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Cryogenic preservation of critically endangered *Cirrhinus reba* fish sperm

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Abstract
The natural population of *Cirrhinus reba* in Bangladesh has been decreasing rapidly and is subsequently categorized as threatened. The study was carried out to develop a suitable protocol for *C. reba* sperm cryopreservation. The sperm activation was observed in 0.1%-1.1% NaCl solution with highest motility 92.5 ± 2.5 (mean±SE) at 0.3% NaCl solution and lowest (zero motility) at 1.1%. Two different extender such as inorganic chemical Alsever's solution, organic egg-yolk citrate and two cryoprotectants namely methanol and dimethylsulfoxide (DMSO) were used in toxicity analysis at 5%, 10% and 15% concentration. The cryopreservation was carried out with three diluents (Alsever's solution plus 10% DMSO, Alsever's solution plus 10% methanol; Egg-yolk citrate plus 5% DMSO) in two steps freezing protocol (from room temperature to -4°C at a rate of 4°C per min and then from -4°C to -80°C at a rate of 10°C per min). Alsever's solution with 10% DMSO showed best performance and producing 85±5.77 and 58.75±3.15% equilibration and post-thaw motility, respectively. This protocol will be helpful to conserve the gene pool of *C. reba*.

Keywords: Diluents, Freezing protocol, *Cirrhinus reba*

1. Introduction
Fisheries in Bangladesh are diverse; there are about 795 native species of fish and shrimp in the fresh and marine waters of Bangladesh and 12 exotic species that have been introduced [18]. In addition, there are 10 species of pearl bearing bivalves, 12 species of edible tortoise and turtle, 15 species of crab and 3 species of lobster [18]. The total number of freshwater fish species occurring in Bangladesh compiled as 250 to 266 species [25, 29]. Although the resources is high the indigenous fish production decreases due to a variety of factors such as diseases, habitat loss as a result of human interaction like urbanization, environmental degradation, pollution, over-exploitation etc. A total of 54 native freshwater fish species of Bangladesh have been declared threatened by IUCN Bangladesh [9]. Among them 12 species are recorded as critically endangered, 28 species are endangered and rest 14 species are vulnerable. The *Cirrhinus reba*, locally known as reba belongs to the family Cyprinidae of the order Cypriniformes is a commercially important indigenous fish species of Bangladesh. It is found throughout the Indian sub-continent including Bangladesh [19], India [31], Nepal [23] and Pakistan [13]. This carp fish is known as Raik, Tatkini, Bata, Laacho and Bhagna in Bangladesh and Suhnee and Sunee in Pakistan [9, 15]. The species is highly priced fish preferred by the consumer of all classes. It is a good source of protein, calcium and low fatty acid, as well as an ideal dietetic food for human consumption [1]. At present, *C. reba* can be a potential candidate for artificial culture in ponds and rivers. Due to over exploitation, siltation, habitat loss and other ecological changes to its habitat, the natural population of *C. reba* in the water body of Bangladesh decreasing rapidly and is subsequently categorized as vulnerable [9]. But more studies on the reproductive biology and stock assessment of the fish are required [7,8]. Size structure (length frequency) in riverine fish reveals many ecological and life-history traits such as the river health, stock conditions and breeding period of the fish [22]. Spawning season of the fish extends from June to August. However, the conservation status of *C. reba* can be improved through effective habitat preservation and increasing public aware-ness, ranching and through cryopreservation (preservation of the genetic material of the fish).

Creation of sperm banks to stock genetic potential of valuable males for selection programmes, or banking for routine sperm handling, are being adopted in some fish farms. Cryopreservation of gametes and embryos is a key issue in the development of reproductive technologies. It provides the possibility to preserve genetics from rare or valuable breeds (gene banking), giving insurance against death, infertility or illness and enabling semen and embryos to be marketed or to be exported. The development of suitable extenders and cryoprotectants is the first step in cryopreservation of fish spermatozoa. To develop a suitable cryopreservation protocol some parameters have to be considered such as appropriate activation media development, immobilization solutions, cryoprotective agents, equilibration time, cooling rates, thawing rates etc. because of differences within and among species. Considering the potentiality of cryopreservation techniques in aquaculture, research on sperm cryopreservation protocol of different freshwater species has been developed in Bangladesh. Cryopreservation of sperm of different threatened fishes like sarpunti [14], veda [27], pabda [28], baim [20] have also been done. However, the aim of this research was to develop a suitable cryopreservation protocol for the critically endangered *C. reba* fish sperm.

