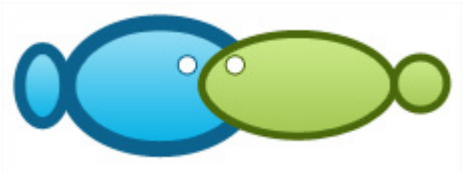

The effect of skim milk combined with 5% of methanol on motiliy, viability, and abnormality of Java barb, *Barbonymus gonionotus* spermatozoa after 24 hours freezing

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Abstract. The effect of skim milk combined with 5% of methanol to preserve spermatozoa for 24 hours at -34°C has been studied. The objective of the study was to determine the optimum concentration of skim milk on preserving spermatozoa in sub-zero freezing. Skim milk used in this study were, 0%, 5%, 10%, 15%, 20%, and 25%, respectively. The sperms were diluted with the combination of 5% methanol, fish ringer and skim milk (1 part of sample + 5 part of solvent) and were equilibrated for 20 minutes at 4°C before freezed for 24 hours at -34°C. After freezed, sample (sperms) were thawed for 13 sec. at 40°C. Spermatozoa viability, motility, and abnormality, were then observed. The results showed that 5% of methanol combined with 20% of skim milk displayed the highest spermatozoa viability (81.75±8.22%), and the highest spermatozoa motility rate (83.23±3.27%). On the other hand, the combination of 5% methanol and 20% of skim milk showed the lowest abnormality (26.25±1.89%). It was concluded that 20% of skim milk combined with 5% of methanol was the optimum concentration for Java barb (*Barbonymus gonionotus*) spermatozoa cryopreservation compared to other skim milk concentration tested.

Key Words: spermatozoa quality, sub-zero freezing, short-term preservation.

Introduction. According to the World Conservation Monitoring Centre (1992), Indonesia has 44 endemic freshwater species. Java barb - *Barbonymus gonionotus* is one out of 44 Indonesia freshwater fish which has an economic value (Sunarma et al 2007). Recently, Java barb is in least concern status (Thinh et al 2012) and it might be worst caused by habitat degradation and exploitation. Cryopreservation, therefore is needed as one out of two strategies to prevent the species extinction.

Cryoprotectant and extender are two factors needed for cryopreservation methods. Cryopreservation using the combination of cryoprotectant, and extender have been reported. Egg yolk, glucose, and honey have been used as the cryoprotectant, while coconut water was used as the extender for preserving African catfish, *Clarias gariepinus* spermatozoa (Muchlisin et al 2015). Besides, Muchlisin et al (2010) in the previous study have been used sugarcane water, soybean solution, and coconut water as the natural extenders for preserving African catfish spermatozoa. In previous study, Ringer solution was also used as the extender for preserving baung, *Mystus nemurus* spermatozoa (Muchlisin & Azizah 2009).

Cryopreservation has been applied to several fish species for example *Oreochromis mossambicus* (Harvey 1983), common carp *Cyprinus carpio* (Horvath et al 2003), rainbow trout *Oncorhynchus mykiss* (Stoss & Donaldson 1983), tropical bagrid catfish *Mystus nemurus* (Muchlisin et al 2004), and Java barb (Abinawanto et al 2013). Abinawanto et al (2011) have been studied the effect of DMSO to protect the *Osphronemus goramy* spermatozoa while kept in the liquid nitrogen for 24 hours. It was also reported that sucrose is suitable as the cryoprotectant and extender for preserving *O. goramy* spermatozoa in -34°C for two days (Abinawanto et al 2012a).

Skim milk is the aqueous part of whole milk after centrifugation from which the cream has been taken (Hurley 2008). Skim milk contains no more than 0.2% milk fat (Smith 2003). According to Singsee et al (2005), protein in skim milk play an important role as the extracellular cryoprotectant from cold shock condition during cryopreservation (Hafez & Hafez 2000). Further, 5% of methanol was used to protect cells intracelulluarly in low temperature and less toxicity compared to DMSO (Singsee et al 2005).

