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CRYOFREEZING OF GRASS CARP (*Ctenopharyngodon idella*) SPERMATOZOA FOR *EX SITU* CONSERVATION

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ABSTRACT

Experiments were conducted to develop and standardize the protocols for cryopreservation of sperm of grass carp (*Ctenopharyngodon idella*). Seven extenders such as Alsever's solution, Urea egg-yolk, Egg-yolk citrate, Kurokura-1, Kurokura-2, 0.9% NaCl and 0.6% Glucose and five cryoprotectants i.e. DMSO, methanol, ethanol, DMA and glycerol were employed for finding suitable combinations. Cryodiluents were prepared by mixing the cryoprotectants at 10% concentration of the extenders (v/v). Milt and cryodiluents were mixed at a ratio of 1:9 for Alsever's solution, Kurokura-1, Kurokura-2, 0.9% NaCl and 0.6% glucose, and 1:4 for urea egg-yolk and egg-yolk citrate. Among the 35 combinations of extenders and cryoprotectants, Alsever's solution with ethanol and methanol, urea egg-yolk and egg-yolk citrate with DMSO found suitable for preservation and it produced 74 ± 2.44%, 72 ± 2.54%, 76 ± 2.44% and 75 ± 2.23% spermatozoan motility at the post-thaw period respectively. Rest of the combinations, on the other hand, produced <60% motility at the post-thaw period and glycerol was found to be clotted after freezing. The dilution of milt with cryodiluent has been tested using six dilution ratios (1:2, 1:4, 1:7, 1:9, 1:15, 1:20) and found that 1:9 dilution ratio produced the highest post-thaw spermatozoan motility with Alsever's solution (>75%) and 1:4 with urea egg-yolk and egg-yolk citrate (>70%). As an optimum level of cryoprotectant, 10% concentration was found effective to produce significantly highest ($P<0.05$) percentage of spermatozoan motility compared to those of other four concentrations (5, 15, 20 and 30%).

Key Words: Grass carp, Cryopreservation, Sperm, Conservation

INTRODUCTION

Bangladesh is endowed with numerous rivers, flood plains, low lands, *haor, baor, beels*, lakes etc. The country is also enriched with endemic fish resources including 260 freshwater fishes and 24 prawn species; 475 marine fishes and 36 shrimp species (DoF,

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2010). Besides, a considerable number of exotic fish species including grass carp have been introduced in this country since 1960 (Rahman, 1985). Ali (1998) stated that 15 exotic species have been introduced and cultured in Bangladesh in order to increase the fish production and it contributed about 11.33% to the total fish production (FRSS, 2008-2009). As an exotic fish grass carp (*Ctenopharyngodon idella*) is a comparatively low priced commodity that is affordable to middle and low income classes of people in Bangladesh and other countries. After silver carp, globally grass carp has the largest production in freshwater aquaculture. Global production of cultured grass carp was only 10527 tonnes in 1950. By 2002 it had reached to 3572825 tonnes, an increase of more than 339 times in 52 years, and accounted for 15.6 percent of global freshwater aquaculture production. During the decade 1993-2002, the average annual growth rate of cultured grass carp production was 10.1 percent globally and 9.9 percent in China (FAO, 2003).

In Bangladesh about 900 hatcheries in public and private sectors are established in order to supply the fry of indigenous and exotic carps for aquaculture. At present, 99% of total seed are produced through induced breeding in the hatcheries (DoF, 2010). However, most of the hatcheries have a tendency to keep a small number of broodstocks and breed them repeatedly to keep the production cost at a minimum. As a result, the quality of fry is deteriorated due to inbreeding, genetic drift and bottleneck effect. Since exotic carps are imported and not available in the local natural environment, maintenance of the genetic diversity of the exotic fish species is currently a challenge. There are few techniques available to conserve the genetic resources, cryofreezing of sperm is the most important and effective means. Therefore, to conserve the existing gene pool of grass carp, an attempt has been taken to cryopreserve the sperm by developing cryopreservation protocol through optimizing cryodiluents, milt dilution and cryoprotectant concentration.

