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*Original article*

# Effect of pre-freezing on motility, viability and abnormality of Nile tilapia fish *Oreochromis niloticus* sperm post cryopreservation

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## Abstract

Nile tilapia *Oreochromis niloticus* is a popular freshwater fish that has been extensively and intensively cultured worldwide. However, cryopreservation of its sperm, especially pre-freezing procedure, has not been properly developed. Therefore, the study aimed to determine the best pre-freezing procedure for cryopreservation of Nile tilapia *Oreochromis niloticus* sperm. The completely randomized design with five treatments and four replications was employed in this study. The tested treatments were T1=4°C → 0°C → -4°C → -10°C → -79°C → -196°C, T2=4°C→ 0°C → -4°C → -10°C → -196°C, T3=4°C → 0°C → -4°C → -196°C, T4=4°C → 0°C → -196°C), and T5=4°C → -196°C, with a 10 min equilibration at each respective temperature. Furthermore, sperm was cryopreserved for two weeks in liquid nitrogen (-179 °C). The results of the ANOVA test showed that pre-freezing had a significant effect on sperm motility, and viability ($P<0.05$), but had no considerable impact on sperm abnormality ($P>0.05$). Treatment T4 exhibited higher motility and viability, but these values were not significantly different from T3 and T5. Based on practical consideration, it was recommended to utilize the T5 pre-freezing procedures (4°C → -196°C) for cryopreservation of Nile tilapia sperm. Considering these results, Nile tilapia sperm can be directly cryopreserved into liquid nitrogen after equilibration at 4°C for 10 min.

**Keywords:** sperm quality, cryoprotectants, DMSO, egg yolk, fish breeding

## Introduction

Nile tilapia is one of the most popular freshwater fish that has been cultured intensively worldwide (Hassan et al. 2013). The availability of high-quality larvae is one of the key factors to successful fish farming (Abidin et al. 2006, Sumarni 2018). Breeding technology for tilapia, including sex reversal and monosex (Abdelhamid 2009, El-Greisy and El-Gamal 2012, Nduku et al. 2022, Silva et al. 2023) as well as genetic engineering, was developed (Yanez et al. 2020, Geletu and Zhao 2022) have been reported. However, cryopreservation technique for sperm has not been intensively studied. This technology can be combined with genetic engineering, sex reversal or mono sex, which have been widely applied in breeding process. The cryopreserved sperm can be used at all the time, regardless of the spawning season, hence, maintaining a consistent quantity and quality. Currently, only two reports have been conducted on this topic. These included the study of Asmad et al. (2011) who investigated sperm cryopreservation of tilapia using the slow freezing method with application of Tris Citric Acid Yolk Extender (TCAYE). The results showed viability and motility rates of 38.61% and 61.39%, respectively. Study by Bozkurt and Yavaz (2016) who employed the same method with a combination of Tris Glucose (TG) + 10% Dimethylacitamide (DMA), reported motility, viability and fertility of 55%, 79%, and 45.7%, respectively.

Cryopreservation methods can be divided into slow and rapid freezing. The slow sperm freezing technique used a gradual decrease in temperature with longer steps and time. This approach employs low concentrations of cryoprotectants (<5%) (Watson 2000). Rapid freezing is a technique with shorter temperature reduction, and it usually applies cryoprotectants with higher concentrations (>5%) (Pegg 2015). However, the usage of low concentration of cryoprotectants in rapid freezing has not been studied.

Several studies have explored the application of both slow and rapid freezing for fish sperm cryopreservation. For instance, the slow freezing technique has been utilized for cryopreservation of catfish sperm (*Clarias gariepinus*), with pre-freezing temperatures of 4°C →, 0°C →, -4°C →, and -79°C → at - 196°C per 5 min (Muchlisin et al., 2015). In snakehead fish (*Channa striata*), 5°C →and -140°C →, were applied at -196°C per 7 min (Mangkunegara et al. 2019), while in depik *Rasbora tawarensis,* two levels such as 4°C →and -110°C →, were utilized at -196°C per 10 min (Muchlisin et al. 2020). The rapid freezing technique has been applied to botia (*Chromobotia macracanthus*) using one level of pre-freezing temperatures, namely 4°C →at -196°C per 120 min (Abinawanto et al. 2018). In this study, a combination of fast and slow rates using low concentrations of cryoprotectants at 5%, was assessed regarding sperm quality of tilapia post cryopreservation. Therefore, the aim of this study was to determine the best pre-freezing procedure for tilapia sperm using DMSO as an intracellular cryoprotectant and egg yolk as an extracellular cryoprotectant both at a low concentration (5%).

