

Preliminary Study to Develop Sperm Cryopreservation Technique of Endangered Rita Catfish, *Rita rita* (Hamilton, 1822)

Muhammad Forhad Ali^{1*}, Md. Abdus Salam², Md. Rafiqul Islam Sarder²,
Mohammad Matiur Rahman² and Md. Fazlul Awal Mollah²

ABSTRACT

The study was carried out to characterize the fresh and cryopreserved sperm of the endangered Rita catfish, *Rita rita* (Hamilton, 1822) in order to develop sperm cryopreservation technique. Sperm was collected by dissecting out the testes and their quality was assessed as sperm motility $96.63 \pm 3.03\%$, volume $2.87 \pm 0.08 \mu\text{L} \cdot \text{g}^{-1}$ of fish, density $3.89 \pm 0.55 \times 10^9 \text{ cells} \cdot \text{mL}^{-1}$ and pH 7.73 ± 0.13 . Activation of sperm motility was evaluated in different concentrations of NaCl solution and highest motility ($96.67 \pm 1.53\%$) and swimming duration ($70.0 \pm 2.0 \text{ min}$) were achieved at 0.4% ($128 \text{ mmol} \cdot \text{kg}^{-1}$). Toxicity of three cryoprotectants- DMSO, methanol and ethanol mixed with three extenders, Alsever's solution, egg-yolk citrate and urea egg-yolk at their 5, 10, and 15% concentration was tested during 5–40 min incubation period. Cryoprotectants at 5 and 10% yielded highest motility during 5 and 10 min of incubation. Sperm processed with Alsever's solution plus 10% DMSO produced significantly ($p < 0.05$) highest equilibration motility ($89.67 \pm 5.51\%$) and when cooled at $10 \text{ }^\circ\text{C} \cdot \text{min}^{-1}$ yielded highest post-thaw motility ($79.33 \pm 5.13\%$). Frozen sperm processed with Alsever's solution, egg-yolk citrate and urea egg-yolk and 10% DMSO was stored in liquid nitrogen Dewar and assessed their quality by examining their post-thaw motility at weekly interval. Six-week investigation demonstrated gradual reduction of motility with the progress of storage period in all three diluents, but significantly ($p < 0.05$) highest motility was observed from Alsever's solution even at the end of investigated storage period.

Keywords: Conservation, Cryopreservation, *Rita rita*, Sperm quality

INTRODUCTION

Bagridae catfish is imperative for viable fisheries and aquaculture industry all over Asia and Africa. This family has comprised of 20 genera with about 232 species globally (Froese and Pauly, 2021) of which six genera with 13 species inhabiting in Bangladesh (Siddiqui *et al.*, 2007). Recently several bagrid catfish species (*Mystus cavasius*, *M. gulio*, *M. tengara* and *M. vittatus*) are farming commercially in Bangladesh (Rahman *et al.*, 2004; Alam *et al.*, 2006; Yesmin *et al.*, 2014) but farming of *Rita rita* (Hamilton, 1822) was not possible due to unavailability of seeds. A preliminary attempt

was taken on artificial insemination on Rita catfish (Mollah *et al.*, 2008), but no successful result was obtained. The Rita catfish is dispersed in the Indian subcontinent, Afghanistan, Myanmar, Nepal and western Thailand (Talwar and Jhingran, 1991; Froese and Pauly, 2007). It is one of the biggest and most eminent freshwater catfish in Bangladesh, attaining 150 cm length (Talwar and Jhingran, 1991). The species contains high protein (17.22–19.55%), lower fat (1.01–2.70%) and improved mineral (0.89–1.07%) and non-bony intramuscular flesh (Mitra *et al.*, 2017). Thus, the catfish is highly accepted as a delicious and nutritious food item. The availability of this species was very high

¹Department of Fisheries, Bangamata Sheikh Fojilatunnesa Mujib Science and Technology University, Jamalpur, Bangladesh

²Department of Fisheries Biology and Genetics, Bangladesh Agricultural University, Mymensingh, Bangladesh

*Corresponding author. E-mail address: forhadali@bsfmstu.ac.bd

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in all native countries until the nineties decade (Tripathi, 1996). But presently, its catch has steadily declined due to the united causes of much natural and manmade interference (World Bank, 2005; IUCN Bangladesh, 2015). Consequently, it was categorized as endangered species for almost a decade in Bangladesh (IUCN Bangladesh, 2015), close to threatened in India (Gupta, 2015) and least concerned in the world (IUCN Bangladesh, 2015). So, it is essential to take urgent actions to conserve the genetic materials of the fish with collective strategies, such as live- and cryo-protective gamete repository. While, the hatchery production of Rita catfish fry has been tough as the species is a seasonal breeder, and asynchronous maturity occurred between male and female broodstock in captivity. Appropriate and skilled use of cryopreserved sperm might support not only to produce fry of Rita catfish for aquafarming but also assurance the maintenance of its genetic resources for further uses and conservation.

The cryopreserved fish sperm has numerous potential applications, such as the protection of biodiversity and the preservation of dying out species (Tiersch *et al.*, 1998), simple sharing of genetic resources, to spawn fish fry with asynchronous ripeness of male/female (Legendre *et al.*, 1996), to help in lowering the expenditure by removing holding male broodstock in hatcheries, and sperm economy (Cabrita *et al.*, 2010). So, spermatozoa cryopreservation procedure for over 230 species of fish (Chao and Liao, 2001; Tiersch *et al.*, 2007; Tsai *et al.*, 2010) including 22 catfish species (Ramirez-Merlano *et al.*, 2010; Viveiros, 2011) were developed and available for numerous applications globally. In Bangladesh, very limited work has been conducted on sperm cryopreservation of several carp species including three threatened catfish species, *Ompok pabda* (Sarder *et al.*, 2013), *Mystus cavasius* (Islam *et al.*, 2016) and *M. vittatus* (Sarder *et al.*, 2017). Though the abundance of Rita catfish is declining fast (Mishra *et al.*, 2009) in all native countries especially in Bangladesh, no initiative was taken to conserve them through cryopreservation so far. Therefore, sperm cryopreservation of the commercially important and endangered Rita catfish could be a safe and sound technique to protect the genetic resources, offering the possibility to conserve reproductive samples from the threat of extinction.

