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Cryopreservation of Spermatophores in the White Shrimp (*Litopenaeus vannamei*)

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ABSTRACT

To study the cryopreservation of male gamete of white shrimp, *Litopenaeus vannamei*, various combinations of factors including proper extender, kind and concentration of three cryoprotectants, four equilibration times, five freezing rates and three thawing temperatures were examined. Practical cryopreservation of spermatophore in white shrimp was carried out. Due to the immobility of spermatozoa of white shrimp, the viability percentages of sperm before and after cryopreservation were assessed by staining the sperm cells with eosin-nigrosin. Best result was obtained in the subgroup in which shrimp spermatophores were treated with Ca-F saline (Calcium-free marine crustacean saline) at 25 °C, equilibrated in cryoprotectant of 5% DMSO for 30 min, frozen at rate of -2 °C/min until -80 °C, maintained for 2 min, and quenched in liquid nitrogen (LN_2, -196 °C). The best thawing was found using water bath of 30 °C. The highest viability of sperm out of cryopreserved spermatophores based on this protocol was 34.4 ± 3.4%. Almost similar sperm viability (33.3 ± 3.9%) was observed in the subgroup cryopreserved at freezing rate of -1 °C/min. For long-term cryopreservation, equilibration in cryoprotectant of 5% DMSO for 30 min at ambient temperature of 25 °C resulted in 49.5 ± 8.3% viability in equilibrated sample, 44.3 ± 6.6% viability in one-day cryopreserved sample, and 33.0 to 37.0% in 10-, 20-, 30-, 40-, 50-, 60-,and 70-day cryopreserved samples. There was no significant difference among the last seven subgroups.

This is the first report on the establishment of cryopreservation protocol for spermatophores of white shrimp based on the systematic combination of favorable factors. Further studies on the application could provide information for commercialization for both research and industrial use.

Keywords: cryopreservation, cryoprotectant, freezing rate, white shrimp, spermatophore

INTRODUCTION

In contrast with the 20th century aquaculture, the 21st century aquaculture should be in pursuit of three objectives: to continue producing affordable aquatic products for specialized markets; to supply high-value aquatic products for specialized markets; and to maintain the germplasm repository of important aquatic species. Breeding ecology as *in situ* (*in vivo*) conservation is essential. Study on cryopreservation of sperm as *ex situ* (*in vitro*) preservation should be undertaken (Chao, 2003).

Cryopreservation is a well-known technology in which liquid nitrogen (-196 °C) is used as refrigerant to maintain functional biomaterial sample during long-term preservation. Different species, various qualities and concentrations of sperm may result in different biochemical or physiological function of cryoprotectants. The banking of spermatophores may provide the safeguards and flexibilities needed by

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domestication programs for the white shrimp. The long-term cryopreservation of sperm of aquatic organisms is well documented (Iwata *et al.*, 1989; Rana, 1995; Alvarez *et al.*, 2003; Ji *et al.*, 2004; Riley, 2004). In Taiwan, sperm from more than 20 fish and shellfish species have been successfully cryopreserved (Chao *et al.*, 1986, 1987, 1993a, 1997, 2007; Gwo, 2000; Gwo *et al.*, 2002).

Although the conditions required for successful cryopreservation of crustacean sperm are less understood, related studies in species such as the freshwater prawn *Macrobrachium rosenbergii* and the black tiger shrimp *Penaeus monodon*, have been reported (Chow *et al.*, 1985; Bart *et al.*, 2006; Vuthiphandchai *et al.*, 2007). White shrimp, *Litopenaeus vannamei,* is one of the economically important marine shrimps in Latin America originally and in Asia recently. The importance of sperm preservation for paternal germplasm repository in this species for various purposes including cross breeding and genetic improvement should not be neglected. In this study, cryopreservation protocol was formulated for white shrimp based on favorable type and concentration of extender, cryoprotectant, equilibration time, and comparison of practical protocols.