## Materials and Methods

### Experimental design

This study was conducted using non-factorial experiment with a completely randomized design. The tested factor was the differences in pre-freezing temperatures with 10 min equilibration time at each. Furthermore, the treatments involved were (T1)=4°C → 0°C → -4°C → -10°C → -79°C → -196°C, (T2)=4°C → 0°C → -4°C → -10°C → -196°C, (T3)=4°C → 0°C → -4°C → -196°C, (T4)=4°C → 0°C → -196°C, and (T5)=4°C → -196°C, and every treatment was performed with four replications.

### Broodstock preparation

A total of 50 males and 20 females tilapia were collected from local farms in Nagan Raya, Aceh province, Indonesia, and were acclimatized for 1 week in a fiber circular tank at the Laboratory of Hatchery and Fish Breeding, Faculty of Marine and Fisheries, Syiah Kuala University. During the acclimatization period, fish were fed a commercial diet twice daily at 8 AM and 4 PM, with a feeding ration of 3% body weight.

### Sperm collection and sperm characteristics analysis

A total of 25 matured male fish were collected from the brood stock tank, and their genital area was cleaned with the tissue paper to ovoid contamination with water and urine. Furthermore, their abdomen was pressured gently from the upper region to the genital pore, and sperm was collected using a sterile syringe. The initial quality of sperm was assessed by evaluating motility rate. Only those with motility higher than 70% from different males were pooled into a beaker glass and stored in an ice box at 4°C before being used for experiment.

The analysis of sperm characteristics included the assessment of color, pH, and viscosity. Color observation was conducted through direct visual inspection, while the pH was checked using a pH meter. The procedure and categorization of sperm consistency followed

the method outlined by Sugito et al. (2016). Finally, sperm concentration was determined using a hemocytometer. All procedures involving animals have followed by the animal ethics guidelines used in research of Universitas Syiah Kuala (Ethic Code No: 958/2015).

### Extender and cryoprotectant preparations

The extender used was Ringer solution, while a combination of 5% DMSO and 5% egg yolk served as cryoprotectants. The composition of the solution was prepared according to procedures outlined by Muchlisin et al. (2004). A total 2.5 ml of DMSO and 2.5 ml egg yolk 120 were added to the extender in a beaker glass until the total volume reached 50 ml. 121 Subsequently, 2.5 ml of sperm was included, achieving a dilution ration of 1: 20 (v/v) and the mixture was thoroughly mixed to ensure homogeneity. The sperm concentration in the diluent was approximately 1.08 – 1.21 x$10^9$ cells/ml. The solution was then distributed into 20 cryotubes (vol. 2 ml) up to a level of 1.5 ml each, and the remaining was kept for the stock.

### Equilibration and pre-freezing steps

The cryotubes were kept in an ice box at the temperature of 4°C for 10 min. Out of which 4 tubes were immersed directly into liquid nitrogen (-179°C) as the experiment P5, while 16 were transferred to a freezer at 0°C for 10 min. Furthermore, 4 tubes were immersed into liquid nitrogen as experiment P4. The other 12 cryotubes were kept in the freezer, and the temperature was reduced to -4°C for 10 min. Following this, 4 cryotubes were immersed into liquid nitrogen as experiment P3, while 9 were kept in the freezer, and the temperature was reduced to -10°C for another 10 min. Then, 4 cryotubes were moved into liquid nitrogen as P2, and the remaining 4 cryotubes were removed from the freezer and then kept at the neck of the nitrogen tank (-79°C) for 10 min, then immersed into liquid nitrogen (experiment P1). The samples were kept in liquid nitrogen for 2 weeks.