The main purpose of the preservation of sperm is to minimize spermatozoa motility in cryopreservation, ensuing in better post-thaw motility; which is attained using accurate extenders and cryoprotectants. The appropriateness of extenders and cryoprotectants varies with fish species, such as dimethyl sulphoxide (DMSO) and dimethylacetamide (DMA) (Ponchunchoovong and Plime, 2010) or Hank's balanced salt solution and DMA (Rani *et al.*, 2016) for striped catfish, *Pangasianodon hypophthalmus* (synonym: *Pangasius sutchi*); fructose and methanol for bagrid catfish, *Hemibagrus wyckioides* (Ratanatrivong *et al.*, 2011), Fish Ringer and DMSO for African catfish, *Clarias gariepinus* (Kamaruding *et al.*, 2012), Alsever's solution and DMSO for Gangetic *Mystus*, *Mystus cavasius* (Islam *et al.*, 2016), and so on. So far, the variety of extenders and cryoprotectants appropriate for the endangered Rita catfish sperm is yet to be known, in spite of the burning requirement of cryopreserved sperm for the task of artificial breeding. The aim of the current investigation was to evaluate general sperm quality, activation of sperm at various osmolalities, the influence of cryoprotectants, and suitability of the combination of diluents with their particular concentrations for the Rita catfish sperm dilution for cryopreservation.

MATERIALS AND METHODS

Experimental fish

To carry out the present study 100 Rita catfish (male: female = 50: 50, length and weight ranging from 25–35 cm and 400–700 g, respectively) were collected from the Old Brahmaputra River, adjoining the Bangladesh Agricultural University, Mymensingh and were stocked in the raceway of the Faculty of Fisheries. The fish were reared for about 12 months by feeding cleaned and chopped chicken viscera once a day in the afternoon at 3–5% of body weight. Three months before the breeding season, local prawns, the flesh of cheaper fish or trash fish and *Loligo* flesh were provided at the same rate in lieu of chicken viscera to enhance gonadal maturation. Matured males were identified and selected based on well developed secondary sexual characters such as flat abdomens and long protruded genital papillae.

Conditioning and inducing of the broods

Thirteen matured males having 36.00 to 41.50 cm (38.92 ± 1.53 cm) length and 700–1,000 g (874.62 ± 102.63 g) weight were shifted from the raceway to the cistern ($216 \times 182 \times 54$ cm³) of the mini-hatchery. They were acclimatized by providing vigorous shower for about 6 h prior to injection with pituitary gland extract (PGE). During acclimatization, no feed was provided. A single dose of 40 mg PGE·kg⁻¹ was given to stimulate the males (Mollah *et al.*, 2008). After the PGE injection, continuous showering was provided for another 20–22 h for better spermiation.

Collection of milt

Sperm of Rita catfish was collected by sacrificing the males and dissected out the testes. Sperm could not be collected by stripping due to the serrated structure of testes (Figure 1). The tips of serrated testes were cut into small pieces by a scissor and the milt was pressed out of testes in a

sterilized dry Petri plate. Motility of fresh sperm was estimated by mixing 1–2 µL of milt with to 8–10 µL water on a glass slide and observed under a microscope at $\times 10$ magnifications. The sperm showing more than 80% motility was mixed with each of the three extender solutions- Alsever's solution, egg-yolk citrate and urea egg-yolk at a ratio of 1:1. Alsever's solution was prepared by dissolving of 0.4% sodium chloride and 0.8% sodium citrate in 100 mL deionized water. The pH was adjusted to 7.8. For egg-yolk citrate preparation a buffer solution was made by dissolving of 0.4% sodium chloride and 0.3% sodium citrate in 100 mL deionized water. Then 80 mL of the buffer was added to 20 mL of egg-yolk. The pH was adjusted to 6.5. In case of urea egg-yolk extender another buffer solution was prepared by dissolving of 0.3% sodium chloride and 0.4% urea in 100 mL deionized water. Finally, 80 mL of the buffer was mixed with 20 mL egg-yolk at pH 6.1. The diluted milt was taken into microcentrifuge tubes (MCT; Eppendorf, Germany) by a micropipette and preserved on ice till further utilization.



Figure 1. Serrated testes of *Rita rita*.

Observation-I: Assessment of general sperm quality of Rita catfish

The basic parameters of sperm collected from 13 males were determined through measuring milt volume ($\mu\text{L}\cdot\text{fish}^{-1}$ and $\mu\text{L}\cdot\text{g}^{-1}$ of body weight), sperm motility (%), density ($\text{cells}\cdot\text{mL}^{-1}$) and pH. Milt volume was determined with the help of a micropipette. Sperm motility was evaluated using a luminous binocular microscope (CX41RF, Olympus Corporation, Tokyo, Japan). About 1–2 μL extended milt was put on a glass slide and 100 μL deionized water was added to activate the motility of sperm. Instantly the sperm motility was monitored under the compound microscope at $\times 10$ and/or $\times 40$ objective lenses. Sperm motility was evaluated three times in five fields for each sample. The motility was recorded as the percentage of active forward motile sperm cell. Sperm concentration (number of sperm cells $\cdot\text{mL}^{-1}$) was determined five times by haemocytometer (Marienfeld, Germany) counting. Sperm pH was determined by using litmus paper (Ranbaxy Fine Chemicals Limited, India).

Observation-II: Sperm activation at diverse osmotic pressure of NaCl

Collected milt from three males for triplicate trials was mixed with three extenders- Alsever's solution, egg-yolk citrate and urea egg-yolk in MCT which were put on ice until use. Twelve graded NaCl solution (from 0.1% to 1.2%) equivalent to osmolalities from 48 to 383 $\text{mmol}\cdot\text{kg}^{-1}$ were prepared through dissolving NaCl in deionized water. The osmolalities of NaCl solutions were measured by an osmometer (Vapro 5600, USA). About 1–2 μL of the suspended milt was mixed with 20 μL of graded solution of NaCl on a glass slide to activate the sperm. Motility of sperm in each graded solution of NaCl was investigated using a microscope (CX41RF, Olympus Corporation, Japan) having a display monitor (Olympus DP 2-SAL, Japan). The percent motility and swimming period of sperm were tracked at different osmotic pressure of NaCl solution. Swimming period was calculated by determining the time variation between initiation and stoppage of motility.

Observation-III: Estimation of cryoprotectants toxicity to sperm

Milt was collected as described earlier from three fish for triplicate trials. The milt was extended at 1:4 for urea egg-yolk and egg-yolk citrate, and 1:9 for Alsever's solution (% v/v). The extended milt was mixed with each of three cryoprotectants - DMSO, methanol or ethanol in 1 mL MCT to make a final concentration of 5, 10, and 15% for each cryoprotectant following the earlier studies of Meni, *Nandus nandus* (Sarder *et al.*, 2012) and Pabda catfish, *Ompok pabda* (Sarder *et al.*, 2013). The toxic effect of these cryoprotectants was evaluated by observing the spermatozoan motility using a microscope at $\times 10$ and $\times 40$ objective lenses from 0 (initial) to 40 min at 5 min intervals.

