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Sperm cryopreservation of Indian major carp, *Labeo rohita*: cryodiluents, sperm: cryodiluent dilution ratio and cryoprotectant concentration

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Abstract

Cryogenic preservation trials of spermatozoa of *Labeo rohita* were carried out. Twenty four cryodiluents (extender + cryoprotectant), with the combination of six extenders such as egg-yolk citrate, urea-egg-yolk, 0.9% NaCl, Kurokura-2, M^a and M^b and four cryoprotectants *viz.* DMSO, glycerol, methanol and ethanol, were used to screen out the suitable cryodiluents. Sperm was preserved in 0.25ml plastic straw in programmable freezer. Two step freezing method was followed. Sperm preserved with egg-yolk citrate and urea-egg-yolk containing 10% DMSO showed best post-thaw motility (80%) followed by 0.9% NaCl (60%) and Kurokura-2(30%) solutions. Sperm with the extenders M^a and M^b clotted at the time of equilibration and also after few days of preservation. Egg-yolk citrate mixed with ethanol and methanol also showed good percentage of motility (80%) but egg-yolk citrate with glycerol showed less sperm motility (>60%).

To determine suitable dilution ratio of milt and cryodiluent two best extender egg-yolk citrate and urea-egg-yolk with four cryoprotectants such as DMSO, glycerol, methanol and ethanol at different ratio *viz* 1:2,1:4,1:7,1:10,1:15 and 1:20 were used. Highest post-thaw motility (>80%) was observed when milt was preserved with egg-yolk citrate containing 10% DMSO at 1:2, 1:4, 1:7 and 1:10 dilutions. Meanwhile using glycerol as cryoprotectants provided less post thaw motility at lower dilution ratio but with the increase of its dilution showed good sperm motility compared with other cryoprotectants. Finally, evaluation on the effect of cryoprotectant concentration on post-thaw sperm motility was conducted. Egg-yolk citrate and four cryoprotectant i.e. DMSO, glycerol, methanol and ethanol with six different concentrations namely 5%, 7%, 10%, 15%, 20% and 30%.were evaluated. Among the cryoprotectants DMSO, methanol and ethanol showed highest post-thaw motility (about 80%) at 7% and 10% concentrations. Although glycerol was not suitable at low concentration but its 20% and 30% concentration levels provided best post-thaw motility. No post-thaw motility was obtained with DMSO at 30% concentration. The overall analysis on cryoprotectant concentration indicated that below 5% and above 20% cryoprotectant concentrations could not be suitable for effective cryopreservation of spermatozoa.

Key words: Cryopreservation, Cryodiluent, *Labeo rohita*

Introduction

Bangladesh is ranked 3rd in aquatic biodiversity in Asia behind china and India, with approximately 300 species of fresh and brackish water species (Hussain and Mazid 2001). Indian major carps are the most extensively cultured fish species in Bangladesh. Among Indian major carps, *L. rohita* is the first ranking fish in aquaculture due to their relatively better test and higher market price. It constitutes nearly 23% of the fish produced through freshwater aquaculture (Alam 2002). In early stage of aquaculture fish farmers were fully dependent on nature for carp seeds but its supply gradually declined due to many factors such as over-exploitation, environmental degradation, blocking or changing of migration routes, introduction of exotic carps etc. These environmental and man-made changes bring genetic degradation in natural population and in some cases it attributes very unfavorable state for some fish species.

In early 1980's many fish hatcheries both in government and private sectors were established. At present about 141 government and 635 private hatcheries are being operated in Bangladesh (Ali 1998). Production of fish seed from government and private hatcheries are quite enough to fulfill the farmers demand. But the quality of seed from hatchery is gradually deteriorated due to lack of scientific management of hatchery including selection and maintenance of brood stock. Most commonly they stock a small number of broods in their farm and make the broods replacement by succeeding generations. So the hatchery carp is virtually closed to genetic exchange with wild stocks (Eknath and Doyle 1990). The maintenance of the genetic biodiversity of indigenous fish is currently a challenge and urgently required to take necessary step for conservation of these species.

In this regard cryopreservation is the simplest and most inexpensive method to preserve genomes that can be used to maintain future conservation options. Establishment of germ plasm repository through cryopreservation is called cryogenic gene bank. As more fish species become threatened or endangered, it is reasonable to establish a regional fish germ plasm repository to preserve the vanishing genetic material as well as to conserve the existing gene pool. Although cryopreservation of fish sperm is a potential technique for improving quality seed production system, no work has been done in Bangladesh so far. It is the first work in Bangladesh with a view to standardize the protocol for cryopreservation of Rohu sperm.

