Sperm motility and fertilization performance of *Nodipecten nodosus* (L., 1758) exposed at two different cryoprotectants

Fausto Silvestri[1,2], Valentina Vitiello[2], Francesco Del Prete[2], Adele Fabbrocini[3], Jaime Fernando Ferreira[4] & Giovanni Sansone[2]

[1]FIPERJ – Fundação Instituto de Pesca do Estado do Rio de Janeiro, Angra dos Reis, Brazil

[2]Laboratorio di Fisiologia Ambientale e degli Organismi Acquatici & Laboratorio di Criobiologia, Università degli studi di Napoli Federico II, Napoli, Italy

[3]Istituto di Scienze Marine – UOS Lesina, Consiglio Nazionale delle Ricerche, Lesina, Italy

[4]Laboratório de Moluscos Marinhos, Universidade Federal de Santa Catarina, Florianópolis, Brazil

Correspondence: F Silvestri, FIPERJ – Fundação Instituto de Pesca do Estado do Rio de Janeiro, Rua do Comércio n°10, CP 23980-560, Centro, Angra dos Reis (RJ), Brasil. E-mail: silvestrifausto@hotmail.com

Abstract

Cryopreservation is a valuable tool for aquaculture as it provides continuous seed production, regardless of the spawning season of the brood stock. The selection of a suitable cryoprotectant with low toxicity and high water solubility is important to avoid membrane injuries and intracellular ice crystallization. This study has been aimed at the assessment of the toxic effects of two usually applied cryoprotectants, 1-2 propylene glycol (PG) and methanol (MetOH), on spermatozoa of the of lion-paw scallop *Nodipecten nodosus,* by evaluating the sperm motility and the development of D larvae after fertilization procedure. Sperm was exposed at room temperature (22°C) for 10, 20 and 30 min to different concentration ranges of two cryoprotectants. Regarding the sperm motility, PG5%, PG7%, MetOH4% and MetOH6% did not show differences compared to control (semen incubated in seawater) ($P < 0.05$). The development of D larvae was not affected by the exposition to PG5%, MetOH 4% and MetOH 6%. These results indicate the potential use of both cryoprotectants for cryopreservation procedures.

Keywords: lion-paw scallop, 1-2 propylene glycol, methanol, cryopreservation

Introduction

Cryopreservation of sperm has been well established for many years in several finfish species, but only in a limited number of shellfish (Chao & Liao 2001). Researches involving semen cryopreservation of marine molluscs are scarce and mainly focused on oysters and abalones (Gwo 2000). Regarding scallops, there are few researches on this topic, most of them developed with Asian species (Xue & Xiang 1995; Yang, Wan, Kong, Liu, Liu & Wang 1999; Li, Li & Xue 2000; Yang, Yang, Liu & Zhou 2007). Other studies were developed with the scallop *Argopecten purpuratus* in Chile (Dupré & Espinoza 2004; Espinoza, Valdivia & Dupré 2010), but only preliminary studies were carried out with the lion-paw scallop *Nodipecten nodosus* (Reis, Pauls, Cabezas & Castro 2003; Zeni, Sotelo, Pauls & Serafim-Junior 2009).

Nodipecten nodosus (Lmk, 1816) has a great economic potential and is commercially produced in the Caribbean and Latin America (Lovatelli, Farías & Uriarte 2008). In Brazil, this species presents an annual production of 14 tons mainly in Rio de Janeiro State (MPA 2011). Currently the seeds production and supply are the main impediments to the expansion of mariculture in Brazil (Rupp, Oliveira-Neto & Guzensky 2008).

In commercial hatcheries, the gamete cryopreservation can provide major benefits such as selective breeding, regular availability of gametes throughout the year, gametes protection from hazards and diseases. It can also facilitate the stocks transportation between hatcheries, improve genetic breeding programmes, reduce costs of brood stock conditioning and provide an important

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tool for protecting the endangered species (Tsai & Lin 2012). Therefore, in the light of these considerations, the optimization of a protocol for the cryopreservation of *N. nodosus* gametes could be of a great benefit for its positive outcomes on the artificial reproduction of this species.

However, cryopreservation protocols are species-specific and all steps need to be tailored for the single biological system to be cryopreserved (Holt 2000a). Cryoprotectant agents (CPAs) are commonly used in cryopreservation procedures to avoid membrane injuries and intracellular ice crystallization. Low molecular weight, high water solubility and mainly a low cellular toxicity are the ideal physicochemical characteristics that these agents should present (Nash 1966): the addition of penetrating cryoprotectant agents at seminal fluids, in effect, may result in cytotoxic effects impairing factors as the sperm motility, cell viability and fertilization capacity (De Baulny, Labbe & Maisse 1999; Holt 2000b). The toxicity of intracellular cryoprotectants mainly depends on their concentration and association with diluents, on the equilibration time and the temperature during loading (Chao & Liao 2001).

