Optimization of spermatozoa cryopreservation of Albino pangasius catfish: cryoprotectants with various concentrations and different equilibration times

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Abstract. This study was aimed to optimize the spermatozoa cryopreservation protocol in Albino pangasius catfish by assessing the effectiveness of different cryoprotectant concentrations and different time of equilibration. The first experiment was conducted to optimize the combination of 10% methanol with different concentration of skim milk (0%, 5%, 10%, 15%, and 20%). The second experiment was aimed to optimize different equilibration time (10, 20, and 30 minutes). Deep freezer was used for the cryopreservation with a temperature of -80 °C at a storage period of 8 days. The first study found that the viability and motility of sperm after cryopreservation was significantly different (P<0.05) with fresh sperm. The best result was obtained at the 10% skim milk concentration with sperm viability of 78 ± 4.35% and motility of 69.66 ± 1.52%. The second experiment identified a significant different (P<0.05) in sperm viability and motility between fresh sperm and sperm post equilibration. The best result was obtained at 10 minutes equilibration time with sperm viability of 76 ± 1.15% and motility of 66 ± 2.64%. The study concluded that the 10% methanol with 10% skim milk, and 10 minutes equilibration time is the best combination for cryopreservation of Albino pangasius catfish spermatozoa.

1. Introduction
Sperm cryopreservation is an essential tool in the attempt to conserve species for artificial reproduction and maintaining genetic resources [1]. There is a need to develop cryopreservation protocols because it can affect sperm quality which can differ between species [2]. Research on optimization of sperm cryopreservation protocols has been carried out on many species such as camel [3], African clawed frog (Xenopus laevis) [4], brown bear [5], and buffalo [6]. The assessment of the success of sperm cryopreservation techniques was conducted by observing the ability of spermatozoa to live after cryopreservation and its fertility level [1].

Sperm cryopreservation has also been successfully carried out for many aquatic biotas such as fish. However, no sperm cryopreservation technique can be developed with certainty for all fish species [7]. Optimization of sperm cryopreservation protocols in aquatic biota is also not much, whereas cryopreservation of aquatic biota sperm can provide benefits such as the availability of gametes
throughout the year, protection of valuable strains, improvement of genetic resources, and help ex situ conservation to preserve endangered genomes [8].

The success of cryopreservation can be influenced by many factors such as diluent solution consisting of extenders and cryoprotectants [9]. Cryoprotectants are compounds that play a role in reducing cell damage by suppressing the formation of ice crystals during the freezing process. Cryoprotectants can be divided into two types, intracellular (permeating cryoprotectant) and extracellular (nonpermeating cryoprotectant). Intracellular and extracellular cryoprotectants work together to protect cells from damage that can be caused during cryopreservation [10]. Optimization of equilibration time and the temperature is also crucial, so cryoprotectants can effectively penetrate cells to minimize toxicity and damage to sperm cells during the freezing process [9].

Cryoprotectants used in cryopreservation of fish sperm such as methanol and skim milk. Methanol or a combination of methanol and skim milk has been used on cryopreservation of sperm of several fish species such as blue catfish: *Ictalurus furcatus* [11], *Sarotherodon mossambicus* [12], Salmonid fishes [10], and zebrafish [13]. During the cryopreservation process problems that often arise are caused by cold shock conditions and the formation of ice crystals. In an attempt to overcome these conditions in addition to optimizing the cryoprotectant concentration used, it can also be done by finding the optimal equilibration time [9].

This study uses the spermatozoa of red-eyed Albino pangasius catfish. The fish species are freshwater fish and well known as ornamental fish. Albino pangasius catfish is a fish that has a gene deviation. Nowadays, farmers are waiting for the emergence of Albino pangasius catfish from blackish gray catfish, and the albino color will usually appear around 10%. However, the mating of adult male and female Albino pangasius catfish parents will also result in the emergence of Albino pangasius catfish. Gene deviations in this species causes them to be easily stressed compared to consumption catfish. Therefore, the control of feed intake, water pH, temperature, as well as disease is needed [14].

