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CRYOPRESERVATION OF SPERMATOZOA OF THREATENED ASIAN STRIPED DWARF CATFISH *Mystus Vittatus* (BLOCH 1794) FOR *EX-SITU* CONSERVATION: AN APPROACH TO PROVERY ALLEVIATION

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ABSTRACT

Cryopreservation is an important ex-situ conservation measure practiced successfully in fishes over the years. The effects of different extenders and cryoprotectants on the viability and fertilizing ability of cryopreserved sperm of Mystus vittatus were investigated in this study in order to develop a cryopreservation protocol. Milt was collected through sacrifice of males and was suspended in extender solutions. The concentration and pH of milt were found to be 7.9×10^9 - 8.1×10^9cells/ml and 8±0, respectively. Different concentrations of NaCl solutions (0.1% to 1.1%) were used to evaluate activation of sperm motility and it was decreased as the concentration of the NaCl solution increased. Sperm motility was completely inhibited at 1.1% and 0.8% NaCl solution with Alsever's solution and Kurokura-2 solution respectively. Two extenders, Alsever's and Kurokura-2 solutions and two cryoprotectants, Dimethyl sulfoxide (Me_2SO) and methanol were employed to preserve the sperm. Ten percent cryoprotectants with both extenders, Alsever's and Kurokura-2 solutions produced better motility after 5 and 10 min incubation. 15% cryoprotectant was found to be toxic to sperm. Alsever's solution with 10% Me_2SO showed better performance producing 77.5±1.4% and 58.8±1.25% equilibration and post-thaw motility than that of 73.8±3.15% and 52.5±2.5% with Kurokura-2 solution plus Me_2SO respectively. Between two diluents, sperm preserved with Alsever's solution plus Me_2SO produced highest fertilization (70.0±7.07%) and hatching (37.5±13.7%), while those preserved with Kurokura-2 plus Me_2SO produced 72.5±2.5% and 29.9±12.5% fertilization and hatching respectively. Fresh sperm yielded 85.0±0% fertilization and 48.0±15.5% hatching. The protocol developed through this study can be applied for long-term preservation of genetic materials of the threatened catfish M. vittatus and the cryopreserved sperm can be used in artificial breeding for broodstock development. Thus the protocol will help to propagate the new generation in hatcheries using cryopreserved sperm and make the fish available in captive culture systems as well as in wild which eventually helps to eradicate poverty.

Keywords: *Mystus vittatus, sperm, cryopreservation, breeding, conservation*

INTRODUCTION

Mystus vittatus (Bloch, 1794), commonly known as Asian striped dwarf catfish is a freshwater fish species belongs to the Bagridae family (order Siluriformes). This species commonly occurs in inland water bodies of Bangladesh, India, Pakistan, Sri Lanka, Thailand, Nepal and Malaysia (Daniels, 2002; Talwar and Jhingran, 1991). *M.vittatus* is an important target fish for small-scale fishermen in Bangladesh (Craig *et al.*, 2004; Kibria and Ahmed, 2005; Hossain *et al.*, 2006). This small, indigenous fish species has high demand as a food fish in fish markets as it has high nutritional value in terms of protein, micronutrients, vitamins and minerals (Ross *et al.*, 2003). Recently it has also got its entry in ornamental fish markets of Bangladesh and India (Gupta and Banerjee, 2012) and has been reported to have moderate export price too (Gupta and Banerjee, 2014).

The total fish production of the country was estimated at 3.684 million metric tons in 2014-15 fiscal year in which inland open water fisheries contribution was 27.79% (DoF, 2016). Recently, Bangladesh has stood fifth in producing freshwater fish globally (FAO, 2014). Fisheries play an important role in the national economy as well as nutritional security. However, over-harvesting of juvenile as well as brood fish from natural waters combined with destructive and unregulated fishing practices has reduced the production of open water fisheries which further led to the extinction of a number of rather valuable native species. Freshwater small indigenous species (SIS) was available in natural waters and fishermen used to catch them for their daily livelihood. But their abundance has been declining at an alarming rate and some of them have become locally extinct. Although *M. vittatus* is listed by IUCN as least concern (IUCN, 2003), its abundance in natural water bodies of Bangladesh is reduced at a severe scale in comparison to a decade before. Recent studies reported *M. vittatus* as threatened species in Bangladesh (Hossain, 2014). Considering the present biodiversity status of *M. vittatus* in nature, conservation is in urgent need to safeguard the species from extinction.

Among *ex-situ* conservation measures, sperm cryopreservation offers an initial and practical application for long-term storage of the parental genetic material for future uses (Bart, 2002). It is hoped that cryopreserved semen could be stored for a few years without deterioration provided proper maintenance and handling measures are well taken care of all time (Armitage, 1987).

