was evaluated under a light binocular microscope by putting 1-2 µl of milt on a glass slide. About 50-100 µl of distilled water was added to milt to activate the sperm and observed the sperm motility. The motility was assessed using a compound microscope with 10X magnifications. The motility was denoted as the percentage of sperm which showed active rotatory movement.

Collection of eggs and fertilization: Female brood fishes of 110-150g body weight were stimulated with PG extract at a dose of 1 mg PG/kg body weight as the first dose and 4 mg PG/kg body weight as the second dose. Stripping was applied to collect eggs into a plastic bowl just after ovulation. Eggs were taken for fertilization using a measuring spoon and the number of eggs in each spoon was counted. This was done to calculate the total number of eggs used for fertilization. Collected eggs were then used to fertilize both fresh and cryopreserved sperm thawed at room temperature (26^{o}C).

Study I: Activation of sperm at various concentrations of NaCl: Promptness of sperm motility was performed at different concentrations of NaCl solution (Sarder *et al.* 2013). After collection, sperm was kept on ice until use. Dilutions of eleven grade of NaCl (from 0.1 to 1.1%) were made by mixing NaCl salt in distilled water. About 1-2 μl of milt suspension was put on a glass slide and 20-25 μl of NaCl solution from the stock of different grades of NaCl were mixed to activate the sperm. Sperm motility was assessed instantly under microscope and the percent motility was documented. The percentage of motility and swimming time of prompted sperm were recorded at different concentrations of NaCl. It was determined by calculating duration from sperm activation to the time when the sperm became inert.

Study II: Evaluation of toxicity effect of different cryoprotectants to sperm: Cryoprotectant toxicity to sperm was assessed according to the method of Yang *et al.* (2007) and Sarder *et al.* (2013). Two extender, Alsever's solution and egg-yolk citrate and two cryoprotectants, DMSO and methanol were used. In case of egg-yolk citrate, milt was diluted at a ratio of 1:4 and for Alsever's solution it was 1:9. Cryoprotectants (DMSO, methanol) were mixed with the milt in such a way to make the final concentration of cryoprotectants of 5, 10 and 15%. The mixture was kept on ice. The toxicity of these cryoprotectants was assessed by monitoring the motility of sperm under a microscope for a maximum period of 45 min with an interval of 5 min.

Study III: Selection of suitable diluents (extender plus cryoprotectant): For selecting suitable extenders and cryoprotectants, Alsever's solution and egg-yolk citrate were used as extenders; DMSO and methanol were used as cryoprtectant. Chemical constituents and preparation protocol of two extenders used for sperm cryopreservation of *L. bata* were different. Egg yolk citrate (pH 6.7) was prepared by using sodium chloride (0.4%) and sodium citrate (0.3%) dissolving in distilled water. Egg yolk was added to the solution at a ratio of 1:4 (egg yolk: buffer). Therefore, 80

ml of buffer was mixed with 20 ml of egg yolk. On the other hand, Alsever's solution (pH 7.9) was prepared by adding sodium chloride (0.4%) and sodium citrate (0.8%) dissolved in distilled water. To prepare the standard diluent (extender+ cryoprotectant) 10% cryoprotectant was added to 90% extender (% v/v). For each diluent at least two replications were maintained to minimize the error. The diluted samples of milt were equilibrated for 5-10 min and 0.22 ml diluted milt was taken into 0.25 ml French straws (Minitüb System, Minitüb, Tiefenbach, Germany). Sealing of the free ends of straws was done manually using a heated crucible tongs. The quality of sperm of all the samples was examined twice: one was before adding of cryoprotectants and another was just before freezing as equilibration motility.

Freezing and thawing of milt: Straws containing diluted milt were kept in the cryo-chamber of a computer controlled-rate freezer (CL 3300) (Cryologic, Pty. Ltd., Australia 1998 & 1999) for freezing. The freezing rate was optimized through several trials. A one-step freezing method was applied where milt samples were subjected to cool from 0°C to -80°C at the temperature falling rate of 10°C/min. Frozen samples were transferred to liquid nitrogen (-196°C) for storage. Post-thaw motility of sperm was checked retrieving the frozen straws from the liquid-N_2 container using a tweezer and thawed at room temperature for 30-40s. One to 2 µl of thawed milt sample was taken every time onto a glass slide and 150-200 µl of distilled water was mixed to activate the sperm. Then percent motility of sperm was measured under a binocular microscope.

