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Short Term Preservation of *Arius arius* (Hamilton -Buchanan) Spermatozoa

Leena Grace Beslin*

Department of Biotechnology, Gogate Jogalker College, Ratnagiri, Maharashtra, India

Abstract

Arius arius, the estuarine fish inhabiting in the south west coast of India shows signs of depletion. Thus the present study standardizes the cryoprotectant by using glycerol, DMSO, ethylene glycol and methanol. The efficiency of these cryoprotectants based on the survival rate was measured and their capability of protecting the sperms in different concentrations was also observed. The observations proved glycerol is the suitable cryoprotectant for the conservation of *Arius arius* spermatozoa.

Introduction

One of the major problems confronting the gamete biology is the change of gamete quality with time and the individual variability in their initial quality and survival. There are evidences to prove that aquatic resources particularly the commercial stocks of fish and shellfish are under serious threat due to overexploitation of certain species and environmental degradation particularly due to pollution in coastal waters. 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 (Yang and Tiersch 2009).

Freezing study consists of two steps such as the choice of a freezing medium which is harmless as possible to spermatozoa and good protection against freezing damages (Horvath et al., 2010). The efficiency of artificial insemination can be improved, either by changing the volume of sperm or by diluting the sperm. Sperm dilution which was performed in saline, tris buffer, ovarian fluid, borax buffer and freshwater has been reported to improve the percentage of fertilized eggs (Van Heerden et al., 1993). Certain key enzymes in spermatozoa are labile to freezing. Thus the ability of various cryoprotectants to stabilize membranes and proteins is necessary for the option of suitable cryoprotectants (Pegg 2007). Chow (1982) improved the cryopreservation technique by using 10% glycerol as a protective agent. The importance of using suitable cryoprotectants has been studied by screening several cryoprotectants such as glycerol, ethylene glycol, methanol and DMSO (Jeyalectumie and Subramoniam, 1989). Several cryoprotectants such as DMSO, glycerol and ethylene glycol have been used for the cryopreservation of fish sperm, since there is no universal cryoprotectant for all the species (Gwo et al., 1991) DMSO, methanol and glycerol at 1 and 1.5M concentrations were used by Rana et al. (1992). 10% cryoprotectants of ethylene glycol, dimethyl sulfoxide and methyl ether were used by İnanan and Yılmaz (2018). 10% and 20% glycerol and DMSO were used by Leena Grace (2013). 20% glycerol and 7.5% DMSO were chosen as cryoprotectants and they were examined using 0.3M glucose either with or without 10% egg yolk (Jorma piironen, 1992).

The aim of spermatozoa preservation is to keep sperms in an inactive condition by providing the milt under low temperature, thus storing the internal energy reserves (Benno Pereira, 1999).The present study thus aims to reduce the cryoprotectant toxicity and to provide the most effective and protective agents for the survival of frozen spermatozoa.

Material and Methods

Collection of milt

The matured males above 300g (20-25 cm length) used for the collection of milt. The testes of matured males were dissected out and removed. They were then blotted dry and weighed. Testis from each fish was homogenized with a glass homogenizer and the seminal plasma was suspended in modified Hank's Balanced Salt Solution (HBSS) (Way man et al., 1996).

Macroscopic evaluation of milt

The color of milt was determined visually. The density of the milt was assessed by photoelectric colorimeter method (John Edwin and Ulaganathan, 1988). pH was determined by digital pH meter.

Microscopic evaluation of milt

A drop of milt in HBSS was placed on a clean slide and activated by a drop of distilled water. The motility of fresh milt was assessed under the low power objective (100 X) of the phase contrast microscope. Motility was evaluated by assessing the number of sperms exhibited a directional upward movement after activation (Aas et al., 1991). Dead sperms were non motile and settled at the bottom. The milt showing more than 90% motility was taken for cryopreservation. Motility of spermatozoa was evaluated by the method of Guest et al. (1976). Sperm cell concentration was estimated by the standard procedure using improved Neubauer counting chamber (Sorrensen, 1979).

Standardization of cryoprotectants

For the present study four different cryoprotectants such as glycerol, DMSO, ethylene glycol and methanol were selected. For each cryoprotectant, the concentrations used were 5%, 7.5%, 10% and 12.5%. These different concentrations were mixed with HBSS and milt in the ratio of 2:1 and equilibrated for 10 minutes in room temperature for the penetration of the cryoprotectants into the sperm cell. After 10 minutes, the milt was stored in -5^{0}C ± 10C. The motility of the spermatozoa was tested between the intervals of 30 minutes up to 3 hrs. The motility was assessed by activating the spermatozoa with distilled water. The slides

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***Corresponding author:** Leena Grace Beslin, Department of Biotechnology, Gogate Jogalker College, Ratnagiri-415 612, Maharashtra, India, Tel: 02227453820, Email: drblgrace@rediffmail.com

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were kept at 37°C in the stage biotherm before counting the motile sperms.