Observation-IV: Suitable diluents selection

For choosing an accurate mixture of extenders and cryoprotectants, three extenders- Alsever's solution, egg-yolk citrate, and urea egg-yolk solution and three cryoprotectants- DMSO, methanol, and ethanol were utilized. Diluents were made by mixing 10% cryoprotectant with 90% extender (% v/v). Semen was collected as described earlier from three fish for triplicate trials in each of the nine diluent combinations. Diluted sperm was permitted to equilibrate for 5–10 min. During equilibration 0.23 mL diluted sperm was taken in each 0.25 mL plastic French straw (Minitüb System, Minitüb, Tiefenbach, Germany) by a micro-pipette. The open tops of the straws were closed manually using heated tongs. After the equilibration period, the motility of sperm was examined and it is known as equilibration motility.

The straws containing diluted sperm was kept in the cryochamber of a computerized freezer (CL-3300) operated by the Cryogenesis software version 4 (Cryologic, Australia 1998 and 1999) to cool the sample from 4 °C to -80 °C using a single-step freezing procedure with declining at 10 °C $\cdot\text{min}^{-1}$. Following freezing, the straws were instantly kept in liquid nitrogen (LN_2) (-196 °C) for cryo-storage. For post-thaw examination of sperm, the straws

were thawed at 25–26 °C (room temperature) for 30–40 s and cut at both ends. About 1–2 µL of sperm was placed on a glass slide and 100–150 µL deionized water was added to activate the sperm. Post-thaw spermatozoa motility was evaluated under microscope and the motility percentage was documented.

Observation-V: Estimation of post-thaw motility of sperm in short-term storage

Based on the above suitable protocol, three diluents, Alsever's solution, egg-yolk citrate and urea egg-yolk each with 10% DMSO were prepared, and sperm was cryopreserved and stored in liquid nitrogen Dewar (Taylor-Wharton, USA). The straws were stored for 6 weeks, and the post-thaw motility of sperm was evaluated at weekly basis. One to two straws were retrieved from the Dewar and thawed at room temperature. The post-thaw motility of thawed sperm was determined under the microscope. The sperm displaying active movement were counted and expressed as percent motility. For each sperm sample, the estimation of sperm motility was conducted in five fields under each observation. The estimation was done for at least three times to minimize any error. The motility of freshly collected sperm extended in respective extenders was also determined as control.

Statistical analysis

Data from the observations II, III, IV, and V were tabulated as percent of motile sperm, and all values were arcsine-transformed prior to analysis using one-way ANOVA in the SPSS version 23. The influences of various extenders, cryoprotectants, cryoprotectant concentration, and their permutations on equilibration and thawing

motility were analyzed using one-way ANOVA. Mean values were segregated by Duncan's multiple range tests (DMRT) and considered significant at $p < 0.05$ (Duncan, 1955).

RESULTS

Observation-I: General sperm quality of Rita catfish

Weights of testes of 13 fish were between 14.00 g and 18.10 g with a mean of 16.10 ± 1.33 g. General sperm quality of Rita catfish are illustrated in Table 1. In this study, almost 90–95% of sampled sperm displayed rotatory movement.

Observation-II: Sperm activation at diverse osmotic pressure of NaCl solutions

Sperm activation motility was evaluated in 12 graded NaCl solutions (osmolalities ranged 48–383 mmol·kg⁻¹). Motility of spermatozoa in all three tested extenders was steady and highest at the osmotic pressure of 128 mmol·kg⁻¹ of NaCl and deemed completely activated. It was reduced by the rise of osmotic force and entirely ceased at 351 mmol·kg⁻¹ in case of urea egg-yolk, and at 383 mmol·kg⁻¹ for both the Alsever's solution and egg-yolk citrate. There was a significant ($p = 0.000$) difference between the full activity and stoppage of sperm motility in all tested extenders (Figure 2).

Swimming duration of sperm

The swimming period of stimulated sperm varied in diverse osmolalities of NaCl solutions. At lower osmolality (48 mmol·kg⁻¹), sperm motility duration was also lower but the motility duration gradually increased and reached peak at 128

Table 1. Sperm characteristics of *Rita rita*.

Parameters	Minimum	Maximum	Mean±SD
Volume (µL·fish ⁻¹)	1,950	3,000	2518.46±331.22
Volume (µL·g ⁻¹ of fish)	2.78	3.00	2.87±0.08
Motility (%)	90	100	96.63±3.03
Concentration (cells·mL ⁻¹)	3.05×10^9	4.55×10^9	$3.89 \pm 0.55 \times 10^9$
pH	7.60	8.00	7.73±0.13

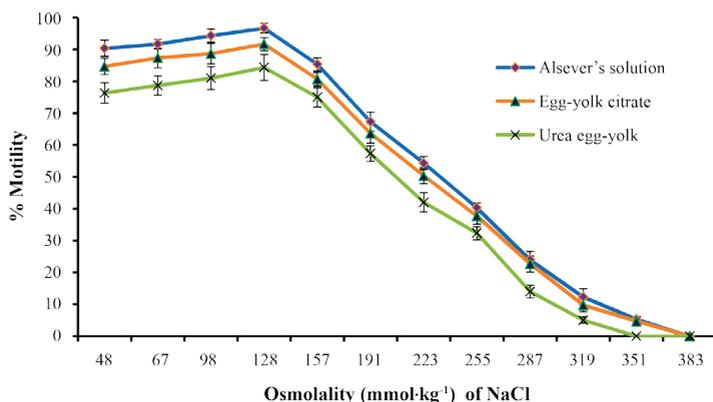


Figure 2. Sperm motility of *Rita rita* at different osmolalities of NaCl solution; Each spot represents mean value from three fish and error bar represents \pm SD.

mmol·kg⁻¹ and again declined with the increase of osmolalities of NaCl in all tested extenders. A considerable ($p = 0.000$) variation was observed in motility duration between 128 and 383 mmol·kg⁻¹ of NaCl for Alsever's solution and egg-yolk citrate; and between 128 and 351 mmol·kg⁻¹ for urea egg-yolk (Figure 3).

Observation-III: Estimation of cryoprotectants toxicity to sperm

Fresh sperm motility of *Rita* catfish prior to incubation by cryoprotectants ranged from 90 to 100% with a mean of $96.63 \pm 3.03\%$. Percent motility of sperm was not varied significantly ($p > 0.05$)

between 5 and 10% cryoprotectants; but it was significantly ($p < 0.05$) varied between 5 and 15%, and 10 and 15% of respective cryoprotectants in all cases (Figure 4, 5, and 6). Sperm motility declined significantly ($p < 0.05$) with the rising of incubation period (0–40 min) in all cases (Figure 4, 5, 6, and 7). Additionally, statistical analysis showed that Alsever's solution with 5% and 10% DMSO did not generate major variation ($p = 0.251$) in the percent motility within 10 min. Nevertheless, significant differences ($p = 0.000$) in motility were exhibited between 5% and 15%; and 10% and 15% of DMSO during 10 min incubation (Figure 4). Sperm motility was not varied significantly ($p > 0.05$) at 10% concentration of DMSO, methanol and ethanol with each extender (Figure 7).