Standardization of cryopreserved sperm with fresh sperm: For fertilization of eggs, 8 straws each containing 0.22 ml diluted sperm were used every time. The total concentration of sperm was calculated as 6.4×10^9 cells/ml. In 8 straws total concentration of sperm cells was 1.024×10^9 cells (sperm preserved with Alsever's+DMSO at 1:9 dilution ratio). So, for standardization of fresh sperm with cryopreserved sperm, 0.16 ml of fresh milt was needed. Similarly, in the case of egg-yolk citrate along with DMSO (dilution ratio at 1:4), 4 straws provided a total number of 1.024×10^9 spermcells, so 0.16 ml fresh milt was needed to equalize the number of fresh sperm with cryopreserved sperm.

Study IV: Effects of cryopreserved sperm on fertilization and hatching of eggs: This experiment was conducted to find out whether the cryopreserved sperm have an effect on fertilization and hatching of eggs. Mature females of 110-150g body weight and males of 90-120g body weight were selected for breeding trials and kept in cisterns ($7\times5\times2.5$ cubic feet) separately for conditioning. Mature females were treated with PG extract for ovulation and eggs were stripped onto a plastic bowl from the ovulated females. Three batches of eggs were prepared where each batch containing about 500 eggs; two batches of eggs were used for fertilization with cryopreserved sperm and one for control. The first batch of eggs with a replication was used to fertilize by 8×0.22 ml of sperm cryopreserved with Alsever's solution plus DMSO and the second batch of

eggs with a replication was fertilized with 4×0.22 ml of cryopreserved sperm preserved with egg- yolk citrate plus DMSO. As control the third batch of eggs with a replication was fertilized with fresh sperm. As the number of cryopreserved sperm (1.024×10^9 sperm) was standardized with fresh sperm, 0.16 ml of fresh milt was used for both trials. After fertilization eggs were transferred to a circular hatching bowl where continuous water supply was provided. It also created a circular motion which prevents the eggs to settle down in the bowl. After about 1h of fertilization, cell division started that was observed under microscope. The properly fertilized eggs were vivid and translucent while unfertilized eggs had opaque appearance and white chorion. To hatch out the fertilized eggs took 16-24 h, hatchlings were counted and transferred to plastic bowls for further rearing. The rate of fertilization was calculated as fertilization percentage which was obtained from the whole number of eggs for different treatments. Similarly, rate of hatching from different treatments was determined as the percentage of hatchlings obtained from entire number of eggs.

Statistical analyses: Data of experiments I and II were analyzed using Independent - samples T-test of Statistical Package for the Social Science (SPSS v 16) and to separate the means Least Significant Difference (LSD) at 5% probability level was used. The effects of different extenders and cryoprotectants and their combinations (Expt. III) on both equilibration and post-thaw motility of sperm were assessed using one-way ANOVA of SPSS (version 16). Duncan's Multiple Range Test (DMRT) at 5% level of significance was used to separate means. For breeding trial (Study IV), Independent-samplesT-test of SPSS (version 16) was used to compare the efficacy of cryopreserved sperm.

Results

Sperm motility activation at various concentrations of NaCl: Sperm motility activation at various concentration of the salt solution (%NaCl) along with distilled water was tested for *L. bata*. It showed that the sperm motility decreased as NaCl concentration increased. Sperm motility in distilled water was $87.5\pm2.5\%$. The sperm motility in 0.1% NaCl solution was 95% which gradually reduced to $82.5\pm2.5\%$ at 0.4% NaCl solution (Fig. 1). As the motility was stable at 0.4% NaCl (128 mOsmol/kg), it was considered as complete activation. At 1.1% NaCl solution the motility was completely inhibited thus considered as complete inhibition. Statistical analysis showed a significant difference ($p<0.05$) between complete activation and inhibition of the NaCl concentration.