MATERIALS AND METHODS

1. Shrimp and Spermatophores

Cultivated male white shrimps (27.86 ± 4.64 g BW and 16.05 ± 0.89 cm TL) reared in private shrimp farm were acclimated to our indoor rearing aquaria for two days and fed with commercial white shrimp pellet feed. The shrimps were fasted in the morning of scheduled experiment. The spermatophores at the base of the 5th pair of walking legs were removed with forceps after partial extrusion.

2. Sperm Concentration

Based on the method described by Lin (1989), trypsin at 62.5 mg/ml was prepared. Spermatophores and vas deference were soaked in the prepared trypsin at 4 ^{o}C for 24 hr. The collected sperms were counted using flow cytometer (Partec PA II). Normal and mature sperms have mean diameter of 5 μm and 7 μm with spike. The sperm concentrations in spermatophore and vas deferens were 0.25 ± 0.23 x 10^6 and 4.19 ± 2.66 x 10^6 cells/ml, respectively. It was found that intact spermatophores were easily removed from shrimp body and maintained as a whole organ to hold sperms of higher quantity. However, vas deferens were fragile and were easily broken allowing sperm to disperse, and therefore were not used for sperm cryopreservation in the succeeding experiments despite of their higher sperm concentration.

3. Optimal Factors

Experiments on cryopreservation of spermatophore in the white shrimp were conducted by examining the effect of each major factor separately and in combination. Viability percentage for immobile sperm were recorded and compared between pre- and post-frozen milts, respectively. In all experimental subgroups, Ca-F saline (Calcium-free marine crustacean saline) was used as extender. In control group, no cryoprotectant was added into Ca-F saline. To evaluate the toxicity of cryoprotectant, 5, 10, 15, and 20 % DMSO (dimethyl sulfoxide), methanol, or glycerol was added respectively into the cryovials containing spermatophores and extender. After 10, 20, 30, and 60 min, the cryoprotectant was removed and spermatophores were rinsed with Ca-F saline twice. Milts were sucked out and sperm cells were stained with eosin-nigrosin before a minimum of 500 cells were examined under microscope for quality assessment (Swanson and Bearden, 1951; Perez-Garnelo *et al*., 2006; Bart *et al.*, 2006; Vuthiphandchai *et al*., 2007). Three replicates were prepared.

To select the best cryoprotectant, 5, 10, 15, and 20% each of DMSO, methanol, and glycerol were prepared and compared using comparative trials of spermatophore freezing to LN_2 (liquid nitrogen, -196 ^{o}C). Similar procedures were run to check sperm viability after freezing.

4. Freezing Protocols and Rates

Three freezing protocols, Protocol A, B, and C, were designed and compared in order to determine their suitability in cryopreservation of white shrimp spermatophores. Program freezer (Planer, KRYO 10 Series II) was used to run the desired freezing rates. After being equilibrated for 30 min at about 25 °C, the spermatophores in 0.5 ml of cryoprotectant such as DMSO (e.g., 5% DMSO with Ca-F saline) was frozen at various rate of -1, -2, -6, -10, and -14 °C/min until -30 °C (Protocol A), until -80 °C (Protocol B), and until -80℃ followed by quenching to LN_2 (Protocol C) (Vuthiphandchai *et al*., 2007). In Protocols A and B, spermatophores were held at -30 and -80 °C respectively for 2 min before thawing and examination for the sperm viability. In Protocol C, they were held at -80 °C for 2 min, then directly quenched in LN_2 at -196 °C and held for 1 hr before thawing. All samples were thawed for 2 min using warm bath at 30 °C. DMSO solution was decanted after thawing, and the spermatophores were rinsed with Ca-F saline twice. Sperm viability was then analyzed. Three replicates were used for each protocol and at every freezing rate.

