The effect of coconut milk in various concentrations on spermatozoa motility of Koi fish (Cyprinus carpio, Linnaeus 1758) 48 hours post-cryopreservation

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Abstract. Research about the effect of various concentrations of coconut milk on spermatozoa quality of Koi Fish (Cyprinus carpio, Linnaeus 1758) 48 hours post cryopreservation has been carried out. The research aims to understand the effect of 5 % methanol and various concentrations of coconut milk (0 %, 2 %, 4 %, 6 %, 8 % and 10 %) on the motility of Koi fish spermatozoa 48 hours post-cryopreservation. Koi fish milt in this research was collected by stripping method and then evaluated microscopically and macroscopically. Results obtained are further assayed statistically using the Sapiro-Wilk normality test, Levene homogeneity test, one way Anova test, and Tukey multiple comparison test. One way Anova test showed that various concentration of coconut milk had significant difference (P < 0.05) of average value of motility on spermatozoa of Koi fish 48 hours post cryopreservation. A combination of 5 % methanol with 6 % coconut milk is the optimum concentration of cryoprotectant because it produced the highest average value of spermatozoa motility (79.13 ± 4.18 %).

Keywords: Coconut milk, cryopreservation, Koi fish, motility, spermatozoa

1. Introduction
Ornamental freshwater fish is one of the best commodities that is high in demand by Indonesian people. One of the leading commodities that are still in demand is ornamental Koi (Cyprinus carpio) [1]. Koi fish has diverse patterns on its body that makes them an attractive ornamental fish for people inside and even outside of the country. Nowadays, Koi fishes are cultured by maintaining and spawning the parental, collecting eggs/larva, and breeding, however the spawning time of female and male Koi fish are different.

One of the ways to overcome that limitation is to store the Koi fish spermatozoa for a long time by cryopreservation. According to Bozkurt [2], cryopreservation is the ex-situ conservation to preserve the genetic materials in sub-zero temperature for a certain time. Cryopreservation consists of freezing process and storing in very low temperature to keep the biological properties from damage [3, 4]. Until now, the cryopreservation technique is used to store many genetic materials, including spermatozoa, ovum, somatic cell, and embryo [5, 6].

Extender and cryoprotectant are two of the important factors in cryopreservation to protect cell from damage caused by ice crystal formation. Extenders commonly used are fish Ringer, glucose, and saline [7]. Cryoprotectant is divided into intracellular cryoprotectant (penetrating cryoprotectant) and
extracellular cryoprotectant (non-penetrating cryoprotectant) [7]. Intracellular cryoprotectant commonly used are dimethyl sulfoxide (DMSO), dimethylacetamide (DMA), ethylene glycol (EG), and methanol [7]. Extracellular cryoprotectant commonly used are polymers, sucrose, egg yolk, and skim milk [8].

The previous cryopreservation study has been reported related to the fish spermatozoa, such as Java Barb [9], Osphronemus goramy [10], Osteochiilus hasseltii [11], Cyprinus carpio [12], and Brown Tout [13]. Methanol was used as cryoprotectant during cryopreservation of Cyprinus carpio spermatozoa [4] and Barbodes gonionotus [14].

Previous studies related to spermatozoa cryopreservation using coconut milk as a cryoprotectant has never been done on fish but has been shown to other species, including Buck [15], Cow [16], and Boar [17]. Coconut milk as extracellular cryoprotectant contains important essential component to protect cells from damage and can be combined with any intracellular cryoprotectant. Combination of 20 % coconut milk with DMSO and EG as a cryoprotectant of Buck spermatozoa shows 54.20 % motility post cryopreservation [15].

However, the effect of 5 % methanol combined with various concentrations of coconut milk on Koi fish spermatozoa motility is still unknown. Pre-research of Koi fish spermatozoa cryopreservation using combination of 5 % methanol and various concentration of coconut milk (0 %, 2 %, 4 %, 6 % and 8 %) shows 72 % motility of Koi fish spermatozoa post-cryopreservation. Therefore the aim of this study was to know the effect of 5 % methanol combined with various concentration of coconut milk among 0 %, 2 %, 4 %, 6 %, 8 % and 10 % on Koi fish spermatozoa motility 48 hours post-cryopreservation.

