Viability of Tor fish spermatozoa (*Tor soro*, Valenciennes 1842) 48-hours cryopreservation: the effects of duck egg yolk as a natural cryoprotectant

P D Wulandari¹, Abinawanto¹,³, J Subagja², A H Kristanto²

¹Departemnt of Biology, Faculty of Mathematics and Natural Sciences, University of Indonesia, Depok, 16424, Indonesia
²Installations for Freshwater Fish Genetic Resources, Ministry of Marine Affairs and Fisheries, Cijeruk, West Java, Indonesia
³Corresponding author: abinawanto.ms@sci.ui.ac.id

Abstract. Tor is endemic freshwater fish in Sunda, locally known as *kancra* or *dewa*, which inhabits rivers with clean and clear water quality. In local customary and cultural perspectives, it is sacred fish whose populations have persistently depleted into scarcity. Sperm cryopreservation is one of many techniques to maintain its survival from extinction by inducing breeding at the reproductive stage. This study was designed to determine the effects of duck egg yolk as a cryoprotectant on the viability of Tor fish spermatozoa after 48 hours of cryopreservation. It employed the stripping method to obtain the fish semen and used diluent solution consisting of fish ringer solution, 10% methanol, and duck egg yolk in various concentrations (0%, 5%, 10%, 15%, 20%, 25%), with 1:10 ratio of sperm to diluent solution. These samples were equilibrated at 5°C for 10 minutes, frozen at -10°C for 48 hours in a freezer, and then defrosted at 40°C for 1 minute. The One-way ANOVA revealed statistically significant differences (p <0.05), and the post-hoc Tukey test also showed statistically significant differences (p <0.05). Because the 10% duck egg yolk produced the highest viability (averagely 83.75±1.71%), this concentration is the best cryoprotectant for optimal Tor spermatozoa cryopreservation.

1. Introduction

Some Tor genus are included in the IUCN (International Union for Conservation of Nature and Natural Resources) red list. The red list contains endangered animals [1]. There are four species of Tor fish in Indonesia, namely *Tor tambroides*, *Tor tambra*, *Tor douronensis*, dan *Tor soro* [2].

*Tor soro* is a freshwater fish of the Cyprinidae family that lives in the upstream habitat of a tropical rainforest with clear water, has a flow of water, rich in oxygen, cool temperature, and rocky [3]. *Tor soro* or kancra fish (West Java) has high economic value, both as consumption and ornamental fish. For some Kuningan (West Java people), *Tor soro* is called dewa fish because of the cultural values attached to it.

However, high demand leads to overfishing which has triggered a decline in population. Keep in mind that the Kancra larvae need four years to grow into adulthood [4]. In addition, natural spawning...
is also constrained by differences in the time of gonad maturation between males and females. Differences in gonad maturation can be overcome by sperm freezing. The success of cryopreservation is determined by extender, cryoprotectant, dilution ratio, freezing rate, and thawing rate [5].

Ringer solution is one of the most common types of extenders used in cryopreservation [6]. As intracellular cryoprotectant, methanol has a very small molecular size that can enter the cell membrane [7]. Meanwhile, duck egg yolk is one of the natural extracellular cryoprotectant that can maintain the stability of the outer cell membrane [7].

Viability is the life force of spermatozoa and an indicator of semen quality. Inspection of spermatozoa viability can be used as an indicator of the integrity of spermatozoa membrane structure [8]. Research using a combination of methanol and various concentrations of duck egg yolk in cryopreservation of kancra has never been done before. This study aimed to evaluate the effect of various concentrations of duck egg yolk (0%, 5%, 10%, 15%, 20%, 25%) on the viability of kancra spermatozoa at 48 hours after cryopreservation.

2. Material and methods
2.1. Fish ringer solution preparation
Fish ringer solution made by dissolving NaCl 0.675 g; KCl 0.3 g; CaCl₂ 0.125 g; and NaHCO₃ 0.1 g in 500 ml of distilled water, modification [6], store in a glass bottle and cover with parafilm, make fresh every three days [9].