Cryopreservation refers to long term preservation and storage of biological materials at very low temperature, usually at -196^{o}C. At 0°C conditions, spermatozoa can be stored for a few hours up to several days depending on the species, while cryopreserved gametes can be theoretically stored between 200 and 32000 years without deleterious effect (Ashwood-Smith, 1980). It is estimated that sperm from 200 fish species has been successfully cryopreserved (Billard *et al.,* 1995). However, appropriate to each species, optimizations of technology are needed.

Unlike terrestrial animals, it is more difficult to standardize the cryopreservation protocols of spermatozoa of fish (Tiersch, 2000). Different species of fish exhibited different responses to the same extenders and cryoprotectants. Therefore, the species-specific and reproducible sperm cryopreservation procedures have to be developed for fish. The establishment of sperm cryopreservation protocols and utilization of frozen sperm of *M. vittatus* in fertilization have not been reported so far in Bangladesh and elsewhere. Therefore, the objective 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. Once the cryopreservation protocol is developed seeds of the species can be generated using cryopreserved sperm in hatcheries and make them available again in nature and culture systems.

RESEARCH METHOD

Experimental fish

Mature brood fish of *M. vittatus* were collected from different sources (*haors*, *baors* and rivers) of Mymensingh district, Bangladesh and were stocked in ponds and cisterns of Faculty of Fisheries, Bangladesh Agricultural University (BAU), Mymensingh and Bangladesh Fisheries Research Institute, Mymensingh, Bangladesh. Fish were reared with a commercial Mega feed containing about 35% protein and it was administered twice a day at 4-5% of total body weight of fish. Organic fertilizer (cow dung) was applied at a rate of 2 kg/decimal and inorganic fertilizers (urea and phosphate) were also applied at a rate of 150 g/decimal, at 15 days interval to increase the natural food production of the pond. Liming was done at a rate of 250 g/decimal/month.

Selection and conditioning of brood fish

Matured male fish were caught from the ponds 4-6 h prior to hormone treatment and kept in cistern for conditioning without any supplementary feed. Additional aeration was ensured by continuous water supply through PVC pipe over the cistern.

Collection of sperm and its quality assessment

Mature male broods of 7-9 g body weight were induced with a single dose of pituitary gland (PG) extract at 2 mg/kg body weight. The induced fish were kept in cisterns for another 7 h and sacrificed to collect testes as the fish possesses small amount of milt which is difficult to collect by stripping. Collected testes were put on a petridish and placed immediately on ice to facilitate low temperature, which avoid the death of sperm cells as well as loss of seminal fluids through evaporation. As the milt was viscous extender solution like Alsever's solution or Kurokura-2 solution was added to the testes and crushed them gently using a scissor so that the sperm is suspended into the extender solution. The chemical constituents of Alsever's solution and Kurokura-2 solution are presented in Table 1. The suspended sperm was collected by a micropipette and put into an eppendorf which was placed on ice until use. The quality of sperm was evaluated by examining its motility under a light microscope. To standardize milt dilution and also to estimate the density of sperm per straw the number of sperm per unit volume was counted using a haemacytometer following the standard counting method. The concentration of sperm ranged 7.9 x 10^9 to 8.1 x 10^9cells/ml.

Table 1. Chemical constituents and preparation procedure of extenders used for cryopreservation of sperm of *M. vittatus*

Extender constituent	Alsever's solution	Kurokura-2 solution
Sodium chloride	0.40%	0.36%
Sodium citrate	0.80%	-
Calcium chloride	-	0.02%
Potassium chloride	-	1.00%
Magnesium chloride	-	0.01%
Sodium hydrogen carbonate	-	0.02%
	Dissolved in 100 ml of distilled water	

Estimation of sperm motility

About 1 to 2 µl of sperm suspension was placed on a glass slide and 100 µl of distilled water was added to the suspension to activate the sperm. The motility of the activated sperm was observed using a compound microscope at ×10 or × 40 magnifications. The motility was expressed as the percentage of sperm which had active rotatory movement. The sperm motility was estimated at least two times with five fields observed at a time.

Collection of eggs and fertilization

Mature females of 16-22 g body weight were induced with a single dose of PG extract at the rate of 4 mg/kg body weight and kept in cistern for about 8 h for ovulation. Eggs were collected by stripping into a plastic bowl immediately 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. Eggs were then fertilized with fresh sperm as well as cryopreserved sperm thawed at room temperature (26 ºC).

