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Sperm motility of kancra fish (*Tor soro*, Valenciennes 1842) after frozen: the effect of soybean milk as a natural cryoprotectant

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Abstract. Sperm cryopreservation is a way of managing and maintaining kancra fish (*Tor soro*) resources that increasingly threatened because of over-exploitation. The optimization of sperm cryopreservation is carried out using a natural cryoprotectant with lower toxicity than a chemical cryoprotectant. The purpose of this study was to analyze the effect of soybean milk as a natural cryoprotectant on sperm motility percentage of kancra fish after 48 hours preservation. The sperm was collected through stripping and was diluted into the soluble liquid of fish ringer + methanol 10% + soybean milk. Importantly, the concentration of soybean used were 0%, 5%, 10%, 15%, 20%, and 25%. The sample was equilibrated at the temperature of 5 °C for 10 minutes, frozen at the -10 °C for 48 hours and thawed at 40 °C for 1 minute. The motility percentage was analyzed using ANOVA and Tukey test. The result showed soybean milk had effects ($p < 0.05$) on the post-cryopreservation sperm motility. Additionally, 5% of soybean milk showed the highest sperm motility ($84.37 \pm 1.54\%$).

1. Introduction

Kancra (*Tor soro*) is a fishery resource that has spiritual value (sacred) in West Java [1], and cultural value in (tribal ceremony) North Sumatra [2]. They also have significance indication in the economic sector, due to its high selling worth that reaches IDR 500,000 per kilogram. This further causes an elevation in the level of exploitation, which contributes to the decline in genetic diversity [2], thus threatening sustainability [3].

Kancra fish resources management and preservation is required in maintenance. The efforts of preserving kancra fish have been done through domestication, yet it shows less optimum result because of the time difference between the maturity period of male and female gonad. Moreover, the males tend to attain this extent of development by the time its body weight reaches 300 g, which is about 700 g for females [4]. The other efforts towards preservation is obtained through sperm cryopreservation, a technique that requires storing sperm by freezing at very low temperatures [5]. In addition, the success of this procedure is evaluated based on a comparison between the quality of sperm before and after the process [6].



Sperm cryopreservation process may reduce the quality of sperm because the freezing process may damage the sperm cells [7]. The addition of cryoprotectant is able to protect sperm from the cold shock [8] and to preserve the cell integrity during cryopreservation [9]. Soybean milk is a natural cryoprotectant which contains lecithin [10], which is able to protect the sperm membrane from cold shock [11].

Fish sperm cryopreservation through soybean milk cryopreservation had been conducted by Yildiz *et al.* [12] on goldfish, and the results demonstrated 10% soybean juice as the optimum concentration. The cryopreservation research on kancra fish sperm using soybean milk as the natural cryoprotectant had never been conducted before, thus, there requires a research which observes the effect of soybean milk towards the sperm motility of kancra fish in 48 hours after cryopreservation.

2. Material and methods

2.1 Time and place

This research was conducted at the Installations for Freshwater Fish Genetic Resources, Cijeruk, Bogor, West Java, between April and July 2019.

2.2 Collection of sperm

Sperm collection was carried out by massaging the abdomen of male fish with mature gonads. During the stripping process the sperm color was observed, and instances where a reddish pigmentation is observed indicates the possibility of blood contamination, due to injury during the process [13].

2.3 Dilution of sperm

Sperm was diluted in a dilution solution that consists of fish ringer + 10% methanol + soybean milk, while the soybean milk concentration used were 0%, 5%, 10%, 15%, 20%, and 25%.

2.4 Cryopreservation process

Samples were equilibrated at a temperature of 5 °C for 10 minutes, frozen at -10 °C for 48 hours, and thawed at 40 °C for 1 minute.

2.5 Observation parameters

Sperm evaluation was conducted (1) macroscopically, this includes the measurement of pH, volume, and color of fresh sperm, and (2) microscopically, through observation and calculation of sperm motility (fresh and cryopreservation sperm), using a 10x40 magnification light microscope, with the help of digital eye-piece and Scopephoto 2.0.4 image driving software.

2.6 Observation of sperm motility

After thawing, about 10 µL of the sperm solution was dropped in the Improved Neubauer count room, and observations were conducted under a microscope, at a magnification of 10x40. In addition, the number of motile, as well as the total spermatozoa was counted [14], and calculations were made using the following formula:

$$\% \text{ Motility} = \frac{\sum \text{ Motile spermatozoa}}{\sum \text{ Total spermatozoa}} \times 100\%$$

2.7 Statistical analysis

Sperm motility percentage was evaluated using the analysis of variance (ANOVA) and also the Tukey test, which required the SPSS software version 16.0.

3. Result and discussion

3.1 Analysis of fresh sperm

Fresh sperm were observed to possess a milky white coloration, with a typical pH of 8.5 and the volume of 2.2 mL per ejaculation, with $85.92 \pm 1.98\%$ fresh sperm motility (Table 1).

