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Optimization of Spermatophores Cryopreservation Protocol of Banana Shrimp (*Penaeus merguiensis*) (De Man, 1888)

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Abstract: The objectives of this study were to determine the effects of different cryoprotectants on sperm viability and optimization of spermatophore cryopreservation protocol for durable storage of Banana shrimp (*Penaeus merguiensis*). Spermatophore suspended for 15 min in Calcium-Free saline (Ca-F saline), used CPA $MgCl_2$ and with concentration (15%), thawing temperature was 27°C. Use 15 min equilibration in room temperature (25°C) overall. Exposure and cooling rate selected as 25, 20, 16, 4, 2, -4, -20, -80, -150°C/10 min. Examination of sperm viability used a modified eosin-nigrosin staining technique. The smallest reductions in apparent sperm viability occurred with $MgCl_2$, however freezing protocol was developed using Ca-F saline containing 15% $MgCl_2$. Spermatophores were cryopreserved using above exposure/cooling rate and -196°C in liquid nitrogen up to 180 days. Mean sperm viability for fresh (93.8±1.3%) and cryopreserved spermatophore held for 24 h and 60 days was 83.5±0.6 and 61±1.2 did not differ ($p>0.05$), however that for spermatophore stored in liquid nitrogen between 90 and 180 days were lower ($p<0.05$) and varied from 55.4±0.3-16.4±1.2. Spermatophores earlier held in liquid nitrogen for 60 and 90 days. However, storage beyond 90 days caused a significant decline ($p<0.05$) in sperm viability. Spermatophores kept for 120 and 150 days had viabilities of 48.9±0.9 and 32.4±0.9%, respectively. Cryopreserved spermatophore stored in liquid nitrogen from 150-180 days had low viabilities (<35%). Mean fertilization rate of *P. merguiensis* females artificially inseminated with cryopreserved spermatophore that had been stored in liquid nitrogen for 7-30 days and for 60-90 days were 73.9±1.5-66.7±3.1 and 67.3±3-64.1±2.1%, respectively whereas that of fresh spermatophore was 88.2±1.5%. Hatching rates of eggs fertilized with cryopreserved spermatophore kept for 7-30 days and for 60-90 days were 77.6±2.5-72.7±3.5 and 81.5±12.1-62.5±1.5 which were not different ($p>0.05$) from those of the control group 76.2±13.5%, respectively. In conclusion, Cryopreserved spermatophore held in liquid nitrogen l<90 days revealed high sperm viability although, for longer periods, sperm viability declined at 180 days.

Key words: Banana shrimp, *Penaeus merguiensis*, spermatophore, cryopreservation, fertilization, hatching

INTRODUCTION

Cryopreservation procedures have been developed for many aquacultured species (Billard and Zhang, 2001) including different salmonids (Lahnsteiner *et al*., 1995; Cabrita *et al*., 2001), carp (Linhart *et al*., 2000), sturgeons (Grunina *et al*., 2006), turbot (Dreanno *et al*., 1997), sea bass (Fauvel *et al*., 1998) and sea bream (Cabrita *et al*., 2005). It has various important applications in biotechnology and reproductive biology such as the development of selective breeding, domestication and conservation of stocks. Indeed, the extensive investigation in this field has allowed cryopreservation of gametes and embryos in vertebrates (Polge *et al*., 1949; Sommerfeld and Niemann, 1999; Rahman *et al*., 2008; Wang *et al*., 2011; Abd-Allah, 2011; Nathanailides *et al*., 2011). In contrast, research in invertebrates has been attempted for a few species and the cryopreservation techniques are still being developed (Bhavanishankar and Subramoniam, 1997; Caffey and Tiersch, 2004). Successful preservation of crustacean spermatophores was first reported in freshwater shrimp, *Macrobrachium rosenbergii* in which sperm viability and successful fertilization were maintained up to 4 days after storage in Ringer's solution at 2°C (Chow, 1982; Chow *et al*., 1985).

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However, chilled storage of crustacean spermatophores was developed by Ishida *et al.* (1986) who stored lobster spermatophores in paraffin oil at 4-7°C for up to 289 days. Furthermore, earlier studies have shown the feasibility of sperm cryopreservation in species like the horseshoe crab (*Limulus polyphemus*) (Behlmer and Brown, 1984), Ridgeback rock shrimp (*Sicyonia ingentis*) (Anchordoguy *et al*., 1988), Edible mud crab (*Scylla serrata*) (Bhavanishankar and Subramoniam, 1997, Jeyalectumie and Subramoniam, 1989), *L. vannamei* (Dumont *et al*., 1992), *Penaeus chinensis* (Ke and Cai, 1996), marine shrimp *Sicyonia ingentis* (Thomas *et al*., 1998), *P. monodon* (Bart *et al*., 2006; Vuthiphandchai *et al*., 2007), *abalone Haliotis diversicolor* and oyster *Crassostrea virginica* (Paniague-Chavez and Tiersch, 2001).

Cryopreservation of shrimp spermatophore is a valuable method for restoration of endangered species as well as a technique for reproductive manipulation for genetic improvement in shrimp. It is also a great resource for the gene bank. Cryopreservation provides a continuous source of tissues and genetically stable living cells for a variety of purposes including research and biomedical processes (Christensen and Tiersch, 2005).

The cryopreservation of spermatophore and those from valuable selected lines would provide and maintain the flexibilities in near future which is needed for domestication programs of this species. In *P. merguiensis* mostly seed production is still largely dependent on wild broodstock which can be irregular in terms of their quality (Othman, 2006).

Presently hatchery operators are facing a serious problem to have year round availability of quality male brood stock of *P. merguiensis*. Previously no research on record in the cryopreservation of *P. merguiensis* however, Bart *et al.* (2006) reported the successful cryopreservation of *P. monodon* spermatophore which kept in liquid nitrogen for 2 days as well as Vuthiphandchai *et al.* (2007) kept spermatophore in liquid nitrogen for 210 days which was a big break through. Pursue of previous studies on crustaceans the development of a long-term storage of cryopreservation protocol of *P. merguiensis* spermatophore is needed to generate a reliable and steady supply of good quality spermatophore for breeding technology. The quality of spermatophore in mature males was often related directly to hatchery holding time (Aiken and Waddy, 1980).

Extended literature review showed that the inspection of different cryoprotectants and sperm viability for spermatophore cryopreservation process in *P. merguiensis* is virtually none (Memon *et al*., 2011). This investigation would cover the changes in percentage viable sperm of *P. merguiensis* samples in different cryoprotectants. This study inspects six CPAs, Dimethyl Sulfoxide (DMSO), Ethylene Glycol (EG), methanol, glycerol, sucrose, magnesium chloride ($MgCl_2$) to choose best one out of them at different percentage of concentrations, cooling rates and as well different equilibration periods under different temperatures. An attempt was thus made to study the cryopreservation of male gametes for long-term storage. Therefore, the achievement of this study would be able to provide a solid base line for cryopreservation process of spermatophore. On the basis of results, it would be able to provide a continuous supply of male gametes and allow implementation of certain economic and management benefits including international transport of good quality spermatophore.

Previously various Cryoprotectants Agents (CPAs) such as Dimethyl Sulfoxide (DMSO), glycerol, propylene glycol, methanol and formamide have been widely used for spermstophore cryopreservation of fin and shell fishes (Christensen and Tiersch, 2005; Bart *et al*., 2006; Vuthiphandchai *et al*., 2007). There are varieties of CPAs are currently available to protect sperm from harmful effects of the cryopreservation process and cell death occurs in rapid freezing due to the formation of intracellular ice (Mazur *et al*., 1972). Exposure to CPAs prior to freezing is an important factor in the cryopreservation of sperm for many species. This effect varies due to the CPAs, time of exposure and concentration (Morris, 1981).

It is well understood that equilibration time is the time of slow cooling which allows the sperm and extender to mix and for slowly decreasing temperature to prepare for the freezing processes. Equilibration time can also avoid a cold shock for sperm during freezing. In one study, Maxwell *et al.* (1997) reported that each phase of cryopreservation and more specifically the rate and length of cooling were found to be responsible for membrane damage. Equilibration times of 10-20 min are most commonly used by Billard and Zhang (2001). As well as cooling rate is the rate of gradually decreasing a temperature during the cryopreservation process which is one of the critical parameters that can affect sperm motility or storage ability of sperm after freezing. Various studies have been reported on changes in the fluidity of the sperm membrane during cooling and subsequent freezing of the gamete (Pettit and Buhr, 1998). Cooling rate also varied among species. For fish sperm, optimal rates have been varied from 5-45°C min^{-1} to 5-80°C but some species showed high post thawed motility with a combination of different cooling rates (Rana and Gilmour, 1996; Sansone *et al*., 2002). Polge (1957) reported that a critical temperature zone (between -15 and -30°C) is responsible

for exerting most of the damage to spermatozoa and if the cooling rates were not optimal, all the cells damaged at 80°C temperature. Besides that cooling rate also can affect the rate of osmosis, diffusion and formation of ice crystals within a cell (Morris, 1981).

The objectives of this study were to determine the effects of different cryoprotectants on sperm viability and optimization of spermatophore cryopreservation protocol for durable storage of *P. merguiensis* spermatophores.