There is a lack of research on spermatozoa cryopreservation, especially the optimization of cryoprotectant concentration and equilibration time in Albino pangasius catfish spermatozoa. The present study observes motility, viability, and morphometry parameters in spermatozoa of Albino pangasius catfish.

2. Material and method

This research is an experimental study using spermatozoa samples from 4 male red-eye Albino pangasius catfish aged 2-3 years. In this study, two experiments were conducted. First, the optimization of cryoprotectant concentrations using skim milk which was divided into five treatment groups: 0%, 5%, 10%, 15%, and 20% of skim milk. Second, the optimization of equilibration time is divided into three treatment groups. Equilibrium time used are 10 minutes, 20 minutes, and 30 minutes. Each treatment group was repeated three times.

2.1 Spermatozoa collection

The collected fish are first injected using GnRH analog (Ovaprim) at a dose of 0.5 ml/kg body weight. Injections were performed intramuscularly one time, and spermatozoa were collected after 10-12 hours of induction. Spermatozoa were obtained by stripping. The semen that comes out was collected in a 15 ml centrifuge tube. Samples were stored at 4°C until further analysis. Spermatozoa were analyzed both macroscopically and microscopically. Spermatozoa were mixed with diluents for cryopreservation.

2.2 First experiment: optimization of skim milk concentration

2.2.1 Making diluent solution

The diluent used consists of: extender, skim milk with 5 different concentrations (0%, 5%, 10%, 15%, and 20%) and 10% methanol. The composition of the diluent solution is 350 μl Ringer fish extender,
50 μl 10% methanol, and 50 μl skim milk with various concentrations. The mixture was stored in a refrigerator (4°C).

2.2.2 Sperm cryopreservation
- Equilibration and Freezing Processes
  A total of 50 μl of cement was mixed with a diluent solution (Ringer) and 10% methanol. Equilibration was carried out at 4-5 °C for 10 minutes [15,16,17]. The freezing process was carried out in a deep freezer at -80 °C for eight days.
- Thawing Process
  Thawing was carried out at 40 °C for 1-2 minutes until it melts (by immersing the cryotube in a water bath) [17].

2.2.3 Microscopic examination
Microscopic examination is performed on fresh and cryopreserved sperm, including:
- Sperm motility
  The semen was diluted before the observation of spermatozoa motility. The dilution was 100 times that was carried out by diluting 10 μL of semen into 90 μL extender. Subsequent dilution took 10 μL from the 10 times dilution and was added to 90 μL activator solution. Furthermore, as much as 10 μL of diluted semen was dropped in the Neubauer chamber. The Neubauer chamber was covered with a glass cover. Spermatozoa motility was observed under a microscope with a magnification of 10 × 40. The percentage of motility was assessed by counting the number of motile spermatozoa divided by the total number of spermatozoa (100 cells).
- Sperm Viability
  Sperm viability examination was carried out by taking 10 μL of semen as a result of dilution 100 times dripped on the slide and mixed with 10 μL of 0.5% eosin-Y solution. Then, the mixture was covered with a cover glass. Observations were made under a light microscope with a magnification of 10 x 40. Live spermatozoa will not be colored by a 0.5% eosin-Y solution, while non-viable spermatozoa will appear red because they have been dyed. A total of 100 spermatozoa cells were observed for this viability test.
- Sperm morphometry
  A total of 10 μl of semen was dissolved in 990 μl extender to obtain 100 times the dilution. Furthermore, 10 μl of the 100 times diluted semen was dropped on the object-glass A. The tip of the object-glass B was placed on one edge of the object-glass A forming an angle of 45°. The semen spread on the object-glass A was fixed using methanol for 3 minutes. The semen was stained with Giemsa's solution for 30 minutes. The semen was then washed using running water and left to dry at room temperature. The spermatozoa were observed under a microscope with a magnification of 10 x 40. Spermatozoa were analyzed using Image-J software by measuring the sperm head length of 100 sperm in each treatment group.

2.3 Second experiment: optimization of equilibration time
2.3.1 Making diluent solution
  The diluent used consists of an extender, 10% skim milk and 10% methanol. The composition of the diluent solution is 350 μl of Ringer fish extender, 50 μl of 10% methanol, and 50 μl of 10% skim milk. The mixture was kept in the refrigerator (4 °C.).

