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The Intracellular Cryoprotectant Effects in Preserving Goramy Spermatozoa after Two Days Sub-Zero Freezing

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Abstract

Abinawanto Abinawanto, Nisa Fitrianingrum, Retno Lestari, Agung Sudaryono, Rita Rostika, and Yushinta Fujaya. 2015. The Intracellular Cryoprotectant Effects in Preserving Goramy Spermatozoa after Two Days Sub-Zero Freezing, *Aquacultura Indonesiana, 16 (1): 16-21*. The spermatozoa quality of goramy after 2 d sub-zero freezing was examined. The quality of spermatozoa examined included motility, viability, and abnormality. We aimed to determine the optimum concentration of glycerol protecting spermatozoa during preservation. We used 0%, 1%, 3%, 5%, 7%, and 9% of glycerol, respectively. Sperms were diluted by the combination of glycerol and fish ringer (1 part of sperm + 3 part of solvent). The dilute sperms were then equiliberated at 4°C for 45 min, and were freezed at -34°C for 2 d. Thawing was then carried out at 30°C for 2 min. Based on Dunnet test, 5% of glycerol was the optimum concentration maintaining spermatozoa motility (75.95±4.76)%.

Keywords: Glycerol; *Osphronemus goramy;* Spermatozoa motility; Sub-zero freezing; Viability and Abnormality

Introduction

According to Sunarma *et al.* (2007), *Osphronemus goramy* is very important commodity of the local freshwater fish in Indonesia. However, goramy fish aquaculture is still traditionally, and cause declining in genetic material quality (Alam *et al.*, 2002). Accordingly, cryopreservation effort is needed as an alternative method for preserving the quality of genetic materials such as spermatozoa, ovum and embryos under low temperature condition (Fickle *et al.*, 2007). Research on cryopreservation of fish spermatozoa has been reported such as in salmon (Kusuda *et al.*, 2005) and catfish (Urbanyi *et al.*, 1999). Cryoprotectant and extender are needed during cryopreservation. Christensen and Tiersch (2005) were reported that glycerol, Dimethyl Sulphoxide (DMSO), Propilene Glycol, and methanol can be used as the cryoprotectant. While, fish ringer have been used as the extender (Park and Chapman, 2005). Horvarth and Urbanyi (2000) have administered glycerol and fish ringer to preserve spermatozoa of *Clarias gariepinus*. Whereas, Kyoung Ho Kang *et al.* (2004) have used 5%, 10%, 15%, and 20% of glycerol for preserving spermatozoa of *Thamnaconus septentrionalis*. On the other hand, Muchlisin *et al.* (2004) have studied the combination effect of 5%, 10%, and 15% of glycerol and fish ringer on *Mystus nemurus* spermatozoa under low temperature. Accordingly, the studies of cryopreservation using variation of cryoprotectant and extender are very important.

Studies of cryopreserved goramy spermatozoa have been reported. Dimethyl Sulfoxide (DMSO) has been used as the cryoprotectant for preserving goramy spermatozoa in liquid nitrogen for 24 h (Abinawanto *et al.*, 2011). Besides, sucrose could also be used as the extender and cryoprotectant (Abinawanto *et al.*, 2012[a]). It has been reported by Abinawanto *et al.* (2012[b]) that skim milk play an important role as the extracellular cryoprotectant. However, currently lack information is available on the glycerol effect as the intracellular cryoprotectant in preserving goramy spermatozoa after 2 d sub-zero freezing.

The aim of this study was to evaluate the intracellular cryoprotectant effects (motility, viability, and abnormality) at the optimum concentration of glycerol as the cryoprotectant.

Materials and Methods

The study was conducted in June 2010 – November 2010, at the Genetics Laboratory, Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok, West Java Provinces, Indonesia

Fish Preparation

Twenty four mature males of *Osphronemus goramy* were bought from the private commercial hatchery, Parung-Bogor, West Java, Indonesia. All fishes were acclimatized for 14 d in one-4000-L square concrete cement fish pond till they attain 2.5-4 kg size in the indoor Aquatic Biology laboratory, Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok, West Java Province. The fishes were grouped of 6 fishes and were stocked in 7 square concrete cement fish pond (1000-L). The fish pond were equipped with closed re-circulation system and a black plastic mesh lid to minimize disturbances and prevent fish from jumping out. Six experimental group based on glycerol concentration were assigned to four times replication in a completely randomized design. Fishes were fed with the commercial diet and leaf of *Allocasia macrorrhiza* two times daily *ad libitum* at 08:00 and 17:00.

