The fertilization of *Tor soro* fish (Valenciennes, 1842) using post cryopreservation sperm: the effect of skim milk as a cryoprotectant

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Abstract. *Tor soro*, an endemic fish from North Sumatera, has a high economic value. Environmental damage causes a decreasing of *Tor soro* populations and leads to the extinction of these fishes in their natural habitat. Cryopreservation is an efficient strategy that can be used to minimize the problem. The success of this approach depends on how effective it is. This study to evaluate the fertilization of *Tor soro* using post cryopreservation spermatozoa 48 hours. The sperms collected by stripping, followed by the dilution using soluble liquid (fish ringer methanol 10%+skim milk) 1:9 comparison. The concentrations of the skim milk that is used in 0%, 5%, 10%, 15%, 20%, and 25%. The equilibration was carried out at the 5 oC for 10 minutes before, then it kept frozen at the temperature of -10 oC for 48 hours. The sperm thawing was carried out at the 40 oC for 1 minute and one hundred egg cells (±1 g) were fertilized using 1.5ml of post cryopreservation sperm. The ANOVA test showed there was a fertility percentage effect of the skim milk on the frozen sperm. The 10% skim milk give an optimum with the highest fertility of (p<0.05) with 91.25±2.22% and motility (p<0.05) in 10% with 82.90±1.40%.

1. Introduction

*Tor soro* is a local fish that is spread in several regions in Indonesia, it is an endemic fish in North Sumatra [1]. *Tor soro* is a freshwater fish species that are economically important and its population value is limited in nature [2]. The population of *Tor soro* in nature is classified as getting smaller, although efforts to conserve this fish in nature have been carried out by the community to keep breeding [3]. The decreased *Tor soro* population in nature is caused by damage to the habitat and overfishing. These problems can be solved by storing superior sperm *Tor soro*, so that superior sperm can be used when the time is needed [4]. Storage of spermatozoa can be by cryopreservation process [5].

Sperm cryopreservation is a storage technique of frozen spermatozoa which aims to maintain cell life [6]. Successful cryopreservation can be seen from the quality of motility after cryopreservation of spermatozoa [7, 8] or the success rate of fertilization [9]. Motility and fertilization have a positive correlation, this is due to a decreased in sperm quality such as changes in motility and duration of sperm movement [10,11].
Factors that influence the success of cryopreservation techniques are changes in temperature during cryopreservation, freezing and thawing, and cryoprotectant that was used [12]. The role of cryoprotectants is to reduce cell damage during the freezing process and the establishment of extracellular ice crystals so that cell viability remains in good condition [13]. Methanol can reduce the freezing of intracellular ice crystals so that the cells do not experience damage [14]. The performance of intracellular cryoprotectants will be more maximal if added with extracellular cryoprotectants, one example is the addition of skim milk.

Skim milk is an extracellular cryoprotectant that is widely used in cryopreservation of spermatozoa [15]. The fat contained in skim milk is not more than 0.5% whereas in whole milk it ranges from 2.4—5.5% [16]. That is because skim milk contains nutrients that can be used by spermatozoa as an energy source [17]. The casein protein content in skim milk plays a role in protecting spermatozoa from cold shock [18]. The two cryoprotectants can interact to complement each other in maintaining cells, so that performance in maintaining spermatozoa will be more optimal [19].

2. Materials and methods

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

2.2. Collection of semen and eggs
The male parent and female mature of *Tor soro*. Intake of semen and eggs was carried out using the hand stripping method. The resulting eggs were collected in clean containers such as bowls or plates with smooth surface so that the eggs are not damaged [20].

2.3. Fish ringer preparation
Preparation of Ringer's solution was carried out by dissolving 3.25 g NaCl; 0.125 g KCl; 0.1755 g CaCl_2·H_2O; and 0.1 g NaHCO_3 in 500 ml of distilled water [21]. Ringer fish solution is stored in the refrigerator at a temperature of 4°C with a maximum storage time of three days.

2.4. Activator preparation
Preparation of the activator solution was done by dissolving 45 mM NaCl (0.2633g NaCl), 5mM KCl (0.0373g KCl), and 30mM Tris HCl pH 8.0 (0.3634g C4H11NO3) in 100 ml of distilled water [22].

2.5. Semen dilution
The used diluent consists of an extender (fish ringet + 10% methanol + skim milk) [23]. With a dilution ratio of 9: 1. The concentration of skim milk used in this study was 0%, 5%, 10%, 15%, 20%, and 25% [24].

2.6. Equilibration and freezing
Equilibration was carried out in a refrigerator at 5°C for 10 minutes [25]. Freezing was done in a deep freezer with a temperature of -10°C for 48 hours [26].

2.7. Thawing
Thawing was done at 40 °C within 1-2 minutes until it melted [25].

2.8. Parameters examined
Macroscopic testing included pH, color of cement and volume of fresh semen. Microscopic testing was performed to determine the motility value of the post-cryopreservation cement using a
microscope (image driving software; Scopephoto 2.0.4). Fertilization testing was performed on eggs using fresh semen as a comparison and using post-cryopreservation semen as a result of research.