2. Materials and Methods

2.1 Brood collection and rearing

The fishes were collected from natural sources of Mymensingh district in April 2014. Mature male (milt or sperm comes after gentle pressure on abdomen) and female (swollen abdomen) of *C. reba* fishes were reared in the ponds of Fisheries Faculty of Bangladesh Agricultural University, Mymensingh, Bangladesh. The fishes were fed twice daily by commercial feed.

2.2 Sperm collection and quantifying sperm motility

The brood males were collected from the pond and acclimatized in the tank of hatchery area after measuring the length (cm) and weight (kg). The broods were injected with pituitary gland extract (dry PG was collected from the local market). After six hours of injection (2 mg kg^{-1}) milt was collected in an eppendorf tube by gentle pressure on abdomen. Sperm concentration was determined in triplicate counts by use of haemacytometer. The milt was diluted 1000 times in distilled water. 10-12µl of the diluted sample was taken on haemacytometer and covered with a cover slip (Marienfeld, Germany) and expressed as the number of cells $x10^9$ ml^{-1}. After measuring the concentration, the motility of sperm in each tube was checked out under the microscope taking about one-two µl of milt on a glass slide with 100-200 µl distilled water.

2.3 Evaluation of sperm and cryopreservation

Sperm samples were exposed to 10 graded dilutions of NaCl solutions. Sperm motility was observed in 0.1 to 1.1% NaCl solutions for activation. This experiment was done to formulate an extender which osmolality should be resembled to the milt and sperm will remain inactive in it during cryopreservation.

To determine a suitable diluent for cryopreservation of sperm of *C. reba*, two extender Alsever's solution, egg-yolk citrate and two cryoprotectants namely methanol and DMSO were used in cryopreservation trials. Cryoprotectant concentrations were maintained according to the percentage (5, 10 and 15%) in the final suspension. Collected milt samples were diluted with the diluents at 1:4 (milt: diluent) for egg-yolk citrate and 1:9 for Alsever's solution. After observing the equilibration motility, the diluted milt was taken into 250µl plastic straws and sealed the open end. Then the straws were placed into the cryochamber and initiated cooling. Computer controlled freezer (CL-3300) was used to freeze the sample using two steps freezing protocol, where milt sample was firstly cooled from ambient temperature (25°C) to -4°C at a rate of 4°C per min and then from -4 to -80°C at a rate of 10°C per min. Finally the frozen straws were placed into the liquid nitrogen (-196°C) for long term preservation. Straws were retrieved from liquid nitrogen container and thawed at room temperature for 30-40 seconds to assess the motility under microscope.

2.4 Statistical Analysis

Data of motility for activation, toxicity and equilibration expressed in percentage were converted to arcsine transformation. Data of activation and toxicity were analyzed with one factor ANOVA of SPSS v 20 and the means were separated by Least Significant Difference (LSD) at 5% level of significance.

3. Results

This research was conducted on *C. reba* fish sperm activation, toxicity analysis on sperm motility, selection on diluents for cryopreservation and observation of the effect of diluents on sperm motility after thawing. The weight of male fishes (N=10) ranged from 46 to 86 g (64.9±14.87, mean ±SD) and total length ranged from 12 to18 cm (15.03±1.91, mean ±SD) were used in this experiment. The collected sperm volume was 0.20 to 1.5 ml. The p^H range from 8.6-8.7 in all samples. Fresh sperm motility was estimated from 85 to 95%.

3.1 Sperm activation

The concentration of sperm of *C. reba* was 6.3± 0.4x 10^9 ml^{-1}. Sperm activation at different concentration of NaCl solution was tested. It was found that the activation was decreased with the increase of NaCl solution. But the highest motility 92.5 ± 2.5% was observed at 0.3% NaCl solution and then gradually decreased and the motility was completely inhibited at 1.1% concentration (Fig.1). A significant difference was found (P=0.000) between complete activation and complete inhibition of sperm at 0.3 and 1.1% NaCl solution, respectively.

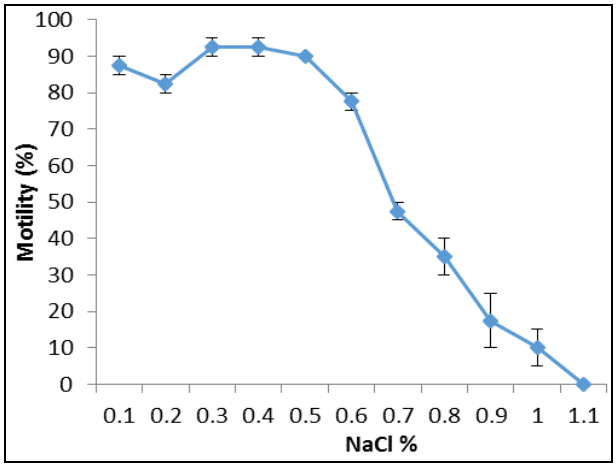

Fig 1: Effect of different concentrations of NaCl solution (0.1 to 1.1%) on *C. reba* fish sperm motility

The swimming duration was different in different NaCl solution. The highest swimming duration (12.50 ±1.55 min) was found at 0.4% NaCl. The swimming duration was decreased with the increase of concentration of NaCl solution (Fig 2). There was a significant difference (P=0.015) between the highest swimming and the lowest swimming duration.

