Cryobiology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/cryo

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

Raphaela Cantarino Ribeiro[a,*], Alexandra Caroline da Silva Veronez[a], Thaís Tristão Tovar[a], Serean Adams[b], Dayse Aline Bartolomeu[c], Clayton Peronico[d], Tatiana Heid Furley[a]

[a] Instituto Aplysia, Rua Júlia Lacourt Penna n 335, Jardim Camburi, 29090-210, Vitória, ES, Brazil
[b] Cawthron Institute, 98 Halifax Street East, Nelson, 7010, New Zealand
[c] Instituto Federal do Espírito Santo – IFES, Rua Augusto Costa de Oliveira, 660, Praia Doce, 29285-000, Piúma, ES, Brazil
[d] Instituto Federal do Espírito Santo - IFES, Avenida Rio Branco, 50, Santa Lúcia, 29056255, Vitória, ES, Brazil

ARTICLE INFO

Keywords:
Technological innovation
Echinodermata
Preservation
Cryopreservation
Cryoprotective agents
Spermatozoa
Egg
Marine invertebrates

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

* Corresponding author.
E-mail addresses: raphaelacr2@gmail.com, raphaela@institutoaplysia.org (R.C. Ribeiro), alexandra@institutoaplysia.org (A.C. da Silva Veronez), thais@aplysia.com.br (T.T. Tovar), serean.adams@cawthron.org.nz (S. Adams), daysealine@hotmail.com (D.A. Bartolomeu), cperonico@ifes.edu.br (C. Peronico), tatiana@institutoaplysia.org (T.H. Furley).

http://dx.doi.org/10.1016/j.cryobiol.2017.10.002
Received 1 February 2017; Received in revised form 9 October 2017; Accepted 10 October 2017

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 is white, female 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