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Short-term Storage of White Shrimp (*Litopenaeus vannamei*) Spermatophores

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Abstract

The objective of this study was to estimate the suitability of extender solutions for maintaining sperm viability in spermatophore of white shrimp (*Litopenaeus vannamei*). *L.vannamei* sperm were exposed to different types of extenders to evaluate their efficiency over various storage periods. The type of suitable extender solution for shrimp spermatophores was observed in spermatophore stored in mineral oil and Ca-F saline. At 720 hr. extended period, the highest percentage of sperm viability was observed in mineral oil (64.33±17.29). Sperm exposed in Ca-F saline, although percent viability was lower than that in mineral oil after 1 month of storage, there was no significant difference at beginning periods. The highest percentage of sperm mortality was observed in phosphate buffer at 24 hr. storage. However, although in this study the best result of short-term storage of *L*. vannamei sperm was observed with sperm preserved in mineral oil extender solution, next study for long-term storage (cryopreservation) the Ca-F saline should be considered as a good alternative for long-term storage due to its sperm cell osmoregulation properties.

Keywords: Spermatophore, Preservation, White shrimp, Aquaculture, Sperm viability

Introduction

Recently, an important methodology for enhancing of fry production capacity in aquatic species has been mainly focused on the use of artificial fertilization technique[1]. Sperm are determined to have an important role in large-scale fry production in crustacean[2]. In the last decade, white shrimp (*L. vannamei*) is one of the most important species that dramatically increase the national value in Thailand. Nowadays, this species has been clearly dropped due to viral infection (White spot syndrome virus; WSSV[3], Taura syndrome virus; TSV[4], Early mortality syndrome; EMS[5], Enterocytozoon hepatopenaei; EHP[6]) in several areas,

especially in the southern part of Thailand[7]. However, there is a bottleneck problem of domesticated brood stock in earthen pond with appearance of non-synchronization during spawning season of male or female. This gives rise to delay of fry production and reduction of spermatophore quality when the broodstock culture period extended with an appearance of spermatophore melanization (black color) [8,9]. This blackening directly affects sperm quality, resulting in lower fertilization and hatching rates.

Therefore, the use of short-term storage techniques is a valuable application for generating a reliable sperm quality source for artificial insemination process. Extender solution for spermatophore storage has been widely used composing of Mineral Oil, Ringer's solution, Phosphate buffer, 0.85% NaCl, and Ca-F saline. Extended solution is associated with substance osmoregulation between inner and outer membrane. Previous research is reported in preservation of *Penaeus monodon* spermatophores at 2-4ºC, result showed that the highest percentage of viable sperm was at 58.3% with 42 days storage period using mineral oil[10] extender. According to Ishida[13] has preserved the lobster sperm (*Homarus*), the result indicated that use of paraffin oil was the most suitable extender solution for maintaining lived sperm cells. Whereas use of Ca-Free saline (Ca-F saline) is also reported for the success of striped bass (*Morone saxatilis*) sperm yielding to >65% sperm motility when sperm were kept in long-term period reported by Guthrie[11]. Ca-F saline has directly affected on acrosomal reaction events under inseminated operation of crustacean spermatophores [12]. However, several researches about white shrimp emphasized infected diagnosis or nutrition, but there are a few researches in preservation of crustacean spermatophores. Hence, this research is a preliminary study to approach the basic information of extender capacity for preserving good quality sperm.

The objective of this study was to estimate the suitability of extender solution to sperm viability, composing of Mineral Oil, Ringer's solution, Phosphate buffer, 0.85% NaCl, and Ca-F saline at different periods of 6, 12, 24 (1 day), 48 (2 days), 96 (4 days), 168 (1 week), 336 (2 weeks), 720 (1 month) hr. This research would be useful for basically important information to enhance the capacity of fry production of *L. vannamei*, and to develop the further cryopreservation protocol.

Material and method

1. Spermatophore collection

In order to get the fresh spermatophore, shrimp broodstock were randomly collected from entrepreneur farm (N= 40), Samut Sakhon province, using from the age of 12-15 months[29]. Collected shrimp were maintained in Styrofoam box supplied with cultured water prior to transportation to laboratory of Burapha University within 1 hour after capture. Sample collection was made by manual stripping around the coxae of the fifth walking leg to take out a pair of spermatophore by forceps (Figure 1). All extruded spermatophores were placed in sterile Petri dish on crush ice (2°C) . Appearance of spermatophore with melanization performance was not considered for experiment.

