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Deep Freezing Trials of *Arius arius* (Hamilton-Buchannan) Spermatozoa

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ABSTRACT

A major technological breakthrough in the field of artificial insemination is the advent of frozen semen technology permitting the preservation of semen for several years and spreading superior germplasm. The present study attempts to conserve the male gametes of the finfish *Arius arius* of the family Aridae inhabiting the estuaries of the south west coast of India. The milt collected from the mature males of *A. arius* was processed and stored at -196°C for a period of 300 days. When fructose was mixed with citrate glycerol, the survivability was 86.2 ± 1.8% on day 0 and 83.85 ± 1.8% survivability on the 300th day of storage. Glucose + citrate glycerol combinations gave a survivability of 85.7 ± 1% on day 0 and 83.25 ± 1.2% on the 300th day of storage. In mannitol citrate + glycerol combinations, the motility on day 0 was 70 ± 1.5% and 65.9 ± 1.7% motility on the 300th day of storage at -196°C. In Tris glycerol + mannitol combinations, the motility on day 0 was 85 ± 1.9% and survivability on the 300th day of storage was 65.9 ± 1.8%. The present results show that glucose-glycerol combinations produced good fertilization rates (above 50%) and that the Tris diluent showed better fertilization than citrate diluent. The fructose + glycerol combination also achieved a level of fertilization comparable to that of glucose + glycerol. Mannitol combinations resulted in lower levels of fertilization. This study proved that cryoresistance may be improved by the introduction of substances which might improve membrane stability and energetics of cells in media used in cryopreservation and also by providing optimum freezing conditions.

KEYWORDS: Cryopreservation, Cryoprotectant, Fertility Fructose, Glucose, Glycerol, Mannitol, Motility.

INTRODUCTION

Cryopreservation is the preservation of live materials particularly the spermatozoa, ova and embryos maintained in viable condition indefinitely at very low temperature of -196°C. At very low temperatures, the cellular viability can be stored in a genetically stable form. Modern techniques for rapid freezing of gametes to very low temperature have proved successful for a variety of animals including fish. Two step freezing methods of first slow cooling at 3^{o} C/ min and second immersion in liquid nitrogen are suitable for cryopreservation (Melo and Godinho 2006). Controlled rate freezing is essential to the successful cryopreservation of eggs and embryos at low cryoprotectant concentrations (Dong *et al.* 2007). For practical reasons, liquid nitrogen (-196°C) is the most commonly used cryogen for the freezing of straws containing diluted sperm. Frozen samples are normally stored in liquid nitrogen containers held either in the vapour phase or immersed under liquid nitrogen (Basavaraja *et al.* 1998). The proper processing of sperm for cryopreservation and the high thawing velocity of frozen pellets are more important factors for good success in fertilization. The motility rate of cryopreserved semen after thawing approximately correlates with the fertilization rate. Therefore the effects of various extender components and cryoprotectants after dilution and thawing of the semen were examined by motility control and fertilization experiments (Tian et *al.* 2008).

The sperm cryopreservation in aquatic animals has not reached the stage of commercial application as seen in domestic mammals. This may partly be due to the problems related to the need for relatively large volume of sperms to fertilize the large number of eggs produced by aquatic animals and non availability of sufficient seed and spawners to produce seed at the desired time (Diwan *et al.* 1994). Unlike mammalian spermatozoa, the duration of sperm motility in fishes is short and last only for a few minutes. Consequently the opportunity for the spermatozoa to fertilize eggs is limited to a brief period (Long *et al.* 2008). Reproductive technologies are being developed to recover a population just from frozen sperm, applying further hybridization programmes of using androgenetic procedures (Babiak *et al.* 2006; Grunina *et al.* 2006). Ideally, a conservation program should include the preservation of sperm, eggs, or embryos and larvae to secure the revival of species or strains. Currently, cryopreservation techniques in fish are mostly applied to sperm (Yang and Tiersch 2009). Cryopreservation is the most efficient method for large-scale, long-term storage of important genetic materials. It extends the time offspring can be produced from individual fish, reduces the need to maintain live populations, and can prevent catastrophic

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loss of irreplaceable research lines (Meng *et al*. 2008). The present study thus aims to conserve the *Arius arius* male gametes through deep freezing at -196°C and standardize the Cryoprotectants.

MATERIAL AND METHODS

Preparation of extenders and cryoprotectants

1. Tris glycerol diluent

24.22 g of Tris hydroxy methyl amino methane and 13.6 g of citric acid were dissolved in 672 ml of double distilled water. Boiled the above solution for 3 min in water bath and cooling down to room temperature and filtered the solution. A pinch of penicillin and streptomycin were added. This was considered as the basic solution.

- 80 ml of basic solution + 0.8 g. of sugar + 7.5% glycerol + 20ml egg yolk.

