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Cryopreservation: Extending the viability of biological material from sea urchin (*Echinometra lucunter*) in ecotoxicity tests

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ABSTRACT

The sea urchin, *Echinometra lucunter,* is widely used in embryo-larval tests for ecotoxicological studies in Brazil and other countries. For each test, sea urchins are collected from the wild and this can cause impact on wild populations and it is limited by the weather and season which in turn limits the ability to carry out the tests. Cryopreservation is a method of live biological material storage at low temperature and can be used for long periods with little decline in viability, reducing the number of animals taken from the wild and enabling testing to be carried out on demand, irrespective of spawning season or location. In this study, 15 combinations of cryoprotective agents (CPAs) were evaluated on spermatozoa, subjected to a rapid cooling curve followed by immersion in liquid nitrogen. Twenty-four CPA combinations were evaluated on eggs subjected to a more gradual cooling curve in nitrogen vapor down to −35 °C and then plunging in liquid nitrogen. Fertilization tests using cryopreserved spermatozoa gave high pluteus larvae yields (≈80%) when concentrations of 10.5% or 13.65% ME2SO or 13.65% ME2SO + 15.75% sucrose were used. The higher concentrations of ME2SO plus sucrose were more effective at maintaining the fertilization capacity of spermatozoa post-thawing. Egg cryopreservation was not successful with 0% fertilization observed post-thawing. The results suggest that it is feasible to implement spermatozoa cryopreservation as technological innovation to create a sperm bank for *E. lucunter,* which can be used in ecotoxicological tests, bringing benefits for researches and contributing to the conservation of the species.

1. Introduction

Aquatic ecotoxicity tests are an effective means to evaluate environmental contamination [13,23] and play an essential role in studies of impacts that chemicals cause to the environment [29]. Recently, in the past few decades, it has gained recognition in Brazil and worldwide as providing biological relevance to chemical data and enriching environmental contamination studies [39]. The sea urchin, *Echinometra lucunter*, is often used in ecotoxicological tests to monitor water pollution [13,40]. It sensitivity at early developmental stages; ease of collection, fast life cycle [13,16,22,31,40], and wide distribution on the Brazilian coast make it an ideal model species for such tests. Unlike most ecotoxicity tests that use organisms cultivated in the laboratory, sea urchins are collected from the environment [1], therefore can cause impact on wild populations and can be limited by weather and season to have high quality biological material and potentially limiting laboratory work. The ability to successfully cryopreserve E. *lucunter* spermatozoa and eggs would benefit ecotoxicology and academic research by enabling the laboratory storage and later use of excess gametes for new research or ecotoxicological tests [2,35]. This technique makes the biological material available at any time of the year and at any location, thereby contributing to reduction of its collection from the environment, to consequent preservation of the species, and at the same time allowing provision of gametes to ecotoxicological laboratories.

Previous studies present different protocols for cryopreservation of tissues, gametes, embryos and larvae for animal breeding and reproduction, and for species conservation [2,6,10,12,33]. In contrast to mammals, fish, and plants, cryopreservation studies of marine invertebrates are limited to a smaller number of cell types and species [35,36]. Protocols are generally species and cell type specific; thus, it is

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important to emphasize that there have been no previous studies involving the cryopreservation of *Echinometra lucunter*, the target species of this study. The usual water toxicity tests using invertebrate marine organisms to evaluate the environmental contamination levels are widely studied and present biological meaning to chemical data, complementing the existing studies on environmental contamination [8,15,39]. However, the availability of high-quality biological material throughout the year is still a limiting factor for this technique [9,17].

Successful cryopreservation generally requires cells to be suspended in solutions containing either permeating or non-permeating cryoprotective agents (CPAs) or both, to prevent intracellular ice formation and reduce damage to cells from shrinkage, dehydration and exposure to high solute concentrations [18,27,37]. Most cryoprotective agents currently used are permeating cryoprotectants. That is, chemicals capable of penetrating the cell membrane. When used, there are two important procedures to follow: (i) gradual addition of the CPA before freezing, and (ii) gradual removal after thawing. Exposure time is also important to allow for equilibration, but also to avoid toxicity. Non-permeating cryoprotectants, such as trehalose and sucrose, are generally less toxic and provide protection by stabilizing cell membranes and reducing the salt concentrations cells are exposed to at a given temperature [18]. The rate of cooling and thawing is also important. Cells cooled too quickly generally freeze ice intracelllulary whereas cells cooled too slowly are exposed to high solute concetrations [26]. Simarlarly, it is now generally accepted that thawing cells rapidly is beneficial, reducing the likelihood of devitrification and recrystallization damage [26]. Thus, the purpose of this study was to investigate the preservation of the gametes of *Echinometra lucunter* using cryo-preservation techniques, and to attempt to evaluate the results in this context firstly, and secondly to consider the application of the results.

