ISSN: 2347-5129
IJFAS 2015; 2(6): 175-181

www.fisheriesjournal.com
Received: 20-05-2015
Accepted: 22-06-2015

Najmus Sakib Khan
Department of Fisheries Biology and Genetics, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh.

Md. Rafiqul Islam Sarder
Department of Fisheries Biology and Genetics, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh.

Mohammed Abdullah Al-Faroque
Department of Fisheries Biology and Genetics, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh.

Md. Fazlul Awal Mollah
Department of Fisheries Biology and Genetics, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh.

Correspondence
Md. Rafiqul Islam Sarder
Department of Fisheries Biology and Genetics, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh.

Standardization of sperm cryopreservation techniques of Indian Major Carp Rohu (*Labeo rohita,* Hamilton 1822)

Najmus Sakib Khan, Md. Rafiqul Islam Sarder, Mohammed Abdullah Al-Faroque, Md. Fazlul Awal Mollah

Abstract
This research dealt with standardization of sperm cryopreservation techniques of *Labeo rohita*. The concentration of sperm ranged from 8.55×10^9 to 8.57×10^9cells ml^{-1}. Activation of sperm motility was decreased with increasing concentration of the extending media and motility was severely inhibited at 1% NaCl. The toxicity of cryoprotectant (DMSO and methanol) to sperm was tested at different concentrations (5%, 10%, 15%) and incubation time (5-35 min). Cryoprotectants with 5 and 10% concentrations produced better motility during 5 and 10 min incubation. Two extenders, Alsever's solution and egg-yolk citrate, and two cryoprotectants, DMSO and methanol were used for cryopreservation of sperm. Alsever's solution with 10% DMSO showed best performance producing 82.5±1.44% and 75.0±2.04% equilibration and post-thaw motility respectively. The fertilization and hatching rates were 62.0±0.80% and 45.5±1.78% for Alsever's solution plus DMSO while those of 50.9±0.73% and 16±5.10% for egg-yolk citrate plus methanol respectively. Fresh sperm yielded 87.3±0.98% and 74.7±2.27% fertilization and hatching respectively. The breeding efficiency of cryopreserved sperm those were preserved with two diluents was compared and a significant variation was found for fertilization (P=0.001) as well as for hatching (P=0.006). The standardized cryopreservation techniques can be used to conserve the existing gene pool of rohu for cryogenic gene banking.

Keywords: Standardization, sperm cryopreservation, *Labeo rohita.*

1. Introduction
Indian major carps such as rohu (*Labeo rohita*), catla (*Catla catla*) and mrigal (*Cirrhinus cirrhosus)* are the prime species for aquaculture in Bangladesh. Earlier aquaculture contributed nearly 40% of total fish production in Bangladesh [17] but in recent time it has been increased to 52.92% [12]. Rohu contributes nearly 17.50% of the total aquaculture production [10] and fetches the highest market price. In the beginning of aquaculture seeds of rohu along with other Indian major carps were used to collect from major rivers but in recent time natural seed production has been seriously reduced due to some environmental and anthropogenic activities. It was reported that a gradual decrease in the population size of many economically important fish species including Indian major carps has taken place in all Bangladeshi rivers [9]. To support the carp aquaculture, about 964 private and public-owned hatcheries have been established all over the country and more than 99% of the total required seeds are produced there using captive reared *L. rohita* broodstocks. Though hatchery produced seeds of rohu are available but the purity of seeds becomes threatened because of inbreeding and hybridization practices by hatchery operators specially in private hatcheries. It is, therefore, necessary to conserve the gene pool of wild stocks of rohu for quality brood production which can be ensured by cryopreservation of gametes.

In Bangladesh, cryopreservation of fish sperm was started in 2004, and protocols of some commercially important indigenous and exotic carps have been developed [28], [16]. A preliminary work on cryopreservation of sperm of *Labeo rohita* was conducted using different diluents and cryoprotectants for development of working protocol [27]. Very recently, sperm cryopreservation protocols of the critically endangered fish species such as olive barb (*Puntius sarana*) [25]; veda (*Nandus nandus*) [29] and pabda (*Ompok pabda*) [30] have been developed. However, the aim of the present research was to standardize the sperm cryopreservation techniques of *L. rohita* and its application in cryogenic gene banking. The specific objectives were to assess: 1) the quality of sperm; 2) the sperm activation at different osmotic pressures; 3) the toxic effects of

cryoprotectants; 4) the combination of extenders and cryoprotectants; and 5) the efficacy of cryopreserved sperm for fertilization of eggs.

2. Materials and Methods

2.1 Experimental fish

Spawn of *L. rohita* were collected from the Halda River and stocked in the ponds in the vicinity of Fisheries Faculty premises, Bangladesh Agricultural University, Mymensingh. Fish were reared with a commercial Mega feed and administered twice a day at the rate of 4-5% of total body weight. Organic fertilizer (cow dung) was applied at a rate of 2 kg decimal^{-1} and inorganic fertilizers, urea and triple super phosphate were applied at the rate of 100g and 50g decimal^{-1} respectively to increase the natural food production of the pond at 1-2 months interval depending upon the feed availability. Liming (250g decimal^{-1}) was also done monthly.

