

Effect of different extenders and cryoprotectants on sperm viability of mud crab *Scylla tranquebarica* for short-term storage

Pengaruh bahan ekstender dan krioprotektan yang berbeda terhadap viabilitas sperma kepiting bakau *Scylla tranquebarica* untuk penyimpanan jangka pendek

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ABSTRACT

The aim of the present study was to determine the extenders such as calcium-free saline, Ringer's solution and phosphate buffer and to determine the effect of different concentrations of cryoprotectants with different duration of exposure for short-term storage of mud crab, *Scylla tranquebarica* sperm cells. A total of 30 mud crabs were used in the present study. In the present study, three treatments with three replicates were tested to determine the suitable extender. Three treatments (dimethyl sulfoxide, glycine and glycerol) were tested to determine the suitable cryoprotectants. Eight treatments (25, 20, 16, 4, 2, -4, -20, and -80°C) were assessed to determine the optimal cooling rate. The result showed the phosphate buffer solution produced the highest sperm viability (82.36 ± 5.84%) after 60 min compared the calcium-free saline and Ringer's solution. Using 5% glycine as the optimal cryoprotectant, sperm viability at -80°C exhibited robust initial viability 95.17 ± 0.92% at 6 hours, 94.11 ± 0.64% at 12 hours, and 93.31 ± 0.51% at 24 hours. However, sperm viability gradually declined to 90.84 ± 2.05% by day 7, 82.82 ± 0.41% by day 14, and continued to decrease by day 21. In a nutshell, the phosphate buffer and 5% glycine proved to be the most effective extender and cryoprotectant in this study.

Keywords: cooling rate, cryoprotectant, extender, *Scylla tranquebarica*, sperm viability

ABSTRAK

Tujuan penelitian ini adalah untuk menentukan pengencer diantaranya saline bebas kalsium, larutan Ringer, dan buffer fosfat serta untuk menentukan efek dari berbagai konsentrasi krioprotektan dengan durasi paparan yang berbeda untuk penyimpanan jangka pendek sel sperma kepiting lumpur, *Scylla tranquebarica*. Sebanyak 30 kepiting lumpur digunakan dalam penelitian ini. Dalam penelitian ini, tiga perlakuan dengan tiga ulangan diuji untuk menentukan pengencer yang tepat. Selain itu, tiga perlakuan (dimetil sulfoksida, glisin, dan gliserol) diuji untuk menentukan krioprotektan yang tepat. Delapan perlakuan (25, 20, 16, 4, 2, -4, -20, dan -80°C) dinilai untuk menentukan laju pendinginan optimal. Hasil penelitian menunjukkan larutan buffer fosfat menghasilkan viabilitas sperma tertinggi (82,36 ± 5,84%) setelah 60 menit dibandingkan larutan garam bebas kalsium dan larutan Ringer. Menggunakan glisin 5% sebagai krioprotektan optimal, viabilitas sperma pada suhu -80°C menunjukkan viabilitas awal yang kuat 95,17 ± 0,92% pada 6 jam, 94,11 ± 0,64% pada 12 jam, dan 93,31 ± 0,51% pada 24 jam. Namun, viabilitas sperma secara bertahap menurun menjadi 90,84 ± 2,05% pada hari ke 7, 82,82 ± 0,41% pada hari ke 14, dan terus menurun pada hari ke 21. Singkatnya, buffer fosfat dan glisin 5% terbukti menjadi ekstender yang paling efektif dan krioprotektan dalam penelitian ini.

Kata kunci: daya tahan, ekstender, kadar penyejukan, kryoprotectant, *Scylla tranquebarica*

INTRODUCTION

Aquaculture is the way to a safe and sustainable source of seafood production to ensure an adequate seafood supply (Yue & Shen, 2022). Therefore, the trade for seafood such as lobsters, shrimp and crabs for export has been increasing significantly recently (Ikhwanuddin *et al.*, 2014a). Among the edible species, genus *Scylla* especially *Scylla tranquebarica*, which commonly called the purple mud crab is the most dominant species in Sabah coastal water and constitutes the primary crustacean fishery resource (Nurul *et al.*, 2016). According to Ikhwanuddin *et al.* (2014b), the crab fishery in Malaysia is yet to be known as a major fishery despite the abundant occurrence of food crabs along the Malaysia coastal waters.

