Fertilization Rate of Giant Grouper (*Epinephelus lanceolatus*, Bloch 1790) Sperm Post-cryopreservation and Application on Hybridization in Different Grouper Species

W Widyaningsih¹, Abinawanto* and D Susandi²

¹ Departement of Biology, Faculty of Mathematics and Natural Science, Indonesia University, Depok 16424, Indonesia
² Industry Engineering, Faculty of Engineering, Majalengka University, Majalengka, Indonesia

*Corresponding author: abinawanto.ms@sci.ui.ac.id

Abstract. Fertilization was doing to measure the success of sperm cryopreservation because it was more informative. Sperm cryopreservation was expected to get sperm that successfully fertilize the egg well. The aim of this study was to determine the sperm fertilization ability of giant grouper (*Epinephelus lanceolatus*, Bloch 1790) 48 hours post-cryopreservation in fertilizing tiger grouper eggs (*Epinephelus fuscoguttatus*, Forskal 1775). Fertilization was carried out artificially using cryopreservation sperm with 6% of glycerol and palm dates concentrates (0%; 5%; 10%; 15%; 20%; and 25%). The tiger grouper brood was previously injected with hormone chorionic gonadotropin at a dose of 500 IU/kg. The results showed that there were significant differences (P < 0.05) on fertilization of tiger grouper eggs with sperm of giant grouper which were cryopreserved with 6% of glycerol and various concentrations of palm date concentrates. Sperm which was cryopreserved with 6% of glycerol and 10% of palm date concentrate has the best fertility ability by showing the highest value of 66.25 ± 3.23%.
1. Introduction

Important parameters in the evaluation of spermatozoa post-cryopreservation are sperm motility and fertility rate [1, 2, 3]. Sperm motility and fertilization ability of sperm post-cryopreservation are to be lower compared with fresh sperm [4]. These cryopreservation process will decrease the quality of sperm, such as duration of sperm movement and sperm motility [5].

Fertilization is the fusion process of oocytes (eggs) with sperm to form zygotes, followed by fusion of both genetic material and its important issues in fish reproductive biology [6, 7]. Fertilization on fish occurs externaly, merging two gametes (sperm and eggs) that happen in the outside of the body of fish. Externally fertilization occurs when the male and female fish close each other then release their eggs and sperm coordinately. The fertilization on fish occurs about 1-2 minutes because of the short duration of motility in the waters [8].

Fertilization process using spermatozoa post-cryopreservation has been carried out on several species of fish, such as African catfish (Clarias gariepinus) [4] and on the application of hybridization between estuary grouper (Epinephelus coioides) and giant grouper (Epinephelus lanceolatus) using spermatozoa post-cryopreservation [9, 10]. Fertilization rate of estuary grouper with giant grouper using sperm cryopreservation combined with some of cryoprotectants and extenders such as marine fish Ringer (MFR), sodium citrate (CT), sodium chloride (NaCl) dimethyl sulfoxide (DMSO) and trehalose was reach up to 57-75% [9].

The fertilization was carried out using two different species of grouper, because of unsynchronizing of maturation. Therefore, hybridization between giant grouper (male) Fig. 1 and tiger grouper (female) is the solution. Hybridization of them was carried out to get the better species from both or have the advantages quality than the parents [9, 19]. Hybridization is one of technique to improve the diversity of genetic of fish and to get the new grouper (hybrid species) [20]. Hybrids species usually has fast growth, resistance to diseases and extreme environmental, and sterile species [21, 22].

The different of this research with another research is on the cryoprotectant. This study used natural cryoprotectant (palm dates) to preserve the sperm, because the content of palm date can improve the quality of spermatozoa and fertility after cryopreservation. According to [18], motility of spermatozoa post-cryopreservation of giant grouper was showed that combination of 6% glycerol and 10% palm dates was the highest post-thaw motility (76.70 ± 6.88%). This study aimed to determine the fertilization rate of giant grouper spermatozoa post-cryopreservation using palm dates as a cryoprotectant on tiger grouper (Fig. 2). The successful of fertilization between giant grouper and tiger grouper is expected to be a solution to overcome the problem of spawning which is unsynchronization. Furthermore, hybridization was doing on giant grouper with tiger grouper, because the overfishing of tiger grouper required the best management, so the resources remains sustainable [24]. Hybridization process on grouper have several benefits, are support grouper conservation and grouper aquaculture by ensuring the availability of grouper seeds on an ongoing basis [23].

2. Research methods

The experiment was conducted at the Hatchery in Grouper Division of Marine Fish Aquaculture, Lampung. Mature tiger grouper was used in this experiment has the body weight and total length in a range of 10 kg and 79 cm, respectively. Spermatozoa of giant grouper was cryopreserved for two days with combination of 6% glycerol and various concentration of palm dates [18]. The concentrations of palm dates used were 0%, 5%, 10%, 15%, 20%, and 25%, respectively.