Fish ringer preparation

Fish ringer solution was prepared according to the method of Ginzburg (1972). A fish ringer stock solution was prepared by dissolving 3.25 g NaCl; 0.125 g KCl; 0.175 g $CaCl_2.2H_2O$; and 0.1 g $NaHCO_3$ with aquabidest up to 500 mL. The extender fish ringer solution was then kept at 4°C.

Activator solution preparation

The activator solution was prepared based on the method of Perchec *et al.* (1995) by diluting 45 mM NaCl, 5 mM KCl, and 30 mM Tris with aquabidest up to 100 mL

0.5% Eosin-Y solution preparation

The 0.5% of Eosin-Y solution was prepared according to the method of WHO (1988), by diluting 0.5 g of the Eosin-Y with aquabidest up to 100 mL.

0.15M of Phosphate buffer solution pH 6.8 preparation

The Phosphate buffer solution was prepared by dissolving 5.34 g $Na_2HPO_4.2H_2O$ with aquabidest up to 200 mLl, and by dissolving 4.08 g KH_2PO_4 with aquabidest up to 200 mLl. $Na_2HPO_4.2H_2O$ solution was then added to KH_2PO_4 solution until the pH reach 6.8. The Phosphate buffer solution was then kept at 4°C.

Giemsa solution preparation

The Giemsa solution was prepared based on WHO (1988), by diluting one part of the Giemsa stock solution and 10 parts of the Phosphate buffer solution pH 6.8. The mixed solution was then filtered by Whatman filter paper number one.

Collection of the ejaculated sperm/semen

The sperm was collected by hand stripping method, 12−15 h after injected intra-muscularly with GnRH-analog (Ovaprim Syndel) at a single-dose of 0.2 mL/kg body weight according to modification method of Sunarma *et al*. (2007), and was put in 1.5 mL of cryotube.

Semen/sperm Dilution

According to Akcay (2004), ratio among the ejaculated semen/sperm and the solvent was 1: 3. The sperm was added to the solvent solution in the cryotube. The compostition of each component of the solvent solution and the ejaculated sperm was seen in Table 1.

Table 1. Composition of Semen/sperm, Fish ringer and glycerol (G) of experimental group

Composition	*Experimental group*					
	C	*1%G*	*3%G*	*5%G*	*7%G*	*9%G*
Semen/Sperm (µL)	50	50	50	50	50	50
Fish Finger Solution (µL)	150	148	144	140	136	132
Glycerol/G (µL)	0	2	6	10	14	18

* C= control group; ** 1% G, 3% G, 5% G, 7% G, 9% G = treatment group.

Semen/sperm Equilibration

The diluted sperm was then equilibrated at 4°C for 45 min (Akcay *et al*., 2004 and Bozkurt *et al.*, 2005).

Semen/sperm Freezing

The diluted sperm was freezing at -34°C for 48 h (Changjiang Huang *et al.*, 2004).

Semen/sperm Thawing

The frozen sperm was incubated at 30°C for 2 min. (Akcay *et al*., 2004 and Bozkurt *et al.*, 2005).

Semen/sperm Evaluation and Spermatozoa Analyses

Fresh semen/sperm color was observed by visual, whereas sperm volume was measured by the cryotube with scale. Fresh sperm pH was measured by standardized pH paper (pH range 5-10). The parameter of the spermatozoa analyzed were spermatozoa viability, abnormality, and motility, respectively. All of the spermatozoa analyses parameters were observed under trinocular microscope (Boeco) equipped with the digital eye piece camera (MDCE-5a). This microscope was connected to the computer equipped by the image driving software (Scopephoto 2.0.4). Spermatozoa motility was analyzed by subjective method (Rurangwa *et al*. (2004), whereas spermatozoa viability and spermatozoa motility were analyzed by Salisbury and VanDemark method (1985).