2.2 Collection of sperm and its quality assessment

Male brood fishes were collected from rearing ponds and conditioned in cistern for 6 hrs before induced with PG extract at a dose of 2 mgkg^{-1} body weight. Sperm was collected by stripping in a 5 ml glass tube. One to two µl of milt was placed on a glass slide and 100 µl of distilled water was added to activate the sperm. The motility of the activated sperm was observed using a compound microscope at x10 or x40 magnification. The motility was expressed as the percentage of sperm which had active forward movement and passive Brownian's movement. The number of sperm was counted using a haemacytometer and it was ranged from 8.55×10^9 to 8.57×10^9 cells ml^{-1}.

2.3 Collection of eggs for *in vitro* fertilization

Matured females having 1.5-2.5 kg body weight were collected from stock ponds and kept in cistern for 6-12 h for acclimatization. Two injections of pituitary gland (PG) extract were administered after acclimatization of females at the dose of 2 and 4-5 mgkg^{-1} respectively at 6 h interval. Eggs were collected by stripping into a plastic bowl immediately after ovulation.

2.4 Experiment I: Sperm motility in various concentrations of NaCl

Activation of sperm motility was tested following the method of Yang *et al* (2007) [36] and Sarder *et al* (2012) [29]. Ten graded dilutions of NaCl solution (from 0.1% to 1%) corresponding to the osmotic pressure ranging 48 to 319 mOsmol kg^{-1} was prepared by dissolving NaCl in distilled water. One to two µl of sperm was placed on a glass slide and 20 µl of NaCl solution from the graded dilutions were added to activate the sperm. The motility of sperm was observed immediately under microscope.

2.5 Experiment II: Evaluation of toxicity of cryoprotectants to sperm

The toxicity of cryoprotectant was evaluated following the procedure of Yang *et al* (2007) [36] and Sarder *et al* (2012) [29]. Two cryoprotectants, DMSO and methanol were mixed with the milt to make the final concentrations of 5, 10 and 15%. The collected milt was diluted at a ratio of 1:4 for egg-yolk citrate, and 1:9 for Alsever's solution. The chemical constituents of two extenders are presented in Table 1. The dilutions between milt and extenders were chosen from previous studies with Indian major carps [18, 28] and common carp [21]. The cryoprotectant was added to sperm at room temperature at different concentrations and assessed the toxicity by monitoring the motility of sperm under microscope during the incubation period of 35 min at 5 min interval.

Table 1: Chemical constituents of different extenders used for cryopreservation of sperm of *L. rohita*.

Extender Constituent	Egg-yolk citrate (pH 6.7)	Alsever's solution (pH 7.9)
Sodium chloride	0.4%	0.4 %
Sodium citrate	0.3%	o.8 %
	Dissolved in 100 ml of distilled water. 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

2.6 Experiment III: Selection of suitable diluents (extenders + cryoprotectants)

Two extenders, Alsever's solution and egg-yolk citrate, and two cryoprotectants, DMSO and methanol were used for cryopreservation of sperm. Diluents were prepared by adding 10% cryoprotectant to 90% extender (% v/v). Milt was diluted at a ratio of 1:9 for Alsever's and 1:4 with egg-yolk citrate. During equilibration (8-10 min) 0.23 ml of diluted milt was drawn into 0.25 ml French plastic straws (Minitüb System, Minitüb, Tiefenbach, Germany) and sealed manually using a heated crucible tongs. The motility of all the fresh sperm samples was observed prior to addition of cryoprotectant and then just before initiation of freezing as equilibration motility.

The straws containing diluted milt were placed in the cryochamber of a computer controlled-rate freezer (CL-3300) equipped with the software Cryogenesis, version 4, for Windows (Cryologic, Pty Ltd., Australia 1998 & 1999) for cooling. One-step freezing protocol was used where milt sample was cooled from 0 ºC to -80 ºC at a decreasing rate of 10ºC per min. Frozen samples were removed from the cryochamber and immediately plunged into liquid nitrogen (-196 ºC) for storage. Frozen straws were retrieved from the LN_2 container dewar using a tweezer and thawed at room temperature (25-26 ºC) for 30-40 sec. One to two µl of milt was placed on a glass slide and post-thaw motility of the sperm was assessed by activating the sperm with 150-200 µl of distilled water.

