The spermatozoa motility of kancra fish (*Tor soro* Valenciennes, 1842) after the frozen process: the application of egg yolk as a cryoprotectant

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Abstract. Kancra is one of the endemic fishes in Indonesia, however, its population keeps decreasing due to overexploitation. One of the ways methods to solve this problem is by implementing cryopreservation. Cryoprotectant has effect on successful cryopreservation. The purpose of this study was to evaluate the effect of free-range chicken egg yolk as a natural cryoprotectant combined with 10% of methanol to motility of spermatozoa that had been used after 48 hours cryopreservation. The egg yolk concentration used were 0%, 5%, 10%, 15%, 20%, and 25%. The sperm was collected by stripping and diluted with methanol and fish ringer, then it was equilibrated at 5 °C for 10 minutes. After that, it was frozen at -10°C for 48 hours and thawed at 40 °C for 1 minute. The spermatozoa motility was analyze using ANOVA and Tukey test. The results show there is a significant effect (p <0.05) on all of egg yolk concentrations. The 5% concentration of free-range chicken egg yolk shows the highest motility percentage of 84.06 ± 1.67%.

1. Introduction
Kancra fish is one of the endemic fishes that comes from the genus *Tor* in Indonesia [1]. The people of North Sumatra call this fish as *Batak* fish, while the West Java call it as *kancra* fish [2]. The people use kancra fish for consumption [3] and traditional ceremonies in North Sumatera [4]. The marked decline in population observed, as a result of exploitation [5]. Furthermore, it is possible to overcome this challenge using some reproduction technology, e.g., cryopreservation.

Cryopreservation is a long-term storage technique conducted in very low temperatures [6], which tends to stop metabolic processes in the cell, making each unit last for a longer time [7]. This procedure is usually performed on germ and embryo cells, with the benefit of species conservation, as in the creation of cryobank [8], and a major supporting factor for success is the use of cryoprotectant [9]. Cryoprotectant is a substance that is highly needed to mitigate the ice crystallization process during cryopreservation process [9]. Egg yolk is an example of natural cryoprotectant [10]. Egg yolk has the benefit of protecting sperm cell membrane from damage due to cold shock [11]. The use of free-range chicken’s egg yolk as a cryoprotectant produces better cryopreservation results compared to domestic chicken’s egg yolk. This is supported by the research previous [12], where a comparison was made between the percentages of post-cryoprotected spermatozoa motility, using cryoprotectants of free-range and domestic chicken egg yolk. The result of the research discovered that the spermatozoa motility post-cryopreservation was better when using free-range chicken’s egg yolk rather than domestic chicken’s.
Related research using a combination of domestic chicken’s egg yolk and methanol had been conducted on the spermatozoa of tawes fish (Barbonymus gonionotus) [13]. There had never been any cryopreservation research which uses the combination of free-range chicken’s egg yolk and methanol on kancra fish. The renewal of this research is the use of cryoprotectant combination of free-range chicken egg yolk and methanol 10% of kancra fish. In a previous study [23] combined glycerol and domestic chicken’s egg yolk used for cryopreserved the sperm of kancra fish. It is expected that by replacing the cryoprotectant used, it will be able to maintain a higher percentage of motility in the post-cyopreservation sperm. Methanol has lower toxic than glycerol [9] and based on the research of Jenice et al. [12] it is proven that free-range chicken egg yolk is better in maintaining sperm motility than domestic chickens egg yolk. Therefore, this research aimed to investigate the effect of free-range chicken’s egg yolk using various concentrations (of 0%, 5%, 10%, 15%, 20% and 25%) towards the motility of spermatozoa post-cryopreservation.

2. Materials and methods

2.1. Time and location
The study was conducted between April and July 2019 in Installations for Freshwater Fish Genetic Resources, Ministry of Marine Affairs and Fisheries, Cijeruk, West Java.

2.2. Collection of sperm
Mature male of kancra fish obtained from Installations for Freshwater Fish Genetic Resources, Ministry of Marine Affairs and Fisheries, Cijeruk, West Java. The ejaculated sperm were collected by hand stripping [13].

2.3. Preparation of the extender fish ringer solution
The fish ringer was prepared by dissolving NaCl 3.25 g, KCl 0.125 g, CaCl2·2H2O 0.175 g and NaHCO3 0.1 g in 500 ml of distilled water [14].

2.4. Preparation of the activator solution
The activator solution was prepared by dissolving 0.2633 g NaCl, 0.0373 g KCl and 0.3634 g C4H11NO3 in 100 ml of distilled water [15].

2.5. Sperm dilution
The ejaculated sperm were diluted with a diluent solution (free-range egg yolk + fish ringer (ekstender) + 10% methanol). Ratio diluted 1:10 [15] with modification. Egg yolk concentration which is used in this study: 0%, 5%, 10%, 15%, 20%, and 25%.

2.6. Equilibration and freezing
Sample was then equilibrated at 5° C for 10 minutes and was frozen at -10 °C for two days (48 hours) [13] with modification.