In this study the effects of different CPAs solution for sperm and for eggs were tested during cryopreservation technique, with an addition method, a freezing temperature curve, a thawing temperature, and spermatozoa concentration (10^8) used for fertilization were evaluated to try to develop protocols for *E. lucunter* gametes. Altogether, five trials were performed in 2015 using separate semen pools from the same broodstock batch, to test twenty-four treatments (interactions between extenders and cryoprotectants concentrations) for female gametes and fefteen treatments (interactions between extender and cryoprotectant concentrations) for male gametes. Trials were carried out in five replicates.

2. Materials and methods

2.1. Organism collection

Adult *E. lucunter* were collected from the rocky shore of Curva da Baleia, at Serra, Espírito Santo (Brazil), and transported in an insulated box to the aquatic ecotoxicology laboratory of Aplysia, at Vitória, Espírito Santo, Brazil. They were placed in tanks containing artificial seawater and maintained under ideal conditions at a temperature of 25 °C, a salinity of 35% with natural photoperiod (12:12) and constant aeration. Broodstock were held for at least 24 h before being used in experiments and were fed with algae collected from the same area at Curva da Baleia during this time.

2.2. Gamete collection

Gamete release was induced by injecting 2–4 mL of 0.5 M potassium chloride (KCl) into the perioral region of each animal (Standard ABNT No. 15350/2012). Animals were placed upside down over beakers containing either artificial seawater or autoclaved artificial seawater and left until spawning. Once spawning, sex was determined from the color of the gametes being released (sperm = white, oocytes = orange, Fig. 1). Males were removed from the water once spawning had begun and sperm collected as concentrated as possible from the aboral region

Fig. 1. Determining sex from the color of the gametes being released. Male (on the left) is white, female (on the right) is orange. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

using a Pasteur pipette. Spermatozoa were then transferred to an Eppendorf microtube (2 mL), and stored on ice to keep them inactive until ready to be used in experiments.

The concentration of eggs to be used for fertilization was defined using the ABT NBR 15350:2012 method. For the male gamete sampling, the dilution consisted of removing a 0.5 mL volume of concentrated and fresh spermatozoa and adding a 24.5 mL volume of artificial seawater. A 0.05 mL volume of fresh SPTZ was removed from this dilution and, then, a 4.85 mL volume of artificial seawater was added to it. The total volume of the solution for the fertilization tests was 5 mL. The concentration of spermatozoa was defined using a Neubauer chamber, with a dilution of a thousand times and, then, a 0,1 mL volume of iodinated lugol was added to fix the spermatozoa. Eggs were freely released from the gonopores and sank to the bottom of the beaker. The quality of the eggs released by each female was validated under a microscope and any that were considered unacceptable (e.g. small size, unrounded, translucent) were not collected. The material was pooled, filtered and siphoned to remove impurities and kept at room temperature (15 °C). Eggs were diluted with artificial sea water at a rate ~2000 eggs/mL. Gametes from at least three males and three females randomly selected were combined for each trial to ensure genetic variability. Eggs concentration were determined using a Sedgwick-Rafter chamber, with a dilution of a hundred time. Sperm concentration was determined by counting a known dilution of fixed sperm on a Neubauer haemocytometer.

2.3. CPA solutions

The concentrations and types of CPA evaluated were selected based on previous studies with marine invertebrates [2–6,11,19,30,32,34]. Reagents were obtained from Sigma-Aldrich Chemicals and Vetec and solutions were prepared with artificial seawater with 35% salinity.

For sperm, 15 CPA solutions were prepared using dimethyl sulfoxide (Me_2SO) as a permeating CPA, with or without trehalose (TRE) or sucrose (SC) as non-permeating CPAs (Table 1). For oocytes, 24 CPA solutions were prepared using the permeating CPAs: ethylene glycol (EG), propylene glycol (PG) and ME2SO with or without TRE.

2.4. Cryopreservation

2.4.1. Spermatozoa

Sperm held on ice, were diluted 1:20 with CPA solutions in four fixed molar steps, 5 min apart to avoid osmotic stress [18]. Diluted sperm at a concentration of 10^9 mL^{-1} were then loaded into 0.25 mL plastic straws and sealed with PVC powder. Straws were then placed on an aluminum rack that had been pre-cooled on ice. Then, the aluminum

Table 1
The concentrations of CPA tested in gamic material. The concentrations selected for the current study are highlighted in bold. TRE = Trehalose; SC = Sucrose.