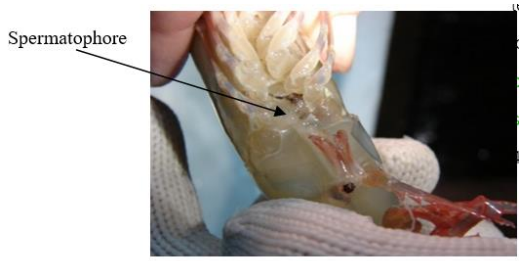

Figure 1. Fresh spermatophore collected from coxae position of the fifth walking leg of *L. vannamei*.

2. Extender solution

In order to determine the appropriate type of extenders for chilled storage of spermatophore, five extender solutions consisted of mineral oil[14] (pH 7.4, 364 mOsm/kg) Krishco Medical Products Pvt Ltd., Qatar, Ringer' s solution[15] (pH 7.8, 728 mOsm/kg) Oxoid™, USA, phosphate buffer[16] (pH 7.3, 669 mOsm/kg) Welgene Fresh Media™, South Korea, 0.85% NaCl[17] (pH 7.7, 491 mOsm/kg) Quality Biological, USA and calcium-free saline (Ca-F saline) [18] (pH 7.4, 403 mOsm/kg) Capricorn Scientific GmbH, Germany were chosen as commonly used extender for preserving in crustacean sperm. Freshly collected spermatophores from mature males were directly plunged into 1.5 ml cryogenic vial that was loaded with 1 ml extender solution containing 0.1% penicillin-streptomycin as antibiotics at the room temperature (25°C) . After spermatophore loading into different solutions, (in each cryotube will be loaded only 1 ml solution: 2 spermatophores) the vials were chilled at 0-4°C in the refrigerator and assessment of viable sperm was evaluated at 6, 12, 24 (1 day), 48 (2 days), 96 (4 days), 168 (1 week), 336 (2 weeks) , 720 (1 month) hr. In each experiment of sperm viability assessment was done with triple replications.

3. Sperm viability assessment

Spermatophore collection was done under room temperature (25°C) by manual stripping and gently ground in glass homogenizer using the proportion of 1 ml of Ca-F saline: 2 spermatophores. Supernatant was transferred into 1 ml Eppendorf tube at room temperature. For staining the samples,

5% eosin was prepared by weighing 0.25 g of eosin and dissolving it in 50 ml of deionized water. Nigrosin (10%) was prepared by weighing 0.5 g of nigrosin and dissolving it in 50 ml of deionized water. Staining solutions were filtered with grade filter papers (9-13 µm) for residual removal. To determine live-dead cell of sperm, about 70 µl of supernatant was immediately removed placing on a slide, and mixed with 100 µl of eosin and nigrosin before fixing the sample with heat (~2 s) . Unstained cells with the blue background of nigrosin were considered as live cells, whereas dead cells show red. Assessment of percentage of live (unstained) sperm was calculated from triple counting of 300 cells in each slide.

4. Sperm count

To assess sperm concentration inside spermatophore, freshly stripped spermatophores were slightly ground with homogenizer. Manually homogenized spermatophores were transferred into 1.5 ml Eppendorf tube containing 1 ml of seawater (10 ppt), and then loaded into the chamber of centrifuge. The tubes were firstly centrifuged at 800 rpm for 10 min, followed by a second centrifugation at 1200 rpm for 15 min (MIKRO 220, Hettich Zentrifugen, Germany). After sample centrifugation, sperm suspension was mixed with deionized water at the dilution ratio of 1: 1000 (sperm suspension: deionized water) . To evaluate sperm density, a small volume (~50 µl) of diluted sperm were placed on hemacytometer before covering with glass slip and counting of sperm was estimated immediately.

5. Statistical analysis

Significant differences among treatments were compared using two-way analysis of variance (ANOVA) . Whereas appearance the output of ANOVA analysis with significant differences, Duncan' s New Multiple Range Test (DMRT) was used to compare of differences within group. The percentage of sperm viability was considered at a 95% confidence level ($P<0.05$) using the Statistical Package for the Social Sciences (SPSS) program (IBM SPSS Statistic Premium Grad Pack 26). All data of treatment were reported with an average of standard error of the mean (SE).

6. Animal ethics

Procedures on animal care were conducted according to the guideline of the Ethics committee of the Hatchery, Department of Aquatic Science, Burapha University.

