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Assessment of Viability of Sperm Cells of *Litopenaeus vannamei* on Cryopreservation

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ABSTRACT

Aiming at assessing the cryopreservation potential of Litopenaeus vannamei *sperm cells, 74 spermatophores were manually extracted from the sexually mature individuals. After the toxicity test to define the cryoprotectant concentration, suspensions of spermatic cells were cryopreserved in the groups in freezing solutions comprising different cryoprotectants such as dimethyl sulfoxide (DMSO) and ethylene glycol (EG) at 10% concentration. Each treatment was divided in subgroups for storage in liquid nitrogen during 0, 30, 60 and 90 days, in triplicate. After thawing at 25ºC for 40 seconds, cell viability in the suspensions was analyzed under the microscope in eosin-nigrosin stain and flow cytometry. There were no significant differences between the cryoprotectants used. For all the treatments, lower and higher mortalities occurred in the 0 and 90 days, respectively. By applying the eosin-nigrosin technique, lower and higher mortalities were 23.17 and 82.11% for DMSO and 29.94 and 83.72% for EG, while the flow cytometry registered mortalities of 2.42 and 55.13% for DMSO and 0.90 and 55.56% for EG. The Spearman correlation coefficient indicated a positive correlation (R=0.91) between the two techniques used. It was concluded that there was a decrease in cell viability within a longer cryopreservation time.*

Key words: cryogenics, semen, marine shrimp, cell viability

INTRODUCTION

As the *Litopenaeus vannamei* species is responsible for the main commercial production of shrimp farm in Brazil and worldwide (FAO 2010), investments in the study of technologies to explore the productive potential of this species are important. The main aim of reproduction biotechnologies is the optimization of male and female gametes and production of individuals with recognized origin, emphasizing genetic inheritance qualities. The techniques of germplasm cryopreservation include storage of the sperm, eggs and embryos and contribute directly to animal breeding programs (Tsai and Lin 2012) such as hybridization, hatcheries, population conservation and genetic selection, making the programs of genetic improvement in the species of economic interest more favorable (Gwo 2000). In this context, semen cryopreservation can be considered an interesting alternative since the resources availability, limitations and chances of failure concerning the material period of storage are observed. Freezing of the semen in liquid nitrogen guarantees cell stability and ensures a permanent semen supply in a reduced space with little manpower, free from seasonal limitations of reproduction, and low cost in the maintenance of broodstock due to the lower number of reproductive males necessary for the culture (Gwo

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2000). Semen cryopreservation of fish is already possible and many manipulation techniques are applied in some species, with special highlights for the salmonidae (Scott and Baynes 1980), siluridae (Legendre et al. 1996), cyprinids (Billard et al. 2004), and characidae (Carosfeld et al. 2003). However, there are only a few studies on crustaceans and penaeidae concerning this matter (Lezcano et al. 2004, Vuthiphandchai et al. 2007; Salazar et al. 2008). The development of methods of semen preservation and the implementation of effective artificial reproduction protocols require a good knowledge of the reproductive biology and seminal characteristics of the targeted species (Ninhaus-Silveira et al. 2006).

For marine shrimps, cryoprotectants dimethyl sulfoxide (DMSO) and ethylene glycol (EG) are widely used (Anchordoguy et al. 1988; Barth et al. 2006; Vuthiphandchai et al. 2007). DMSO, when compared with glycerol, proline and trehalose, presents low toxicity to the spermatic cells of *Sicyonia ingentis* (Anchordoguy et al. 1988). For *Penaeus monodon*, Vuthiphandchai et al. (2007) reported that DMSO, etylenoglicol and sucrose had low toxicity to embryos, with survival ranging of 68.0 to 82.3%. DMSO, etylenoglicol and methanol were used to cryopreserve the spermatophores of the same specie for 48h, and the higher survival (79.7 ± 0.4%) was observed with DMSO (5%) (Bart et al. 2006). Thus, this work aimed at checking the possibility of cryopreservation for the semen of the *Litopenaeus vannamei* species for long periods (0, 30, 60 and 90 days) in two distinct cryoprotectant agents, viz. dimethyl sulfoxide (DMSO) and ethylene glycol (EG) with the analysis of cell viability adopting two complementary techniques of eosine-nigrosine stain and flow cytometry.

MATERIALS AND METHODS

Biological material

The shrimps used were the *Litopenaeus vannamei* species, which were specific pathogen free (SPF) notified by the World Organization for Animal Health (OIE) lineage, provided by the Genearch Aquacultura LTDA (located in Rio do Fogo/RN, Brazil) and cultivated in a super intensive biofloc system to an average size of 42.6 g. After the maturation period, when animals were fed eight times per day, with mussel (3x), squid (3x) and ration Inve Breed-S (2x), visibly health males were selected. Were considered healthy the males which had bright color, no external symptoms of diseases and no melanized spermatophore.

