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Research Article
Extender and Cryoprotectant Assessment to Maximize the Competence of Cryostorage in *Glossogobius giuris* (Hamilton-buchanan) Spermatozoa

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Abstract

Background and Objective: The species which are found only in fewer numbers is believed to be endangered and the conservation of these rare organisms turns out to be highly imperative. The present study elucidates the conservation of male gametes of *Glossogobius giuris* through the cryopreservation technique. **Material and Methods:** The milt from matured males was collected and processed by the addition of two diluents such as tris glycerol and citrate glycerol along with additional sugars namely glucose, fructose and mannitol. Then the spermatozoa with cryoprotectants were processed and stored in liquid nitrogen for a period of 300 days. The liquid nitrogen protects the spermatozoa due to its deep freezing nature at -196°C. **Results:** The survivability of tris glycerol glucose combination ranged from 99±3.2-96.7±3.9% during the storage period. In tris glycerol fructose combinations, the motility range was 99.1±1.9 and 95.9±0.9%. The observations of motility and fertility tests showed that the tris glucose-glycerol and fructose-glycerol combinations proved to be superior cryoprotectants. Survivability of mannitol with citrate glycerol was 75±1.2 and 65±3.7%. In the current study this proved the least motility score. **Conclusion:** From this study it was suggested that glycerol glucose and fructose in tris combinations gave maximum protection to the finfish *Glossogobius giuris* sperms. The buffer citrate glycerol also performed well next to tris glycerol. These were documented to be non-toxic to fish milt.

Key words: Cryopreservation, motility, glycerol, mannitol, glucose, fructose, cryoprotectant and fertility

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Competing Interest: The author has declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The indigenous fish production decreases due to a variety of factors such as diseases, habitat loss as a result of human interaction like urbanization, environmental degradation, pollution, over-exploitation, etc. Due to this cryopreservation of fish sperm and the development of genetic selection for protecting valuable gametes has been gaining demand. Techniques of cryopreservation applied to aquatic organisms have direct application to aquaculture and conservation of threatened species as well as genetically modified or improved eggs and embryos[1]. At very low temperatures (-196°C) the cellular viability can be stored in a genetically stable form. Modern techniques for rapid freezing of gametes to very low temperatures have proved successful for a variety of animals including fish[2]. 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 also limited to a brief period[3]. The fish gamete cryopreservation protocols of the rainbow trout reviewed 14 extenders with the fertilizing ability durable for 2 years[4]. Babiak *et al.*[5] obtained 90.6% hatching rate on fertilization using cryopreserved semen. New approaches in the cryopreservation of fish embryos exploration obtained 83.4 and 78.9% fertilization rates for the spermatozoa conserved for one year and eight months in different cryoprotectants[6]. The sperm physiology and quality studies reported that the post-thaw motility as similar to that of the fresh semen[7].

Extender with egg yolk proved to be the most efficient medium which provides 74.5% of maximum hatching rate for the fish sperm[8]. Since there is no universal cryoprotectant for all the species, several cryoprotectants such as DMSO, glycerol and ethylene glycol were used for the cryopreservation of fish sperm[9], DMSO, methanol and glycerol at 1 and 1.5 M concentrations were used in different trials[10]. Cabrita *et al.*[11] studied the effect of external cryoprotectants successfully stored certain cultivable marine fish spermatozoa for 240 days using simple diluents like sodium citrate, NaCl, KCl, CaCl, $MgSO_4$ and marine ringer solution.

The cryopreservation of gametes of few aquatic animals has met with success when compared with the situation in other terrestrial animals and the practical application was achieved in mammals[12]. Many aquatic organisms were endangered in the natural environment due to habitat destruction and pollution stress[13]. Permanent storage of gametes paves the way to preserve the stalk of these endangered aquatic species. The present study thus aimed to conserve the male gametes through deep freezing at -196°C and standardize the cryoprotectants for *Glossogobius giuris* spermatozoa which are reported to be least concern in the Western Ghats of India

MATERIAL AND METHODS

Study area: The present study was carried out from January-October, 2018. The period of study was a total of 10 months. The site selected for the current study was the Thiruvananthapuram area at Kerala coast of India.

