Honey effect on sperm motility of kancra fish (*Tor soro* Valenciennes, 1842) after 48 hours freezing

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**Abstract.** Kancra (*Tor soro*) is a freshwater endemic fish found in specific place of Indonesia, with a drastic decrease in its population due to the varying gonad maturity times between males and females. However, the use of cryopreservation is effective for the synchronization of their gametes using cryoprotectants. Therefore, this study aimed to determine the effect of honey concentration (0%, 5%, 10%, 15%, 20%, and 25%) using 10% of methanol in the spermatozoa motility of kancra fish 48 hours after cryopreservation. The study was conducted in the Research and Development Institute for Freshwater Fish Genetic Resources (Balai Besar Perikanan Budidaya Ikan Tawar, BBPBAT), in Cijeruk, Bogor, West Java. The sperm were collected through the stripping method followed by the evaluation of spermatozoa motility 48 hours after cryopreservation. Furthermore, it was equilibrated for 10 minutes at a temperature of 5 ºC, frozen at 10 ºC for 48 hours, and thawed for 1 minute at 40 ºC. The data were analyzed using ANOVA followed by the Tukey test. Additionally, the ANOVA test showed that honey had effects (P<0.05) on the motility percentage after cryopreservation with a 5% optimum concentration using 85.97±1.91%.

**1. Introduction**

Kancra fish (*Tor soro*) is one of freshwater endemic fish which is found in specific parts of Indonesia [1]. Batak people, North Sumatra, commonly know *T. soro* called “ihan” which used as requirement in traditional marriage of ceremonies. *T. soro* in around Kuningan, West Java is named as kancra fish or god fish [2]. This kind endemic species plays important role in ecosystem. If its population slowly decreases, it can cause further imbalance ecosystems.

*Tor soro* is known as a good commodity in fisheries [3]. The best taste of its meat; delicious and tasteful lead the selling becomes high-priced [2]. A fatty component in *T. soro* is lower than any others fish; 2 essential fatty acids, DHA 4.74%, and EPA about 0.31%. That reason makes *T. soro* as of consumption freshwater which is popular in Indonesia [4].

Decreasing population of *T. soro* in Indonesia drastically was from 16% until 13% in year 2017 [5]. It was caused by imbalance between low stock availability, consumption level, and expensive selling price (Rp 350,000-1,500,000 for each kg). Various way of preservation has been done, example by restocking in Toba Lake and location of tourist attraction, Cibulan, Darmaloka, Pesawahan, West Java. Another way of preservation comes with ex situ conservation; hatchery and breeding of Batak fish *Neolissochilus thienemanni* and *T. soro*, genetic resource research, morphometric characterization, and feed nutrition but unfortunately all of them were unsuccessful [6].
Cryopreservation is an alternative means of storing sperm at low temperatures which requires cryoprotectant to maintain the cell structures after the freezing process [7]. Cryopreservation is helpful to facilitate fish management also female and male gamete synchronization as a genetic selection program and species conservation [8]. Although cryoprotectant protects cells, it also harms it due to its toxic characteristics [9], with the freezing degree determined by the concentration and types of cryoprotectant towards the crystallization process. This research aimed to evaluate the optimum concentration of honey as a natural and extracellular cryoprotectant to maintain sperm motility 48 hours after cryopreservation.

2. Material and method

2.1 Liquid preparation

The sperm and honey stored are thawed (diluted) due to their thick volume, with the diluent solvent consisting of honey (which has been thawed), Fish Ringer, and methanol. The honey dilution used to add Fish ranger in this study were in concentrations of 0%, 5%, 10%, 20%, and 25%. This was dissimilar to the use of fish ringer made by mixing NaCl 0.135 g, KCl 0.06 g, CaCL₂ 0.025 g, MgCl₂ 0.035, and NaHCO₃ 0.02 g to the 100 ml of aquades (distilled water). Sperm motility required a type of solvent known as an activator, which was made by combining 0.2633 g of NaCl, 0.0373 g of KCl, and 0.3634 g of C₄H₁₁NO₃ to 100 ml of aquades (distilled water) [10] with modification.