MATERIALS AND METHODS

Source of animals: Sexually matured *P. merguiensis* male specimens were collected from Kota Kuala Muda, Palau Sayak, Kedah, Malaysia (5°39"N; 100°19"E). A total of 972 males with mean Body Weight (BW) of 24.2±3.84 g and mean Total Length (TL) of 14.4±0.5 cm and 66 female with mean BW of 28.1±6.1 g and mean TL of 15.4±0.6 cm were used throughout the study. They immediately transported to the marine hatchery, at the Institute of Tropical Aquaculture, University Malaysia Terengganu (UMT) in an aerated condition. Precautions were taken to reduce the external stress to the brood stocks by providing ambient environmental conditions during transportation.

Spermatophore collection: Specimens were weighed and selected with sign of a clear white swelling around the coxae at the base of the fifth walking leg (pereiopods). Left and right spermatophores did not differ in weight (0.048-0.092 g). Only non-melanized spermatophores were selected for preservation studies as reported by Dougherty and Dougherty (1990). Slight pressure was applied with the thumb between the abdomen and the base of the fifth walking leg to eject out the spermatophores. The protruded spermatophores were pulled out with a pair of sterile forceps and each spermatophore was weighed, before transferred into glass homogenizer (High speed variable speed reversible, Glas-col, Terre Havte In USA) with 200 μL of Ca-F saline.

Optimal freezing medium: In this study, six different Cryoprotectants (CPAs), namely; Dimethyl Sulfoxide (DMSO), Ethylene Glycol (EG), methanol, glycerol, sucrose and magnesium chloride ($MgCl_2$) (Sigma Chemicals, St. Louis, MO, USA) were used to choose the best one, based on their extensive use for cryopreservation of fish and shrimp sperm. Examination of cryoprotectant was conducted in two groups; A and B in which each cryoprotectant was prepared at four concentrations as 5, 10, 15 and 20% (v/v) as well in Group A equilibration periods were; 5, 15, 30 and 60 (25°C) min using sterile Calcium Free saline (Ca-F saline) as an extender medium. The Group B used three low temperatures (-4, -20 and -80) and three exposures (6, 12 and 24 h) for spermatophore preservation process. Also in control group, the freshly collected spermatophores were immersed in Ca-F saline without cryoprotectants.

Cooling rates: Different cooling rates were applied in earlier studies as described by Anchordoguy *et al.* (1988), Jeyalectumie and Subramoniam (1989), Bart *et al.* (2006) and Vuthiphandchai *et al.* (2007). In this study, modified protocol was used to cryopreserve the shrimp spermatophore. For spermatophore cryopreservation, $MgCl_2$ (15%) concentration was selected as the ideal cryoprotectants on the basis of its high live sperm viability. In this study, 12 protocols were applied with 3 exposure times and at 9 cooling temperatures (Table 1). Six replicates were conducted for each cooling rate within each treatment. Spermatophores collected from males were transferred directly to 10 mL of Ca-F saline (25°C) where they were held for 5 min. Each spermatophore was then transferred to 0.5 mL of cryoprotectants solution ($MgCl_2$ 15% in Ca-F saline) which was inserted in a 1.8 mL cryovial at room temperature (25°C) for 15 min equilibration. Vials were capped and spermatophores were cooled at 10 min exposure in each step Stage 1st (25, 20, 16, 4, 2°C) in Stage 2nd (-20, -80°C) in 3rd stage (-100 to -150°C) and then stored in liquid nitrogen (-196° C) for 24 h. These cooling rates were achieved by using air condition room (Panasonic CS PC9 JKH Malaysia),

Table 1: Different cooling rate

Protocols	Time (min)	Temprature (°C)									
A	5	25	20	16	4	2	-4	-150	-196	-	-
B	5	25	20	16	4	2	-4	-20	-150	-196	-
C	5	25	20	16	4	2	-4	-20	-80	-150	-196
D	5	25	20	16	4	2	-4	-80	-150	-196	-
E	10	25	20	16	4	2	-4	-150	-196	-	-
F	10	25	20	16	4	2	-4	-20	-150	-196	-
G	10	25	20	16	4	2	-4	-20	-80	-150	-196
H	10	25	20	16	4	2	-4	-80	-150	-196	-
I	15	25	20	16	4	2	-4	-150	-196	-	-
J	15	25	20	16	4	2	-4	-20	-150	-196	-
K	15	25	20	16	4	2	-4	-20	-80	-150	-196
L	15	25	20	16	4	2	-4	-80	-150	-196	-

Normal fridge (Haier Refrigerator HR-250 T.SL Malaysia), deep freezers (-20 EKOFRIGOLAB 1500 Angelantoni and -80 Thermo Scientific Forma 700 Series) and liquid nitrogen vapors -150 and LN -196° C (Thermo Scientific Model 8033).

Freezing protocol for long-term storage of spermatophore: Spermatophores were subsequently transferred to 0.5 mL of cryoprotectants solution (15% of $MgCl_2$ in Ca-F saline) inside 1.8 mL cryovial. After 15 min equilibration in room temperature (25°C) and exposure with cooling points selected as 10 min exposure in each step Stage 1st 25, 20, 16, 4, 2°C in Stage 2nd -20, -80°C in 3rd stage -100 to -150°C (liquid nitrogen vapor) and immediately stored in liquid nitrogen -196°C for 6, 12, 24 h, 7, 30, 60, 90, 120, 150 and 180 days. Frozen spermatophore were thawed at 27°C for 2 min and evaluated by using vital stains, up to 180 days storage in liquid nitrogen. Cryopreserved spermatophores were stored in liquid nitrogen to evaluate over time sperm viability during long-term storage. At the beginning of the experiment, freshly collected spermatophores were examined by sperm viability and served as controls. Other 2nd group of freshly collected spermatophore was cryopreserved at the beginning of experiment in same day and the percentage of viable sperm was evaluated after storage in liquid nitrogen for 6 and 12 h. Moreover, the 3rd group cryopreserved spermatophore was evaluated at 24th h and the fourth batch evaluated after 7th day after this randomly sampled once every month during a long-term storage of 180 days for assessment of sperm viability. Cryopreserved spermatophores were thawed in a water bath at 27°C for 2 min.

Effect of thawing temperature: Spermatophores were cooled with $MgCl_2$ at a freezing rate, by three stages. In 1st stage 25, 20, 16, 4, 2°C in Stage 2nd -20, -80°C and in 3rd stage -100 to -150°C (liquid nitrogen vapor) and immediately stored in liquid nitrogen -196°C. The cryopreserved spermatophores were kept in liquid nitrogen for 24 h prior to evaluating the effect of thawing temperatures on sperm viability. Frozen spermatophores were thawed at five different thawing temperatures such as 25, 27, 29, 31 and 33°C for 2 min and viability of sperm were recorded in six replicates. Thawing of spermatophore was done by using Water bath MEMMERT Germany and CORNING (temperature controller digital PC-420D USA).

Sperm viability assessment of frozen sperm: In this study, fully mature male's broodstock were used as control group. Spermatophore earlier stored in -196°C liquid nitrogen tank at Institute of Marine Biotechnology (IMB) laboratory UMT as well as scanning performing in the Institute of Oceanography (INOS) UMT. For control group fresh spermatophore were collected. As well as cryopreserved spermatophore (6, 12, 24 h and 7, 30, 60, 90, 120, 150 and 180 days) were used, after the thawing at 27°C/2 min. Fresh and frozen spermatophore individually transferred into glass homogenizer (High speed variable speed reversible, Glas-col, Terre Havte In USA) or manually by using Mortar and pestle with 200 μL of Ca-F saline.

Statistical analysis: Data ware analyzed as factorial CRD (2 factors or more). Analyses of Variance (ANOVA) were analysis using MSTAT C program. The factors involved were CRD; different concentration, CPAs and/or temperature. Means for individually factor were test by LSD ($p>0.05$) and the interaction were test by Duncan. Parameters means were support by Pearson correlation (2 tailed). Percentages of viable sperm were calculated by following equation:

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

RESULTS

Optimal freezing medium: In this study, result showed that in Group A, the means of different concentration on sperm viability which are shown in Fig. 1, the highest percentage of viability was recorded at 5 and 10% as 73.12±13.92 and 71.44±13.29%, respectively. Whereas, lowest viability was recorded at 20% concentration as 56.59±15.28%. The control was 86.5±8.54% at 0% concentration.