Therefore, performing of toxicological tests with cryoprotectants in pre-freezing step is essential to select the most effective one, able to preserve sperm quality, which keeps thus characteristics similar to those it had before the exposition. This kind of preliminary study is then an essential step to develop an effective protocol and ensure the success of cryopreservation procedures.

Substances such as 1-2 propylene glycol (PG) and methanol (MetOH) are commonly used as cryoprotectants to freeze marine organisms' gametes. These substances showed good results in the male gametes freezing of fish (Gwo, Weng, Fan & Lee 2005; Marco-Jiménez, Garzón, Peñaranda, Pérez, Viudes-de-Castro, Vicente, Jover & Asturiano 2006; Tian, Chen, Ji, Zhai, Sun, Chen & Su 2008), oysters (Paniagua-Chavez & Tiersch 2001; Dong, Huang, Eudeline & Tiersch 2005; Dong, Huang, Eudeline, Allen & Tiersch 2006; Kawamoto, Narita, Isowa, Aoki, Hayashi, Komaru & Ohta 2007; Adams, Smith, Taylor, Tervit & McGowan 2013) and mussels (Di Matteo, Langellotti, Masullo & Sansone 2009), but at this moment the effects of these substances on the physiological parameters of *Nodipecten nodosus* spermatozoa are still unknown.

Thus, this study has been aimed to verify the toxicological effects of these cryoprotectants to spermatozoa of the lion-paw scallop *N. nodosus* in relation to different concentrations and exposure periods, to set up a cryopreservation protocol for this biological system.

Materials and methods

Animals

Experiments were performed in December 2012 with mature scallops collected in a commercial farm situated at Porto Belo, Santa Catarina State, southern of Brazil (27°07′S, 48°32′W). The scallops were kept in a recirculation aquaculture system under controlled conditions (water temperature $18 \pm 1°C$; salinity 35 ± 1 psu) to maintain the gonads at the ripe stage until the experiments were performed; for this aim, the scallops were fed daily a mixture of *Isochrysis galbana* and *Chaetoceros calcitrans* with a total concentration of 5×10^9 cells ind^{-1} day^{-1}, ratio 1:4 in continuous flux.

The monitoring of gametes quality was carried out during all experimentation to assess the effect of rearing conditions on sperm fitness. Daily observations were performed to observe the presence of spawning in the water.

Gametes collection

Sperm and eggs were obtained from scallops induced to spawn by thermal shock cycles (Rupp & Poli 1994). Gametes were separately collected from each animal and immediately filtered using a 18 μm mesh for spermatozoa and 30 μm one for eggs; subsequently, they were diluted in UV-sterilized seawater (SSW, previously filtered through 0.45 μm filters) up to an adjusted concentration of 30×10^6 cells mL^{-1} for males and 3×10^3 mL^{-1} for females, for further analysis.

Assessment of semen and eggs quality

The evaluation of semen quality was performed immediately after spawning, on samples of sperm suspension in SSW (temperature $22 \pm 1°C$, salinity 35 ± 1 psu). Samples of 10 μL were placed on a glass microscope slide, without a cover slide and motility was assessed by subjective analysis by Olympus ITM2 microscope with phase-contrast lens (200× total magnification). Semen quality was evaluated in terms of motility, on the basis of

the percentage of RVF (rapid, vigorous and forward moving) motile spermatozoa.

The sperm samples were video recorded and then motility was visually assessed by each of three independent and trained workers. The sperm samples with similar motility characteristics were used to constitute sperm pools of similar quality. Only sperm pools with motility percentage greater than 70% and coming from at least five scallops were used in the experiments.

The quality evaluation of female gametes was performed by macro and microscope analysis. The macroscopic analysis was performed by observation of morphological aspects of the female gonadal portion (Sühnel, Lagreze, Bercht, Ferreira, Carneiro-Schaefer, Magalhães & Maraschin 2010) and microscopic analysis was based on the morphological aspects of oocytes as tawny in colour and of rounded shape (De la Roche, Marín, Freites & Vélez 2002).