MATERIALS AND METHODS

The experiment was conducted in the genetics laboratory, mini hatchery and wet laboratory of the Department of Fisheries Biology and Genetics, Bangladesh Agricultural University, Mymensingh. The methods that have been followed were mentioned below :

Collection and maintenance of brood fish

More than 30 brood fish of grass carp each weighing about 2-4.5 kg were collected from good hatchery sources and stocked in the pond in the vicinity of Fisheries Faculty premises. A supplemental feed comprising of mustard oil cake, wheat bran and rice bran was provided at the rate of 4-5% body weight two times a day. Vitamin- E was supplemented to the feed for enhancing gonadal development of fish. Besides, green grass and leaves of banana were also provided to the fish. Inorganic and organic fertilizers were applied regularly to increase the natural food (phytoplankton and zooplankton) production.

Collection and observation of milt quality

Milt was collected from matured males after being induced with pituitary gland (PG) extract. Pure and concentrated white-coloured milt was collected in an eppendorf tube by applying gentle abdominal pressure and kept on ice. The quality of milt samples were checked under the binocular light microscope by placing 1-2 µl of milt on a glass slide with about 100-200 µl tap water. Milt samples containing more than 80% motility were considered as study sample.

Preparation of milt for cooling

Collected milt samples were diluted with cryodiluents at different ratios depending on the type of extender. For determining suitable cryodiluent, seven extenders, Alsever's solution, urea egg-yolk, egg-yolk citrate, Kurokura-1, Kurokura-2, 0.9% NaCl and 0.6% Glucose were used. Similarly five cryoprotectants such as DMSO (Dimethyl sulphoxide), methanol, ethanol, DMA (Dimethyl acetamide) and glycerol were used. Cryodiluents were prepared by mixing 10% cryoprotectant with 90% extender (% v/v). Milt and cryodiluents were mixed at a ratio of 1:9 for Alsever's solution, Kurokura-1, Kurokura-2, 0.9% NaCl and 0.6% Glucose, and 1:4 for urea egg-yolk and egg-yolk citrate. For equilibration about 10 min times was allowed and during the equilibration cryodiluent mixed milt was drawn into 0.25 µl French straws using a pipette. The opening end of the straws was sealed using heat. The straws were placed in the cryochamber of the control rate freezer for cooling. Two- step freezing protocol was used where milt sample was cooled from the ambient temperature (25^{o}C) to -4^{o}C at a decreasing rate of 4^{o}C per min and then from -4^{o}C to -80^{o}C at a decreasing rate of 10^{o}C per min. After that samples were removed from the cryochamber, loaded into the canisters and finally placed into the liquid nitrogen (-196^{o}C) dewar for long-term preservation.

For optimizing suitable dilution ratio between milt and cryodiluent, milt was diluted with each of the cryodiluents at five different ratios such as 1:2, 1:4, 1:7, 1:9 ,1:15 and 1:20 in the second experiment. Three extenders (Alsever's solution, urea egg-yolk and egg-yolk citrate) and four cryoprotectants (ethanol, methanol, DMSO and DMA) which performed well in the first experiment were used in this experiment.

Similar to the second experiment, three extenders and five cryoprotectants were used to determine the suitable cryoprotectant concentration in the third experiment. Five different concentrations such as 5%, 10%, 15%, 20% and 30% were tested. Procedures for collection, preparation and cooling of sperm used for the first experiment were remained same in the second and third experiments.

Statistical analyses

The equilibrium and post-thawed motility of spermatozoa were analyzed using two-factor randomized complete block design through computer software package (MSTAT).

RESULTS AND DISCUSSION

Effect of cryodiluent on sperm motility at equilibrium and post-thaw period

Motility of sperm at equilibrium (the time that allows the cryodiluent to enter into the cell before freezing) and post-thaw period was checked and a positive correlation (r=0.83) was observed (Fig. 1). ANOVA showed that cryodiluent had significant effect on the spermatozoan motility at post-thaw *(*P<0.01*)* period (Table 1). The extenders and cryoprotectants selected for this experiment were chosen from previous studies (Sin, 1974; Chao *et al*., 1975; Shirohara *et al*., 1982; Kumar 1988 and 1989 and Magyary *et al*., 2000) and among the 35 combinations (extender + cryoprotectant), Alsever's solution with ethanol and methanol, urea egg-yolk and egg-yolk citrate with DMSO produced the highest spermatozoan motility at the post-thaw period (>72%). The three extenders Alsever's solution, egg-yolk citrate and urea egg-yolk solutions were found suitable and could be explained to have specific concentration to balance the osmotic pressure of spermatozoa. The reason behind using egg-yolk citrate and urea egg-yolk is that, the egg-yolk provides a significant protection to the membrane and is called 'membrane stabilizer'. The LDL (Low density lipoprotein) fraction associated with cell membrane significantly provides protection of sperm against injury during cryogenic freezing (Babiak *et al.,* 2000). Again it allows greater and more prolonged survival of sperm in the fertilization media (Billard, 1970). The Na-citrate fraction of Alsever's solution associated with cell membrane may provide protection against injury during cryogenic freezing.