Results

The macroscopic and microscopic qualities of milt of A. arius are presented in Table 1. The color of the milt was translucent. The sperm density of *A. arius* was 48% and the pH of the milt was 7. At '0' hour of collection, the percentage mobility of spermatozoa was 98.35±3.2. The motility duration was 4.8 ± 1.25and the spermatozoa concentration was 2. 95 ´ 109.

Table 1: Macroscopic and microscopic evaluation of the fresh milt of *A.arius*

Sl. No.	Parameter	A.arius
1	Color of milt	translucent
2	Density (% transmittance)	48
3	PH	7.5
4	Sperm count/ml	2.95 x 109
5	Motility % at '0' hour	98.35 ± 3.2
6	Motility duration (minutes)	4.8 ± 1.2

The percentage survivability of spermatozoa of *A. arius* at different concentrations of glycerol in –5 ± 10C is presented in Table 2. The motility in 5% glycerol at 0 minute was 90.5 ± 2.5% and at 180 minutes it was 78.2 ± 1.8%. In 7.5% glycerol, the percentage motility was 94.5 ± 2.7% during 0 minute and 92.8 ± 2.3% at 180 minutes of storage. When 10% glycerol was used, at 0 minute the motility was 90 ± 3.2% and at 180 minutes it was 78.3± 1.9%. In 12.5% concentration of glycerol, at 0 minute the motility registered was 89 ± 2.8% and at 180 minutes it was 73.7 ± 3.3%. The survival rate showed significant variation between different concentrations of glycerol and different storage period at 5% level (Table 2a).

Table 2: Percentage survivability of *A.arius* spermatozoa in different concentrations of glycerol at -5±1°C

Storage period (minutes)	Concentration of glycerol ± S.E			
	5%	7.50%	10%	12.50%
0	90.5 ±2.5	94.5 ±2.7	90 ±3.2	89 ±2.8
30	89.3 ±0.9	93.8 ±2.8	88 ±2.8	85 ±2.6
60	86.5 ±1.3	93.5 ±3.5	85 ±3.2	80.5 ±3.2
90	84.2 ±2.5	93.3 ±3.2	83 ±1.8	79.2 ±1.0
120	82.5 ±1.8	93 ±1.8	82 ±2.8	78 ±1.8
150	80 ±1.6	93 ±2.9	80 ±3.2	75 ±3.9
180	78.2 ±1.8	92.8 ±2.3	78.3 ±1.9	71.2 ±3.8

Table 2a: Two way ANOVA showing percentage survivability of *A.arius* spermatozoa in different concentrations of glycerol at ±1°C

Source of Variation	SS	Df	MS	F	P-value	F crit
Rows	349.4236	6	58.23726	10.64458	4.25E-05	2.661302
Columns	702.0182	3	234.0061	42.77153	2.14E-08	3.159911
Error	98.47929	18	5.471071			
Total	1149.921	27				

The results of the percentage survivability of the spermatozoa of *A. arius* in different concentrations of DMSO at –5 ± 1° C are presented in Table 3. In 5% DMSO at 0 minute, the motility was 91 ± 0.9% and at 180 minutes, it declined to 73.2 ± 3.9%. When 7.5% DMSO was used, the 0 minute motility was 90 ± 1.8% and 77.7 ± 3.3% was noticed at 180 minutes. In 10% DMSO, the survivability was 88 ± 2.8% at 0 minute and 64.3 ± 3.1% at 180 minutes. When 12.5% DMSO was used, the motility rate for 0 minute was 87 ± 1.9% and at 180 minutes it was 61.1 ± 3.5%. The survivability showed significant variation between different concentration of DMSO and different storage periods at 5% level (Table 3a).

The results of the percentage survivability of the spermatozoa of *A. arius* in different ethylene glycol concentrations at –5 ± 1o C are presented in Table 4. During the storage period of 0 minute, the motility was 88 ± 1.5% in 5% ethylene glycol and at 180 minutes, it was 71 ± 3.5%. In 7.5% ethylene glycol at 0 minute the motility recorded was 89 ± 1.9% and 73 ± 1.8% motility was observed at 180 minutes. In 10% ethylene glycol, the motility was 88.6 ± 2% at 0 minute and it was 67.8 ± 3% at 180 minutes. When 12.5% ethylene glycol was used, the percentage motility was 88 ± 1.2% at 0 minute and 64 ± 3.2% at 180 minutes of storage. The survival rate showed significant variation between different ethylene glycol concentration and at different storage periods at 5% level (Table 4a).

