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Goramy spermatozoa quality after sub-zero freezing: The role of coconut water as the cryoprotectant

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Abstract. *Abinawanto, Putri PE. 2017. Goramy spermatozoa quality after sub-zero freezing: The role of coconut water as the cryoprotectant. Cell Biol Dev 1: 1-5.* The coconut water effect combined with 5% of glycerol for preserving goramy spermatozoa at -34 °C for 48 hours has been studied. The study's objective is to find the best combination among concentrations of coconut water on 0%, 21%, 23%, 25%, 27%, and 29%, respectively, combined with 5% of glycerol for maintaining good spermatozoa motility, viability and minimizing spermatozoa abnormality. One part of semen/sperm was mixed with three parts of solvent (5% glycerol + fish ringer + coconut water) and equilibrated at 4°C for 45 min. The diluted sperm were then frozen at -34 °C for 48 h. Finally, cryopreserved sperms were thawed at 30 °C for 3-5 min. Spermatozoa quality was evaluated before and after sub-zero freezing. Based on the Kruskal-Wallis test, spermatozoa motility and viability were higher than control ($P<0.05$), while the spermatozoa abnormality was not significantly different compared to control ($P>0.05$). Twenty-five percent of coconut water combined with 5% of glycerol was the best combination for preserving spermatozoa motility (80.36±1.54)% and spermatozoa viability (82±1.86)%, and also minimized spermatozoa abnormality (10±1.03)%.

Keywords: Coconut water, cryoprotectant, *Osphronemus goramy*, spermatozoa quality, sub-zero freezing

INTRODUCTION

According to the Ministry of Maritime Affairs and Fisheries, Republic of Indonesia data, Indonesian fish consumption increased by 5% in 2013 (KKP 2013). *Osphronemus goramy* (Lacepede 1801) is Indonesia's most popular and commercial freshwater fish (Saparinto 2008). Goramy fish production in Indonesia was about 280,079,000 kg in 2000 (Dunia Ikan 2008). However, nowadays, goramy fish is cultured conventionally because of the limited male and female parents. Therefore, they usually put male and female parents together in one pond for a long time, which will be caused inbreeding. Hence, the genetic materials' quality was reduced (Alam et al. 2002).

Cryopreservation is used to maintain genetic materials for a long period. According to Bozkurt (2005), cryopreservation is the ex-situ conservation effort to preserve genetic materials in sub-zero temperatures for a certain time. The genetic materials cryopreserved include spermatozoa, ovum, somatic cells, and embryo (Simione, 2003).

Cryoprotectants and extenders are two important solutions to protect cells from ice crystals during freezing (Muchlisin 2005). Based on Jamieson (1991), glycerol, methanol, and dimethyl sulphoxide (DMSO) are common intracellular cryoprotectants used for cryopreserved fish spermatozoa. On the other hand, milk, egg yolk, and sugar were used as extracellular cryoprotectants (Jamieson 1991). According to Routray et al. (2007), the extender is also needed to support cells with additional nutrition and maintain osmotic pressure during cryopreservation. Saline solution and fish Ringer solution are two extenders usually used for fish spermatozoa cryopreservation (Muchlisin 2005).

The previous cryopreservation study has been reported related to the fish spermatozoa, such as in *Barbonymus gonionotus*, Java Barb (Abinawanto et al. 2013), *Osphronemus goramy* (Abinawanto et al. 2011, 2012a, 2012b), *Osteochiius hasseltii* (Sunarma et al. 2007), *Cyprinus carpio* (Akcay et al. 2004), tilapia's fish (Chao et al. 1987), rainbow trout (Stoss and Donaldson 1983), carp (Harvey 1983; Horvath et al. 2003; Withler 1982), and salmonid (Harvey et al. 1982).

Dimethyl Sulfoxide (DMSO) was used as the cryoprotectant during the cryopreservation of goramy spermatozoa for 24 hours (Abinawanto et al., 2011). Besides, sucrose (Abinawanto et al. 2012a) and skim milk (Abinawanto et al. 2012b) were also used as a cryoprotectant for goramy spermatozoa. However, the effect of coconut water in many variations of concentration combined with 5% of glycerol on the spermatozoa quality of goramy was still unknown. Accordingly, the aim of the study was to find the suitable concentration of coconut water among 0%, 21%, 23%, 25%, 27%, or 29% in preserving spermatozoa motility and viability and in reducing the spermatozoa abnormality during cryopreservation.