And for this reason, research on carp cryopreservation is initiated. By standardizing a suitable protocol and establishing cryopreserved gene bank good quality sperm could be supplied to both government and private hatcheries as required and thus, genetically improved carp seeds could be made available to the rural fish farmers.

Materials and methods

Male and female broods were collected from different sources (Halda river and different hatcheries) and stocked in the ponds located in the Fisheries Faculty Field Complex, Bangladesh Agricultural University, Mymensingh. Broodstocks were fed two

times a day at 5% of their total body weight. Vitamin-E was supplemented with the given feed to enhance the gonad development.

Expt. 1. Suitable extenders and cryoprotectants

Collection of milt

Mature male with desired phenotypic characteristics were selected and induced by injecting PG extract to get sufficient amount of milt. Excess moisture, urine, gut extrudes and mucus were wiped from the area of the genital pore with absorbent paper. Gentle abdominal pressure was applied to collect the milt. Micropipette and tips were used at the time of collection of milt. The collected milt was transferred to eppendorfs or plastic tubes and stored on ice to prevent quality deterioration during further processing.

Evaluation of sperm

After collection the quality of sperm was checked under microscope by placing 1-2μl diluted sperm on glass slide. Only samples containing more than 80% motile cells by eye-estimation were used for cryopreservation.

Estimation of sperm

Number of sperm cells in a unit volume of milt sample was essential to count for standardizing the degree of dilution and estimating the density of sperm per straw for maintaining desired level of egg: sperm ratio during fertilization. The concentration of sperm was calculated using the following formula:

No of cells/ml=

$$\text{Total no of sperm in 5 large squares X dilution factor} \; \frac{\text{Counting factor (Volume)}}{\text{No. of small squares counted}}$$

Cryodiluents

Dilution of sperm was done by adding cryodiluent to the milt sample at specified ratios. Cryodiluents consist of extenders and cryoprotectants. The extender is a solution that contains organic and inorganic chemicals which increase the efficacy of cryopreservation of sperm. However, cryoprotectants are mixed with extender to protect the cell from damage during cooling and freezing. Six extenders namely Egg-yolk citrate, Urea-egg-yolk, Kurokura-2, M^a, M^b, and 0.9% NaCl and four cryoprotectants viz. DMSO, glycerol, methanol and ethanol were used throughout the experiment. The constituents of these extenders are given in Table 1.

Dilution and preparation of milt for cooling

Collected milt was diluted with the cryodiluent (extender + cryoprotectant) at different ratios depending on the type of extender. In case of egg-yolk citrate, urea-egg yolk, M^a and M^b milt was diluted with the cryodiluent at a ratio of 1:4 (milt:

cryodiluent) and for Kurokura-2 and 0.9% NaCl milt was diluted at a ratio of 1:9 (milt: cryodiluent). Cryodiluents were prepared by mixing 10% cryoprotectant (e.g. DMSO, glycerol, ethanol and methanol) and 90% extender by volume. The sperm and cryodiluent were kept at 0°C prior to dilution.

Equilibration, filling and sealing of straws

The diluted sperm was equilibrated for 10 min at room temperature (at 15°C to 20°C) and the motility of the equilibrated sperm was checked under microscope before filling straws.

Equilibrated milt sample was loaded into the straw by using pipette and tips and free end of the straw was heat sealed. The straws were finally placed in the controlled rate freezer and initiated for cooling.

Freezing

Computer controlled freezer (CL 300) was used to freeze the samples using the following program. Two step freezing method was applied i.e. firstly the milt was cooled from +20°C to -4°C at a rate of 4°C per minute, then from -4°C to -80°C at a rate of 10°C per minute and finally transferred into liquid nitrogen.