The percentage survivability of spermatozoa of *A. arius* in different methanol concentrations at -5 ± 10° C is presented in Table 5. When 5% methanol was used, the motility at 0 minute was 67 ± 1.7% and 43.8 ± 1.9% at 180 minutes of storage were recorded. In 7.5% methanol concentration,

Table 3: Percentage survivability of *A.arius* spermatozoa in different concentration of DMSO at -5±1°C

Storage period (minutes)	Concentration of DMSO ± S.E			
	5%	7.50%	10%	12.50%
0	91 ±0.9	90 ±1.8	88 ±2.8	87 ±1.9
30	86 ±1.8	87.2 ±1.9	85 ±2.5	84.2 ±3.2
60	85.5 ±3.2	86 ±1.8	83.3 ±2.8	80 ±3.9
90	84.3 ±1.9	85.3 ±1.9	80 ±3.9	77.3 ±2.8
120	82 ±4.2	83 ±3.2	75 ±4.2	73 ±2.9
150	77 ±1.9	79 ±2.8	70.2 ±1.9	69.2 ±3
180	73.2 ±3.9	77.7 ±3.3	64.3 ±3.1	61.1 ±3.5

Table 3a: Two way ANOVA showing percentage survivability of *A.arius* spermatozoa in different concentration of DMSO at -5±1°C

Source of Variation	SS	Df	MS	F	P-value	F crit
Rows	1156.242	6	192.707	3.59E+01	4.58E-09	2.661302
Columns	306.7657	3	102.2552	1.91E+01	8.07E-06	3.159911
Error	96.56929	18	5.36496			
Total	1559.577	27				

Table 4: Percentage survivability of *A.arius* spermatozoa in different concentration of ethylene glycol at -5±1°C

Storage period (minutes)	Concentration of ethylene glycol± S.E			
	5%	7.50%	10%	12.50%
0	88 ±1.5	89 ±1.9	88.6 ±2	88 ±1.2
30	86.2 ±1.8	87.9 ±3	85.3 ±2.8	86 ±3.2
60	85 ±0.9	84.3 ±3.2	83.8 ±2.6	82.8 ±4
90	82 ±1.8	83.1 ±1.9	80.1 ±1.9	79.1 ±3
120	80.1 ±3.4	80 ±1.3	74.3 ±1.2	70.2 ±1
150	74.2 ±1.5	76.2 ±2.3	70.2 ±1.5	67.1 ±1.5
180	71 ±3.5	73 ±1.8	67.8 ±3	64 ±3.2

Table 4a: Two way ANOVA showing percentage survivability of *A.arius* spermatozoa in different concentration of ethylene glycol at -5±1°C

Source of Variation	SS	Df	MS	F	P-value	F crit
Rows	1314.117	6	219.0195	58.3558	8.03E-11	2.661302
Columns	114.5754	3	38.19179	10.17586	0.000382	3.159911
Error	67.55714	18	3.753175			
Total	1496.25	27				

Table 5: Percentage survivability of *A.arius* spermatozoa in different concentration of methanol at -5±10°C

Storage period (minutes)	Concentration of methanol ± S.E			
	5%	7.50%	10%	12.50%
0	67 ±3	65 ±1.2	61 ±1.3	60 ±1
30	60 ±1	60 ±3.2	53 ±1.5	51 ±1.5
60	53.2 ±1.8	54.2 ±3	45 ±1.8	45 ±2
90	50.3 ±2.2	50.1 ±3.8	44 ±1.6	43.1 ±3
120	45.7 ±2.8	44.6 ±2.9	40.3 ±1.7	40 ±0.7
150	44.8 ±1.9	42 ±0.9	36.8 ±1	38.2 ±1.9
180	43.8 ±1.7	40 ±0.7	34.5 ±1.9	33.9 ±1.7

Table 5a: Two way ANOVA showing percentage survivability of *A.arius* spermatozoa in different concentration of methanol at -5±10°C

Source of Variation	SS	Df	MS	F	P-value	F crit
Rows	1936.889	6	322.8149	247.1498	2.76E-16	2.661302
Columns	328.1268	3	109.3756	83.73887	9.12E-11	3.159911
Error	23.51071	18	1.306151			
Total	2288.527	27				

the motility was 65 ± 1.2% at 0 minute and 40 ± 0.7% survivability at 180 minutes. In 10% methanol, the survivability was 61 ± 1.3% at 0 minute and 34.5 ± 1.9% at 180 minutes. When 12.5% methanol was used, the motility 60 ± 1% at 0 minute and 33.9 ± 1.7% survivability at 180 minutes of storage. The survival rate showed significant variation between different concentrations of methanol and different storage periods at 5% level (Table 5a).