5. Effect of Thawing Rate on the Viability of Sperm in Spermatophores

Spermatophores collected from male spawners were first soaked in 10 ml of Ca-F saline at 25 ℃ for 5 min and then transferred into cryovials with 0.5 ml of cryoprotectant solution (5% DMSO with Ca-F saline). They were equilibrated at 25 ℃ for 30 min, and frozen at the desired rate (e.g. -14 °C/min) until it reached -80 °C. They were held for 2 min before being directly quenched in LN_2 and stored for 24 hr. In order to determine the optimal thawing rate, warm water baths set at 30, 50, and 70 °C for 2 min were adopted. After thawing, sperms were sucked out immediately and stained with eosin-nigrosin. Three replicates were examined.

6. Protocol for Long-term Cryopreservation

The ultimate purpose of this study is to optimize the methods for long-term cyropreservation of spermatophores of white shrimp in laboratory and to establish a referable protocol for practical application.

In control group, the spermatophores collected from the white shrimp were treated in extender solution Ca-F saline for approximately 5 min and examined. In experimental groups, all samples were treated with Ca-F saline (Calcium-free marine crustacean saline) at 25 °C for 30 min, equilibrated in cryoprotectant of 5% DMSO for 30 min, frozen at rate of -2 °C/min until -80 °C, maintained for 2 min, and quenched in liquid nitrogen (LN_2, -196 °C) for trials of long-term cryopreservation. The viability of sperm from spermatophores cryopreserved for 1, 10, 20, 30, 40, 50, 60, and 70 days was recorded respectively to evaluate the feasibility of long-term cryopreservation.

RESULTS

1. Shrimp and Spermatophores

Spermatophores of bright-white to brownish colors were obtained from live white shrimps. Result indicated that melanized spermatophores (brownish or darken color) were poor of quality and therefore discarded. In addition, males with blackened necrotic spermatophores, which were reported to be of poor function (Lin, 1989), were not used in this study.

A total of 278 intact and good-quality spermatophores out of 332 samples were found useful in this study. Left and right spermatophores show an average difference in weight of 0.101 ± 0.071 g and could be equally adopted.

2. Toxicity of Selected Extender and Cryoprotectants

Live and dead sperms were distinguished by their color after staining with eosin-nigrosin. Live sperm was not stained and bright against the red background; dead sperm was stained by eosin and became red or dark blue.

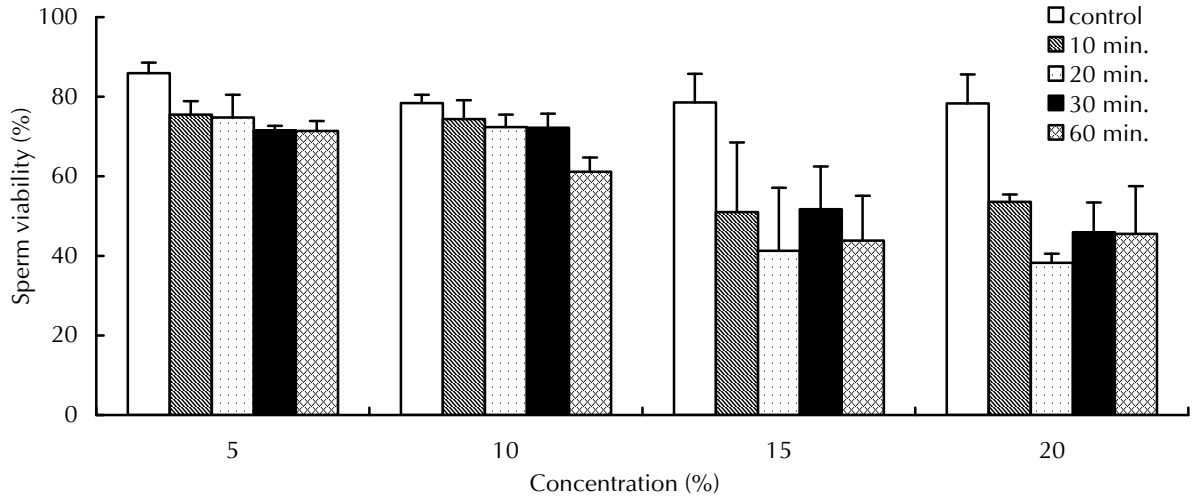

Fig. 1 Percentage of viable sperm of white shrimp (*Litopenaeus vannamei*) spermatophores pretreated with DMSO at different concentrations for various exposure duration. In each control group, freshly collected spermatophores were immersed in Ca-F saline without cryoprotectant (n=3).