Results

1. Effect of short-term storage with five extender solutions on sperm viability

Spermatophores preserved in all extenders had sperm viability percentage above 90 % ($P<0.05$) during the first six hours (Figure 2. and Table 1.). The average sperm viability in mineral oil and Ca-F saline was not

significantly different in sperm viability when sperm were exposed at 120 hr. and 210 hr. as shown in Figure 2. Storage period of spermatophore in mineral oil for 48 hr. showed the highest sperm viability percentage (95. 26±12. 74%) whereas phosphate buffer showed no sperm viability (0%) for 24 hr. At the last period (1 month) of sperm storage, mineral oil showed the highest sperm viability percentage when compared to all other extenders (Table 1). There was no significant difference (P>0.05) between sperm stored in mineral oil and Ca-F saline beginning from 6 to 48 hr. (P>0.05), as seen in Figure 2. Although at 24 hr. storage observing the percentage of sperm viability stored in 0. 85% NaCl was higher and significantly different than that in Ringer' s solution (P<0.05), the viability percentage in 0.85% NaCl gradually decreased (P>0.05) by 48 hours of storage. However, the result of extender solution showed that prolonging the longest viable period of sperm was observed in mineral oil and Ca-F saline (at 1 month). High percentages of sperm viability preserved in both mineral oil and Ca-F saline solutions were compared with different storage periods. Significant difference of sperm kept in Ca-F saline seem to be dramatically reduced (P<0. 05) when the exposure duration extended from 2 weeks (64%) to 1 month (18%). Importantly, there was no significant difference in percentage of viable sperm in mineral oil and Ca-F saline at 6, 12, 24 (1 day) 48 (2 days), 96 (4 days), or 168 (1 week) of storage.

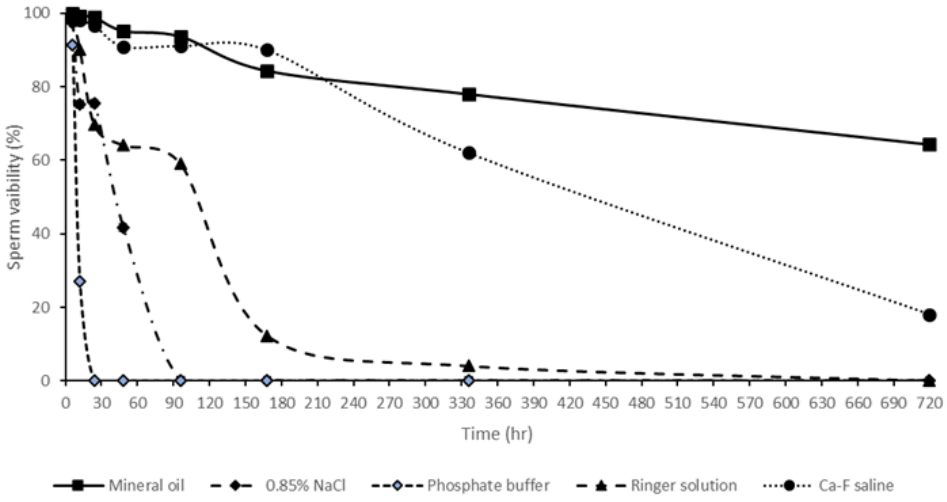

Figure 2. Viable sperm of stripped spermatophores from *L. vannamei* during short-term preservation with different extenders, and exposure periods

Table 1. Percentage of viable sperm (mean±SE) in stripped spermatophores from *L. vannamei* during short-term preservation with five different extenders.

Time (hr.)	Extenders				
	Mineral Oil	0.85% NaCl	Phosphate Buffer	Ringer Solution	Ca-F saline
6	99.89±0.45^{a,1}	97.48±4.91^{a,1}	91.47±4.65^{a,1}	99.22±0.66^{a,1}	99.66±017^{a,1}
12	99.31±0.10^{a,1}	75.15±32.42^{b,2}	26.96±20.58^{b,3}	90.17±10.42^{a,1}	98.11±5.55^{a,1}
24 (1 day)	98.96±9.52^{a,1}	75.47±38.29^{c,3}	-	69.74±29.79^{b,2}	96.69±64.55^{a,1}
48 (2 days)	95.26±12.74^{ab,1}	41.8±26.12^{d,3}	-	64.14±27.60^{c,2}	91.02±8.75^{a,1}
96 (4 days)	93.71±37.41^{ab,1}	0.07±14.65^{de,3}	-	59.02±18.99^{c,2}	91.11±6.83^{ab,1}
168 (1 week)	84.38±9.82^{ab,1}	-	-	12.29±56.75^{d,2}	90.04±24.6^{ab,1}
336 (2 weeks)	78.04±18.18^{ab,1}	-	-	4.07±8.21^{e,3}	62.01±2.14.^{c,2}
720 (1 month)	64.33±17.29^{c,1}	-	-	-	18.04±7.35^{d,2}

Differences in superscript letters in the column indicate significant difference ($p<0.05$).

Differences in superscript numbers in the row indicate significant difference ($p<0.05$).