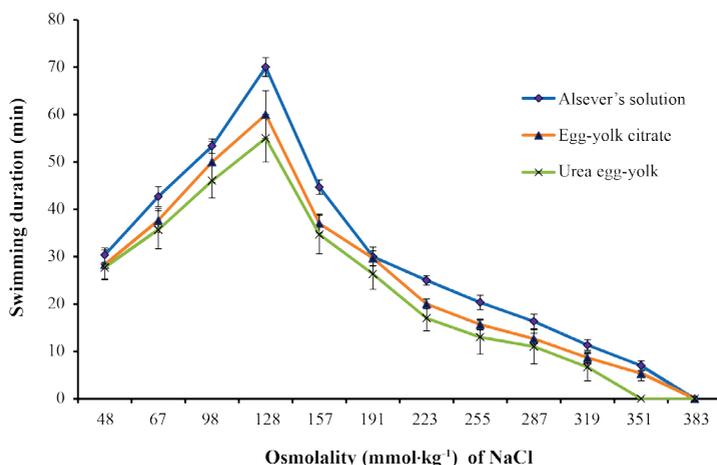


Figure 3. Sperm motility duration of *Rita rita* at various osmolalities of NaCl solution; Each spot represents mean value from three fish and error bar represents \pm SD.

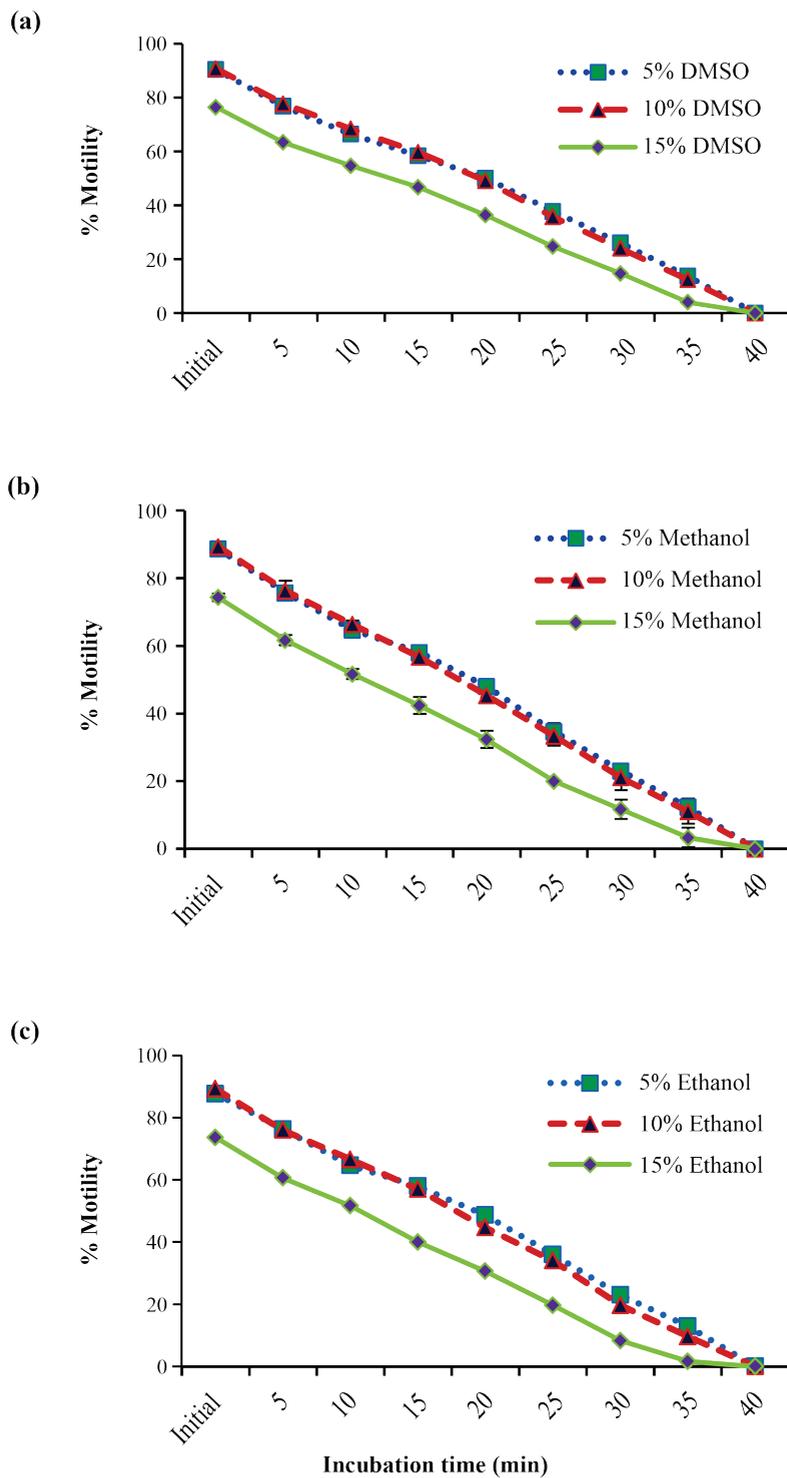


Figure 4. Sperm motility of *Rita rita* during 40 min incubation in three cryoprotectants, (a) DMSO; (b) Methanol; and (c) Ethanol, each at 5% (squares), 10% (triangles) and 15% (diamonds) concentrations along with Alsever's solution; Each spot represents mean value from three fish and error bar represents \pm SD.

