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Evaluation of toxicity of cryoprotectant to the sperm of mohashol fish, *Tor tor* (Hamilton) at various concentrations of the salt solution

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Abstract
Cryogenic gene banking is a prime necessity for conserving the genetic constitutes of vulnerable, endangered and critically endangered native and exotic fish species. Cryogenic freezing of fish gametes is the first step to develop such a gene bank. In the study, attempts have been taken to develop a cryogenic freezing protocol of spermatozoa of Mohashol, *Tor tor* as it is considered as a critically endangered fish species by IUCN. Juvenile and brood fish of *Tor tor* were collected from the Someshwari River of Netrokona district and domesticated in captive condition at Bangladesh Agricultural University campus. During breeding season, sperms were collected from hormone induced males by stripping. The milt was very sticky. The concentration of sperm was $4.47x10^{10}$ cells per ml and pH was 8.6±0.5. To evaluate the toxicity of cryoprotectant, sperm was incubated with DMSO and methanol at 5, 10 and 15% concentrations for a period of 5 to 55 min. The motility of sperm decreased with the increase of cryoprotectant concentration (5, 10 and 15%) and 10% DMSO with Alsever's solution could be the best combination for cryopreservation of *T. tor*. sperm at 10 min (83.0±2.74).

Keywords: toxicity, sperm motility, cryoprotectant

1. Introduction
Bangladesh is enriched with numerous rivers, flood plains, low lands, haor, baor (ox-bow lakes), beels, lakes etc. The country is also enriched with endemic fish resources including 260 freshwater fishes and 24 prawn species, 16 exotic freshwater species; 475 marine fishes and 24 shrimp species (DoF 2016) [7]. Among 260 freshwater fish species of the country, 12 have been categorized as critically endangered, 28 as endangered and 14 as vulnerable (IUCN Bangladesh 2015) [11] and stocks of some fish species have been depleted to below replaceable levels.

Conservation programs require large populations to ensure biodiversity but for threatened and endangered species the numbers of fish are steadily decreasing. Cryopreservation can help in both of these situations. Cryopreservation is a process by which biological cells or tissues are preserved at subzero temperatures resulting in a radical decrease in the rate of metabolic processes and the ability to store samples for extended periods (Armitage 1987) [1]. The availability of frozen sperm is a proven technique for developing, maintaining, and distributing genetic improvement in livestock, and provides great unexploited potential for fish breeding.

Fig 1: Mohashol, *Tor tor* (Hamilton 1822)

Mohashol *(Tor tor)* (Figure 1) has unique taste and deserves special mentioning. The abundance of *T. tor* has decreased to such an extent that very seldom they are reported to be caught in the region of Netrokona and Sunamganj districts. And as such not many people are lucky enough to taste the delicious flesh of *T. tor*. The mohashol (*T. tor*) including several other species of *Tor* have also been recognized as endangered fish in India, Pakistan, Nepal and Bhutan, in Southern Asia, Afghanistan, Thailand and Malaysia (Desai 2003, Rahman *et al*. 2005, Sharma 2003, Gurung *et al.* 2002, Bazaz and Keshavanath 1993) [6, 15, 17, 8, 4]. Several attempts have been made to conserve the fish in different countries (Patil and Lakra 2005, Nguyen *et al.* 2006, Ingram *et al*. 2005, 2007) [14, 13, 9, 10] but in Bangladesh research work have yet been done.