However, the results of extender solutions showed prolonging the longest viable period of sperm observed in mineral oil and Ca-F saline (at 1 month). High percentages of sperm viability preserved in both of mineral oil and Ca-F saline solutions were compared with different storage periods. Significant difference of sperm kept in Ca-F saline seem to be dramatically reduced ($P<0.05$) when the exposure duration extended from 2 weeks (62% viable sperm) to 1 month (18%). Importantly, there was no significant difference in the percentage of viable sperm between mineral oil and Ca-F saline at storage durations of 6, 12, 24 (1 day) 48 (2 days), 96 (4 days), or 168 (1 week).

Discussions

In this experiment, different extender solutions were used for short-term preservation of *L. vannamei* spermatophore. We knew that extender solutions and exposure times are related with living sperm in spermatophore. Although extender solutions prolong cell viability during short-term preservation process, the effect of osmotic pressure to sperm cell damage must be carefully determined. Our results, mineral oil showed less lethal effects to *L. vannamei* sperm. This may be due to moisture properties affecting on balanced cell osmoregulation, which helps maintain live cells during preservation using hydrophobic

property for protecting fluid hydrolysis process[10], as shown in Figure 3A. In contrast, dead cells are shown in Figure 3B. A report about the direct effect of cryoprotectant to osmotic pressure in black tiger shrimp (*P. monodon*) sperm during cryostorage led to a lot of sperm cell death by Vuthiphandchai[19]. Similarly, Nimrat[10] indicated that use of mineral oil maintained the most suitable extender solution for prolonging sperm viability of *P. monodon* spermatophore reaching to 42 days storage period. This information suggested that the preservation of spermatophore at low temperature (4ºC) in suitable extender solution could prolong the viability of sperm cells inside spermatophore during preservation[11]. There was a decline of sperm viability percentage and spermatophore damage with an expanding spermatophore wall layer preserved in 0.85% NaCl, phosphate buffer and ringer solution after 4 days of storage. According to Braga[8] and Daimond[20] discussed about the damage of spermatophore at outer layer wall related with mineral composition of extender solutions enriching nitrogen (N) and phosphorous (P) compounds, which can penetrate into spermatophore layer to combine with seminal fluids. Hence, combination of these elements can cause bacterial proliferation within the layer of spermatophore, and reduce the percentage of sperm count as well as viable and normal sperm[21,22,23]. Regardless of inferior capacity of Ca-F saline media compared to mineral oil, Ca-F saline was better than 0.85% NaCl, ringer solution and phosphate buffer for preserving spermatophore. Most studies on determination of preservation of crustacean spermatophore have been focused on Ca-F saline. Generally, this buffer was used to maintain osmotic stress between intracellular and extracellular of sperm cells for long-term preservation because the absence of Ca^{2+} element in Ca-F saline would not induce an acrosome reaction. Appearance of Ca^{2+} molecule in extender solution is directly associated with the signal transduction for production of cyclic adenosine monophosphate (cAMP) by adenylyl cyclase activity as well as phosphorylation of tyrosine protein to express response at the region of plasma membrane of sperm cell by opening Ca^{2+} channels for acrosomal reaction process[24].

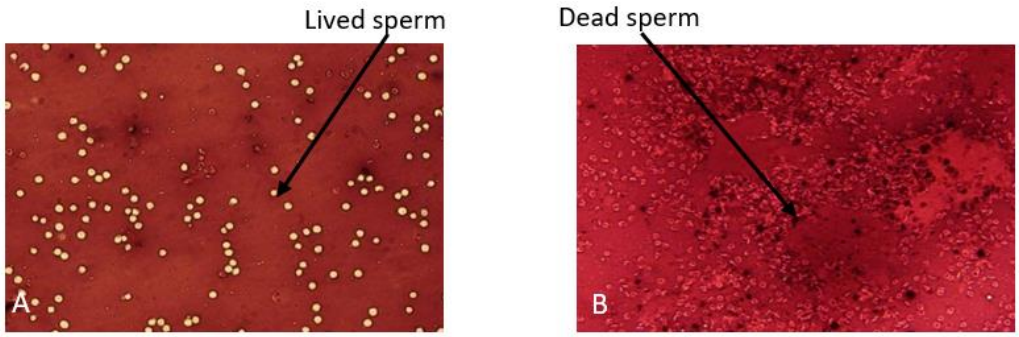

Figure 3. The photograph shows the sperm staining with eosin and nigrosine for *L. Vannamei*. sperm unstaining with red background shows live sperm (B) whereas dead cell (A) show red, under the light microscope with 10X magnification.

Preservation of *L. vannamei* spermatophore under low temperature (0-4ºC) with mineral oil is the most suitable extender solution for prolonging sperm cell viability. Mineral oil has been popularly used for reducing sperm cell damage during short-term preservation of several species of marine shrimp spermatophore[25,26,27,28]. However, an achievement with use of mineral oil solution for short-term storage in *L. vannamei* would be an importantly basic information for developing long-term storage protocol, such as cryopreservation.

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