Discussion

Attempts to preserve living cells in a frozen state had inspired the idea that vital processes can be suspended at low temperature. It is a fact that the act of freezing produces destructive effects. The damages are caused by internal ice crystal formation which often affects the structure of the spermatozoa. Thus it is necessary to withdraw the solute concentration as water from the suspended medium. This could be achieved by suspending the sperm in an ideal extender which affords cryoprotection during freezing (Leena Grace, 2013).

Osmotic pressure is the major factor of the diluent which influences the survival of cells. When osmotic pressure increases, it leads to the death of all the cells. HBSS was used successfully frozen the spermatozoa of Crassostrea virginica (Zell et al., 1979). In the present study, Hank's phosphate buffered salt solution (HBSS) was used as the extender to dilute the spermatozoa with different cryoprotectants.

The semen must be diluted to an optimum range as the direct preservation of milt without any diluent results in zero fertilization rate (Padhi and Mandal, 1995). In general glycerol was reported as the best cryoprotectant for mammalian and marine fish spermatozoa (Scott and Baynes, 1980). In the present study glycerol gave the highest survival rate and the percentage motility was 95.5 ± 1.5 proving that 7.5% glycerol was the best cryoprotectant for A. arius sperms. The protection by glycerol is partially due to its ability to penetrate the membranes. This permeability of glycerol stabilizes internal membranes and minimizes osmotic stress. According to İnanan and Yılmaz (2018) concentration of glycerol between 5 and 10% at ambient temperature produces optimal results. Glycerol gave better protection to sperms than DMSO. However, the protective ability of both cryoprotectants was related to the concentration (Pegg 2007).The above findings were supported by the present study in which 7.5% gave the high score of motility.

DMSO performed better next to glycerol in the present study. 7.5% DMSO achieved 77.7 ± 3.31% survivability in A. arius. For salmonids and other freshwater fishes, DMSO acted as a best cryoprotectant (Erdahl and Graham, 1980). A lesser cryoprotective character of DMSO than glycerol was reported by Serafini and Marrs (1986). The present study supported the views of these authors by protecting the sperms next to glycerol. However, DMSO is toxic to the cells. It is detrimental to the embryo produced from the cryopreserved milt containing DMSO (John Edwin and Ulaganathan, 1980). DMSO requires lesser concentration and shorter pre treatment (Leena Grace 2013).

In the cryopreservation of carp spermatozoa, a combination of DMSO + sucrose gave better results than DMSO alone (Yang and Tiersch 2009). It was found that the addition of trehalose or glucose resulted in reducing the toxicity of DMSO to embryos (Horvath et al., 2010).

Ethylene glycol ranked third in the preservation of spermatozoa. The concentration of ethylene glycol had the significant role in the preservation of sperms. For *A. arius* spermatozoa, 5% and 7.5% ethylene glycol afforded more or less a similar protection and the motility percentage was 71 ± 3.5 and 73 ± 1.8% respectively. The ethylene glycol gave only limited success for the sperms of salmonids, carp and cod (De Baulry et al., 1997; Horvath and Urbanyi, 2000). The presented study also gave limited protection while comparing glycerol and DMSO. However, Erdahl and Graham (1980) reported good survivability score with ethylene glycol for the preservation of rainbow trout. Gwo et al. (1991) found ethylene glycol was superior to DMSO. But in the current study, the DMSO was found to be superior to the ethylene glycol.

Methanol was the least protective agent for the preservation of *A.arius* spermatozoa. During the short term storage, the motility percentage decreased considerably when the concentration of methanol was increased and the motility was 43.8 ± 1.7% for *A. arius* at 5% methanol. The above results proved that methanol was not suitable for long term storage of fish spermatozoa. The current study is in agreement with the suggestions of Kuldeep et al. (1995) that the methanol was toxic to the sperm cells even at low concentrations. Zhang and Rawson (1992) however pointed out that methanol was more effective than DMSO and ethane diol for zebra fish embryo cryopreservation under frozen circumstances. The post thaw motility rate of sperm of zebra fish protected by methanol was higher than that of DMSO or glycerol (Harvey et al., 1982). Thus from the present findings 7.5% glycerol was a superior cryoprotectant in frozen technology.

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