2.2 Sperm preparation

The sperm was obtained from T.soro by stripping it at the Research and Development Institute for Freshwater Fish Genetic Resources (Balai Besar Perikanan Budidaya Ikan Tawar, Cijeruk, West Java), in Cijeruk, West Java. A syringe size of 3 ml was used as the media to collect the fish sperm which was then transferred to two microtubes. The first was measured to determine its pH, volume, and color, while the second was transferred to the cryotube filled by the previous diluent solvent (diluted Fish Ringer, methanol, and honey) [10] according to their previous concentration in ratio of 1:10.

2.3 Freezing, thawing (diluting), and observation of motility

The observation was carried out for fresh sperm and post-cryopreservation motility. The sperm mixed with the diluent solvent (honey, methanol, and Fish Ringer) was put into cryotubes and equilibrated for 10 minutes at a temperature of 5°C [10]and put into the freezer for two days at -10°C. After 48 hours, the sperm was thawed (diluted) for microscopic analysis. This was conducted by removing it from the freezer for 5 seconds and inserting it into warm water at a temperature of 40°C for 1 minute [11]. The thawed sperm was re-thawed using Fish Ringer with a ratio of 1:4 and analyzed under a microscope to calculate its motility. The observation was conducted by putting 10 μl of sperm into Improved Neubauer with its motility analyzed under a light microscope with magnificence of 10 x 40 connected to Scopephoto 2.0.4, an image drawing software. The motility percentage was calculated using the formula below [12]:

\[
\text{% Motility} = \frac{\sum \text{moving sperm}}{\sum \text{total of sperm}} \times 100
\]

2.4 Statistic analysis

The data were counted based on the 4 repetitions, with the significant difference statistically counted using ANOVA continued by Tukey test (P<0.05). All statistical analysis was performed using SPSS Version 16.0 of 2007.
3. Results and discussion

3.1 Analysis of fresh sperm

Some characteristics of fresh sperm (shown in Table 1) were milky white in color, with pH 8.5, and an average total volume of 1.5 ml per ejaculation. The moving and living sperm is seen in Figure 1, with an average motility percentage of 85.92 ± 1.91.

Table 1. Motility of fresh sperm.

| Physical and chemical characteristics | Microscopic Analysis |
|-------------------------------------|----------------------|
| Volume (ml) | pH | Color | Motility(%) |
| 1.5 | 8.5 | Milky White | 87.76 |
| | | | 83.36 |
| | | | 85.70 |
| | | | 86.87 |
| Avg | | | 85.92 |
| Stdev | | | 1.91 |

Figure 1. The image of viable and non viable sperm. (A) showed the viable sperm, while (B) showed the non viable sperm.

3.2 The analysis of sperm after freezing process

The motility after the freezing process had higher motility than the fresh sperm with honey concentrations of 69.30 ± 1.88%, 85.92 ± 1.91%, 82.18 ± 1.90%, 79.85 ± 1.80%, 75.67 ± 1.94%, and 71.41 ± 1.83%. The result of fresh sperm is shown in Table 2, while all the results of the percentage are shown in Figure 2.

Table 2. Sperm motility after the freezing process.

| Concentration | Fish Ringer (%) | Honey (%) |
|---------------|----------------|-----------|
| 0%           | 69.30d         |           |
| 5%           |                | 85.92a    |
| 10%          |                | 82.18ab   |
| 15%          |                | 79.85bc   |
| 20%          |                | 75.67c    |
| 25%          |                | 71.41d    |

Different rate showed a significant difference (P<0.05)
One of the sperm parameters measured after freezing was motility [9]. Its percentage was measured because the motility was claimed to be more informative to determine the sperm quality, right after fertilization [13]. The result of post-freezing observation tends to be different which is capable of being influenced by species, extender solvent, cryoprotectant used, reproduction period, and feed of each individual [14].

The motility result of fresh sperm was 69.30±1.88%, with a decrease in percentage of sperm motility after 48 hours of cryopreservation. The highest motility was 87.76% of the 5% honey concentration. The cold shock during the freezing process led to a decrease of motility and damaged the DNA, integrity of plasma membrane, its functionality, and ATP capable of causing low fertilization power [15][16].