Many coastal fishing communities in Malaysia started to culture mud crabs in floating cages and pen enclosure in mangrove forests due to high selling prices in Malaysia. The spawning of mud crabs also depends on the season which prevents the production of mud crabs continuously produce for whole year. The availability of good sperm and eggs does not occur the same time thus constraining the production if mud crabs which in turn decreasing the profitability of mud crab production (Ikhwanuddin *et al.*, 2014b). It becomes harder to accomplish a consistent production rate because climate change can be a major challenge for mud crab farmers (Dutta *et al.*, 2022). Additionally, *S. tranquebarica* has been informed in Sabah coastal waters and it is the commercially crustaceans in the trade markets as demand that has been reported to be increased every year (Fatimah *et al.*, 2019).

In order to increase the number of mud crabs in a population, an alternative source of supply such as sperm cryopreservation is needed to further expand mud crab aquaculture by external artificial insemination to maximize the sustainable yield from wild stocks (Ikhwanuddin *et al.*, 2014b). The testes and vas deferens are visible in mud crabs and develop gradually until maturity (Islam *et al.*, 2018). Therefore, there are few studies on cryoprotectants for the sperm in other crustacean's species such as edible rock lobster, *Panulirus homarus* (Sasikala & Meena, 2009), banana shrimp, *Peneus merguensis* (Memon *et al.*, 2012), white shrimp, *Litopenaeus vannamei* (Uberti *et al.*, 2014; Castelo-Brance *et al.*, 2015), mud spiny lobster, *Panulirus polyphagus* (Fatimah

et al., 2016), mud crab, *S. tranquebarica* (Fatimah *et al.*, 2018) and banana shrimp, *Fenneropenaeus merguensis* (Nimrat *et al.*, 2022). Thus, the objectives in the present study were to determine the effect of different extenders such as calcium-free saline, Ringer's solution and phosphate buffer and to determine the effect of different concentrations of cryoprotectants (such as dimethyl sulfoxide, glycine, and glycerol) with different duration of exposure for short term storage of *S. tranquebarica* sperm cells.

MATERIALS AND METHODS

Sample and study site

The study was held at Shrimp Hatchery, Borneo Marine Research Institute, Universiti Malaysia Sabah. A total of 30 mud crabs were used in the present study. The sample was taken from the wild and obtained from Kota Kinabalu fish markets. Three treatments (calcium-free saline, phosphate buffer and Ringer's solution) with three replicates were tested to determine the suitable extender. Three treatments (dimethyl sulfoxide, glycine and glycerol) with two replicates were tested to determine the suitable cryoprotectants. Eight treatments (25, 20, 16, 4, 2, -4, -20, and -80°C) with two replicates were assessed to determine the optimal cooling rate. A temperature of 26°C was used for thawing the sperm sample.

Determination of suitable extenders

Calcium-free saline (Ca-F saline), Ringer's solution and phosphate buffer solution were tested in this study. Ca-F saline, Ringer's solution and phosphate buffer were prepared two days before the collection of samples and stored in the refrigerator. The carapace width (CW) and body weight (BW) of the mature males of *S. tranquebarica* were measured. The mud crab was dissected and the vas deferens was removed to get the sperm sample (Figure 1).

An extender (Ca-F saline, Ringer's solution and phosphate buffer) was added to the testes and homogenized using a mortar and pestle. Immediately, transferred the sperm into 2 mL microcentrifuge tube and each microcentrifuge tube were chilled for 5, 15, 30 to 60 min at 25°C. For each incubation, 50 µL of sperm was stained with eosin-nigrosin and transferred onto a Neubauer hemocytometer to observe the sperm viability under the light microscope.