Collection of experimental fishes: Experimental animals were collected from the Veli Lake in the southwest coast of India. Twenty matured males above 300g (20-25 cm length) were used for the collection of milt. Females were maintained in separate tanks for the test of fertility. Three replicates were used in experimentation.

Collection of milt: The matured males were smoothly caught using hand nets. The selected fishes were blotted dry and weighed. By abdominal massage, the milt was collected and homogenized with a glass homogenizer. The seminal plasma was suspended in modified Hank's Balanced Salt Solution (HBSS)[14].

Microscopic evaluation of milt: For activation, a drop of milt with HBSS was taken on a clean slide and a drop of distilled water was added. Fresh milt's motility was assessed under the low power objective (100 X) of the phase-contrast microscope. The number of sperms exhibited a directional upward movement after activation evaluated motility score[15]. Nonmotile sperms settled at the bottom were dead ones. The milt having more than 90% motility score was taken for further cryopreservation. To get the average count, Computer Assisted Semen Analysis (CASA) and the manual method of counting were used. The motility of spermatozoa was evaluated by the method of Diwan *et al.*[16].

Dilution of milt for deep freezing: Undiluted semen is not suitable for freezing and so it has to be diluted with a suitable extender. Dilution of milt was carried out for limiting the number of spermatozoa during motility assessment and minimizes the number of sperms in fertilization[17]. In the water medium concentration of sperms enhance successful fertilization because it is diluted in the water current but in artificial fertilization, we should limit the sperms to avoid the freezing shock during long-term storage of spermatozoa in liquid nitrogen and for the reason of conservation. Direct immersion of spermatozoa creates mass mortality of cells due

to non-acclimatized frozen environment. So by the addition of suitable diluents and cryoprotectants the processed milt was diluted in the present study[18].

Extender and cryoprotectant preparation

Tris glycerol diluents: The basic solution was prepared by adding 13.6 g of citric acid and 24.22 g Tris hydroxymethyl aminomethane were dissolved in 672 mL of double distilled water. It was boiled for 3 min in a water bath and cooled down to room temperature and the solution was filtered. A pinch of penicillin and streptomycin were added:

- 0.8 g glucose+7.5% glycerol+20 mL egg yolk+80 mL of basic solution
- 0.8 g fructose+7.5% glycerol+20 mL egg yolk+80 mL basic solution
- 0.8 g mannitol+7.5% glycerol+20 mL egg yolk+80 mL basic solution

Citrate glycerol diluents: Basic solution was prepared by dissolving 32 g sodium citrate in 1000 mL double distilled water. A pinch of penicillin and streptomycin were added:

- 1.6 g glucose+7.5% glycerol+10 mL egg yolk+90 mL basic solution
- 1.6 g fructose+7.5% glycerol+10 mL egg yolk.+90 mL basic solution
- 1.6 g mannitol+7.5% glycerol+10 mL egg yolk+90 mL basic solution

All the above solutions were mixed gently and warmed about 40°C before adding into the milt.

Dilution of milt with the diluents: Dilution was made by adding 5-6 mL of the diluent into the milt and then poured the whole content of the milt back into the conical flask containing the diluent at room temperature. Dilution was done by adding one part milt and 5 parts diluents[19].

Processing the diluted milt for Cryopreservation

Equilibration of milt: In the present study, 30 min equilibration time was afforded at room temperature in order to allow good penetration of the cryoprotectant into the spermatozoa[18]. It was found that the milt stored for a few hours before freezing gives better post thaw motility and fertility. This pre-freeze storage period is termed as equilibration time.

Cooling of milt: For avoiding cold shock, the milt should be diluted and cooled gradually to 5°C. Slow cooling is required for enhanced survivability of spermatozoa. Then the milt was transferred to cold handling unit in which the temperature was maintained at 5°C up to one hour[20]. So the cooling rate of 5°C was attained to milt after the addition of cryoprotectants.

