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Biochemical Changes of Total Protein, Glucose, Lactate Dehydrogenase and Total Lipid in the Cryopreserved Sperm of Mud Spiny Lobster, *Panulirus polyphagus*

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ABSTRACT

The study was aimed to determine the biochemical changes of total protein, glucose, lactate dehydrogenase (LDH) and total lipid in the cryopreserved sperm of mud spiny lobster, *P. polyphagus*. The mean sperm viability, total protein, glucose, LDH and lipid for control and cryopreserved sperm that stored in liquid nitrogen from control, 6, 12, 24 h, 7, 30 and 60 days were 94.91±2.17% (control), 80.58±1.33, 77.08±2.53, 76.49±3.10, 63.32±7.08, 62.93±3.72 and 60.91±10.78% (sperm viability), 21.44±3.52, 18.82±0.23, 17.79±0.80, 16.98±0.48, 16.65±0.07, 15.03±2.53 and 12.16±2.02 mg mL⁻¹ (total protein), 0.28±0.012, 0.26±0.004, 0.20±0.035, 0.15±0.062, 0.11±0.036, 0.07±0.036 and 0.04±0.013 mg mL⁻¹ (glucose), 72.65±7.88, 64.38±6.40, 64.24±4.80, 62.84±7.20, 62.11±3.84, 49.94±2.78, 38.31±3.29 IU L⁻¹ (LDH) and 8.77±0.05, 4.87±0.25, 4.69±0.29, 4.37±0.02, 4.16±0.17, 2.87±0.29 and 2.83±0.26 mg mL⁻¹ (total lipid). There were significant differences in sperm viability, total protein, glucose, LDH and total lipid between cryopreserved periods (p<0.05). As a conclusion, the sperm viability and biochemical changes of total protein, glucose, LDH and total lipid were determined in the cryopreserved sperm for control, 6, 12, 24 h, 7, 30 and 60 days. The storage duration in *P. polyphagus* cryopreserved sperm was also determined until 60 days and suitable for further breeding program.

Key words: Total protein, glucose, lactate dehydrogenase (LDH), total lipid, cryopreserved sperm

INTRODUCTION

Spiny lobster is usually distributed in the Indo-West Pacific region and is considered as one of the world's most important valuable seafood, fetching high market demand in Asia, Europe and America (Williams, 2007). Amongst the key constraints in understanding of fisheries management and development of mud spiny lobster, *P. polyphagus* is the scarcity of information for fishery management purposes (Ikhwanuddin *et al.*, 2014). The problems in *P. polyphagus* breeding technology are low survival rate in the larvae rearing of pueruli stage, hard to maintain from pueruli until juvenile stages and difficult to get the mature broodstock especially male *P. polyphagus* (Fatihah *et al.*, 2014a, b).

There were a few studies about the cryopreservation of lobster sperm cells with the first study by Talbot and Helluy (1995) on *Homarus americanus* and the recent study on *P. homarus*

by Sasikala and Meena (2009). The biochemical assessment of sperm from different breeding animals facilitates the understanding of many diverse transformations that occur throughout the sperm cryopreservation until artificial insemination (Bailey, 2012). Sperm motility and viability are the physiological parameters of sperm. Sperm motility is a key condition for purpose of the quality and fertilizing ability of sperm (Billard, 1978; Stoss, 1983). For assessment of sperm quality, the evaluation of biochemical parameters in sperm was used (Nynca *et al.*, 2012). To assess crustacean sperm viability and fertilizing capability, numerous tests have been used such as trypan blue stain exclusion (Wang *et al.*, 1995; Bhavanishankar and Subramoniam, 1997), eosin-nigrosin stain response (Bhavanishankar and Subramoniam, 1997), hypo-osmotic sensitivity (Bhavanishankar and Subramoniam, 1997), sperm-egg attachment (Behlmer and Brown, 1984), biochemical analysis (Bhavanishankar and Subramoniam, 1997), induction of sperm acrosome reaction (Anchordoguy *et al.*, 1988) and fertilization assay (Behlmer and Brown, 1984).