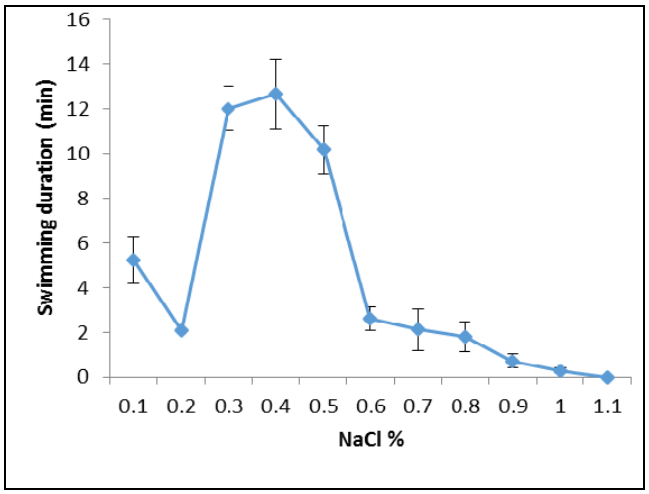

Fig 2: The swimming duration of sperm of *C. reba* in different concentrations of NaCl solution (0.1 to 1.1%)

3.2 Evaluation of toxicity of cryoprotectant concentration

In the combination of Alsever's solution and cryoprotectant (DMSO and methanol) at 5, 10 and 15% concentrations the sperm motility was observed upto70 min of incubation period. Alsever's solution with 5 and 10% DMSO produced 85±5 and 87.5 ±2.5% motility at 5 min of incubation and motility was decreased to 80±5 and 82.5±2.5% within 10 min (P=0.690). At 15% DMSO sperm motility was 77.5±2.5% at 5 min incubation and it was reduced to 72.5±2.5% at 10 min. No significant differences was found between 5 and 15% (P=0.308); and 10 and 15% (P=0.313) concentration.

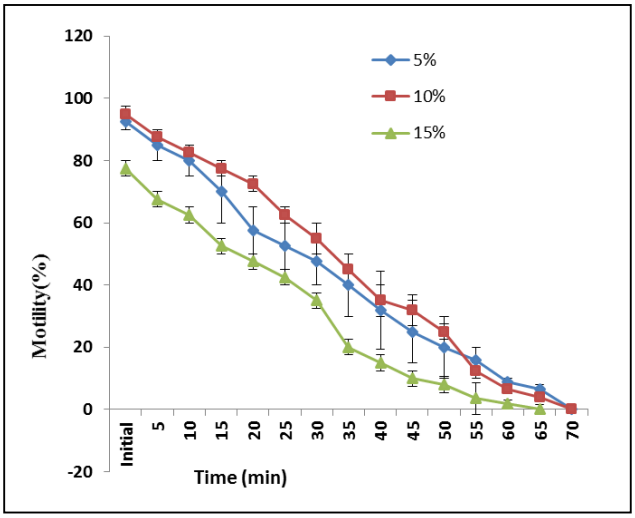

Fig 3: The motility of sperm of *C. reba* suspended in Alsever's solution was incubated with DMSO at 5, 10 and 15% concentration

In combination with Alsever's solution 5 and 10% methanol produced 57.5±2.5 and 72.5±2.5% motility at 5 min of incubation and it was reduced to 52.5±2.5 and 67.5±2.5% at 10 min(P=0.150). A comparatively poor result was shown by 15% than 5 and 10%, the motility was 52.5±2.5 and 47.5%±2.5% at 5 and 10 min incubation, respectively. Statistical analysis showed no significant difference between 5 and 15% methanol (P=0.293) at 10 min of incubation.