Maintenance of broodstock

The animals used in this experiment were kept in the maturation sector of the LCM for a period of 45 days. A hundred animals were stored, 1 male:1 female proportionally, in six tanks of 6000 L, through an artificial photoperiod of 13hs of light: 11hs of darkness. A daily renewal of 150% water was performed in the tanks to maintain water quality and to eliminate remaining food particles. The animals were fed eight times a day (8:00h, 10:00h, 12:00h, 14:00 h, 17:00h, 21:00h, 24:00h and 3:00h) *ad libitum* with fresh food (squid 3 times a day and shellfish 2 times a day) and commercial diet (2 times) for maturation (Breed S, INVE®; Salt Lake City, USA) offered alternately. Salinity and temperature were maintained at 330-350 $g.L^{-1}$ and 28-29ºC, respectively; whereas dissolved oxygen and pH were maintained at 6 -7 $mg.L^{-1}$ and approximately 7.0, respectively.

Preparation of gametes

After biometry of sexually mature individuals used in the test, totally mature spermatophores were manually extracted through a light squeeze in the terminal segment according to directions in the methodology used by Lezcano et al. (2004), with discard of the melanized ones. All spermatophores were weighted and submitted to mechanical agitation in 1 mL of sterile seawater (SSW, 350 $g.L^{-1}$) in order to obtain a suspension of fresh sperm (Bhavanishankar and Subramoniam 1997).

Effect of cryoprotectant agents on the cell integrity

Aiming at the identification of any toxic effects caused by the cryoprotectants, 5, 10, and 20% DMSO and EG (2:1 proportion) in SSW were added to the sperm suspension. The samples containing cryoprotectants in different concentrations were analyzed in triplicate, with three samples obtained from three distinct spermatophores, and the sperm suspension in SSW used as control. Optical microscopy was used to asses the viability of the sperm (400 x magnification), with a minimum cell count number of 100 cells per sample with the aid of a Neubauer chamber at 5, 15 and 30 minutes after exposure to the cryoprotectant solutions (equilibration time to allow total penetration of the cryoprotectant

agent). Considering the presence of a spiked form structure in the normal and viable shrimp spermatic cells, viability was expressed as a percentage of cells presenting a spike among the total number of cells observed (with and without spike and dead cells).

Experimental design

Once ideal concentrations of cryoprotectant agents were determined in the sperm cell suspensions, collections for the cryopreservation were made. Of a total of 74 spermatophores, two were obtained by cryopreservation of the sperm suspension without any cryoprotectant agent and used as control. The remaining 72 spermatophores were divided in the treatments with two distinct cryoprotectants after a selection based on cryoprotectant toxicity tests carried out for penaeidae by diverse authors (Gwo, 2000; Alfaro et al. 2001; Lezcano et al. 2004; Vuthiphandchai et al. 2007). One group of spermatophores was submitted to DMSO and the other to EG, both at 10% concentration and an equilibration time of 10 minutes, based on their prior toxicity tests. For each treatment, the spermatophores were divided in four sub-groups for storage in liquid nitrogen for 0, 30, 60, 90 days. The time zero treatment consisted of immersion and immediate withdrawal of the samples in the liquid nitrogen. The treatments were carried out in triplicate, and each triplicate was composed by a pool of three spermatophores (Table 1).

Table 1 - Experimental design for cryopreservation of spermatophores of *Litopenaeus vannamei*. Cryoprotectants used Dimethylsulfoxide (DMSO) and Ethyleneglycol (EG) were tested during different time periods.

Treatment (cryoprotectant)	Cryopreservation period (days)	Number of spermatophores
Control	-	2
DMSO	0	9
	30	9
	60	9
	90	9
EG	0	9
	30	9
	60	9
	90	9

Sample preparation for cryopreservation

Aliquots of 500 µL were obtained from the prepared sperm cell suspensions as above mentioned and transferred afterwards to cryotubes duly identified containing 500 µL of a freezing solution (5% of egg yolk and 0.2 M of sucrose) (Lezcano et al. 2004). Later, 25 µL of cryoprotectants were added (DMSO or EG to the 10% final concentration) and the solution was left to stabilize for 5 minutes; next, 25 µL of cryoprotectants were added, following the treatment directions to complete the 10% final concentration, totalizing 50 µL of cryoprotectants. The control was performed with the cellular suspensions containing a freezing solution (5% of egg yolk and 0.2 M of sucrose) without the addition of cryoprotectants. The material was taken to the pre-freezing stage in which cryovials was maintained 3.0 cm above the liquid nitrogen layer for about 5 minutes as proposed by Akarasanon et al. (2004), and immediately submerged in liquid nitrogen where they were stored in the containers at -196ºC. The containers were replenished periodically for the maintenance of an adequate liquid nitrogen level.