Effects of cryoprotectants on sperm motility

The pool of the selected spermatozoa was incubated at 1:10 (v v^{-1}) with SSW and added to the following cryoprotectants (Sigma–Aldrich, Saint Louis, MO, USA): 1–2 propylene glycol (PG) in final concentrations of 5%, 7%, 10% and 15% (v v^{-1}) and methanol (MetOH) in final concentrations of 4%, 8% and 10% (v v^{-1}). After 10, 20 and 30 min of incubation at room temperature (22 ± 1°C), the evaluations of sperm motility were performed. Semen samples incubated in SSW without CPAs at the same temperature for 10, 20 and 30 min were used as control group. The results were expressed as percentage of RVF motile spermatozoa (mean ± standard deviation values).

Effects of cryoprotectants on the fertilization sperm performance

The effect of CPAs on the fertilization performance was evaluated on sperm suspensions diluted and incubated as previously described. At the end of each incubation time, the sperm aliquots were added to egg suspension at a rate of 20:1 in 50 mL test tubes for the fertilization. Egg and sperm suspensions without CPAs were again used as the control group.

One hour after fertilization, the suspensions were checked under microscope for the presence of cleavage (Velasco, Barros & Acosta 2007). The

embryos were counted and distributed at a concentration of 10 mL^{-1} in triplicate test tubes containing seawater and incubated for 24 h at room temperature (22 ± 1°C). At the end of this time, 0.5 mL of buffered formalin (4%) was added to the test tubes and the embryos were examined for abnormalities. The number of normally and abnormally developed embryos was counted in a Sedgwick Rafter chamber by microscope (100×). The results were expressed in percentage of normal D-shaped larvae on sample aliquots of 200 μL.

Statistical analysis

Each experiment, carried out in triplicate, was repeated at least three times ($n \geq 9$). Statistical analyses were performed using *Statistica 8.0*. A paired sample *t* Student's test was used to analyse significant differences between two sample groups (Zar 2010). One-way ANOVA was used to evaluate the effects of different CPAs concentrations with the same equilibration times. A value of $P < 0.05$ was chosen as the level for significance.

Results

Effects of cryoprotectants on sperm motility

The controls, represented by *Nodipecten nodosus* sperm activated in SSW without the presence of cryoprotectants, showed intense motility exhibiting more than 80% of RVL spermatozoa and keeping these motility values also after 30 min from dilution/activation (Fig. 1).

Spermatozoa exposed to 5% and 7% PG showed values of RVL motility higher than 60% also after 30 min of incubation. 1-2 propylene glycol was significantly toxic compared to control when added at equal or greater than 10% concentrations ($P < 0.05$): significant reductions in the percentage of RVL motility were observed after 10 and 20 min of incubation with 10% PG and they increased after 30 min. The 15% PG was significantly toxic compared to control group ($P < 0.01$) already after 10 min of exposure, with values of RVL motility lower than 30%.

Regarding the MetOH, this cryoprotectant did not produce any apparent effect on the sperm motility after 30 min of exposure when used at final concentration of 4%, while at 6%, it induced a falling of sperm motility after 20 min of incuba-
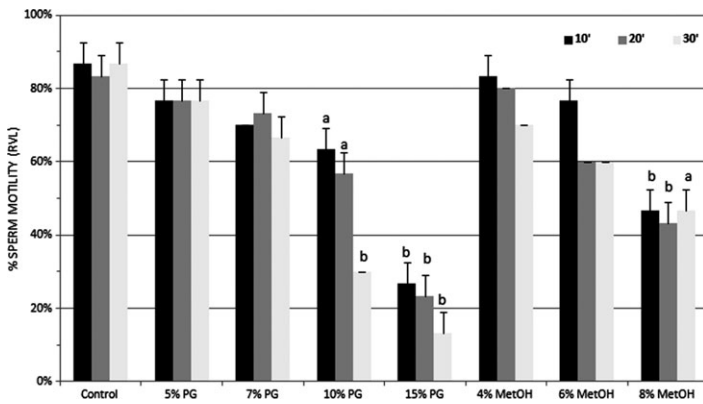
Figure 1 Effects of propylene glycol (PG) and methanol (MetOH) on lion-paw scallop sperm motility (mean ± SD, $n \geq 9$) after 10, 20 and 30 min of exposure. Columns represent the exposure times (min). Control represents motility of semen not exposed to CPAs. Bars with different letters indicate significant differences ($P < 0.05$).

tion. The motility of sperm exposed to 8% MetOH was significantly lower than the control after all incubation times. At this concentration, high percentages of not motile spermatozoa were registered.

Effects of cryoprotectants on the fertilization sperm performance

Fertilization procedures performed with semen not exposed to cryoprotectans resulted in values of 73 ± 7%, 71 ± 8% and 68 ± 5% of normal shaped D larvae formation respectively, after 10, 20 and 30 min from activation (Fig. 2).