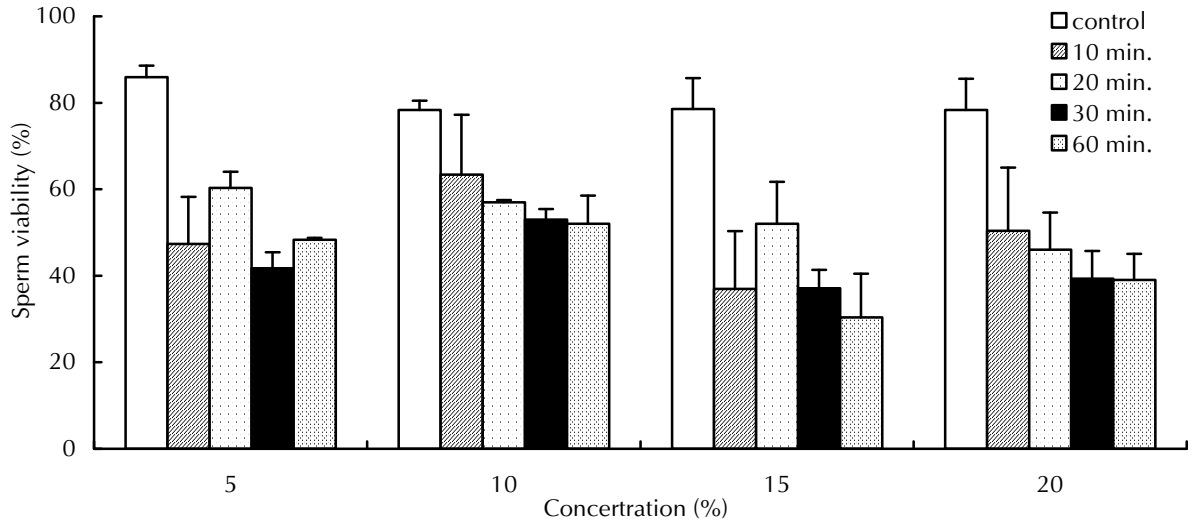

Fig. 2 Percentage of viable sperm of white shrimp (*Litopenaeus vannamei*) spermatophores pretreated with Glycerol at different concentrations for various exposure duration. In each control group, freshly collected spermatophores were immersed in Ca-F saline without cryoprotectant (n=3).

Survival rate of sperms obtained from grinding spermatophores treated in advance with extender Ca-F saline for 10, 20, 30, and 60 min was 85.9 ± 2.7, 78.4 ± 2.1, 78.6 ± 7.2 and 78.0 ± 7.3%, respectively. These were favorable for treating spermatophore after being removed from shrimp and before being frozen.

In each of the cyroprotectant tested, high survival rates of spermatohphores were observed at 5% DMSO subgroups: 75.5 ± 3.4% after treatment for 10 min and 71.4 ± 2.5% after 60 min (Fig. 1), 10% glycerol subgroups: 63.4 ± 13.8% and 56.1 ± 7.2% after treatment for 10 and 60 min, respectively (Fig. 2) and 5 and 15% methanol subgroups: 59.1 ± 5.2 and 58.1 ± 2.7% for 10 and 20 min, respectively (Fig. 3). Overall, DMSO shows the least toxicity during pretreatment and was selected for the succeeding sub-experiments.