Thawing

Thawing after varied time intervals (0, 30, 60 and 90 days) was performed by withdrawing the bottles from the containers and immersing them in 20-25ºC water for 40 seconds, based on the methodology of Akarasanon et al. (2004). After the homogenization and re-suspension in a solution of 0.2 M sucrose, the material was prepared for the assessment of cell viability.

Assessment of cell viability

The sperm solution samples were divided in the aliquots for viability assessment using the semen smears eosine-nigrosine stained according to Jeyalectumie and Subramoniam (1989) and flow cytometry analysis (FACS), following the protocol proposed by Lezcano et al. (2004). For the first one, 25 µL of eosin (0.5%) was added to 25 µL of nigrosin (10%) in 50 µL of sperm solution. The 100 µL solution was transformed in a smear, stained in a microscope blade and air-dried for observation in a 400 x magnifying optic microscope. Eosin-stained cells (rosaceous) were considered dead while there was no coloration in the live cells, but rather a translucent condition. The survival average percentage was obtained by counting a minimum of 100 cells per blade (two repetitions per sample). For the flow cytometry, a new sample collection of spermatophores was conducted, following the same procedure to be used as reference sample of fresh material in the

flow cytometer so that it could later recognize the sperm cell standard to be assessed.

Once the FACS conditions were optimized, thawed cell suspensions were analyzed after incubation with propidium iodide (PI) (Sigma 5 $\mu g.mL^{-1}$) in the dark for 10 min at atmospheric temperature, as directions in the protocol of the manufacturer. The cells with breached membranes were penetrated by the PI and emit fluorescence, while complete cells could not be marked. Approximately 20,000 events were analyzed in each sample, and the fluorescence was analyzed through a 610 nm filter. The data registered in the FACS was analyzed with the *FlowJo* 7.5.6 versionsoftware and expressed as survival percentages.

Statistical analyses

Data on percentages obtained in the cryoprotectant toxicity and survival tests after cryopreservation through flow cytometry and eosine-nigrosine stain were transformed in arcsine and submitted to the Variance Analysis in Repeated measures ANOVA ($\alpha<0.05$). After a significant difference could be seen between the treatments, the Student Newman Keuls (SNK) test was used for the separation of averages ($\alpha<0.05$). The correlation analysis between the two methodologies was analyzed through the Spearman coefficient with a significance level of 0.05 (Zar 1999).

RESULTS AND DISCUSSION

The cryoprotectant toxicity pretests results displayed no significant difference in the viability of sperm cells between DMSO and EG cryoprotectants at 5 and 10% concentrations and equilibration times of 5 and 15 min (Fig. 1). However, there was a reduction in cell survival when the samples were exposed to the cryoprotectants for 30 min. Similarly, significant reductions (p=0.0042) were observed when the 20% concentration of cryoprotectant was used in all the equilibration times.

Different equilibration times (10, 20, 30 and 60 min) were tested with several cryoprotectants for the *Penaeus monodon* semen (Vuthiphandchai et al. 2007). In the present work, the mortality of sperm cells changed as the cryoprotectant concentrations and exposition times increased and the optimal concentration and equilibration time were 10% and 10 minutes, respectively.

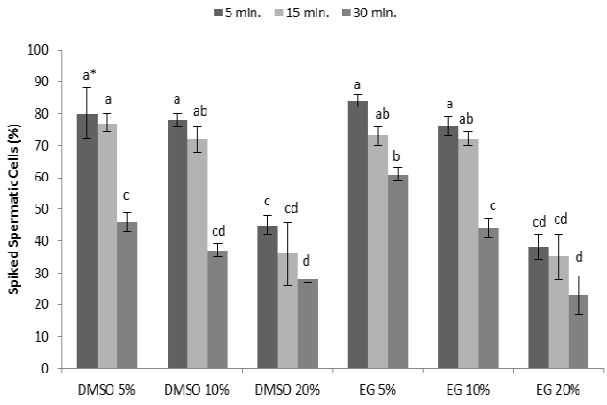

Figure 1 - Integrity of sperm cells *Litopenaeus vannamei* after exposure to cryoprotectants dimethylsulfoxide (DMSO) and ethylene glycol (EG) at different concentrations (5%, 10% and 20%) for different equilibration times (5, 15 and 30 min). Cells with spike presence were considered intact. Different letters indicate significant differences between treatments by SNK test for separation of means ($p<0.05$).