Data analysis

All data were analyzed by Kruskal-Wallis and Dunnet's multiple comparison test (Zar, 1974) using a statistic program of SPSS version 13 for Windows. All probability values were set at 0.05 level of significance.

Results

Fresh semen (FS) were milky white, pH 8.03±0.05, and 0.45±0.10 mL of volume per ejaculate (Table 2). The viable or motile spermatozoa showed green color (Figure 1) on the sperm head, while the non-viable spermatozoa showed pink or red color on the sperm head (Figure 1). Both of viable and non-viable spermatozoa were found in all treatment groups and control. However, percentage of spermatozoa viability were different quantitatively either among treatment groups or between control and treatment groups. On the other hand Figure 2 were shown the normal spermatozoa (A) and many variations of abnormal spermatozoa such as macrocephalus (B), microcephalus, folding tail spermatozoa (C), and broken tail spermatozoa. Either the normal or abnormal spermatozoa morphologically were also found in all treatment groups and control. However, the percentage of spermatozoa normal and abnormal were different, among treatment groups aor between control and treatment groups.

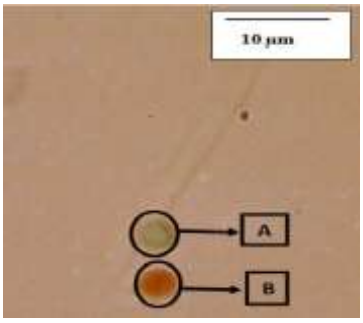

Figure 1. Spermatozoa viable (A); spermatozoa non-viable (B); 10x40 162x95 mm (96 x 96 DPI)

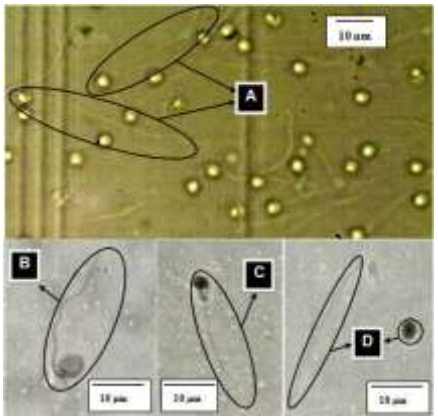

Figure 2. Normal spermatozoa (A); Macrocephalus spermatozoa (B); Folding tail spermatozoa (C); Broken tail spermatozoa (D); 10x40 162x204 mm^2 (96 x 96 DPI)

The percentage of post-thaw spermatozoa motiliy in control; and in various glycerol concentration of 1%, 3%, 5%, 7%, and 9%, were: (59.31 ± 6.38)%, (63.83 ± 4.79)%, (66.43 ± 6.82)%, (75.95 ± 4.76)%, (68.90 ± 4.65)%, and (69.93 ± 8.16)%, respectively (Table 3). Post-thaw spermatozoa viability in control; and in various glycerol concentration of 1%, 3%, 5%, 7%, and 9%, were: (71.33 ± 5.39)%, (76 ± 6.10)%, (73.83 ± 6.43)%, (75.50 ± 5.43)%, (70.50 ± 6.22)%, and (69.83 ± 14.23)%, respectively (Table 3). On the other hand, post-thaw spermatozoa abnormality in control; and in various glycerol concentration of 1%, 3%, 5%, 7%, and 9%, were: (15.33 ± 5.57)%, (19.50 ± 4.04)%, (19.17 ± 5.95)%, (14.83 ± 2.79)%, (16.50 ± 3.83)%, and (19.67 ± 3.50)%, respectively (Table 3).

Table 2. Fresh Semen/sperm evaluation and spermatozoa analyses of *Osphronemus goramy* spermatozoa, before freezing

Paramater	Color	Volume (mL)	pH	Viability (%)	Abnormality (%)	Motility (%)
	Milky white	0.45±0.10	8.03±0.05	85.16±1.94	15.67±2.16	74.12±5.26

Values are means ± SD of four replicates.