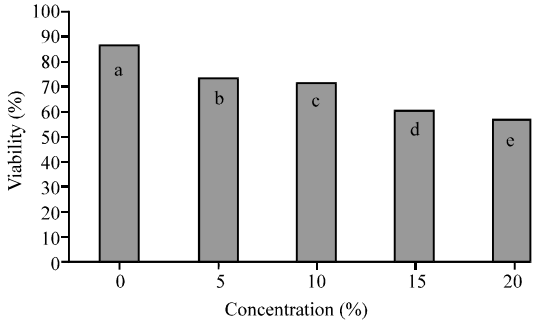

Fig. 1: Means compression effect of different concentration on sperm viability (n = 100 referred 200 spermatophore). Different letters indicate significant difference among concentration ($p>0.05$) (Mean (%): 0 = 86.5, 5 = 73.12, 10 = 71.44, 15 = 60.38 and 20 = 56.59)

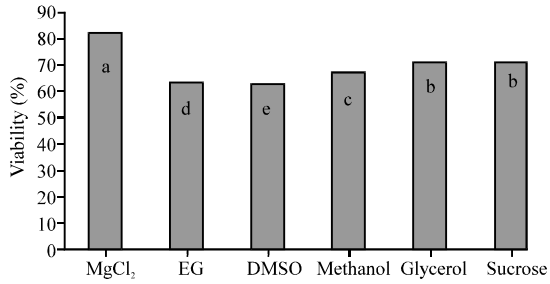

Fig. 2: Means compression effect of different CPAs on sperm viability (n = 100 referred 200 spermatophore). Different letters indicate significant difference among CPAs (p>0.05) (Mean (%): $MgCl_2$ = 81.92, EG = 63.63, DMSO = 62.51, Methanol = 67.32, Glycerol = 71.24 and Sucrose = 71.02)

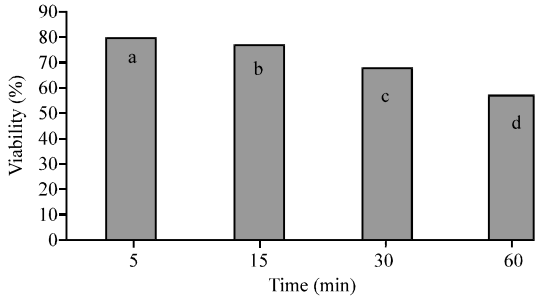

Fig. 3: Means compression effect of different equilibrium times on sperm viability (n = 100 referred 200 spermatophore). Different letters indicate significant difference among concentration (p>0.05) (Viability (%): 5 = 79.2, 15 = 76.25, 30 = 67.05 and 60 = 55.92)

Table 2: Percentage of sperm viability of *P. merguiensis* spermatophores shows interaction between different CPAs and concentration

	Concentration (%)				
CPAs	*0 (control)	5	10	15	20
$MgCl_2$	86.5±8.7^{ab}	87.5±8.70^{a}	84.9±9.90^{b}	78.2±8.70^{c}	72.6±9.40^{e}
EG	86.5±8.7^{ab}	63.3±11.2^{hi}	69.8±12.7^{f}	51.2±7.70^{k}	47.4±7.70^{l}
DMSO	86.5±8.7^{ab}	74.9±11.8^{d}	71.1±11.5^{ef}	41.7±5.00^{m}	38.3±6.50^{n}
Methanol	86.5±8.7^{ab}	70.1±14.6^{ef}	60.6±11.2^{j}	59.8±10.9^{j}	59.7±11.5^{j}
Glycerol	86.5±8.7^{ab}	71.5±12.7^{ef}	71.1±11.5^{ef}	65.3±10.9^{gh}	61.7±12.2^{ij}
Sucrose	86.5±8.7^{ab}	71.4±12.4^{ef}	71.1±11.5^{ef}	66.2±13.0^{g}	59.9±150^{j}

Values are mean±SD (n = 100 referred 200 spermatophore), mean value with different subscripts letters in the same column were significantly different (p<0.05).*Control 0 concentration only Ca-F saline. Spermatophores maintained at room temperature overall 25°C

Table 3: Percentage of sperm viability of *P. merguiensis* spermatophores shows interaction between different various concentration and equilibrium time

	Time			
Concentration (%)	5	15	30	60
0	93.83±0.38^{a}	92.53±0.40^{a}	84.8±0.780^{b}	74.83±0.90^{e}
5	82.42±1.27^{c}	81.28±1.25^{c}	72.5±1.870^{f}	56.28±1.68^{i}
10	81.11±0.98^{c}	79.22±1.49^{d}	70.67±1.51^{f}	54.78±1.49^{i}
15	70.75±2.10^{f}	66.49±2.46^{g}	55.64±1.89^{i}	48.62±1.70^{k}
20	67.89±2.24^{g}	61.75±2.34^{h}	51.64±1.84^{j}	45.08±1.94^{l}

Values are mean±SD (n = 100 referred 200 spermatophore), mean value with different subscripts letters in the same column were significantly different (p<0.05).*Control 0 concentration only Ca-F saline. Spermatophores maintained at room temperature overall 25°C

Table 4: Percentage of sperm viability of *P. merguiensis* spermatophores shows interaction between different various CPAs and equilibrium time

	Time**			
CPAs	5	15	30	60
$MgCl_2$	90.0±4.10^{a}	88.2±7.30^{a}	78.7±9.70^{cd}	70.7±6.90^{fg}
EG	72.2±14.2^{ef}	70.4±16.2^{fg}	61.1±15.9^{i}	51.1±13.4^{lm}
DMSO	70.4±19.2^{fg}	67.1±22.2^{h}	62.1±21.4^{i}	50.6±16.2^{m}
Methanol	80.2±9.70^{c}	73.8±11.9^{e}	61.1±13.1^{i}	54.4±11.7^{jk}
Glycerol	82.3±6.70^{b}	77.9±9.20^{d}	68.7±12.1^{gh}	56.1±10.4^{j}
Sucrose	80.2±8.20^{c}	80.2±8.20^{c}	70.9±9.93^{fg}	52.8±13.1^{kl}

Values are mean±SD (n = 100 referred 200 spermatophore), mean value with different subscripts letters in the same column were significantly different (p<0.05) spermatophores maintained at room temperature overall 25°C

Figure 2 shows the means of different CPAs on sperm viability. The highest viability was recorded in $MgCl_2$ as 81.92±10.61%. It was 31.05% higher than DMSO which was recorded as 62.51±21.17%.

Table 2 shows the best treatment for cryoprotectants recorded in $MgCl_2$. It showed higher percentage of viability at 5 and 10% concentrations as 87.5±8.7 and 84.9±9.9%, respectively. It was not different (p>0.05) from that for control as 86.5±8.7%. However, it was higher than that in other CPAs (p<0.05). Lower sperm viability than control was also observed in EG, DMSO, methanol glycerol and sucrose. Percentage of viability was reduced (p<0.05) to 38.3±6.5% within 20% DMSO; 47.4±7.7% EG; as compared to the control (86.5±8.7%). An increase of concentration in $Mgcl_2$ to 15 and 20% reduced (p<0.05) the viability percentage as 78.2±8.7 and 72.6±9.4% at 15 and 20% concentration, respectively.

Figure 3 shows the mean viabilities in different equilibrium times. The highest viability was recorded as 79.2±13.09% at 5 min and the lowest as 55.92±13.96% at 60 min equilibrium time. Percentage of viability was reduced (p<0.05) to 76.25±15.18 and 67.05±15.5% between 15 and 30 min.

Table 3 shows the mean viability percentages in interactions between concentration and equilibrium time. Highest sperm viability 82.42±1.27, 81.11±0.98 and 81.28±1.25% was observed when equilibrium time and concentration was 5 min at 5% and 15 min at 10% was not different (p>0.05) from that at 0% and was (p<0.05) >30 and 60 min equilibrium and 15 and 20% concentration.

Table 4 shows the mean viability percentages in interactions between CPAs and equilibrium time. Highest sperm viabilities as 90±4.1 and 88.2±7.3% were observed when equilibrium at 5 and 15 min of $MgCl_2$ was (p<0.05) >30 and 60 min equilibrium. However, viability decreased significantly when it reached to 60 min equilibrium in DMSO (50.6±16.2%). Viability of other

Table 5: Percentage of sperm viability of *P. merguiensis* spermatophores shows interaction between different various CPAs and concentration

		Concentration (%)			
CPAs	0 (Control)	5	10	15	20
DMSO	31.74±7.45^{o}	59.52±28.03^{i}	62.20±31.84^{h}	64.79±31.95^{f}	63.27±33.10^{gh}
$MgCl_2$	31.74±7.45^{o}	71.42±10.20^{d}	78.60±11.06^{c}	84.20±7.640^{a}	82.42±8.450^{b}
Glycerol	31.74±7.45^{o}	16.20±24.46^{r}	18.78±27.53^{q}	20.55±27.86^{p}	17.77±23.92^{q}
Sucrose	31.74±7.45^{o}	62.56±24.23^{h}	64.16±26.4^{fg}	67.78±25.91^{e}	62.63±26.86^{h}
EG	31.74±7.45^{o}	48.48±31.87^{l}	51.21±31.63^{k}	53.24±33.86^{j}	48.95±7.450^{l}
Methanol	31.74±7.45^{o}	42.32±35.85^{n}	45.15±38.54^{m}	47.94±38.95^{l}	44.7±38.1200^{m}

Values are mean±SD (n = 750 referred 1500 spermatophore). Mean value with different subscripts letters in the same column were significantly different ($p<0.05$) spermatophores maintained at room temperature overall 25°C

Table 6: Percentage of sperm viability of *P. merguiensis* spermatophores shows interaction between different CPAs and the exposures time

	Time (h)*		
CPAs	6	12	24
DMSO	66.1±23.9^{e}	53.8±30.1^{h}	48.9±34.7^{j}
$MgCl_2$	74.1±18.6^{a}	72.1±21.8^{b}	62.8±22.2^{e}
Glycerol	31.6±30.7^{k}	20.6±21.5^{n}	10.7±10.8^{o}
Sucrose	64.4±22.1^{d}	56.2±27.3^{g}	52.8±29.2^{i}
EG	57.5±23.6^{f}	53.8±30.3^{h}	28.8±29.6^{l}
Methanol	55.3±31.8^{g}	48.8±34.3^{j}	23.0±28.1^{m}

Values are mean±SD (n = 750 referred 1500 spermatophore), mean value with different subscripts letters in the same column were significantly different ($p<0.05$) spermatophores maintained at room temperature overall 25°C

CPAs was significantly lower than that of $MgCl_2$ at all equilibrium periods. In Group B, Fig. 4 shows the mean viabilities of different concentrations. The highest sperm viability 56.42±35.36% was recorded at 15% and concentration was higher ($p<0.05$) than control 31.74±7.43%. However, lowest was recorded as 50.08±32.31%.