Barth et al. (2006) while working with the giant tiger prawn, *P. monodon*, used DMSO at 5 and 10% as cryoprotectant. Results showed that DMSO at 5% offered better results when sperm survival reached 79.7%. An increase in the DMSO concentration to 10% resulted a reduction in sperm survival. The toxic potential of cryoprotectant agents in sperm survival obtained in this study was also found by Chow et al. (1985). After testing seven different equilibration times (0, 10, 15, 30, 60, 120 and 180 minutes) with 10% glycerol for *Macrobrachium rosenbergii*, they concluded that the equilibration times were of utmost importance to guarantee a total penetration of the agent in the biological material. However, they emphasized that an extended contact of sperm cells with the cryoprotectant agent could cause a negative effect, multiplying its toxicity, since after 60 minutes of equilibration time the viability decreased significantly. This was because after an extended exposition to cryoprotectants, the sperm cells started to degrade them, generating potentially toxic metabolites, a limiting factor for a successful utilization (Fahy 2010).

The mortality estimates obtained through the application of the eosine-nigrosine stain technique were similar for DMSO and EG cryoprotectants (p=0.496) at the 10% concentration. For the two

cryoprotectants applied and the two techniques used to assess the cell viability, the decrease in cell survival as cryopreservation times increased was evident, which meant that the cell mortality increased as the time of material immersion in the liquid nitrogen was extended (Fig. 2). Statistical analyses of flow cytometry data also revealed no significant differences between DMSO and EG cryoprotectants (p=0.0524) (Fig.3).

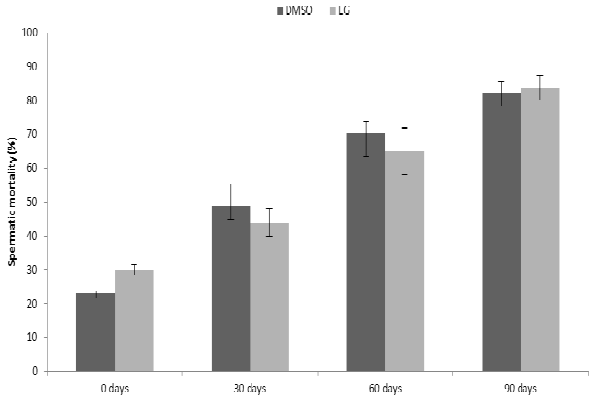

Figure 2 - Mortality percentage of *Litopenaeus vannamei* spermatic cells after application of the eosine-nigrosine stain technique, after cryopreservation with dimethyl sulfoxide (DMSO) and ethylene glycol (EG) during different periods (0, 30, 60 and 90 days).

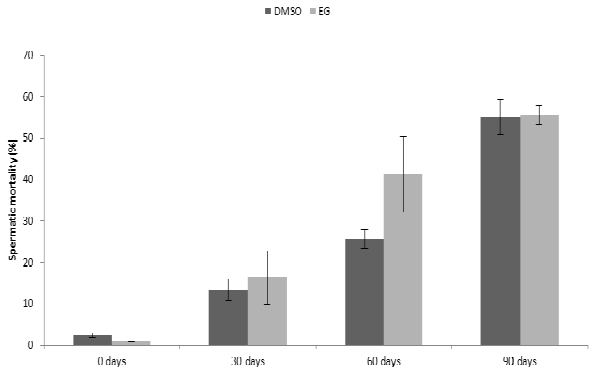

Figure 3 - Positive *Litopenaeus vannamei* spermatic cells for Propidium Iodide (PI positive, considered injuried or dead after application of the flow cytometry technique) after cryopreservation with dimethyl sulfoxide (DMSO) and ethylene glycol (EG) along different time intervals (0, 30 60 and 90 days).

Semen storage time was relatively short if practical needs and the applications of cryopreservation were considered, since their function was to keep the viable material stored and available for long periods of time. However, taking into consideration the experimental character of the study, few works in this area have tested time intervals above 30 days of freezing, since they were in general interested in generating cryopreservation protocols and not in assessing the freezing effect on the long term. Thus, the protocols must be better investigated and described, mainly, so that they could be reapplied to avoid the randomness of results. It could be said that this study was a relevant step in the assessment of protocols as well as of the effects of cryopreservation through different time intervals on the semen of penaeidae shrimps, such as studies of Diwan and Shoji (1999) and Nimrat et al. (2008).