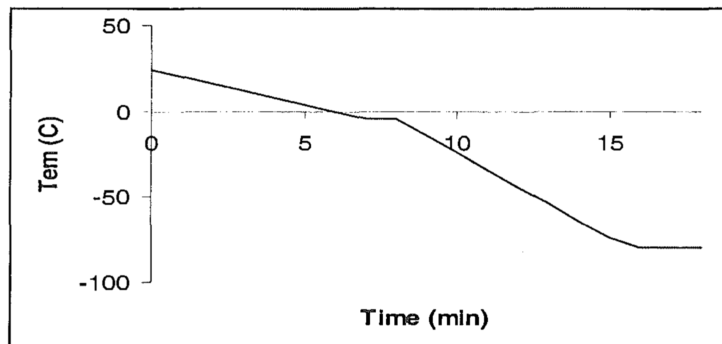

Fig.1. Schematic diagram showing two step freezing protocol.

Thawing

Straws were retrieved from the LN dewar using tweezer and thawed in room temperature. One to two μl of post thawed milt sample placed on to glass slide and activated by adding conditioned water to assess the motility using microscope.

Statistical analysis

The data were analyzed using two factor randomized complete block design. Significant differences presented in the table are based on Duncans Multiple Range Test (DMRT)

Table 1. Chemical constituents of different extenders used for sperm cryopreservation of *L. rohita*

Extender/Constituent	I	II	III	IV	V	VI
	Ma	Mb	Kurokura-2	Urea-egg-yolk	Egg-yolk citrate	0.9% NaCl
Sodium chloride	730 mg	850 mg	360 mg	0.3%	0.4%	0.9 g NaCl dissolved in 100 ml distilled water.
Urea				0.4%	—	
Sodium citrate				—	0.1%	
Sodium bicarbonate	500 mg	500 mg	20 mg	Dissolved in 100 ml of distilled water. Pancromycin (1000 iu/ml)and streptomycin (1000 iu/ml) were added to the buffer solution. Egg yolk was added to the buffer at a ratio of 1:4 (egg yolk: buffer). Therefore, 80 ml of buffer was mixed with 20 ml of egg yolk.	Dissolved in 100 ml of distilled water. Pancromycin (1000 iu/ml)and streptomycin (1000 iu/ml) were added to the buffer solution. Egg yolk was added to the buffer at a ratio of 1:4 (egg yolk: buffer). Therefore, 80 ml of buffer was mixed with 20 ml of egg yolk.	
Fructose	500 mg					
Mannitol	500 mg	-				
CaCl2			22 mg			
KCl			1000 mg			
MgCl2			8 mg			
Vegetable lecithin	750 mg	1500 mg				
	Dissolved in 100 ml distilled water					

Exp.2. Suitable sperm: cryodiluent dilution ratio

For this experiment, except the dilution ratio between milt and cryodiluent, all other procedures such as milt collection, quality assessment, dilution, equilibration, freezing and thawing were same as mentioned in the Expt. 1. Three extenders (Egg-yolk citrate, Urea-egg yolk, 0.9% NaCl) were mixed with four cryoprotectants (DMSO, glycerol, ethanol and methanol) and thus twelve cryodiluents were prepared. The milt was diluted with each of the cryodiluents at six different ratios such as 1:2, 1:4, 1:7, 1:10, 1:15 and 1:20 and cryopreserved following the procedures mentioned above. The post-thaw motility of the sperm in different ratios was checked under microscope and recorded the results.

Expt. 3. Suitable concentration of cryoprotectant in the cryodiluent

To determine the suitable cryoprotectant concentration in the cryodiluent four cryoprotectants were mixed with one exyender at six different concentrations namely 5%, 7%, 10%, 15%, 20%, and 30% by volume (v/v). During cryopreservation all other procedures i.e. from milt collection to preservation, were same as Expt. 1.

Results

Evaluation of fresh sperm

For each of the experiments the quality of milt was checked and the number of sperm/ml of milt was estimated. The survivability of sperm in the original milt was close to 100% even after 6-10 hrs of collection as they kept in ice. The number of sperm per ml of fresh milt was ranged from 5.33×10^8 to 20×10^{10}.

Mobility of spermatozoa

Mobility of spermatozoa can be categorized into three such as progressive movement, oscillatory movement and rotatory movement in this experiment. In the present study about 80-90% rotatory movement was recorded from the samples preserved with egg-yolk citrate and urea-egg-yolk and 10% DMSO. The highest progressive movement (40-50%) was found for the samples preserved with 0.9% NaCl and Kurokura-2 and 10% DMSO. However, after cryopreservation this oscillatory movement was completely absent.