	CPA	Individua Concentration (%)	Mix Concentration (%)	Dilution Water
Sperm	*Me_2SO (ME2SO)*	**7.35, 10.5, 13.65**	+ **TRE 5.25, 15.75**	**Artificial Seawater**
		7.35, 10.5, 13.65	+ **SC 5.25, 15.75**	
Egg		14, 20	+ TRE 60	Artificial seawater, distilled water
	PG	14, 20	+ TRE 60	Artificial seawater, distilled water
	EG	20	+ TRE 60	Distilled water, Artificial seawater

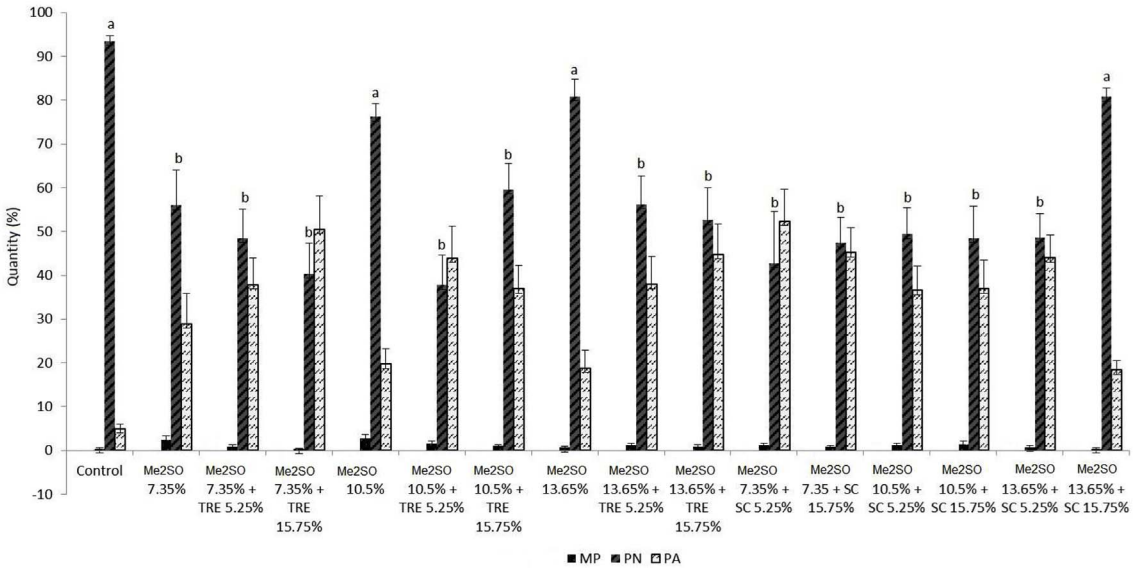

Fig. 2. Post-thaw rate of embryo-larval development after 42 h (morula to prism stage - MP, normal pluteus - PN, and abnormal pluteus - PA). Rate of normal pluteus larvae for 7.35%, 10.5% and 13.65% ME2SO associated or not to trehalose or sucrose after thawing and fertilization of fresh eggs diluted in artificial seawater at 35%. The control sample evaluated the capacity of fertilization and development of normal pluteus larvae from fresh spermatozoa diluted in artificial seawater at 35%. Data are expressed as the mean ± standard error. Values with different superscript letters are significantly different ($p < 0.05$; Dunnett's test).

rack was inserted into a freezing system, partially filled with LN_2 in. This gave a cooling rate of ~15 °C min^{-1}. After 10 min of cooling, the straws were immersed in N_2L and transferred to the cryovessel, in which they were stored (at least an hour) until thawing. The straws were thawed in water bath at 21 °C–22 °C for 30 s and CPA was removed by adding ASW in 3 fixed steps, 5 min apart to avoid osmotic injury.

2.4.2. Eggs

Aliquots of concentrated eggs were diluted 1:1 with each test CPA solution at room temperature. The solution was added in three fixed molar steps that were 5 min apart to reduce the risk of osmotic injury. Diluted eggs (~2000 mL^{-1}) were then aspirated into 0.25 mL straws (5 replicates per treatment) and sealed with PVC powder. The TK 4000 programmable freezer (TK Equipamentos para Reprodução®, Brazil) was programmed to cool the eggs at a temperature curve that started at 4 °C, followed by a 2 min rest, then a cooling rate of 1 °C min^{-1} to −12 °C, followed by a 2 min rest, and then at a cooling rate of 0.5 °C min^{-1} to −35 °C followed by a final rest of 2 min; followed by plunging in liquid nitrogen at the end. For the egg thawing, the straws were immersed in a water bath at 21 °C–22 °C for 30 s. CPA was removed by adding ASW (35% salinity) in three fixed molar steps, 5 min apart [18].