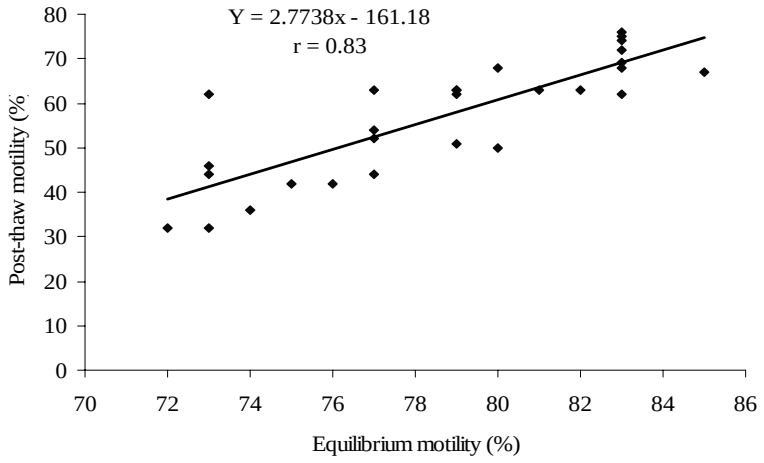

Fig. 1. Relationship between equilibrium and post-thaw motility of *Ctenopharyngodon idella* sperm

The cryoprotective agent (also called cryophylactic substance) is a crucial factor that functions by reducing the thermal shock. High water solubility and low toxicity to cells and easy penetration to cells are essential considerations for a chemical to be cryoprotective. Kumar (1989) reported satisfactory performance with Alsever's solution. Alvarez *et al*. (2003) also found good results with Alsever's solution and 10% DMSO. DMSO was found more effective than other cryoprotectants, and the reason could be of its more permeability to cells (Whittingham, 1980) where DMSO penetrates rapidly into the cellular membrane (Rao, 1989) and brings a quick balance in between the intra and

extra-cellular fluid concentration. It has been established that the use of cryoprotectants such as glycerol significantly suppresses the sperm motility of all sturgeon species while 10-15% DMSO acts as the best cryoprotectant (Cherepanov *et al.*, 1993). The lowest percentage of equilibrium motility and post-thaw motility was observed in DMA with 0.6% glucose, 0.9% NaCl (32 ± 2%). Glycerol provided less motility during carp sperm preservation (Kumar, 1988 and 1989 and Magyary *et al*., 2000) and it was found clotted with extenders in the present study. Harvey and Ashwood-Smith (1982) mentioned the poor performance of glycerol gave in terms of post-thaw motility because glycerol penetrates slowly into the cell membrane. Methanol was used as a best cryoprotectant in the cryopreservation of Indian major carp embryos than DMSO and glycerol in a number of cell types (Ahammad *et al*., 1998). In the present study ethanol and methanol showed moderate results at post-thaw period.

Effect of dilution ratio on post-thaw sperm motility

Further investigations were performed using different dilution ratios between milt and cryodiluent (extenders, Alsever's solution, egg-yolk citrate solution, urea egg-yolk solution and cryoprotectants, DMSO, ethanol, methanol and DMA) for observing its any effect on post-thaw motility. The dilution ratios tested were 1:2, 1:4, 1:7, 1:9, 1:15 and 1:20 for each of the three extenders and cryprotectants. The statistical analyses showed that the percent motility of spermatozoa at the post-thaw period was significantly (P<0.05) higher with ethanol and methanol than other cryoprotectants in dilution ratios of 1:9 and 1:4 (Fig. 2). In case of ethanol and methanol, highest percentage of spermatozoan motility (>70%) was scored from 1:9 dilution with Alsever's solution and from 1:4 dilution with egg-yolk citrate and urea egg-yolk in post-thaw period. The study also revealed that the post-thaw motility of grass carp spermatozoa was lower when spermatozoa were mixed with the diluents at the ratio of 1:2, 1:15 and 1: 20. In a preliminary study, walleye spermatozoa were diluted at the dilution ratios (semen: extender) of 1:5, 1:9 and 1:15, and best results were obtained from 1:15 (Bergeron *et al*., 2002). Dreanno *et al.* (1997) found no significant effect on post-thaw motility when turbot sperm was diluted at the ratios of 1:1, 1:2, 1:4 and 1:9. In the present study higher dilution beyond 1:9 and lower dilution beyond 1:4 reduced the motility, and the extremely low and high dilutions were presumably harmful to sperm. However, it was disagreed with the findings of Horton *et al.* (1967) who found that as the dilution of milt decreased, the degree of motility and the percentage of motile salmon sperm increased.