Effect of extender and cryoprotectant on motility of sperm at the equilibrium period

When the milt mixes with cryodiluent and draws into the straws the equilibration of cells takes place. The equilibration period can be varied with species. Before freezing of sperm i.e. during the equilibration period the motility of sperm was checked in every trial because it has an effect on post thaw survivability of sperm. Evaluation of sperm motility in different cryodiluents at equilibrium period is shown Table 2.

Table 2. Average motility of sperm at the equilibrium and post-thaw period

Extender	Cryoprotectant	Dilution ratio (milt: diluent)	% motility at equilibrium period	% motility at Post-thaw period
Egg-yolk citrate	DMSO	1:4	85±7.90	80±6.12
	Glycerol		80±7.90	60±12.74
	Methanol		80±7.90	80±11.72
	Ethanol		75±7.90	80±6.12
Urea-egg-yolk	DMSO	1:4	90±7.35	80±12.74
	Glycerol		75±15	70±7.90
	Methanol		80±6.12	70±10.70
	Ethanol		80±4.90	60±5.60
M^a	DMSO	1:4	10±3.53	5±1.25
	Glycerol		10±7.90	0
	Methanol		10±5.34	5±2.45
	Ethanol		10±7.90	5±3.53
M^b	DMSO	1:4	5±6.45	0
	Glycerol		5±3.53	5±1.95
	Methanol		5±10.90	0
	Ethanol		0	0
0.9% NaCl	DMSO	1:9	65±9.35	60±7.90
	Glycerol		30±7.90	5±3.53
	Methanol		40±10.90	10±6.90
	Ethanol		30±5.90	20±7.80
Kurokura-2	DMSO	1:9	50±5.30	30±11.90
	Glycerol		30±7.90	0
	Methanol		40±5.30	10±3.53
	Ethanol		30±8.70	20±10.90

Statistical analysis showed that extenders and cryoprotectants had significant influence on the motility of sperm at the equilibrium period (F- ratio= 194.8604 & $p<0.01$ and F-ratio =7.4520 & $p<0.05$ respectively). Among six extenders and four cryoprotectants egg-yolk citrate and urea-egg-yolk gave best sperm motility at the equilibrium period (about 80% and more). The Kurokura-2 and 0.9% NaCl provided poor equilibrium motility (20% to 60%) and very few or in some cases no motility was observed in M^a and M^b. Among the cryoprotectants 10% DMSO was found more suitable with egg-yolk citrate and urea-egg-yolk though other cryoprotectants were also effective in both the extenders. For Kurokura-2 and 0.9% NaCl, 10% DMSO combination produced highest equilibrium motility (>60%) compared to other cryoprotectants. There was a positive relation ($r=0.705$ and $Y=0.7505x-3.233$) between the equilibrium motility and post- thaw motility (Fig. 2).

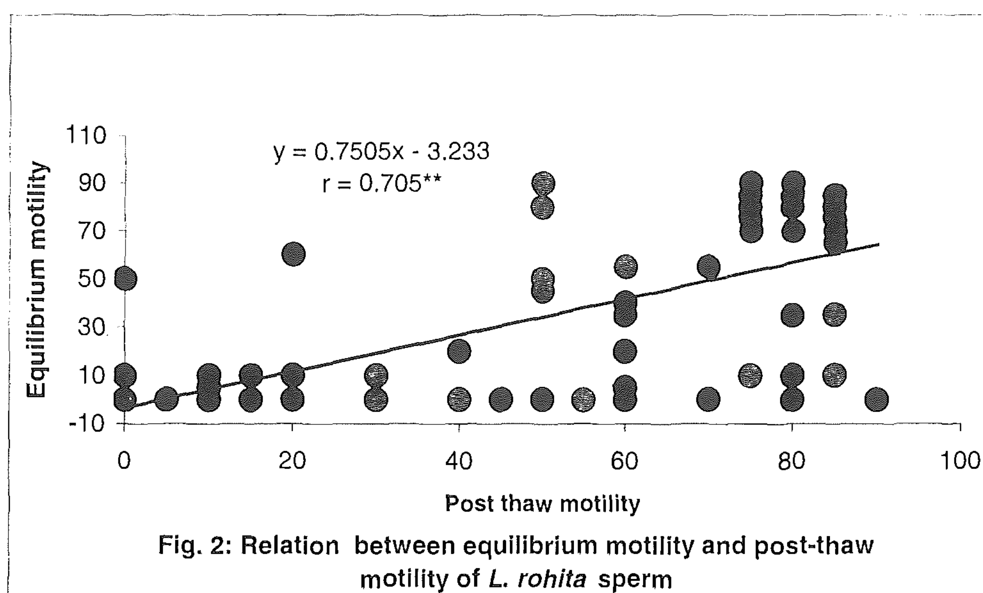

Fig. 2: Relation between equilibrium motility and post-thaw motility of *L. rohita* sperm