2.4.3. Fertilization capacity

The fertilization test was used to assess the fertilization capacity of sperm and eggs. Three controls were performed: to evaluate the fertilization capacity of the egg in artificial seawater before cooling (control 1), to evaluate the fertilization capacity of the egg in autoclaved seawater (to prevent contamination) before cooling (control 2), and to evaluate the fertilization capacity of the sperm before cryopreservation (control 3). To evaluate cryopreserved sperm, fresh eggs were used in fertilization tests. However, no tests were performed on thawed eggs since 100% of the eggs were lysed upon thawing. Parallel was carried an embryo development test with fresh material, having taken 0.1 mL/h of material incubated to evaluate on optical microscope development of fertilized material until complete 42 h of incubation.

For each trial, a volume of 0.1 mL was removed from a concentrated fresh egg suspension and added to 4.85 mL of artificial seawater. Sperm were diluted (10^6 concentration) 50 fold in artificial seawater and then 0.05 mL of diluted sperm suspension was added to the eggs to give a total volume in the fertilization test of 5 mL. The test was concluded after 42 h of incubation, and samples were fixed by formaldehyde buffered with borax, and then analyzed using an optical microscope. Analysis of the microscope slides comprised 100 embryos/replicates and visual classification based on embryonic development, as follows: morula to prism stage (MP), normal pluteus (PN) and abnormal pluteus (PA).

The results were expressed in quantity of number of normal pluteus larvae calculated for each treatment. For cryopreserved spermatozoa treatments, an analysis of fertilization capacity was performed. The test was concluded after 42 h of incubation.

2.5. Statistical analysis

Statistical analyses were performed using Sigma Stat 12.5. Data were checked for variance normality and homogeneity. The mean values for the treatments were compared by analysis of variance (ANOVA) followed by Dunnett's test. The significance level applied was at 95%

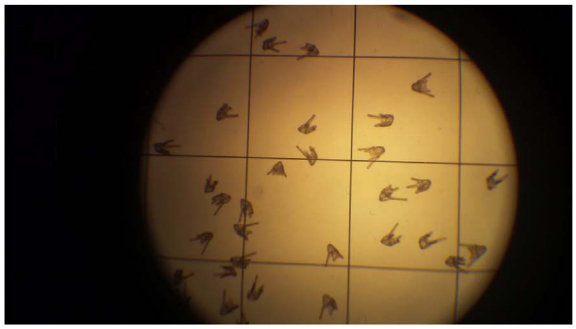

Fig. 3. Optical microscope image after 30 h incubation of fresh material fertilization on development embryo-larval test on a Sedgewick Rafter Chamber.

($\alpha = 0.05$).

3. Results

3.1. Sperm cryopreservation

Cryopreservation of sperm reduces fertilization capability (Fig. 2). During the embryo-larval development, the normal pluteus larvae rate was low except for treatments with 10.5% ME2SO, 13.65% ME2SO, and 13.65% ME2SO + 15.75% SC, in which the rates were lower than 20%. Observing the embryo-larval development of the control group, it was noticed that the fertilized egg reached pluteus larvae stage 30 h after fertilization (Fig. 3). However, when cryopreserved sperm were used, embryos were still developing after 42 h of incubation (Fig. 2).

The concentration of ME_2SO affected the success of the cryopreservation, as observed in the treatments with 7.35% ME_2SO with or without TRE, in which the results were inferior when compared to control samples (Fig. 2). Similar results were found for treatments with 10.5% and 13.65% ME_2SO plus TRE. The treatments with 10.5% and 13.65% ME2SO concentration resulted in approximately 76% and 80% of normal pluteus, respectively (Fig. 2).

The treatments with ME_2SO plus sucrose significantly differed from the control samples (Fig. 2), except for the treatment with 13.65% ME_2SO + 15.75% SC, which resulted in approximately 80% of normal pluteus larvae (Fig. 2).

3.2. Egg cryopreservation

For the *E. lucunter* eggs, the temperature happened to be a limiting factor in the cryopreservation process, as 100% of the eggs lysed at temperatures below −10 °C.

4. Discussion

The study developed a method of storage in laboratory for *Echinometra lucunter* spermatozoa, aiming at overcoming the seasonality of these organisms and reinforcing the use of embryo-larval biotests with sea urchins for assessing water contamination levels. According to Fabbrocini [17], the success of the cryopreservation protocol depends on interaction of many variables. Therefore, an experimental design involving multiple factors allows a more efficient search towards the best conditions for success. The sensitivity of the cryopreservation process varies according to the species and the cell type studied [24,25,37]. However, it is possible to extrapolate the protocol from one species to others, by adjusting the cryopreservation steps according to the result from each species studied. The spermatozoa cryopreservation protocol presented by Fabbrocini [17] was extrapolated and adjusted, and proved to be quick and easy to be carried out. It was the most efficient for the species studied (*Echinometra lucunter)* than to the *Paracentrotus lividus* species.