Our previous study shown the benefit of skim milk for protecting spermatozoa of *O. goramy* when freezed at -34^{o}C for 24 h (Abinawanto et al 2012b). However, the combination effect of methanol and skim milk for preserving Java barb spermatozoa in sub-zero freezing has not been reported. The present study, therefore is to evaluate skim milk effect combined with 5% of methanol on spermatozoa quality of Java barb when freezed in -34^{o}C for 24 h.

Material and Method. The study was conducted in December 2008 – May 2009, at the Genetics Laboratory, Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok, West Java Provinces, Indonesia. Mature males of Java barb were obtained from a private commercial hatchery, Bogor, West Java, Indonesia, and were transported to the indoor Aquatic Biology laboratory, Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok, West Java Province. Thirty fishes were acclimatized in laboratory conditions and maintained with a commercial diet for 14 days in a 2000-L square concrete cement tank. After acclimatization, the fishes were grouped into 7 groups consisted of 4 fishes per group with an average initial weight of 147.5±16.58 g, and were stocked in 7 square concrete cement fish pond (1000-L). The tanks were equipped with continuous aeration. Seven treatment groups were assigned to four times replication in a completely randomized design. Fishes were fed two times daily *ad libitum* at 08:00 and 17:00.

Preparation of the extender fish ringer solution. Extender fish ringer solution was prepared in the laboratory based on the method of Ginzburg (1972). A stock of extender fish ringer 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 distilled water up to 500 mL. The extender fish ringer solution was then kept at 4°C.

Preparation of the solvent solution. Solvent solution was prepared according to the method of Harvey (1983). The extender fish ringer solution was mixed with the methanol in the cryotube, and was kept at 4°C. Skim milk was incubated at 90°C for 1 minute in the waterbath and was kept at 25°C (room temperature) until cooling down. Skim milk was then added to the mixing solution of fish ringer and methanol.

Preparation of the activator solution and 0.5% Eosin-Y solution. The activator solution was prepared by diluting 45 mM NaCl, 5 mM KCl, and 30 mM Tris with distilled water up to 100 mL (Perchec et al 1995). The 0.5% of Eosin-Y solution was prepared by diluting 0.5 g of the Eosin-Y with distilled water up to 100 mL (WHO 1988).

Preparation of the 0.15M of phosphate buffer solution pH 6.8. The phosphate buffer solution was prepared by dissolving 5.34 g $Na_2HPO_4.2H_2O$ with distilled water up to 200 mL, and by dissolving 4.08 g KH_2PO_4 with distilled water up to 200 mL. $Na_2HPO_4.2H_2O$ 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.

Preparation of the Giemsa solution. The Giemsa solution was prepared by diluted 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 (WHO 1988).

Semen/sperm collection. The sperm were collected from individual male donor weighing 150 g by abdominal gently stripped method (Muchlisin et al 2010) and placed in 2-mL vials (Cryogenic storage vial, Nalgene and Nunc International).

Semen/sperm dilution. Fresh sperm suspension was diluted in the mix solution (diluents) containing fish ringer solution, 5% of methanol and skim milk as seen in Table 1. The composition of mix solution was adopted from Salisbury & VanDemark (1985). The dilution ratio among the fresh sperm and the mix solution was 1:5 according to Harvey (1983).

Table 1

Composition of semen/sperm, methanol, fish ringer solution and skim milk of experimental group

Composition	*Experimental group*						
	C1	*C2*	*5%SM*	*10%SM*	*15%SM*	*20%SM*	*25%SM*
Semen/sperm (µL)	40	40	40	40	40	40	40
5% methanol (µL)	0	10	10	10	10	10	10
Fish Ringer solution (µL)	200	190	180	170	160	150	140
Skim milk/SM (µL)	0	0	10	20	30	40	50

* C1 and C2 = control group; ** 5% SM, 10% SM, 15% SM, 20% SM, 25% SM = treatment group.