Figure 5 shows the mean viabilities of different CPAs. The highest sperm viability 69.67±21.48% was recorded in $MgCl_2$. However, lowest was recorded in glycerol 21.0±24.08%.

Table 5 shows the mean viability percentages in interactions between CPAs and concentrations. Highest sperm viabilities as 84.2±7.64 and 82.42±8.45% were observed when concentration at 15 and 20% of $MgCl_2$ was ($p<0.05$) >5 and 10% concentrations. However, viability of other CPAs was significantly lower than that of $MgCl_2$ at all concentrations.

Table 6 shows the mean viability percentages in interactions between CPAs and exposures. Highest sperm viabilities as 74.1±18.6 and 72.1±21.8% were observed when exposure at 6 and 12 h of $Mgcl_2$ was ($p<0.05$) >24 h exposure. However, lowest was recorded in glycerol 10.7±10.8%. Viability of other CPAs was significantly lower than that of $MgCl_2$ at all exposures.

Figure 6 shows the mean viabilities of different low temperatures. The highest sperm viability as 61.18±30.45% was recorded at -20°C. However, lowest was recorded at -4 and -80°C as 59.04±28.33 and 26.7±25.14%, respectively.

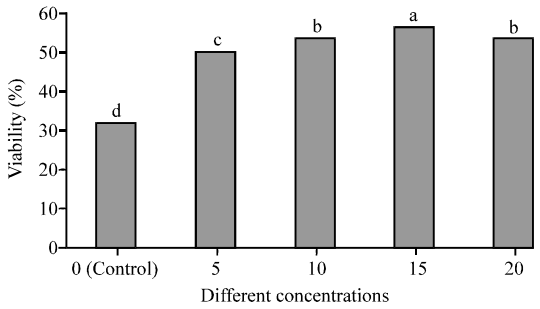

Fig. 4: Means compression effect of different concentration on sperm viability (n = 750 referred 1500 spermatophore). Different letters indicate significant difference among equilibrium times ($p>0.05$) (Mean viability (%): 0 = 31.74, 5 = 50.08, 10 = 53.35, 15 = 56.42 and 20 = 53.29)

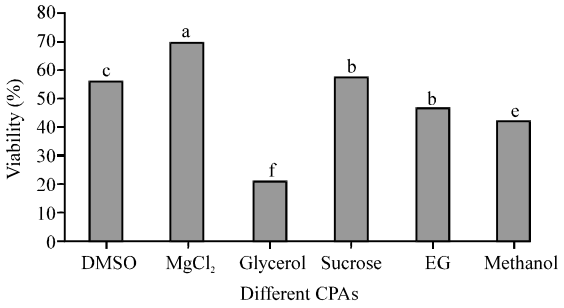

Fig. 5: Mean compression effect of different CPAs on sperm viability (n = 750 referred 1500 spermatophore). Different letters indicate significant difference among CPAs ($p>0.05$) (Mean viability (%): DMSO = 56.3, $MgCl_2$ = 69.67, Glycerol = 21, Sucrose = 57.77, EG = 46.72 and Methanol = 42.37)

Figure 7 shows the mean viability percentages in interactions between low temperatures and different concentrations. Highest mean of sperm viabilities as 71.29±27.55 and 70.79±29.83% were observed when concentration was 15% at -4 and -20°C was ($p<0.05$) >5, 10 and 20% concentration.

Figure 8 shows the best treatment for cryoprotectants recorded in $MgCl_2$. It shows higher percentage of

Table 7: Percentage of mean sperm viability of *P. merguiensis* spermatophores shows interaction between different temperatures, CPAs and concentrations

		Concentration (%)			
CPAs	0 (Control)*	5	10	15	20
-4					
DMSO	33.06 t	76.7±4.70^{ij}	84.9±5.70^{bc}	88.1±6.20^{a}	85.5±7.600^{bc}
$MgCl_2$	33.06 t	65.4±10.6^{l}	73.7±13.7^{k}	85.6±5.80^{bc}	79.1±11.10^{ghij}
Glycerol	33.06 t	44.9±23.1^{rs}	51.4±25.3^{op}	53.5±24.9^{no}	46.01±21.5^{qr}
Sucrose	33.06 t	77.8±6.50^{ij}	81.8±5.60^{ef}	86.3±4.80^{ab}	80.9±12.30^{fg}
EG	33.06 t	47.3±31.8^{q}	50.3±30.8^{p}	54.2±31.6^{n}	43.5±34.40^{r}
Methanol	33.06 t	49.7±35.9^{p}	53.2±38.2^{no}	60.1±37.9^{m}	52.9±38.70^{no}
-20					
DMSO	32.59 t	78.5±11.8^{hij}	80.9±10.9^{fg}	83.5±10.8^{cde}	84.3±10.40^{bcd}
$MgCl_2$	32.59 t	76.7±7.60^{ij}	85.1±7.90^{bc}	86.7±7.80^{ab}	85.8±7.100^{abc}
Glycerol	32.59 t	3.01±3.90	4.5±4.90	7.5±7.10^{z}	6.7±6.100^{z}
Sucrose	32.59 t	77.1±11.7^{ij}	80.8±9.40^{fg}	82.4±9.20^{def}	78.3±13.10^{hij}
EG	32.59 t	79.4±10.1^{ghi}	82.01±9.1^{ef}	85.9±7.20^{abc}	84.6±8.700^{bc}
Methanol	32.59 t	72.4±11.6^{k}	78.4±9.90^{hij}	78.8±10.7^{ghij}	76.7±10.20^{j}
-80					
DMSO	29.55 u	23.4±15.1^{v}	20.8±17.1^{wxy}	22.7±15.2^{vw}	19.9±16.80^{xy}
$MgCl_2$	29.55 u	72.1±8.90^{k}	77.0±7.20^{ij}	80.3±7.70^{fgh}	82.3±4.600^{def}
Glycerol	29.55 u	0.6±1.90	0.42±1.2	0.7±1.80	0.61±1.60
Sucrose	29.55 u	32.8±15.9^{t}	29.8±13.9^{u}	34.6±15.6^{t}	28.7±10.30^{u}
EG	29.55 u	18.8±10.6^{y}	21.4±11.6^{vwx}	19.6±13.8^{xy}	18.7±14.40^{y}
Methanol	29.55 U	4.9±8.90	3.9±6.50	5.1±7.90	4.4±6.700

Values are mean±SD (n = 750 referred 1500 spermatophore), mean value with different subscripts letters in the same column were significantly different ($p<0.05$) spermatophores maintained at room temperature overall 25°C.*Control without CPA only Ca-F saline

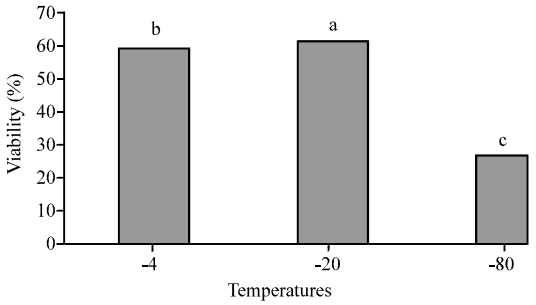

Fig. 6: Mean compression effects of different low temperatures on sperm viability (n = 750 referred 1500 spermatophore). Different letters indicate significant difference among the temperatures ($p>0.05$) (Viability (%): -4 = 59.04, -20 = 61.18 and -80 = 26.7)

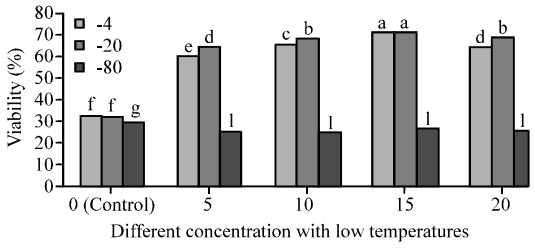

Fig. 7: Percentage of mean sperm viability of *P. merguiensis* spermatophores shows interaction between different concentrations with interaction between low temperatures (n = 750 referred 1500 spermatophore). Different letters indicate significant difference among the temperatures ($p>0.05$) (−4: 33.06, 60.29, 65.89, 71.29, 64.67; −20: 32.59, 64.51, 68.6, 70.79, 69.42; −80: 29.55, 25.44, 25.56, 27.17 and 25.78)

viability at every low temperature at -4, -20 and -80°C as 67.4±20.94, 73.4±22.07 and 68.3±21.01%, respectively. However, it was higher than that in other CPAs ($p<0.05$). Table 7 percentages of viable sperm exposed with 15 and 20% concentrations of the DMSO and $MgCl_2$ which was higher ($p<0.05$). Viability of sperm did not change significantly with the temperatures at -20°C $MgCl_2$ with 10, 15 and 20% as an overall average value of 85.1±7.9, 86.7±7.8 and 85.8±7.1%. At -80°C $MgCl_2$ also showed higher percentage of viability at 15% concentration (80.3±7.7%).