Effects of different cryoprotectant concentrations on post- thaw sperm motility

It was found that among the five cryoprotectant concentrations, 10% concentration was most effective in most of the extenders and it produced significantly higher motility than those of other concentrations (Fig. 3). The highest post-thaw motility (>73%) was achieved at 10% concentration of ethanol and methanol with three extenders (Alsever's solution, egg-yolk citrate and urea egg-yolk). Increase of cryoprotectant concentration at more than 10% caused a rapid decline in post-thaw motility and a very poor motility was observed at 30% concentration. A mixed findings were reported by Yao *et al.* (2000) where motility of post-thawed ocean pot sperm increased with the increase of DMSO level and peaked when DMSO level reached to 20% (v/v) in extender. They also found that further increase of DMSO above 20% caused a rapid decline in post-thawed sperm motility and

no motility was reported at 30% concentration. Similar assumption was postulated by Rana and McAndrew (1989) that the motility could not be initiated when the spermatozoa were suspended in 30% and 40% methanol or DMSO. The four cryoprotectants used in the study found to be suitable for cryopreservation but their higher concentrations seemed toxic that might be resulted from interactions between the cryoprotectants and some components of diluents.

Table 1. Average equilibrium and post-thaw motility of *Ctenopharyngodon idella* sperm mixed with different cryodiluents

Extenders	Cryoprotectant	Dilution ratio	Equilibrium motility (%)	Post-thaw motility (%)
Alsever's solution	Ethanol	1:9	83 ± 2	74 ± 2.45
	Methanol	1:9	83 ± 2	72 ± 2.55
	DMSO	1:9	77 ± 2	63 ± 2
	DMA	1:9	73 ± 2	62 ± 1.22
	Glycerol	1:9	74 ± 2.45	-
Urea egg-yolk	Ethanol	1:4	85 ± 2.34	67 ± 2
	Methanol	1:4	83 ± 2	68 ± 2.55
	DMSO	1:4	83 ± 2	76 ± 2.45
	DMA	1:4	79 ± 1	51 ± 3.32
	Glycerol	1:4	81 ± 1	-
Egg-yolk citrate	Ethanol	1:4	82 ± 1.22	63 ± 2
	Methanol	1:4	83 ± 2	69 ± 3.32
	DMSO	1:4	83 ± 2	75 ± 2.24
	DMA	1:4	77 ± 2	52 ± 3.74
	Glycerol	1:4	80 ± 1.58	-
Kurokura-1	Ethanol	1:9	79 ± 2.45	63 ± 2
	Methanol	1:9	81 ± 3.32	63 ± 2
	DMSO	1:9	79 ± 2.45	62 ± 1.22
	DMA	1:9	80 ± 1.58	50 ± 3.16
	Glycerol	1:9	79 ± 1	-
Kurokura-2	Ethanol	1:9	79 ± 2.45	63 ± 2
	Methanol	1:9	83 ± 2	62 ± 2
	DMSO	1:9	80 ± 1.58	68 ± 3.74
	DMA	1:9	77 ± 2	54 ± 2.45
	Glycerol	1:9	79 ± 2.45	-
0.9 % NaCl	Ethanol	1:9	76 ± 2.45	42 ± 2
	Methanol	1:9	73 ± 2	46 ± 2.45
	DMSO	1:9	74 ± 2.45	36 ± 2.45
	DMA	1:9	73 ± 2	32 ± 2
	Glycerol	1:9	74 ± 2.45	-
0.6 % Glucose	Ethanol	1:9	73 ± 2	44 ± 2.45
	Methanol	1:9	75 ± 2.23	42 ± 2
	DMSO	1:9	77 ± 2	44 ± 2.45
	DMA	1:9	72 ± 2	32 ± 2
	Glycerol	1:9	81 ± 2.45	-
Level of significance			*	**

