EFFECT OF VARIOUS CRYOPROTECTANTS ON PRESERVATION OF BLACK TIGER Penaeus monodon SHRIMP SPERMATOZOA

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ABSTRACT

The development of cryopreservation technique on tiger shrimp Penaeus monodon broodstock spermatophore has been carried out to support the artificial insemination. This study aims to determine the effect of three cryoprotectants (methanol, dimethylsulphoxide (DMSO), and glycerol) for long term storage of tiger shrimp Penaeus monodon spermatozoa. Spermatophores were collected from the wild broodstocks through electrical shock. Spermatozoa were obtained by homogenizing the spermatophores using a Radnoti micro homogenizer in Ca-free saline solution containing one of three cryoprotectants (methanol, dimethylsulphoxide, and glycerol) separately at the concentration of 5%. One mL of each cryoprotectant containing spermatozoa with the density of $1.02 \times 10^6$ cell/mL was transferred into a cryovial and cryopreserved at room temperature, -20°C, and -196°C for 5, 10, and 30 days. The apparent sperm viability (ASV) of cryopreserved spermatozoa was monitored after treated. Thawing of cryopreserved spermatozoa was carried out in a 30°C water bath for two minutes. The result showed that the best apparent sperm viability was obtained at the using of glycerol at -196°C in liquid nitrogen, even after the thirty days of cryopreservation time period with the ASV of $0.82 \times 10^6$ cells/mL (80.39%). Meanwhile two other cryoprotectants displayed the ASV of $0.54 \times 10^6$ cells/mL (56.86%), and $0.23 \times 10^6$ cells/mL (22.55%) for DMSO and methanol, respectively. In turn, the control showed the lowest ASV with the ASV of $0.01 \times 10^6$ cells/mL (1.27%). The ASV showed by this glycerol exhibited a significant difference (P<0.05) to that of methanol, DMSO, and control.

KEYWORDS: cryoprotectant; Penaeus monodon; spermatozoa; temperature

INTRODUCTION

The tiger shrimp domestication process still has several constrains including the very low number of mating spawner from culture in the tank so that the eggs fertility could not be yielded (Lante & Laining, 2016). One of the alternatives which can be achieved to improve the eggs fertility of tiger shrimp is through artificial insemination (AI) technique. The AI had been carried out by Research Institute for Brackish water Aquaculture and Fisheries Extension (RIBAFE) and given a good result particularly on the wild shrimp broodstock in 2014. Nevertheless, there has been often occurred a constrain in the applying of this AI such as un-sincronized gonad maturation between male and female broodstock as well as spermatophore supplying (donor) and molting cycle of female broodstock (receiver).

To overcome this problem, it is required cryopreservation technique to preserve spermatophore at the time of female broodstock ready to be spawned. Study on cryopreservation particularly spermatozoa has been started since 1957 and more than 230 aquatic organism species have been investigated (Dong et al., 2007). The successful of sperm cryopreservation technique has been reported on more than 200 species (Figel & Tiersch, 1997; Tiersch, 2000). However, only a limited number of reports exist on successful cryopreservation of Shrimp (Bart et al., 2006). Cryopreservation of spermatophore of tiger shrimp Penaeus monodon using various of cryoprotectants such as methanol (MeOH), dimethylsulphoxide (DMSO), and ethylenglycol has been also reported. Nevertheless, their effects on the survival rate of sperm cells still gave a various result (Gwo, 2000; Wayman & Tiersch, 2000; Chao & Liao, 2001).

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The successful of spermatophore cryopreservation is determined by several factors such as extender, cryoprotectant, dilution ratio, freezing, and thawing rate as well as extender solution (Billard et al., 1995). The aims of this research was to determine the effect of three cryoprotectants (methanol, dimethylsulphoxide (DMSO), and glycerol) for cryopreservation of tiger shrimp Penaeus monodon spermatozoa. The best cryoprotectant obtained will be used for spermatophore cryopreservation of tiger shrimp broodstock in supplying of spermatophore for artificial insemination.

**MATERIALS AND METHODS**

This study was conducted on the tiger shrimp breeding Station of Research Institute for Brackish Water Aquaculture and Fisheries Extension (RIBAFE)-Barru District South Sulawesi Province. Spermatophores were collected from wild male broodstock which is catched from Aceh waters. Extender was used from Ca-free saline solution. Meanwhile, there are three kinds of cryoprotectants used i.e. dimethylsulphoxide (DMSO), methanol (MeOH), and glycerol solution.