Packaging of milt: In the present study, straw technique was followed which has several advantages such as uniform capacity, protection and no contamination over other packaging systems. French straws of 0.5 mL capacity were used for filling the milt[21]. Different colored straws were used for different combinations of cryoprotectants for easy identification. The straws were arranged swiftly on the freezing rack. Filling of milt and sealing of straws were done by the french type single straw filler and sealer. The entire processing was carried out in 5°C[21].

Freezing of straws: Direct immersion in liquid nitrogen is normally lethal to fish sperm and so gradual cooling is necessary to attain best post thaw viability[22]. Freezing of straws was first done in liquid nitrogen vapor at-180°C. The freezing rack was placed on a wide mouth container particularly planned for vapour freezing[23]. Within about 10 min, the inside straw temperature reached-140°C. After that the straws were shifted to goblets and put in liquid nitrogen for storage at-196°C. The milt samples were stored for 300 days. Motility of the cryopreserved milt was evaluated once between 30 days.

Thawing of straws: In order to prevent cell injury by water recrystalization at warmer temperature thawing must be conducted quickly[24]. The samples were thawed in a temperature range of 30-35°C by dipping the straws in warm water for 5 min.

Evaluation of post freeze viability of Spermatozoa: After thawing the samples in 35°C, they were transferred into the test tube by cutting both ends of straws. A drop of semen was placed in the center of the slide with a drop of distilled water and covered with a cover slip. The slide was first placed in stage biotherm of 37°C. Then 200 sperms from five random fields were counted by the help of phase-contrast microscope and CASA examination[19]. By using the following formula the percentage viability of sperm cells was calculated:

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

Fertility studies: Mature females of *Glossogobius giuris* were induced to spawn by injection of 0.3 mL kg^{-1} body weight of ovaprim. After 12 hrs, eggs were collected in glass petri dishes by abdominal pressing. An average of 100 eggs was deposited in each beaker. 0.5 mL of cryopreserved milt after thawing was mixed with the eggs gently. After thorough mixing, water was added to the beaker and the eggs were washed well by changing the water for every 30 min for oxygenation[24]. The fertilization test was also conducted with the control by the addition of fresh milt. Following fertilization, the eggs were allowed to harden in water. The rate of fertilization was assessed by estimating the percentage of egg development up to the neurula stage after 20 sec during the observation of optic vesicles with the help of a dissection microscope[25].

RESULTS

In tris glycerol glucose combination on the 0 day, the survivability was 99±3.2 and 96.7±3.9% on the 300th day of storage. In tris glycerol fructose combinations, the motility at 0 day was 99.1±1.9% and on the 300th day, it was 95.9±0.9%. When mannitol was mixed with tris glycerol the survivability of the spermatozoa of *G. giuris* on the 0 day was 80±2.3% and the motility on the 300th day of storage was 66.3±2.8%. In glucose citrate glycerol combinations, the motility on the 0 day was 95.5±3 and 93±0.7% motility on the 300th day of storage. When fructose was mixed with citrate glycerol, the motility on the 0 day was 95±1.9 and 92.43±1.9% was on the 300th day of storage. In mannitol with citrate glycerol, the survivability on the 0 day was 75±1.2 and 65±3.7% motility on 300th day at -196°C (Table 1).

Glucose with tris glycerol showed 73±3.9% fertilization in control and 53±4.9% fertilization in the cryopreserved sperm stored for 300 days. In fructose tris glycerol combinations reported 70±4.7% fertilization in control and 50.7±4.6% fertilization in the cryopreserved milt stored for a period of 300 days (Table 2). In mannitol tris glycerol combination, 60.3±5.2% fertilization in control semen and 30.7±4.8% fertilization were recorded in the cryopreserved milt preserved for 300 days. When glucose was mixed with citrate glycerol, it gave a fertilization rate of 70±4.7% in control and 48.3±4.6% in the cryopreserved milt stored for 300 days. In fructose citrate glycerol combination, a fertilization rate of 69±3.9% in control and 45±3.8% in the cryopreserved spermatozoa stored for 300 days was obtained. In mannitol-citrate glycerol combinations, a fertilization rate of 52±4.8% in control and 24±3.9% was noticed in