3. Freezing Protocols and Rates

After freezing the samples from ambient temperature to -30 °C, the best survival rate of 60% and above were observed in the subgroups with

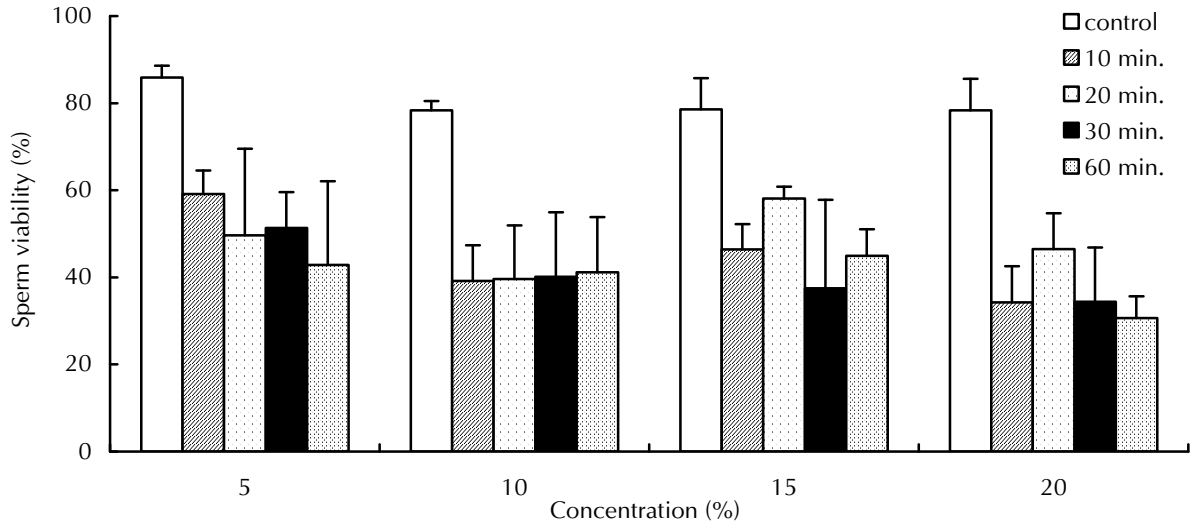

Fig. 3 Percentage of viable sperm of white shrimp (*Litopenaeus vannamei*) spermatophores pretreated with Methanol at different concentrations for various exposure duration. In each control group, freshly collected spermatophores were immersed in Ca-F saline without cryoprotectant (n=3).

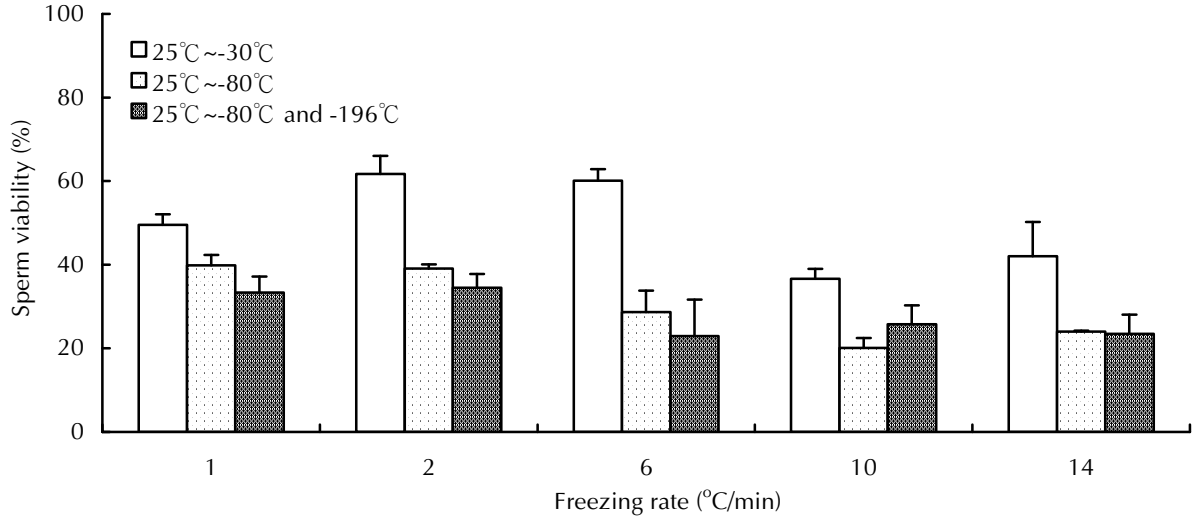

Fig. 4 Percentage of viable sperm of white shrimp (*Litopenaeus vannamei*) spermatophores frozen with different freezing protocols (n=3).