Figure 9 shows the mean viabilities of different exposures. The highest sperm viability was achieved at 6 h exposure as 58.18±28.78%. However, lowest was recorded at 12 and 24 h of exposure as 50.89±31.85 and 37.85±32.46.

Figure 10 shows the mean viability percentages in interactions between different exposures and different concentrations. Highest mean of sperm viabilities as 65.8±30.87 and 64.07±31.1% were observed when concentration at 10 and 15% which appeared ($p<0.05$) >5 and 20% concentration. Viability in other exposure was significantly lower than ($p<0.05$) that of above.

Table 8: Percentage of mean sperm viability of *P. merguiensis* spermatophores shows interaction between different exposure time, CPAs and concentration

		Concentration (%)				
Time (h)	CPAs	0 (Control)	5	10	15	20
6	DMSO	40.39±3.28	70.22± 21.16^{gh}	73.18±22.58^{ef}	74.42±23.61^{e}	72.37±23.19^{efg}
	$MgCl_2$	40.39±3.28	77.08±8.1900^{d}	84.4±6.8100^{b}	86.5±6.4700^{a}	82.14±9.540^{c}
	Glycerol	40.39±3.28	26.9±33.2800	30.64±36.86	33.24±36.43	26.95±29.44
	Sucrose	40.39±3.28	72.0±16.8500	72.56±19.42	73.6±19.580	63.34±24.75
	EG	40.39±3.28	60.36±25.990	63.38±23.79	64.65±23.64	58.88±24.86
	Methanol	40.39±3.28	57.75±33.430	60.25±36.19	62.39±35.74	55.77±33.37
12	DMSO	30.98±2.44	58.11±28.700	58.01±31.51	61.41±31.53	60.72±33.12
	$MgCl_2$	30.98±2.44	73.86±5.4400^{ef}	82.08±6.720^{c}	86.93±5.600^{a}	86.71±6.590^{a}
	Glycerol	30.98±2.44	15.25±20.530	18.13±23.98	19.38±24.86	19.38±24.31
	Sucrose	30.98±2.44	60.46±22.630	62.74±26.85	64.01±29.31	62.69±29.50
	EG	30.98±2.44	57.14±29.400	59.39±29.17	61.84±33.49	59.64±33.96
	Methanol	30.98±2.44	48.8±33.8900	52.18±36.57	56.12±39.30	55.87±38.76
24	DMSO	23.84±3.38	50.23±30.200	55.41±37.33	58.54±37.54	56.71±39.54
	$MgCl_2$	23.84±3.38	63.3±10.6600	69.3±12.060	79.15±8.070^{d}	78.42±6.870^{d}
	Glycerol	23.84±3.38	6.44±8.7300	7.56±9.960	9.04±10.72	6.97±8.670
	Sucrose	23.84±3.38	55.24±28.980	57.19±29.97	65.72±27.31	61.86±26.66
	EG	23.84±3.38	27.93±29.990	30.87±31.39	33.24±34.49	28.34±36.16
	Methanol	23.84±3.38	20.4±29.4600	23.02±33.00	25.32±31.48	22.45±32.36

Values are mean±SD (n = 750 referred 1500 spermatophore), mean value with different subscripts letters in the same column were significantly different ($p<0.05$).*Control 0 concentration only Ca-F saline. spermatophores maintained at room temperature overall 25°C

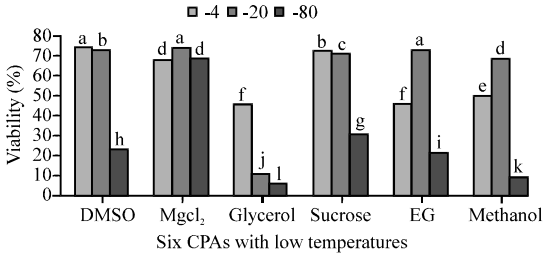

Fig. 8: Percentage of mean sperm viability of *P. merguiensis* spermatophores shows interaction between different CPAs between low temperatures (n = 750 referred 1500 spermatophore). Different letters indicate significant difference among the temperatures ($p>0.05$) (−4: 73.7, 67.4, 45.8, 72.0, 45.7, 49.8; −20: 72.0, 73.4, 10.9, 70.2, 72.9, 67.8; −80: 23.3, 68.3, 6.4, 31.1, 21.6 and 9.6)

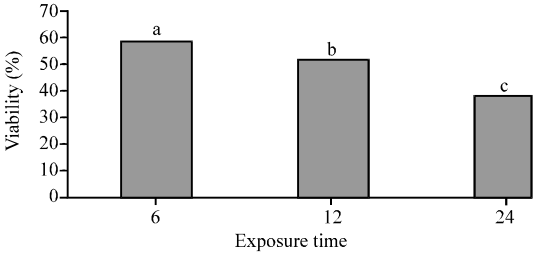

Fig. 9: Mean compression effects of different exposures time (min) on sperm viability (n = 750 referred 1500 spermatophore). Different letters indicate significant difference among the temperatures ($p>0.05$) (Viability (%): 6 = 58.18, 12 = 50.89 and 24 = 37.85)

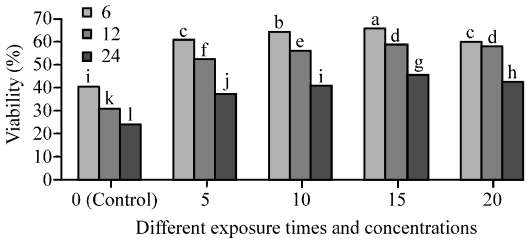

Fig. 10: Mean compression effects of different exposures time (min) and different concentrations percentage on sperm viability (n = 750 referred 1500 spermatophore). Different letters indicate significant difference among the temperatures ($p>0.05$) (6: 40.39, 60.72, 64.07, 65.8, 59.91; 12: 30.98, 52.27 55.42, 58.28, 57.5; 24: 23.84, 37.26, 40.56, 75.17, 42.460

Figure 11 shows the mean viability percentages in interactions between exposures time and CPAs. Highest mean of sperm viabilities in $MgCl_2$ as 74.1±18.59 and 72.11±21.86% were observed when exposures 6 and 12 h was ($p<0.05$) >24 h exposure. Viability in other CPAs was recorded which appeared significantly lower than ($p<0.05$) in all exposures.

Table 8 shows the best treatment for cryoprotectant recorded in $MgCl_2$. It showed higher percentages of viability at 5, 10, 15 and 20% concentration; in 6 h exposure as 77.08±8.19, 84.4±6.81, 86.5±6.47 and 82.14±9.54%. At 12 h exposure, it showed 73.86±5.44, 82.08±6.72 and 86.93±5.6 and 86.71±6.59%. Finally, at 24 h exposure it remained higher as 63.3±10.66, 69.3±12.06 and

Table 9: Percentage of mean sperm viability of *P. merguiensis* spermatophores shows interaction between different exposure time, temperatures and concentration

		Concentration				
Time (h)	Temperature	0 *(Control)	5	10	15	20
6	-4	40.66±2.71^{q}	72.92±8.870^{g}	78.87±7.950^{cd}	80.67±7.21^{ab}	67.36±12.43^{i}
	-20	42.38±1.47^{p}	73.71±30.88^{g}	77.18±30.94^{ef}	79.36±29.55^{bc}	77.78±29.85^{de}
	-80	38.14±3.64^{r}	35.53±25.75^{tu}	36.15±26.80st	37.38±26.98^{rs}	34.59±26.83^{u}
12	-4	32.79±2.94^{v}	69.22±11.77^{h}	75.8±11.550^{f}	81.37±12.89^{a}	80.6±13.170^{ab}
	-20	30.05±1.16^{w}	64.64±28.94^{j}	67.58±29.78^{i}	69.87±30.28^{h}	69.47±29.85^{h}
	-80	30.1±1.640^{w}	22.95±24.25^{y}	22.89±26.16^{y}	23.6±28.180^{y}	22.43±28.24^{y}
24	-4	25.73±2.75^{x}	38.73±33.53^{r}	42.99±35.89^{g}	51.83±38.74^{n}	46.04±41.31^{o}
	-20	25.35±1.53^{x}	55.18±25.12^{m}	61.06±27.64^{l}	63.15±27.65^{k}	61.00±27.69^{l}
	-80	20.42±2.53^{z}	17.85±25.11	17.63±26.26	20.52±28.30^{z}	20.33±30.14^{z}

Values are mean±SD (n = 750 referred 1500 spermatophore), mean value with different subscripts letters in the same column were significantly different ($p<0.05$).*Control 0 concentration only Ca-F saline. Spermatophores maintained at room temperature overall 25°C

Table 10: Percentage of mean sperm viability of *P. merguiensis* spermatophores shows interaction between different exposure time, temperatures and CPAs