2.1. Mature female selection

Mature female grouper were taken from floating net cage in Marine Fish Aquaculture, Lampung and transferred to a hatchery. The mature female gonad was characterized by a large and soft stomach and red urogenital holes around it [11]. The grouper was induced by hormone chorionic gonadotropin (hCG) twice with a different of 3 hours and 500 IU/kg of dose. Giving these hormones serves to stimulate ovulation of tiger grouper, so it can help the release of eggs from the body of fish [26].
2.2. Collecting of eggs
The eggs of tiger grouper were collected by abdominal massage of the females after 8 hours of second induced of hormone [12]. This step was taken at the ovulation time but supported by the hormone. Extreme care was taken to avoid contamination of blood, urine, and feces. The eggs were collected into a smooth small container to prevent demage of eggs (Fig. 3) [13].

2.3. Fertilization process
Fertilization was taken with fresh sperm and spermatozoa post-cryopreservation as a comparison. The cryopreservation sperm were thawing by immersing the cryogenic tube in a waterbath at 45°C for 30 seconds. Fertilization process was carried out by pouring eggs into a Petri dish, with the number of eggs in a Petri dish around 100-200 eggs [14] with modification. The eggs were mixed with the sperm (fresh and post-cryopreservation) by gently stirring for 3 minutes until it was mixed and poured to the incubation. The incubation must be connected to the aeration hose (Fig. 4) and air stone during the fertilization process which is adjusted to provide oxygen [14, 9]. The quality of water in the incubation must be controlled with salinity conditions of 30-32 ppt, temperature 28-29°C, oxygen content of 7.5-7.8 mg/L and pH around 8.2-6.6 [14,9].

Figure 1. Giant grouper (Epinephelus lanceolatus).

Figure 2. Tiger Grouper (Epinephelus fuscoguttatus).

Figure 3. The process of eggs collecting.

Figure 4. Fertilization process. This process was done in the container with modification and connected with aeration hose.
2.4. The fertilization rate
The fertilized egg will be clear (transparent), round and float in the water, while the unfertilized egg was milky white and settles in the water [11]. Percentage of fertility was done after 2 hours (morula phase) of fertilization and calculated as the formula below:

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

2.5. Statistical analysis
The statistical analysis was carried out using SPSS version 20.0 software. All fertilization data were presented as mean ± SD, and differences were represented by the letter identification methods (a,b). The fertilization rate of giant grouper sperm post-cryopreservation with tiger grouper was analyze by one way ANOVA. The significance level P < 0.05.

3. Results and discussion
Fertility rate of hybrid species (giant grouper x tiger grouper, named Cantang) with fresh sperm was 80-83% (Table 1). Hybridization of grouper was done before, especially in giant grouper and tiger grouper which have fertility rate about 86.8% [14]. Furthermore, in another species of grouper which is tiger grouper and batik grouper (named Cantik) with the percentage of fertilization was 82.90% [20]. The percentage of fertilization of these hybrids including the good result.

| N   | Fertilization rate (%) |
|-----|------------------------|
| 1   | 81.75                  |
| 2   | 83.76                  |
| 3   | 80.24                  |
| 4   | 82.58                  |
| Avg. | 82.08                 |
| Stdev. | 1.48                  |

The average of fertilization rate of fresh semen was more than 80%, it means almost the eggs was fertilized.

Cryopreservation of spermatozoa of giant grouper using palm dates as a cryoprotectant was showed the good results, with an average value of motility rate 68-76% (Table 2). The highest value was showed at 10% of palm dates treatment, which is 76.70 ± 1.54 % [18]. Post-thaw spermatozoa of giant grouper was fertilized the eggs of tiger grouper. Fertilization rate of post-thaw spermatozoa of giant grouper in control (0%) and in various palm dates concentration of 0%, 5%, 10%, 15%, 20%, 25%, were: 54.37 ± 4.27%, 58.12 ± 6.88%, 66.25 ± 3.23%, 61.25 ± 3.23%, 58.12 ± 4.27%, 56.87 ± 4.27% (Table 2). This study presented that it was possible to produce hybrids grouper from sperm post-cryopreservation or fresh sperm from giant grouper sperm and tiger grouper eggs.

The fertilization of giant grouper and tiger grouper was taken by artificially. Artificial fertilization in fish has been widely applied as a fish hatchery business. This provides advantages including the implementation schedule of fertilization can be adjusted according to needs, fertilization media can be arranged, and the results of fertilization are more easily controlled so as to get more larvae compared to natural fertilization [25].

Based on the observations made, the eggs of tiger grouper were clear (transparent) (Fig. 5) and the fertilized eggs has a perfect structure and actively dividing. According to [12] fertilized eggs have a normal morphology, which is transparent and round. The percentage of fertility is influenced by several factors, one of which is the quality of spermatozoa. A good spermatozoa were determined by motility, viability, and abnormality. The percentage of motility is the most important parameter in the success of fertilization. So that the percentage of motility and fertility ability has a good correlation [1].
Cantang grouper eggs that have been fertilized by sperm post-cryopreservation give the good results with a combination of 6% glycerol and various concentrations of palm dates. It was showed by the high fertility rate and a good develop of the eggs that have been fertilized (Fig. 5.c). The embryo of the eggs was successfully formed until the tail of the grouper was able to come out of the egg yolk (Fig. 5.d). The good conditions of the environment will affect the development of embryo and hatchability of eggs. Hatching rate was required of time and influenced by a supportive of the environment such as salinity. The high salinity (> 30 ppt) is a good condition for the growing of the eggs [15].