MATERIALS AND METHODS

Preparation of fish

Fifteen mature males of *Osphronemus goramy* were brought from the private commercial hatchery, Parung-Bogor, West Java, Indonesia. All fishes were acclimatized for 14 d in a one-4,000-L square concrete cement pond until they attained 2.5-3.5 kg in the indoor Aquatic Biology laboratory, Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok, West Java Province. The fishes were grouped into 6 fishes and were stocked in a 7-square concrete cement fish pond (1,000-L). The fish pond was equipped with a closed re-circulation system and a black plastic mesh lid to minimize disturbances and prevent fish from jumping out. Six experimental groups based on coconut water concentration were assigned four replications in a completely randomized design (Table 1). 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). First, 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 (2010) 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 reached 6,8. The Phosphate buffer solution was then kept at 4 °C.

Giemsa solution preparation

The Giemsa solution was prepared based on WHO (2010) by diluting one part of the Giemsa stock solution and 10 parts of the phosphate buffer solution at 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 8-10 hours after being injected intra-muscularly with GnRH-analog (Ovaprim Syndel) at a single dose of 0.2 ml/kg body weight according to the modification method of Sunarma et al. (2007) and was put in 1.5 mL of cryotube.

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

Composition	Experimental group					
	C	21% CW	23% CW	25% CW	27% CW	29% CW
Semen/sperm (µL)	50	50	50	50	50	50
Fish finger solution (µL)	140	98	94	90	86	82
Coconut water/ CW (µL)	0	42	46	50	54	58
5% glycerol/G (µL)	10	10	10	10	10	10

Note: * C= control group (0% CW); ** 21% CW, 23% CW, 25% CW, 27% CW, 29% CW = treatment group

Semen/sperm dilution

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

Semen/sperm equilibration

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

Semen/sperm freezing

The diluted sperm was frozen at -34°C for 48 h (Huang et al. 2004).

Semen/sperm thawing

The frozen sperm was incubated at 30°C for 3-5 min. (Akcay et al. 2004 and Bozkurt et al. 2005).

Semen/sperm evaluation and spermatozoa analysis

Visual observation was carried out on the fresh semen/sperm color, and the cryotube with a scale measured sperm volume. The standardized pH paper (pH range 5-10) measured sperm/semen pH. The spermatozoa viability, abnormality, and motility were observed under a trinocular microscope (Boeco) equipped with the digital eyepiece camera (MDCE-5a). This microscope was also connected to the computer equipped with image driving software (Scopephoto 2.0.4). The Rurangwa et al. (2004) method was used to analyze the spermatozoa motility, whereas the spermatozoa viability and motility were analyzed by Salisbury and Van De Mark 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 a 0.05 level of significance.

RESULTS AND DISCUSSION

Results

Fresh semen (FS) was milky white, pH 8.06±0.05, and 0.57±0.10 mL of volume per ejaculate (Table 2).

Figure 1 showed that non-viable spermatozoa showed red on the sperm head (A), while the viable spermatozoa showed green color on the sperm head (B). Both viable and non-viable spermatozoa were found in all treatment groups and control. However, the percentage of spermatozoa viability was quantitatively different, either among treatment groups or between control and treatment groups. Both normal and abnormal spermatozoa morphologically were found in all treatment groups and control (Figure 2). However, the percentage of spermatozoa normal and abnormal were different among treatment groups or between control and treatment groups. Table 3 shows the percentage of spermatozoa abnormality, viability, and motility two days after sub-zero freezing.

The percentage of spermatozoa motiliy after freezing in control; and in various coconut water concentration of 21%, 23%, 25%, 27%, and 29%, were: (71.42±4.01)%, (72.22±2.96)%, (77.39±2.26)%, (80.36±1.54)%, (77.13±2.59)%, and (74.09±1.98)%, respectively (Table 3). The spermatozoa viability in control; and in various coconut water concentration of 21%, 23%, 25%, 27%, and 29%, two days after sub-zero freezing, were: (72±3.42)%, (75±3.36)%, (79±2.22)%, (82±1.86)%, (80±1.47)%, and (77±1.96)%, respectively (Table 3). On the other hand, the spermatozoa abnormality after freezing, in control; and in various coconut water concentration of 21%, 23%, 25%, 27%, and 29%, were: (11±1.83)%, (11±1.41)%, (12±1.04)%, (10±1.03)%, (11±0.83)%, and (11±0.75)%, respectively (Table 3).

Based on the Kruskal-Wallis test, there was a significant effect ($P<0.05$) of various concentrations of coconut water on spermatozoa viability and motility, respectively, to days after sub-zero freezing. On the other hand, there was no significant effect of coconut water on reducing spermatozoa abnormality ($P>0.05$) compared to the control (Table 3). According to the Dunnet test, 25% coconut water concentration showed the highest percentage of spermatozoa viability (82±1.86)% and motility (80.36±1.54)%, respectively.