2.7 Experiment IV: Efficacy of cryopreserved sperm for fertilization of eggs

Eggs were fertilized with fresh and cryopreserved sperm. Eggs were collected from ovulated females and divided into six groups (approximately 500 eggs in each group) for fertilization. Four groups of eggs were fertilized by cryopreserved sperm which were preserved with two diluents and two groups with fresh sperm as control at room temperature (26-27 ºC) so that each group had two replications. For control, adequate amount of fresh milt (motility 90-95%) collected from male fish was used. The frozen straws stored in liquid nitrogen for 12 to 24 h, after thawing at room temperature for about 30-40s, were cut at both ends and mixed with egg. Each batch of eggs was fertilized with cryopreserved sperm from 5 straws where each of the straw contained 230 µl diluted milt, so egg to sperm ratio was $1:1.44x10^7$. Small amount of tap water and 0.4% NaCl solution was added to activate the sperm for fertilization. Egg and sperm mass was mixed, washed carefully 3-4 times with tap water and finally transferred to circular bowls where continuous water

supply was maintained. After 1 h of fertilization some eggs were collected from both cryopreserved sperm and control groups and observed the progress of cell division under a microscope. After 18 to 24h of fertilization the eggs hatched out, individual hatchling was counted and transferred to plastic bowls for rearing. The fertilization and hatching rates of eggs were calculated.

2.8 Statistical analyses

Data of experiments I, II and III expressed in percentage were converted to arcsine transformation. Data of experiments I and II were analyzed with one factor ANOVA of SPSS v 16 and the means were separated by Least Significant Difference (LSD) at 5% level of significance. The effects of different extenders, cryoprotectants and their combinations on equilibration and post-thaw motility were analyzed using two-factor ANOVA of MSTAT followed by Duncan's Multiple Range Test at 5% level of significance. Since the fresh sperm concentration was not standardized during breeding trials, statistical comparisons were not made between fresh and cryopreserved sperm. However, Student's t-test was applied to compare the fertility of cryopreserved sperm for two diluents.

3. Results

3.1 Evaluation of sperm quality

Sperm was collected from the males through gentle pressure on abdomen for each trial. The concentration of sperm was found between 8.55×10^9 to 8.57×10^9 cells ml^{-1} with a pH of 7.0±0. The motility of fresh sperm was 90-95% and they were motile for a maximum of 98±3.49s. The sperm displayed active forward movement and passive Brownian movement during motility test.

3.2 Sperm motility in various concentrations of NaCl

Motility of sperm activated with NaCl solutions (osmolalities, 48 to 319 mOsmol kg^{-1}) decreased with increasing concentration of NaCl and it was nearly stopped at 319 mOsmol kg^{-1}. The motility of sperm at 0.1% NaCl (pH-5.58) solution was 95±0% which reduced to 90±0% at 0.4 % NaCl (pH-6.94). Then the motility decreased at a faster rate as the concentration of NaCl increased and reached to 0.5±0.03% at 1% NaCl (pH 9.04) (Fig. 1). Motility of sperm at 0.4% NaCl was stable and considered to be complete activation. Motility of sperm was completely inhibited at 1% NaCl. A significant difference (P=0.00) was observed between the complete activation and inhibition of sperm motility at 0.4% and 1% NaCl solutions respectively.

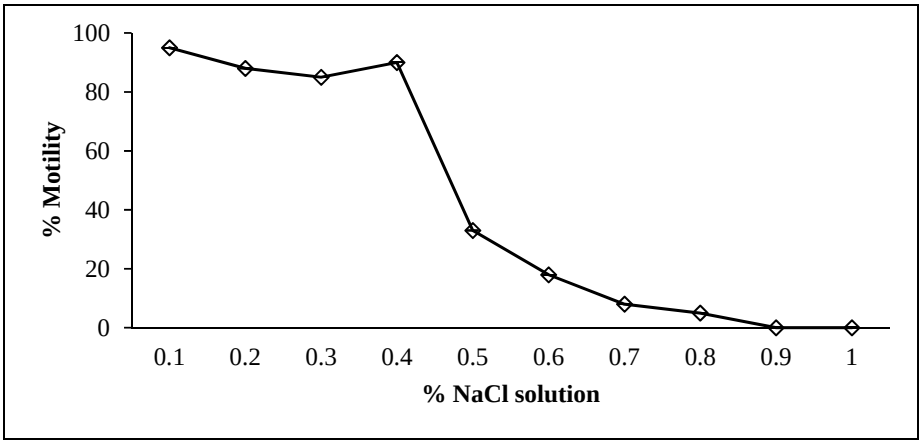

Fig 1: Motility of sperm of *L. rohita* in different concentrations of NaCl solution.

3.3 Swimming duration of sperm in different NaCl solution

The swimming duration of activated sperm varied in different NaCl concentrations and the duration was severely reduced at 0.5% NaCl (pH-7.29). The highest swimming duration of sperm was estimated as long as 102±1.0s at 0.4% NaCl and lowest 3±0s at 0.5% NaCl (Fig. 2). A significant (P=0.00) decrease in swimming duration was observed between two concentrations of NaCl, i.e. at 0.4% and 0.5% concentration.