freezing rate of -2 °C/min and -6 °C/min (Fig. 4). Further freezing to -80 °C, the survival rate was 40 ± 1.1% and 28.6 ± 5.1% in the subgroups with freezing rate of -2 °C/min and -6 °C/min, respectively. When the samples were finally quenched to targeted temperature of -196 °C, survival rate was slightly lower than that at -80 °C and remained to be best in the subgroups with freezing rate of -2 °C/min. In general, freezing rate of -2 °C/min seemed to be favorably effective and was chosen for the follow-up experiments.

4. Effect of Thawing Temperature on the Survival of Sperm

In thawing the frozen spermatophores from -196 °C, the best survival rate of 34.9 ± 9.7% was found at thawing temperature of 30 °C (Fig. 5), which was significantly higher ($P < 0.05$) than at 50 and 70 °C. Thus, thawing temperature of 30 °C was chosen for the follow-up experiments.

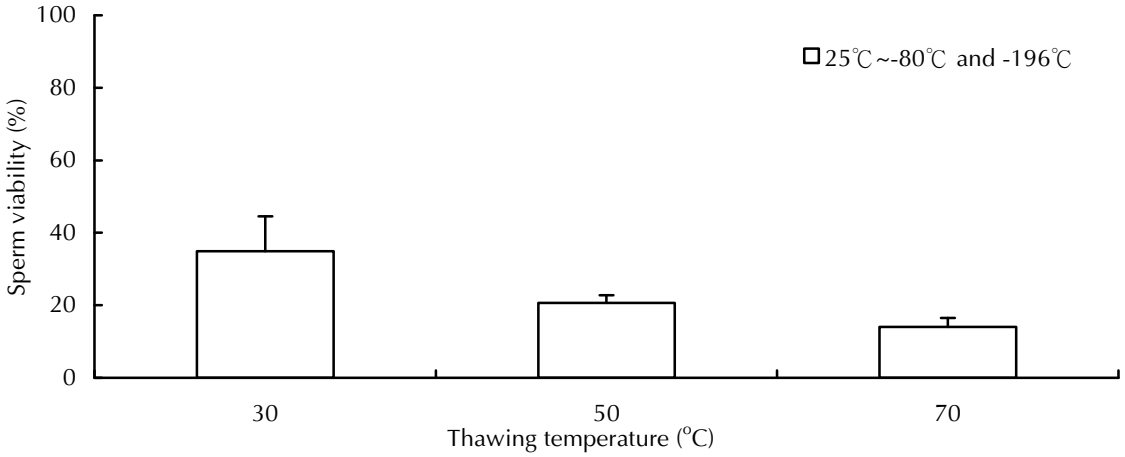

Fig. 5 Effect of thawing temperatures on viability of frozen-thawed sperm of white shrimp (*Litopenaeus vannamei*). Cryopreserved spermatophores were stored in liquid nitrogen for 24h prior to thawing and evaluation of sperm viability (n=3).

