Effect of date palm (*Phoenix dactylifera* L.) on spermatozoa viability of kancra fish (*Tor soro* Valenciennes 1842) 48 hours post cryopreservation

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Abstract. Kancra is an endemic fish used in traditional functions in different parts of Indonesia. However, the population of the fish is decreasing due to environmental damage and excessive fishing. Cryopreservation of sperm of kancra fish is one of the most effective approaches to solving this problem. One of the success factors of cryopreservation is cryoprotectant. This study aims to evaluate the date palm juice as a natural cryoprotectant on the viability percentage of kancra fish after 48 hours of post cryopreservation. The sperm was collected by stripping and was diluted in the soluble liquid (fish ringer solution+ methanol 10%+ date palm juice). The date palm juice concentration used were 0%, 5%, 10%, 15%, 20% and 25%. The sperm was equilibrated for 10 minutes at the 5°C temperature before it was frozen for 48 hours at the -10 °C. The thawing was done for 1 minute at 40 °C. Data were analyzed using ANOVA and followed by Tukey test. The result showed there was an effect of date palm juice (p<0.05) on the viability percentage of sperm post cryopreservation. The 10% concentration of date palm juice was optimum (p<0.05) with the percentage of viability 80.75 ± 1.19%.

1. Introduction
Kancra (*Tor soro*) fish is a freshwater fish which possesses high economic value [1]. Kancra fish is widespread in several regions in Indonesia, such as Sumatra, Java, and Kalimantan [2]. In West Java, kancra fish can be found in Kuningan or Sumedang regions. Kancra fish, in Kuningan region, is considered as a god fish [3]. The population of kancra fish in the nature decreases due to overfishing [4]. To anticipate the extinction of kancra fish population, several alternatives are required in which one of them is cryopreservation process [5].

Cryopreservation is a genetic material storage technique in low temperature [6]. Cryopreservation is able to preserve the viability of cell in low temperature so that the cell can be used whenever needed without being limited to space and time [7]. An important factor required for the cryopreservation process is cryoprotectant. Cryoprotectant protects sperm from heat and cold shock during the cryopreservation process. The use of cryoprotectant may prevent crystallization within the cell during cryopreservation process [8]. Cryoprotectant made of natural materials is more recommended to be used for the cryopreservation because it does not contain high toxic [9]. Therefore, there needs to be a research on the use of natural materials as the cryoprotectant, one of which is date palm.
Date palm are fruit which mostly composed by simple sugars such as glucose, fructose, and sucrose. The sugar content of date palm reaches 88% [10]. High sugar content in date palm provide energy during cryopreservation [11]. In addition, date palm are good to use as cryoprotectant for cryopreservation because they contain high fiber and low in fat [12]. The cryopreservation research which uses date palm as natural cryoprotectant on kancra fish has never been conducted. Therefore, this research aimed to evaluate the effect of date palm juice as the natural cryoprotectant using various concentrations towards the viability of kancra fish’s sperm in 48 hours post-cryopreservation.

2. Material and methods
2.1 Time and location
The study was conducted between April and July 2019 at the Installations for Freshwater Fish Genetic Resources, Ministry of Marine Affairs and Fisheries, Cijeruk, West Java, Indonesia. Kancra fish used were obtained from this same center.

2.2 Sperm collection
The sperm was obtained by stripping the gonad of matured and ripe male of the fish [13].

2.3 Fish ringer preparation
The fish ringer preparation was carried out by dissolving 0.325 g NaCl, 0.0124 g KCl, 0.0175 g CaCl₂·2H₂O and 0.01 g NaHCO₃ in 100 ml distilled water [14].

2.4 Activator dilution preparation
The activator solution was also prepared by dissolving 0.2633 g NaCl, 0.0372 g KCl, and 0.3634 g C₄H₁₁NO₃ in 100 ml of distilled water [15].

2.5 Sperm dilution
The sperm was diluted with the fish ringer and then date palm juice added with 10% methanol. Also, the concentration of date palm juice used were 0%, 5%, 10%, 15%, 20%, and 25%.

2.6 Equilibration and freezing
The equilibration process was performed at a temperature of 5 °C for 10 minutes followed by freezing at -10 °C for 48 hours [16].

2.7 Post-thawing parameters examined
The thawing was carried out on a water bath at the temperature of 40 °C for 60 seconds [13]. Then, the samples were evaluated both macroscopically and microscopically. The microscopic observations involved using a light microscope with the aid of an eye-piece digital linked to image driving software on the computer, to observe the sperm viability while the macroscopic included observing the sperm color, volume, and pH.