### Thawing and sperm motility analysis

After two weeks, the cryotubes were removed from the tank and thawed in a water batch (30°C) for 3 min then analyzed for sperm quality post cryopreservation (Bozkurt & Yavaş 2016). The thawing process started with T5 followed by T4 and T3.

### Sperm motility

Motility and viability were determined pre-cryopreservation and post-cryopreservation. Motility rate was 143 calculated by placing a drop of sperm onto a glass slide, then 2 drops of water were added for activation. Observation was conducted under a stereo microscope connected to a 145 CCD camera with 400x magnification. Sperm were classified as motile when they move 146 agile forwards. The counting was performed by randomly selecting approximately 100 sperm 147 from 5 field of view, then the values were averaged. Sperm motility was recorded for two minutes in five fields of view at the four corners and the center of the slide using 149 recording software (Optilab Viewer 3.0). The recorded data was saved and calculated later.

### Sperm viability and abnormality

A drop of sperm sample was placed on an object slide, then 1 drop of 0.2% eosin solution was added. The sample was rubbed with the fringe of another object slide to make a smear which was then allowed to dry for 30 min. Subsequently, observation was conducted under a stereo microscope at a 400X magnification. The viable sperm are transparent, while those that were dead appeared red due to absorbing eosin (Boonthai et al. 2016). Viability was calculated by randomly selecting 100 sperm samples from 5 fields of view, and then the results were averaged. At the same time, sperm abnormalityy was identified by observing the shape of the head and tail.

### Data analysis

The color, pH and viscosity data were presented in the table and analyzed descriptively. Meanwhile, those associated with sperm motility, viability and abnormality were subjected to One-way of Analysis of Variance (one-ways ANOVA), followed by a Duncan Post-hoc 165 multiple range test using SPSS software (ver. 22.0) to determine the best treatment.

## Results

Fresh sperm had milky white color, pH 7.5, high viscosity with concentration of 21.51 x $10^9$ cells/ml. The motility and viability of fresh sperm were 80.6% and 95.6%, respectively (Table 1). The ANOVA test showed that pre-freezing had a significant effect on sperm motility and viability ($p<0.05$), but did not affect their abnormality ($p>0.05$). The Duncan's multiple range test showed that the highest motility and viability were recorded in experiment T4 with values of 49.3% and 58.3%, respectively. These were significantly different with T1 (29.1% and 47.06%, respectively), and T2 (32.2% and 54.2%, respectively). However, the values were not different significantly from those

Table 1. Characteristic of nile tilapia *Oreochromis niloticus* sperm used in this experiment.

| Sperm characteristics | Description |
|---|---|
| Color | Milky white |
| pH | 7.5 |
| Viscosity | High |
| Concentration (cells/ml) | 21.51±1.4 x $10^9$ |
| Motility (%) | 80.6±0.8 |
| Viability (%) | 95.6±2.07 |
| Abnormality (%) | 1.20±1.6 |

Table 2. Sperm motility, viability, and abnormality of Nile tilapia sperm post cryopreservation with different pre-freezing steps. The average±SD at the same column followed by different superscripts is significantly different ($p<0.05$).

| Treatment | Motility (%) | Viability (%) | Abnormality (%) |
|---|---|---|---|
| T1 | 29.1±1.0$^a$ | 47.1±3.7$^a$ | 2.2±0.8$^a$ |
| T2 | 32.2±3.6$^a$ | 54.2±0.9$^a$ | 2.1±0.9$^a$ |
| T3 | 46.2±1.3$^b$ | 55.0±0.7$^b$ | 1.9±0.4$^a$ |
| T4 | 49.3±0.4$^b$ | 58.3±2.1$^b$ | 1.3±0.4$^a$ |
| T5 | 49.1±0.1$^b$ | 56.4±2.4$^b$ | 1.9±0.2$^a$ |

Note: T1=4°C → 0°C → -4°C → - 10°C → -79°C → -196°C, T2=4°C → 0°C → -4°C → -10°C → -196°C, T3=4°C → 0°C → -4°C → -196°C, T4=4°C → 0°C → -196°C, T5=4°C → -196°C with 10 min of the equilibrium time at every tested pre-freezing temperature.

of T3 (46.2% and 55.0%, respectively) and T5 (49.1% and 56.4%). The lowest sperm abnormality was observed in T4 (1.33%), but it was not significantly different among the treatments (Table 2).