Study by Jeyalectumie and Subramoniam (1989) found that there were fluctuations in the biochemical composition of spermatophores and seminal plasma during cryopreservation at certain subzero temperature conditions. Besides, fluctuations in LDH activity of *S. serrata* seminal plasma through cryopreservation were also observed (Jeyalectumie and Subramoniam, 1989). In the crab, *Paratelphusa hydrodromous*, LDH enzyme showed the significant fluctuation through spermatophore storage (Jeyalectumie and Subramoniam, 1986). In addition, LDH activity also can be used as an indicator of injured fish sperm which guide to the leakage of LDH from sperm into seminal plasma (Ciereszko and Dabrowski, 1994). Amaral *et al.* (2011) showed that an exogenous glucose improves long-standing the sperm motility, viability and mitochondrial function in human. The sperm viability and biochemical components such as total protein, total free sugar and total lipid in the control and cryopreserved samples were estimated once in 5 days up to twenty five days (Sasikala and Meena, 2009).

Before this, the assessment of the cryopreserved sperm for biochemical changes was determined by Sasikala and Meena (2009) and Jeyalectumie and Subramoniam (1989). However, there is no assessment techniques established to assess the cryopreservation protocol in mud spiny lobster, *P. polyphagus*. To determine the viability of cryopreserved sperm after long term storage, biochemical assessment of cryopreserved sperm is required. Therefore, the objective of the present study was to determine the viability of *P. polyphagus* cryopreserved sperm through biochemical changes (total protein, glucose, LDH and total lipid) during long storage durations (60 days).

MATERIALS AND METHODS

Panulirus polyphagus with body weight, 638.9 g and carapace length of 11.8 cm was used in the present study. Testis was collected and transferred directly into 5 mL of Ca-F saline where they were homogenized manually using mortar and pestle. After the testis became a suspensions sperm, the sperm suspensions and cryoprotectant (10% glycine) were taken with ratio 1:3 into cryovials (2 mL). The cryovials were capped and cooled at 15 min exposure in each step for 1st stage (25, 20, 16, 4, 2, -4°C), 2nd stage (-20, -80°C) and 3rd stage (-100 to -150°C) and immediately stored in liquid nitrogen (-196°C) for 6, 12, 24 h, 7, 30 and 60 days. Frozen sperm were thawed at 26°C for 30 sec. Besides, freshly sperm suspensions were examined for sperm viability as a control treatment. The sperm viability and biochemical changes of fresh and frozen from *P. polyphagus* after 6, 12, 24 h, 7, 30 and 60 days were examined. The biochemical changes of cryopreserved sperm were examined for total protein, glucose, LDH and total lipid. To determine the sperm viability, percentages of live sperm were calculated by following equation:

$$\text{Percentages of live sperm} = \frac{\text{Observed number live sperm}}{\text{Total number of sperm observed}} \times 100$$

Total protein concentration: Protein concentration was measured by method of Lowry *et al.* (1951). Absorbance was measured at 595 nm. Protein concentration was determined by comparison of the samples to the standard curve prepared using the protein standards.

Glucose concentration: Glucose concentration was determined using Glucose (GO) Assay Kit (GAGO-20, Sigma-aldrich). Absorbance was measured at 540 nm. For calculation of glucose assay kit, for standards, absorbance at 540 nm (y axis) versus mg of glucose (x axis) was plotted. While for sample, mg glucose from standard curve was determined. If there was dilution of the samples, multiply the mg glucose by the dilution factor in the sample preparation.

Lactate dehydrogenase (LDH) activity: Lactate Dehydrogenase (LDH) was determined using Lactate Dehydrogenase Assay Kit (KA1653, Abnova). The sample was measured with OD_{565 nm} (OD_{S0}) and again after 25 min (OD_{S25}).

Calculation of results:

$$\begin{aligned}\text{LDH activity} &= \frac{\text{OD}_{S25} - \text{OD}_{S0}}{\epsilon_{\text{mtt}} \cdot I} \times \frac{\text{Reaction vol } (\mu\text{L})}{\text{Time sample vol } (\mu\text{L})} \times n \\ &= 43.68 \times \frac{\text{OD}_{S25} - \text{OD}_{S0}}{\text{OD}_{S25} - \text{OD}_{S0}} \times n (\text{IU L}^{-1})\end{aligned}$$

OD_{S25} and OD_{S0} are OD_{565 nm} values of sample at 25 and 0 min. ϵ_{mtt} is the molar absorption coefficient of reduced MTT. I is the light path length which is calculated from the calibrator. OD_{CAL} and OD_{H2O} are OD_{565 nm} values of the Calibrator and water. Reaction Vol and Sample Vol are 200 and 10 μL , respectively. N is the dilution factor. Unit definition: 1 Unit (IU) of LDH will catalyze the conversion of 1 μmole of lactate to pyruvate per min at pH 8.2.