Determination of suitable cryoprotectants

Dimethyl sulfoxide (DMSO), glycine and glycerol were prepared before dissecting the crabs. For preparation of cryoprotectants, each cryoprotectant solution was diluted into 5% (v/v) and 10% (v/v) using phosphate buffer, the optimal extender that has been tested before as the extender medium. First of all, the body weight (BW) and carapace width (CW) in mature male of *S. tranquebarica* were measured. The mud crab was dissected and the vas deferens was removed to get the sperm sample.

A cryoprotectant (5 and 10% DMSO, 5 and 10% glycine and 5 and 10% glycerol) was added to the testes at a ratio of 1:3 and homogenized using a mortar and pestle. Immediately, the sperm was transferred into 2-mL microcentrifuge tube and each microcentrifuge tube was incubated for 5, 15, 30 to 60 min at 25°C and of 6, 12, and 24 h at -4, -20 and -80°C. 50 µL of sperm was stained with eosin-nigrosin, and transferred onto a Neubauer hemocytometer to observe the viability of the sperm under the light microscope (Vuthiphandchai *et al.*, 2007).

Determination of sperm viability and quantity

The sperm was put into 2 mL microcentrifuge tube and 5% and 10% cryoprotectant solution was added at a ratio of 1:3. Each microcentrifuge tube was chilled for 5, 15, 30 to 60 min at 25 °C and 6, 12, and 24 h at -4, -20 and -80°C. For each incubation, 50 µL of sperm was stained with eosin-nigrosin and put onto a Neubauer hemocytometer to observe the viability of the sperm (Vuthiphandchai *et al.*, 2007; Fatimah *et al.*, 2019). Live sperm remained unstained against the red background, while dead sperm exhibited

pink stained membranes (Ikhwanuddin *et al.*, 2014b). To determine the sperm quantity, the amount of sperm present in five of the 25 squares on the haemocytometer with complete sample coverage are counted. The mean sperm counts in the 25-square grid (0.1 µL) was multiplied by 10⁴ cells/mL (Fatimah *et al.*, 2015; Fatimah *et al.*, 2016).

$$\text{Sperm quantity (count)} \quad \text{Mean counts of 5 total squares} \\ \text{(sperm mL)} \quad = \times 25 \times 10^4 \text{ cells/mL} \times \text{dilution} \\ \text{of sperm}$$

Development of cryopreservation protocols

The suspension (testes homogenized with glycine 5%) was mixed at a ratio of 1:3 in a microcentrifuge tube. The microcentrifuge tube was subjected to eight different cooling protocols (25, 20, 16, 4, 2, -4, -20°C) for 15 min each (Fatimah *et al.*, 2015), followed by the storage at 6 h, 12 h, 24 h, day 7 and day 14, and the sperm viability was checked. After cryopreservation, the sperm suspension was thawed for 30 s in water bath at 26°C. After that, the sperm viability was observed. For each incubation, 50 µL of sperm was stained with eosin-nigrosin and transferred onto a Neubauer hemocytometer to observe the sperm viability under light microscope (Vuthiphandchai *et al.*, 2007).

Data analysis

One-way ANOVA were used for all treatments and significant values were cross-examined using Tukey's post hoc test. Two-way ANOVA were used to evaluate the significance of sperm viability between the duration of exposure and the concentrations of cryoprotectants solution at room temperature.

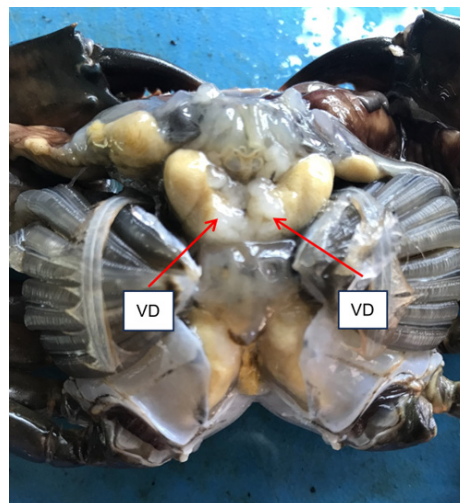


Figure 1. Reproductive system of mature male in mud crab, *Scylla tranquebarica*, VD: Vas deferens.