* Significant at 5% level of confidence, ** Significant at 1% level of confidence

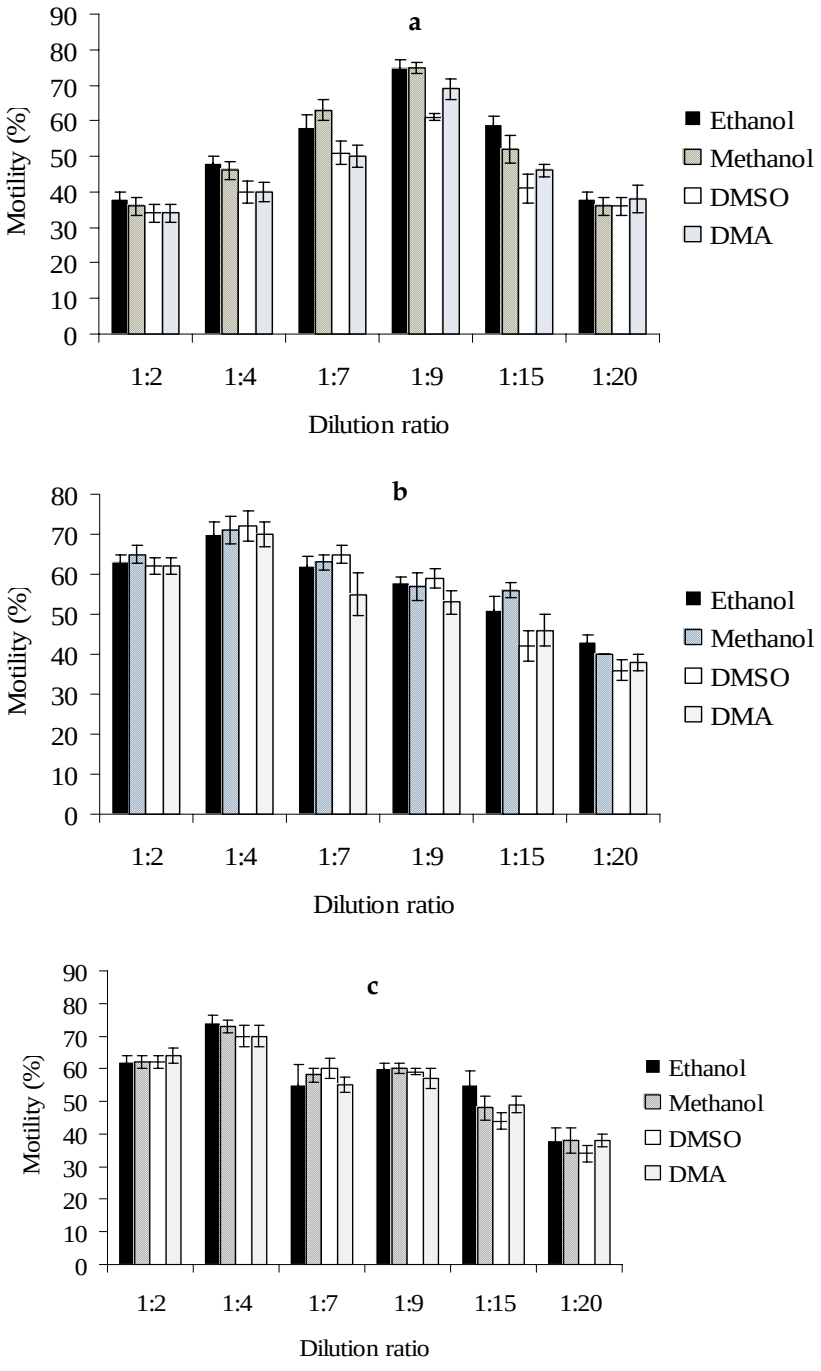

Fig. 2. Effect of different dilution ratios on post-thaw motility of *Ctenopharyngodon idella* sperm. a) Alsever's solution, b) urea egg-yolk and c) egg-yolk citrate mixed with ethanol, methanol, DMSO and DMA