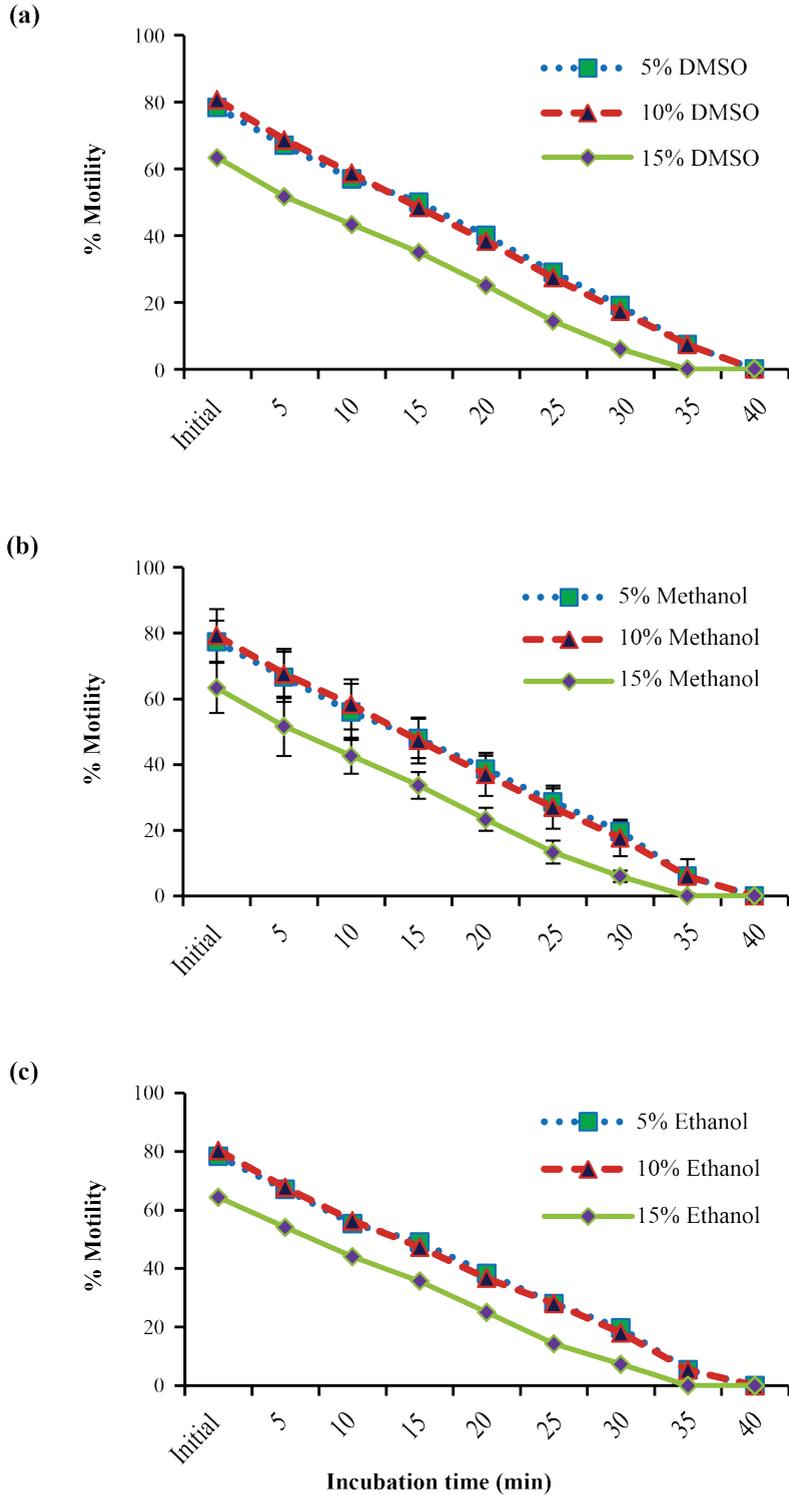


Figure 5. Sperm motility of *Rita rita* during 40 min incubation in three cryoprotectants, a) DMSO; (b) Methanol; and (c) Ethanol, each at 5% (squares), 10% (triangles) and 15% (diamonds) concentrations with Egg-yolk citrate; Each spot represents mean value from three fish and error bar represents \pm SD.

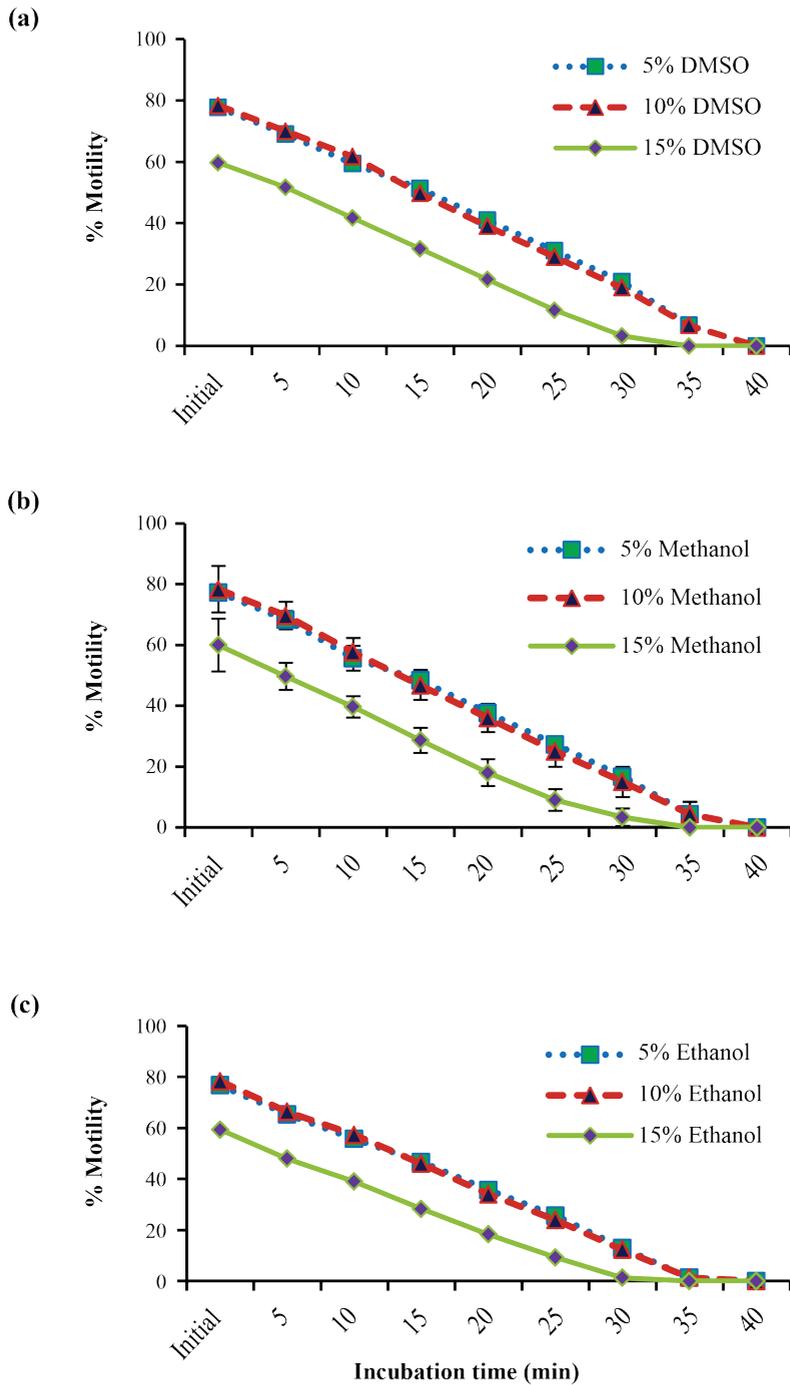


Figure 6. Sperm motility of *Rita rita* during 40 min incubation in three cryoprotectants, a) DMSO, (b) Methanol and (c) Ethanol, each at 5% (squares), 10% (triangles) and 15% (diamonds) concentrations along with Urea egg-yolk; Each spot represents mean value from three fish and error bar represents \pm SD.

