3. (Suci Lestari) The Percentage of embryo viability after 48h sperm cryopreservation effect of various natural cryoprotectant

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The Percentage of embryo viability after 48h sperm cryopreservation: effect of various natural cryoprotectant

The Percentage of embryo viability after 48h sperm cryopreservation: effect of various natural cryoprotectant

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Abstract. Natural cryoprotectant is an important factor needed to protect cell compound. Its proper usage is based on toxicity which is influenced by its type, concentration, temperature, and exposure period of the compound. This study aims to evaluate the best concentration period of natural cryoprotectant, with the utilized sperm obtained from *Tor soro* fish using the stripping technique. It was further diluted with a solution consisting of fish ringer, methanol 10%, and natural cryoprotectant in a ratio of 1:10. The natural cryoprotectant used were honey, brown sugar, and date palm juice, at 10 % concentration. Furthermore, the sperm was stored in the liquid nitrogen at -196 ºC for 48 h, melted at 36 ºC for 1 minute, and mixed with 100 eggs for fertilization. The percentage of embryo viability was analyzed using ANOVA and Tukey test. The result showed an effect of natural cryoprotectant on frozen sperm towards the percentage of embryo viability ($p<0.05$). The honey cryoprotectant is a natural compound that produces the highest embryo viability percentage at 1080 minutes after fertilization with a total of 94.20 ± 2.79 ($p<0.05$).

1. Introduction

Cryopreservation is a long-term storage technique for conserving biological materials without damages at low temperatures. The stored material is usually in the form of sperm cells, ova, or tissue, which also aims to provide a guaranteed source of genetic material for scientific purposes. In fish groups, it used for aquaculture purposes [14], such as for biodiversity conservation. Fish sperm freezing techniques overcome problems regarding spawning time differences between males and females, unbalanced sex ratios, and sperm handling. The maintenance of gametes in the form of sperm tends to reduce the cost of hatching and contribute to the preservation of effective genetic resources over a prolonged period. It is also properly utilized in studies regarding breeding and hybridization [5]. The success associated with the cryopreservation of fish sperm produces over 200 species of fish used for cryobanking [27].

Tor soro is an economically important fish group found in the freshwater of the *Cyprinine* tribe located in West Java Indonesia. It is a close relative of the tambra fish (*Tor tambroides*) and based on the red list of endangered species, published by IUCN in 1990, it is among the 29 species of fishes threatened with extinction along with all Genus Tor [13]. Due to population growth which was followed by an increase in the consumption rate of food [15] including animal protein, intensive freshwater fishing activities [22], and changes in habitat characteristics, there is a decrease in the

number of freshwater fish species, including *Tor soro* [22]. Therefore, there is adequate need for restocking and conservation.

Based on the research Hezavehei *et al.* [12], sperm cryopreservation causes a decrease in plasma membrane integrity and DNA damage which affects motility, viability, fertilization ability, and leads to low hatching rate [20]. It also causes damages due to cold shock [11] and the formation of ice crystals [12], therefore, there is adequate need for the provision of compounds capable of protecting cells during cryopreservation. This is known as cryoprotectant, which consists of natural and artificial chemicals. According to Muchlisin *et al.* [16], natural cryoprotectants are considered to be more environmentally friendly, easily prepared, inexpensive, and tend to be non-toxic. Its toxicity is one of the successful factors [8, 11] in fish sperm cryopreservation [16]. Each cell of a different species has varying cryoprotectant tolerance levels.

The selected cryoprotectants are brown sugar, honey and sari kurma. Selection of cryoprotectants based on organic chemical content and molecular weight. Brown sugar contains glucose and sucrose, while honey and sari kurma contain fructose and glucose. According to the research of Abinawanto *et al.* [4], 0.5% sucrose concentration showed the highest post-thaw sperm motility (81.62 ± 4.19), post-thaw sperm viability (82.17 ± 2.56) and glucose showed the highest post-thawing sperm motility (88.45%) and sperm viability (59.80%) in a concentration of 6% [2]. The high percentage of motility and viability of post-thaw sperm can be indicated as a success factor in fertilization.The molecular weight for glucose, fructose and sucrose are 180,154 g/mol, 180,155 g/mol, and 342,295 g/mol respectively. They are grouped in the extracellular cryoprotectant group, due to their inability to pass directly through the cell membrane owing to their large molecular size. Extracellular cryoprotectants work by covering the outside environment of the cell [12], thereby preventing damage to its membranes due to the formation of ice crystals, during the process.

Based on the above description, a study was conducted to evaluate the optimal concentration of three different types of natural cryoprotectants in *Tor soro*. The percentage of embryo viability was used to evaluate the sperm function after cryopreservation after calculation.

2. Material and methods

2.1 Semen collection

Semen was collected at the Freshwater Fisheries Germplasm Research Installation, Cijeruk, West Java, with Male *T. soro* sperm obtained from April-July according to natural spawning time. Sperm was extracted by stripping it with the help of syringe and injected into a microtube containing stock solution. The sperm is then inserted into a cryotube using a micropipette.

