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Fertility of cryopreserved common carp (*Cyprinus carpio*) spermatozoa

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Abstract: The study was aimed at standardization of cryopreservation protocol for the common carp, (*Cyprinus carpio*) spermatozoa for using in the artificial propagation. Three extenders (Alsever's solution, egg-yolk citrate and 0.9% NaCl) were combined with three cryoprotectants (ethanol, methanol and DMSO). Two-step freezing protocol (5^{o}C to -4°C at a rate of 4°C per minute then -4°C to -80°C at a rate of 10°C per minute and finally held for 10 min) was carried out in a computer-controlled freezer where 0.25 ml straws containing spermatozoa were stored in a liquid nitrogen container at -196^{o}C. Alsever's solution with ethanol, Alsever's solution with methanol and egg yolk citrate with DMSO gave better motility (%) in both pre-freeze (93.33±1.05, 90±1.83 and 88.33±2.11, respectively) and post-thaw (80±4.28, 74.17±3.96 and 76.67±4.59, respectively) periods whereas, 10% cryoprotectant gave the best motility during post-thaw period. Considering both pre-freeze and post-thaw motilities of the common carp spermatozoa, 1:9 was the best ratio for the milt and cryodiluents. The egg yolk citrate with DMSO yielded the best fertilization (37.67±1.76%) and hatching (28.67±1.85%) compared to other combinations under study.

Key words: *Cyprinus carpio*, spermatozoa, cryopreservation, cryoprotectant, cryodiluent, fertility.

Introduction

The common carp (*Cyprinus carpio* Linnaeus, 1758) belongs to Cyprinidae, the largest freshwater teleost family (Nelson, 1994), and most extensively cultured fish species in the world. It is one of the most important fish species in global aquaculture sharing 13% (3.4 million tonnes) of the total global freshwater aquaculture production (FAO, 2004). The fish has acclimatized to a wide range of habitats and environmental conditions and therefore enjoys a world-wide distribution. As many as 15 exotic fishes (Ali, 1998) have been introduced into Bangladesh, of which the common carp is the most important one and is being extensively cultured throughout the country. To keep the production cost to a minimum, hatchery owners in Bangladesh maintain limited number of broods to minimize effective breeding numbers. As a result, the quality and the growth performance of fry produced in hatcheries are deteriorated due to inbreeding, genetic drift and bottleneck effects. Therefore, the maintenance of the genetic biodiversity of indigenous as well as exotic fish species is currently a challenge to increase the fish production (Ormerod, 2003).

Successful cryopreservation of fish sperm in 200 species has been reported around the world (Tiersch, 2000). The technique may be used to increase the number of offspring from genetically superior males and also in the effective transportation of semen and for a year-round supply of male gametes. Furthermore, cryopreservation of fish sperm can increase the economic utilization of males and is a prerequisite for the establishment of gene banks (Munkittrick and Moccia, 1984).

In Bangladesh crypreservation protocols for a number of aquacultured species have been developed (Rafiquzzaman, 2004; Sarder, 2004). Salam (2005) developed a cryopreservation protocol for common carp sperm in Bangladesh but with a very poor hatching rate. The protocol, therefore, needs to be standardized for a better success rate that depends on the post-thaw motility, fertilization and hatching improvement. The present study was conducted to standardize the cryopreservation protocol for *C. carpio* spermatozoa emphasizing their fertilizing and hatching ability.

Materials and Methods

Husbandry of broodstock: Mature males and females of C. carpio were stocked and reared in the ponds of Fisheries Field Laboratory Complex, Bangladesh Agricultural University, Mymensingh. Brood stocks were fed twice a day at 5% of their total body weight.

Milt collection: Selected male fishes were brought into the cistern from the pond for conditioning before 6 hrs of hormone treatment. Then the broods were injected with pituitary gland (PG) extract at the rate of 2 mg PG/kg body weight for easy collection of milt samples. Milt was collected in vials discarding watery or bloody portion and stored immediately in

ice. The quality of milt was checked by observing the motility percentage under light microscope (Novex K-range, Holland) at 400×.

Extenders and cryoprotectants: Three extenders viz. Alsever's solution, egg-yolk citrate and 0.9% NaCl in combination with three cryoprotectants: ethanol, methanol and dimethylsulphoxide (DMSO), were used for cryopreservation of spermatozoa. Milt was diluted with extenders and cryoprotectant was added to prevent cryoinjury and finally equilibrated for 15 min in the ice box (4°C).

Dilution of milt samples: The milt samples were diluted with cryodiluents at two ratios, namely 1:9 (milt: cryodiluent) was maintained for 0.9% NaCl and Alsever's solution, whereas 1:4 ratio was maintained for egg-yolk citrate.