2. Materials and Methods

The cryopreservation of Mohashol (*Tor tor*) spermatozoa at liquid nitrogen temperature (-196 ºC) by standardizing the suitable combination of extender and cryoprotectant, optimal milt-diluent ratio and cryoprotectant concentration. The duration of the experimental period was February to May 2016. The materials and methods are described under the following headings:

2.1 Experimental fish

To conduct this study, the collection of fish was the first step. The experiments were conducted in the Laboratory of the Department of Fisheries Biology and Genetics under the Faculty of Fisheries, Bangladesh Agricultural University (BAU), Mymensingh. One hundred and fifty fish of *Tor tor* were collected from wild sources (collection place) and maintained in circular tank with the provision of continuous water supply and aeration for conditioning for 6 h. Two rectangular earthen ponds each of 30.0 decimal area, having inlet and outlet facilities were used for stocking of fish. After conditioning *Tor tor* were stocked in a density of 2, 0000 kg/ha and reared in ponds for domestication. The ponds were dried and exposed to the sunlight for better mineralization, escape of toxic gases and to make free from aquatic weeds, harmful aquatic insects, predatory and weed fishes. Lime was used at the rate of 1kg per decimal. Then the pond was filled with water and after seven days, urea and triple super phosphate (TSP) were applied at the rate of 200g and 100g per decimal respectively. In pond, the fish were reared with the commercial supplementary feed having 30% protein at the rate of 2 to 6% of body weight up to maturation. The fish were fed two times a day at 09.00 and 17.00 h and the amount of feed were adjusted on the basis of fish weight. Broods of Mohashol were reared in ponds in the vicinity of Fisheries Faculty premises.

2.2 Collection of fish gametes

2.2.1 Selection of mature male fish

Mature males were selected on the basis of their desired phenotypic characteristics mainly genital papilla. Before selecting male fish, a gentle pressure was applied on abdomen to remove some milt, which indicated the maturity of fish. Mature male broods were collected from the stock ponds for getting quality spermatozoa.

2.2.2 Conditioning of brood fish

Selected male fishes were brought to the circular tank from the pond for conditioning before 6h of hormone treatment. During the period of conditioning, no feed was supplied to them. This was done for more effectiveness of hormone and to keep the fish in better condition after collection of milt.

2.2.3 Inducing with hormone injection

The brood fishes were induced by pituitary gland (PG) extract. Male broods were induced so that sufficient amount of milt was collected easily at a time.

2.2.4 Preparation of inducing agents

Dry carp pituitary glands (PG, available in the market) were used to induce the fish. The required amount of PG as per dose was weighed, homogenized in a tissue homogenizer with about 1.5 ml of distilled water. The suspension was centrifuged for 5 minutes at 3000 rpm. The supernatant was poured in a cubate and diluted with distilled water to obtain the desired quantity for injection. Then the solution was loaded into a 3 ml hypodermic syringe for injection.

2.2.5 Collection of sperm and checking their motility

Firstly, excess moisture, urine, gut extrudes and mucus was wiped from the genital area of male *Tor tor* with absorbent paper. Gentle pressure was applied (Figure 2) on abdomen from anterior to posterior direction before collecting milt to remove some milt for avoiding contamination with urine and water. When the milt was concentrated and whitish in colour, 5 ml-sized glass tubes were held against the tip of the genital papillae and sperm were collected into the tubes and weighed. Watery or bloody milt and residues were discarded and tubes were weighed again to measure the actual quantity of milt. The collected milt was stored on ice to prevent quality deterioration during further processing. The quality of sperm in each tube was checked under the microscope taking about 1-2 µl of milt on a glass slide. Samples containing more than 80% motile cells by eye-observation under microscope were used for cryopreservation.

Fig 2: Collection of milt from *Tor tor* male

2.3 Evaluation of the toxicity of cryoprotectants and determination of suitable cryoprotectant concentration to the extender

For determining the toxicity of cryoprotectant, a series of concentrations of cryoprotectant were tested. Milt was diluted at a ratio of 1:4 for urea-egg-yolk and egg-yolk-citrate, 1:9 for Alsever's solution. The cryoprotectants (DMSO and methanol) were mixed with the milt to make the final concentration of 5, 10 and 15%. The mixture was kept on ice. The toxicity of these cryoprotectants was measured by monitoring the motility of sperm under microscope at different incubation times of 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 and 55 min. The toxicity was evaluated following the method of Yang *et al*. (2007) [19].