2.7. Thawing
Thawing was carried out at 40 °C for 60 seconds [13] with modification.

2.8. Post-thaw parameters examined
Observations performed included (1) Macroscopic evaluation, which consists of color, volume and pH assessment. Sperm color measurements were carried out visually, Volume’s sperm by inserting sperm into the scale eppendorf and pH measurements used a pH meter (2) Microscopic examination of motility spectrum, using a light microscope, with the aid of an eye-pie, digitally linked to an image driving software (Scopephoto 2.0.4).
2.9. The motility rate
Observation of motility was performed on fresh sperm and post-cryopreservation sperm. The observation was carried out by placing 10 μl of the sperm on an Improved Nauber, then subsequently observed under a microscope, with a magnification of 10 x 40. The percentage of spermatozoa were calculated as the formula [16] and analysis with ANOVA and Tukey test [17]. All statistical analyses were performed using SPSS Version 16.0 of 2007:

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\text{% Motility} = \frac{\sum \text{Number of motilitied sperms}}{\sum \text{Number of sperm counted}} \times 100\%
\]

3. Results and discussion
3.1. Fresh sperm analysis
Fresh sperm had milky-white coloration, with 8.5 pH and a volume of 1.5 ml per ejaculation. The average percentage of fresh sperm motility is 89.42 ± 1.34 % (Table 1). Spermatozoa motility is a very influential parameter that determined the quality of sperm [16]. The motility percentage of fresh sperm affects the success of cryopreservation. The motility of fresh sperm that is feasible for cryopreservation is above 70% [18]. Based on the observation, the mean percentage of fresh sperm’s motility reached 89.42 ± 1.34%.

| Table 1. Fresh Sperm Profile |
|-----------------------------|
|                             |
| Physical-chemical characteristic | Microscopically analysis |
| Volume (ml) | pH | Color       | Motility (%) |
| 1.5           | 8.5 | Milky-white | 89.42 ± 1.34 |

3.2. Sperm analysis after freezing
On post-cryopreservation, motility evaluation was observed to be lower, in contrast with the fresh spermatozoa, and the percentage value for the varying concentrations of 0%, 5%, 10%, 15%, 20%, and 25%, were 56.20 ± 2.54%, 84.06 ± 1.67%, 77.74 ± 2.67%, 72.69 ± 2.48%, 70.14 ± 1.94% and 57.04 ± 2.76%, respectively. The percentage result is shown in Table 2. After cryopreservation, the motility decreased. The motility percentage decrease of the spermatozoa post-cryopreservation reached more than 5%. The highest percentage of the sperm’s motility post-cryopreservation was 84.06 ± 1.67%. This research is supported by the other research [17] which also proved that the decrease of sperm motility percentage post-cryopreservation might even reach up to 15%.

| Table 2. Post-cryopreservation of spermatozoa motility |
|------------------------------------------------------|
| Treatments (%) | Egg yolk (%) |
|----------------|--------------|
| 0%             | 56.20 ± 2.54a |
| 5%             | 84.06 ± 1.67d |
| 10%            | 77.74 ± 2.67c |
| 15%            | 72.69 ± 2.48bc |
| 20%            | 70.14 ± 1.94b |
| 25%            | 57.04 ± 2.76a |

Different letters in the column indicate significant differences (P <0.05)
Based on ANOVA analysis, it was found that there was a significant effect (p < 0.05) from various concentrations of free-range chicken’s egg yolk on the spermatozoa post-cryopreservation. Moreover, the tukey’s evaluation also showed significant differences (p < 0.05) in 5% egg yolk concentration toward all treatments of spermatozoa motility post-cryopreservation (Figure 1). 5% concentration of free-range chicken’s egg yolk was the optimum concentration to preserve the motility of spermatozoa post-cryopreservation. The motility percentage using 5% concentration of free-range chicken’s egg yolk reached 84.06 ± 1.67%, while for treatments that did not add free-range chicken’s egg yolk (0%) only reached 56.20 ± 2.54%. This was because free-range chicken’s egg yolk contained low-density lipoprotein (LDL) which played an active role in maintaining the stability of sperm cell membrane in the crystallization process during cryopreservation [19]. The protection mechanism of the egg yolk towards spermatozoa during cryopreservation is suspected to come from the phospholipid contained in the LDL. Phospholipid will create a layer that is able to protect the spermatozoa surface and even replace the damaged spermatozoa membrane due to extreme temperature change [20].

The previous research about cryopreservation had been conducted on gourami fish by using 7% dimethyl sulfoxide (DMSO) without the addition of a natural cryoprotectant. The result showed that the highest motility percentage only reached 68.58% [21]. Meanwhile, other researchers attempted to combine 10% methanol and egg yolks on botia fish (Chromobotia macracanthus), and the highest percentage for post-cryopreservation sperm motility was 96.43 ± 1.49% [22]. Cryopreservation research on kancra fish had been conducted previously, using a combination of 10% glycerol and egg yolk, which only preserved the sperm motility percentage at 76.7% [23].

**4. Conclusion**

The research concludes that the combination of 10% methanol and 5% concentration of free-range chicken’s egg yolk is able to preserve the spermatozoa motility of kancra fish up to 84.06 ± 1.67%.

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