Freezing protocol: A computer-controlled freezer (CL 3300, Australia) was used to freeze the samples. Two step freezing protocol was used. Firstly the milt was cooled from 5°C to - 4°C at a rate of 4°C per minute (Ramp 1 minute), then -4°C to -80°C at a rate of 10°C per min and held for 10 min. Afterwards the straws were taken out from the cryochamber and plunged into the liquid nitrogen (-196°C). Whenever needed, straws were retrieved from the liquid nitrogen and thawed in water bath at 40ºC for 7 sec. One µl of post thawed milt sample was placed on a glass slide and activated with distilled water (24 mOsmol/kg) to assess the motility under microscope.

Suitable milt cryodiluent ratio: Considering the better performance of three combinations designed as Cryo-1 (Alsever's solution with ethanol), Cryo-2 (Alsever's solution with methanol) and Cryo-3 (egg yolk citrate with DMSO), milt was mixed with the cryodiluents at eight different ratios, such as 1:1, 1:2, 1:4, 1:5, 1:7, 1:9, 1:12, and 1:15.

Suitable cryoprotectant concentration in the cryodiluent: In order to determine the suitable cryoprotectant concentration, the cryoprotectants were mixed with Alsever's solution and egg-yolk citrate at three concentrations viz. 5%, 10%, and 15% by volume (v/v).

Fertilization and hatching: Trials were conducted to find out the effects of cryopreserved spermatozoa on fertilization and hatching rate. Three best combinations of the cryodiluents mentioned above were used for fertilization trial. Stripped eggs were divided into four batches and each batch contained approximately 800 eggs. Three batches were used for the cryopreserved sperm and one for control. Three batches of eggs were fertilized with 15×230 µl of cryopreserved milt solution and the same amount of fresh milt was used for control. For proper insemination, 10 ml of tap water (31 mOsmol/kg) was added and mixed with feather for 1 min. Then the fertilized eggs were washed carefully 3-4 times with fresh milk to reduce the stickiness of eggs and transferred into the previously marked incubation bowls. The fertilization rates were calculated as the percentage of fertilized eggs obtained from the total number of eggs after 22-24 hrs. The hatching rates were calculated as the percentage of larvae obtained from total number of eggs, either from thawed or fresh sperm.

Statistical analyses: Percent motility values were subjected to arcsine transformation prior to statistical analysis. The effects of different extenders and cryoprotectants combinations, cryoprotectant concentrations and dilution ratios on both pre-freeze and post-thaw motility of sperm, fertilization and hatching rates were analyzed using one-factor ANOVA (Analysis of Variance). Means were separated by Duncan's Multiple Range Test (DMRT) and a value of $P<0.05$ was considered as being statistically significant. Data were expressed as mean ± SD and analyzed using SPSS (version 11.5).

Results and Discussion

Suitable extender and cryoprotectant combinations: The efficiency of extenders and cryoprotectants on the viability of spermatozoa in C. carpio at the pre-freeze and post-thaw periods are presented in Table 1.

Results shows that Alsever's solution with ethanol or methanol and egg-yolk citrate with DMSO gave significantly ($P<0.05$) higher motility (%) compared to other combinations at post-thaw period. Alsever's solution was found to be the best extender for cryopreservation of carps in Bangladesh (Rahman, 2005). Moreover, Salam (2005) reported Alsever's solution as the best extender for common carp sperm cryopreservation, which is an agreement with the present research. In respect to post-thaw motility, the other combinations were thought to be unsuitable for sperm cryopreservatoion of *C. carpio*. Effective extenders and cryoprotectants are needed to standardize the sperm cryopreservation protocol for each of the fish species. Cryoprotectants must be designed to prevent cryoinjury caused due to the ice formation and dehydration when the spermatozoa are frozen and thawed (Ohta Izawa, 1996). DMSO and methanol were found effective as cryoprotectants for sturgeon (Urbanyi *et al*., 2003) and paddlefish (*Polyodon spathula*) spermatozoa (Mims *et al*., 2000).

Table 1. Effects of three extenders and cryoprotectants each on the pre-freeze and post-thawed motility of spermatozoa in *C. carpio*

Cryodiluents		Pre-freeze	Post-thaw
Extenders	Cryoprotectants	motility (%)	motility (%)
Alsever's	Ethanol	93.33±1.05[a]	80±4.28[a]
solution	Methanol	90±1.83[a]	74.17±3.96[a]
	DMSO	83.17±1.64[a]	43.33±7.26[b]
Egg-yolk	Ethanol	81.67±3.33[b]	32.5±10.63[b]
citrate	Methanol	77.5±2.14[b]	45.83±8.41[b]
	DMSO	88.33±2.11[a]	76.67±4.59[a]
0.9%	Ethanol	74.17±3.00[c]	16.67±5.43[c]
NaCl	Methanol	75±2.89[c]	6.67±4.22[c]
	DMSO	80.83±3.75[b]	24.17±6.64[c]

Mean values on each column assigned with different superscripts were found to be significantly different (p<0.05).