Total lipid analysis: Total lipid was measured based on Sulfo-phospho-vanillin method by Barnes and Blackstock (1973). Absorbance was measured at 540 nm against the blank on a spectrophotometer (UV Spectrophotometer Shimadzu UV-1800). A calibration curve was constructed using cholesterol as a standard (Modified from Fatima *et al.*, 2013).

Statistical analysis: Data were expressed as Means $\pm$ standard deviation. Among the treatments were tested using one-way ANOVA and significant values were cross examined using Turkey's *post-hoc* test at $p < 0.05$.

RESULTS

The mean sperm viability for control and cryopreserved sperm that stored in liquid nitrogen from control, 6, 12, 24 h, 7, 30 and 60 days were 94.91 $\pm$ 2.17% (control), 80.58 $\pm$ 1.33, 77.08 $\pm$ 2.53, 76.49 $\pm$ 3.10, 63.32 $\pm$ 7.08, 62.93 $\pm$ 3.72 and 60.91 $\pm$ 10.78% respectively. There were significant differences in sperm viability between cryopreserved periods ($p < 0.05$) (Fig. 1). The total protein of *P. polyphagus* cryopreserved sperm for control, 6, 12, 24 h, 7, 30 and 60 days were 21.44 $\pm$ 3.52,

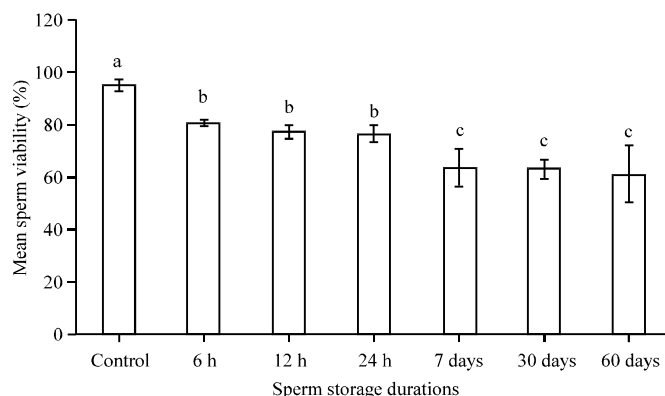


Fig. 1: Mean sperm viability of *P. polyphagus* cryopreserved sperm for control and different sperm storage durations (6, 12 and 24 h, 7, 30 and 60 days)

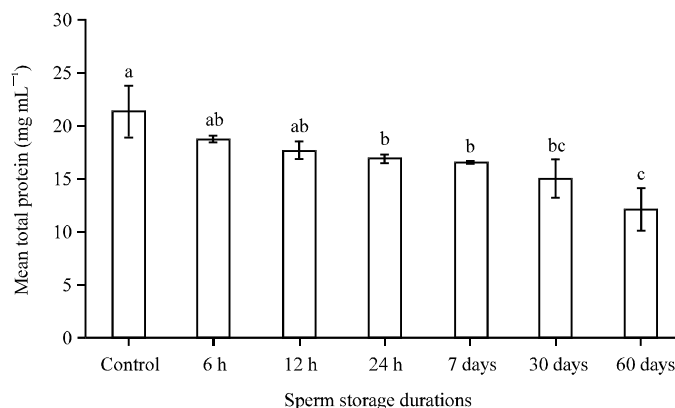


Fig. 2: Mean total protein of *P. polyphagus* cryopreserved sperm for control and different sperm storage durations (6, 12 and 24 h, 7, 30 and 60 days)

18.82±0.23, 17.79±0.80, 16.98±0.48, 16.65±0.07, 15.03±2.53 and 12.16±2.02 mg mL⁻¹ respectively. There were significant differences in total protein between cryopreserved periods ($p < 0.05$) (Fig. 2).