2.3.2 Equilibration process
  A total of 50 μl of semen was mixed with diluent (ringer), skim milk, and methanol. Equilibration at 4-5 °C for 10 minutes (group 1), 20 minutes (group 2), and 30 minutes (group 3). Fresh spermatozoa and the three treatment groups of equilibration time were analyzed for sperm motility, viability, and morphometry as in the 1st experiment.
2.3.3 Data analysis
The data were tested for normality using Saphiro-Wilk. Data homogeneity were tested using Lavene. After that, a one-way ANAVA test was carried out and continued with the Tukey test. Statistical analysis was performed with SPSS 24 software with P <0.05 considered significant. All data were expressed in mean ± S.D.

3. Results and discussion
3.1 First experiment: optimization of cryoprotectant concentrations
3.1.1 Macroscopic examination of sperm

| Parameter | Mean   |
|-----------|--------|
| Volume (ml) | 2.5 ± 0.64  |
| Color      | White milk |
| pH         | 8       |
| Smell      | Typical |
| Concentration | 7.2×10^9 |

3.1.2 Examination of spermatozoa motility
Motility of spermatozoa after thawing with five different skim milk concentrations is presented in Figure 1. Based on the analysis, a significant difference (P <0.05) was found between the motility of fresh sperm (71.33 ± 0.57%) with a concentration of 4 skim milk K 0% (20.33 ± 2.08%), K 5% (25.66 ± 2.51%), K 15% (48.33 ± 2.51%), and K 20% (30.33 ± 2.08%) against the motility of spermatozoa albino pangasius catfish. The motility of sperm cryopreserved with 10% concentration of skim milk (69.66 ± 1.52%) did not show a significant difference with motility of fresh spermatozoa (p > 0.05). The best albino pangasius catfish sperm motility was obtained in the treatment of 10% skim milk concentration (69.66 ± 1.52%).

![Figure 1](image_url)

Figure 1. Motility of spermatozoa of albino pangasius catfish using skim milk with various concentrations. Different superscripts indicate significant differences with fresh sperm (P<0.05).

3.1.3 Examination of spermatozoa viability
Viability of spermatozoa after thawing with five different skim milk concentrations is presented in Figure 2. There is a significant difference (P <0.05) between fresh sperm viability (82 ± 1%) with 4 concentrations of skim milk K 0% (47 ± 2.64%), K 5% (22 ± 5.29%), K 15% (37 ± 2.64%), and K 20% (44 ± 1%) on spermatozoa of albino pangasius catfish. The viability of sperm cryopreserved with 10% concentration of skim milk (78 ± 4.35%) did not show a significant difference with viability of fresh spermatozoa (p > 0.05). Skim milk with a concentration of 10% produces the best viability of spermatozoa compared to 4 other concentrations (0%, 5%, 15%, and 20%).
Figure 2. Viability of spermatozoa Albino pangasius catfish using different skim milk concentrations. Different superscripts indicate significant differences with fresh sperm (P <0.05).

3.1.4 Morphometry examination of spermatozoa
Morphometry of spermatozoa after thawing with five different concentrations of skim milk are presented in Table 2. There are significant morphometry differences in sperm head size of albino pangasius catfish in the five concentrations of skim milk.

Table 2. Morphometry of fresh spermatozoa and post cryopreservation spermatozoa

| Treatment          | Skim milk concentration |
|--------------------|-------------------------|
|                    | 0 %                    |
|                    | 5 %                    |
|                    | 10 %                   |
|                    | 15 %                   |
|                    | 20 %                   |

| Mean (µm)          |
|--------------------|
| 10.73±1.75         |
| 9.37±1.63          |
| 11.29±2.11         |
| 11.25±2.29         |
| 10.89±1.93         |
| 10.39±2.12         |

Note: average head length of 100 spermatozoa counted.

Figure 3. Mean Length of spermatozoa head of albino pangasius catfish uses different skim milk concentration.