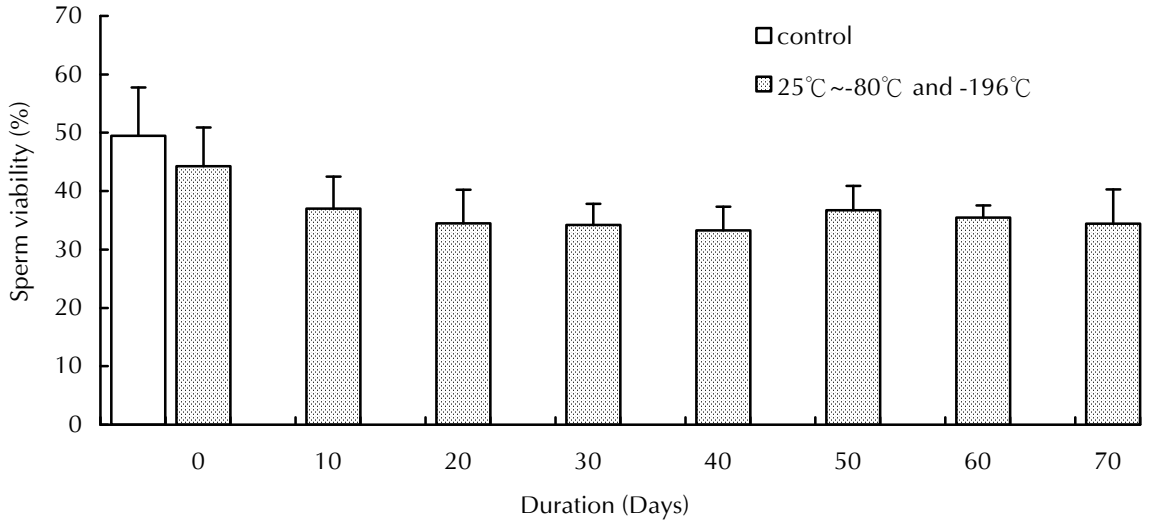

Fig. 6 Percentage of viable sperm of white shrimp (*Litopenaeus vannamei*) spermatophores held in liquid nitrogen during continuous long-term storage period up to 70 days. Fresh spermatophores on day 0 were examined for baseline data of sperm viability without freezing and served as the control, while that of day 0 cryopreserved group was referred to cryopreserved spermatophores held in liquid nitrogen for 1 h (n=3).

5. Long Term Cryopreservation of Spermatophore

Survival rate of sperm treated with Ca-F saline as extender and 5% DMSO as cryoprotectant and equilibrated for 30 min at 25 °C was 49.5 ± 8.3%, in comparison with 44.3 ± 6.6% in one-day cryopreserved and 30 °C thawed sample (Fig. 6). The survival rates after being preserved for 10, 20, 30, 40, 50, 60, and 70 days ranged between 33.0 to 37.0% without any significant variance ($P > 0.05$).

DISCUSSION

Through our experiment, an optimized protocol to preserve spermatophores of white shrimp was established. In this preliminary study, we found the optimal pretreatment and protocol to preserve spermatophores of white shrimp in 0.5 ml cryovials. Application of Ca-F saline as extender and 5% DMSO as cryoprotectant, followed by proper equilibration time of 30 min, utilization of optimal freezing rate of -2 °C/min to bring temperature of

sperm mixture to -80 °C and then holding for 2 min before being further quenched in LN_2. Thawing of cryopreserved spermatophores in water bath temperature at 30 °C is recommended. Dumont *et al*. (1992) reported -30 °C freezing of *P. vannamei* spermatophore and obtained a 43% fertilization rate but did not cryopreserve the spermatophore.

A useful method for the cryostorage of spermatophores in the white shrimp, *Litopenaeus vannamei*, was developed. It was found that DMSO was the least toxic cryoprotectant to white shrimp sperm, at concentration of 5, 10 15 and 20%. We have therefore used 5% DMSO throughout this study for our evaluation. The effectiveness of DMSO for cryopreservation of sperm has been reported in various groups of animals, including fish (Chao and Liao, 2001; Chao *et al.*, 1987, 2007); shellfish (Iwata *et al*., 1989; Gwo *et al*., 2002), mud crab (*Scylla serrata*) (Jeyalectumie *et al*., 1989), and shrimps (*Sicyonia ingentis* and *Penaeus monondon*) (Anchordoguy *et al.*, 1988; Vuthiphandchai *et al*., 2005; Bart et al., 2006). In other studies, glycerol was found to be most effective for sperm cryopreservation of freshwater shrimp, *Macrobrachium rosenbergii* (Chow *et al*., 1985; Akarasanon *et al*., 2004) and horseshoe crab, *Limulus polyphemus* (Behlmer and Brown, 1984). In freezing spermatic mass of *L. vannamei*, highest sperm viability was found with methanol in comparison with DMSO, glycerol, and ethylene glycol (Lezcano *et al*., 2004). Ethylene glycol was proven useful for the cryopreservation of embryos and larvae of tiny marine shrimp (Newton and Subramoniam, 1996). Vuthiphandchai *et al*. (2005) indicated that DMSO was favorable for cryopreservation of embryos of black tiger shrimp (*P. monodon*) with regard to cryoprotectant toxicity. Species specific differences in tolerance to cryoprotectants are evidenced judging from the results of these studies as well as our work.