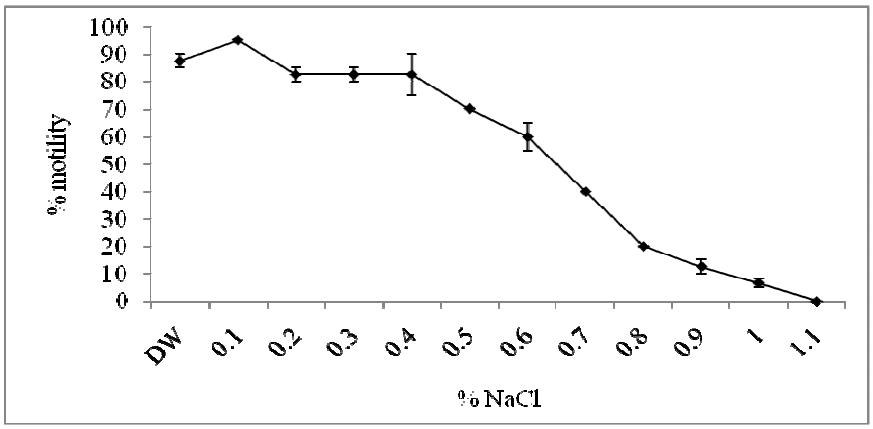

Fig. 1. Motility of sperm of *L. bata* in different concentrations of NaCl solution (0.1 to 1.1%). Sperm was suspended in Alsever's solution during collection. Data are presented as mean ± SE.

Swimming duration of sperm in different concentrations of NaCl: The duration of swimming of activated sperm reduced with the increase of NaCl solution. The swimming duration was estimated as long as 18.54±2.54 min at 0.1% NaCl and the highest was recorded as 19.2±2.02 min at 0.3% NaCl. The swimming was totally ceased at 1.1% NaCl (Fig. 2). The swimming duration of activated sperm in distilled water was 3.6±0.4 min. Statistical analysis revealed that there was a significant difference (p<0.05) between maximum swimming duration and complete inhibition.

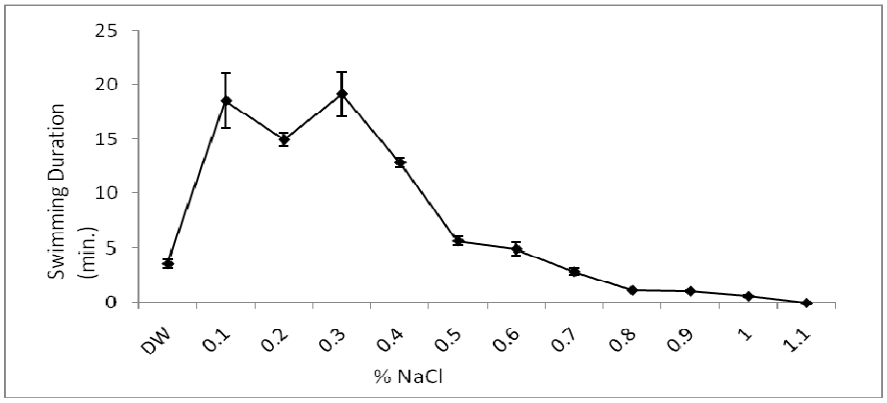

Fig. 2. The swimming duration of sperm of *L. bata* in different concentrations of NaCl solution (0.1 to 1.1%). Data are presented as mean±SE.

Determination of toxicity of various cryoprotectants: Fresh sperm motility of *L. bata* just before incubation with cryoprotectants was 85-90%. Motility of sperm decreased as the concentration of cryoprotectants (5, 10 and 15%) and incubation time (5 to 25 min) increased. At 5 min incubation of sperm in Alsever's solution with 5% DMSO 80±5% motility was recorded which reduced to 70±10% at 10 min. About 72.5±2.5%

motility was found from 10% DMSO with Alsever's solution at 5 min incubation that reduced to 62.5±2.5% at 10 min (Table I). No significant difference (p>0.05) was observed between 5% and 10% cryoprotectant during 10 min incubation.

In the case of egg-yolk citrate, 5% DMSO produced 57.5±2.5% motility at 5 min incubation which dropped to 51.5±3.5% at 10 min. At 10% DMSO sperm motility was recorded as 50±5% at 5 min incubation that reduced to 45.0±5.0% at 10 min. 15% DMSO produced 47.5±2.5% motility at 5 min which dropped to 37.5±2.5% at 10 min (Table I). No significant difference (p>0.05) was found between 5% and 10% cryoprotectant during 10 min incubation.