Table 1: Percentage motility of *G. giuris* spermatozoa in different combinations of diluents at -196°C

Storage period (days)	Tris glucose A	Citrate glucose B	Tris fructose C	Citrate fructose D	Tris mannitol E	Citrate mannitol F
0	99.0±3.2	95.5±3.0	99.1±1.9	95.0±1.9	80.0±2.3	75.0±1.2
30	98.5±2.9	95.0±3.9	98.5±3.2	94.5±3.2	77.2±1.9	71.3±4.0
60	98.4±3.2	95.0±3.7	98.0±4.0	94.4±3.0	76.0±3.0	70.0±1.9
90	98.3±1.9	94.7±2.6	97.7±1.8	94.0±2.8	74.0±1.2	69.3±3.7
120	98.2±1.3	94.2±3.7	97.0±1.5	93.9±3.9	70.3±3.0	69.0±2.2
150	98.0±1.9	94.0±3.0	97.0±1.9	93.5±2.7	68.3±3.0	68.5±2.0
180	97.9±3.0	93.9±2.7	96.8±1.5	93.5±3.9	68.0±3.2	68.0±0.9
210	97.8±1.5	93.5±2.9	96.5±3.9	93.3±3.2	67.0±3.0	67.92±0.8
240	97.5±1.9	93.3±2.0	96.3±3.2	93.12±0.7	67.0±1.2	67.0±0.3
270	97.0±3.0	93.2±1.8	96.0±3.9	92.95±0.8	66.5±2.2	65.8±1.8
300	96.7±3.9	93.0±0.7	95.9±0.9	92.43±1.9	66.3±2.8	65.0±3.7

Table 2: Fertilization rate of the control and cryopreserved milt of *G. giuris* in different combinations of tris glycerol diluent at -196°C

	Fertilization percentage±S.E	
Tris glycerol	Control milt	Cryopreserved milt
Glucose	73.0±3.9	53.0±4.9
Fructose	70.0±4.7	50.7±4.6
Mannitol	60.3±5.2	30.7±4.8

Table 3: Fertilization rate of the control and cryopreserved milt of *G. giuris* in different combinations of citrate glycerol diluents

	Fertilization percentage±S.E	
Citrate glycerol	Control milt	Cryopreserved milt
Glucose	70.0±4.7	48.3±4.6
Fructose	69.1±3.9	45.0±3.8
Mannitol	52.0±4.8	24.0±3.9

Table 4: Tukey HSD statistical analysis to prove the significance of *G. giuris* spermatozoa in different combinations of diluents at -196°C

Treatments pair	Tukey HSD Q statistic	Tukey HSD p-value	Tukey HSD interference
A vs B	1.2362	0.8999947	Insignificant
A vs C	2.1366	0.6391107	Insignificant
A vs D	1.1099	0.8999947	Insignificant
A vs E	5.6088	0.0026057	**p<0.01
A vs F	6.2479	0.0010053	**p<0.01
B vs C	0.9003	0.8999947	Insignificant
B vs D	0.1263	0.8999947	Insignificant
B vs E	6.8451	0.0010053	**p<0.01
B vs F	7.4841	0.0010053	**p<0.01
C vs D	1.0266	0.8999947	Insignificant
C vs E	7.7454	0.0010053	**p<0.01
C vs F	8.3845	0.0010053	**p<0.01
D vs E	6.7187	0.0010053	**p<0.01
D vs F	7.3578	0.0010053	**p<0.01
E vs F	0.6391	0.8999947	Insignificant

**Highly significant, A-F is given in Table 1 , A: Tris glucose, B: Citrate glucose, C: Tris fructose, D: Citrate fructose , E: Tris mannitol, F: Citrate mannitol

cryopreserved semen stored for 300 days (Table 3). The statistical significance between different diluents and cryoprotectants were interpreted in Table 4. The statistical data analyzed by Turkey's HSD test proved that tris mannitol and citrate mannitol performed least and it showed highly significant variation between all other cryoprotectants used in the present study.