3.2 Second experiment: optimization of equilibration time
3.2.1 Spermatozoa motility examination
The motility of spermatozoa after equilibration with three different equilibration times is presented in Figure 4. There is a significant difference (P <0.05) between fresh spermatozoa motility and the three equilibration times. The motility of fresh spermatozoa (83.66 ± 0.57%) was significantly different from the motility of spermatozoa with 10 minutes (68 ± 2.64%), 20 minutes (56 ± 1%), and 30
minutes (37 ± 1%) equilibration time. The best equilibration time was 10 minutes (68 ± 2.64%) compared to other equilibration times.

\[\text{Figure 4. Motility of spermatozoa of Albino pangasius catfish using different equilibration times. Different superscripts indicate significant differences with fresh sperm (P <0.05).}\]

3.2.2 Spermatozoa viability examination
Viability of spermatozoa after equilibration with three different equilibration times is presented in Figure 5. There is no significant difference in fresh spermatozoa viability (81 ± 3.60%) with sperm equilibrated within 10 minutes (76.33 ± 1.15%) with a value (p> 0.05). The viability of fresh spermatozoa was significantly different with the viability of spermatozoa with 20 minutes (48 ± 1.73%) and 30 minutes (30.33 ± 1.52%) equilibration time (p <0.05). The present study showed that the best viability of spermatozoa is 10 minutes of equilibration time (76.33 ± 1.15%).

\[\text{Figure 5. Viability of spermatozoa albino pangasius catfish using different equilibration times. Different superscripts indicate significant differences with fresh sperm (P <0.05).}\]

3.2.3 Morphometry examination of spermatozoa
Morphometry of spermatozoa after equilibration with three different equilibration times is presented in Table 3. Based on the results of data analysis, we found differences in sperm head length size between the three equilibration time treatments.
Table 3. Morphometry of spermatozoa after equilibration

| Treatment | Equilibration Time | Mean (µm)  |
|-----------|--------------------|------------|
| Fresh sperm | 10 minutes | 45±2.05  |
|            | 20 minutes      | 9.33±2.0   |
|            | 30 minutes      | 10.23±1.88 |
|            | 40 minutes      | 10.22±2.49 |

Note: average size of head length of 100 spermatozoa that are counted.

Figure 6. Mean Length of spermatozoa head of albino pangasius catfish uses different equilibration times.

3.3 Discussion

Cryopreserved sperm of Albino pangasius catfish showed a decrease in sperm motility and viability compared to fresh sperm. Methanol and skim milk were used in the present study as cryoprotectants. Skim milk with a concentration of 10% has the best value in motility (69.66 ± 1.52%) and viability (78 ± 4.35%). Fish spermatozoa can only survive for a few minutes after being expelled from the body due to the phenomenon of osmotic injury and the limited amount of energy that can be used. Spermatozoa obtain energy by utilizing energy reserves in their bodies or utilizing energy sources from outside[18].

In this study, before cryopreservation, heating of skim milk at a temperature of 90-97 °C for 1 minute[19]. The purpose of heat is to reduce the toxic effects of lactenin contained in milk. The heating process causes the deactivation of catalase enzymes contained in milk, which can metabolize methanol. Methanol metabolism can occur during the process of equilibration and thawing. Methanol metabolism occurs when there is an enzyme catalase, which is widely found in the cytoplasm of cells. The enzyme turned into formaldehyde which will then be converted into formic acid, which is toxic and damages cells. The deactivation of this enzyme makes methanol unable to turn into formaldehyde so that it can reduce the toxicity of methanol [20].

Post-cryopreservation sperm morphometry study showed that in the first experiment, there was a change in the average size of the post-thawing sperm head length in 100 sperm counted for each treatment compared to fresh sperm. Morphologically, the sperm is divided into three main parts, namely the head, neck, and tail. At the head, there is a nucleus that is protected by cell membranes. In this nucleus, there is genetic information that will be transmitted to the next generation. The neck part (middle part) is the part that connects the tail to the head. It arranged by microtubules containing fiber substances. The fiber substances are composed by dynein protein which has ATPase activity that serves to provide energy for sperm. The tail of the sperm functions as a means of movement of sperm to swim towards the egg. Changes in the dimensions of the sperm head length are thought to be caused by changes in the sperm chromatin structure. The surface area of the sperm head tends to decrease after thawing, as results in the treatment of 0% skim milk. This decrease in surface area is caused by changes in the structure of sperm chromatin. It is suspected that cryopreservation can induce
chromatin condensation. The condensation of sperm chromatin can cause morphometric changes in the sperm head [21].