**Male Broodstock Maturation**

Mature male of tiger shrimp Penaeus monodon with the body weight of 80 to 102 ± 8.55 g and the body length of 19.5-21.9 ± 1.5 cm were selected based on their health and physical conditions. The selected shrimp were individually tagged using labeled plastic tags around the eyestalk and then reared in the maturation tank 3.0 m³ tank in volume with the density of 15 ind./tank. Water salinity, temperature, and water depth were maintained at 32%-35% 28°C-29°C, and 50 cm, respectively. During rearing period, males broodstock were fed fresh feed consisted of squid, marine bloodworms, and oyster at amount of 15% of ration tank 3.0 m³ tank. Water salinity, temperature, and water depth were changed the water at amount level of 80%.

**Collection of Spermatophore**

The matured males indicated with a clear white swelling around the coxae at the base of the fifth walking leg were selected from the maturation tank. The selected males were measured their weight and length. Spermatophores were taken out through electrical shock using transformer set up at 5 mA and 8-12 V following the protocol as described by Sandifer et al. (1984). The transformer was connected with an electrode placed near the gonophores at the base of the 5th pereiopods applied usually for two seconds. The electrical shock stimulates contraction surrounding the terminal ampullae expelling a single spermatozoa from each gonophore. Spermatophores were removed with forceps and placed into an eppendorf tube filled with 1 mL of Ca-free saline solution known its weight. Other to prevent infection, a small drop of iodin 5% was applied to the genital pore of the tested shrimp. The males were then reared back in the maturation tank.

**The Observation on Spermatophore and Spermatozoa**

The released spermatophore was weighted using an electrical balance (Sartorius, Germany). Subsequently, to release spermatozoa, the spermatophores were manually homogenized with Ca-free saline solution in a glass tissue grinder (Radnoti, USA). The spermatozoa observation was tested with staining technique using ethylene blue. A ten µL of ethylene blue were added into 50 µL sperm suspension. Briefly, 10 µL of this spermatozoa suspension were aspirated into a pipette, put on a haemocytometer and covered by coverslip. Number of spermatozoa was calculated under a microscope with a 40x objective (Olympus DP21, Japan). The sperm cells were snapped and counted the number using Image J software.

The estimation of sperm cells number was calculated using the equation as followed:

\[
\text{The sperm cell quantity (cell/mL)} = \sum n_i p \times 10^4
\]

Note:

- \(N\) = Total of sperm cells
- \(p\) = Dilution

Apparent sperm viability (ASV) post cryopreservation was counted by the following formula:

\[
\text{ASV } (\%) = \sum \frac{n_i}{n_j} \times 100
\]

Note:

- \(n_i\) = Sperm cells at the observation time
- \(n_j\) = Total of sperm cells initial number (fresh)

Characterization of the sperm cell was observed for the number of total sperm cell and percentage of normal cell. The cells with spherical body shape and straight and elongate spike was considered as normal cell, while sperm cells with irregular form and bent, double, or missing spike was considered as abnormal cell (Talbot et al., 1989).

**Cryopreservation of Spermatozoa**

Prior to cryopreservation, spermatozoa were separately stocked with the density of 1.02 ± 10^6 cells/mL in 50 mL of cryoprotectant solution (methanol...
(M), dimethylsulphoxide (D), and glycerol (G)) at the concentration of 5% in Ca-free saline solution. The similar spermatozoa were also stocked into 50 mL of Ca-free saline solution only as the control (S). One mL of each sperm-cryoprotectant solution was transferred into cryovials labeled according to the cryoprotectant code (M, D, G, and S) performed with 10 replicates. The cryovials containing spermatozoa were cryopreserved at room temperature, -20°C and -196°C in liquid nitrogen. Sperm cells cryopreserved at -20°C previously kept at the temperature of 4°C-5°C for one hour. Similar to sperm cells cryopreserved at -20°C, sperm cells cryopreserved at -196°C in liquid nitrogen, it was kept at the temperature 4°C-5°C for one hour, -20°C for overnight and continued to -80°C for overnight to prevent sperm cell shock.

Viability Monitoring of Cryopreserved Sperm

The viability of cryopreserved sperm was monitored at 5, 10, and 30 days of cryopreservation period. To determine the viability of cryopreserved sperm, the samples were thawed at a 30°C water bath for two minutes. When the sperm suspensions melted completely, the viability of sperm cells was determined using the method mentioned before.

RESULTS AND DISCUSSION

The wild tiger shrimps Penaeus monodon used were 20 shrimps with weight and length ranged from 85-107 g and 18.0-22.5 cm, respectively. The weight, length, number and weight of spermatophore were summarized in Table 1.