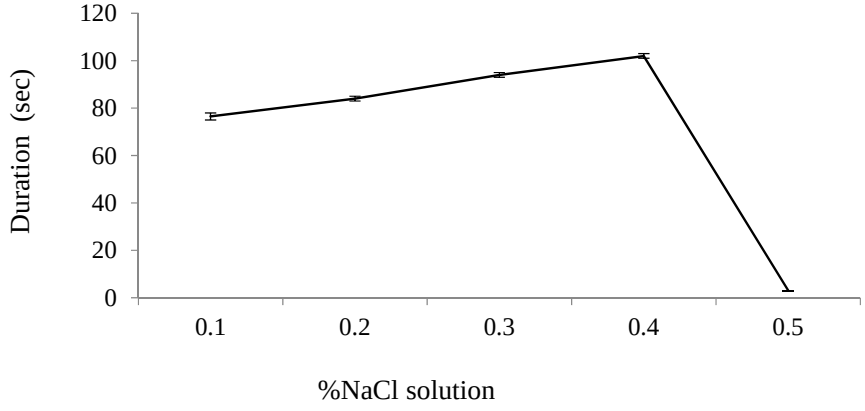

Fig 2: Duration of swimming of *L. rohita* sperm in NaCl activation solution.

3.4 Evaluation of toxicity of cryoprotectants to sperm

The motility of fresh sperm before incubation with cryoprotectants was 90-95%. The motility of sperm decreased with increasing cryoprotectant concentration (5, 10 and 15%) and incubation time (5-35 min). Alsever's solution with 5% cryoprotectants produced 72.5 to 77.5% motility at 5 min incubation which decreased to 52.5% at 15 min incubation (P=0.000). Sperm exposed to 10% cryoprotectants for 5 min showed 70 to 77.5% motility which reduced to 40 to 50% after 15 min of incubation (P=0.000) (Table 2).

Sperm suspended in egg-yolk citrate showed 72.5% motility at 5 min incubation with 5% cryoprotectants and motility reduced to 52.5 to 57.5% at 15 min incubation (P=0.028). Sperm motility at 10% concentration was 65 to 72.5% at 5 min incubation which decreased to 40 to 57.5% at 15 min incubation (P=0.028) (Table 2). Sperm incubated with 15% cryoprotectants along with both extenders showed poor motility from the beginning of the incubation and motility ceased completely within 25 to 30 min of incubation in many cases.

Table 2: Motility of sperm of *L. rohita* at different concentrations of cryoprotectants and incubation times using Alsever's solution and egg-yolk citrate. Data are presented as mean ± SE.

Cryoprotectant concentration		Sperm motility (%) Incubation time (min)							
		Initial (0)	5	10	15	20	25	30	35
		Alsever's solution							
DMSO	5%	87.5±2.5	77.5±2.5	72.5±2.5	52.5±2.5	42.5±2.5	30±0	7.5±2.5	0
	10%	85±0	77.5±2.5	72.5±2.5	40±5	27.5±2.5	12.5±2.5	2.5±2.5	0
	15%	62.5±2.5	37.5±2.5	27.5±2.5	20±0	10±0	10±0	0	0
Methanol	5%	80±5	72.5±2.5	62.5±2.5	52.5±2.5	42.5±2.5	35±0	12.5±2.5	0
	10%	77.5±2.5	70±0	60±0	50±0	37.5±2.5	25±5	12.5±2.5	0
	15%	60±5	42.5±2.5	35±0	20±0	10±0	0	0	0
		Egg-yolk citrate							
DMSO	5%	82.5±2.5	72.5±2.5	62.5±2.5	52.5±2.5	40±0	30±5	12.5±2.5	0
	10%	70±2.5	65±2.5	50±2.5	40±5	25±5	10±2.5	0	0
	15%	52.5±2.5	42.5±2.5	32.5±2.5	22.5±2.5	12.5±2.5	0	0	0
Methanol	5%	82.5±2.5	72.5±2.5	60±5	57.5±2.5	50±0	42.5±0	0	0
	10%	77.5±2.5	72.5±2.5	62.5±2.5	57.5±2.5	45±5	27.5±2.5	0	0
	15%	22.5±2.5	12.5±2.5	5±0	0	0	0	0	0

3.5 Selection of suitable combination of extenders and cryoprotectants

The highest motility of the equilibrated sperm was recorded from Alsever's solution plus DMSO (82.5±1.44%) followed by Alsever's solution plus methanol (76.3±1.25%). Sperm preserved with egg-yolk citrate had less equilibration motility (75±2.04 to 77.5±3.22%) compared to that of Alsever's solution. Highest post-thaw motility (75.0±2.04%) was recorded from both Alsever's solution plus DMSO and egg-yolk citrate plus methanol followed by 71.3±1.25% and 70±2.04% from egg-yolk citrate plus DMSO and Alsever's solution plus methanol respectively. The statistical analysis showed that there were no significant (P>0.05) effects of any extenders and cryoprotectants, and their combinations (diluents) on equilibration and post-thaw motility of sperm (Figure 3). Duncan's Multiple Range Test revealed that Alsever's solution plus DMSO to be the best diluent for preservation of sperm of rohu.