Table 3. Viability, Abnormality, and Motility of *Osphronemus goramy* spermatozoa, 48 h after sub-zero freezing

Paramater	*Experimental group*					
	C	*1%G*	*3%G*	*5%G*	*7%G*	*9%G*
Viability (%[1])	71.33±5.39[a]	76±6.10[a]	73.83±6.43[a]	75.5±5.43[a]	70.5±6.22[a]	69.83±14.23[a]
Abnormality (%)	15.33±5.57[a]	19.5±4.04[a]	19.17±5.95[a]	14.83±2.79[a]	16.5±3.83[a]	19.67±3.50[a]
Motility (%)	59.31±6.38[a]	63.83±4.79[a]	66.43±6.82[ab]	75.95±4.76[b]	68.90±4.65[ab]	69.93±8.16[ab]

Values are means + SD of four replicates. Mean values having the same superscript are not significantly different (P >0.05).

Based on Kruskal-Wallis test, there were not significant effect (P>0.05) of various concentration of glycerol on post-thaw sperm viability and abnormality, respectively, but not on post-thaw motility (P<0.05) compared to control (Table 3). According to the Dunnet test, the concentration of 5% of glycerol showed the highest post-thaw sperm motility (75.95 ± 4.76)%, although was significant different with other concentration group of 3%, 7% and 9% of glycerol, respectively.

Discussion

The concentration of 5% glycerol was shown the highest percentage spermatozoa motility (75.95 ± 4.76)%, 2 d after sub-zero freezing. This finding was similar with the previous study (Horvarth and Urbanyi, 2000) when they preserved spermatozoa of *Clarias gariepinus*. On the other hand, post thaw motility in this study was lowered compared our previous study (80.98%; Abinawanto *et al.* 2012[b]; 96.10%; Abinawanto *et al.,* 2013). However, post thaw motility in this study was higher than *Brachydanio rerio* (51%; Harvey *et al*., 1982), *Oreochromis mossambicus* (70%; Harvey, 1983), tilapian's fish (40%; Chao *et al*., 1987), *Cyprinus carpio* (55%; Akcay *et al*., 2004), *Osteochiius hasseltii* (63.33%; Sunarma *et al*., 2007), and *Osphronemus goramy* (68.58%; Abinawanto *et al*., 2011). Post thaw viability in the treatment group of 5% glycerol was 75.5 ± 5.43%, although was not significant different compared with other treatment groups and control, statistically. However, this finding was higher than previous reported in spermatozoa of *Mystus nemurus* (60%; Muchlisin *et al.,* 2004), *Cyprinus carpio* (20%; Withler, 1982; 58%; Horton and Otto, 1976), and *Osphronemus goramy* (63.5%; Abinawanto *et al.,* 2011). Post thaw viability in this study on the other hand was lowered than our previous work in *Barbonymus gonionotus* spermatozoa (77.25%; Abinawanto *et al*., 2009; 85.50; Abinawanto *et al.,* 2013). The effect of 5% of glycerol can declined the post thaw abnormality (14.83 ± 2.79)%, better than other treatment group of glycerol concentration and control group, although those results were not significant different, statistically. Our previous study showed the higher spermatozoa abnormality (29%; Abinawanto *et al.,* 2011) when using the combination of 13% of DMSO + 189M extender. Post thaw abnormality in *Barbonymus gonionotus* spermatozoa was higher (45%) when preserved in the combination of 6% of glucose + 10% of methanol (Abinawanto *et al.,* 2009). However, the post thaw abnormality showed mearly similar (14%) when the spermatozoa of *Barbonymus gonionotus* protected by the combination of 13% of egg yolk + 10% of methanol (Abinawanto *et al.,* 2013).

Either all of treatment groups or control were shown the viable spermatozoa, motile spermatozoa, and abnormal spermatozoa, visually. However, the percentage of spermatozoa viability, motility and abnormality were different among treatment groups, or between control and treatment groups. The optimum condition of the treatment group (percentage of glycerol) was shown by the lowest percentage of spermatozoa abnormality (by reduced percentage of spermatozoa abnormality) after sub-zero freezing for 2 d. Furthermore, the highest percentage of viability or motility of spermatozoa among the treatment groups or between control and treatment groups 2 d after sub-zero freezing also as the indicator of the

optimum condition of percentage of glycerol as the cryoprotectant.

Conclusion

The data obtained in the present study indicate that 5% of glycerol was the optimum concentration as the cryoprotectant for preserving *Osphronemus goramy* spermatozoa at sub-zero freezing for 2 d.

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