Experiment I. Activation of sperm at various concentrations of NaCl

Test of activation of sperm motility was performed at various concentrations of NaCl solution (Sarder *et al.*, 2013). After collection of sperm from crushed testes, it was suspended in a suitable extender and kept in an eppendorf which was placed on ice. Eight male fish were sacrificed for each combination of extender and cryoprotectant, where two replications were maintained for each combination. Twelve graded dilutions of NaCl solution (from 0.1% to 1.2%) were prepared by dissolving NaCl salt in distilled water. About 1-2 µl of sperm suspension 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 instantly under a microscope. The percentage of motility and swimming duration of activated sperm were recorded at different concentrations (from 0.1% to 1.2%) of NaCl solution. It was determined by calculating time difference from sperm activation to the time when the sperm became or near to immotile.

Experiment II. Evaluation of toxicity effect of different cryoprotectants to sperm

The toxicity of cryoprotectant to sperm was assessed following the method of Yang *et al.* (2007) and Sarder *et al.* (2013). Milt was collected from 16 fish with two extenders for duplicate trials. It was diluted at a ratio of 1:9 for both Alsever's and Kurokura-2 solution. The cryoprotectants (Me_2SO and methanol) were mixed with the milt to make the final concentration of cryoprotectant of 5, 10, and 15% respectively. The toxicity of these cryoprotectants was assessed by monitoring the motility of sperm during the 60 min incubation period with 5 min intervals.

Experiment III. Selection of suitable diluents (extender plus cryoprotectant)

During refrigeration extender maintains the viability of sperm and cryoprotectant helps in protecting the sperm from cold shock during freezing. Two extenders, Alsever's solution and Kurokura-2 solution and two cryoprotectants such as Me_2SO and methanol were used. The diluents were prepared by adding 10% cryoprotectant to 90% extender (% v/v). For each combination of extender and cryoprotectant, two replications were maintained and milt was collected from more than 30 fish and pooled to avoid the sampling error. After collection of milt, it was diluted with Alsever's solution or Kurokura-2 solution at the ratio of 1:9. 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. 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) by pipetting and the free ends of them were sealed manually using a heated crucible tongs.

The straws containing diluted milt were placed in the cryochamber of a computer controlled-rate freezer (CL-3300) (Cryologic, Pty Ltd., Australia 1998 & 1999) for cooling. One-step freezing protocol was used where milt sample was cooled from 0 ^{o}C to -80 ^{o}C at a decreasing rate of 10 ^{o}C per min. After freezing, samples were removed from the cryochamber and immediately plunged into liquid nitrogen (-196 ^{o}C) for storage. Frozen straws were retrieved from the LN_2 container using a tweezer and thawed at room temperature (25-26 ^{o}C) for 30-40 sec. The post-thaw motility of the sperm was assessed by placing 1 to 2 ml of milt onto a glass slide and activated them by adding 100-150 ml of distilled water. The motility was examined under a light microscope.

Experiment IV. Effects of cryopreservation on fertilization and hatching rates of eggs

To evaluate the fertility and hatching rates breeding trials were conducted using fresh and cryopreserved sperm with eggs. Eggs were collected from one or two ovulated females (depending on egg number) for each breeding trial by stripping into a plastic bowl and divided into three batches (approximately 500 eggs in each batch) for fertilization with sperm preserved with two different diluents and one batch with fresh sperm as control. After thawing for about 30-40 sec at room temperature, the frozen straws were cut at both ends and sperm was mixed with eggs. Each batch of eggs was fertilized with cryopreserved sperm from 5 straws (7.9×10^9 cells/ml), where each of the straw contained 230 µl diluted milt. Small amount of tap water was added to create a natural condition for the sperm thereby it can swim over the eggs and can fertilize easily. The fertilized eggs were then placed into hatching tray where continuous water supply was maintained from above through porous PVC pipe. To observe cell division of eggs of both cryopreserved and control groups, eggs were taken under microscope after 1 h of incubation. The fertilized eggs looked vivid and transparent while unfertilized eggs looked turbid and possessed white chorion. After 22-26 h of incubation at about 26 °C of water temperature, the eggs were hatched out. Individual hatchling was counted carefully to obtain the hatching rate. The fertilization rates were calculated as the percentage of fertilized eggs obtained from the total number of eggs. Similarly, the hatching rates were calculated as the percentage of larvae obtained from total number of eggs for cryopreserved or fresh sperm.