		Cryoprotectants					
Time (h)	Temperature	DMSO	$MgCl_2$	Glycerol	Sucrose	EG	Methanol
6	-4	74.91±18.410^{cd}	68.51±15.35^{klm}	69.13±16.47^{ijkl}	68.88±15.90^{jkl}	56.38±11.58^{r}	70.78±16.62^{ghi}
	-20	82.21±20.29^{a}	80.95±20.01^{a}	16.71±13.95	80.95±19.65^{a}	81.55±19.78^{a}	78.1±18.790^{b}
	-80	41.23±4.230^{n}	72.85±18.14^{ef}	9.03±14.91	43.3±9.0900^{t}	34.67±6.390^{v}	17.05±13.31
12	-4	69.92±18.96^{ijk}	75.43±22.64^{cd}	46.99±8.310^{o}	72.86±20.73^{ef}	70.59±19.38^{hij}	71.96±20.93^{fgh}
	-20	72.39±22.12^{efg}	73.04±22.37^{ef}	8.87±11.32	68.82±20.85^{jkl}	71.98±21.83^{fgh}	66.83±19.65^{mn}
	-80	19.23±7.650^{z}	67.88±20.06^{lm}	6.02±12.14	26.85±8.530^{w}	18.82±8.250^{z}	7.58±11.86
24	-4	76.19±26.34^{c}	58.18±20.63^{q}	21.24±4.340^{y}	74.15±25.46^{de}	10.01±10.34	6.6±10.410
	-20	61.3±19.720^{p}	66.17±21.49^{n}	6.98±9.880	60.94±19.47^{p}	65.18±21.50^{no}	58.33±17.78^{q}
	-80	9.34±8.480	64.06±23.77^{o}	4.08±8.300	23.21±11.89^{x}	11.33±8.260	4.08±8.300

Values are mean±SD (n = 750 referred 1500 spermatophore), mean value with different subscripts letters in the same column were significantly different ($p<0.05$).*Control 0 concentration only Ca-F saline. Spermatophores maintained at room temperature overall 25°C

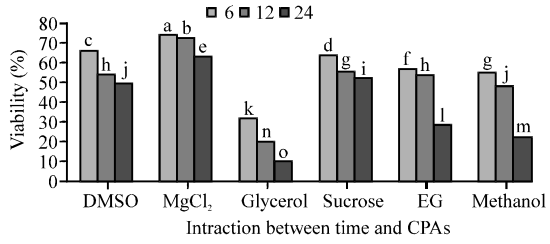

Fig. 11: Mean compression effects of different exposures time (min) and CPAs exposures time (min) and different concentrations percentage on sperm viability (n = 750 referred 1500 spermatophore). Different letters indicate significant difference among the temperatures ($p>0.05$) (6: 66.12, 74.1, 31.62, 64.38, 57.53, 55.31; 12: 53.85, 72.11, 20.62, 56.17, 53.8, 48.79; 24: 48.94, 62.8, 10.77, 52.77, 28.84 and 23.01)

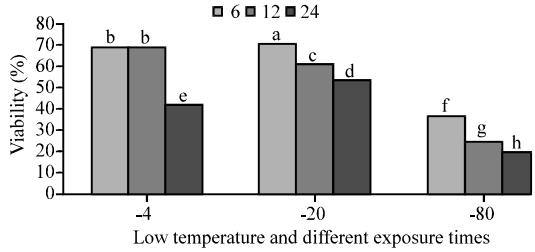

Fig. 12: Mean compression effects of different exposures time and low temperatures on sperm viability (n = 750 referred 1500 spermatophore). Different letters indicate significant difference among the temperatures ($p>0.05$) (6: 68.1, 70.08, 36.36; 12: 67.96, 60.32, 24.4; 24: 41.06, 53.15 and 19.35)

79.15±8.07 and 78.42±6.87%, respectively. Though, it was higher than that in other CPAs ($p<0.05$). Lower sperm viability was also observed in DMSO, glycerol, sucrose, EG and methanol. Lowest sperm viability was observed in glycerol as 6.97±8.67% when concentration was 20% and exposure time was 24 h.

Figure 12 shows the mean viability percentages in interactions between exposures time and low temperatures. Higher viability percentage was recorded as 70.08±30.41% at 6 h exposure and temperature was -20°C.

Viability in other low temperatures were significantly lower than ($p<0.05$) in remaining exposures. Table 9 shows the mean viability percentages in interactions between exposures time, low temperatures and concentration in different percentages. The best treatment recorded at 6 h exposure with -4 and -80°C in 15% as 80.67±7.21 and 79.36±29.55%; at 12th h exposure with -4°C in 15% it came as 81.37±12.89% and lowest sperm viability was observed at 24th h exposure with -80°C as 17.63±26.26%.

Table 10 shows the mean viability percentages in interactions between exposures time, low temperatures

and different CPAs. The best treatment for cryoprotectants recorded in DMSO, $MgCl_2$, sucrose, EG and methanol. It showed higher percentage of viability at 6th hour exposure with above CPAs at -20°C as 82.21±20.29, 80.95±20.01, 80.95±19.65 and 81.55±19.78 and 78.1±18%. $MgCl_2$ showed higher percentage of viability when exposure was 12th and 24th h at every temperature. Lowest sperm viability was observed in glycerol as 4.08±8.3% when exposure was 24th h and temperature was -80°C.

Cooling rates: The effects of cooling rates immersion in LN for 24 h were examined (Fig. 13). In this study, 12 protocols were applied. They were further distributed in 3 exposures as 5, 10 and 15 min. Every exposure was further distributed in 4 different temperatures (Table 1).

Highest post-thaw sperm viability was recorded at E, F and G protocols as 81.93±4.75, 76.73±1.05 and 84.3±2.90%, respectively. Lowest post-thaw sperm viability was observed in protocol C as 24.77±2.55%.

Cryopreservation of spermatophore in different time duration: Successful cryopreservation of spermatophore in liquid nitrogen was achieved by protocol G with cooling rates as 25, 20, 16, 4, 2, -4, -20, -80, -150°C/10 min before storing in liquid nitrogen. Optimal thawing was in 27°C water bath for 2 min, this yielded live sperm after storage in liquid nitrogen for 180 days. Figure 14 shows, at 24th h it had an average viability as 83.5±0.6% which was not different ($p>0.05$) from that for fresh spermatophores (93.8±1.3%). The cryopreserved spermatophore which kept up to 60 days had an average viability as 61±1.2%. However, storage beyond 90 days caused a significant decline ($p<0.05$) in sperm viability. Crypreserved spermatophore stored in liquid nitrogen from 120-180 days had viabilities 48.9±0.9 and 16.4±1.2%, respectively.

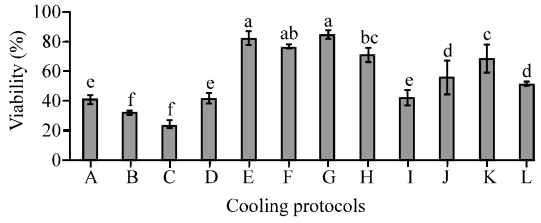

Fig. 13: Mean compression effects of different cooling protocols on sperm viability (n = 18 referred 36 spermatophore). Different letters indicate significant difference among the temperatures ($p>0.05$) (Mean (%): 41.05, 32.6, 24.77, 42.55, 81.93, 76.73, 84.3, 70.8, 42.3, 55.83, 68.4 and 51.83)

Fertilization rate: There was no significant difference ($p>0.05$) in the fertilization rate of cryopreserved spermatophores which were kept in liquid nitrogen for 6 and 24 h and for 7 and 90 days, compared to fresh spermatophores. Average fertilization rate of *P. merguiensis* females artificially inseminated by using SHDAI with cryopreserved spermatophores that had been stored in liquid nitrogen for 6th and 24th h were 87.2±3.2 and 86.8±5.1% and for 7 and 90 days were 73.9±1.5 and 64.1±2.1%, respectively. Whereas, fresh spermatophores was recorded 88.2±1.5% (Fig. 15).

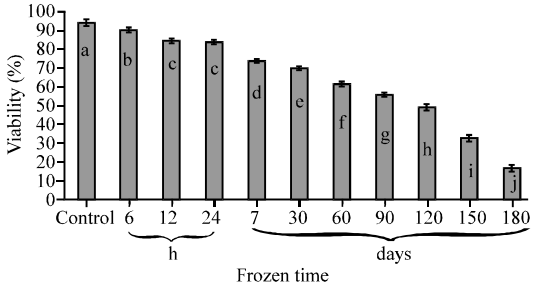

Fig. 14: Percentages of viable sperm of *P. merguiensis* spermatophores after long term storage (180 days) which were stored for long-term storage in LN -196°C fresh spermatophore served as control (n = 33 referred 66 spermatophore). Different letters indicate significant difference among the temperatures ($p>0.05$) (Viability (%): 93.79, 89.6, 84.15, 83.5, 73.37, 69.59, 61, 55.35, 48.98, 32.43 and 16.44)

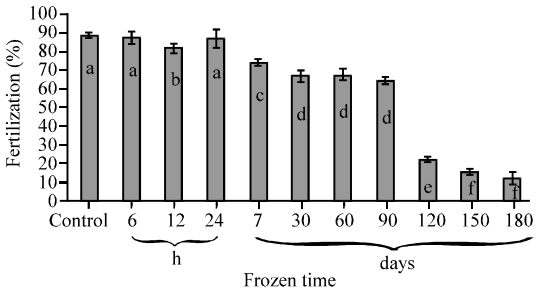

Fig. 15: Fertilization rate of *P. merguiensis* spermatophores after long term storage (180 days) which were stored for long-term storage in LN -196°C fresh spermatophore served as control (n = 33 referred 66 spermatophore). Different letters indicate significant difference among the temperatures ($p>0.05$) (Viability (%): 88.22, 87.24, 81.6, 86.84, 73.93, 66.72, 67.32, 64.1, 21.72, 14.93 and 11.71)