Cryopreservation process. The vials were labeled, and allowed to equilibrate for 20 min in refrigerator (4°C) to allow time for the sperm to be exposed to the diluents before freezing (Muchlisin & Azizah 2009). The tubes were then placed in the freezer (-34^{o}C) for 24 h (Huang et al 2004).

Evaluation of motility, viability, and abnormality. After 24 h, samples were thawed in a water bath at 40^{o}C for 13 sec (Horvath et al 2003). Seals were broken and samples were diluted in tap water at dilution ratio 1:20 (sperm:tap water; v/v) according to the Muchlisin & Azizah 2009. Then, one drop (10 µL) of thawed sperm was placed onto improved Neubauer equipment for assessing sperm motility, and another drop (10 µL) for assessing sperm viability, while the remining of thawed sperm (10 µL) was used for assessing sperm abnormality.

Percentage of motility, viability, and abnormality were observed with a light microscope (Boeco BM-180 SP, Germany) connected with the digital eyepiece camera (MDCE 5ª) using Soft Imaging software analysis (Scopephoto 2.0.4). Spermatozoa motility was analyzed by subjective method (Rurangwa et al 2004), whereas spermatozoa viability and spermatozoa abnormality were analyzed by Salisbury & VanDemark method (1985). The abnormalities were evaluated base on head morphology (swelled or rupture) and flagellum performance (present, absent or ruptura). Fresh semen/sperm color was observed by visual, whereas sperm volume was measured by the vial with scale. Fresh sperm pH was measured by pH paper (pH range 5-10).

Statistical 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 and Discussion. Fresh semen (FS) were milky white, pH 7.02±0.15, and 0.47±0.11 mL of volume (Table 2). Java barb spermatozoa had rounded head and a flagellum (tail). The viable or motile sperm showed green color (Figure 1) on the sperm head, while the non-viable sperm showed pink or red color on the sperm head (Figure 1). Most of the spermatozoa in fresh milt have a normal structure. On the other hand, there were also many variations of abnormal head spermatozoa were shown such as macrocephalus, microcephalus, and spermatozoa with two heads (Figure 2). Besides, there were many variations of abnormal flagellum (tail) spermatozoa shown in Figure 3. However, these abnormalities occure naturally.

The percentage of sperm motility, viability and abnormality of fresh semen (FS) were: 81.36%, 86% and 23.5%, respectively (Table 2). While post-thaw spermatozoa motiliy in control-1; C1 (0% methanol; 0% skim milk), control-2; C2 (0% skim milk) and in various skim milk concentration of 5%, 10%, 15%, 20%, and 25%, were: 67.3%,

74.6%, 72.68%, 75.03%, 76.08%, 83.23%, and 81.11%, respectively (Table 3). Post-thaw spermatozoa viability in control-1; C1 (0% methanol; 0% skim milk), control-2; C2 (0% skim milk) and in various skim milk concentration of 5%, 10%, 15%, 20%, and 25%, were: 63.25%, 50.25%, 49.25%, 53.75%, 61.75%, 81%, and 66.50%, respectively (Table 3). On the other hand, Post-thaw spermatozoa abnormality in control-1; C1 (0% methanol; 0% skim milk), control-2; C2 (0% skim milk) and in various skim milk concentration of 5%, 10%, 15%, 20%, and 25%, were: 39.25%, 41%, 31.25%, 29.75%, 28.25%, 26.25%, and 29.75%, respectively (Table 3).

Based on Kruskal-Wallis test, there were a significant effect of various concentration of skim milk on post-thaw sperm viability and abnormality ($p < 0.05$). According to the Dunnet test, the concentration of 20% of skim milk + 5% of methanol showed the highest post-thaw sperm viability (81.75)%, and the lowest post-thaw abnormality (26.25)%.