2.4 Statistical analyses
Data was presented as percentage of motile cells and all per cent motility values were converted to arcsine transformation before statistical analysis. Data was analyzed using Independent-samples T test of Statistical Package for the Social Science (SPSS v 16) and the means were separated by Least Significant Difference (LSD) at 5% probability level.

3. Results
The sperm was collected from *Tor tor* in a 1.5 ml eppendorf tube. During experiment mobility of sperm was examined all the time under microscope. Mobility of sperm can be categorized into two types: forward movement and Brownian movement. About 80-90% forward movement and 10-20% Brownian movement was recorded from the sample with different activation solution (0.1-1.0% NaCl). The number of spermatozoa per ml ranged from $4.47x10^{10}$ to $9.65x10^{10}$. The motility of fresh sperm of *T. tor* before incubation with cryoprotectants was 92.0±2.74%. The motility of sperm decreased with the increase of cryoprotectant (DMSO and methanol) concentration (5%, 10% and 15%). Sperm suspended with Alsever's solution showed 78±5.70% motility at 5% DMSO during 10 min incubation which completely inhibited at 30 min. At 10% DMSO, sperm motility was recorded as 83.0±2.74% during 10 min incubation and it completely inhibited at 45 min. (Figure 3). Statistical analysis showed that in association with Alsever's solution, 5% and 10% DMSO did not produce any significant difference (P=0.115) between them during 10 min incubation. However, significant differences (P<0.001) in sperm motility were observed between 5% and 15% and 10% and 15% of DMSO at 10 min incubation.

When methanol was used, comparatively lower motility was obtained. The motility was totally inhibited during 15 min incubation at 5% concentration of methanol, while 22±5.70% motility was recorded from 10% methanol at 10 min incubation and total inhibition at 15 min of incubation (Figure 4). In presence of Alsever's solution, significant differences (P=0.003), (P=0.001), (P<0.001) in motility were observed between 5% and 10%, 5% and 15%, 10% and 15% methanol during 10 min of incubation respectively.

The combination of egg-yolk citrate with 5% and 10% DMSO produced 32±7.58% and 33±2.74% motility at 10 min of incubation respectively. At 15% DMSO, sperm showed very poor motility from the very beginning and completely stopped at 10 min (Figure 5). When methanol was used, 15% concentration showed very poor result but its 5 and 10% concentration produced comparatively better motility (Fig. 6). In association with egg-yolk citrate solution, 5% and 10% DMSO showed insignificant differences (P<0.789) between them, but significant differences (P<0.001) were observed between 5% and 15% and 10% and 15% DMSO during 10 min incubation In case of methanol, sperm motility between concentrations of 5% and 10%, 5% and 15% and 10% and 15% were significantly different (P<0.001) during 10 min of incubation.

In case of urea-egg-yolk, 5% DMSO produced 39.0±9.62% motility at 10 min incubation which was stopped at 20 min incubation. At 10% DMSO sperm had 45.0±10.61% motility at 10 min incubation which gradually reduced to 2.0% at 25 min incubation. 15% DMSO produced poor result from the beginning of incubation and only 13.0±4.47% motility was observed during 10 min of incubation which was inhibited at 15 min incubation (Figure 7). In presence of urea-egg-yolk solution, sperm motility in DMSO concentration between 5% and 15%; and 10% and 15% showed significant differences (P=0.036) and (P<0.001) respectively during 10 min of incubation. Sperm motility at concentration between 5% and 10% was not significantly different (P=0.220). When methanol was used along with urea-egg-yolk, no motility was observed at 10 min incubation (Figure 8).