RESULTS AND DISCUSSION

Result

Suitable extenders

Based on Figure 2, the sperm viability significantly reduced as time increased. An extender of phosphate buffer solution (PBS) produced the highest sperm viability ($82.36 \pm 5.84\%$) after 60 min exposure and it was similar with the rates of viability observed after 15 min exposure to Ca-F saline and 30 min to Ringer's solution. However, Ca-F saline and Ringer's solution still produced high sperm viability after 60 min exposure, which was $77.27 \pm 2.94\%$ and $80.91 \pm 3.14\%$, respectively.

Suitable cryoprotectants

Effects of cryoprotectant exposure on sperm viability at room temperature (25°C)

The cryoprotectants of 10% glycine, 5% glycine and 10% DMSO produced the highest mean sperm viability with $95.11 \pm 0.76\%$, $94.77 \pm 0.28\%$, and $93.14 \pm 1.32\%$, respectively after

5 min exposure. Interestingly, the sperm viability after 60 min exposure was only slightly reduced to $90.38 \pm 0.98\%$, $88.33 \pm 0.75\%$, and 92.25% , respectively. However, 10% glycerol gave the lowest mean sperm viability after the sperms were exposed at 5 min ($88.98 \pm 1.34\%$), whereas 5% glycerol produced the lowest mean sperm viability after 60 min exposure ($76.14 \pm 0.88\%$). From Table 1, the sperm viability at 5% glycerol and 5% glycine significantly reduced as time increased.

Effects of cryoprotectant exposure on sperm viability at -4°C

Based on Table 2, there was no significant difference observed for the viability of sperm at -4°C. Quite similar mean sperm viability, at $95.13 \pm 0.17\%$, $95.06 \pm 1.04\%$, and 95.63 ± 0.13 , were produced after 6, 12 and 24 h when using 5% glycine. On the other hands, 5% DMSO also produced high mean sperm viability after exposure to 6, 12 and 24 h, $95.90 \pm 2.57\%$, $94.95 \pm 1.92\%$, and $91.40 \pm 1.66\%$, respectively.

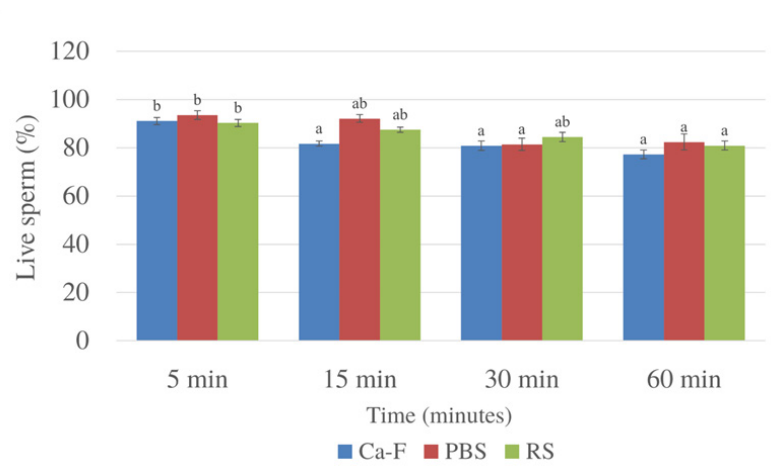


Figure 2. Mean of sperm viability in *S. tranquebarica* after exposure to the extenders (Ca-F saline, phosphate buffer and Ringer's solution) in different durations (5, 15, 30, and 60 min) at room temperature (25°C).

Table 1. Mean *S. tranquebarica* sperm viability after exposure to 5 and 10% cryoprotectants (DMSO, glycerol and glycine) in different durations (5, 15, 30 and 60 min) at room temperature (25°C).