Effect of extender and cryoprotectant on motility of sperm at the post-thaw period

To assess the motility of sperm straws were removed from the liquid nitrogen after a few days of preservation and thawed at room temperature and checked under the microscope shown in Table 2. The ANOVA showed significant influence of extender and cryoprotectant on motility percentage of post-thawed sperm (F-ratio= 194.86 & p<01 and F-ratio=7.45 & p<0.05 respectively). DMRT showed that egg-yolk citrate and urea-egg-yolk was significantly better extender and DMSO was the best cryoprotectant for cryopreservation of rohu sperm. Milt preserved with egg-yolk citrate and urea-egg-yolk with DMSO showed highest post-thaw sperm motility (80%). Methanol and ethanol mixed with egg-yolk citrate also showed good motility. However, glycerol with urea-egg-yolk and egg-yolk citrate showed less motility (70% & 60%). In case of 0.9% NaCl and Kurokura-2 extenders, all of their combinations with the cryoprotectants showed very poor post-thaw sperm motility. The worst condition (mostly 0% motility) was found when sperm preserved with M^a and M^b and their respective combinations with the cryoprotectants.

Effect of dilution ratio on post-thaw sperm motility

For this experiment milt was diluted with the eight cryodiluents, originated from the combination of two best extenders (selected from Expt.t 1) such as egg-yolk citrate and urea-egg-yolk and four cryoprotectants *viz* DMSO, methanol, ethanol and glycerol at six different ratios such as 1:2,1:4,1:7,1:10,1:15 and 1:20. It was observed that the dilution ratio did not affect significantly on post-thawed sperm motility except the 1:2 dilution ratio. Most of the cryoprotectants performed well, however, glycerol with both egg-yolk citrate and urea-egg-yolk, and ethanol with urea-egg-yolk showed very poor performance. It was also observed that motility percentage increased with the increasing

of dilution ratio between glycerol and egg-yolk citrate and urea-egg-yolk, and ethanol with urea-egg-yolk. Highest post-thaw motility scored was >80% with egg-yolk citrate containing 10% DMSO at 1:2, 1:4, 1:7 and 1:10 ratios The results of the effect of dilution ratio on post-thaw sperm motility are presented in the following (Figs 3a to 3d)