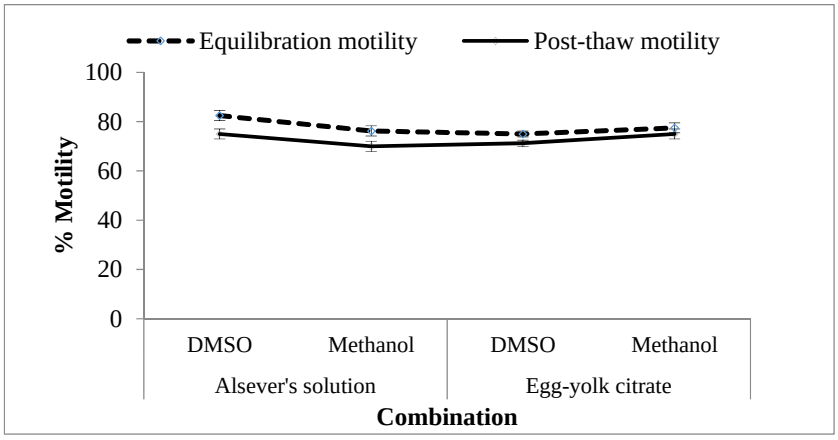

Fig 3: Equilibration and post-thaw motility of sperm of *L. rohita* at different combinations of extender and cryoprotectant.

3.6 Effects of cryopreserved spermatozoa on fertilization and hatching of eggs

Cryopreserved sperm (preserved with two diluents, Alsever's solution plus DMSO and egg yolk-citrate plus methanol) and fresh sperm were used for fertilization of eggs. The post-thaw motility of sperm was recorded as 75.0±2.04% from Alsever's solution plus DMSO and 75.0±2.04% from egg-yolk citrate plus methanol during fertilization of freshly collected eggs. Sperm preserved with Alsever's solution plus DMSO produced the highest fertilization (62.0±0.8%) and hatching (45.5±1.78%),

and those preserved with egg-yolk citrate plus methanol produced 50.9±0.73% and 16.0±5.1% fertilization and hatching respectively. Fresh sperm used as control yielded 87.3±0.98% and 74.7±2.27% fertilization and hatching respectively (Figure 4). The breeding efficiency of cryopreserved sperm for two diluents was compared and a significant variation was found for fertilization (P=0.001) as well as for hatching (P=0.006). No statistical analyses were made between the breeding performance of fresh and cryopreserved sperm as the concentration of fresh and cryopreserved sperm was not standardized

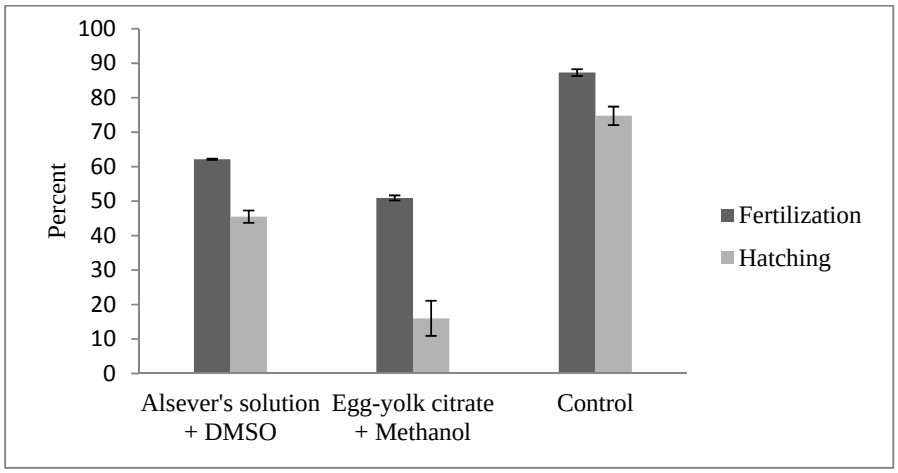

Fig 4: Fertilization and hatching of eggs of *L. rohita* using cryopreserved and fresh sperm.

4. Discussion

Assessment of the quality of sperm is pre-requisite for cryopreservation trial, which can be done by observing motility and concentration of sperm [31]. In the present study the concentration of fresh sperm was between $8.55\times10^9$and 8.57×10^9 cells ml^{-1} and the motility of sperm was recorded 90-95%. They were motile for a maximum of 98±3.49s. The sperm had two kinds of mobility, active forward movement and passive Brownian movement. The swimming duration of sperm upon activation was 102±1.0s at 0.4% NaCl solution which was higher than the value (90±5.5s) obtained from the study of Verma *et al.* (2009) [34]. The pH of milt of *L. rohita* was 7.0±0 which was more or less similar to that of Verma *et al.* (2009) [34] as 7.3±0.06. The osmolality of seminal plasma of *L. rohita* was reported 269±5.5 $mOsmolkg^{-1}$ in which spermatozoa remain immotile [34].

It is important to assess the sperm activation and motility before formulating a suitable extender solution. Fish seminal fluids generally have high concentrations of K^+, Na^+ and Cl^- ions, and low concentrations of Ca^{2+} and Mg^{2+} ions [23, 33]. Alavi and Cosson (2006) [2] reported several factors which affect sperm motility are pH, temperature, ions and osmolality, where pH is considered as the most important sperm activating factor in fish species [32]. Although fish sperm are characteristically immotile in the testis [22] they become activated on release into aquatic environment [1, 5].