When methanol was used with Alsever's solution at 5, 10 and 15% concentration, mean sperm motility were 55±5, 50±10 and 40±5% at 10 min of incubation respectively (Table I). Along with egg-yolk citrate solution 5, 10 and 15%methanol produced 50±5 and 47.5±2.5 42.5±2.5% sperm motility at 10 min of incubation respectively (Table I). Methanol at 15% concentration produced poor results with both Alsever's solution and egg-yolk citrate from the beginning of incubation. No significant difference (p>0.05) was noticed between 5% and 10% cryoprotectant at 10 min incubation. Similarly, no significant difference was also observed between 5% and 15% (p>0.05) and 10% and 15% (p>0.05) cryoprotectant used with Alsever's solution and egg-yolk citrate respectively. Since 5 and 10% cryoprotectant seemed less toxic to sperm, the later one was applied in the subsequent experiments.

Table I. Motility of *L. bata* sperm at different concentrations of cryoprotectants and incubation times using the extenders: Alsever's solution, and Egg-yolk citrate.

Extender	Cryoprotactant		Time (Min.)								
			Initial	5	10	15	20	25	30	35	40
			% motility								
			Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean
Alsever's solution	DMSO	5%	92.5±2.5	80.0±5.0	70.0±10	45.0±5.0	32.5±7.5	20.0±10	12.5±7.5	3.5±1.5	0
		10%	82.5±2.5	72.5±2.5	62.5±2.5	47.5±2.5	25.0±0.0	12.5±2.5	2.5±2.5	0	
		15%	72.5±2.5	57.5±2.5	40.0±5.0	22.5±2.5	12.5±2.5	2.5±2.5	0		
	Methanol	5%	80.0±5.0	65.0±5.0	55.0±5.0	37.5±2.5	20.0±5.0	10.0±5.0	4.0±4.0	0	
		10%	77.5±7.5	62.5±7.5	50.0±10	35.0±10	22.5±7.5	12.5±7.5	5.0±5.0	2.5±2.5	0
		15%	70.0±5.0	55.0±5.0	40.0±5.0	27.5±2.5	17.5±2.5	7.5±2.5	2.5±2.5	0	
Egg-yolk citrate	DMSO	5%	62.5±2.5	57.5±2.5	51.5±3.5	40.0±5.0	30.0±5.0	17.5±2.5	10.0±5.0	2.5±25	0
		10%	62.5±2.5	50±5.0	45.0±5.0	35.0±5.0	27.5±2.5	15.0±5.0	7.5±2.5	2.5±2.5	0
		15%	57.5±2.5	47.5±2.5	37.5±2.5	25.0±5.0	17.5±7.5	10.0±5.0	2.5±2.5	0	
	Methanol	5%	70.0±5.0	60.0±5.0	50.0±5.0	42.5±2.5	32.5±2.5	20.0±5.0	7.5±2.5	0	
		10%	62.5±2.5	57.5±2.5	47.5±2.5	40±5.0	27.5±7.5	20.0±5.0	7.5±2.5	0	
		15%	57.5±2.5	52.5±2.5	42.5±2.5	37.5±2.5	25.0±5.0	15.0±5.0	5.0±5.0	0	

Selection of suitable diluents: The suitability of the extenders and cryoprotectants was assessed on the basis of sperm motility at equilibration and post-thaw period. The highest equilibration motility; 86.66±3.33% was recorded from Alsever's solution with DMSO followed by 68.33±4.41% from egg-yolk citrate with DMSO. In the case of post-thaw motility, the highest average motility 70±2.88% was recorded from Alsever's solution with DMSO. Egg-yolk citrate along with DMSO produced less post-thaw motility 50±2.89% than that with Alsever's solution. Though Alsever's solution and egg-yolk citrate along with methanol produced good equilibration motility (ranged from 55-65%) but poor post-thaw motility (<40%) was obtained (Fig. 3). So, both the diluents were discarded from breeding trials.