Statistical analyses

Data of experiments (I, II, III) were presented as percentage of motile cells. Data of experiments I and II were analyzed using Independent-samples T-test of SPSS (version 16) and the means were separated by Least Significant Difference (LSD) at 1% and 5% level of probability. The effects of different extenders and cryoprotectants and their combinations (experiment III) on both equilibration and post-thaw motility of spermatozoa were analyzed with SPSS (version 16) computer based software and the variations among the data series were analyzed by using Duncan's Multiple Range Test (DMRT) and one way ANOVA (analysis of variance). The effect of cryopreserved sperm on the fertilization and hatching rates (experiment IV) were analyzed by independent-samples T-test.

RESULTS

Activation of sperm motility at various concentrations of NaCl

The activation of sperm motility was tested at various concentration of salt solution (% NaCl solution) and found that the activation of sperm motility decreased with the increase of NaCl concentration. About 80 -90% forward movement and 10-20% Brownian movement of sperm were recorded from the samples preserved with different NaCl solutions. The motility of sperm suspended with Alsever's solution was recorded as 87.5±1.25% at 0.1% NaCl and 75.0±0% at 0.4% NaCl solution. The motility decreased at a faster rate as the concentration of NaCl increased reaching 5.0±0% at 1% NaCl and become 0% at 1.1% NaCl solution (Figure 1). Almost all the sperm became activated at 0.4% NaCl solution so more or less complete activation can be considered at 0.4% NaCl concentration. The complete inhibition of sperm was recorded from 1.1% concentration of NaCl for Alsever's solution. Similar to Alsever's solution, the motility of sperm suspended in Kurokura-2 solution decreased at a faster rate with the increase of NaCl concentration reaching zero at 0.8% NaCl (Figure 2). The more or less complete activation and complete inhibition of sperm were recorded from 0.4% and 0.8% NaCl solution for Kurokura-2 solution. A significant difference (P=0.002) was observed between the more or less complete activation and complete inhibition, i.e. 0.4% and 1.1% for Alsever's solution and 0.4% and 0.8% for Kurokura-2 solution respectively.

Swimming duration of sperm at various concentrations of NaCl

The swimming duration of activated sperm was different at various NaCl concentrations and was used as suspending medium the highest swimming duration of sperm was estthe duration was rigorously reduced with the increase of NaCl concentration. When Alsever's solution imated as long as 20.18±1.12 min at 0.2% NaCl and it became zero at 1.1% NaCl solution (Figure 3). Again, sperm suspended in Kurokura-2 solution demonstrated its highest swimming duration of about 22.67±0.67 min at 0.2% NaCl

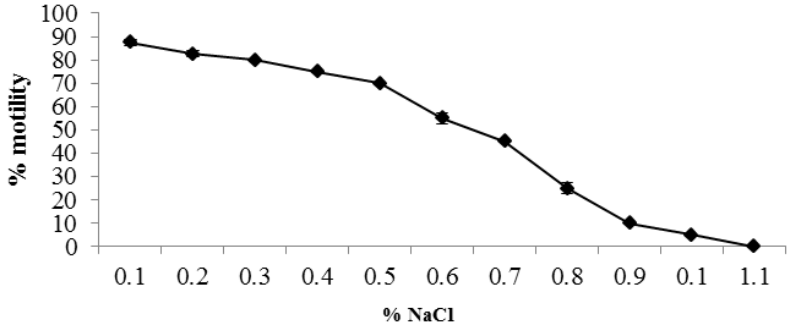

Figure 1. The motility of sperm of *M. vittatus* in different concentrations of NaCl solution (0.1 to 1.1%) which was suspended in Alsever's solution during collection.

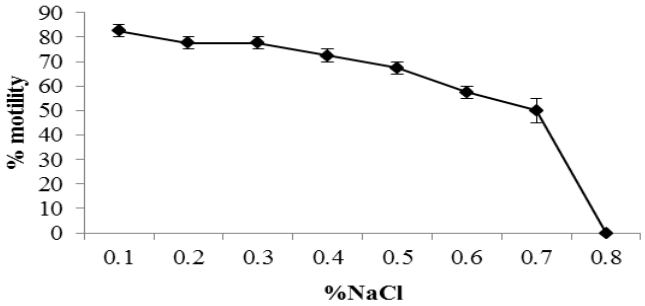

Figure 2. The motility of sperm of *M. vittatus* in different concentrations of NaCl solution (0.1 to 0.80%) which was suspended in Kurokura-2 solution during collection

but the motility completely ceased at 0.8% NaCl solution (Figure 4). Statistical analysis showed that there were significant differences (p=0.003 and p=0.001) between maximum swimming duration and complete inhibition when suspended in Alsever's solution and Kurokura-2 solution respectively.