2. Experimental method

The sperm ejaculates from four males of Cyprinus carpio were collected by hand stripping, and sperm were diluted by coconut milk-fish Ringer using a 1:9 ratio according to Hovarth et al. [18]. The concentration of coconut milk used in this study were 2 %, 4 %, 6 %, 8 %, 10 % and 0 % as control, respectively. Sperm were equilibrated at 4 °C for 20 minutes and then frozen at -34 °C for 48 hours. Sperm were thawed at 40 °C for 90 seconds. After thawing the sperm were evaluated by utilizing the light microscope with the support of digital eye-piece linked to the computer assisted by image driving software. The sperm motility and some physical and chemical characteristics of semen such as color, volume, and pH were evaluated. The one-way ANOVA followed by Tukey’s test were applied to evaluate any significant differences among treatments and to reveal the optimum concentration of coconut milk to get the best spermatozoa quality after frozen.

3. Results and discussion

3.1. Physical and chemical characteristics

The fresh semen’s color was milky-white, and the pH of semen was 7.9. Fresh semen volume was 0.52 ± 0.12 mL per ejaculate (table 1). A similar result was also discovered in the prior research where the color of sperm was milky white with pH 8.6 and sperm volume was 0.56 ± 0.23 mL [18]. According to Muchlisin [19], the range of optimum semen pH is 7.2 to 8.2.

| Color        | Volume (mL) | pH  | Motility (%) |
|--------------|-------------|-----|--------------|
| Milky-white  | 0.52 ± 0.12 | 7.9 | 81.40 ± 1.36 |
Table 2. Sperm analysis after freezing. The values are means ± SD of four replicates, with superscript letter denotes significant differences (P < 0.05).

| Parameter | Coconut milk concentration |
|-----------|---------------------------|
|           | 0 % | 2 % | 4 % | 6 % | 8 % | 10 % |
| Motility (%) | 65.00 ± 2.55<sup>a</sup> | 66.05 ± 6.75<sup>a</sup> | 72.82 ± 3.03<sup>b</sup> | 79.13 ± 4.18<sup>b</sup> | 65.99 ± 1.29<sup>ab</sup> | 61.27 ± 1.70<sup>a</sup> |

3.2. Fresh sperm analysis

The motile sperm showed green color or transparent on sperm head (data not shown). The spermatozoa motility before freezing was 81.40 ± 1.36 % (table 1). This present study showed lower mean semen percentage than previous study [10], which motility was 87.00 ± 5.00 %. The spermatozoa in this study was considered optimal to be short term-cryopreserved because it showed the motility percentage was more than 80 % [2, 20].

3.3. Sperm analysis post-cryopreservation

The percentage of spermatozoa motility post-cryopreservation in 5 % methanol and various coconut milk concentration of 0 % (control), 2 %, 4 %, 6 %, 8 % and 10 %, were: 65.00 ± 2.55 %, 66.05 ± 6.75 %, 72.82 ± 3.03 %, 79.13 ± 4.18 %, 65.99 ± 1.29 % and 61.27 ± 1.70 %, respectively. The percentage of spermatozoa motility can be seen on table 2. Based on one way ANOVA test, there were significant effects (P < 0.05) of methanol 5 % and various coconut milk concentrations on spermatozoa motility 48 hours post-cryopreservation. There were also significant differences (P < 0.05) among treatment groups on spermatozoa motility based on Tukey test.

The concentration of 6 % coconut milk showed the highest percentage of spermatozoa post thaw motility (79.13 ± 4.18%) 48 hours post-cryopreservation. This finding was similar with the previous study [13] when they cryopreserved Koi fish spermatozoa using combination of 10 % DMSO and 10 % egg yolk as cryoprotectant (78.60 ± 0.70 %). On the other hand, post thaw motility found in this study was higher compared to previous study [18] of Cyprinus carpio spermatozoa cryopreservation using combination of methanol and glucose as cryoprotectant (63.00 ± 9.00 %). However, post thaw motility in this study was lower compared to previous study [14] about cryopreservation of Barbodes gonionotis using 10 % DMSO and 5 % methanol as cryoprotectant (83.60 ± 3.20 %). The difference of post thaw motility percentage can be caused by the difference of species or cryoprotectant used in the study.

4. Conclusion

Combination of 5% methanol and various coconut milk concentrations (0 %, 2 %, 4 %, 6 %, 8 %, and 10 %) as cryoprotectant affected the motility of Koi fish (Cyprinus carpio, Linnaeus 1758) spermatozoa 48 hours postcryopreservation. Combination of 5 % methanol and 6% coconut milk was the optimum concentration to preserve sperm motility (79.13 ± 4.18 %).

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