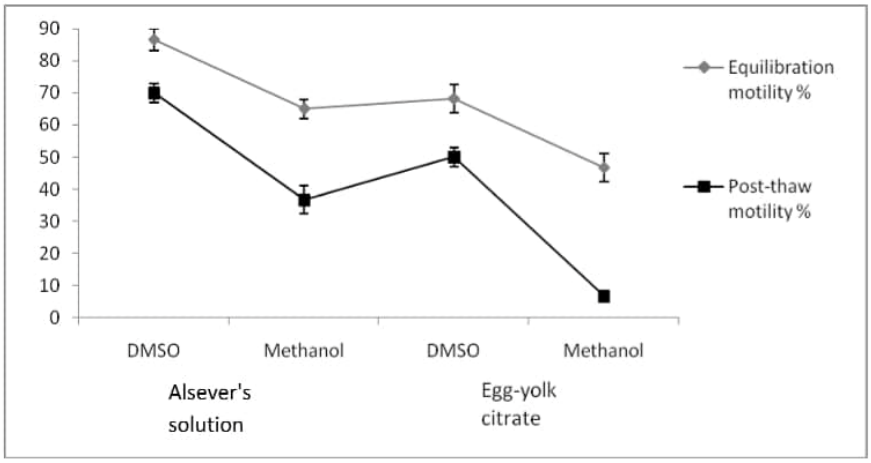

Fig. 3. Equilibration and post-thaw motility of sperm of *L. bata* at different combinations of extenders and cryoprotectants. Data are presented as mean ± SE.

The statistical analysis revealed a significant variation ($p<0.05$) between Alsever's solution plus DMSO and Alsever's solution plus methanol, for equilibration and post-thaw motility. However, no significant difference ($p>0.05$) was observed from egg-yolkcitrate with both DMSO and methanol during equilibration period but the post-thaw motility demonstrated a significant difference ($p<0.05$) between two diluents. Duncan's Multiple Range Test revealed that Alsever's solution plus DMSO was the best combination for the preservation of *L. bata* sperm.

Effect of cryopreserved sperm on fertilization and hatching: To see the efficacy of cryopreserved sperm eggs were fertilized with both cryopreserved as well as fresh sperm. The post-thaw motility of sperm preserved in Alsever's solution plus DMSO was 70±2.88% and for egg-yolk citrate plus DMSO was 50±2.89% during fertilization of eggs. The highest fertilization, 87.5±2.5% and hatching, 18.4±6.8%, was recorded from sperm preserved with Alsever's solution plus DMSO and those preserved with egg-yolk citrate solution plus DMSO produced 82.5±2.5% and

16.4±5.8% fertilization and hatching, respectively. In contrast, fresh sperm produced 92.5±2.5% and 63.6±6.6% fertilization and hatching respectively (Fig. 4). Statistical analyses were performed between the breeding performances of fresh and cryopreserved sperm as the concentration of both fresh and cryopreserved sperm was standardized. In the case of Alsever's plus DMSO and control, no significant difference ($p>0.05$) was revealed between these two groups in terms of fertilization but a significant difference was observed in terms of hatching ($p<0.05$). Similar result was obtained between egg-yolk citrate plus DMSO and control for fertilization ($p>0.05$) and hatching ($p<0.05$).

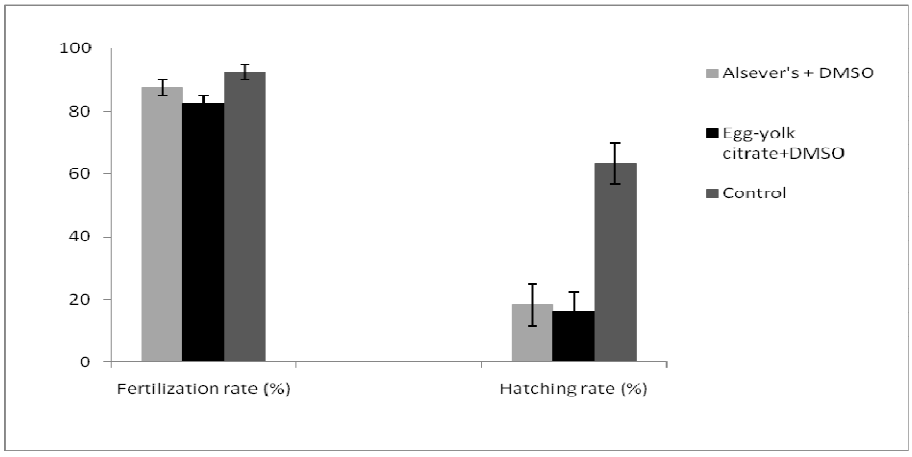

Fig. 4. Fertilization and hatching of eggs of *L. bata* using cryopreserved and fresh sperm. Data are presented as mean ± SE.