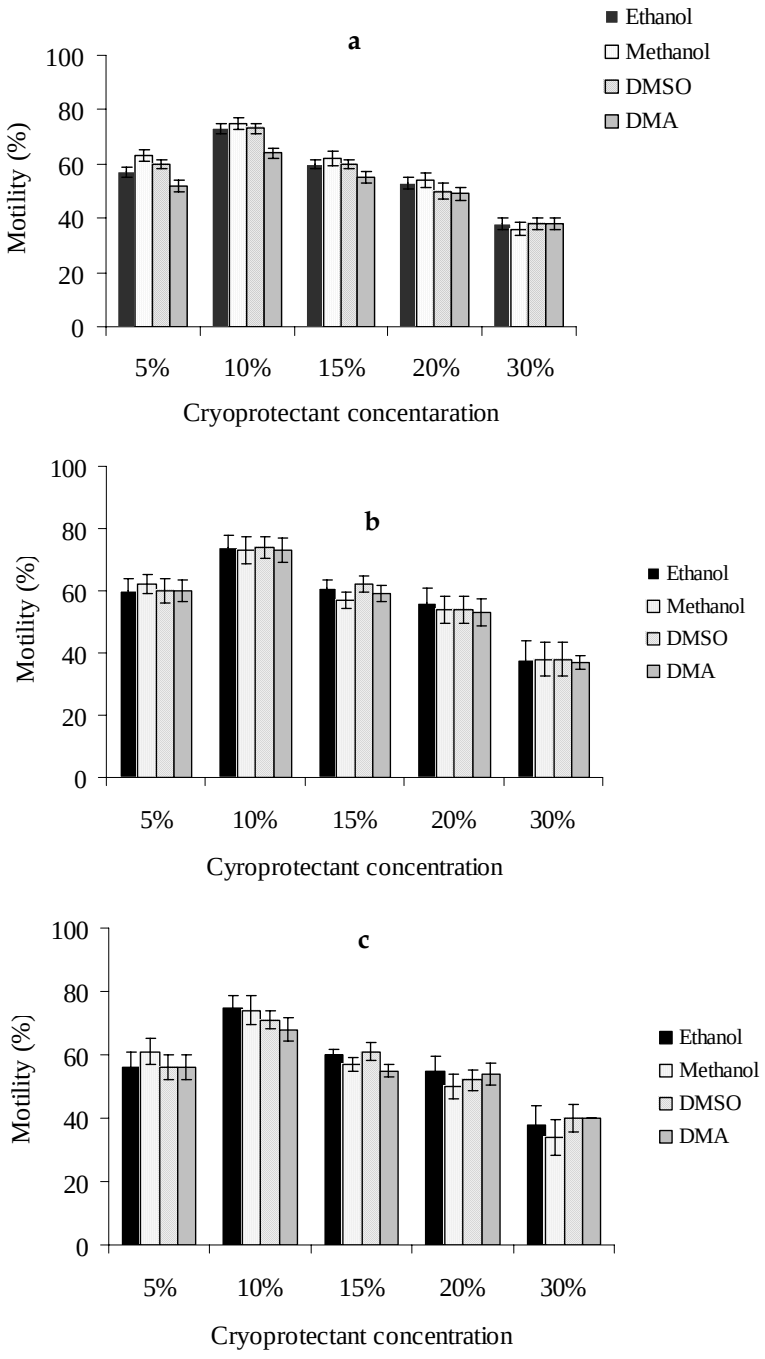

Fig. 3. Effects of different cryoprotectant concentrations on post-thaw motility of *Ctenopharyngodon idella* sperm. a) Alsever's solution, b) urea egg-yolk and c) egg-yolk citrate mixed with ethanol, methanol, DMSO and DMA

CONCLUSION

In breeding trials, sperm preserved with the cryodiluents did fertilize eggs but could not produce hatchlings. The reason for failure of hatching of eggs was not clear but the consequences of the improper thawing technique of cryopreserved spermatozoa prior to fertilization or any unwanted error in fertilization process could be responsible. Cryopreservation protocol for grass carp spermatozoa has been developed and standardized through this study but further research need to be conducted especially on improvement on fertilization and hatching of eggs with cryopreserved sperm before demonstrating full protocol to the hatchery operators. The development of such protocols for cryopreservation of sperm may facilitate sustainability in aquaculture and the establishment of a national facility for gene banking of fish gametes and DNA material.

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