Besides, the glucose of *P. polyphagus* cryopreserved sperm for control, 6, 12, 24 h, 7, 30 and 60 days were 0.28±0.012, 0.26±0.004, 0.20±0.035, 0.15±0.062, 0.11±0.036, 0.07±0.036 and 0.04±0.013 mg mL⁻¹ respectively. There were significant differences in glucose between cryopreserved periods ($p < 0.05$) (Fig. 3). The enzyme activity of lactate dehydrogenase (LDH) for control, 6, 12, 24 h, 7, 30 and 60 days were 72.65±7.88, 64.38±6.40, 64.24±4.80, 62.84±7.20, 62.11±3.84, 49.94±2.78, 38.31±3.29 IU L⁻¹ respectively. There were significant differences in enzyme activity of lactate dehydrogenase between cryopreserved periods ($p < 0.05$) (Fig. 4). The total lipid of *P. polyphagus* cryopreserved sperm for control, 6, 12, 24 h, 7, 30 and 60 days were 8.77±0.05, 4.87±0.25, 4.69±0.29, 4.37±0.02, 4.16±0.17, 2.87±0.29 and 2.83±0.26 mg mL⁻¹ respectively. There were significant differences in total lipid between cryopreserved periods ($p < 0.05$) (Fig. 5).

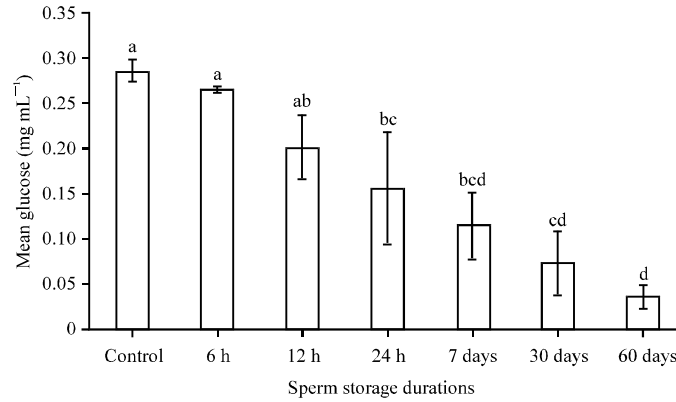


Fig. 3: Mean glucose of *P. polyphagus* cryopreserved sperm for control and different sperm storage durations (6, 12 and 24 h, 7, 30 and 60 days)

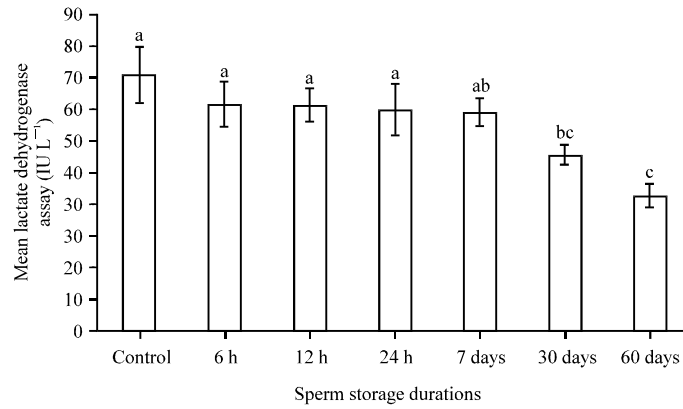


Fig. 4: Mean lactate dehydrogenase (LDH) of *P. polyphagus* cryopreserved sperm for control and different sperm storage duration (6, 12 and 24 h, 7, 30 and 60 days)

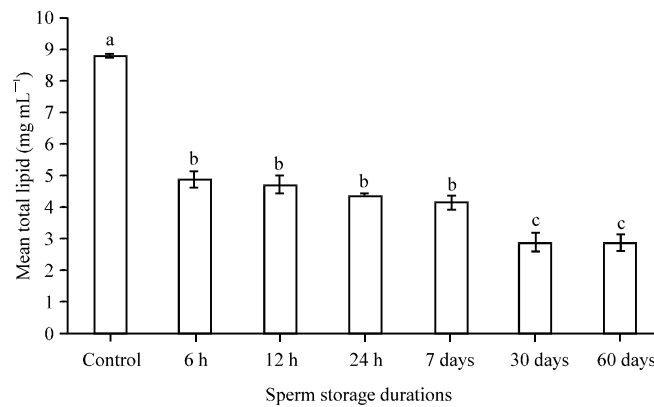


Fig. 5: Mean total lipid of *P. polyphagus* cryopreserved sperm for control and different sperm storage durations (6, 12 and 24 h, 7, 30 and 60 days)