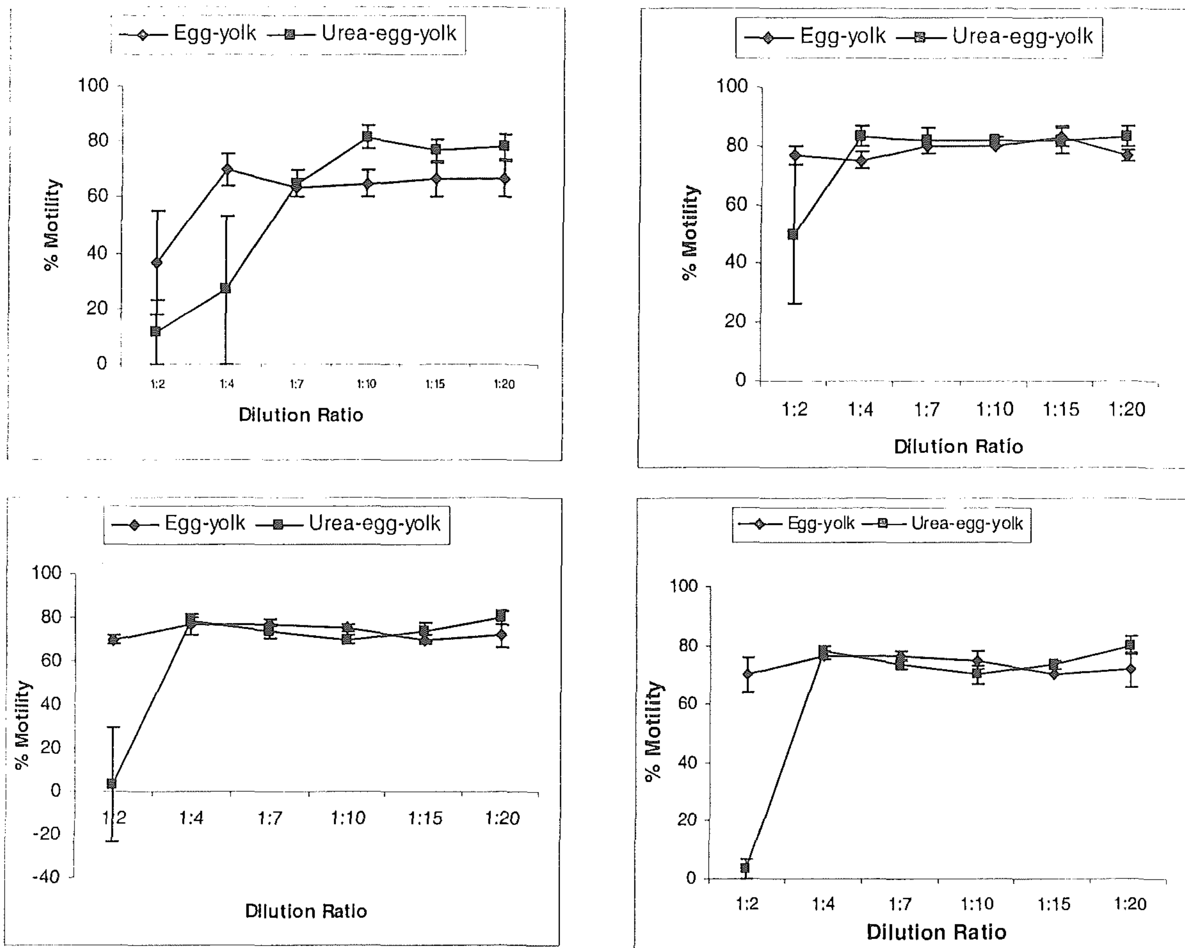

Fig. 3. Effects of different dilution ratios (milt: cryodiluent) on the viability of cryopreserved *Labeo rohita* spermatozoa when egg-yolk citrate and urea-egg-yolk used as extender. Each value (average % motility) represents the data from three replicates.

a. 10% DMSO as cryoprotectant, b. 10% glycerol as cryoprotectant,
c. 10% methanol as cryoprotectant, d. 10% ethanol as cryoprotectant

Effect of different concentration of cryoprotectant on post-thaw motility

In this experiment four cryoprotectants with different concentrations such as 5%, 7%, 10%, 15%, 20% and 30% in cryodiluents were used to determine the suitable concentration of cryoprotectant. In case of DMSO, methanol and ethanol the highest post-thaw motility (80%) was achieved at 7% and 10% concentration levels. Both 20% and 30% glycerol concentrations levels provided best post-thaw motility. No post-thaw motility was observed in case of 30% DMSO. Apart from this, 30% ethanol provided few percentage of post-thaw sperm motility. From this study it has been concluded that

below 5% and above 20% concentrations of the cryoprotectants could severely affect the cryopreservation efficiency (Fig.4).

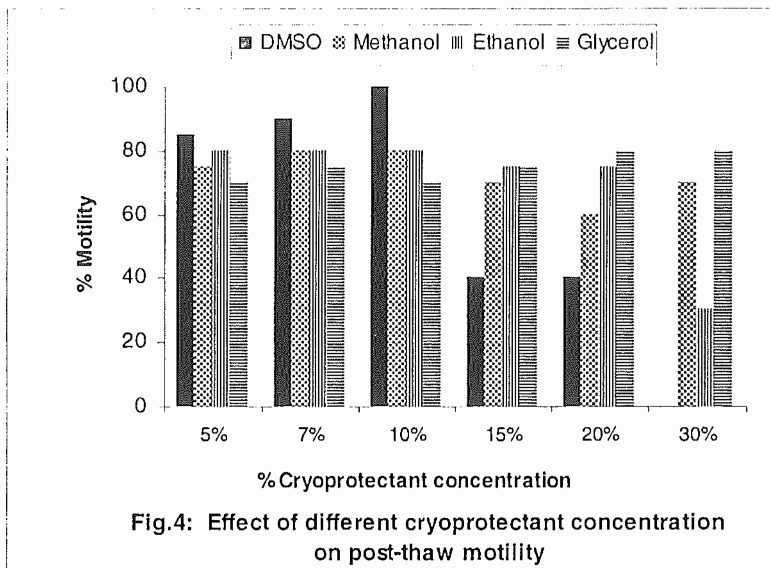

Fig.4: Effect of different cryoprotectant concentration on post-thaw motility

Fertilization trials

Two trials on fertilization of eggs with the cryopreserved and pure sperm were carried out. Both trials could be considered as preliminary trials. However, some success (20%) in terms of fertilization and hatching were achieved.