In the present study the motility of sperm decreased with increasing concentration of the extending solution and the motility decreased significantly at 1% NaCl (osmolality of 319 $mOsmolkg^{-1}$). Yang *et al* (2007) [36] reported that the activation of sperm motility of zebra fish decreased as the osmotic pressure of Hanks' balanced salt solution increased over the range of 20-603 $mOsmolkg^{-1}$. Similarly, motility of sperm of *N. nandus* and *O. pabda* decreased with the increase of osmolality of the extending solution and was completely inhibited at almost 319 $mOsmolkg^{-1}$ [29, 30]. Alavi and Cosson (2005) [1] reported that osmolalities of seminal plasma of most freshwater cyprinids are within a range of 230-346 $mOsmolkg^{-1}$ and rohu represents the cyprinids with the osmolality of 269±5.5 $mOsmolkg^{-1}$ [34]. So, the extending solution used for cryopreservation of sperm of rohu should have osmolality similar to that of seminal plasma.

The cryoprotectants, DMSO and methanol at 5 and 10% concentrations produced better motility of sperm of rohu during 5 and 10 min incubation but 15% concentration seemed toxic and yielded poor motility which is similar to the findings of Yang *et al.* (2007) [36] and Sarder *et al.* (2012, 2013) [29, 30]. Leung (1987) [20] observed the best post-thaw motility of barramundi sperm at 5% DMSO while 10% cryoprotectant was effectively used by Withler and Lim (1982) [35]. Sperm of Olive barb (*Puntius sarana*) incubated with 5% DMSO and methanol produced longer motility but an acute toxicity was observed at 15% concentration [25]. Cryoprotectants (DMSO, methanol, ethanol) at 5 and 10% concentration performed better during cryogenic freezing of *N. nandus* and *O. pabda* spermatozoa [29, 30].

During preservation of sperm highest equilibration (82.5±1.44%) and post-thaw (77.5±3.22%) motility were obtained from the diluent, Alsever's solution plus DMSO which proved its suitability for preservation of sperm. It is assumed that Alsever's solution has an optimal osmolality to balance the osmotic pressure of spermatozoa and the Na-citrate fraction of Alsever's solution associated with cell membrane may provide protection against injury during cryogenic freezing. An accepted performance of Alsever's solution in cryopreservation of Indian major carp sperm was reported by Kumar (1988) [18]. Similarly, Alvarez *et al.* (2003) [3] obtained good results from Alsever's solution with 10% DMSO during preservation of silver carp (*Hypophthalmichthys molitrix*) sperm. Along with Alsever's solution DMSO produced highest post-thaw motility in the present study which is considered as a common and effective cryoprotectant for cryopreservation of fish sperm [19] and cell lines [37]. Rao (1989) [26] suggested that along with Alsever's solution DMSO might have positive impact on preservation as it penetrates rapidly into the cellular membrane and brings a quick balance between the intra and extra-cellular fluid concentrations[8]. Egg-yolk citrate, the other extender also produced satisfactory level of equilibration and post-thaw

motility but these values were comparatively lower than those obtained from Alsever's solution. Glogowski *et al.* (1996) [13] reported that the protective action of egg-yolk is species-specific and depends on the constituents of the extender and the cryopreservation procedure [14]. Like DMSO, methanol also performed well and could also be a suitable cryoprotectant for cryopreservation of *L. rohita* sperm. Methanol has been found suitable for cryopreservation of sperm of zebra fish [36], Olive barb [25] and *Nandus nandus* [29]. Sperm of tropical bagrid catfish (*Mystus nemurus*) preserved in 10% methanol had the highest post-thaw motility (58%) compared with those preserved in other cryoprotectants, DMSO, ethanol and glycerol at 5, 10 and 15% concentrations [24].

Fertilization and hatching of eggs using cryopreserved sperm is the main purpose of development of a cryopreservation protocol. The efficacy of cryopreserved sperm is testified by assessing the fertilization ability of sperm to an egg [15]. Two extenders (Alsever's solution, egg-yolk citrate) and two cryoprotectants (DMSO, methanol) were used for cryopreservation of sperm in experiment III but successful fertilization and hatching was obtained from Alsever's solution plus DMSO and egg-yolk citrate plus methanol. The cryopreserved sperm indicate its potentiality through fertilization and hatching of eggs but the fertilization and hatching rates were much lower than that of control. The actual reasons of low fertilization and hatching are difficult to explain but some basic damages might occur in spermatozoa during cryopreservation that affect spermatozoa function such as motility, plasma membrane integrity and functionality, ATP content, DNA damage etc. and might cause low fertilization [7]. Sarder *et al.* (2013) [30] reported 78-80% fertilization and 39-42% hatching of Pabda (*Ompok pabda*) when eggs were fertilized with sperm preserved with Alsever's solution plus DMSO.