Discussion

In the present study sperm motility of *L. bata* was observed under different concentrations of NaCl (0.1%-1.1%). It showed that the motility decreased with the increase of the concentration of the extending solution. Motility of sperm decreased significantly at 1.1% NaCl i.e. the osmolality of seminal plasma seemed around the concentration. Alavi and Cosson (2005) reported that the osmolality of seminal plasma of most freshwater cyprinids are within a range of 230-346 mOsmol/kg. After activation, the sperm of *L. bata* showed active motility in both extenders. The duration of swimming of *L. bata* sperm also decreased in the same manner as activation did. The highest swimming duration was 19.15±2.02 min at 0.3% NaCl. Then swimming time was reduced sharply with the increase of NaCl concentration that was zero at 1.1% NaCl. However, Zebra fish showed a reverse situation where longer period swimming of sperm was found in higher osmolalities (Yang *et al*. 2007). Longer period swimming ability of sperm in extending medium offers an opportunity to have a good egg-sperm interaction that increases the chance of higher fertilization rate as well as hatching.

To lessen the stress on cells during cooling and freezing, cryoprotectant is added to sperm and also to protect the sperm cells from cold-shock through external crystal formation or cryo-injury. Chao (1991) reported that without cryoprotectant very few spermatozoa survived at very low temperature due to ice crystal formation. Although cryoprotectant is essential for preservation; it is likely to be toxic to the cell with the increasing concentration and incubation time. So, the concentration of cryoprotectant needs to be optimized. In the present study, 5% and 10% DMSO and methanol produced good results in most of the cases during 5 and 10 min incubation but 15% concentration produced worst result. In both fresh and marine water fish species, 10% DMSO has been used as a cryoprotectant because of its excellent permeation capacity (Withler and Lim 1982). A 10% (v/v) cryoprotectant concentration of the diluent was maintained as it showed effective for many species (Shirohara *et al.* 1982, Withler and Lim 1982, Chao *et al.* 1975, Daly *et al.* 2008, Nahiduzzaman *et al.* 2011). DMSO is well known and popular cryoprotectant, has been successfully used for the cryopreservation of sperm of salmonids (Erdahl *et al.*1984); *Cyprinus carpio* (Withler 1980); *Pangasius sutchi*, *Puntius gonionotus* (Withler 1980); *Labeo rohita*, *Catla catla*, *Cirrhinus mrigala* (Routray *et al.* 2006). DMSO and methanol as low molecular weight molecules were selected as cryoprotectants in the present study with the view that they would pass through the cell membrane easily and minimize the stress on the cells during cooling and freezing. Olive barb (*Puntius sarana*) sperm incubated with 5% DMSO and methanol showed higher motility and an acute toxicity was found at15% concentration had (Nahiduzzaman *et al.* 2011).

Dilution ratio between sperm and diluents is another important phenomenon that decides the life of the spermatozoa during cryopreservation. Extenders and cryoprotectants are added to the gametes at a particular ratio which differ from species to species depending on their gametes concentration. Several authors have used different dilution ratios ranging from 1:1 to 1:1000 at varying results (Lubzens *et al.* 1997, Scott and Bayens 1980).

An important pre-requisite for the successful cryopreservation protocols is the selection of suitable extender and cryoprotcetant. In this study, Alsever's solution and egg-yolk citrate were applied with DMSO and methanol. Between the two cryoprotectants used, 10% DMSO with Alsever's solution gave significantly better result compared to other combinations of extender and cryoprotectant at the post-thaw level. Alsever's solution with DMSO provided the highest equilibration and post-thaw motility of sperm which confirmed its suitability for cryopreservation of sperm of the species. It also indicates that Alsever's solution contains the osmotic pressure similar to that of sperm. Alsever's solution contains the Na-citrate fraction associated with cell membrane may provide defense against injury during cryogenic freezing. A satisfactory performance with Alsever's solution in sperm preservation of Indian major carp was reported by Kumar (1989). Satisfactory results from Alsever's solution with 10% DMSO in the preservation of silver carp (*Hypophthalmichthys molitrix*) sperm was also

reported (Alvarez *et al*. 2003). Sarder *et al*. (2012 & 2013) reported the best performance of Alsever's sloution with 10% DMSO among the diluents when cryopreservation was conducted with the sperm of *Nandus nandus* and *Ompok pabda*. The dilution ratio between milt and diluent was selected 1:4 for egg-yolk citrate and 1:9 for Alsever's solution. The dilution ratio was selected from the studies of Magyary *et al*. (2000); Kumar (1988 & 1989); Shirohara *et al*. (1982).