Discussion

Cryopreservation of *Labeo rohita* spermatozoa

Although Indian major carps are the main culture species in Bangladesh, India and Nepal but very little attention was paid for cryopreservation of its sperm. So far only two works have been reported on IMCs sperm cryopreservation (Kumar 1988 and 1989).

Based on earlier studies (Chao *et al.* 1975, Withler 1982, Shirohara *et al.* 1982) the dilution ratio of milt: cryodiluent was selected 1:4 for egg-yolk citrate, urea-egg-yolk, M^a and M^b and 1:9 for Kurokura-2 and 0.9% NaCl during determination of suitable extender-cryoprotectant combination (Kumar 1988 and 1989, Magyary *et al.* 2000). Cryoprotectant concentration was maintained 10% (v/v) by volume in the trials as it was reportedly common and effective for many species (Chao *et al.* 1975, Withler 1982; Shirohara *et al.* 1982). It was observed that egg-yolk citrate and urea-egg-yolk with 10% DMSO, methanol and ethanol produced best post-thaw motility, whereas glycerol provided less motility. Although DMSO was considered as most successful cryoprotectant in many cases (Rao 1989), methanol and ethanol also exhibited satisfactory performance in terms of post-thaw motility in this experiment. When egg-yolk is used as extender it acts as an extracellular cryoprotectant. Egg-yolk improves post-thaw motility because the low density lipoprotein fraction (LDL) associates with

cell membranes and provides protection against injury during the cryopreservation process (Babiak *et al.* 2001). When DMSO is used as cryoprotectant it penetrates rapidly into the cellular membrane (Rao 1989) and brings a quick balance in between the intra and extra-cellular fluid concentration. On the other hand, glycerol gave poor performance in terms of post-thaw motility because glycerol penetrates slowly into the cell membrane (Harvey and Ashwood-Smith 1982).

Coagulation of thawed semen was common for the extenders M^a and M^b with all the cryoprotectants. Several reasons have been attributed for these clump formation after thawing the stored semen. These are improper concentration of cryoprotectant (Withler, 1982) and inadequate concentration of the semen prior to their immersion in liquid nitrogen (Chao *et al.* 1975). The inorganic extender 0.9% NaCl and Kurokura-2 with 10% DMSO exhibited low post-thaw motility. It was further deteriorated when these extenders mixed with methanol, ethanol and glycerol. The absence of egg-yolk in the cryodiluent might be responsible for this poor performance.

To determine suitable dilution ratio of milt and cryodiluent six ratios (1:2, 1:4, 1:7, 1:10, 1:15 and 1:20) were tried and among them 1:7, 1:10, 1:15 and 1:20 responded well although there were differences between them. The study also revealed that post-thaw motility of sperm was lower when sperms were diluted with the diluents at the ratio of 1:2. This finding was not always consistent with other species. In a preliminary study, walleye spermatozoa was diluted at the dilution ratios (semen: extender) of 1:5, 1:9 and 1:15, and best results were obtained at 1:15 dilution ratio (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. Another interesting finding was obtained from the present study that when milt was diluted with 1:2 ratio most of the cases post-thaw motility was poor especially for glycerol. However, glycerol exhibited exceptionally better post- thaw motility at the dilution ratio of 1:20. Since glycerol is very densed and viscous in nature its higher dilution reduces the viscosity and increases its permeability to cell, therefore glycerol in 1:20 ratio could exhibit higher post-thaw motility.

From this experiment it was observed that 7% and 10% of DMSO, methanol and ethanol could produce 80% post-thaw motility, which was highest among the six cryoprotectant concentrations. Best post-thaw motility of barramundi sperm with 5% DMSO concentration was reported by Leung (1987). Gwo *et al.* (1991) reported no significant differences in post-thaw fertility when 15% and 20% concentrations of DMSO were used to preserve Atlantic croaker spermatozoa. No post-thaw motility was observed in case of 30% DMSO and in some cases 30% methanol and ethanol were also very detrimental. Similar assumption was postulated by Rana and McAndrew (1989) that motility could not be initiated in spermatozoa suspended in 30% and 40% methanol or DMSO. Apart from the three cryoprotectants, glycerol with low concentration was found not suitable but its 20% and 30% concentration provided best post-thaw motility. It was an agreement with the findings of Piironen (1993) who stated that 20% glycerol might provide better cryoprotection under conditions for freezing pellets of brown trout and Arctic char.