Table 11: Percentages of viable sperm of *P. merguiensis* spermatophores were kept in liquid nitrogen for 24 h and frozen spermatophores were thawed different thawing temperatures and time duration (min) (n = 38)

	Viability (%)				
Temperature	1 (min)	2 (min)	3 (min)	4 (min)	5 (min)
25	24.77±2.35^{hijk}	41.27±3.26^{cd}	30.43±6.940^{fgh}	20.93±0.51^{ijk}	20.13±1.55^{ijk}
27	25.93±7.51^{ghij}	82.27±4.17^{a}	58.43±11.08^{b}	38.07±6.72^{cde}	21.70±2.14^{ijk}
29	21.53±1.77^{ijk}	43.20±2.63^{c}	37.03±4.020^{cdef}	30.23±1.05^{fgh}	26.90±1.57^{ghi}
31	21.03±2.37^{ijk}	36.37±1.86^{cdef}	34.90±1.310^{def}	26.73±3.80^{ghi}	21.40±1.95^{ijk}
33	18.93±1.39^{ijk}	34.10±1.97^{def}	33.13±2.670^{efg}	26.07±3.92^{ghij}	17.40±2.55^{k}

Values are mean±SD (n = 38 referred 76 spermatophore), mean value with different subscripts letters in the same column were significantly different ($p<0.05$). *Control 0 concentration only Ca-F saline. Spermatophores maintained at room temperature overall 25°C

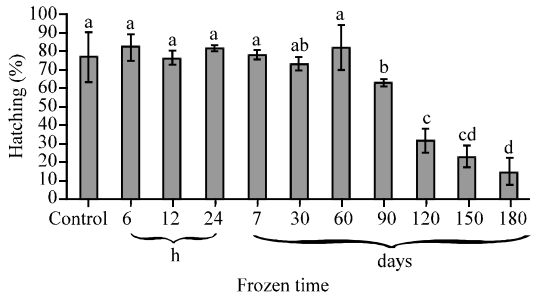

Fig. 16: Hatching rate of *P. merguiensis* spermatophores after long term storage (180 days) which were stored for long-term storage in LN -196°C fresh spermatophore served as control (n = 33 referred 66 spermatophore). Different letters indicate significant difference among the temperatures ($p>0.05$) (Viability (%): 76.2, 81.6, 76.08, 81.21, 77.62, 72.72, 81.51, 62.5, 31.42, 22.71 and 13.93)

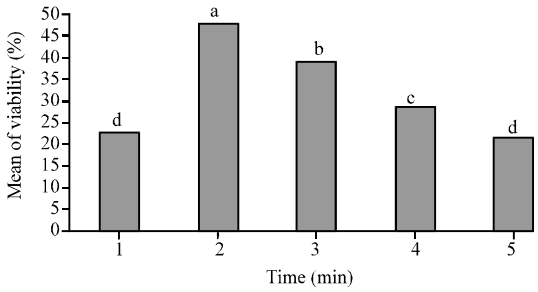

Fig. 17: Mean compression effects of different time duration (min) on sperm viability of *P. merguiensis*, spermatophores were kept in liquid nitrogen for 24 h (n = 38). Different letters indicate significant difference among the temperatures ($p>0.05$) (Viability (%): 22.44, 47.44, 38.79, 28.41 and 21.51)

Hatching rates: Cryopreserved spermatophores kept for 6 and 24 h were 81.6±7.2 and 81.2±1.5%, then for 7 and 90 days were 77.6±2.5 and 62.5±1.5% which were not different ($p>0.05$) from those of the control group (76.2±13.5%) (Fig. 16).

Thawing temperature: Figure 17 shows the highest mean of thawing time duration was recorded as 47.44%. In this study, five time duration were applied 1-5 min. Figure 18 shows the higher mean value of thawing treatment in Group B as 45.28%. In this study, five treatments (thawing temperatures) were examined as 25, 27, 29, 31 and 33°C. From Table 11, it can be seen that each thawing temperature it had significantly different sperm viability.

Highest sperm viability as 82.27±4.17% was observed after thawing at 27°C for 2 min. At higher and lower thawing temperatures, survival declined significantly. Lower sperm viability was observed as 17.40±2.55% in which cryopreserved spermatophores thawed at 33°C for 5 min. Cryopreserved spermatophores thawed at 25°C for 2 min had mediatory sperm viability of 41.27±3.26%. No complete mortality of sperm was recorded at any thawing temperatures.

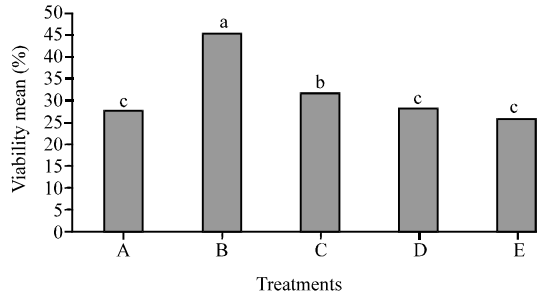

Fig. 18: Mean compression effects of different thawing temperatures (A-E) on sperm viability of *P. merguiensis*, spermatophores were kept in liquid nitrogen for 24 h (n = 38). Different letters indicate significant difference among the temperatures ($p>0.05$) (Viability (%): 27.51, 45.28, 31.78, 28.09, 25.93)

Artificial insemination: The AI process was carried out by using SHDAI. A total of 99 shrimps were used. There were 33 male specimens and females 66 used for this study. From them, 78% (n = 52) females successfully accepted spermatophore. However, no difference recorded between fresh sperm and cryopreserved sperm-

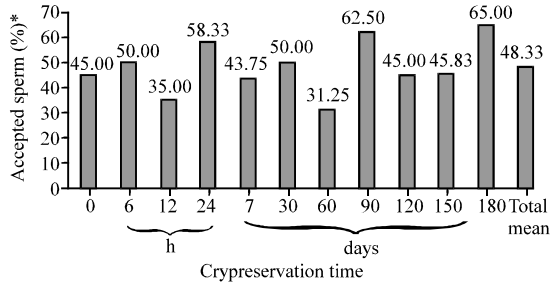

Fig. 19: Percentages of accepted sperm of *P. merguiensis* spermatophores were kept in liquid nitrogen in different time duration (n = 99)**. Used fresh spermatophore as a control. Every group used three female and percentage refer the accepted spermatophore during AI process (*refer all over mean of accepted spermatophore during artificial insemination) (n = 99) **referred 66 spermatophore extracted from 33 male and placed in each female

acceptance in different time periods. The mean of accepted spermatophore in shrimp was 48.4%. Whereas fresh spermatophores was recorded 45%. There was no significant difference ($p>0.05$) from those of the control group (Fig. 19).

DISCUSSION

Optimal freezing medium: This study showed that the percentage of viable sperm decreased significantly as concentrations of cryoprotectants, exposure time and temperatures increased with all treatments. Same observation reported by Vuthiphandchai *et al.* (2007) in *P. monodon*. In this study, an attempt was made to find out the best cryoprotectants with least toxic effect on the spermatophore of *P. merguiensis* through percentage viability counts. Six cryoprotectants were selected to evaluate such as Dimethyl Sulfoxide (DMSO), Ethylene Glycol (EG), methanol, glycerol, sucrose and $MgCl_2$.

In Group A; the analysis in between concentration and CPAs shows that $MgCl_2$ at 5 and 10% concentration gave higher percentage of sperm viability (87.5±8.7 and 84.9± 9.9%) at room temperature (25°C) and control showed 86.5±8.7% viability. The analysis in between CPAs and equilibration also proved viability percentage was higher at 5 and 15 min (90±4.1 and 88.22±7.3%). Whereas, 5% concentration of DMSO had quite higher viability (74.9±11.8%). However, viability declined at high exposure times (30 and 60 min) which was observed as 70.4±19.2 and 50.6±16.2% which was near to the findings reported by Bart *at el.* (2006). Vuthiphandchai *et al.* (2007) in their study the *P.monodon* sperm viability was decreased in DMSO even at 5% concentration for 60 min exposure (63.1±5.6%). In present study, EG; methanol; glycerol and Sucrose were also had lower viability percentage at 60 min exposure as 51.1±13.4, 54.4±11.7, 56.1±10.4 and 52.8±13.1%. As comparing to present study, Bart *at el.* (2006) and Vuthiphandchai *et al.* (2007) stated in their study that *P. monodon* sperm viability was decreased in EG was when 10 min exposure in 5% concentration (43.5±2.1%) and Methanol at 5% in 10 min rather increases (54.9±5.4%).

In Group B; the analysis of CPAs in between different low temperatures and concentration was observed. $MgCl_2$ viability remains constant in -4°C from 10-20% concentration viability percentage was observed as 73.7±13.7-79.1±11.1%. DMSO and glycerol also had higher viability. Whereas sucrose, EG and methanol was extremely lower. $MgCl_2$ was very high in -20°C at 10-20% concentration the viability percentage was observed (85.1±7.9 and 85.8±7.1%) , DMSO was (80.9±10.9 and 84.3±10.4%); EG was (82.01±9.1 and 84.6±8.7%) sucrose (80.8±9.4 and 78.3±13.1%) and methanol (78.4±9.9 and 76.7±10.2%) indicates no significant differences ($p>0.05$). Low sperm viability was observed in glycerol (4.5±4.9 and 6.7±6.1%). However, at -80°C only $MgCl_2$ viability percentage was constant in 5-20% concentration (72.1± 8.9 and 82.3±4.6%) which was higher ($p<0.05$) than other CPAs.