3.1. 0.5% eosin-Y solution preparation
An eosin-Y 0.5% solution made by dissolving 0.5 g eosin-Y in distilled water up to 100 ml [10].

3.2. Collection of the ejaculated semen/sperm
Adult male kancra (Tor soro) obtained from the Installations for Freshwater Fish Genetic Resources, Cijeruk, West Java. The semen obtained through hand stripping in the abdomen. The abdomen has been cleaned with tissue paper to avoid the presence of urine and mucus into the semen.

3.3. Semen/sperm dilution
The ratio of semen and diluent is 1:10, modification [11]. The diluent consists of a fish ringer (extender) solution, 10% methanol (intracellular cryoprotectant), and duck egg yolk (extracellular cryoprotectant) as seen in Table 1.

The concentrations of duck egg yolk used in this study were 0%, 5%, 10%, 15%, 20%, and 25%, modification [12].

| Experimental Group | Composition |
|--------------------|-------------|
|                    | Semen/Sperm (µl) | Methanol 10% (µl) | Fish Ringer Solution (µl) | Duck Egg Yolk (µl) |
| 0% EY              | 50           | 500                | 4500                      | 0                   |
| 5% EY              | 50           | 500                | 4250                      | 250                 |
| 10% EY             | 50           | 500                | 4000                      | 500                 |
| 15% EY             | 50           | 500                | 3750                      | 750                 |
| 20% EY             | 50           | 500                | 3500                      | 1000                |
| 25% EY             | 50           | 500                | 3250                      | 1250                |

0% = control group; 5% EY, 10% EY, 15% EY, 20% EY, 25% EY = treatment group
3.4. Semen/sperm equilibration, freezing, and thawing

Cryotubes containing diluted semen samples were stored at 5°C for 10 minutes (equilibration) so that the samples adapted to the temperature and osmotic changes in cell fluids. Freezing is done by putting the equilibration cryotube in the freezer at -10°C for 48 hours. Thawing is carried out 48 hours after cryopreservation by immersing the frozen cryotube in a 40°C waterbath for 60 seconds.

3.5. Semen/sperm quality evaluation

Evaluation is carried out before (fresh semen) and after the cryopreservation. The fresh semen evaluation includes macroscopic evaluation (volume, pH, color) and microscopic (viability).

Percentage of spermatozoa viability is known from the number of viable spermatozoa (not stained with 0.5% eosin-Y solution) per 100 spermatozoa at each test. 10 µL of cement sample produced 100 times dilution was dropped on a sliding glass and 0.5% eosin-Y was added (1:1 ratio). The mixture is then homogenized using cover glass and reviewed using slide glass with an angle of 45°. The mixture is closed using a glass cover so that the review does not bubble. The review results were observed under a light microscope with a magnification of 10x40 [13].

Meanwhile, the post-cryopreservation semen is only evaluated microscopically. Microscopic evaluation is carried out using a light microscope and digital eyepiece camera connected to the computer through Soft Imaging software analysis (Scopephoto 2.0.4.). Viable spermatozoa will not be stained by 0.5% eosin-Y solution, while the non-viable spermatozoa become red due to the entry of eosin into the cell due to damage to the cell membrane [13]. The percentage of viability is calculated using the formula [14]:

\[
\% \text{ Viability} = \frac{\sum \text{viable spermatozoa}}{\sum \text{total spermatozoa}} \times 100\%
\]

3.6. Statistical analysis

The research data is tabulated and tested for normality and homogeneity. One-way ANOVA analysis and Tukey [15] test using the SPSS version 16 for Windows statistical program carried out on normally distributed and homogeneous data. All probability values were set at level of significance of 0.05.

3. Result and discussion

3.1. Result

Semen of the kancra is milky white. The results of this study indicate that the average pH of the kancra semen is 8.5. This study used 2.17 ml of semen derived from three adult male kancra in which each fish produced as much as 1.5 - 2.5 ml of semen. The results of the macroscopic evaluation of fresh macaques are shown in Table 2.