Cryoprotectants	Time (min)			
	5	15	30	60
5% DMSO	90.52 ± 1.82^a	90.64 ± 1.45^a	90.19 ± 2.23^a	89.06 ± 2.31^a
10% DMSO	93.14 ± 1.31^a	92.35 ± 2.91^a	92.33 ± 1.01^a	92.25 ± 2.35^a
5% Glycerol	91.64 ± 2.32^b	93.14 ± 2.09^b	84.62 ± 3.15^{ab}	76.14 ± 0.88^a
10% Glycerol	88.97 ± 1.33^a	85.90 ± 3.63^a	81.46 ± 0.97^a	81.70 ± 1.49^a
5% Glycine	94.77 ± 0.28^b	91.86 ± 1.65^{ab}	91.39 ± 1.42^{ab}	88.33 ± 0.74^a
10% Glycine	95.11 ± 0.76^a	92.64 ± 2.76^a	91.53 ± 1.63^a	90.38 ± 0.98^a

Meanwhile, 10% glycerol at $86.11 \pm 3.93\%$, $84.66 \pm 3.56\%$, and $82.76 \pm 4.88\%$ had the lowest viability of sperm after exposure to 6, 12 and 24 h, respectively.

Effects of cryoprotectant exposure on sperm viability at -20°C

By using 10% DMSO as a cryoprotective agent, the viability of sperm was significantly decreased to $88.98 \pm 1.84\%$, $80.74 \pm 2.44\%$, and $76.28 \pm 4.20\%$ after 6, 12, and 24 h exposure, respectively. Based on the result from Table 3, 10% glycerol had the lowest mean sperm viability

after 6 h exposure meanwhile, 10% DMSO had the lowest mean sperm viability after 24 h exposure ($76.29 \pm 4.21\%$).

Effects of cryoprotectant exposure on sperm viability at -80°C

By using 5% glycine as cryoprotective agent, the highest mean sperm viability was observed at $90.34 \pm 1.08\%$, $89.23 \pm 0.97\%$, and $93.97 \pm 0.43\%$, respectively after 6, 12, and 24h exposure, and the sperm viability reduced significantly as time increased. However, 10% glycerol gave the lowest viability of sperm after exposing to 24 h ($81.58 \pm 3.42\%$) (Table 4).

Table 2. Mean *S. tranquebarica* sperm viability after exposure to 5 and 10 % cryoprotectants (DMSO, glycerol and glycine) in different durations (6, 12, and 24 h) at 4°C.

Cryoprotectants	Time (hour)		
	6	12	24
5% DMSO	95.80 ± 2.58^a	94.94 ± 1.91^a	91.40 ± 1.66^a
10% DMSO	92.20 ± 3.66^a	87.36 ± 2.21^a	86.66 ± 6.20^a
5% Glycerol	91.65 ± 3.08^a	92.88 ± 2.56^a	90.33 ± 1.28^a
10% Glycerol	86.11 ± 3.93^a	84.66 ± 3.56^a	82.76 ± 4.88^a
5% Glycine	95.13 ± 0.17^a	95.06 ± 1.04^a	95.63 ± 0.13^a
10% Glycine	95.13 ± 0.17^a	92.68 ± 2.65^a	89.55 ± 1.96^a

Table 3. Mean *S. tranquebarica* sperm viability after exposure to 5 and 10 % cryoprotectants (DMSO, glycerol and glycine) in different durations (6, 12, and 24 h) at -20°C.

Cryoprotectants	Time (hour)		
	6	12	24
5% DMSO	95.02 ± 1.66^a	94.74 ± 2.58^a	91.51 ± 1.91^a
10% DMSO	88.98 ± 1.84^b	80.74 ± 2.44^{ab}	76.28 ± 4.20^a
5% Glycerol	94.41 ± 1.71^a	90.69 ± 4.71^a	86.55 ± 4.99^a
10% Glycerol	85.19 ± 0.00^a	83.33 ± 2.62^a	79.44 ± 6.27^a
5% Glycine	90.95 ± 1.07^a	87.15 ± 4.70^a	83.30 ± 1.00^a
10% Glycine	92.07 ± 0.50^a	92.82 ± 3.31^a	90.86 ± 10.13^a

Table 4. Mean *S. tranquebarica* sperm viability after exposure to 5 and 10 % cryoprotectants (DMSO, glycerol and glycine) in different durations (6,12, and 24 h) at 80°C.