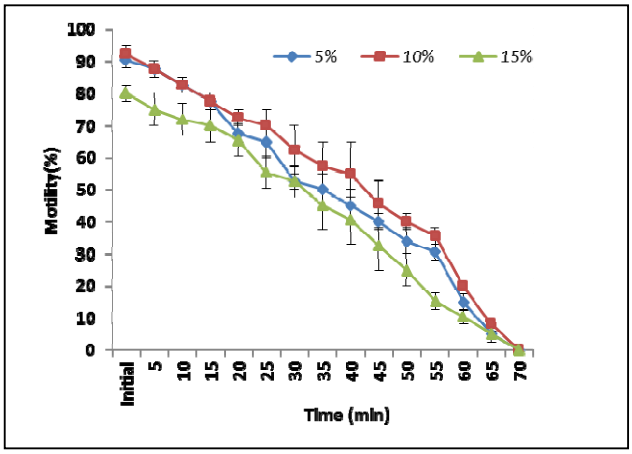

Fig 4: The motility of sperm of C. *reba* suspended in Alsever's solution and incubated with methanol at 5, 10 and 15% concentration

The sperm motility was very low when suspended in egg-yolk citrate and cryoprotectant (DMSO, methanol) at 5, 10 and15% compared to Alsever's solution. The motility gradually decreased from initial to complete ceased at 35 min. The 5 and 10% DMSO produced 37.5±2.5 and 45±5% at 5 min of incubation which reduced to 32.5±2.5 and 35±5% at 10 min (P=0.015). Egg-yolk and 15% DMSO produced 32.5±2.5% motility at 5 min which was decreased to 25±5% at 10 min. No significant difference was observed between 5 and 15% DMSO (P=0.024) at 10 min of incubation.

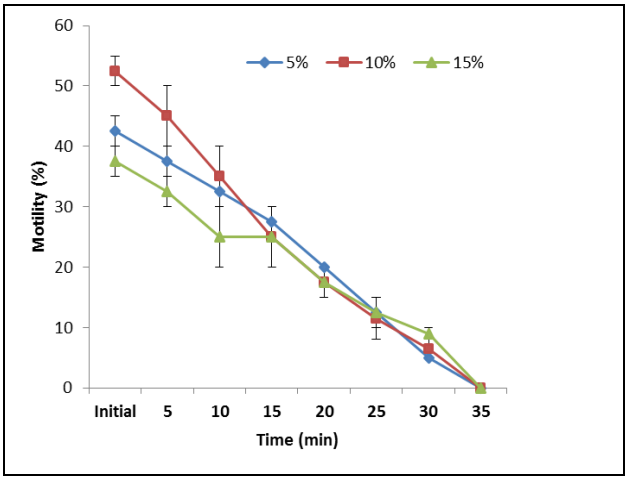

Fig 5: The motility of sperm of *C. reba* suspended in egg-yolk cit1rate solution and incubated with DMSO at 5, 10 and 15% concentration.

Methanol at 5 and 10% with egg-yolk citrate produced 42.5±2.5 and 37.5±2.5% motility within 5 min of incubation and it was reduced to 27.5±2.5 and 27.5±2.5% at 10 min. Poor motility was observed at 15% methanol which produced, 27.5±2.5 and 22.5±2.5% motility at 5 and 10 min, respectively. No significant difference was observed between 5 and 10% (P=0.70); and 5 and 15% (P=0.313) methanol at 10 min of incubation period.

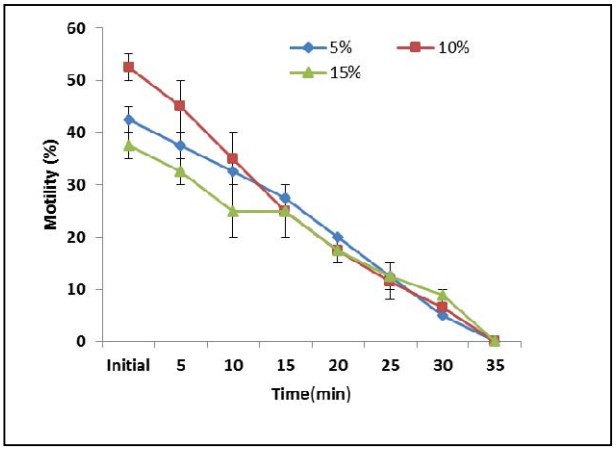

Fig 6: The Motility of sperm of *C. reba* suspended in egg-yolk citrate solution and incubated with methanol at 5, 10 and 15% concentration

3.3 Selection of suitable diluents for *C. reba* fish sperm

In toxicity analysis Alsever's solution and DMSO combination given the best motility than methanol combination. Egg-yolk citrate and DMSO combination gave better motility than methanol. Therefore, cryopreservation of *C. reba* sperm was done with these two cryoprotectants. Evaluation of equilibration and post thaw motility was presented in Table 1. Post-thaw motility is one of the most important indicators of the success of a freezing protocol. There was a significant difference between equilibration motility and post-thaw motility (P=0.001) with Alsever's solution plus 10% DMSO combination. Similarly, cryoprotectant effect was significantly found in post-thaw motility at Alsever's solution and 10% methanol (P=0.001). The sperm sample that contained 10% DMSO and Alsever's solution had significantly higher post-thaw motility (58.75±3.15%) than Alsever's plus 10% methanol and egg-yolk citrate plus 5% DMSO combination as observed 25±3.54 and 15±5% respectively.