Percentage of viability is one of the sperm quality parameters. These parameters indicate the number of viable spermatozoa. Viable spermatozoa are bright green, while non-viable spermatozoa are red (Figure 1). The average viability percentage of fresh spermatozoa of kancra was 85.25 ± 2.22% (Table 1). This value is close to the average viability percentage of fresh spermatozoa of tawes (Java barb) by 82.57 ± 5.38% [16]. Viability percentage above 80% is a requirement for spermatozoa to be used in cryopreservation [17].

The percentage of spermatozoa viability of kancra after 48 hours of cryopreservation can be seen in Table 3. The average percentage of the viability of spermatozoa of kancra fish after 48 hours of cryopreservation was 52.25 ± 2.22% (KT 0%), 72 ± 2.94% (KT 5%), 83.75 ± 1.71% (KT 10%), 73.25 ± 1.71% (KT 15%), 71.5 ± 1.29% (KT 20%), and 70, 25 ± 2.50% (KT 25%).

Based on one way Anova test, there was significant effect (p<0.05) of various concentration of duck egg yolk on the post-thaw sperm availability, compared to control (10% methanol, 0% duck egg yolk).
According to the Tukey multiple comparison test, the concentration of 10% duck egg yolk showed the highest post-thaw sperm availability (83.75±1.71%).

3.2. Discussion
The highest viability value (83.75 ± 1.71%) of kancra spermatozoa after 48 hours of cryopreservation resulted from 10% duck egg yolk concentration. This value is 1.5% lower than the viability of fresh semen. On the other hand, there are significant differences and an increase in the average value of spermatozoa viability by 31.5% against 0% duck egg yolk concentration.

In another study, the use of 10% DMSO resulted in a 75.8% common carp spermatozoa viability value [18]. The use of standalone-intracellular cryoprotectant (without extracellular cryoprotectant) drastically reduces viability.

The use of 10% methanol and 0.5% sucrose produces the viability of gurame (*Osphronemus goramy*) spermatozoa of 82.17 ± 2.56% [19]. The use of 10% methanol and 5% egg yolk resulted in the viability of tawes (*Java barb*) spermatozoa of 85.50 ± 3.11% [16]. The average viability values of gurame and tawes spermatozoa were not significantly different because they both used intracellular and extracellular cryoprotectants.

![Figure 1. Viable spermatozoa and non-viable spermatozoa.](image-url)
Table 2. Fresh semen (spermatozoa) profile.

| Physical-chemical characteristic | Microscopic characteristic |
|----------------------------------|---------------------------|
| No. | Volume (ml) | pH | Color       | No. | Viability (%) |
|-----|-------------|----|-------------|-----|---------------|
| 1.  | 2.5         | 8.5| Milky white | 1.  | 83            |
| 2.  | 1.5         | 8.5| Milky white | 2.  | 84            |
| 3.  | 2.5         | 8.5| Milky white | 3.  | 86            |
|     |             |    |             | 4.  | 88            |
| Average | 2.17     | 8.5|             |     | 85.25         |
| St. Dev. |          |    |             |     | 2.22          |

Table 3. Post-thaw and fresh spermatozoa quality.

| Duck Egg Yolk Concentration (%) | Viability (%) |
|---------------------------------|---------------|
| 0                               | 52.25±2.22a   |
| 5                               | 72±2.94b      |
| 10                              | 83.75±1.71c   |
| 15                              | 73.25±1.71b   |
| 20                              | 71.5±1.29b    |
| 25                              | 70.25±2.50b   |

Different letters in each column show significant difference (p<0.05)

4. Conclusion
It is concluded that the combination of 10% methanol + 10% duck egg yolk showed the highest post-thaw viability (83.75±1.71%). Further, work will be focused on fertility rate, hatching rate, and survival rate after in vitro fertilization.

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