As a counterpart for this test, in which the survival difference between DMSO and EG in both tests for the cell viability was not observed, Anchordoguy and collaborators (1988) reported a higher sperm survival when they used DMSO as cryoprotectant in comparison with other agents (glycerol, trehalose, sucrose and proline) for the *Sycionia ingentis* sperm. A more recent study by Salazar et al. (2008), as well as this study also found no significant differences between the cryoprotectant agents, viz. DMSO, glycerol, methanol and ethylene glycol at 10%, working with the sperms of *Litopenaeus vannamei.* In a study conducted with *M. rosenbergii,* the sperm quality displayed a significant decline under the cryopreservation at -20ºC with EG at 20%. When submitted to -196ºC, the decline was more (Akarasanon et al. 2004), a fact that could be also related to the toxic potential of cryoprotectants when submitted to higher temperatures.

S. serrata shrimp spermatophores were cryopreserved at -196ºC for up to 30 days, displaying a sperm survival of 89% assessed by the eosine-nigrosine stain technique and by the utilization of DMSO as cryoprotectant (Jeyalectumie and Subramonian 1989). These survival values were better than this study although assessed by the same eosine-nigrosine technique (51.11%). However, they displayed similar results when survival was assessed by the flow cytometry (86.63%). It must be emphasized that the above-mentioned test used complete

spermatophores and not the cell suspensions as in the present study.

The Spearman test revealed a positive correlation (0.91) between the two methods of assessment applied for sperm integrity ($p<0.05$). Although cell integrity analysis obtained through flow cytometry displayed higher survival results, both have demonstrated identical mortality profiles after an elapse of cryopreservation time. Direct comparison of results between the distinct studies can be problematic, if not impossible, due to the high variability in sample preparations, stain methods and protocols of flow cytometry (Tiersch and Green 2011). The same authors report that among the 15 studies that had evaluated membrane integrity by the use of fluorescent marker SYBR 14/ Propidium Iodide in the last 10 years, there were considerable variations, or fails in the reports of details about the procedures such as sperm concentration, stain concentration, and how other time parameters were gathered such the time elapsed between the collection, or thawing and the analysis itself, treatment intervals, and the time interval between the stain and the flow cytometry assessment.

In fact, during the cytometry analyses, there was a certain difficulty in identifying a single homogeneous cell population that could characterize a unique cell type (in this case, *L. vannamei* sperm cells). One of the possible reasons for this limitation could be the method used to obtain the cellular suspension, in which the complete spermatophore was agitated in a vortex motion to stimulate the release of cells. However, fragments of the spermatophore membranes themselves got loose and joined the cell suspension. Such fragments might have been caught by the flow cytometer together with the cells and resulted in this heterogeneity of populations registered by the cytometer. Thus, the methods that can keep the samples more homogeneous must be explored such as filtering, or separation of the material that ensure that only sperm cells are assessed.

Another fundamental aspect is related to the way the biological material itself is submitted to low temperatures. Lezcano et al. (2004) assessed cryopreservation protocols and results of complete spermatophores, sperm masses and cell suspensions and obtained better results concerning the preservation of the complete spermatophore by using methanol at 10%, with both the techniques of morphotype analysis and flow cytometry. This indicated that the membrane involving the spermatophore might be protecting the sperm cells submitted to cryopreservation, while in suspension, cells were more exposed to thermal shock and changes in cell osmolarity and in the environment.

In general, cryoprotectant agents used as well as equilibration times and freezing rates, which share a definitive role in the cryopreservation success, seem to be well defined for certain species. However, the biggest problem seems to be survival analysis and cell viability. The positive correlation found between the application of eosine-nigrosine stain and flow cytometry techniques indicated that both the techniques could be complementary. However, some methodological adjustments should be considered, from the operational time needed to align the flow cytometer conditions to the time of sample exposition to atmospheric temperatures and stain intervals. In fact, simultaneous handling of many samples makes it difficult the execution of each step in optimal time conditions, generating a range of results in cryopreservation studies.

Finally, emphasis should be given to the fact the limited success obtained in many species of penaeidae reproduction in captivity could be related with reproductive problems in the males, since most studies with shrimps describe females, a gap to be fulfilled with the development of studies concerning spermatophore production and sperm quality (Nimrat et al. 2005) as well as of biotechnologies of assessment for sperm quality.

In conclusion, there was no difference observed between the two cryoprotectants tested, DMSO and EG on results for cell survival, Cell viability decreased as storage time in liquid nitrogen increased and flow cytometry and eosine-nigrosine stain techniques were highly correlated, indicating that both could be used with high probability for confidence in the analyses of sperm cells viability. Due to the considerably lower cost of the eosine-nigrosine technique, it still seemed to be the more advantageous technique for the objectives considered in this work.

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