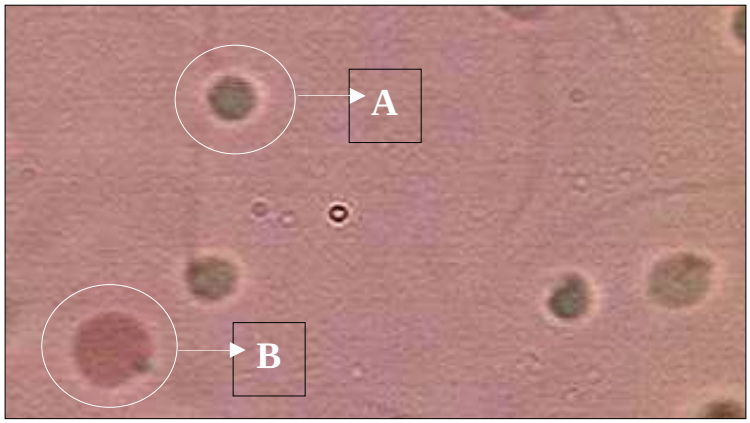

Figure 1. Spermatozoa viable (A); spermatozoa non-viable (B); 10x40;(96 x 96 DPI).

Table 2

Fresh semen/sperm evaluation and spermatozoa analyses of Java Barb spermatozoa, before freezing. Values are means±SD of four replicates

Color	*Volume (mL)*	*pH*	*Viability (%)*	*Abnormality (%)*	*Motility (%)*
Milky white	0.47±0.11	7.0±0.15	86±2.71	23.5±1.9	81.36±6.07

Table 3

Viability, abnormality, and motility of Java Barb spermatozoa, 24 hours after sub-zero freezing. Values are means±SD of four replicates. The different superscript in the same column denotes significant differences ($p < 0.05$)

Paramater	*Experimental group*						
	C1	*C2*	*5%SM*	*10%SM*	*15%SM*	*20%SM*	*25%SM*
Viability (%)	63.25 ± 11.81^{a}	50.25 ± 9.81^{b}	49.25 ± 6.02^{c}	53.75 ± 12.07^{b}	61.75 ± 14.77^{a}	81.75 ± 8.22^{d}	66.55 ± 8.99^{e}
Abnormality (%)	39.25 ± 10.24^{a}	41 ± 10.98^{b}	31.25 ± 2.06^{c}	29.75 ± 0.5^{d}	28.25 ± 1.5^{e}	26.25 ± 1.89^{f}	29.75 ± 4.57^{d}
Motility (%)	67.3 ± 12.18^{a}	74.66 ± 14.72^{a}	72.68 ± 14^{a}	75.03 ± 2.04^{a}	76.08 ± 6.78^{a}	83.23 ± 3.27^{a}	81.11 ± 5.22^{a}