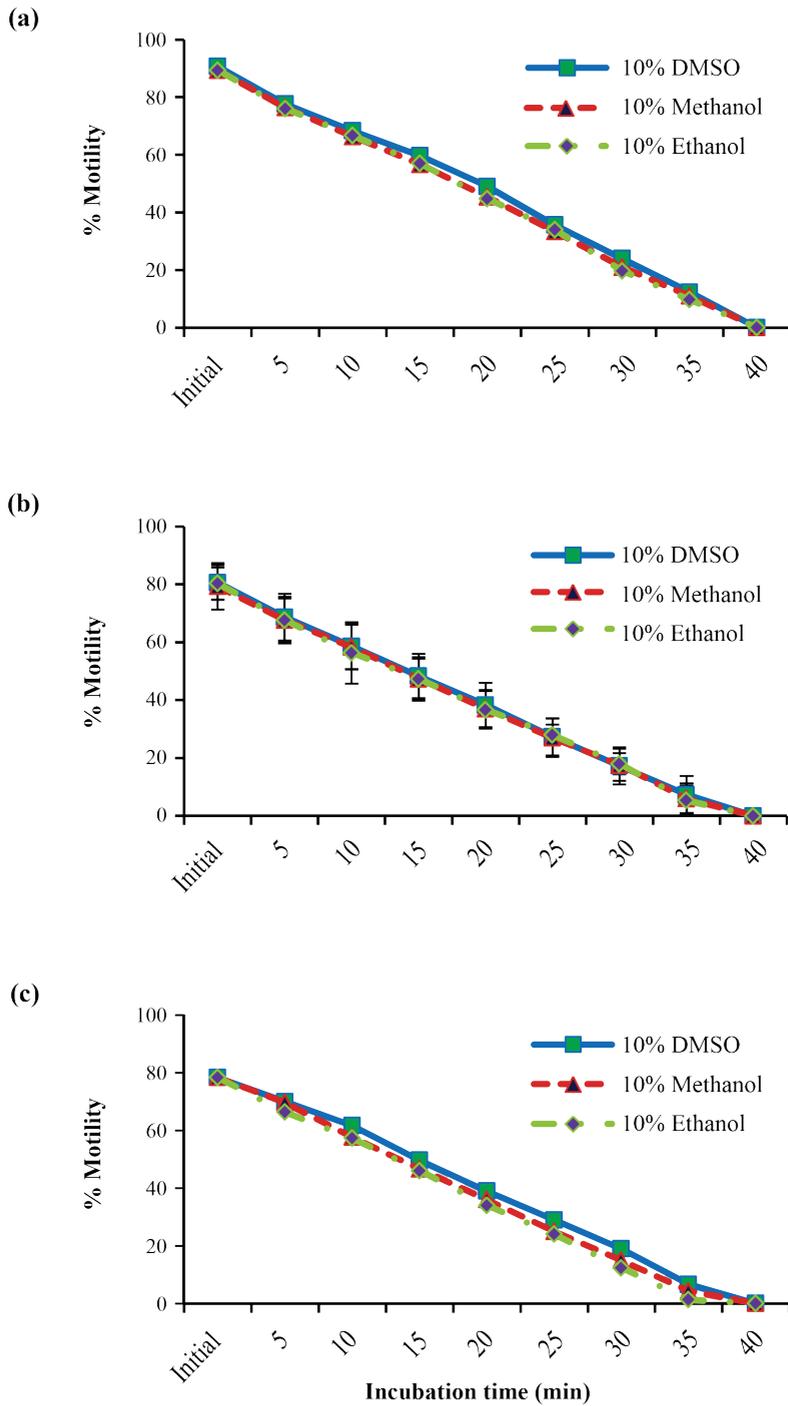


Figure 7. Sperm motility of *Rita rita* exposed to 10% cryoprotectants of DMSO (squares), Methanol (triangles) and Ethanol (diamonds) each with: (a) Alsever's solution; (b) egg-yolk citrate; and (c) urea egg-yolk; Each spot represents mean value from three fish and error bar represents \pm SD.

Observation-IV: Selection of suitable diluents

Alsever's solution and DMSO produced significantly ($p < 0.05$) highest equilibration motility ($89.67 \pm 5.51\%$) followed by Alsever's solution with methanol ($80.00 \pm 5.00\%$) and ethanol ($75.67 \pm 4.04\%$) (Table 2). Along with the three cryoprotectants, the equilibration motility of sperm in egg-yolk citrate (62.33 ± 2.52 to $73.67 \pm 4.04\%$) and urea egg-yolk (57.67 ± 2.52 to $65.33 \pm 4.51\%$) were lower than that with Alsever's solution (Table 2). Alsever's solution plus 10% DMSO had the highest performance ($p < 0.05$) of thawed sperm motility ($79.33 \pm 5.13\%$). Egg-yolk citrate and urea egg-yolk had comparatively poor performance in post-thaw motility ranging from 42.33 ± 2.52 to $66.00 \pm 4.48\%$ (Table 2). All diluent mixture showed significant influence

($p < 0.05$) on sperm motility during equilibration period and post-thawing phase. Among all the diluents, Alsever's solution plus 10% DMSO might be the best combination revealed by DMRT.

Observation-V: Post-thaw sperm motility estimation in short-term storage

Post-thaw sperm motility from the initial to sixth week cryostored period was considerably lower ($p < 0.05$) than that of the control (Table 3). Cryopreservation generated a significant ($p < 0.05$) drop in sperm motility from the initial (79.33 ± 5.13 to $45.67 \pm 1.15\%$) to the sixth week in all cases (Table 3). While Alsever's solution plus 10% DMSO showed noticeably ($p < 0.05$) a better sperm motility compared to other diluents (Table 3).

Table 2. Effects of various combinations of extenders and cryoprotectants on equilibration and post-thaw motility of spermatozoa of *Rita rita*.

Extender	Cryoprotectants (10%)	Dilution ratio	Equilibration motility (%)	Post-thaw motility (%)
Alsever's solution	DMSO	1:9	89.67 ± 5.51^a	79.33 ± 5.13^a
	Methanol		80.00 ± 5.00^b	70.00 ± 5.00^b
	Ethanol		75.67 ± 4.04^b	63.67 ± 4.04^b
Egg-yolk citrate	DMSO	1:4	73.67 ± 4.04^{bc}	66.00 ± 4.48^b
	Methanol		67.67 ± 2.52^{cd}	55.67 ± 4.04^c
	Ethanol		62.33 ± 2.52^{de}	50.33 ± 4.51^{cd}
Urea egg-yolk	DMSO	1:4	65.33 ± 4.51^d	54.33 ± 6.03^c
	Methanol		61.67 ± 3.51^{de}	50.33 ± 4.51^{cd}
	Ethanol		57.67 ± 2.52^e	42.33 ± 2.52^d

Note: Mean \pm SD ($n = 3$) in the same column having different superscripted letters are significantly ($p < 0.05$) different.