Breeding was conducted using cryopreserved sperm preserved with two different diluents (Alsever's solution+DMSO and egg-yolk citrate solution+DMSO) but highest hatching rate was obtained from Alsever's solution plus DMSO. Cryopreserved sperm showed successful fertilization and hatching of eggs but the results were inconsistent between trials. Variation of fertilization and hatching in subsequent breeding trials was reported by Rana and McAndrew (1989) where fertilization rates varied from 38.7 to 93.4% of control after 13 months of storage of tilapia spermatozoa. Similar results were reported by Sarder (2004), during cryopreservation of sperm of Indian Major Carps. Hossain and Sarder (2009) observed fluctuation in fertilization and hatching rates in silver carp, *Hypophthalmichthys molitrix*. Similar result was also observed in mrigal, *Cirrhinus cirrhosus* (Sarder *et al*. 2009). In the present study, though fertilization rates of cryopreserved sperm was satisfactory in compare to control but the hatching rates of cryopreserved sperm was significantly lower than that of control. The lower fertility could be occurreddue to several reasons such as (i) the lower motility of post-thawed sperm, (ii) the lower percentage of motile sperm due to freezing and thawing and (iii) lower fertility regardless of motility of cryopreserved sperm (Ohta *et al*. 1995).

Fertilization and hatching of eggs by cryopreserved sperm have been successful but need further research especially on improvement of fertilization and hatching rates with cryopreserved sperm.

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Literature Cited

Alavi, S.M.H. and J. Cosson, 2005. Sperm motility in fishes. I. Effects of temperature and pH: a review. *Cell. Biol. Intern,* 29: 101–110.

Alavi, S.M.H. and J. Cosson, 2006. Sperm motility in fishes. II. Effects of ions and osmolality: a review. *Cell. Biol. Intern.*, 30: 1–14.

Alvarez, B., R. Fuentes, R. Pimentel, Z. Abad, E. Cabrera, E. Pimentel and A. Arenal, 2003. High fry production rates using post-thaw silver carp (*Hypophthalmichthys molitrix*) spermatozoa under farming conditions. *Aquaculture*, 220: 195-201.

Ashwood-smith, M.J., 1980. Low temperature preservation of cells, tissues and organs. Low temperature preservation of medicine and biology. Pitman medical. Kent, UK. 19-44.

Billard, R. and M. P. Cosson, 1992. Some problems related to the assessment of sperm motility in freshwater fish. *J. Expt .Zool.*, 261: 122-131.

Chao, N., H.P. Chen and I. Chiuliao, 1975. Study on cryogenic preservation of grey mullet sperm. *Aquaculture,* 5: 389-406.

Chao, N.H., 1991. Fish sperm cryopreservation in Taiwan: Technology advancement and extension efforts. Int. Symp. Rep. Biol. Aqua., Taipei: Taiwan Fisheries Research Institute.

Daly, J., D. Galloway, W. Bravington, M. Holland and B. Ingram, 2008. Cryopreservation of sperm from Murray cod, *Maccullochella peelii peelii*. *Aquaculture,* 285: 117-122.

Hossain, M.S and M.R.I. Sarder, 2009. Cryogenic freezing of silver crap spermatozoa for conservation of gene pool. *Prog Agricul.* 20 (1 & 2): 99-106.

Kumar, K., 1988. A comparative study of various extenders for cryopreservation of carp sepermatozoa. *Indian J. Anim. Sci.*, 58(11): 1355-1360.

Kumar, K., 1989. Studies on cryogenic preservation of carp spermatozoa *In:* Das and Jhingran (eds.), Fish Genetics in India, Today and Tomorrows Publishers, New Delhi.199-206.