2.2 Cryopreservation

Semen was diluted with a solution containing natural cryoprotectant, such as brown sugar, honey, and sari kurma, fish ringer (NaCl3.25 g, KCl 0.125 g, $CaCl_2.2H_2O$ 0.175 g, and $NaHCO_3$ 0.1 g in 500 mL aquades) and 10% methanol [1], in the ratio of 1:10. The concentration for each cryoprotectant is 10%, and the sample was sprayed on liquid nitrogen vapor for 15 seconds, wrapped in aluminum foil and stored in liquid nitrogen for 48 hours. Cryopreservation semen is evaluated by fertilizing an egg.

2.3 Embryo viability

Embryo viability is evaluated by counting the number of yellow embryos at 1080 minutes after fertilization. Also, the number of eggs used per treatment amounted to 100.

2.4 Statistical analysis

Data analysis in this study was conducted using Analysis of Variance (ANOVA) with an error rate of 0.05. Further tests were carried out using the Tukey Test to identify which treatments were significantly different.

3. Results and discussion

The results showed that the highest percentage of embryo viability was found in the natural cryoprotectant type of honey with 94.20 ± 2.79 ($p < 0.05$). Based on research, it is known that fresh T. semen is milky white, and thick, with a pH of 8.5.

Table 1. 48-hour embryo viability post cryopreservation

Treatment	Repetition					Average
	1	2	3	4	5	
Honey	95.00	97.00	92.00	90.00	97.00	94.20±2.79[a]
Brown sugar	80.00	58.00	69.00	67.00	64.00	67.60±7.23[b]
Sari Kurma	57.00	58.00	47.00	61.00	58.00	56.20±4.79[c]

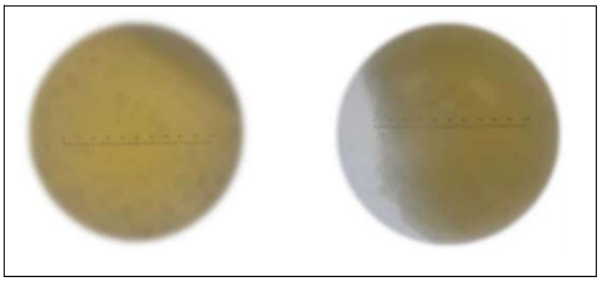

Figure 1. Egg appearance after fertilization at minute 1080, (a) embryo viability and (b) died embryos

Based on figure 1, there is a difference between viability and dead embryos. A yellow characteristic is seen in embryo viability with many congregated (morula phase) [6] cells marked with black spots. Dead embryos have a milky white appearance and damage to the cell membrane, therefore some areas look empty.

Several studies report that sperm cryopreservation has a damaging effect on DNA and causes a significant decrease in the quality of spermatozoa [12]. DNA integrity is a concern during cell freezing because cryopreservation readily changes the nature of the mitochondrial membrane and increases the production of ROS, which can further result in DNA oxidation, resulting in high-frequency single and double-stranded DNA breaks [21].In addition, defects in DNA repair enzymes have been reported as other reasons for DNA damage after freezing [7].thereby reducing the percentage of embryo viability.

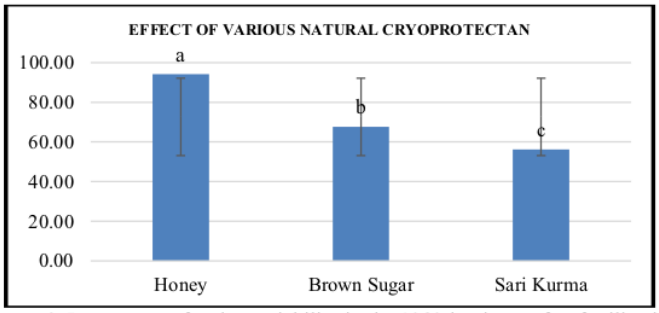

Figure 2. Percentage of embryo viability in the 1080th minute after fertilization

The results showed a significant difference between the three natural cryoprotectants. Based on Tukey's multiple comparison test, honey has the highest average value of the number of embryo viability which is 94.20 ± 2.79 ($p < 0.05$) at 1080 minutes. It comprises of fructose which is a chemical component of the seminal plasma constituents needed by spermatozoa. Spermatozoa which are stored at low temperatures, show decreased motility and stop when the temperature is a few degrees above the freezing level. At low temperatures, the metabolic process continues although it is likely to run slowly in aerobic conditions. Fructose is converted to lactic acid to produce ATP, and the process is better than fructolysis which in turn is better than glycolysis [23].

Besides fructose, honey also contains antioxidants that are important for reproduction [10]. According to Erejuwa *et al.* [10], antioxidants contained in honey significantly increase the concentration of SOD (Superoxide Dismutase) in semen. SOD plays a role in counteracting the increase in ROS (Reactive Oxygen Species) which occurs during the cryopreservation process. Increased ROS [24] causes oxidative stress conditions which result in a decreased sperm quality after cryopreservation due to DNA damage [12]. ROS attacks purine and pyrimidine bases, causing DNA damage and in addition, it initiates apoptosis in sperm which causes capcase enzymes to be activated [26]. Therefore, Honey has one potential chemical component to be used as a natural cryoprotectant.According to research Abinawanto *et al*. [3] showed that honey has a significant effect on the level of motility (80.48 ± 7.18%).

4. Conclusion

Based on this study, further research needs to be carried out on the basic components of honey, brown sugar, and palm juice which have great potential to be used as natural cryoprotectants, especially in cryopreservation of *T. soro* sperm. In addition, molecular analysis is needed to determine DNA damage which prevents eggs from hatching.

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