2. Citrate glycerol diluent

32 g of sodium citrate was dissolved in 1000 ml double distilled water. A pinch of penicillin and streptomycin were added. This was the basic solution.

- 90 ml basic solution + 1.6 g. sugar + 7.5 % glycerol + 10ml egg yolk.
- Dilution ratio of 1:5 (one part milt and 5 parts diluent) was made following the method of Zell *et al*. (1979).

Processing the milt for Cryopreservation

In the present study, 30 min equilibration time was provided in room temperature in order to allow good penetration of the cryoprotectant into the spermatozoa (Stoss 1983). After equilibration the milt was transferred to cold handling unit in which the temperature was maintained at 5°C up to one hr. French straws of 0.5 ml capacity were used for filling the milt as straw technique which has several advantages over other packaging systems (John Edwin and Ulaganathan 1988). The entire processing was carried out in 5°C.

Freezing of straws

Freezing of straws was first done in liquid nitrogen vapour of -180°C. The freezing rack was placed on a wide mouth container especially designed for vapour freezing within about 10 min, the inside straw temperature reached -140°C. After 10 min, the straws were transferred to goblets and submerged in liquid nitrogen for storage at –196°C. The milt samples were stored for a period of 300 days. The motility of the cryopreserved milt was assessed once in 30 days interval.

Evaluation of post freeze viability of Spermatozoa

In the present study, the samples were thawed in a temperature range of 30 to 35°C by plunging the straws in warm water. After thawing, a drop of semen was placed in the centre of the slide with a drop of distilled water and covered with a cover slip. The slide was placed in stage biotherm of 37°C. 200 sperms from five random fields were counted by the help of phase contrast microscope By using the following formula the percentage viability of sperm cells were calculated.

$$\textbf{Percentage viability} = \frac{\text{Number of live sperm cells}}{\text{Total number of sperm cells}} \textbf{ x 100}$$

The rate of fertilization was assessed by estimating the percentage of egg development up to the neurula stage with the help of a dissection microscope.

Experimental design and statistical analyses

The diluted semen from matured males of *Arius arius* were packed in 0.5ml straws. For each cryoprotectant 50 straws were preserved. For each cryoprotectant a peculiar colour straw was used for easy identification. The data treatment was made on a percentage basis, two way anova analysis was used as significant tests for differentiating data. For statistical analysis mat lab Software is used.

RESULTS

When glucose was used with tris glycerol, the survivability at the 0 day was 99.2 ± 1.5% and 97.4 ± 1.9% motility was on the 300th day of storage. In fructose, tris glycerol combinations, the survivability at 0-day was 98 ± 1.8% and motility at the 300th day of storage was 95 ± 1.5%. In tris glycerol, mannitol combinations, the motility on the 0 day was 85 ± 1.9% and survivability on the 300th day of storage was 65.9 ± 1.8%. The survival showed no significant variation between different concentrations of tris glycerol and significant difference between storage

periods at 5% level (**Table 1**, **1a**).When glucose was mixed with citrate glycerol, the survivability was 85.7 ± 1% on the 0-day and 83.25 ± 1.2% on the 300th day of storage. When fructose was mixed with citrate glycerol, the survivability was 86.2 ± 1.8% on the 0-day and 83.85 ± 1.8% survivability on the 300th day of storage. In mannitol citrate glycerol combinations, the motility on the 0-day was 70 ± 1.5% and 65.9 ± 1.7% motility on the 300th day of storage at -196°C. The survival rate showed significant variation between different citrate glycerol combination and different storage periods at 5% level (**Table 2, 2a**).

In glucose tris glycerol combination, a fertilization percentage of 77 ± 5.6 in the control and 56.5 ± 3.7 on the 300 days cryopreserved semen. In fructose tris glycerol combination, the fertilization observed was 75 ± 3.5% in the control and 54.2 ± 5.3% in cryopreserved semen stored for a period of 300 days. In mannitol tris glycerol combination, the fertilization in control was 63.2 ± 3.9% and 32.3 ± 4.6% in the 300 days cryopreserved semen. The fertilization rate showed significant variation between tris glycerol combinations and significant variation between control and cryopreserved milt at 5% level (**Table 3, 3a**). When glucose was mixed with citrate glycerol, a fertilization rate of 75.3 ± 4.8% in control and 50 ± 5.2% in the cryopreserved milt stored for a period of 300 days were obtained. In fructose citrate combination, a fertilization rate of 75 ± 4.9% in control and 49 ± 3.7% was obtained with the cryopreserved spermatozoa stored for a period of 300 days. In mannitol citrate glycerol combination, a fertilization rate of 58 ± 3.9% in control and 27 ± 5.1% in cryopreserved milt stored for a period of 300 days were obtained. The fertilization rate showed significant variation between different citrate glycerol combinations and between control and cryopreserved milt at 5% level (**Table 4, 4a**).