DISCUSSION

Organic buffer Tris hydroxymethyl amino methane prolonged the life of sperms at room temperature which acted to be an intracellular buffer and less toxic in the critical temperature during freezing[22]. Optimum pH was adjusted by manipulating the concentration of the buffer salts. The next diluent used in the present study was sodium citrate and the optimum pH was adjusted by the addition of citric acid to 7.4 which also preserved the sperms by arresting the metabolic activity[23]. The optimum concentration of egg yolk and antibiotics such as penicillin and streptomycin were also used to control pathogens. The most effective means of protecting spermatozoa against the effects of cold shock is by providing lecithin, proteins, lipoproteins and similar compounds found in egg yolk or milk[24].

In the current study, tris glycerol combination with glucose gave maximum motility to *G. giuris* spermatozoa (96.7±3.9%). Due to the energy supplying nature of glucose, the post-thaw survival rate of spermatozoa was increased. Glucose serves as a most important source of energy for metabolic processes in mammalian cells. Since polar molecules cannot be transported across the plasma membrane, carrier proteins called glucose transporters are needed for cellular uptake[25]. Since fructose is a monosaccharide, it also afforded greater protection (95.9±0.9%) like glucose. However, the addition of polysaccharide reduced the motility score due to the membrane intactness of the cells to the complex sugar. The significance was manifested in the present study by turkey's test. The greater permeability of glycerol may not be able to carry these substances to the interior. The decrease in percentage motility (66.3±2.8) in mannitol was suggested to be the complex nature of sugar interferes with the protective nature of glycerol[26].

The citrate glycerol performed well in glucose combination as like the tris diluent (93±0.7%). Fructose combination also showed a similar survival rate (92.43±1.9%). In mannitol combination, the citrate glycerol afforded less protection like tris diluent. Addition of sugars to an extender system may enhance not only the quality of the extender media but also the duration of the storage[27]. Grace[19] showed that complex sugars do not readily penetrate to the interior of cells. The current study also showed that mannitol may not penetrate into the interior of sperms and it gave poor protection and survival rate.

The minimum equilibration period is enough for the penetration of cryoprotectant into the interior of sperm cells owing to the minute size of the sperm[24]. The preserved samples were thawed by immersion in a water bath of 30-35°C which provided the warming rate of approximately 10°C/min. Cabrita *et al.*[11], also thawed the spermatozoa in 30°C and obtained good motility score for six species of freshwater finfishes. Horvath *et al.*[20], thawed the straws in a water bath at 35°C for 5-6 sec and reported good fertilization rate. In the present study the insemination with straw frozen milt was attempted after thawing in 30-35°C[25]. The developmental stages before gastrulation were the sensitive stage, so the rate of fertility was noted in the neurula stage[27]. The present findings agreed with the reports of Grace[18] which showed that the post-thaw fertility of cryopreserved semen depends on the frozen-thawed semen motility. The present investigation achieved the cryopreservation success with the selected *G. giuris* in glycerol fructose combinations.

CONCLUSION

The current study accomplished the cryopreservation success of *G. giuris* Spermatozoa. It is concluded that tris glucose-glycerol and fructose-glycerol combinations proved to be superior cryoprotectants to this technique. The citrate

glycerol combinations also performed well next to tris diluents. Statistical insignificance between these two diluents also proved both are better ones. The optimum conditions and concentrations confirmed by the present investigation also suggested basic concentration of diluents also to germplasm conservation of other finfishes.

SIGNIFICANCE STATEMENT

This study discovered the diluents and cryoprotectants suitable for the selected finfish that can be beneficial for the fish farmers and innovators of the conservation programs. This study will help the researchers to uncover the critical areas of deep-frozen technology regarding spermatozoa preservation of finfishes that many researchers were not able to explore. Thus a new scope on the preservation of aquatic organisms may be indoors in the near future

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