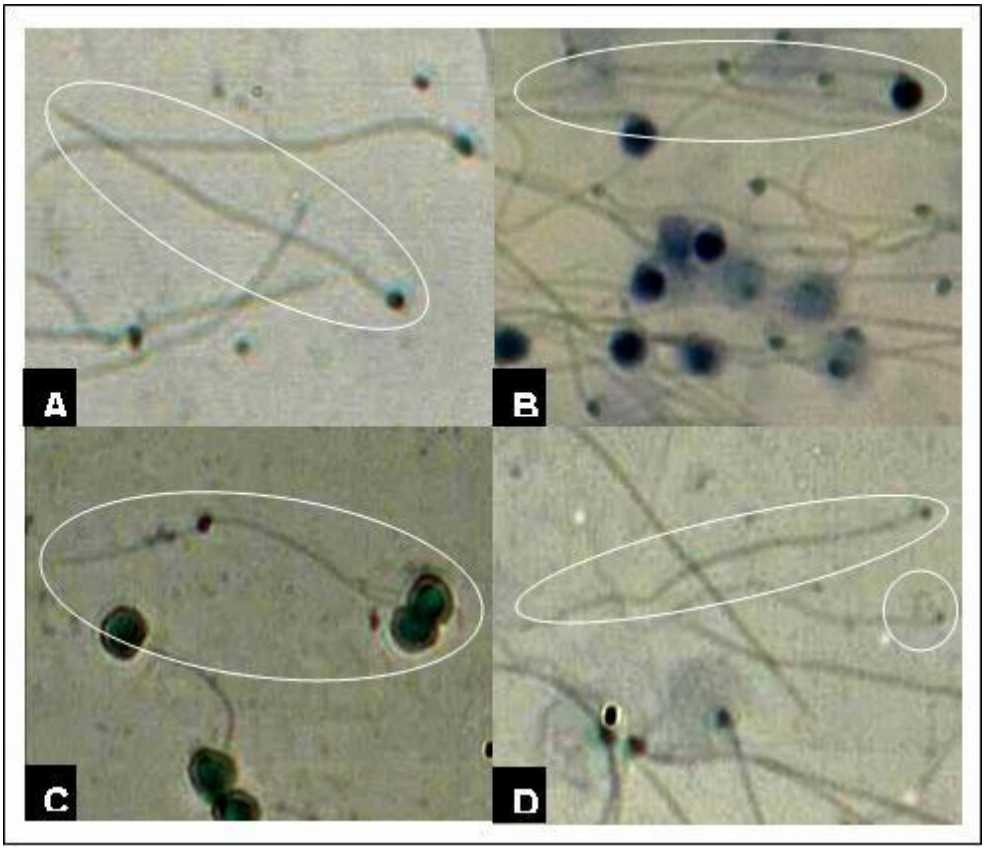

Figure 2. Normal spermatozoa (A); Macrocephalus spermatozoa (B); Two head spermatozoa (C); Microcephalus spermatozoa (D); (96 x 96 DPI).