Among wild shrimp, 20 of the 30 shrimp given an electrical shock presented spermatophore, commonly carrying two spermatophores with the average weight of 0.08 g (Table 1). From the Table 1, it can be seen that there was no correlation between shrimp weight and spermatophore weight. For example, in shrimp weight of 85 and 107 g the spermatophores weight of 0.08 g and 0.06 g. In this study, the average spermatoza number contained in one spermatophore was 4.75 x 10^6 cells/mL. Based on these data, it was revealed that spermatophore weight could not be indicator for spermatoza number in the spermatophore.

The initial sperm cell density used was 1.02 x 10^6 cell/mL and there was obtained 97.08% of normal sperm cells. Among four types of abnormal spermatozoa, the most frequent were missing spike. The similar result was investigated by Pratoomchat et al. (1993) reporting that spermatozoa without spike was a type abnormality which is the most frequent in P. monodon namely more than 95% of the totality of abnormal spermatozoa. Nevertheless, an abnormal spermatozoa does not mean that it is infertile.

In each of the cryoprotectant tested, the highest sperm cell quantity and mean apparent sperm viability (ASV) cryopreserved at room temperature was observed at methanol (MeOH) 81.37 ± 0.40% after treatment for five days and 55.88 ± 0.12% after 10 days. The lowest was obtained at glycerol 37.52 ± 0.64% after treatment for five days while, dimethyl sulphoxide (DMSO) and control (without cryoprotectant) showed the mean ASV of 80.39 ± 0.30% and 38.23 ± 0.34% after treatment for five days. Nevertheless, after treatment for 30 days the highest mean ASV was exhibited by glycerol 24.51 ± 0.98% and three other cryoprotectants (methanol, dimethyl sulphoxide, and control) showed a drastically ASV decreasing (> 90%) even after 10 days and 30 days of treatment, spermatozoa without cryoprotectant (control) exhibited lysis cells (Figure 1).

In turn, cryopreservation of spermatoza at the temperature of -20°C in glycerol showed the highest ASV at day 5 (85.29 ± 0.55%) even after treatment for 10 (80.39 ± 0.34%) and 30 days (67.64 ± 0.30%). The lowest ASV at the same treatment time was exhibited by the control (without cryoprotectant) 1.96 ± 0.45% 1.27 ± 0.44% and 0.98 ± 0.65% respectively. Two other cryoprotectants, methanol and dimethyl sulphoxide also displayed a pure ASV of below 30% after treatment for five days (20.58 ± 0.61% and 26.47 ± 0.55%), 10 days (7.84 ± 0.34% and 7.84 ± 0.45%) and 30 days (2.94 ± 0.50% and 2.94 ± 0.50%) (Figure 2).

The highest mean ASV was also shown by glycerol at the temperature of -196°C. The highest mean ASV observed after treated for 5, 10, and 30 days were 91.17 ± 0.69% 89.22 ± 0.56% and 80.39 ± 0.45% respectively. Meanwhile, the lowest ASV was still exhibited by the control (without cryoprotectant) 3.92 ± 0.44% 1.96 ± 0.34% and 1.27 ± 0.54% after treatment for 5, 10, and 30 days, respectively. Meanwhile, dimethyl sulphoxide and methanol displayed the mean ASV of 77.45 ± 0.30, 70.58 ± 0.96, 56.86 ± 0.05, and 70.59 ± 0.25, 48.03 ± 0.55, and 22.55 ± 0.27% respectively. These mean ASV shown by dimethyl sulphoxide and methanol at the temperature of -196°C were higher than these mean ASV indicated by these two cryoprotectants at the temperature of -20°C. Nevertheless, MeOH resulted a drastic decreasing of mean ASV (67.60%) from day-5 until day-30 (Figure 3).
Table 1. Weight, length, number and weight of spermatophore of wild black tiger *P. monodon* shrimp used for cryopreservation experiment

| Weight (g) | Length (cm) | Spermatophore number | Spermatophore weight (g) |
|------------|-------------|-----------------------|--------------------------|
| 85         | 21          | 2                     | 0.08                     |
| 107        | 22.5        | 1                     | 0.06                     |
| 94         | 21.4        | 2                     | 0.08                     |
| 90         | 19.3        | 2                     | 0.07                     |
| 85         | 20.7        | 2                     | 0.07                     |
| 107        | 21.3        | 2                     | 0.09                     |
| 99         | 20.4        | 2                     | 0.08                     |
| 88         | 18          | 2                     | 0.07                     |
| 84         | 22.3        | 2                     | 0.09                     |
| 99         | 21          | 1                     | 0.07                     |
| 84         | 20.2        | 2                     | 0.08                     |
| 96         | 20.9        | 2                     | 0.08                     |
| 107        | 22.5        | 2                     | 0.06                     |
| 103        | 22.4        | 2                     | 0.08                     |
| 98         | 21.7        | 2                     | 0.07                     |
| 104        | 21.4        | 2                     | 0.08                     |
| 92         | 21          | 2                     | 0.08                     |
| 80         | 20.1        | 2                     | 0.08                     |
| 90         | 20.6        | 2                     | 0.08                     |
| 93         | 20.4        | 2                     | 0.08                     |