2.9. Evaluation of motility
Spermatozoa motility testing was performed using subjective methods [27]. Total of 10 μl of diluted cement was dropped into an improved neubaeur chamber and observed under a microscope with a magnification of 10 x 40. The formula for calculating the percentage of motility is as follows:

\[
\% \text{ Motility} = \frac{\sum \text{motile spermatozoa}}{\sum \text{total spermatozoa}} \times 100%
\] [28].

2.10. Evaluation of Fertilization
The fertilized egg was marked with brightly colored yolk and there is a cleavage pole. The fertilization calculation formula is as follows:

\[
\% \text{ Fertilization} = \frac{\sum \text{fertilized eggs}}{\sum \text{total eggs}} \times 100%
\] [29].

3. Results and discussion

3.1. Motility analysis
Measurement of spermatozoa in macroscopic way is a general characteristic that measured by traditional and in easier way [29]. The percentage of fresh sperm motility of Tor soro at this research was 81,11 ± 1,31%. Level motility of the sperm is being said have good quality for cryopreservation if the level is above 70% [30, 31]. While the percentage of sperm motility post-cryopreservation at this research is around 59,90 ± 1,40%. Observation at motility of fish spermatozoa is parameter commonly use for determining the quality of spermatozoa from fish because this aspect is important in fertilization [32].

| Repetition | 0%   | 5%   | 10%  | 15%  | 20%  | 25%   | Fresh  |
|------------|------|------|------|------|------|-------|--------|
| 1          | 71,42| 69,89| 83,76| 75,05| 77,41| 71,33 | 89,77  |
| 2          | 69,57| 67,05| 82,29| 76,43| 78,11| 69,45 | 90,59  |
| 3          | 71,90| 70,50| 84,32| 74,52| 79,40| 70,23 | 88,41  |
| 4          | 70,79| 68,41| 81,24| 77,55| 80,54| 68,72 | 87,68  |
| Average    | 70,92| 68,96| 82,90| 75,89| 78,87| 69,93 | 89,11  |
| SD         | 1,01 | 1,55 | 1,40 | 1,37 | 1,39 | 1,11  | 1,31   |

Table 1. Motility spermatozoa of Tor soro.
3.2. Fertilization analysis

The results of this study indicate that the fertilize fresh *Tor soro* is 99.25 ± 1.5%. The results showed that there was a significant effect (p<0.05) on the fertilization capability of *Tor soro* 48 hours after cryopreservation using of skim milk with various concentrations (0%; 5%; 10%; 15%; 20%; 25%) and the addition of 10% methanol. 10% of methanol and skim milk used as cryoprotectants in this study succeeded in maintaining the ability of post-cryopreservation spermatozoa in *Tor soro* in fertilization with a yield of 71.25% - 91.25%. Based on the results of research conducted obtained the average value of fertilization of eggs fertilized spermatozoa 48 hours post-cryopreservation highest at 10% skim milk concentration that is 91.25 ± 2.21. This was proven by the ANOVA test showing that the results of 48-hour post-cryopreservation spermatozoa fertilization were significantly different (p<0.05).

| Repetition | 0%  | 5%  | 10% | 15%  | 20%  | 25%  | Fresh |
|------------|-----|-----|-----|------|------|------|-------|
| 1          | 76,00 | 70,00 | 92,00 | 81,00 | 76,00 | 71,00 | 100    |
| 2          | 82,00 | 75,00 | 90,00 | 79,00 | 79,00 | 73,00 | 100    |
| 3          | 80,00 | 74,00 | 89,00 | 82,00 | 73,00 | 72,00 | 97     |
| 4          | 79,00 | 69,00 | 94,00 | 80,00 | 79,00 | 69,00 | 100    |
| **Average** | 79,25 | 72,00 | 91,25 | 80,50 | 76,75 | 71,25 | 99,25  |
| **SD**     | 2,50 | 2,94 | 2,22 | 1,29 | 2,87 | 1,71 | 1,50 |
These percentage results are lower when compared with the percentage value of fertilization of eggs fertilized by fresh spermatozoa Tor soro. This is consistent according [33] to which states that the rate of fertilization in spermatozoa post-cryopreservation is lower than the rate of fertilization in fresh spermatozoa. Decrease in post-cryopreservation spermatozoa fertilization can be caused by spermatozoa cryopreservation processes such as dilution ratio, type of extender used, low sperm motility after thawing and decreased percentage of motile sperm during freezing and thawing [34, 35].

4. Conclusions
The combination of 10% methanol and 10% skim milk produced the highest percentage of motility 82.90 ± 1.40%, while the concentration of 10% methanol and 10% skim milk showed the best fertilization results of 91.25 ± 2.21%.

5. References
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