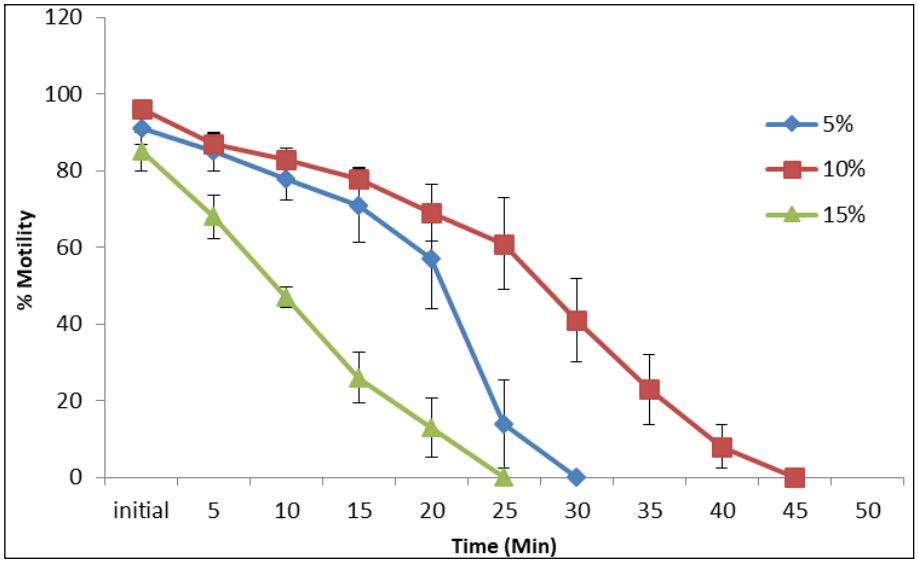

Fig 3: The motility of sperm of *Tor tor* suspended in Alsever's solution with DMSO at 5, 10 and 15% concentration

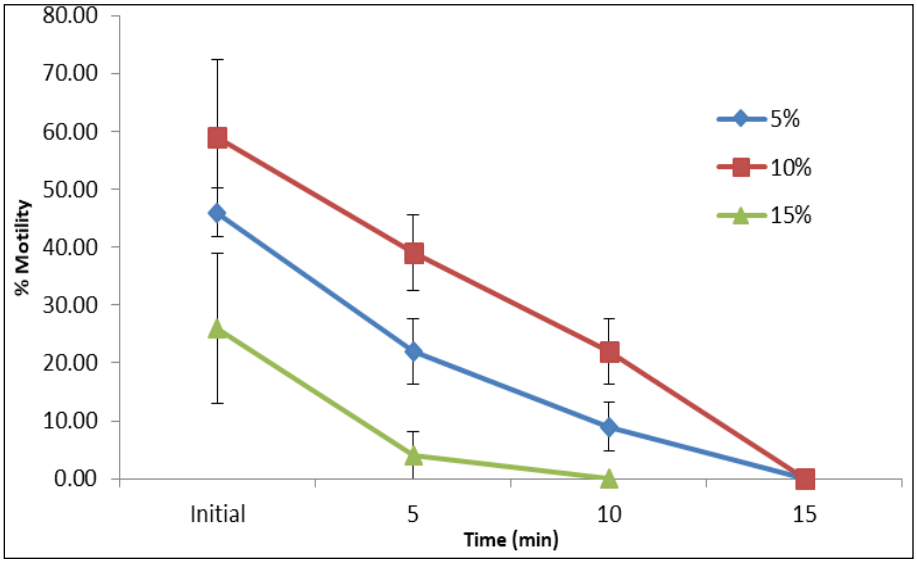

Fig 4: The motility of sperm of *Tor tor* suspended in Alsever's solution with methanol at 5, 10 and 15% concentration