Sperm preserved with Alsever's solution plus methanol and egg-yolk citrate plus DMSO did not respond to fertilization though the frozen-thawed sperm had good motility. The reason of negative response of egg-yolk citrate was not clear but cryopreservation efficiency might be decreased due to presence of chicken egg-yolk in extenders though it produced hatchlings in many species [4]. However, during the process of dilution with extender, freezing and thawing, spermatozoa might have lost some of their potential motility which might be recovered after appropriate incubation conditions [6]. Fresh sperm was used as control for fertilization to assess the quality of eggs. Dong *et al.* (2007) [11] conducted experiment using fresh sperm for ensuring the treatment effects and comparing the result of various studies. Though breeding trials were conducted using both cryopreserved and fresh sperm in the present study, fertilization and hatching rates of eggs from cryopreserved sperm were not compared with fresh sperm as the number of fresh sperm was not standardized with cryopreserved one.

5. Conclusion

Cryopreservation protocol for *L. rohita* spermatozoa has been developed through this study. This protocol will definitely facilitate to conserve the existing gene pool of rohu as well as other relevant fish species. The developed protocol suggests formulating an extending medium comprising of Alsever's solution with 10% DMSO or egg-yolk citrate plus 10% methanol and the osmolality of the extending medium should be around 319 mOsmolkg^{-1}. One-step freezing from 0 ^{o}C to -80 ^{o}C at a decreasing rate of 10^{o}C/min is suitable to obtain a good post-thaw motility of sperm. Successful fertilization and hatching of eggs using cryopreserved sperm have been possible but further research work need to be done to improve the fertilization and hatching rates of eggs before the protocol is suggested to apply in commercial hatchery production.

6. Acknowledgements

The authors gratefully acknowledge the PIU-BARC (NATP: Phase-1) for providing financial support to conduct this research work.

7. References

1. Alavi SMH, Cosson J. Sperm motility in fishes. I. Effects of temperature and pH: A review. Cell Biology International 2005; 29:101-110.
2. Alavi SMH, Cosson J. Sperm motility in fishes. II. Effects of ions and osmolality: a review. Cell Biology International 2006; 30:1-14.
3. Alvarez B, Fuentes R, Pimentel R, Abad Z, Cabrera E, Pimentel E Arena A. High fry production rates using post-thaw silver carp (*Hypophthalmichthus molitrix*) spermatozoa under farming conditions. Aquaculture 2003; 220:195-201.
4. Babiak I, Glogowski J, Kujawa R, Kucharczyk D, Mamcarz A. Cryopreservation of sperm from Asp *Aspius aspius*. Progressive Fish Culturist 1998; 60:146-148.
5. Billard R, Cosson MP. Some problems related to the assessment of sperm motility in freshwater fish. Journal of Experimental Zoology 1992; 261:122-131.
6. Billard R, Cosson J, Perchec G, Linhart O. Biology of sperm and artificial reproduction in carp. Aquaculture 1995; 129:95-112.
7. Cabrita E, Sarasquete S, Martinez-Paramo S, Robles V, Beirao J, Perez-Cerezales S, Harraez MP. Cryopreservation of fish sperm: applications and prospective. Journal of Applied Ichthyology 2010; 26:623-635.
8. Ciereszko A, Ramseyer L, Dabrowski K. Cryopreservation of yellow perch semen, Progressive Fish Culturist 1993; 55:261–264.
9. Das P, Barat A. Fish habitat degradation necessitating conservation. Environmental Science 1990; 4:85-89.
10. DoF. Fishery Statistical Yearbook of Bangladesh 2009-2010. Fisheries Resources Survey System, Department of Fisheries, Ministry of Fisheries and Livestock, Matshya Bhaban, Dhaka, 2011, 43.
11. Dong Q, Huang C, Tiersch TR. Control of sperm solution is necessary for standardization of sperm cryopreservation in aquatic species: evidence from sperm agglutination in oysters. Cryobiology 2007; 54:87-98.
12. Fisheries Resources Survey System (FRSS). Fisheries Statistical Year Book of Bangladesh. Department of Fisheries, Ministry of Fisheries and Livestock, 2011-12, 44.
13. Glogowski J, Babiak I, Goryczko K, Dobosz S. Activity of asparate aminotransferase and phosphatase in cryopreserved trout sperm. Reproduction, Fertility and Development 1996; 8:1179-1184.
14. Glogowski J, Babiak I, Luczynski MJ, Luczynski M. Factors affecting cryopreservation efficiency and enzyme activity in northern pike, *Esox lucius* sperm. Journal of Applied Aquaculture 1997; 7(4):53-67.
15. Honeyfield DC, Krise WF. Measurement of milt quality and factors affecting viability of fish spermatozoa. *In:* Tiersch, T.R. and P.M. Mazik (eds.), Cryopreservation in Aquatic species, World Aquaculture Society, Baton Rouge,