The reason of high survival rate of sperm collected from the spermatophores frozen to -30 °C (protocol A) might be because that the intact spermatophores were not completely frozen under our experimental freezing rates and thus most of the spermatozoa inside the spermatophore were still alive. However, it indicated that there was no fetal damage of the sperm in treated spermatophores down to -30 °C along our protocol. Usually, supercooled spermatophores can not be stored for long, as compared to totally frozen spermatophores. With the ultimate concern to fulfill long-term cryopreservation, the latter can be kept in LN_2 for reasonably longer period of time, thus a long-term storage of useful genome. With regard to the result of the protocols B and C, the optimum freezing rate of -2 °C/min conforms with the results of Lin *et al.* (1993) and Wood *et al.* (2004) who reported that slow freezing rates helped generate favorable extracellular ice crystal, while fast freezing rates increased the probability of intracellular ice formation that might affect the organelle.

The survival rate of sperm from frozen or pre-frozen spermatophores was determined by eosin-nigrosin staining method followed by light-microscopy examination to count the live and dead cells. The results suggest that the method is well accepted and whether there is any possibility of further improvement remains to be studied in the future.

The optimal protocol to cryopreserve sperm in this experimental species was established by the present study and could be regarded as an ideal model of cryopreservation of sperm for other marine shrimp species.

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白蝦精莢之超低溫保存探討

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摘　要

本研究進行白蝦雄性配子之超低溫冷凍保存試驗，考慮各種可能產生影響之因子如適用的稀釋液、三種抗凍劑的種類及濃度、平衡時間、五種降溫速率、冷凍方法及三種解凍速率, 加以一一測試並比較。白蝦的精子不具游動性，其冷凍保存前後結果以 eosin-nigrosin 染色法評估其活存率百分比。實驗結果顯示，白蝦精莢之最佳超低溫冷凍保存效果之稀釋液、降溫流程、抗凍劑種類及抗凍劑濃度為：以 Ca-F saline 作為稀釋液，濃度 5% 之 DMSO 當作抗凍劑，於室溫平衡 30 min 後，以 -2 ℃/min 之速率，將精莢降溫至 -80 ℃，維持 2 min，之後再直接投入液態氮保存。樣品在 30 ℃水浴中解凍，活存率為最高，達 34.4 ± 3.4%。其次佳者為：以-1 ℃/min 之速率降溫，活存率亦可達 33.3 ± 3.9%，與前者並無顯著差異。在白蝦精莢長期保存試驗中，以濃度 5% 之 DMSO 當作抗凍劑，室溫平衡 30 min 之分組經前處理後活存率為 49.5 ± 8.3%；採用上述最佳流程保存 1 天後之活存率為 44.3 ± 6.6%，其後隨著保存時間每 10 天觀察一次，直到第 70 天其活存率介於 33 ~ 37% 之間，尚屬穩定，並無顯著差異 ($P < 0.05$)。

經由本研究探討所得的結果，除了建立冷凍白蝦精莢目前最佳條件組合，並可提供作為日後白蝦雄性配子超低溫冷凍保存供實際應用時之參考。

關鍵詞：超低溫冷凍保存、抗凍劑、降溫速率、精莢、白蝦

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