4.1. Egg cryopreservation

The egg has proven to be extremely sensitive to temperature variations. When lower than −10 °C, these temperatures caused cell membrane rupture even in the presence of cryoprotectants tested in different concentrations and associated or not to trehalose. Thus, it has proved to be impossible to cryopreserve eggs of the species studied based on this methodology. Previous studies on cryopreservation of mussel eggs, the *Perna canaliculus* [4,28], suggest that this cell type is particularly difficult to cryopreserve. Invertebrate eggs present high sensitivity to temperatures below zero as well as to exposures to cryoprotective agents [41]. Cryopreservation studies with oysters (*Crassostrea gigas* and *Crassostrea rhizophorae*) show that, the more advanced the fertilized egg development, more resistant it becomes to the cryopreservation process [19,30].

4.2. Spermatozoa cryopreservation

The success of the spermatozoa cryopreservation protocol needs to ensure the cell recovery (physiologically and morphologically) after thawing, to ensure higher rates of fertilization capacity and normal embryonic development until the pluteus larval stage is reached. In this study, a cryopreservation protocol for the sea urchin (*Echinometra lucunter*) spermatozoa was developed, to allow its storage in laboratories. Special attention was given, in this study, to conservation of the spermatozoa functions after cryopreservation. The analysis of the sperm function included the fertilization capacity, as well as the capability of originating normal pluteus larvae after fertilization.

The recovery of physiological and morphological functions after thawing results from a response that combines multiple factors, such as type and concentration of the cryoprotectant, cooling rates, freezing rates, and thawing rates [10,14,20].

Results showed that the highest ME_2SO concentration (10.5% and 13.65%) was more effective for maintaining the fertilization capability of the spermatozoa after freezing and thawing in water bath than the lower concentrations, which goes against the results proposed by Adams [2], according to whom the best fertilization capacity rates were found applying the lowest ME_2SO concentrations (2.5%–7.5%). In such concentrations (10.5% and 13.65%), it was also possible to observe higher rate of normal pluteus larvae (approximately 80%), as well as with 13.65% ME_2SO + 15.75% SC concentration; thus, obtaining an association between the permeating and non-permeating CPAs, as well as protecting the spermatozoa against injuries during the cryopreservation steps. Such results are still higher than those presented by Fabbrocini [17], who obtained 50% of normal pluteus larvae after the cryopreservation steps.

According to the Standard ABNT NBR [1], the ecotoxicological test results are considered valid if the well-developed pluteus larvae percentage at control sample is equal to or greater than 80%. Thus, the results were satisfactory for the application of the technique in the ecotoxicological tests. The low fertilization capacity of the spermatozoa for other treatments can be result from the injuries that may have occurred either during cryopreservation or previous steps. Cryopreservation itself can generate slow, abnormal cells. According to Mazur [25] and Pegg [37], the sensitivity to the cryoprotective agent and to the freezing curve varies according to the species and the cell type studied, as there is also variation in the responses throughout the cryopreservation process. According to Bellas and Paredes [9], it is important for the temperature to be gradually decreased so as to avoid cell lysis. Another important factor for success of the technique is the use of the most concentrated spermatozoa in cryopreservation so as to reach the best fertilization rates once the semen is heated [2,3,20,21]. This is a common practice not only for aquatic species [2,3,20,21], but it is also used for mammals, having become common practice nowadays [7].

5. Conclusion

It is possible to conclude that the studies aiming at enabling egg storage in laboratory are scarce due to the challenge of working with this cell type, which hinders the development of an efficient technique. More studies have to be performed regarding egg storage period at −10 °C to determine the maximum storage period for the *Echinometra lucunter* species.

With adjustments in the steps of the spermatozoa cryopreservation protocol and adaptations to the species studied, it is possible to extrapolate cryopreservation protocols among different sea urchin species.

The *E. lucunter* spermatozoa proved to be a rather resistant cell under a cryopreservation process, developing normal pluteus larvae after water-bath thawing and fertilization. For treatments that reached a rate of normal pluteus higher than 80%, it is possible that such biological material may be used for ecotoxicological tests [38], in order to evaluate environmental parameters, such as effluent monitoring, which brings new benefits for research and contributes to the preservation of the species.

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