The cryoprotectants showed distinct effects on *N. nodosus* D larvae formation. No significant differences compared to control group were observed in percentage of normal shaped D larvae obtained from semen exposed to PG at 5% concentration. Higher concentrations of PG produced significant decreases in percentages of normal D larvae ($P < 0.05$) with values lower than 50%, the toxic effect was showed already after 10 min of sperm incubation and the increase in exposure time did

not induce any significant decrease in percentages of normal shaped D larvae. Fertilization procedures carried out with semen exposed to 4% and 6% MetOH produced more than 50% of normal shaped D larvae also after 30 min of incubation. Similar values of normal shaped D larvae also were recorded using semen exposed to 8% MetOH for 10 min but, using this concentration, rates of normal D larvae decreased to 28% and 27% after 20 and 30 min of exposure respectively.

Discussion

Cryoprotectants are known to be able to avoid most part of cryoinjuries, however at high concentrations, they become toxic to biological material (Leung & Jamieson 1991): they can cause deleterious effects in the osmotic balance or several biochemical lesions such as inactivation or denaturation of specific enzymes, disruption of transmembrane ionic pumps, or other related perturbations of cellular structure and function, by implication, are most likely due to the direct interaction of the cryoprotectant with proteins and
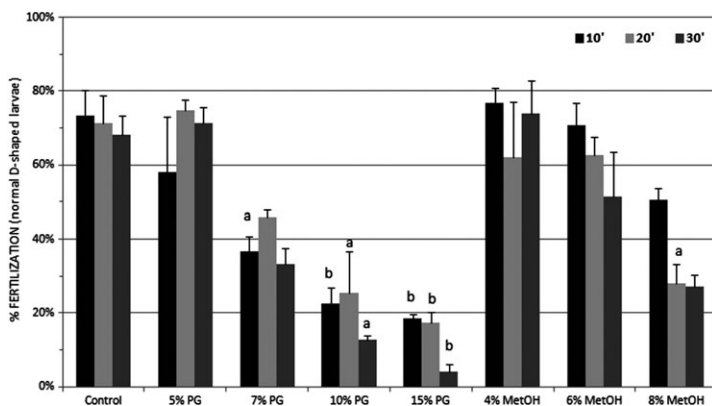

Figure 2 Effects of semen exposure to propylene glycol (PG) on lion-law scallop normal shaped D larvae formation (mean ± SD). Columns represent the exposure times (min). Control represents the percentage of normal shaped D larvae fertilized by semen not exposed to CPA. Bars with different letters indicate significant differences ($P < 0.05$).

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biological membranes (Arakawa, Carpenter, Kita & Crowe 1990; Gwo 2000; Espinoza *et al.* 2010). On the other hand, the toxicity level of each cryoprotectant is species-specific and no one can be defined overall or absolutely more toxic than another (Holt 2000a; Chao & Liao 2001; Gwo, Chen & Cheng 2002). Therefore, the assessment of their level of toxicity is a fundamental step during the honing of a cryopreservation protocol.

Dimethylsulphoxide and glycerol are the most common cryoprotectants used in the cryopreservation of sperm from aquatic species, giving interesting results also for some bivalve species (Adams, Smith, Roberts, Janke, Kaspar, Tervit, Pugh, Webb & King 2004; Lyons, Jerry & Southgate 2005; Acosta-Salmón, Jerry & Southgate 2007); moreover, several studies have been conducted using PG and MetOH for the sperm cryopreservation of marine species (Fabbrocini, Lavadera, Rispoli & Sansone 2000; Hubálek 2003; Zhang, Zhang, Liu, Xu, Wang, Sawant, Li & Chen 2003; Yang, Carmichael, Varga & Tiersch 2007), including some bivalve and scallop species (Xue & Xiang 1995; Gwo *et al.* 2002; Acosta-Salmón *et al.* 2007; Di Matteo *et al.* 2009; Adams *et al.* 2013), even if sometimes with contrasting results.

PG and MetOH could be useful to protect *Nodipecten nodosus* spermatozoa from structural and physiological injuries occurring during freezing process. In fact, in this study, the toxic effects of these cryoprotectants on motility and fertilization performance of *N. nodosus* sperm were clearly observed only in the higher concentrations of the tested cryoprotectants.

1-2 propylene glycol until 7% concentration did not show any significant effect for all exposure times when sperm motility was evaluated but, on the other hand, produced a strong reduction in percentage of normal D larvae compared with control group already after 10 min of incubation, showing a low conformity between the two parameters.