Different cryopreservation method causes the different size of spermatozoa head. Cryopreservation methods are known to have effects on sperm motility, viability, fertility, and chromatin structure. The cryopreservation protocol can affect the level of spermatozoa damage, such as cryoprotectant concentration, cooling rate, equilibration time, and melting temperature [1,2,8,9]. Possible modification of the protocol can also cause sperm head morphometric changes. An average increase in sperm head length at 5%, 10%, 15%, and 20% is thought to be caused by peeling off or decaying the plasma membrane around the head. When the plasma membrane is released, water will enter the head layer, so that the sperm head swells. The decay of the plasma membrane can be caused by conditions such as heat which can be caused by the use of high temperatures (in this study 40°C) during the thawing process.

The second experiment was aimed to assess the best equilibration time using a combination of 10% methanol cryoprotectant and 10% skim milk obtained results in decreased sperm motility and viability after equilibration. The best motility and viability were collected at 10 minutes equilibration compared with 20 and 30 minutes equilibration. Difference equilibration time causes differences in spermatozoa motility. At the time of optimum equilibration, the highest spermatozoa motility will be achieved, because optimum equilibration will provide an opportunity for cryoprotectants to protect sperm from the effects of cold shock. Methanol which is an intracellular cryoprotectant penetrates spermatozoa cells to form a balanced intracellular concentration with skim milk as an extracellular cryoprotectant [9].

The highest motility was obtained at 10 minutes equilibration. It may occur due to the equilibration time of 10 minutes is enough time for methanol to enter the cell membrane and keep cell organelles from cold shock conditions that can cause spermatozoa mortality. At the equilibration time of 20 and 30 minutes, motility and viability becomes low due to the emergence of the toxic nature of methanol due to prolonged contact with spermatozoa with methanol, the toxic nature of methanol can damage the structure of the plasma membrane and cell organelles, causing an inhibition of energy metabolism, the absence of energy supply will cause spermatozoa cannot move and die[22]. Also, the equilibration time of 10 minutes provides sufficient time for the balance of the chemical potential of intracellular and extracellular water. At the equilibration time of 20 and 30 minutes, there is no balance between the chemical potential of intracellular and extracellular fluid, so that during thawing cell wall damage will result in spermatozoa mortality.

Results of sperm morphometry after equilibration showed that there was a change in the size of the average length of the sperm head compared with fresh sperm. There is an association between sperm head size change with cryopreservation method. The difference of cryoprotectant concentration and different equilibration time used can cause a different level of spermatozoa damage. The decrease in surface area due to cryopreservation can cause changes in the structure of sperm chromatin which induces chromatin condensation. The condensation of chromatin structure causes changes in the morphology of the sperm head [21,22].

4. Conclusion
The skim milk concentration and equilibration time affected the quality of spermatozoa of albino pangasius catfish after cryopreservation. The concentration of skim milk 10% and equilibration time of 10 minutes showed the best results on the quality of spermatozoa compared to other treatments.

5. References
[1] Zilli L, and Vilella S 2012 Effect of cryopreservation on bio-chemical parameters, DNA integrity, protein profile and phosphorylation state of proteins of seawater fish spermatozoa In Current Frontiers in Cryobiology IntechOpen
[2] Esteso M C, Toledano-Díaz A, Castaño C, Pradiee J, Lopez-Sebastián A, and Santiago-Moren J 2018 Cryobiology 80 12-17
Acknowledgement
The first author thanks the Indonesia Endowment Fund for Education (LPDP) for providing the research project fund. The first author also thanks to Dr. Eni Kusrini as the field supervisor and the Head of Research Institute for Ornamental Fish Culture in Pancoran, Depok for permission in conducting the research at the institute.