Previously, NaCl 125, $NaHCO_3$ 20, KCl 30, $MgCl_2$ 2.5, $CaCl_2$ 1, pH 8.5 solutions were used for the eel sperm cryopreservation and post-freezing surviving spermatozoa was 24.17±9.73% as reported by Perez *et al.* (2000) and Asturiano and Perez (2003). Another study was carried out by Scott and Cecily (2004) in which movement of the sperm motility was observed as 20.0±4.2% which was tested by using dilute concentration including $MgCl_2$ in frog.

In previous study by Anchordoguy *et al.* (1988) DMSO was found to be more effective cryoprotectant than glycerol, sucrose, proline and trehalose at 5% concentration for freezing *S. ingentis* sperm. Moreover, higher concentrations achieved contradictory results in mud crab *S. serrata* (Bhavanishankar and Subramoniam, 1997). Furthermore DMSO provided good protection at concentrations of 5-20% in echinoderm (sea urchin and sand dollar), oyster, small abalone and polychaeta sperms (Lannan, 1971; Hughes, 1973; Bougrier and Rabenomanana, 1986; Yankson and Moyse, 1991; Bury and Olive, 1993). A study by Dunn and McLachlan (1973) also revealed that the effective range of DMSO concentration was 20-30% for starfish sperm.

However, Behlmer and Brown (1984) indicated that 5% glycerol is a better cryoprotectant than DMSO for horseshoe crab *L. polyphemus* semen. Glycerol at a concentration of 10% gave successful results for freezing freshwater shrimp *M. rosenbergii* spermatophore and horseshoe crab *L. polyphemus* (Chow *et al*., 1985; Akarasanon *et al*., 2004).

Mazur (1981) reported that dextrans, glycols, starches, sugars and polyvinylpyrrolidone provides considerable cryoprotection in a variety of biological systems.

Furthermore, previous studies conducted by Asahina and Takahashi (1978) showed that EG was effective in protecting oyster and sea urchin sperms. EG has been successfully used for the cryopreservation of embryos and larvae of marine shrimp (Newton and Subramoniam, 1996).

Earlier investigations showed that methanol was less toxic to the spermatozoa of *Scylla serrata* than DMSO, EG and glycerol at three physiological temperatures (15, 23 and 30°C) but failed to provide sufficient cryoprotection (Bhavanishankar and Subramoniam, 1997). However, in the study for freezing of *L. vannamei* spermatic mass, highest sperm viability was observed with methanol as compared to DMSO, glycerol and ethylene glycol tested (Lezcano *et al*., 2004). Moreover, methanol has been reported to be the best cryoprotectant for cryopreservation of *P. japonicas* nauplii and zoea (Gwo and Lin, 1998). As well as methanol which was the least toxic cryoprotectants to sperm of *S. serrata* did not provide effective cryoprotection with the concentrations tested by Bhavanishankar and Subramoniam (1997). Also methanol did not protect oyster sperm as reported by Iwata *et al*. (1989).

Cryopreservation of spermatophore: In the present study a successful method for cryopreservation of *P. merguiensis* spermatophore have developed. Because $MgCl_2$ was the least toxic to sperm, it was subsequently used at a concentration of 15% in the cryopreservation studies. Previously no extensive studies were carried out to determine the efficiency of $MgCl_2$ in spermatophore cryopreservation. However, it was used in combination with other cryopreservents. In this study $MgCl_2$ solely used as a cryopreservetive agent and the observed results were very sound.

The high percentage of viable sperm from spermatophore cooled in liquid nitrogen up to 6 h was not surprising. It is likely that the spermatophore was not completely frozen which allowed the spermatozoa to survive, regardless of freezing rates. High percentage of viable sperm confirmed an optimum freezing rate of 4°C/10 min. Woods *et al*. (2004) noted that slow freezing rates generated toxic extracellular ice crystals whereas fast freezing rates increased the probability of intracellular ice formation. The use of cooling rate in the present study provided an easier cryopreservation procedure. Although, the optimum freezing rate during cryopreservation is highly species-dependent as reported by Suquet *et al*. (2000) and Gwo (2000). Slow freezing has also been successful in other decapod species such as cryopreservation of sperm suspension in *P. monodon* (Bart *at el*., 2006; Vuthiphandchai *et al*., 2007). Cryopreservation of sperm suspension in *L. vannamei* (Lezcano *et al*., 2004), *S. serrata* (Billard *et al*., 1995), *S. ingentis* (Anchordoguy *et al*., 1988), *L. polyphemus* (Behlmer and Brown, 1984) and cryopreservation of spermatophores in *M. rosenbergii* Chow *et al*. (1985), Akarasanon *et al*. (2004) and *S. serrata* (Jeyalectumie and Subramoniam, 1989).

Arun and Subramoniam (1997) reported that freezing rates from -1 to -3°C min^{-1} caused critical damages to *P. monodon* larvae whereas higher freezing rates (-5 to -30°C min^{-1}) resulted in 85-95% photo motile survivors. Although, the equilibration period in most sperm cryopreservation studies is usually applied at low temperature (Billard *et al*., 1995; Jeyalectumie and Subramoniam, 1989; Clarke *et al*., 2003), it was also successfully applied at room temperature (25°C) in the present study. Therefore, there was a higher level of tolerance to cryoprotectants induced cell damage by *P. merguiensis* spermatophore than by other aquatic species where low temperature has been reported to reduce it (Asahina and Takahashi, 1978; Robertson *et al*., 1988; Billard *et al*., 1995). A similar observation was also reported by Bart *at el*. (2006), Chow *et al*. (1985) and Vuthiphandchai *et al*. (2007). They successfully cryopreserved spermatophore of *M. rosenbergii* and *P. monodon* after equilibration at the room temperature. In the present study, equilibration for 15 min was enough to allow penetration of cryoprotectants into the spermatophore as the enhanced period of equilibration is a requirement for cryopreservation of a large biological system (Vuthiphandchai *et al*., 2005). Successful cryopreservation of *L. vannamei* spermatophore and spermatic mass was reported with an equilibration time of 1 h whereas that of sperm suspension was 15 min Lezcano *et al*. (2004). The noteworthy stability in sperm viability up till 90 days of storage shows good indication. Similarly, Vuthiphandchai *et al*. (2007) reported crypreserved spermatophores held for <60 days (87.3±4.1%). Akarasanon *et al*. (2004) reported high sperm viability and fertilizing ability of cryopreserved spermatophore of *M. rosenbergii* after 150 days although,

values were significantly lower than those of control. Anchordoguy *et al*. (1988) showed that sperm viability of *S. ingentis* did not decrease after 1 month in liquid nitrogen. Bart *et al*. (2006) reported high fertilization capacity of post-thaw cryopreserved spermatophore of *P. monodon* (79.9±3.7%) after 48 h in liquid nitrogen. Chow *et al*. (1985) reported successful fertilization of *M. rosenbergii* artificially inseminated with cryopreserved spermatophore. A successful storage of cryopreserved spermatophore of *M. rosenbergii* by Chow *et al*. (1985), *S. serrata* by Jeyalectumie and Subramoniam (1989) and *S. ingentis* by Anchordoguy *et al*. (1988) in liquid nitrogen for 30-31 days has also been reported. In this study, the presence of high fertilization and hatching rates after AI ensured the application of cryopreserved spermatophore for the breeding of *P. merguiensis*. Cryopreserved spermatophore of *P. merguiensis* provides a continuous supply of male gametes and allows implementation of definite financial and management profit including international transport of good quality spermatophore and selective breeding programs. Therefore, additional studies should require evaluating the spermatophore cryopreservation protocol on domesticated stock of different strains or different individuals of the same strain of *P. merguiensis* for the benefit of aquaculture.

CONCLUSION

It can thus be concluded that the best CPA concentration is 15% in every equilibrium time, exposure and temperature for the process of spermatophores cryopreservation of *P. merguiensis*. Since, survival of the spermatophore is equally high at 25, -4, -20 and -80°C with $MgCl_2$, it is possible to use the normal refrigeration (-4°C) for a short term storage purpose, thereby could reduce the cost of aquaculture input in connection to *P. merguiensis*/ shrimp hatchery.

The development of cryopreservation protocols of *P. merguiensis* spermatophore was recognized after optimization of cryoprotectants, equilibration period and freezing and thawing rates. *P. merguiensis* spermatophore were successfully cryopreserved with a freezing rate of an initial temperatures of 25°C /15 min (20, 16, 4, 2, -4, -20, -80, -150°C/10 min before storing in liquid nitrogen (-196°C). Cryopreserved spermatophore held in liquid nitrogen <90 days revealed high sperm viability although, for longer periods, sperm viability declined at 180 days. Cryopreserved spermatophore kept for 30, 60 and 90 days had rates of fertilization and hatching which were nearby comparable to those of fresh spermatophore. The method described in this study for long-term storage of *P. merguiensis* spermatophore represents a major advancement in studies with involvement in development of cryopreservation protocol.

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