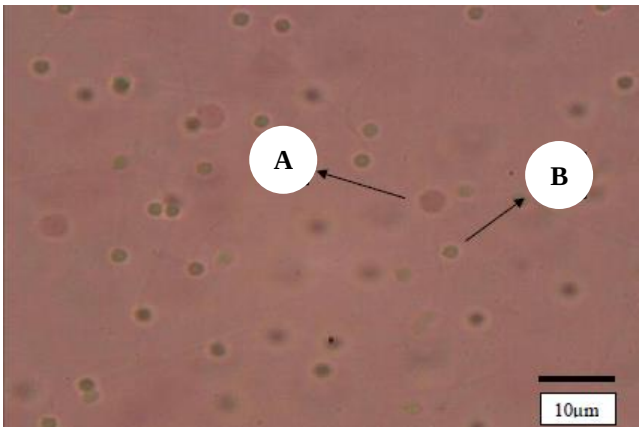

Figure 1. Spermatozoa non-viable (A); spermatozoa viable (B); 10x40 162x95mm (96 x 96 DPI)

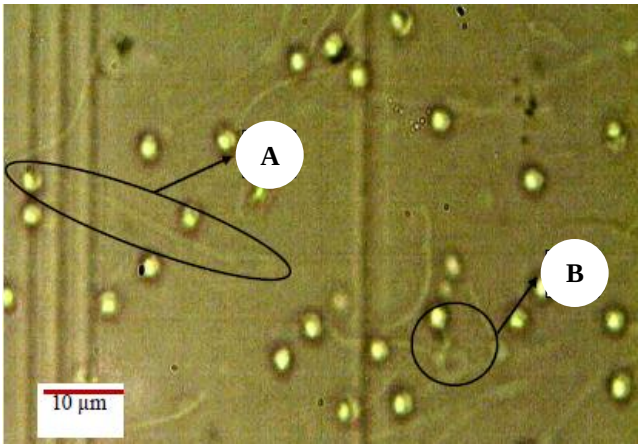

Figure 2. Normal spermatozoa (A); Round tail spermatozoa (B); 10x40 162x204 mm^2 (96 x 96 DPI)

Table 2. Fresh semen/sperm evaluation and spermatozoa analyses of goramy spermatozoa before freezing

Parameter	Color	Volume	Parameter	Color	Volume
Semen/sperm	Milky white	0.57±0.10	8.06±0.05	84±3.16	13±0.63

Note: Values are means ± SD of four replicates

Table 3. The percentage of the spermatozoa abnormality, viability, and motility of goramy 48 hours after sub-zero freezing

Parameter	Experimental group					
	C	21%CW	23%CW	25%CW	27%CW	29%CW
Viability (%)	72±3.42^a	75±3.36^b	79±2.22^{bc}	82±1.86^c	80±1.47^{bc}	77±1.96^{bc}
Abnormality (%)	11±1.83^a	11±1.41^a	12±1.04^a	10±1.03^a	11±0.83^a	11±0.75^a
Motility (%)	71.42±4.01^a	72.22±2.96^a	77.39±2.26^{bc}	80.36±1.54^b	77.13±2.59^c	74.09±1.98^c

Note: Values are means ± SD of four replicates. Mean values having the same superscript are not significantly different ($p > 0.05$)

Discussion

The concentration of 25% coconut water was shown to have the highest percentage of spermatozoa motility (80.36±1.54)%, two days after sub-zero freezing. This finding was similar to 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. 2012b; 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), tilapia'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 it was not statistically significantly different compared with other treatment groups and control. However, this finding was higher than previously 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). On the other hand, post-thaw viability in this study was lower than in 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 decline the post-thaw abnormality (14.83 ± 2.79)%, better than another treatment group of glycerol concentration and control group, although those results were not significantly different statistically. Our previous study showed a higher spermatozoa abnormality (29%; Abinawanto et al. 2011) when using the combination of 13% DMSO + 189M extender. Post-thaw abnormality in *Barbonymus gonionotus* spermatozoa was higher (45%) when preserved in the combination of 6% glucose + 10% methanol (Abinawanto et al. 2009). However, the post-thaw abnormality showed nearly similar (14%) when the spermatozoa of *Barbonymus gonionotus were* protected by the combination of 13% of egg yolk + 10% of methanol (Abinawanto et al. 2013).

Either treatment groups or control was visually shown as viable spermatozoa, motile spermatozoa, and abnormal spermatozoa. However, the percentage of spermatozoa viability, motility, and abnormality differed among treatment groups or between control and treatment groups. Therefore, 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 two days. Furthermore, the highest percentage of viability or motility of spermatozoa among the treatment groups or between control and treatment groups two days after sub-zero freezing is also an indicator of the optimum condition of a percentage of glycerol as the cryoprotectant.

The data obtained in the present study indicate that 25% coconut water combined with 5% glycerol showed the highest spermatozoa motility and spermatozoa viability and reduced spermatozoa abnormality two days after sub-zero freezing.

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