Figure 1. Mean apparent sperm viability (%) treated for 5, 10, and 30 days with different cryoprotectants at the concentration of 5% at room temperature (mean ± standard deviation). Note: GA (glycerol); MA (methanol); DA (dimethylsulphoxide); and SS (Ca-free saline solution/control).
From Figure 1, 2, and 3 after treatment 30 days, it was obtained that sperm cells cryopreserved with glycerol and kept at the temperature of -20°C and -196°C and those cryopreserved with DMSO at -196°C exhibited apparent cell viability > 50% Among them, sperm cells cryopreserved using glycerol was found to be the highest (80.39%). This ASV value of spermatozoa treated with glycerol showed a significant difference (P<0.05) to that of spermatozoa treated with methanol, dimethylsulphoxide and control (without cryoprotectant). The using of methanol, dimethylsulphoxide and glycerol as a cryoprotectant in this experiment, all of them displayed a significant difference to the control (without cryoprotectant). The similar result was showed by Chaves et al. (2014) reporting that Litopenaeus vannamei sperm cryopreserved with 5% and 10% of glycerol and DMSO demonstrated the apparent sperm viability (ASV) of
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78.37% and 97.42% or > 50% and there was not found any difference between glycerol and DMSO at the same concentration. It indicated that glycerol had a low toxic effect to shrimp sperm. However, the using of DMSO at the concentration 5% and the higher concentrations of 10%, 15% and 20% resulted in the greater mortality in the cryopreservation of Panaeus monodon sperm (Bart et al., 2006; Vuthiphandchai et al., 2007). The similar pattern was also obtained by Memon et al. (2012) who got the higher mortality when DMSO at the concentrations of 5%, 10%, 15% and 20% was used for cryopreservation of another shrimp, Panaeus merguiensis sperm. The high sperm cell mortality in this study showed that MeOH at the concentration of 5% gave a negative effect on Panaeus monodon sperm. The same observation was also exhibited by Bart et al. (2006) at the similar species shrimp. There was obtained no any survival observed.

In Panaeus monodon sperm cells cryopreserved for 30 days with DMSO there was a significant decrease in apparent sperm viability (ASV) being more prominent from day-0 to day-5 with the decreasing of 22.50% giving a significant difference to day 10 and 30. In turn, treatment with glycerol did not show any difference between day-5 and day-10. However, ASV in day-5 and day-10 significantly differed to that in day-30. The same observation was obtained in the study L. vannamoe done by Chaves et al. (2014) reporting the decreasing of ASV approximately 27% between day 15 and 30 although there was a difference in the freezing method. Fast freezing procedures is able to cause substantial damage to the cell and high sperm mortality suggesting that gradual cooling protocols must lead to better outcomes. However, in this study cooling to 4°C and freezing to -196°C in two steps (> 4°C > 20°C > -196°C) has no any negative effect on Panaeus monodon sperm cells. However, the cooling rate was done manually in the refrigerator and freezer. Therefore, it might have a bit influence on the result obtained.

There are many factors affecting the success of cryopreservation technique such as cooling and freezing rate, type and concentration of cryoprotectant, final concentration of freezing, extenders, and much more (Kartha, 1985; Ikhwanuddin et al., 2015). If freezing is very late, cells will be more dehydrated so that the concentration of electrolyte substance in the cell is to be high. In contrast, if fast freezing rates increase, the probability of intracellular ice formation will be leading to cryo-injuries. The choice of cryoprotectant is dependent on the species in crustacean. According to Anchordoguy et al. (1988), compared to glycerol, sucrose, proline, and trehalose, DMSO is a more effective cryoprotectant for marine shrimp Sicyoniaingentis. In turn, for tiger shrimp Panaeus monodon glycerol is a more effective cryoprotectant than DMSO at the concentration of 5% as the result of the present study. The high of sperm cells which lived at the glycerol treatment were due to the good protection on the presence of spermatozoa damage during the cryopreservation process (cryoinjury) in which glycerol itself had a function as permeating cryoprotectant.

CONCLUSION

Based on this present study, the best cryoprotectant for cryopreservation of tiger shrimp Panaeus monodon spermatozoa for a long time storage was glycerol at the temperature of -196°C in liquid nitrogen with the apparent sperm cell (ASV) of 80.39%.

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