Kurokura, H., R. Hirano, M. Tomita and M. Iwahashi, 1984. Cryopreservation of carp sperm. *Aquaculture,* 37: 267-273.

Lubzens, E., N. Daube, I. Pekrasy, Y. Magnus, A. Cohen, F. Yusefovich and P. Feigin, 1997. Carp (*Cyprinus carpio L.*) spermatozoa cryobanks- strategies in research and application. *Aquaculture,* 155: 13-30.

Magyary, I., A. Dinnyes and B. Urbanyi, 2000. Cryopreservation methods for sperm of the common carp. *In*: cryopreservation in Aquatic species. Tiersch, T.R. and P.M. Mazik (eds.).World Aquaculture Society, Baton Roige, Louisiana. 288-289.

Mahfuz, M.S., M.B. Hossain and M.H. Minar, 2012. Biochemical composition of an endangered fish, *Labeo bata* from Bangladesh water. *Am. J. Food Techn.*, 7: 633-641.

Morisawa, M and K. Suzuki, 1980. Osmolality and potassium-ion: their roles in initiation of sperm motility in teleosts. *Science,* 210: 1145–7.

Nahiduzzaman, M., M.M. Hassan, U.H. Khanam, S.N.A. Mamun, M.A.R. Hossain and T.R. Tiersch, 2011. Sperm cryopreservation of the critically endangered olive barb (Sarputi) *Puntius sarana* (Hamilton, 1822). *Cryobiology*, 62: 62-67.

Ohta, H., H. Shimma and K. Hirose, 1995. Relationship between fertility and motility of the Amago salmon, *Oncorhynchus masouishikawae*. *Fish Sci.*, 61: 886-887.

Rana, K.J. and B.J. McAndrew, 1989. The viability of cryopreserved *Tilapia spermatozoa*. *Aquaculture,*76: 335-345.

Routray, P., A.K. Choudhary, S.N. Dash, D.K. Verma, C. Dash, P. Swain, J.K. Jena, S.D. Gupta and N. Sarangi, 2006. Cryopreservation of dead fish spermatozoa several hours after death of *Labeo rohita* and its successful utilization in fish production. *Aquaculture,* 261: 1204–1211.

Sarder M.R.I., S.K. Saha and M.F.M. Sarker, 2013.Cryopreservation of sperm of an indigenous endangered fish, Pabda *(Ompok pabda)*. *N. Am. J. Aqua.*, 75:114–123.

Sarder, M.R.I., 2004. Application of cryopreservation in the Indian major carps (*Catla catla, Labeo rohita, Cirrhinus cirrhosus*) and tilapia (*Oreochromis niloticus*) for their quality seed production. *Bangladesh J. Fish.* (Special Issue), 27: 10.

Sarder, M.R.I., F. Rahman, M.S. Hossain and M.S. Samad, 2009. Cryogenic freezing of silver barb spermatozoa for gene pool conservation. *Prog. Agricul.* 20(1 & 2): 117-124.

Sarder, M.R.I., M.F.M. Sarker and S.K. Saha, 2012. Cryopreservation of sperm of fish species *Nandus nandus* for ex-situ conservation. *Cryobiology*, 65:202–209.

Scott, A.P and S.M. Baynes, 1980. A review of the biology, handling and storage of salmonid spermatozoa. *J. Fish Biol.*, 17: 707-739.

Shirohara, J., T. Canto and J.M.F. Rlmendras, 1982. A comparative study of various extenders for milk fish, *Chanos chanos* (Forssakal) sperm preservation. *Aquaculture*, 28: 339-346.

Withler, F.C., 1980. Chilled and cryogenic storage of gametes of Thai carps and catfishes.Canadian Technical Report of Fisheries and Aquatic Sciences.948: 15.

Yang, H., C. Carmichael, Z.M. Varga and T.R. Tiersch, 2007. Development of a simplified and standardized protocol with potential for high-throughput for sperm cryopreservation in Zebra fish *Danio rerio*. *Theriogenology*. 68: 128-136.

Yang, H., L. Hazlewood, R.B. Walter and T.R. Tiersch, 2006. Effect of osmotic immobilization on refrigerated storage and cryopreservation of sperm from a viviparous fish, the green swordtail *Xiphophorus helleri*. *Cryobiology*, 52: 209-218.

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