Table 1. Analysis of fresh sperm before being frozen The mean value \pm SD out of four repetitions

Macroscopically analysis			Microscopically analysis
Color	pH	Volume (mL)	Sperm motility (%)
Milky White	8.5	2.2	$85.92 \pm 1.98\%$

Ancra fresh sperm has milky white color, the pH level of 7.6—7.9, sperm volume 3.92 mL, and sperm motility 89.5% [6]. Color of fresh sperm showed similar results from previous research, but pH, volume, and motility of fresh sperm showed different results from previous research conducted by Zairin Jr et al. [6]. The sperm color of freshwater fish species is generally white and milky white. Sperm that have a reddish or yellowish color is usually caused by contamination of bodily fluids such as blood, urine, and feces. The contamination of sperm causes a decrease in sperm quality and cannot be used for cryopreservation [8].

Freshwater fish sperm have a basic pH. The pH is an important parameter in preparing extenders for cryopreservation. The volume of kancra sperm differs between the same species, depending on the state of maturity rather than size. Sperm motility is the most common parameter used to evaluate sperm quality [8]. Sperm that can be used for cryopreservation are sperm with motility of more than 80% [12]. Based on the result, fresh sperm obtained can be used for cryopreservation.

3.2 Analysis of sperm after cryopreservation

The percentage of sperm motility evaluated after the process of cryopreservation, utilizing the soybean milk at a concentration of 0%, 5%, 10%, 15%, 20%, and 25%, were $55.84 \pm 2.92\%$, $84.37 \pm 1.54\%$, $76.70 \pm 2.15\%$, $71.74 \pm 2.37\%$, $63.92 \pm 2.89\%$, and $58.51 \pm 2.29\%$, respectively, as shown in Table 2.

Table 2. Percentage of sperm motility after 48 hours of cryopreservation using natural cryoprotectants in soybean milk The mean value \pm SD out of four repetitions

Treatments	Sperm motility (%)
Soybean milk 0%	$55.84 \pm 2.92\%^a$
Soybean milk 5%	$84.37 \pm 1.54\%^d$
Soybean milk 10%	$76.70 \pm 2.15\%^c$
Soybean milk 15%	$71.74 \pm 2.37\%^c$
Soybean milk 20%	$63.92 \pm 2.89\%^b$
Soybean milk 25%	$58.51 \pm 2.29\%^a$

Based on the result, the percentage of post-cryopreservation sperm motility was observed to have comparably decreased, in contrast with the fresh variety (Figure 1). In addition, analysis using ANOVA and Tukey test demonstrated significant differences between treatment groups ($p < 0.05$). Treatment with 5% soybean milk exhibited the highest average motility percentage, which was $84.37 \pm 1.75\%$. According to Paula *et al.* [15], $>80\%$ sperm motility percentage showed good sperm condition and higher number of motility reflects better sperm quality.

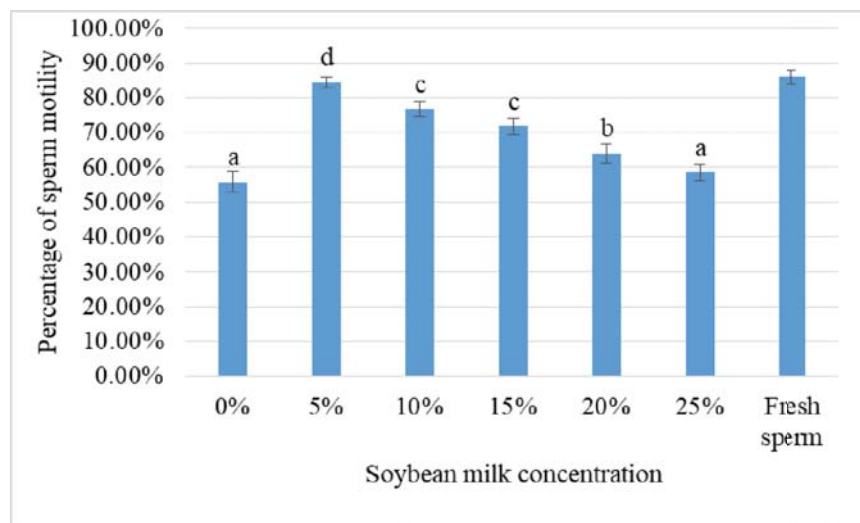


Figure 1. Percentage of sperm motility after 48 hours of cryopreservation using soybean milk as a natural cryoprotectant

Soybean milk is a natural cryoprotectant contains lecithin, which is a similar constituent in egg yolk [16]. Soybean milk also has lower contamination risk compared to egg yolk, so it can be used as a substitute for egg yolk that is widely used as a natural cryoprotectant. [16, 10, 17]. Lecithin can protect the integrity of the phospholipid membrane during cryopreservation [18, 19], so the sperm can be protected from cold shock [19]. The addition of cryoprotectant during cryopreservation may prevent cryoinjuries and preserve the quality of sperm [16, 20].

The use of 5% soybean milk cryoprotectant for kancra fish sperm cryopreservation showed higher sperm motility percentage compared to previous research conducted by Zairin Jr et al. [6] which used 10% egg yolk cryoprotectant (83.33%). This research result showed that soybean milk cryoprotectant has better advantages compared to egg yolk in preserving sperm motility during cryopreservation. This practice has also previously been performed in carp [12], ram [16, 21], goat [21], bovine [22], cattle [11, 23], rabbit [24], buck [25].

4. Conclusion

In conclusion, it was established that the natural cryoprotectant of 5% soybean milk shows the highest percentage of sperm motility ($84.37 \pm 1.54\%$).

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