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References

Alam, M.F., 2002. Socioeconomic aspects of carp production and consumption in Bangladesh. *In:* Penman, D.J., M.G., Hussain, B.J. McAndrew and M.A. Mazid (eds). Proc. Workshop on Genetic Management and improvement strategies for Exotic carps in Bangladesh, BFRI, Mymensingh, Bangladesh, pp. 83-89.

Ali, M.L., 1998. Fishery resources development and management technique. Fish week" 98 compendium. Department of Fisheries, Ministry of Fisheries and Livestock, Government of the peoples republics of Bangladesh, Dhaka, pp. 1-10.

Babiak, I., J. Glogowski, K. Goryczko, S. Dobosz, H. Kuzminski, J. Strzezek and. E. Demianowicz, 2001. Effect of extender composition and equilibration time of fertilization ability and enzymatic activity of rainbow trout cryopreserved spermatozoa. *Theriogenology*, 56 : 177-192.

Bergeron, A., G. Vandenberg, D. Proulx, and J.L, Bailey, 2002. Comparison of extenders, dilution ratios and theophylline addition on the function of cryopreserved Walleye semen.*Theriogenology* 57:1062-1071.

Chao, N., H.P. Chen, and I. Chiuliao, 1975. Study on cryogenic preservation of Grey mullet sperm. *Aquaculture*, 5 : 389-406.

Dreanno, C., M. Suquet, , L. Quemener, J. Cosson, F. Fierville, Y. Normant and R. Billard, 1997. Cryopreservation of turbot (*Scophthalmus maximus*) spermatozoa. *Theriogenology*, 48: 589-603.

Eknath, A.E. and W. Doyle, 1990. Effective population size and rate of inbreeding in aquaculture of Indian major carps. *Aquaculture*, 85: 293-305.

Gwo, J. C., Strawn, K., Longnecker, M. T. and Arnold. C. R., 1991. Cryopreservation of Atlantic Croaker spermatozoa. Aquaculture, 94: 355-375.

Harvey, B. and M.J. Ashwood-Smith, 1982. Cryoprotectant penetration and supercooling in the Eggs of Salmonid fishes. *Cryobiology*, 19: 29-40.

Hussain, M. G. and M. A. Mazid, 2001. Genetic improvement and conservation of carp species in Bangladesh. Bangladesh Fisheries Research Institute and International Centre for Living Aquatic Resources Management. 74p.

Kumar, K., 1988. A comparative study of various extenders for cryopreservation of carp spermatozoa. *Indian J. Animal Sciences*, 58(11): 1355-1360.

Kumar, K., 1989. Studies on cryogenic preservation of carp spermatozoa. Fish Genetics in India, (Eds.) Das & Jhingram, 199-206 p.

Magyary, I., A. Dinnyes, and B. Urbanyi, 2000. Cryopreservation methods for sperm of the common carp. *In*: cryopreservation in Aquatic species. Tiersch, T. R. and P. M. Mazik, Editors. World Aquaculture society, Baton Roige, Louisiana. pp. 288-289.

Piironen J., 1993. Cryopreservation of sperm from brown trout (*Salmo trutta m. lacustris l.*) and Arctic charr (*Salvelinus alpinus* L.). *Aquaculture*, 116: 275-285.

Rana, K. J. and B. J. McAndrew, 1989. The viability of cryopreserved tilapia spermatozoa. *Aquaculture*, 76: 335-345.

Rao, K. G.,1989. Cryopreservation of carp sperm. Fish Genetics in India. pp193-198.

Shirohara, J., J.R.T. Canto and J. M.F. Rlmendras, 1982. A comparative study of various extenders for milk fish, *Chanos chanos* (For) sperm preservation. *Aquaculture*, 28 : 339-346.

Withler, F.C., 1982. Cryopreservation of spermatozoa of some freshwater fishes cultured in South and Southeast Asia. *Aquaculture*, 26: 395-398.

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