DISCUSSION

The present study showed the effect of cooling or freezing at low temperature (-196°C) on sperm viability, total protein, glucose, LDH and total lipid of *P. polyphagus* cryopreserved sperm. Sperm motility is one of the parameters generally considerably affected by the freezing process (Li *et al.*, 2010). In both seminal plasma of fresh and frozen-thawed sperm, AcP, LDH, AS, β -N-AGase and protein concentration were detected in Persian sturgeon, *Acipenser persicus* sperm (Aramli *et al.*, 2014). In the present study, biochemical changes of total protein, glucose, LDH and total lipid were detected in *P. polyphagus* body before and after cryopreservation. The result in freezing process considered injury to cellular structures, such as plasma membrane, nucleus, mitochondria and flagellum (Partyka *et al.*, 2010; Blesbois *et al.*, 2005; Watson, 1995; Donoghue and Wishart, 2000). In the cryopreservation process of *P. polyphagus*, the sperm viability and biochemical changes decreased because when it was stored for a long period of times, the sperm structures were injured and it caused more sperm to die than alive.

The sperm viability of *P. polyphagus* cryopreserved sperm that frozen in slow temperature with initially at room temperature to liquid nitrogen decreased in control, 6 h until 60 days (94.91% (control), 80.58% (6 h) and 60.91% (60 days)). Study by Hughes (1973) reported a distinguished decrease in sperm viability throughout the cryopreservation of oyster sperm. The sperm viability of Banana shrimp, *Penaeus merguensis* spermatophores that cryopreserved in 15% magnesium chloride also decreased in control, 6 h until 180 days (93.79% (control), 89.60% (6 h) and 16.44% (180 days)) (Memon *et al.*, 2012). Besides, the sperm viability of freeze-thawed sperm of Edible rock lobster, *Panulirus homarus* were found to be uppermost (90.6 until 61.40%) once stored in 5% DMSO and glycerol combination up to 25 days of cryopreservation (Sasikala and Meena, 2009). After the cryopreservation, the sperm velocity, motility, fertilization and hatching abilities of common carp were observably decreased (Li *et al.*, 2010).

After the cryopreservation, organic constituents decreased in *P. homarus* sperm (Sasikala and Meena, 2009). In the present study, total protein, total lipid, glucose and LDH of *P. polyphagus* cryopreserved sperm decreased after freezing in liquid nitrogen for 6, 12, 24 h, 7, 30 and 60 days. In addition, study by Sasikala and Meena (2009) showed that total protein, total free sugar and total lipid decreased after freezing in liquid nitrogen for 5, 10, 15, 20 and 25 days. The loss of protein portion from the sperm cells after cryopreservation of *Salmo salar* L. was due to leakage through the cell membrane into the outer medium (Yoo *et al.*, 1987).

LDH is an enzyme that helps to generate energy. In the absence of oxygen, formation of lactate allows glycolysis to proceed and for another cycle of the reaction catalyzed by GAPDH (Miki, 2007). During the cryopreservation, there was disruption of energy supply (LDH) which subsequently affected the sperm motility (Aramli *et al.*, 2014). In addition, LDH indicators for damage to the plasmalemma and the midpiece area of the sperm structures in the cryopreserved sperm (Aramli *et al.*, 2014). In Rainbow trout, *Oncorhynchus mykiss* cryopreserved sperm, LDH leakage from damaged sperm correlated negatively with the ability of cryopreserved sperm to fertilize eggs (Babiak *et al.*, 2001). In the present study, LDH also decreased and negatively for the ability of sperm viability of *P. polyphagus* cryopreserved sperm.