## Discussion

The fresh sperm analysis showed that the average percentage of motility, viability, and abnormality was 80.6%, 95.6%, and 1.2%, respectively. Bozkurt and Secer (2005) stated that for the purpose cryopreservation, the initail motility should be higher than 70%. Therefore, the fresh sperm of tilapia used in this study had good quality and was suitable for cryopreservation.

The best sperm quality was obtained at treatment T4 with pre-freezing step and total equilibration time of 4°C → 0°C → -196˚C and 20 min, respectively. However, it was not significantly different from the T3 (4°C → 0°C → -4°C → -196°C) and T5 (4°C → -196°C) with 10 and 30 min total equilibrations, respectively. These treatments (T3, T4, and T5) were categorized under rapid freezing processes. Based on practical consideration, the recommended pre-freezing rate was treatment T5, where tilapia sperm became frozen immediately after 10 min equilibration at 4°C. This was presumed because a fast and concise pre-freezing can reduce the amount of ice microcrystal formation in cells. Morphologically, the micro ice crystals were pointed and sharp (Vetrakova et al. 2019), such that they caused damage to sperm, leading to highly abnormal cells as recorded in this study. These results aligned with previous studies that have reported that a short and rapid pre-freezing can reduce the formation of micro-ice crystals outside (storage medium) and inside sperm cell, thereby minimizing cell death and resulting in lower abnormality (Rurangwa et al. 2004, Agarwal 2011, Morris et al. 2012).

Fast freezing triggered temperature shock, leading to severe dehydration of cells and potential consequences such as excessive plasmolysis, changes in pH, and osmotic pressure that may result in cell membrane lysis (Zidni et al. 2020, Comizzoli et al. 2022). Therefore, presence of cryoprotectants was very important in protecting sperm from the temperature shock. In this study, 5% DMSO and 5% egg yolk were employed as permeable and non-permeable cryoprotectants to reduce the impact of temperature shock. The ability of cryoprotectants to protect cell membranes was an indication of their good interaction with cell membranes. The interactions reduced cell membrane damage when the state changes from liquid to solid (frozen) form, as well as during subsequent thawing process, when it returns to a liquid structure during the thawing process (Stoss 1983).

In this study, intracellular (DMSO) and extracellular cryoprotectants (egg yolk) were utilized at low concentrations (5%). According to Kostaman and Suatama (2006), the combination of these cryoprotectants was effective in maintaining the integrity of sperm cell

membrane, providing reliable protection during freezing and thawing processes (Maulida et al. 2021, Muchlisin et al. 2023, Nurlaili et al. 2023). This was because the intracellular cryoprotectants entered sperm cell pushed water out and replaced it, such that the formation of micro ice crystals were minimized (Kusumaningrum et al. 2002, Hine et al. 2019, Bebas and Gorda 2020). The external cryoprotectants provide protection from temperature shocks outside the cell during freezing and thawing (Herdis and Darmawan 2012, Ariantie et al. 2013, Nouri Gharajalar et al. 2016). The study showed that fast freezing was possible using their combination at low concentrations. Rapid freezing often utilized high concentrations (Pegg 2015), which increased their toxicity effect (Muchlisin and Azizah 2009, Best 2015). Therefore, it is essential to freeze sperm quickly to avoid the negative effect of cryoprotectants. The results of this study indicated that tilapia sperm can be frozen quickly with low concentrations of cryoprotectants (5%).

According to observations, most of the physical damage to sperm (abnormality), occurred in the tail region. In some instances, the tail was broken or completely absent. The treatment T1 exhibited higher abnormal sperm count, which could be attributed to the formation of a large number of micro crystals.

## Conclusions

In conclusion, pre-freezing process had a significant impact on sperm motility and viability but had no significant effect on abnormality. The results showed that tilapia sperm could be cryopreserved directly in liquid nitrogen after equilibration at 4°C for 10 min.

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