Table 3. Comparison between fresh and thawed sperm motility (%) in various diluents during cryostored periods. (milt samples diluted in 10% DMSO with the three extenders separately used in this experiment; Control = freshly collected milt+respective extender)

Cryostored duration (weeks)	Alsever's solution	Egg-yolk citrate	Urea egg-yolk
Control	96.33 ± 1.53^{a1}	94.00 ± 2.00^{a1}	92.67 ± 2.52^{a1}
Initial	79.33 ± 5.13^{b1}	66.00 ± 4.48^{b2}	54.33 ± 6.03^{b3}
1	77.33 ± 1.15^{bc1}	65.00 ± 1.00^{bc2}	53.00 ± 1.00^{bc3}
2	75.67 ± 1.15^{bcd1}	63.67 ± 1.53^{bcd2}	51.67 ± 1.15^{bcd3}
3	74.33 ± 2.08^{cde1}	63.00 ± 1.00^{bcde2}	50.33 ± 0.58^{bcd3}
4	73.00 ± 2.00^{cde1}	61.67 ± 1.53^{cde2}	49.00 ± 1.00^{cde3}
5	72.33 ± 1.53^{de1}	60.67 ± 1.15^{de2}	47.67 ± 1.15^{de3}
6	71.00 ± 2.00^{e1}	59.33 ± 1.15^{e2}	45.67 ± 1.15^{e3}

Note: Means with different superscripted letters in each column and different superscripted numbers in each row denoted significant ($p < 0.05$) differences.

DISCUSSION

This is the first attempt to cryopreserve the spermatozoa of the endangered and commercially important Rita catfish.

The general sperm quality of Rita catfish

Successful cryostoring of milt relies on sperm quality, evaluated through examining seminal fluid composition, semen volume, sperm motility and density (Rurangwa *et al.*, 2004). Noteworthy, motility is a vital function of sperm, by which they actively reach and enter the egg for internal and external fertilization (Islam and Akhter, 2011). The average motility of fresh spermatozoa of the Rita catfish was more than 95% and thus marked the good fertilizing capacity of the sperm. Spermiation of Rita catfish was very similar to that of European catfish *Silurus glanis* ($2.8 \mu\text{L}\cdot\text{g}^{-1}$) induced with the same hormone source (carp pituitary extract) (Linhart and Billard, 1994). Sperm concentration of Rita catfish in the present study fall within the range reported for *Clarias gariepinus* ($3.5\pm 1.8\times 10^9$ to $8.6\pm 2.0\times 10^9$ cells $\cdot\text{mL}^{-1}$) (Mansour *et al.*, 2004). Milt pH is concerned with the flagellar activity monitoring and the optimum pH varies with species of fish, similar to the seminal fluid (Viveiros, 2011). The pH value of Rita catfish milt was 7.73 ± 0.13 which was similar with that of *C. gariepinus* (pH = 7.7) (Hoysak and Liley, 2001). However, a fairly higher pH value has been reported as 8.0 in *Mystus nemurus* (Muchlisin *et al.*, 2004). It is wise to mention that several biological characteristics of brooders, for instance age, length, weight (Vuthiphandchai and Zohar, 1999; Hoysak and Liley, 2001), keeping environment (temperature, photoperiod, nourishment, undesirable components, and animal wellbeing and health) (Williot *et al.*, 2000), methods and hormone used in induced breeding, breeding season (Linhart and Billard, 1994; Hajirezaee *et al.*, 2010) contribute to the variation in the quality of sperm.

Selection of suitable activation solution for sperm of Rita catfish

Fish spermatozoa cryostorage involves with activation motility which is the unique criterion of sperm. Sperm motility is affected by a number

of factors including pH, temperature, ions and osmolality (Cosson, 2004; Alavi and Cosson, 2006) which guide to activation of axonemal movement. Commonly, spermatozoa of freshwater fish become active in hypotonic fluid and of marine fish in hypertonic fluid (Cosson, 2004; Tiersch *et al.*, 2004). In this investigation, activation motility of Rita catfish spermatozoa was evaluated in 0.1% to 1.2% NaCl solutions in three extenders (Alsever's solution, egg-yolk citrate and urea egg-yolk) at diverse osmolality of 48 to 383 mmol $\cdot\text{kg}^{-1}$. The motility of sperm increased up to 128 mmol $\cdot\text{kg}^{-1}$ and became stable i.e., complete activation of sperm occurred at this osmolality, which gradually declined with the rise of osmolality and entirely ceased at 351 mmol $\cdot\text{kg}^{-1}$ in urea egg-yolk and at 383 mmol $\cdot\text{kg}^{-1}$ in Alsever's solution and egg-yolk citrate. A similar motility pattern of sperm was also reported in the endangered Pabda catfish *Ompok pabda* using the same extending media (Sarder *et al.*, 2013). Seminal fluid osmotic pressure of freshwater fish is nearly 230 to 346 mmol $\cdot\text{kg}^{-1}$ (Alavi and Cosson, 2005). Therefore, the osmolality of the extending medium is supposed to have near that of seminal plasma (Mongkonpunya *et al.*, 1992). The percentage of motility of Rita catfish sperm was comparatively higher in Alsever's solution than urea egg-yolk and egg-yolk citrate in all cases. It might be owing to matching the pH value of Alsever's solution (7.8) with Rita catfish sperm (7.73 ± 0.13), while pH was a bit lower in urea egg-yolk (6.1) and egg-yolk citrate (6.5). The low pH may be accounted for causing low motility of the sperm (Alavi and Cosson, 2005). This would confirm the secured preservation of spermatozoa and its utilization in induced spawning. In this study, the sperm motility persisted higher duration in low osmotic pressure (48 to 128 mmol $\cdot\text{kg}^{-1}$), from 27.67 ± 2.52 to 70.00 ± 2.00 min, which steadily declined by the enhancement of osmotic pressure of the extending media and became stopped at 351 mmol $\cdot\text{kg}^{-1}$ in presence of urea egg-yolk and at 383 mmol $\cdot\text{kg}^{-1}$ in case of Alsever's solution and egg-yolk citrate. A similar sperm motility period was observed with the same extenders in endangered Meni *Nandus nandus* (Sarder *et al.*, 2012). In this investigation, the swimming period was significantly ($p<0.05$) longest at higher pH contained by Alsever's solution (pH 7.8). The swimming duration of sperm

was varied with the pH of extending media and the highest sperm swimming period was recorded at pH 8.0 in the examining series from 6.0 to 9.0 in Persian sturgeon (*Acipenser persicus*) (Alavi *et al.*, 2004). The motility action of sperm in extending media was recorded in a good number of fish where the span of motility differed with species (Alavi and Cosson, 2005; 2006). The motility capability of sperm for a more duration in extending media provides a chance to make a better egg-sperm interaction which amplifies the opportunity for a higher fertilization rate.