Cryoprotectants	Time (hour)		
	6	12	24
5% DMSO	89.90 ± 0.84^a	87.84 ± 2.91^a	83.81 ± 5.30^a
10% DMSO	89.13 ± 7.17^a	90.12 ± 6.24^a	84.26 ± 2.04^a
5% Glycerol	94.11 ± 2.80^a	93.36 ± 3.20^a	85.28 ± 1.23^a
10% Glycerol	83.84 ± 3.34^a	83.13 ± 6.89^a	81.58 ± 3.42^a
5% Glycine	90.34 ± 1.08^a	89.23 ± 0.97^{ab}	93.97 ± 0.43^b
10% Glycine	89.23 ± 0.97^a	86.29 ± 4.64^a	85.18 ± 12.87^a

Development of cryopreservation protocols

By using 5% glycine as the best cryoprotectant, the study was further up until 21 days by storing the sperm at -80°C after the sperm was chilled at 25, 20, 16, 4, -4, and -20 for 15 min each to observe the sperm viability. Based on Figure 3, the mean sperm viability was decreased significantly to $95.17 \pm 0.92\%$, $94.11 \pm 0.64\%$, $93.31 \pm 0.51\%$, $90.84 \pm 2.05\%$ and 82.82 ± 0.41 after 6 h, 12 h, 24 h, 7 days, 14 days and 21 days exposure, respectively.

Discussion

The selection of a suitable extender and cryoprotectant for *S. tranquebarica* sperm was able to preserve the sperm up until -80°C . The concentration of cryoprotectants, time duration, and cooling temperature also affect the sperm viability of *S. tranquebarica*. Phosphate buffer gave the highest mean of sperm viability for *S. tranquebarica* and it is chosen as the best extender to preserve the crab's sperm. The similar result was obtained in the Banana shrimp, *Fenneropenaeus merguensis* (Memon *et al.*, 2012) and the mud spiny lobster, *P. polyphagus* (Fatihah *et al.*, 2016). Otherwise, study by Fatihah *et al.* (2018), used the calcium-free saline (Ca-F saline) as the extender in mud crab, *S. tranquebarica*.

Besides, the sperm cryopreservation protocol of the mud crab, *S. serrata*, also used phosphate buffer as an extender, as well as glycerol, DMSO and trehalose as cryoprotectants to preserve the sperm (Jeyalectumic & Subramaniam, 1989). By using phosphate buffer as standard diluent

together with DMSO, glycerol, methanol and glucose as cryoprotective agents was also successful in sperm cryopreservation protocol of the Edible rock lobster, *P. homarus* (Sasikala & Meena, 2009). The present study showed the effectiveness of cryoprotectants and the duration of exposure for sperm differ in *S. tranquebarica*. The function of cryoprotectant is to protect the sperm cells from the formation of intracellular ice crystals and displace the movement of water molecules through the cells from excessive dehydration during the freeze storing (Hu *et al.*, 2017).

There are two types of cryoprotectants, which are intracellular and extracellular. Intracellular cryoprotectants such as dimethyl sulfoxide (DMSO), glycine, methanol and glycerol can penetrate into the cell to prevent the ice crystals formation that can cause membrane rupture. Meanwhile, extracellular cryoprotectants such as sucrose, trehalose and dextrose cannot pass through into the cells and it helps to improve the osmotic imbalance that occurs during freezing (Thompson, 2012). In this study, the cryoprotective agents such as DMSO, glycine and glycerol were used at concentrations of 5 and 10% to preserve the *S. tranquebarica* sperm. Similar studies were done by Ikhwanuddin *et al.* (2015), where DMSO, glycerol and methanol were used as cryoprotectants for mud crab, *S. olivacea*.