Table 1 Percentage motility of *A.arius* spermatozoa in different Combinations of tris glycerol diluent at -196^{o}C

Storage period (days)	Combinations of tris glycerol ± Standard error		
	Glucose	Fructose	Mannitol
0	99.2 ±1.5	98 ±1.8	85 ±1.9
30	99 ±1.7	97.5 ±2.7	75 ±1.7
60	98.7 ±3.2	97.1 ±3.2	70.32 ±1.2
90	98.5 ±3.9	96.5 ±1.8	69.1 ±1.8
120	98.2 ±3.7	96.5 ±1.9	67.5 ±1.5
150	98.3 ±1.9	96.4 ±1.7	67 ±1.8
180	98.3 ±1.7	96.2 ±1.2	67 ±1
210	97.9 ±2.8	96 ±3	66.7 ±1
240	97.8 ±2.2	95.4 ±2.8	66.5 ±3.2
270	97.5 ±1.2	95 ±2.1	66.1 ±3.7
300	97.4 ±1.9	95 ±1.5	65.92 ±1.8

Table 1a Two way ANOVA showing percentage motility of *A.arius* spermatozoa in different combinations of tris glycerol diluent at -196^{o}C

Source of Variation	SS	Df	MS	F	P-value	F crit
Rows	165.8868	10	16.58868	1.8893	0.108437	2.347875
Columns	5623.61	2	2811.805	320.2391	6.48E-16	3.492829
Error	175.6066	20	8.780331			
Total	5965.103	32				

Table 2 Percentage motility of *A.arius* spermatozoa in different combinations of citrate glycerol diluent at -196^{o}C

Storage period (days)	Combinations of citrate glycerol ± Standard error		
	Glucose	Fructose	Mannitol
0	85.7 ±1	86.2 ±1.8	70 ±1.5
30	85 ±3.2	86 ±1.9	69 ±1.9
60	85 ±1.9	85.8 ±1.9	68 ±3.9
90	84.6 ±3.2	85 ±1.2	67.5 ±1.9
120	84.5 ±4	84.3 ±1.9	67 ±1.8
150	84.3 ±4	84.3 ±1.2	67 ±1.2
180	84.5 ±1.5	84.2 ±3	66.9 ±1
210	84.3 ±3	84.1 ±3	66.5 ±3
240	84 ±3	84 ±3.2	66.3 ±0.7
270	83.7 ±3.2	83.92 ±1.9	66 ±1.7
300	83.25 ±1.2	83.85 ±1.8	65.9 ±0.8

Table 2a Two way ANOVA showing percentage motility of *A.arius* spermatozoa in different combinations of citrate glycerol diluent at -196^{o}C

Source of Variation	SS	Df	MS	F	P-value	F crit
Rows	25.68133	10	2.568133	16.62255	1.36E-07	2.347875
Columns	2191.927	2	1095.963	7093.753	3.06E-29	3.492829
Error	3.089939	20	0.154497			
Total	2220.698	32				

Table 3 Fertilization rates of the control and cryopreserved milt of *A.arius* in different combinations of tris glycerol diluent

Tris glycerol combinations of	Fertilization percentage ± Standard error	
	Control milt	Cryopreserved milt
Glucose	77 ±5.6	56.5 ±3.7
Fructose	75 ±3.5	54.2 ±5.3
Mannitol	63.2 ±3.9	33.2 ±4.6

Table 3a Two way ANOVA showing fertilization rate of the control and cryopreserved milt of *A.arius* in different combinations of tris glycerol diluent

Source of Variation	SS	Df	MS	F	P-value	F crit
Rows	411.79	2	205.895	14.12013	0.066137	19.00003
Columns	847.2817	1	847.2817	58.10595	0.016778	18.51276
Error	29.16333	2	14.58167			
Total	1288.235	5				

Table 4 Fertilization rate of the control and cryopreserved milt of *A.arius* in different combinations of citrate glycerol diluent

Citrate glycerol combinations of	Fertilization percentage ± Standard error	
	Control milt	Cryopreserved milt
Glucose	75.3 ±4.8	50 ±5.2
Fructose	75 ±4.9	49 ±3.7
Mannitol	58 ±3.9	27 ±5.1

Table 4a Two way ANOVA showing fertilization rate of the control and cryopreserved milt of *A.arius* in different combinations of citrate glycerol diluent

Source of Variation	SS	Df	MS	F	P-value	F crit
Rows	524.4633	2	262.2317	54.27354	0.018092	19.00003
Columns	1128.882	1	1128.882	233.6423	0.004253	18.51276
Error	9.663333	2	4.831667			
Total	1663.008	5				

DISCUSSION

Cryoresistance may be improved by the introduction of substances which would improve membrane stability, energetics of cells into the media used in cryopreservation and also by providing optimum conditions of freezing. The serum must be diluted to an optimum range as the direct preservation of milt without any diluent results in zero fertilization rates (Padhi and Mandal 1995). In the current study, tris glycerol combination with glucose afforded maximum motility to *A.arius* spermatozoa (99 ± 3.2%).When monosaccharide glucose was added to the cryoprotectant, the post thaw survival rate of spermatozoa was increased due to the energy supplying nature of glucose. Due to the greater permeability of glycerol into the interior of cells, glucose may be carried in to the sperm cells even though it is a high molecular weight compound (Zhang *et al*. 2003).