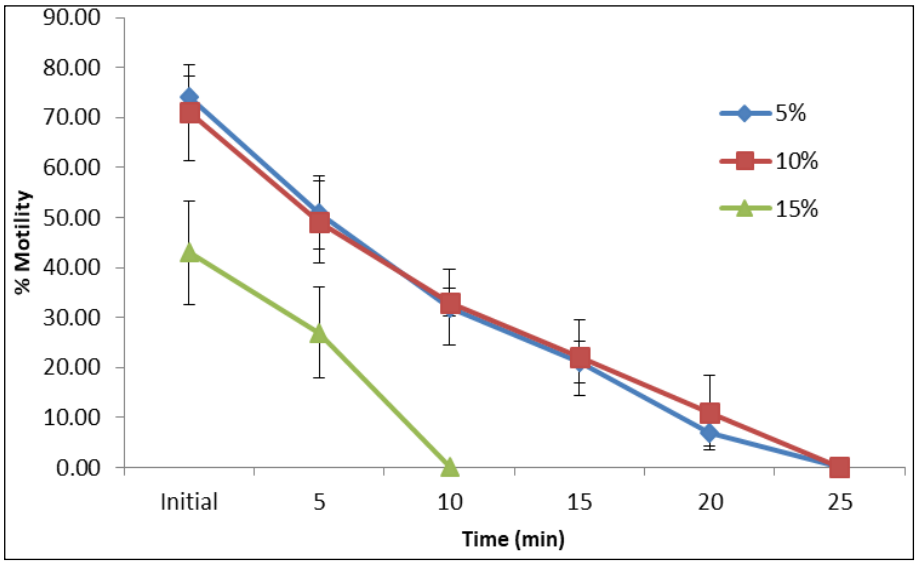

Fig 5: The motility of sperm of *Tor tor* suspended in egg-yolk citrate solution with DMSO at 5, 10 and 15% concentration

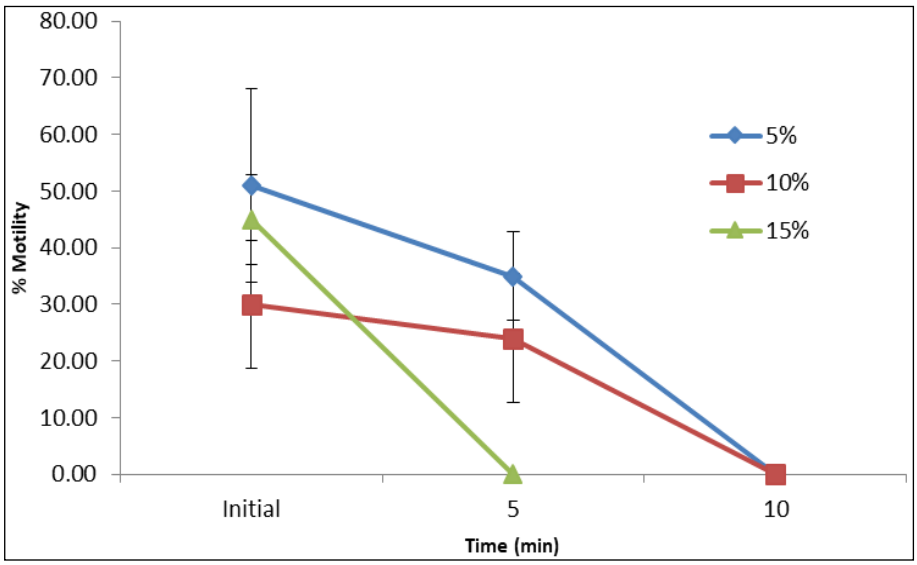

Fig 6: The motility of sperm of *Tor tor* suspended in egg-yolk citrate solution with methanol at 5, 10 and 15% concentration