Throughout the cold storage, the protein component in the ovine seminal plasma undergoes qualitative changes (Garner and Ehlers, 1971). In the bovine sperm, there was a leakage of lipids into the seminal plasma from the cryopreserved sperm (Pickett and Komarek, 1964). Study by Li *et al.* (2010) showed the decrease of protein in common carp sperm subsequent

cryopreservation was generally the effect of protein leakage from sperm into the extracellular medium. Total protein and total lipid in *P. polyphagus* cryopreserved sperm decreased because the leakage of cellular membranes in sperm during the cryopreservation protocol. The leakage of cellular membranes in *P. polyphagus* cryopreserved sperm caused the permeability was more or less constant. In the present study, when the sperm viability decreased, the biochemical changes of total protein, glucose, LDH and glucose also decreased. When the sperm viability was higher, thus the percentage of fertilization process was also higher. The sperm viability and biochemical changes influenced each other for further fertilization process.

Study by Memon *et al.* (2012) showed the percentage of sperm viability and fertilization rate of *P. merguensis* at day 60 were 61.00 and 67.32%. In the present study, the percentage of *P. polyphagus* sperm viability was 60.91% at day 60. Thus, the fertilization rate of *P. polyphagus* cryopreserved sperm also can be higher. The biochemical changes of total protein, LDH and total lipid did not much change but, there were more changes of biochemical in glucose.

Where, in the present study, glucose was still present ($0.04 \pm 0.013 \text{ mg mL}^{-1}$) in *P. polyphagus* cryopreserved sperm after stored in liquid nitrogen at day 60. In the presence of glucose, the good sperm quality samples in human was determined and still had viable sperm although immotile after 16 days (Amaral *et al.*, 2011). Glucose was benefit to human sperm for optimal capacitation and fertilization (Williams and Ford, 2001). Urner and Sakkas (1996) showed that the presence of glucose was essential for sperm penetration into zona-free oocytes in mice. For fertilization in mice (Hoppe, 1976; Fraser and Quinn, 1981) and rat (Tsunoda and Chang, 1975; Niwa and Iritani, 1978), glucose appears to be advantageous. Study by Pickett and Komarek (1964) showed that total lipid of bovine sperm was 3.60 mg mL^{-1} before freezing and 3.55 mg mL^{-1} after freezing. In the present study, total lipid of *P. polyphagus* before freezing (control treatment) was 8.77 mg mL^{-1} while after freezing for 6 h was 4.87 mg mL^{-1} .

In the cryopreservation of *P. homarus*, total protein of fresh sperm was 14.00 mg mL^{-1} while after 25 days freezing in combination of 5% DMSO and glycerol was approximately 8.00 mg mL^{-1} . In the present study, total protein of *P. polyphagus* before freezing (control treatment) was 21.44 mg mL^{-1} while after freezing for 30 days was 15.03 mg mL^{-1} . From the present study, total protein, total lipid and LDH were not much change until 60 days and the sperm viability was more than 50% (60.91%). Glucose was more changes until 60 days but it was still present in the cryopreserved sperm of *P. polyphagus*. Thus, at day 60, *P. polyphagus* cryopreserved sperm was still the suitable storage duration for fertilization based on the sperm viability and biochemical changes in the present study.

CONCLUSION

In the present study, the sperm viability of *P. polyphagus* cryopreserved sperm was determined and the biochemical changes of total protein, total lipid, glucose and LDH during long storage durations (60 days) were also determined. The biochemical changes of total protein, glucose, LDH and total lipid in the cryopreserved sperm decreased after 60 days. Fresh sperm and cryopreserved sperm showed the different constituent of biochemical changes. Biochemical changes of cryopreserved sperm were lower than fresh sperm because the cryopreserved sperm was stored in liquid nitrogen for a long period. Thus, it is important to know the biochemical changes of cryopreserved sperm for further researches such as artificial insemination, in vitro insemination, gynogenesis and others. Furthermore, the present study showed that the suitable storage duration

was until 60 days with the mean sperm viability at $60.91 \pm 10.78\%$. The present study confirmed that the biochemical composition changed after being stored for 6, 12 and 24 h, 7, 30 and 60 days and there were not much difference in the biochemical composition of total protein, total lipid, LDH than glucose in the cryopreserved sperm.

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