The combination of fructose also provided the motility more or less similar to glucose combinations in *A. arius* spermatozoa (95 ± 1.5). Since fructose is a monosaccharide, it also afforded greater protection like glucose. However, the addition of polysaccharide reduced the motility score due to the membrane intactness of the cells to

the complex sugar. This reduced the protective agents in the diluents to the interior of cells. The greater permeability of glycerol may not be able to carry these substances to the interior. The decrease in percentage motility of *A.arius* (65.92 ± 1.8) in mannitol is believed to be the complex sugar interferes with the protective nature of glycerol.(Yang and Tiersch 2009).

The citrate glycerol combination fructose is slightly better than glucose, but the motility score decreased than tris glycerol (83.85 ± 1.8%) in *A. arius* spermatozoa. In mannitol combination, the citrate glycerol afforded less protection like tris diluent for both the species. It was found that the addition of glucose or simple sugars reduces the toxicity of cryoprotectants (Melo and Godinho 2006). Addition of sugars to an extender system may enhance not only the quality of the extender media, but also the duration of the storage (Ponniah *et al*., 1999). Dumont *et al*. (1992) also proved that glycerol with sucrose increases the post-thaw motility of spermatozoa. Achordoguy *et al*. (1988) showed that sugars stabilize protein and phospholipid membranes subjected to freeze and thaw. Penetration of trehalose to the interior of cells will not happen because complex sugars do not readily penetrate to the interior of cells. Current study also showed that mannitol may not penetrate into the interior of sperms because it gave poor survival rate.

Glycerol penetrates the spermatozoa, checks the rise of electrolytes and prevents mechanical damage. Glycerol prevents lipoprotein denaturizing and protects physico-chemical integrity of cells. Addition of sugars in the current study served as an additional energy source for spermatozoa. Additional sugar is utilized by spermatozoa due to increased glycolysis in chilled semen (Melo and Godinho 2006. Kari *et al*. (2007) found that the storage time at –196^{o}C did not affect the capacity of spermatozoa and Sperm stored for four years yielded higher fertilization rate similar to the first year of storage. Linhart and Billard (1992) observed fertilization rates at different storage periods in glycerol were better than dimethyl sulfoxide (DMSO). The present results showed that the glucose glycerol combinations produced good fertilization rates (above 50%) and that tris diluent showed better fertilization than citrate diluent. Fructose + glycerol combination also achieved a fertilization rate next to glucose + glycerol. Mannitol combinations recorded less fertilization. The present findings agree with the reports of Lahnsteiner (2000) that the post thaw fertility of cryopreserved semen depends on the frozen thawed semen motility. The dramatic decline in fish populations necessitates urgent action to enable gametes and embryo cryopreservation as an aid to conservation. However, high yolk content and low membrane permeabilities have frustrated their successful cryopreservation, by limiting water removal and cryoprotectant penetration (Kopeika *et al*. 2005). Retention of the genetic resource and the opportunity to ensure species survival and possible restocking depends on our ability to conserve relevant material. While the motility of frozen–thawed sperm cryopreserved with 13.3% EG (ethylene glycol), 13.3% glycerol, 13.3% MeOH (methanol) and 13.3% DMF (dimethylformamide) was less than 5%, no significant differences were observed in the motility between fresh sperm and frozen–thawed sperm cryopreserved with 13.3% DMSO (dimethyl sulfoxide), 13.3% PG (propylene glycol)(Tian *et al*. 2008). Experiments were conducted to test the feasibility of using 5-mL straws for the cryopreservation of paddlefish (*Polyodon spathula*) sperm. In experiment 1, the effects of 5% or 10% methanol as a cryoprotectant in combination with cooling times of 5 or 7 min on paddlefish sperm stored in 5-mL straws were evaluated for fertilization and hatching rates. Highest fertilization rate of 48 ± 5% (mean ± SE) and hatching rate of 47 ± 10% were observed using sperm cryopreserved with 5% methanol and a 5-min cooling time in liquid nitrogen vapors. However, fertilization and hatching rates were significantly lower with cryopreserved sperm than with fresh sperm (Horvath *et al*. 2010).

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