Furthermore, DMSO and glycerol are most common cryoprotective agents used in crustacean sperm (Bhavanishankar & Subramaniam, 1997). Therefore, the selection of suitable cryoprotectants

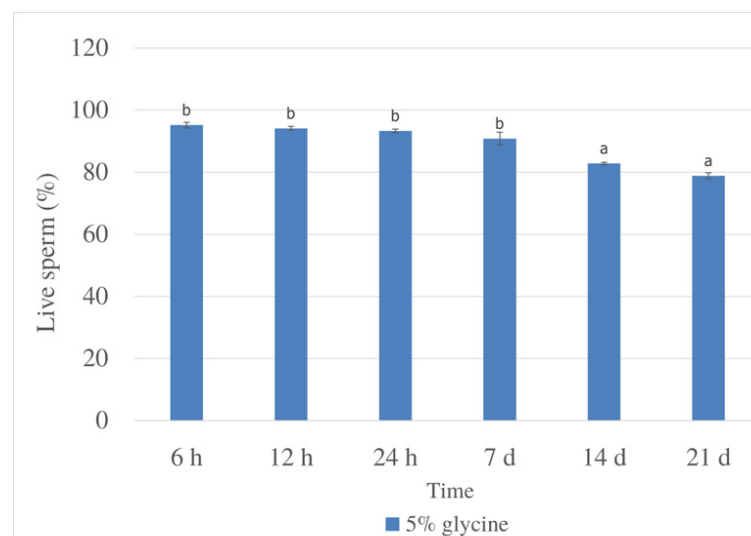


Figure 3. Mean *S. tranquebarica* sperm viability after exposure to 5 % glycine in different durations (6 h, 12 h, 24 h, day 7 and day 14) at -80°C .

will lower the motility of gamete after the cryopreservation. The effectiveness of DMSO for cryopreservation of sperm has been reported in various group of animals, including shrimps (*P. monodon*) (Vuthiphandchai *et al.*, 2007), mud crab (*S. olivacea*) (Ikhwanuddin *et al.*, 2015). Besides, a combination of 5% DMSO and 10% glycerol gave the best result to preserved the sperm of *P. homarus* (Sasikala & Meena, 2009).

For the cryopreservation protocol for shrimps, the cryoprotectant of 15% magnesium chloride was used for *P. merguensis* (Memon *et al.*, 2012). Glycine has a low molecular weight of 75.07 g/mol, non-toxic and less expensive. It penetrates sperm cells and minimizes membrane rupture during slow-freezing protocol. Therefore, 5% glycine was used as the cryoprotectant in this study as it gave high sperm viability. The usage of glycine as cryoprotectant of sperm suspension was also successful in mud spiny lobster, *P. polyphagus* (Fatihah *et al.*, 2016). In the present study, there are only measured the sperm viability using the different extenders and cryoprotectants. Therefore, the present study was not continued the artificial fertilization because if doing the artificial fertilization, it must need a female that ovary with stage 4 and there are many problems during the artificial fertilization.

Previous study by Noorbaiduri & Ikhwanuddin (2015) showed the artificial crablets production of orange mud crab, *S. olivacea* (Herbst, 1796) though *in-vitro* fertilization technique. This study was conducted to explore the artificial crablets production through *in-vitro* fertilization technique on orange mud crab, *S. olivacea* through the determination of (1) Fertilization rate and (2) Embryonic developments from the manipulation of sperm mass with stage 4 ovary (Noorbaiduri & Ikhwanuddin, 2015). Besides, Hassan *et al.* (2019) also measured the sperm viability of Malaysian Horseshoe Crab, *Tachyplues gigas* from Pantai Balok, Kuantan, Pahang for wild and captive *T. gigas*. Furthermore, previous study by Fatihah *et al.* (2016) showed the develop a cryopreservation protocol for sperm of *P. polyphagus* and the successful cryopreservation of *P. polyphagus* sperm using Ca-F saline as an extender and 10 % glycine as a cryoprotectant.

CONCLUSION

Based on this study, the best extender and cryoprotectant for the sperm cryopreservation of *S. tranquebarica* was phosphate buffer and 5%

glycine as it produced the highest percentage of live sperm after 24 h exposure at -80°C ($93.97 \pm 0.43\%$). As recommended, further studies should be done by lengthening the duration of the freezing sperm in liquid nitrogen (-196°C).

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