Cryoprotectants toxicity to sperm of Rita catfish

Cryoprotectants have the capacity to defend the spermatozoa at cold and warming shock dealings and avoid cellular dryness throughout cooling, freezing and thawing or from cryogenic injury (Muchlisin, 2005; Yang *et al.*, 2007). So, a cryoprotectant is required for protection; conversely, it is toxic to the sperm. The suitability of cryoprotectants for sperm cryopreservation relies on the fish species and the toxicity tolerance degree to cryoprotectant concentration. Generally cryoprotectants utilized from the previous times to guard catfish spermatozoa are dimethyl sulfoxide (DMSO), dimethyl acetamide (DMA), methanol, ethanol, and glycerol typically at 10% (v:v), ranging 5 to 15% (Muchlisin *et al.*, 2004; Viveiros, 2011). During toxicity assay, the consequences of cryoprotectants, DMSO, methanol and ethanol on spermatozoa motility were accounted. Five and 10% concentration provided improved motility in 5 and 10 min exposure for nearly all cases and 15% concentration appeared lethal. Moreover 5% concentration created equivalent outcomes to 10% cryoprotectants, but better thawing motility were observed at 10% cryoprotectant. The findings corroborated with that of endangered *O. pabda* (Sarder *et al.*, 2013). On the basis of toxicity tests, 10% cryoprotectants were chosen in the following freezing investigations.

Selection of suitable diluents for sperm of Rita catfish

The fundamental aim of preserving sperm is to minimize motility of sperm throughout storage,

generating high post-thaw motility; it is attained by using accurate extenders and cryoprotectants combination. The fitness of diluent varies with fish species (Muchlisin, 2005). So, the selection of an appropriate mixture of extender and cryoprotectant is a vital part of the fruitful preservation of sperm. The cryoprotectants used in this experiment generated acceptable ranks of sperm motility in the selected extenders during equilibration, though the thawing motility was significantly declined in several events. Alsever's solution including DMSO presented radically ($p < 0.05$) greatest equilibrium ($89.67 \pm 5.51\%$) and thawing ($79.33 \pm 5.13\%$) motility than other combinations. Additionally, DMSO was used as a perfect preference of cryoprotectant owing to its fast access to cells and comparatively less toxic at low temperatures (Leung and Jamieson, 1991). The outcomes confirmed that 10% DMSO was the finest selection of cryoprotectant as in *C. gariepinus* (Hoysak and Liley, 2001) and *S. glanis* (Linhart *et al.*, 2005). Sodium-citrate portion in Alsever's solution related to the membrane of cell may furnish defense against damage throughout cryopreservation and the dextrose present in Alsever's solution can give more cryoprotection (Bozkurt *et al.*, 2009). The sperm motility of catfish differed among extenders and mixing rates. The superior sperm motility was observed in Alsever's solution at 1:9 than at 1:4 ratios in egg-yolk citrate and urea egg-yolk. Similar results were observed in Meni fish (Sarder *et al.*, 2012), Pabda catfish (Sarder *et al.*, 2013), and Asian dwarf catfish (Sarder *et al.*, 2017). Many experiments have been conducted on milt-diluent ratios and the results varied among fish species with different extenders (Ritar and Campet, 2000; Muchlisin *et al.*, 2004).

Freezing protocol

Sperm are very much sensitive to cryoinjuries that commonly occurred in pre-freezing and post-thawing between 0 and $-40\text{ }^{\circ}\text{C}$ (Muchlisin, 2005). In the present study, single-step cooling procedure from $4\text{ }^{\circ}\text{C}$ to $-80\text{ }^{\circ}\text{C}$ at $10\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$ was applied which was also supported by several previous studies on *P. bocourti* (Kainin *et al.*, 2014) and *M. vittatus* (Sarder *et al.*, 2017). The optimal freezing temperature was declined precisely at 5 to $11\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$. In major freezing protocols, catfish

sperm usually cooled to a low temperature prior to plunging it into LN₂ such as -80 °C in Channel catfish (Tiersch *et al.*, 1994) and African catfish (Horváth and Urbányi, 2000). In the current study, the thawing temperature was used as 25–26 °C and it differed from the thawing temperature (25–40 °C) of the above-mentioned studies especially the upper limit of the temperature.

Sperm quality during cryogenic storage

This investigation exposed that the post-thaw sperm quality of the endangered Rita catfish had high motility after six weeks of cryostorage in all three extenders combined with 10% DMSO. Considerably ($p < 0.05$) superior thawed sperm motility was recorded in Alsever's solution over egg-yolk citrate and urea egg-yolk throughout the storage period. It might be happened due to the similar pH value of milt (7.73 ± 0.13) and the Alsever's solution (7.8). Conversely, a gradual decline (significant, $p < 0.05$ in many cases) in thawing motility was observed in all diluents with the progress of storage time. It is a normal phenomenon but owing to the recurrent opening of the LN₂ Dewar (shortage of storage Dewars as they are used for other research) might accelerate the declining rate. Several studies on catfish sperm cryopreservation were conducted to develop the protocol, and fertilizing and hatching capability of the stored frozen sperm were tested within a week of storage but the post-thaw motility, and their quality were not affected (Linhart *et al.*, 2005; Sarder *et al.*, 2013; Islam *et al.*, 2016). Variation in sperm motility of *P. sutchi* was significant ($p < 0.05$) after 20 days at -196 °C and 30 days at -20 °C storage (Rani *et al.*, 2016). The motility of sperm decreased remarkably in cryopreserved sperm of Channel catfish after two weeks as well as 22 weeks (Tiersch *et al.*, 1994) of storage. In African catfish, reduced sperm motility has been reported after 10 months of preservation in 11% DMSO (Rurangwa *et al.*, 2001).

CONCLUSION

It was the first attempt to develop the sperm cryopreservation protocol for the endangered Rita catfish. The basic characteristics of sperm, for instance, milt volume, concentration, pH and sperm motility were investigated and found within acceptable ranges as observed in other catfish species. Alsever's solution with 10% DMSO was found as potential diluent for cryopreservation of sperm of the threatened catfish. However, further research needs to be conducted for assessing the capability of cryopreserved sperm through fertilization of eggs.

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