Toxicity evaluation of cryoprotectant to sperm

Fresh sperm motility of *M. vittatus* before incubation with cryoprotectants was 90-95%. With the increase of cryoprotectant concentration (5, 10 and 15%) and incubation time (5-60 min), sperm motility decreased for the cryoprotectants, Me_2SO and methanol. Alsever's solution with 5% Me_2SO produced 77.5±2.5% motility at 5 min incubation which reduced to 72.5±2.5% at 10 min incubation. Sperm exposed to 10% Me_2SO for 5 min showed 80±5% motility which remained same after 10 min of incubation and then

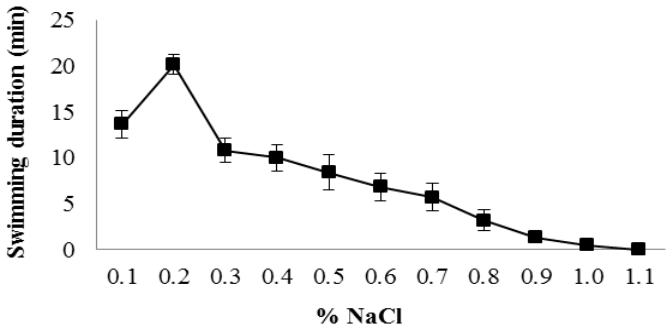

Figure 3. The swimming duration of sperm of *M. vittatus* at various concentration of NaCl solution (0.1-1.1%) those were suspended in Alsever's solution during collection

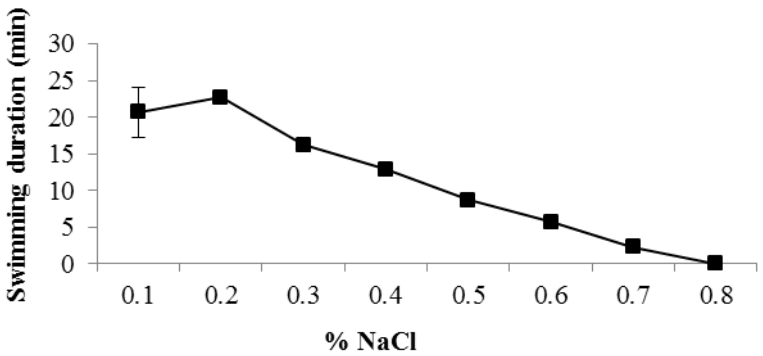

Figure 4. The swimming duration of sperm of *M. vittatus* at various concentrations of NaCl solution (0.1-0.8%) those were suspended in Kurokura-2 solution during collection

decreased gradually. At 15% concentration of Me_2SO, the motility of sperm was very low and it was 54±4% and 47.5±2.5% after 5 and 10 min of incubation respectively (Table 2). No significant difference (P=0.312) was observed between 5% and 10% of Me_2SO after 10 min of incubation.

Sperm suspended in Kurokura-2 solution demonstrated 87.5±2.5% motility at 5 min incubation and 82.5±2.5% at 10 min incubation with 5% Me_2SO. Sperm suspended with Kurokura-2 solution plus 10% Me_2SO showed 82.5±2.5% motility after 5 min of incubation but it decreased to 80±5% after10 min of incubation. At 15% concentration of Me_2SO sperm motility with both the extenders was fairly good just after addition but motility reduced remarkably after 10-15 min of incubation (Table 2). No significant difference (P=0.698) was observed between 5 and 10% cryoprotectants at 10 min incubation.

When methanol was used comparatively poor results were obtained from both Alsever's solution and Kurokura-2 solution. Methanol at 5% concentration with Alsever's solution produced 62.5±2.5% motility after 10 min of incubation where 10% methanol produced 60.0±10% motility during the same incubation time. Kurokura-2 with both 5% and 10% methanol produced 77.5±2.5% motility when incubated separately for 10 min. At 15% concentration of methanol, Kurokura-2 with methanol produced 67.5±2.5% motility at 10 min incubation (Table 2). No significant difference (P=0.831, 1.000) was observed between 5% and 10% cryoprotectants at 10 min incubation. Similarly, no significant differences were also observed between 5% and 15% (P=0.089, 0.106) and 10% and 15% (P=0.312, 0.106) cryoprotectants used with Alsever's solution and Kurokura-2 solution respectively**.** Though 5% and 10% cryoprotectants produced less toxicity to sperm, the later one was applied in the subsequent experiments.