Louisiana, USA, 2000, 49-58.
16. Hossain MS, Sarder MRI. Cryogenic freezing of silver carp spermatozoa for conservation of gene pool. Progressive Agriculture 2009; 20(1-2):99-106.
17. Hussain MG, Mazid MA. Genetic Improvement and Conservation of Carp Species in Bangladesh. Bangladesh Fisheries Research Institute, Mymensingh and WorldFish Center, Penang, 2001, 71.
18. Kumar K. A comparative study of various extenders for cryopreservation of carp sepermatozoa. Indian Animal Science 1988; 58(11):1355-1360.
19. Lahnsteiner F, Berger B, Horvath A, Urbanyi B, Weismann T. Cryopreservation of spermatozoa in cyprinid fishes. Theriogenology 2000; 54:1477–1498.
20. Leung LKP. Cryopreservaton of spermatozoa of the barramundi, *Lates calcarifer* (Teleostei: Centropomidae). Aquaculture 1987; 64:243-247.
21. Magyary I, Dinnyes A, Urbanyi B. Cryopreservation methods for sperm of the common carp. *In*: cryopreservation in Aquatic species. Tiersch, T. R. and P. M. Mazik (Editors). World Aquaculture society, Baton Roige, Louisiana, 2000, 288-289.
22. Morsawa M, Suzuki K. Osmolality and potassium-ion: their roles in initiation of sperm motility in teleosts. Science 1980; 210:1145–7.
23. Morisawa M, Suzuki K, Shimizu H, Morisawa H, Yusuda K. Effect of osmolality and potassium on motility of spermatozoa from freshwater cyprinid fish. Experimental Biology 1983a; 107:95-103.
24. Muchlisin ZA, Hashim R, A. Chong S.C. Preliminary study on the cryopreservation of tropical bagrid catfish (*Mystus nemurus*) spermatozoa; the effect of extender and cryoprotectant on the motility after short-term storage. Theriogenology 2004; 62:25-34.
25. Nahiduzzaman M, Hassan MM, Khanam UH, Mamun SNA, Hossain MAR, Tiersch TR. Sperm cryopreservation of the critically endangered olive barb (Sarputi) *Puntius sarana* (Hamilton, 1822). Cryobiology 2011; 62:62-67.
26. Rao KG. Cryopreservation of carp sperm. *In:* Das and Jhingran (eds.) Fish Genetics in India, Today and Tomorrows Printers and Publishers, New Delhi, 1989, 193-198.
27. Sarder MRI, Rahman AKM, Samad MS, Nazrul KMS, Bhuiyan MKJ. Cryopresevation of sperm of *Labeo rohita* (Hamilton, 1822) and its use in fertilization and hatching of eggs. Progressive Agriculture 2011; 22(1-2):123-137.
28. Sarder MRI, Rafiquzzaman SM, Islam MF. Cryopreservation of spermatozoa of mrigal, *Cirrhinus cirrhosus* with a view to minimize inbreeding and hybridization. J. Bangladesh Agricultural University 2009; 7(1):211-218.
29. Sarder MRI, Sarker MFM, Saha SK. Cryopreservation of sperm of an indigenous endangered fish species *Nandus nandus* (Hamilton, 1822) for ex-situ conservation. Cryobiology 2012; 65:202-209.
30. Sarder MRI, Saha SK, Sarker MFM. Cryopresevation of sperm of an indigenous endangered fish, Pabda Catfish *Ompok pabda*. North American Journal of Aquaculture 2013; 75:114-123.
31. Suquet M, Omnes MH, Normant Y, Fauvel C. Assessment of sperm concentration and motility in Turbot (*Scophthalmus maximus*). Aquaculture 1992; 101:177–185.
32. Stoss J. Fish gamete preservation and spermatozoa physiology. *In*: Fish Physiology, Volume 9B. Hoar, W. S., Randall, D. J. and Donaldson, E. M. (Eds.) Academic press, New York, 1983, 305-350.
33. Tan-Fermin JD, Miura T, Adachi S, Yamauchi K. Seminal plasma composition, sperm density, sperm motility and milt dilution in the Asian bagrid catfish, *Clarias macrocephalus* (Gunther). Aquaculture 1999; 171:323-38.
34. Verma DK, Routray P, Dash C, Dasgupta S, Jena JK. Physical and biochemical characteristics of semen and ultrastructure of spermatozoa in six carps. Turkish Journal of Fisheries and Aquaculture Sciences 2009; 9:67-76.
35. Withler FC, Lim LC. Preliminary observations of chilled and deep-frozen storage of grouper (*Epinephelus tauvina*) sperm. Aquaculture 1982; 27: 389-392.
36. Yang H, Carmichael C, Varga ZM, Tiersch TR. Development of a simplified and standardized protocol with potential for high-thoughput for sperm cryopreservation in Zebrafish *Danio rerio*. Theriogenology 2007; 68:128-136.
37. Zhang T, Rawson DM. Studies of on cryopreservation of luc gene transfected bluegill sunfish fibroblast cell line. Cryoletter 2007; 23:191-196