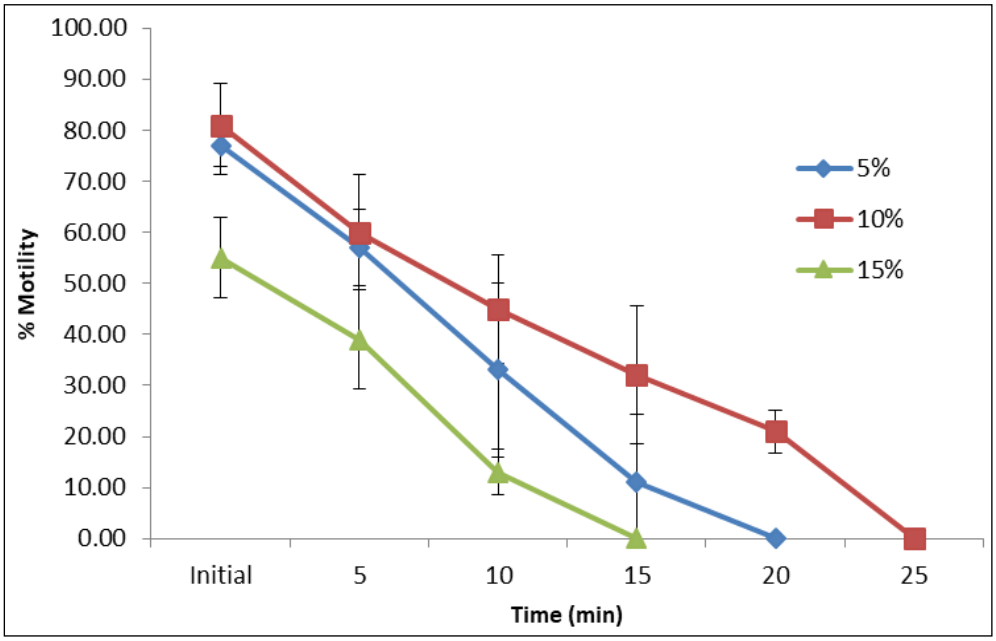

Fig 7: The motility of sperm of *Tor tor* suspended in urea egg-yolk citrate solution with DMSO at 5, 10 and 15% concentration

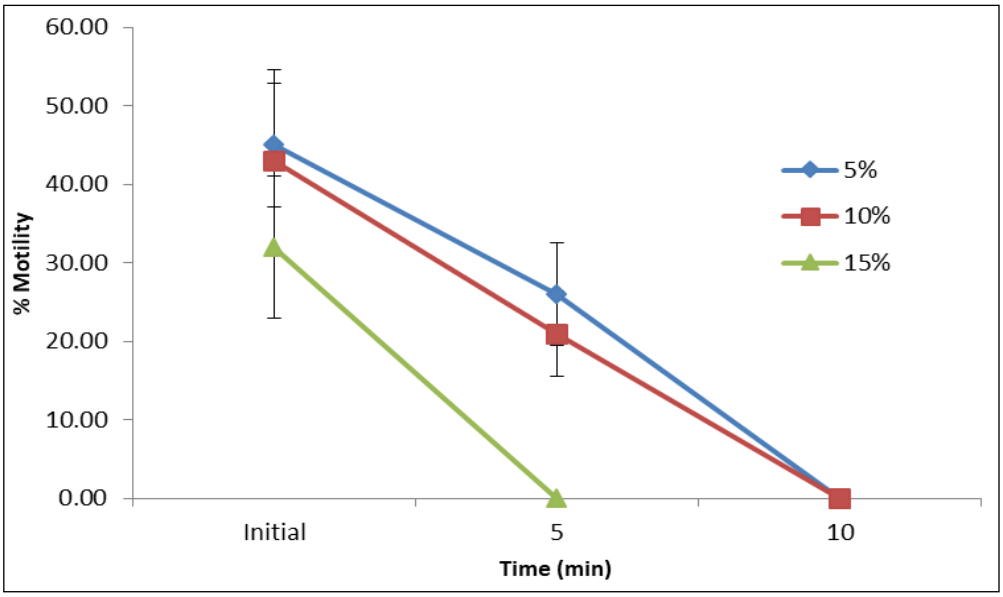

Fig 8: The motility of sperm of *Tor tor* suspended in urea egg-yolk citrate solution with methanol at 5, 10 and 15% concentration