Determination of optimal milt dilution ratio: Post thaw motility at 1:9 (methanol and DMSO with Alsever's solution, ethanol with egg yolk citrate) and 1:7 (methanol with Alsever's solution) ratios responded well although there were significant differences (P<0.05) between them (Fig. 1 a, b). The study also revealed that the post-thaw motility of common carp spermatozoa was much lower when mixed with the diluents at the ratio of 1:15. Analysis of Variance (ANOVA) of effect of dilution ratio on post-thawed motility (%) of C. carpio spermatozoa are presented in Table 2.

Table 2. Analysis of Variance (ANOVA) of effect of dilution ratio on post-thawed motility (%) of *C. carpio* spermatozoa

Source	DF	SS	MS	F
Extender	1	847.30	847.30	146.11***
Cryoprotectant	2	335.83	167.92	28.96***
Dilution ratio	7	3742.78	534.68	92.20***
Extender×Cryoprotectant	2	191.26	95.63	16.49***
Extender×Dilution ratio	7	139.98	19.99	3.45**
Dilution×Cryoprotectant	14	910.29	65.02	11.21***
Extender×Dilution ratio×Cryoprotectant	14	384.41	27.46	4.74***

***P<0.001, **P<0.01; DF: Degree of Freedom; SS: Sum of Square; MS: Mean Square

McPartlin *et al*. (2008) reported the milt dilution ratio is very important fish sperm to survive after cryopreservation. In a preliminary study, the best results were achieved when common carp spermatozoa were diluted Kurokura medium at 1:5 ratio (Linhart and Rodina, 2000).

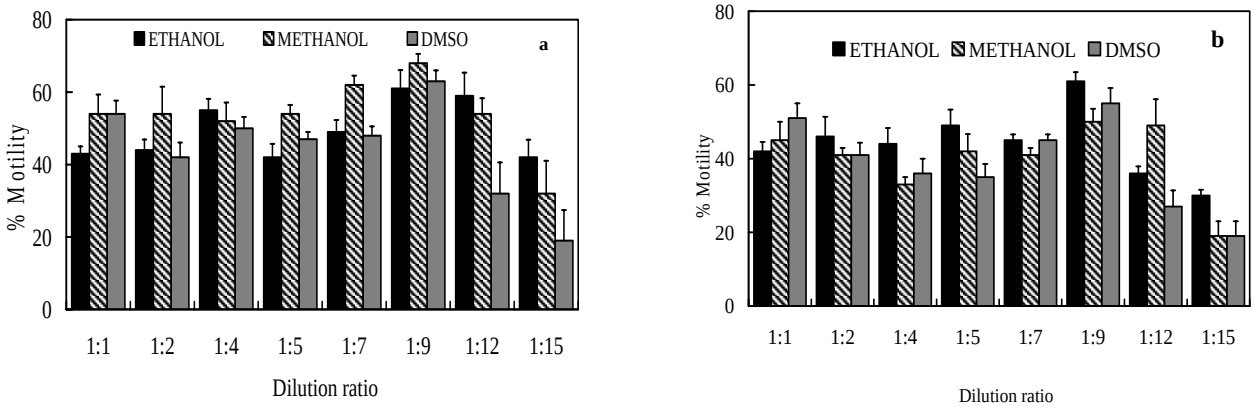

Fig. 1. Effects of dilution ratio (milt: cryodiluents) on post-thaw motility (%) of spermatozoa, diluted with (a) Alsever's solution and (b) egg yolk citrate

Determination of optimal concentration of cryoprotectant in the cryodiluent (v/v): All the three cryoprotectants performed better at their 10% concentraton. The motility of sperm at 10% concentration differed significantly (P<0.05) with the motility of sperm at 5% and 15% concentration. ANOVA of effect of cryoprotectant concentration on post-thawed motility (%) of *C. carpio* spermatozoa are presented in Table 3.

The study reveals that higher and lower concentration of cryoprotectant is equally detrimental for sperm viability (Fig. 2a, b). Therefore, 10% DMSO could be the best option

Table 3. Analysis of Variance (ANOVA) of effect of cryoprotectant concentration on post-thawed motility (%) of *C. carpio* spermatozoa

Source	DF	SS	MS	F
Extender	1	374.71	374.71	56.81***
Cryoprotectant	2	403.85	201.93	30.62***
Cryoprotectant concentration (CC)	2	2307.49	1153.75	174.92***
Extender×Cryoprotectant	2	4.18	2.09	0.32
Extender×CC	2	20.21	10.11	1.53
Cryoprotectant×CC	4	175.12	43.78	6.64***
Extender×Cryoprotectant×CC	4	40.15	10.04	1.52

***P<0.001, **P<0.01; DF: Degree of Freedom; SS: Sum of Square; MS: Mean Square

for sperm cryopreservation of *C. carpio* spermatozoa. This corresponds with previous observations on mirror carp (Ergun, 2004) and yellow perch, *Perca flavescens* (Ciereszko *et al*., 1993).