Selection of suitable diluent

The highest motility of the equilibrated sperm was recorded from Alsever's solution with Me_2SO (77.5±1.4%) followed by Kurokura-2 solution with Me_2SO (73.8±3.15%). Highest post-thaw motility (58.8±1.3%) was also recorded from Alsever's solution plus Me_2SO followed by Kurokura-2 solution plus Me_2SO (52.5±2.5%). Other two diluents, Alsever's plus methanol and Kurokura-2 plus methanol showed very poor equilibration and post-thaw motility (<40% and <15% respectively) (Figure 5). A significant variation (P=0.000) was observed between two diluents, Alsever's solution plus Me_2SO and Alsever's solution plus methanol for equilibration and post-thaw motility. Similarly significant difference (P=0.000) was also obtained from Kurokura-2 solution with both Me_2SO and methanol for equilibration and post-thaw motility. Having analysis Duncan's Multiple Range Test revealed that Alsever's solution plus Me_2SO was the best combination for cryopreservation of *M. vittatus* sperm.

Fertilization and hatching of eggs with cryopreserved sperm

Sperm preserved with different diluents were used for fertilization of eggs but only two diluents responded positively. The post-thaw motility of sperm was recorded as 58.8±1.3% from Alsever's solution plus Me_2SO and 52.5±2.5% from Kurokura-2 solution plus Me_2SO during fertilization of freshly collected eggs. Sperm preserved with Alsever's solution plus Me_2SO produced the highest fertilization (72.5±2.5%) and hatching (37.5±13.7%) and those preserved with Kurokura-2 solution plus Me_2SO produced 70±7.07% fertilization and (29.9±12.5%) hatching (Figure 6). Fresh sperm used as control yielded 82.5±2.5% and 55±6% fertilization and hatching respectively.

Breeding efficiency of cryopreserved sperm preserved with two diluents was compared and no significant difference was observed for fertilization (P=0.677) as well as for hatching (P=0.472). 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.

DISCUSSION

Sperm motility of freshwater fish species is typically initiated when milt is diluted in water (or in another hypotonic solution), by a decrease on the osmolality of the extracellular medium or the K^+ ion concentration, and an increase in the concentration of free Ca^{++} ions (Morisawa *et al.*, 1983). A review of previous reports indicates conflicting roles of these ions in inhibiting and activating fish spermatozoa, confirming that many characteristics of fish spermatozoa are species-specific. For example, K^+ ion inhibits

Table 2. Motility of sperm of *M. vittatus* at different concentrations of cryoprotectants and incubation time

Extender	Cryoprotectant		Time (Min.)												
			Initial(0)	5	10	15	20	25	30	35	40	45	50	55	60
			% motility												
Alsever's solution	DMSO	5%	82.5±0	72.5±2.5	72.5±2.5	67.5±2.5	65±5	62.5±2.5	57.5±2.5	52.5±2.5	47.5±2.5	42.5±2.5	37.5±2.5	35±0	27.5±2.5
		10%	85.0±5	80±5	80±5	75±5	70±5	67.5±7.5	65±5	60±5	57.5±7.5	50±10	37.5±12.5	32.5±12.5	27.5±12.5
		15%	62.5±2.5	50±0	47.5±2.5	42.5±2.5	37.5±2.5	32.5±2.5	27.5±2.5	22.5±2.5	16±4	15±5	11.5±3.5	7.5±2.5	4±4
	Methanol	5%	72.5±2.5	70±0	62.5±2.5	57.5±2.5	55±5	52.5±2.5	45±5	40±5	35±5	30±5	27.5±2.5	22.5±2.5	17.5±2.5
		10%	70.0±5	62.5±7.5	60±10	57.5±7.5	52.5±7.5	50±10	45±10	42.5±12.5	37.5±12.5	30±10	25±5	20±5	14±6
		15%	57.5±2.5	52.5±2.5	45±5	42.5±2.5	37.5±2.5	32.5±2.5	27.5±2.5	20±0	17.5±2.5	12.5±2.5	9.0±1	4±1	0
Kurukura-2	DMSO	5%	90.0±0	87.5±2.5	82.5±2.5	80±0	75±5	70±5	65±5	57.5±2.5	52.5±2.5	50±5	47.5±2.5	40±0	32.5±2.5
		10%	87.5±2.5	82.5±2.5	80±0	77.5±2.5	72.5±2.5	70±0	60±0	52.5±2.5	47.5±2.5	42.5±2.5	37.5±2.5	35±5	27.5±2.5
		15%	82.5±2.5	82.5±2.5	72.5±2.5	67.5±2.5	62.5±2.5	60±5	57.5±2.5	50±5	42.5±2.5	35±±5	27.5±7.5	20±5	12.5±2.5
	Methanol	5%	87.5±2.5	82.5±2.5	77.5±2.5	75±5	72.5±2.5	67.5±2.5	65±0	62.5±2.5	57.5±2.5	57.5±2.5	55±5	55±0	50±5
		10%	82.5±2.5	82.5±2.5	77.5±2.5	77.5±2.5	72.5±2.5	72.5±2.5	67.5±2.5	62.5±5	60±2.5	57.5±5	55±2.5	52.5±5	50±0
		15%	72.5±2.5	72.5±2.5	67.5±2.5	62.5±2.5	60±5	57.5±2.5	52.5±2.5	47.5±2.5	47.5±2.5	42.5±2.5	37.5±2.5	30±0	17.5±2.5