Table 1: Motility of sperm of *C. reba* at different combinations of extender and cryoprotectant during cryopreservation.

Cryodiluent	Concentration (%)	Equilibration motility (%)	Post- thaw motility (%)
Alsever's solution+DMSO	10	85±5.77	58.75±3.15
Alsever's solution + methanol +Methanol	10	63.75±5.54	25±3.54
Egg-yolk citrate +DMSO	5	45±5	15±5

4. Discussion

The creation of sperm banks of the selected stock to prevent outbreaks, catastrophes and genetic drift is essential to develop genetic selection programmes in commercial aquaculture, but it is also necessary for conservation of strains or species in danger of extinction, until the environmental conditions are recovered. The *C. reba* fish sperm can be cryopreserved in suitable cryodiluent from the extinction. The dilution ratio is important for cryopreservation [4]. Dilution of the cryoprotectants causes a significant increase in fragility when the cells are exposed to hypo-osmotic shock. Cryoprotectants must be designed to prevent cryoinjury caused due to the ice formation and dehydration when the spermatozoa are frozen and thawed [16]. In the present research 1:9 dilution ratio (milt:diluent) for Alsever's solution was maintained and found higher motility than others. In this study the sperm activation was done with the different concentration of NaCl. The sperm showed the forward movement. Highest movement was observed in 0.4% NaCl solution upto 12 min and 50 second. Whereas only 35 seconds movement was observed in *Puntias sarana* [14], species.

Effective extenders and cryoprotectants are needed to standardize the sperm cryopreservation protocol for each of the fish species. Alsever's solution was found to be the best extender for cryopreservation of carps [25]. In this study Alsever's solution gave the best result as extender for cryopreservation of *C. reba* sperm. A good result from Alsever's solution with 10% DMSO for preservation of silver carp (*H. molitrix*) sperm was also reported by Alvarez *et al*. (2003) [2]. DMSO has been considered as a common and effective cryoprotectant for cryopreservation of fish sperm [11, 3, 6, 12] and cell lines [35]. In this study, a solution with 10% DMSO was the most efficient cryoprotectant, and a similar concentration was suggested for the cryopreservation of sperm of Olive barb, *Puntius sarana* [14], and common carp [26, 30]. When DMSO is used as cryoprotectant it penetrates rapidly into the cellular membrane [21], and brings a quick balance in between the intra and extra-cellular fluid concentration. DMSO has also been considered to be the most efficient cryoprotectant for use in the cryopreservation of marine fish sperm because of its low toxicity and its protection of sperm during cooling due to its capacity for reducing ice formation by lowering the freezing point of intracellular fluid [17]. Methanol was found a far more efficient cryoprotectant for common carp sperm than DMSO [6]. They first reported on the utilization of methanol as a successful cryoprotectant. Methanol has been found suitable for cryopreservation of sperm of Olive barb [14]. A similar concentration (10% DMSO) was suggested for the cryopreservation of red snapper [24] and mangrove red snapper sperm [34]. In other commercial marine species, satisfactory results were achieved with 10% DMSO for the cryopreservation of cobia *Rachycentroncanadum* [5], common snook [33] and fat snook sperm [32]. During cryopreservation highest equilibration motility (85±5.77) and post-thaw motility (58.75±3.15) was found from Alsever's solution and 10% DMSO concentration. In Indian major carp,rohu (*Labeo rohita*) equilibration and post-thaw motility of sperm was found as 82.5±1.44% and 77.5±3.22% respectively [10].

5. Conclusion

This study was conducted to develop cryopreservation protocol for *Cirrhinusreba*fish sperm. The findings from this experiment are that the Alsever's solution and 10% DMSO will be suitable diluent (extender + cryoprotectant) for this fish sperm with a cooling rate of 0ºC to -80ºC at a decreasing rate of 10ºC/min. This protocol will be helpful to conserve the gene pool of *C. reba*. However, further research needs to be performed for standardizing thawing and fertilization techniques, suitable egg-sperm ratio etc.

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