Statistical analysis showed a significant effect on the use of various concentrations of honey. A vivid difference (P<0.05) was obtained from the Tukey test of sperm motility right after cryopreservation (Figure 2), with 5% concentration used during the maintenance and freezing process. The percentage of 5% honey was approximately 85.92±1.91%, while the concentration without honey (0%) was 69.30±1.88%. It was, however, due to the functional role of the cryoprotectant, to protect the sperm from external object and cold shock [17].

The honey used in the research contained 65.65% reducing sugar, comprising fructose and glucose as one of the important ingredients in most cement extenders [18]. Honey was also known to contain disaccharide, monosaccharide, polysaccharide, and oligosaccharide [19].

Sperm needs the monosaccharide energy provided by honey, which acts as a non-penetrating agent. The addition of fructose and glucose, plasma protein, urea, mineral salt, and fat, functions as sperm nutrients [20]. Furthermore, its antibacterial and antioxidant contents were substantially used to maintain the motility, viability, and integrity of the membrane during the post-thawing process [11]. Especially, since honey was considered a strong antioxidant to maintain the cells of the organs due to the reactive oxygen species (ROS) [21]. Conversely, the addition of honey as the energy and nutrient source, along with the physiological solvent were expected to support sperm life and its motility during the storage stage and after post-thawing.

Sperm cryopreservation using various honey concentrations as the cryoprotectant has been implemented in some fish species, with honey as the liquid material for its sperm storage with a motility effect and life endurance of 26.23% for 48 hours after observation [22]. Another research was conducted on the sperm cryopreservation of gouramy fish using honey concentration of 0.1%, 0.3%, 0.5%, 0.7%, and 0.9%. The result of the research showed maximum motility and viability up to 80% with 0.7% honey concentration [10].
However, studies and further research need to be conducted to evaluate a more informative result related to the sperm cryopreservation of *T. soro* and to determine the best type of natural cryoprotectant to simplify the process.

4. Conclusion

The use of 5% honey was the best extracellular cryoprotectant to maintain sperm motility after the freezing process.

5. References

[1] Weber M and de Beaufort L F 1916 The fishes of the Indo-Australian Archipelago (Leiden: E. Brill. Ltd.) p 404

[2] Tjahjo D W H, Setiadi E, Kartamihardja, Hardjamulia A, Suhenda N, Sadili D, Subagio Mand Sukadi M F 1995 *Pros Semhas Penelitian Perikanan Air Tawar 1993-1994* (Indonesia: Balai Penelitian Perikanan Air Tawar) p 8

[3] Rumondang A M 2017 *IJFA* 2 23-26

[4] Haryono H and Tjakrawidjaja A H 2006 *Biodiversitas* 7 59-62

[5] KKP (Kementerian Kelautan dan Perikanan) 2015 *Lap Akhir Pem Seb JADDI* 1-134

[6] Redjeki S 2007 *IJFA* 2 23-26

[7] Haryono H and Tjakrawidjaja A H 2006 *Biodiversitas* 7 59-62

[8] Cabrita E, Sarasquete S, Martinez-Paramo S, Robles V, Beirao J, Perez-Cerezales S and Harraez M P 2010 *J of Applied Ichthyology* 26 623-635

[9] Galo J M, Streit-Junior D P, Oliveira C A, Povh J P, Fornari D C, Digmayer M and Ribeiro R P 2019 *Brazilian J of Bio* 79 438–445

[10] Abinawanto, Pratiwi I A and Lestari R 2017 *AACL Bioflux* 10 156-163

[11] Ansari A H 2012 Repeated Freeze Thawing for Assessment of Semen Freezeability, Homologous Artificial Insemination (AIH) (USA: Springer) p 117-184

[12] Muchlisin Z A, Nadiah W N, Nadiya N, Fadli N, Hendri A, Khalil M and Siti-Azizah M N 2015 *Czech J of Anim Sci* 60 1-10

[13] Purdy P H 2006 *Small Rumin Res* 63 215-225

[14] Fuller B J 2004 *Cryo Letters* 25 375-388

[15] Rahardhianto A, Abdulgani N and Trisyani N 2012 *J Sains dan Seni ITS* 1 58-63

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