Data are presented as mean ± SE

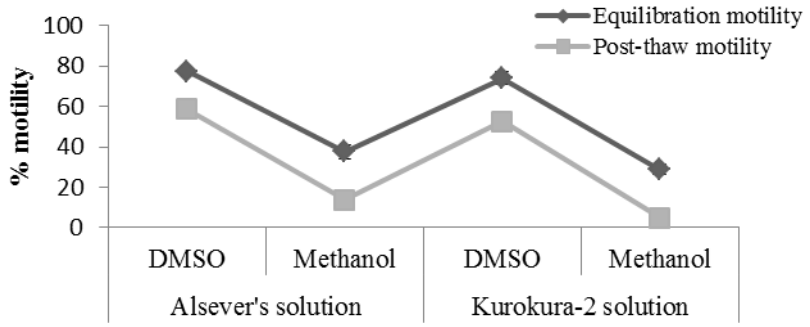

Figure 5. Equilibration and post-thaw motility of sperm of *M. vittatus* at different combinations of extenders and cryoprotectants

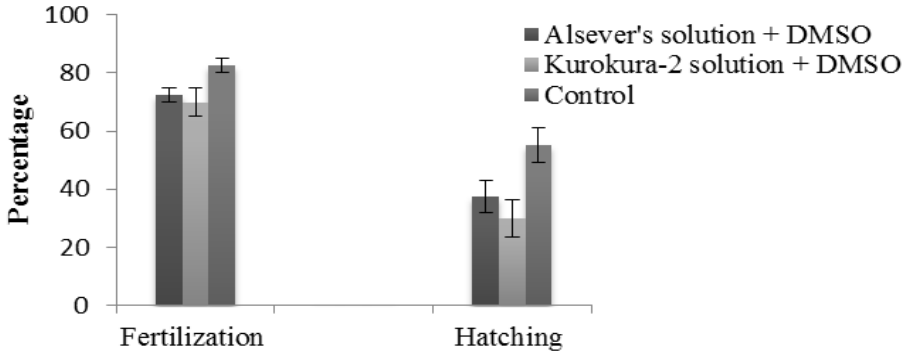

Figure 6. Fertilization and hatching of eggs of *M. vittatus* using cryopreserved and fresh sperm

the spermatozoa activation in rainbow trout (Baynes *et al*., 1981), and ayu (Tsuji *et al*., 2000). Fish sperm are usually immotile in the testis, and motility is controlled by factors such as osmolality, ions, temperature, and pH. Osmolality is the dominant factor in most species studied. Temperature is the most important environmental factor affecting the activity of poikilothermic animals such as fish, and in this context fish sperm, in particular the membranes (Farkas *et al*., 2001). According to Buda *et al.* (1996) temperature affects cellular membranes and cells respond accordingly to achieve a new equilibrium between the environment and the physicochemical properties of their membranous structure. Temperature was recorded 26±2°C during the study period. Furthermore, several researchers have reported a pH effect on sperm motility. In many species, pH is involved in the control of flagellar movement and the optimal pH values seem to be species-specific according to the seminal plasma pH which has been reported as 7.7 in *C. gariepinus* (Horvath and Urbanyi, 2000, Urbanyi *et al*., 1999), 8.0 in *M. nemurus* (Muchlisin *et al.*, 2004), 8.3 in *P. hypophthalmus* and 8.2 in *P. gigas* (Mongkonpunya *et al.*, 2000). The pH value 8.0 was recorded for *M. vittatus* spermatozoa.

In this experiment, the activation of sperm of *M. vittatus*, suspended with two extenders, Alsever's solution and Kurokura-2 solution were tested at different concentration of NaCl solution and found that the motility was decreased with the increase of NaCl concentration. The motility of sperm was >70% at up to 0.5% NaCl and it became zero at and beyond 1.1% NaCl solution while suspended in Alsever's solution. The motility of sperm suspended in Kurokura-2 solution was >65% at up to 0.5% NaCl and motility became zero at and beyond 0.8% NaCl solution.