A lower molar mass and a quick cellular permeability make MetOH toxic already at low concentrations, thus this cryoprotectant is usually tested using percentages lower than other cryoprotectants. Nonetheless, *Nodipecten nodosus* spermatozoa showed good tolerance at MetOH, producing values of normal D larvae higher than 50% after 30 min exposure at concentrations until 6%, but giving good values of normal D larvae also at higher tested concentration after 10 min of exposure. In addition, samples of semen exposed to MetOH showed a better fertilization performance with higher values of normal D larvae when compared to PG.

A trend similar to that recorded in this study was observed for Mediterranean mussel *Mytilus galloprovincialis* sperm exposed to increasing concentrations of PG (Di Matteo *et al.* 2009) with a progressive reduction in sperm motility. In the European flat oyster *Ostrea edulis*, the motility of sperm exposed to PG at concentrations from 5% to 15% was significantly lower than motility of samples exposed to MetOH at concentrations from 4% to 10% (Vitiello, Carlino, Del Prete, Langellotti & Sansone 2011). The same trend was registered for cryopreserved semen of the Pacific oyster *Crassostrea gigas* (Dong *et al.* 2006).

Regarding the PG, positive results were recorded in experiments with sperm of eastern oyster *Crassostrea virginica* (Paniagua-Chavez & Tiersch 2001): the highest fertilization rate was obtained with 10% PG (v v^{-1}), although the highest motility was found with 15% PG (v v^{-1}). Also Reis *et al.* (2003) observed up to 70% of progressive sperm motility after thawing using lower concentrations of PG and dimethyl sulfoxide (Me$_2$SO), although lower survival rates were observed. For the mangrove oyster *Crassostrea rhizophorae*, MetOH was more toxic than PG in the assessment of normal D larvae obtained from normal oocytes fertilized using exposed sperm (Nascimento, Leite, Araújo, Sansone, Pereira & Espírito-Santo 2005; Sansone, Nascimento, Leite, Araújo, Pereira & Mariani 2005). Similar results were obtained on *Crassostrea gigas*: normal oocytes were not fertilized by sperm cryopreserved using MetOH, while sperm frozen using PG at 5% and 10% concentrations achieved fertilization rates higher than 20% (Tervit, Adams, Roberts, McGowan, Pugh, Smith & Janke 2005). Moreover, the MetOH, as well Me$_2$SO, showed good results for the cryopreservation of the Chinese scallop *Chlamys farreri* sperm (Xue & Xiang 1995).

Finally, expressive results were found on sperm cryopreservation of Japanese pearl oyster *Pinctada fucata martensii* using MetOH at 10% concentration (v v^{-1}) (Kawamoto *et al.* 2007).

In contrast with what reported above, Molinia and Swan (1991) observed a decrease in the radius of curvature and track velocity of *Saccostrea commercialis* sperm exposed at 2.5% and 5%

MetOH, and PG resulted very toxic for small abalone *Haliotis diversicolor supertexa* sperm at 5% concentration (Gwo *et al.* 2002).

Scarce sperm motility values were recorded also for black-lip pearl oyster *Pinctada margaritifera* using PG as cryoprotectant (Lyons *et al.* 2005; Acosta-Salmón *et al.* 2007), even if, in these studies the cryoprotectants toxicity before thawing was not evaluated.

It is worth to point out as a successful cryopreservation protocol depends on the balance of a series of factors, not only the type and concentration of the cryoprotectant but also the type of diluent and its interaction with cryoprotectant, and the rates of cooling, freezing and thawing (Gwo 2000; Chao & Liao 2001), so that for each cryopreservation protocol a cryoprotectant can perform better in respect of another as a consequence of the integrate effect of all the involved factors, resulting sometimes in very different outcomes.

For this reason, all the key phases of a cryopreservation procedure need to be specifically optimized, each time taking into account the interaction of the various factors involved.

On the basis of the results obtained in this study, future efforts should be directed towards the elaboration of cryopreservation protocols for *N. nodosus* sperm using both the tested cryoprotectants, taking into account their different effects on the evaluated parameters. In fact, while PG showed lower toxicological effects in respect of MetOH for sperm motility parameter, methanol exhibited better results in the normal shaped D larvae formation.

Despite these results represent an important baseline to development of a new cryopreservation protocol, the effectiveness of protection of both cryoprotectants in the freezing and thawing steps yet to be assessed to find the adequate cryoprotectant to *N. nodosus* sperm.

Acknowledgments

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