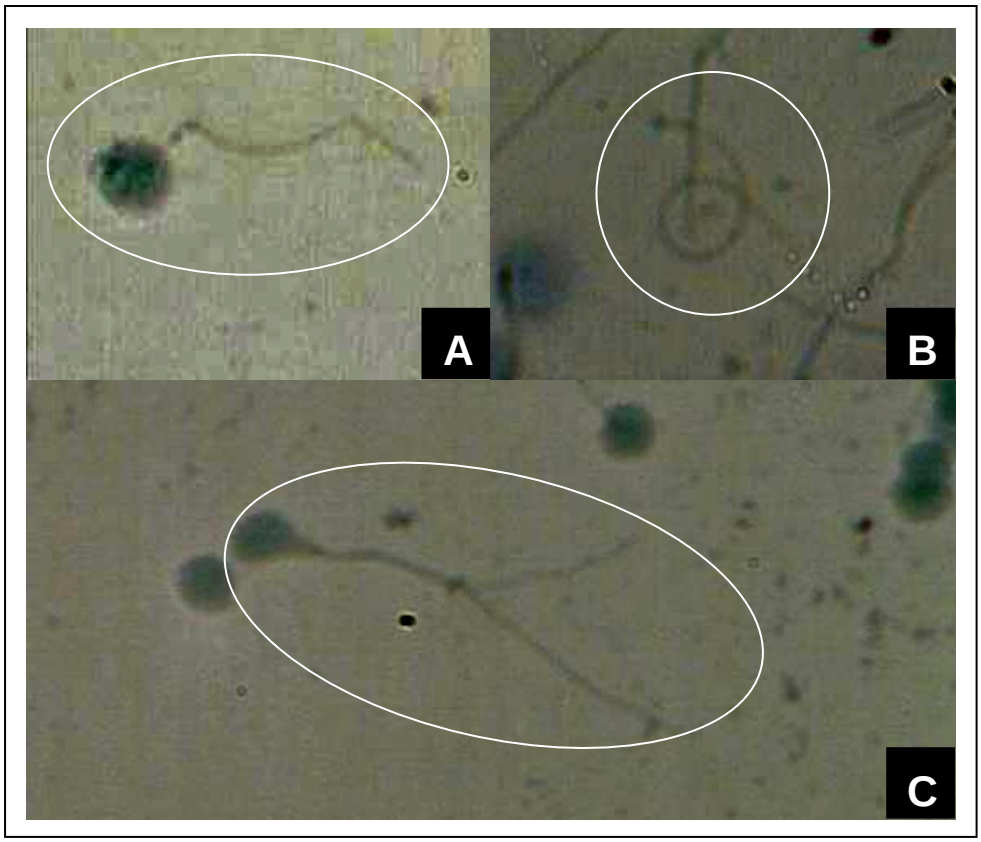

Figure 3. Cut tail spermatozoa (A); round tail spermatozoa (B); two tails spermatozoa (C); (96 x 96 DPI).

The effect of 20% of skim milk + 5% methanol on the percentage of spermatozoa motility 24 hours post-cryopreservation was higher than those observed in other fish species such as *M. nemurus* (Muchlisin & Azizah 2009), *O. goramy* (Abinawanto et al 2012b), *Brachydanio rerio* (Harvey et al 1982), *O. mossambicus* (Harvey 1983), tilapian's fish (Chao et al 1987), *Cyprinus carpio* (Akcay et al 2004), and *Osteochilus hasseltii* (Sunarma et al 2007). Cryoprotectant and extender are two out of many factors causing the differences of spermatozoa quality after sub-zero freezing, such as 10% of metanol (Muchlisin & Azizah 2009); 5% of methanol + 15% of skim milk (Stoss & Donaldson 1983; Chao et al 1987); 13% of DMSO + 189M extender (Abinawanto et al 2011); 10% of methanol + 15% of egg yolk (Abinawanto et al 2012a); 6% of glucose + 10% of methanol (Abinawanto et al 2009), and 15% of DMSO (Sunarma et al 2007; Horton & Otto 1976). The combination of 20% of skim milk + 5% methanol has also maintained the percentage of spermatozoa viability (81.75%). This result (spermatozoa viability) was higher than our previous study (Abinawanto et al 2009). Besides, the viability was also higher compared to other species like, *C. carpio* (20%, Withler 1982; 58%, Horton & Otto 1976), and *O. goramy* (63.5%, Abinawanto et al 2011). Furthermore, the combination of 20% skim milk + 5% methanol has also maintained the percentage of spermatozoa abnormality (26.25%). The spermatozoa abnormality in this study (26.25%) was lower compared to our previous study using 6% of glucose + 10% of methanol (Abinawanto et al 2009), and also lower than other species like, *O. goramy* (Abinawanto et al 2011) and *M. nemurus* (Muchlisin & Azizah 2009). Thawing procedures at 40^{o}C for 13 sec was effective for 2 mL of cryogenic tubes. We choose 40°C because this is easy to achieve using heating devices in our temperature conditions. Methanol as an intracellular cryoprotectant significantly improved motility of cryopreserved sperm. Methanol was employed as successful intracellular cryoprotectant in *M. nemurus* (Muchlisin & Azizah 2009); *B. rerio* (Harvey et al 1982); *O. mossambicus* (Harvey 1983); tilapian's fish (Chao et al 1987); *C. carpio* (Horváth et al 2003), *O. goramy* (Abinawanto et al 2011; Abinawanto et al 2012a; Abinawanto et al 2012b), Java barb (Abinawanto et al 2009; Abinawanto et al 2013).

Conclusions. This study demonstrated that the combination of 5% of Methanol and 20% of skim milk is recommended to use as a cryoprotectant for Java barb, *Barbonymus gonionotus* spermatozoa for short term storage. However, the fertilization study is yet to be clarified for further investigations.

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