2.8 Viability observation (microscopic)
The viability observation was performed by diluting sperm in a fish ringer at a ratio of 990:10. Then 10 μl of the diluted sperm was placed on the glass object, after which 40 μl of eosin-Y coloring was added to it, making it 1:4. The viability observations were later carried out using a light microscope at a magnification of 10x40. On the microscope, viable sperm will not absorb the added color, hence it remains clear. However, the non-viable sperm will absorb color and then turn red. Finally, the viability percentage of the sperm was calculated using the following formula:

\[
\% \text{ Viability} = \frac{\sum \text{Number of viable sperm}}{\sum \text{Number of sperm counted}} \times 100\%
\]
3. Result and discussion

3.1 Fresh sperm analysis

Based on observations, the fresh sperm produced during one ejaculation was 2.5 ml and it was milky white in color with a pH of 8.5. Also, the average percentage of fresh sperm viability was $87.75 \pm 1.15\%$ as shown in Table 1.

| Physical-chemical characteristic | Microscopically analysis |
|---------------------------------|--------------------------|
| Volume (ml)                     | pH | Color   | Viability (%) |
| 2.5                             | 8.5| Milky white | 86          |
|                                 |    |          | 88.5        |
|                                 |    |          | 87.5        |
|                                 |    |          | 89          |
| Avg                             | Milky white | 87.75    |
| Stdev                           | 1.15                                    |

In general, viable sperm good for cryopreservation usually have an average percentage of sperm viability of 80% [17]. Sperm viability can be seen based on the sperm cell’s color. Viable cell would not be colored by the eosin-Y, while non-viable sperm will be colored by it (Figure 1). It is caused the integrity of the cells membrane. Viable sperm has good cell integrity so not all substances are able to enter the cell membrane, while non-viable sperm will lose its permeable selective nature so substances such as eosin-Y would be able to enter and color the cell [18].

3.2 Post cryopreservation sperm analysis

The viability of post-cryopreservation sperm were smaller compared with fresh sperm. The percentage value of post-cryopreservation sperm viability using natural cryoprotectants of date palm juice with concentrations of 0%, 5%, 10%, 15%, 20%, and 25% were $65.38 \pm 1.25\%$; $74 \pm 1.22\%$; $80.75 \pm 1.19\%$; $75 \pm 1.78\%$; $69.13 \pm 1.65\%$; and $69 \pm 1.22\%$ respectively. Based on ANOVA test results showed that various concentration of date palm juice (0%, 5%, 10%, 15%, 20%, and 25%) was significantly different ($P < 0.05$) to the percentage of viability sperm of kancra fish 48 hours post cryopreservation and followed by Tukey test also showed significant differences ($P < 0.05$) in 10% date palm juice concentration towards all treatments (Figure 2).

| FR (%) | Date Palm (%) |
|--------|---------------|
| 0%     | 65.38 $\pm 1.25\%$ $^d$ |
| 5%     | 74.00 $\pm 1.22\%$ $^b$ |
| 10%    | 80.75 $\pm 1.19\%$ $^a$ |
| 15%    | 75.00 $\pm 1.78\%$ $^b$ |
| 20%    | 69.13 $\pm 1.65\%$ $^c$ |
| 25%    | 69.00 $\pm 1.22\%$ $^c$ |

Different letters in the column indicate significant differences ($P <0.05$)

The observations revealed that the average percentage of post-cryopreservation sperm viability decreased compared with that of the fresh sperm. The fresh sperm percentage viability peaked at 87.75 $\pm 1.15\%$ while the highest percentage for post-cryopreservation sperm was 80.75 $\pm 1.19\%$. This decrease in the percentage of post-cryopreservation sperm viability could be as a result of cryoinjury. This cryoinjury is a condition of cell damage caused as a result of the freezing process leading to the formation of intracellular and extracellular ice crystals. These ice crystals, apart from causing cell damage, also result to the death of the cell [19]. The highest average sperm viability post-cryopreservation was found on the treatment which used date palm juice concentration of 10%, which
was 80.75 ± 1.19%; while the lowest percentage was found on treatment which did not use date palm juice, which was 65.38 ± 1.25%. Previous research on cryopreservation fish sperm using date palm juice as cryoprotectant has not been conducted. However, other research on cryopreservation using combination of 10% methanol and sugar as cryoprotectant was found in Osphronemus goramy with the highest percentage of viability was 82.17 ± 2.56% [20].

The combination of date palm juice with 10% methanol was effective by forming a layer on the outside of the cell and replacing the fluid inside the cell which was lost due to dehydration during the process of equilibration and freezing. The date palm juice has a large molecular size, therefore, it cannot enter the cell. However, the sugar content of date palm juice replaced the liquid around phospholipids and then bonded to the phosphate group on phospholipids [21]. The methanol was effective in the sense that it replaced the fluids lost by dehydration during the equilibration and freezing processes. Then, as it enters the cell to replace lost fluid, it reduces its freezing point. Also, the date palm juice and methanol prevent the formation of ice crystals on the cell membranes, which could cause damage or death to the membrane. Based on previous research, methanol was a cryoprotectant with lower toxicity than DMSO and glycerol. Sperm cryopreserved using DMSO lose
the ability to fertilize [22]. Moreover, methanol as an internal cryoprotectant significantly improved motility of cryopreserved sperm [20].

4. Conclusion
The combination of 10% date palm juice concentration shows the highest average percentage of post-cryopreservation sperm viability which was 80.75 ± 1.19%.

5. References
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