Table 2. Post-thaw spermatozoa profile.

| Concentration (%) | n  | Motility (%) | Fertilization (%) |
|-------------------|----|--------------|-------------------|
| 0%                | 1  | 64.28        | 60.00             |
|                   | 2  | 67.74        | 50.00             |
|                   | 3  | 69.51        | 52.50             |
|                   | 4  | 70.59        | 55.00             |
| Avg.              |    | 68.03        | 54.37             |
| Stdev.            |    | 2.76         | 4.27              |
| 5%                | 1  | 75.67        | 65.00             |
|                   | 2  | 71.05        | 55.00             |
|                   | 3  | 68.18        | 50.00             |
|                   | 4  | 71.11        | 62.50             |
| Avg.              |    | 71.50        | 58.12             |
| Stdev.            |    | 3.09         | 6.88              |
| 10%               | 1  | 76.92        | 67.50             |
|                   | 2  | 75.00        | 62.50             |
|                   | 3  | 78.68        | 70.00             |
|                   | 4  | 76.19        | 65.00             |
| Avg.              |    | 76.70        | 66.25             |
| Stdev.            |    | 1.54         | 3.23              |
| 15%               | 1  | 77.59        | 62.50             |
|                   | 2  | 67.74        | 65.00             |
|                   | 3  | 72.79        | 60.00             |
|                   | 4  | 74.57        | 57.50             |
| Avg.              |    | 73.17        | 61.25             |
| Stdev.            |    | 4.12         | 3.23              |
| 20%               | 1  | 70.83        | 60.00             |
|                   | 2  | 69.11        | 62.50             |
|                   | 3  | 74.07        | 57.50             |
|                   | 4  | 72.00        | 52.50             |
| Avg.              |    | 71.50        | 58.12             |
| Stdev.            |    | 2.08         | 4.27              |
| 25%               | 1  | 63.41        | 62.50             |
|                   | 2  | 68.98        | 57.50             |
|                   | 3  | 70.89        | 52.50             |
|                   | 4  | 70.08        | 55.00             |
| Avg.              |    | 68.34        | 56.87             |
| Stdev.            |    | 3.37         | 4.27              |

The percentage of motility spermatozoa of giant grouper with palm dates in various concentration, and the application on hybridization with tiger grouper eggs. Hybridization was express into a value of fertility rate. Values with the same superscript letter were not significantly different.
Figure 5. The eggs of cantang grouper (hybrids). This figure describes the eggs hybrids grouper using fresh sperm and sperm post-cryopreservation. There was unfertilized egg (a) and fertilized egg (b) with fresh sperm. The fertilized egg was showed by the cleavage of the egg (1). The egg with cryopreserved with sperm post-cryopreservation has fertilized by showed at the gastrula phase (c) and well develop to the next phase (d). It was proved with the discharge of the tail (3) from the egg yolk (2). Observation of the egg was doing under microscope with magnification of 10 x 40.

Figure 6. Histogram of fertility rate.
The percentage of fertility of grouper was increasing at the concentration of palm dates 0% to 10% which is about 58%, but has decreasing at the high concentration of palm dates (15% to 25%) which is about 61% to 56% (Fig. 6). The fertility rate of grouper with spermatozoa two days post-cryopreservation has the highest average value at 10% of palm dates treatment, which is 66.25 ± 3.23%. Fertility rate and motility rate have the high values at the same concentration of palm dates. It was a good correlation, because with the high value of motility followed by the high value of fertility. The motility of giant grouper sperm can fertilized well the egg of tiger grouper.

Fertilization ability on hybrid grouper (between giant grouper and tiger grouper) using spermatozoa two days post-cryopreservation has the lowest value (under 70%). The fertility rate of spermatozoa post-cryopreservation usually has the low rate than the fresh semen. These because of the weak of motility after thawing. The low of spermatozoa motility can caused by damage of spermatozoa such as abnormal of morphology while cryopreservation. The abnormal spermtamoza post-cryopreservation will affect the motility, plasma membrane integrity, and triphosphate adenine content (ATP). The demage of spermatozoa can caused by several factors, were cold shock, cooling rate, solvent of composition, and osmotic pressure. The factors will affect the function of cells such as membrane stability and demage of oxidation [16,17].

4. Conclusion
Based on the research, we can concluded that the spermatozoa two days post-cryopreservation can fertilize the eggs of tiger grouper. The combination of 6% glycerol and 10% palm dates showed the highest fertility rate (66.25 ± 3.23%). This study was demonstrated that palm dates can be used as a cryoprotectant for sperm cryopreservation of giant grouper, and successfully fertilized the egg to produce the qualified of hybrids. These fertilization can be used as a guide for the cultivator of grouper or related institutions to produce the hybrids species with affordable prices and easy obtained of natural cryoprotectant. Further work should examine hatching rate and survival rate after invitro fertilization, and compare it with non-hybrid grouper.

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