The selection of the extenders and cryoprotectants was done on the basis of earlier studies of Kumar (1988 and 1989) on Indian major carps, Linhart and Rodina (2000) on common carp and Sarder *et al.* (2012 and 2013) on *Ompok pabda* and *Nandus nandus.* Highest equilibration (77.5±1.44%) and post-thaw motility

(58.75±1.25%) were obtained from the combination of Alsever's solution plus Me_2SO which proved its suitability for preservation of sperm of the fish. It is assumed that Alsever's solution has an optimal osmolality to balance the osmotic pressure of spermatozoa. The Na-citrate fraction of Alsever's solution associated with cell membrane may provide protection against injury during cryogenic freezing. Besides selection of diluents, the dilution ratio between milt and diluent was selected as 1:9 for both Alsever's and Kurokura-2 solutions from the above studies.

Me_2SO 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. The cryoprotectant concentration was maintained at 10% (v/v), as it was found effective during the toxicity test and also commonly used for many species (Chao *et al.,* 1975, Shirohara *et al.,* 1982, Daly *et al.,* 2008 and Nahiduzzaman *et al.,* 2011). In this study, Me_2SO at 5% concentration produced better results in some cases with different diluents compared to 10% Me_2SO, which coincided with Leung (1987) who found the best post-thaw motility of barramundi sperm at 5% Me_2SO. 10% Me_2SO gave acceptable motility though motility decreased with the increase of Me_2SO concentration (> 10%) and 15% Me_2SO gave least motility (Yang *et al.,* 2007 and 2010; Sarder *et al.,* 2012 and 2013). Between the two cryoprotectants Me_2SO showed the best performance producing highest post-thaw motility which was expected as Me_2SO has been a common and effective cryoprotectant for preservation of fish sperm (Lahnsteiner *et al.,* 2000 and Billard *et al.,* 2004) and cell lines (Zhang and Rawson, 2002). The reports suggested that along with Alsever's solution Me_2SO 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 (Ciereszko *et al.,* 1993). Methanol, on the other hand, produced comparatively poor post-thaw motility though it has been found suitable for cryopreservation of sperm of zebrafish (Yang *et al.,* 2007) and Olive barb (Nahiduzzaman *et al.,* 2011). As Me_2SO found to be the suitable cryoprotectant with both extenders (Alsever's and Kurokura-2 solution), it was further used in evaluating fertilization and hatching success of cryopreserved sperm of *M. vittatus.*

Fresh sperm used as control is most important for ensuring the treatment effects and thus result of various studies can be compared effectively (Dong *et al.,* 2007). Fertilization and hatching rates of eggs by cryopreserved sperm proved its potentiality for artificial breeding, but these were not consistent rather fluctuated in different trials. The reasons of fluctuation i.e. low fertilization and hatching rates could be difficult to explain but some basic damages might occur in sperm during cryopreservation which affect sperm function such as motility, plasma membrane integrity and functionality, ATP content, DNA damage etc. and might cause low fertilization (Mazur,1984, Cabrita *et al.,* 2010, Figueroa *et al.,* 2015). Variability in fertilization and hatching of eggs of *Ompok pabda* and *Nandus nandus* was reported by Sarder *et al.* (2012 and 2013). Chao *et al.* (1987) reported that the cryoinjuries occur during pre-freezing and post-thawing, at the temperature range between 0°C and -40 °C. Other causes of cryoinjuries include pH fluctuation, ice crystal formation, osmotic pressure, and cryoprotectant toxicity.

In the present study fertilization and hatching rates of eggs by frozen-thawed sperm were not compared with those obtained from fresh sperm as the number of sperm between fresh and cryopreserved groups was not standardized. Fertilization of eggs from fresh sperm (>85%) and cryopreserved sperm (70-72 %) approved the quality of eggs but it was not reflected in hatching specially from cryopreserved sperm. The results indicated that during fertilization cryopreserved sperm was strong enough but cryopreservation related factors could cause less hatching rate (37.5% and 29.9% in Alsever's and Kurokura-2 solution respectively).

CONCLUSION

To develop the cryopreservation protocol of sperm of *M. vittatus,* the preliminary attempts those were taken in this study seemed successful. Fertilization and hatching of eggs have been successful but need further research especially on improvement of fertilization and hatching rates with cryopreserved sperm. The protocol developed in the experiment will be useful for conserving biodiversity of *M. vittatus* and developing quality broodstock for production of seeds in hatcheries. Once the seeds are available in hatcheries farmers can culture them in different grow-out systems and make profit which ultimately improves their poor livelihood.

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