Effects of cryopreserved spermatozoa on fertilization and hatching rate in *C. carpio*: Fertilization of the eggs with fresh sperm were 88% whereas cryopreserved sperm (Cryo-1, Cryo-2, Cryo-3) were 32%, 35% and 37%, respectively. The hatching rates ranged from 23.29% to 35.35% relative to control (Table 4).

Among the combinations, Cryo-3 (egg-yolk citrate with DMSO) gave the best hatching rate of 35.35 % relative to control. Fertilization and hatching rates between cryopreserved and fresh sperm were significant ($p< 0.05$) different. The results suggest that hatching rate has to be improved for cryopreserved spermatozoa of common carp. Cryoinjury might damage the cell and that could happen after thawing the spermatozoa which might result lower hatching rate (Muchlisin, 2004).

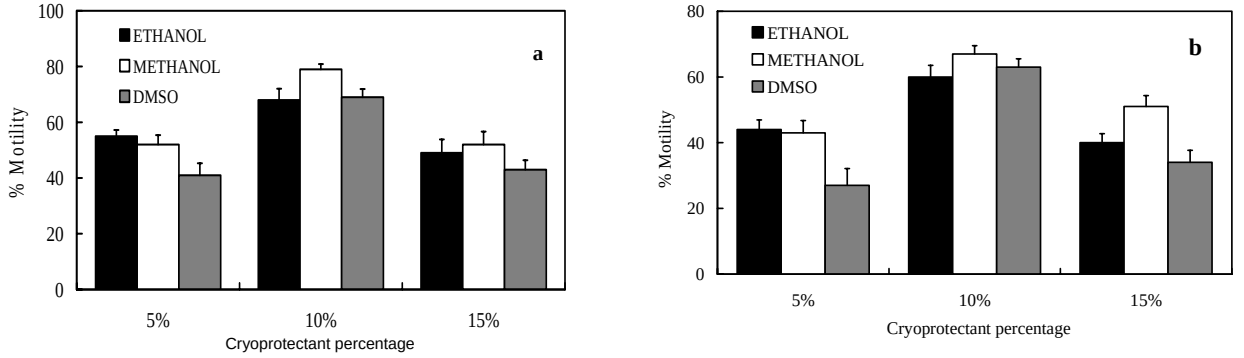

Fig. 2. Effects of cryoprotectant concentration on post-thaw motility of spermatozoa, diluted with (a) Alsever's solution and (b) egg-yolk citrate

In the fertilization trials with common carp, Linhart *et al*. (2000) found significant differences between fresh and thawed spermatozoa both for fertilization (68±11% and 56±10%, respectively) and hatching rate (50±18% and 52 ± 9%, respectively). Lahnsteiner *et al.* (2003) showed that the fertilization rate of cryopreserved spermatozoa was similar with the fresh sperm although differences were observed in the hatching rate. Similar assumptions were also made that fertilization and hatching rate can be decreased after cryopreservation compared to the fresh sperm (Honeyfield and Krise, 2000).

Table 4. Fertilization and hatching success of C. carpio eggs with cryopreserved and fresh spermatozoa

Attributes	Fresh	Cryo-1	Cryo-2	Cryo-3
Fertilization rate(%)	88.33±1.67[a]	32.22±0.91[b]	35.33±1.20[b]	37.67±1.76[c]
Hatching rate(%)	81.1±3.09[a]	18.89±1.11[b]	21.00±1.53[b]	28.67±1.85[c]
Fertilization (%) relative to control	100	36.47	39.99	42.64
Hatching (%) relative to control	100	23.29	25.89	35.35

Cryo-1. Alsever's solution+ethanol; Cryo-2. Alsever's solution+methanol; Cryo-3. Egg-Yolk citrate+DMSO. Mean±values in rows of different superscript are significantly different by DMRT at P<0.05.

Methanol at 10% concentration is the best suited cryoprotectant for *C. carpio* sperm cryopreservation. The study demonstrates that that the spermatozoa of common carp could be preserved successfully through cryogenic freezing to be used for spawn production. This technology could promote genetic and breeding studies, aiding in dispersal of genetically improved germplasm of *C. carpio* in Bangladesh.

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