4. Discussions

Studies on selection of suitable cryoprotectants and extenders are important as spermatozoa are very sensitive cells and are easily affected by diluents. Cryoprotectant is added to extender to minimize the stress on cells during cooling and freezing and also protect the cells from cold-shock through external crystal formation or cryoinjury. The efficacy of the cryopreservation is greatly increased if the pre-frozen milt is diluted with suitable diluent (extender + cryoprotectant). Although cryoprotectant is essential for preservation it is dissimilarly toxic to the cell. There is a difference in permeability of cryoprotectants to cells and the toxicity tolerance level of cells also depends on cryoprotectant concentration. So, concentration of cryoprotectant needs to be optimized.

Cryoprotectant (DMSO and methanol) concentration was maintained at 5, 10 and 15% (v/v) by volume in the present trials as it was reportedly common and effective for many species (Withler and Lim 1982; Shirohara *et al*. 1982) [19, 18]. The choice of the cryoprotectants was made on the basis of earlier study conducted by Yang *et al.* (2007) [20] on zebrafish. DMSO and methanol were used with different concentrations and the sperm suspension was incubated with the cryoprotectants for 5 to 50 min. Alsever's solution plus DMSO at 10% concentrations produced best motility during 5 and 10 min incubation in most cases, but15% concentration produced comparatively poor results. On the other hand, Alsever's solution plus methanol at 10% concentration produced better motility compared to 5% and 15% in most cases, but in case of egg-yolk citrate solution methanol at 5% produced better results. Chew *et al.* (2010) [5] obtained good results from 10% DMSO during preservation of Malaysian Mahseer (*Tor* spp.) sperm. Basavaraja *et al.* (2006) [3] also reported that DMSO at 10% resulted in higher post-thaw spermatozoa motility (46.7%) than 5% or 15% (up to 33.3%) after 385 days of cryopreservation for Mahseer (*Tor khudree*). In cryopreservation of common carp (*Cyprinus carpio*) sperm best motility was obtained when DMSO, methanol, ethanol and DMA were used at 10% concentration (Sarder *et al.* 2009) [16]. Similarly, Armitage (1987) [1]

obtained good results from Alsever's solution with 10% DMSO during preservation of silver carp (*Hypophthalmichthys molitrix*) sperm. Sperm of Olive barb (*Puntius sarana*) incubated with 5% DMSO and methanol produced longer motility and 15% concentration had an acute toxicity (Nahiduzzaman *et al.* 2011) [12]. In case of marine fish glycerol has been proved as an effective cryoprotectant while DMSO is accepted for cryopreservation of sperm of freshwater fishes (Yu *et al.* 2004) [21]. Bibiak *et al.* (1999) [2] reported 77.3% fertilization from northern pike sperm cryopreserved with 15% DMSO.

In the present study, methanol showed good performance at 5% concentration along with urea-egg yolk solution; and at 10% concentration with egg-yolk citrate solution but both of their performances were poor than DMSO. Sperm was motile up to 50 min in Alsever's solution plus 10% DMSO whereas motility existed for 10 min in methanol. Like DMSO, methanol also showed decreased motility with the increase of concentration (Zhang and Rawson 1995) [22]. However, the present study concluded that the motility of sperm decreased with the increase of cryoprotectant concentration (5, 10 and 15%) and suggested that 10% DMSO with Alsever's solution could be the best combination for cryopreservation of *T. tor*.

5. Conclusions

In this experiment the toxicity of cryoprotectants to sperm was tested. Two cryoprotectants (DMSO and methanol) were used with 5, 10 and 15% concentrations and the sperm suspension was incubated with them for 5-55 min. The motility of sperm decreased with the increasing concentration of cryoprotectant and with incubation time for DMSO and methanol. In most of the cases the motility of sperm decreased with the increase of cryoprotectant concentration (5, 10 and 15%) and suggested that 10% DMSO with Alsever's solution could be the best combination for cryopreservation of T. tor.

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