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Cryobiology 51 (2005) 113–117
CRYOBIOLOGY
www.elsevier.com/locate/ycryo

Brief Communication

Selection of cryoprotectants based on their toxic effects on oyster gametes and embryos ☆

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Received 12 January 2004; accepted 14 April 2005
Available online 15 June 2005

Abstract

Cryopreservation is a valuable tool for aquaculture by providing continuous seed production, regardless of the spawning seasons. This study aimed to select the least toxic among the cryoprotectants dimethyl sulfoxide (Me_2SO), propylene glycol (PG), and methanol (MET) based on their toxicological effects on *Crassostrea rhizophorae* gametes and trochophores. They were exposed for 10, 20, and 30 min to a range of concentrations of those cryoprotectants. The end-point was EC15–24 h (effective concentration which causes abnormalities in 15% of the population exposed to the cryoprotectants for 24 h), recently determined as the chronic value (the concentration at which chronic effects are first observed) for *C. rhizophorae* embryonic phases. There were no significant differences ($p > 0.05$) among the exposure times in Me_2SO toxic effects to either gametes or trochophores. For MET, the increase in exposure time resulted in higher toxicity for gametes, but not for trochophores, while for PG there was a significant ($p > 0.05$) increase in toxicity with the increase of exposure for trochophores and spermatozoa, but not for oocytes. For gametes, MET was the most toxic among the cryoprotectants, while PG was the most toxic for trochophores.

Keywords: Cryoprotectants; Toxic effects; Cryopreservation protocols; Oyster gametes; Trochophores

The feasibility of commercial cultivation of the mangrove oyster *Crassostrea rhizophorae* depends on the conditioning of brood stock, to obtain seeds through hatchery practices. Cryopreservation of gametes or embryos would help to avoid the costs

☆ Statement of funding: Grant No. 910230/00-00 from CNPq/CNR (Bilateral Agreement Brazil/Italy).
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0011-2240/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.cryobiol.2005.04.006

necessary to manage the conditioning brood stock. For seed production, thawing of cryopreserved material necessarily follows the cryopreservation. The addition of cryoprotectants can minimize cell damage associated with ice formation [5,11]. Permeability and low toxicity are the most important properties inherent to these substances. Both properties are associated with biological characteristics and may vary largely among organisms. Therefore, to select the best cryoprotectant, it is important to determine the concentration and exposure time that minimize toxicological effects to the specific biological material to be cryopreserved.

Techniques for cryopreservation of bivalve gametes and embryos still need to be refined [9]. Different protocols for cryopreservation of spermatozoa have been suggested even for related species such as *Crassostrea gigas*, *Crassostrea cucullata*, *Crassostrea iredalei*, and *Crassostrea tulipa* [13]. The important variable in these protocols is the cryoprotectant toxicity, which is related to its concentration and exposure. The aim of this study was to determine the toxicity of different cryoprotectants to *C. rhizophorae* gametes and embryos in terms of their concentration and exposure times, to contribute for the cryoprotectant selection before the establishment of the cryopreservation protocols.

Mature oysters *C. rhizophorae* were collected at Barra dos Carvalhos, Bahia (13039′05″S; 38057′59″W), an area free from industrial or domestic waste. Oysters were brought to the laboratory, and kept overnight in filtered and aerated seawater, at the same salinity level (28 ppt) and temperature (26 ± 10 °C), as in the field. Gametes were obtained by stripping the oysters. Eggs and sperm were placed separately into 2 L glass beakers containing sterilized glass fibre (GF/C) and filtered-seawater (maintained at field temperature and salinity level). Oocyte density in the suspension was determined by counting three samples taken under agitation with a perforated plunger. The density was then adjusted to 10^4 oocytes/L [12]. Part of each gamete suspension was reserved to generate embryos (from unexposed gametes), utilized as control in the experiments; the other part was used to provide gametes for the exposure tests. These consisted in exposing the gametes for 10, 20, and 30 min to cryoprotectants (dimethyl sulfoxide, propylene glycol, and methanol), diluted (v/v) in sea water, at concentrations of 0 (control), 5, 10, 15, and 20%. The exposed oocytes were fertilized by unexposed sperm and vice versa. Fertilization was done by adding 2 ml of a concentrated (turbid) sperm suspension to the oocyte suspension. One hour after fertilization, the suspensions were checked at light microscopy for the presence of cells in division. The embryos from both origins were counted and distributed at a concentration of 10/ml in triplicate test tubes containing seawater and left for 24 h at room temperature (26 ± 20 °C). After this period, 0.5 ml of buffered formalin (4%) was added to the test tubes and the material was examined for abnormalities. The number of normally and abnormally developed embryos was counted.

Trochophores were obtained 8 h after fertilization of unexposed gametes. They were exposed for 10, 20, and 30 min to the cryoprotectants dimethyl sulfoxide—Me_2SO, propylene glycol (PG), and methanol (MET), diluted in sea water (v/v) at concentrations of 0 (control), 5, 10, 15, and 20%. After exposure, the trochophores were retained in a plankton net (36 μm size), washed, and kept in triplicate test tubes, at a density of 10/ml of sea water (28‰, 26 °C) for 24 h. The trochophores were then preserved by the addition of formalin to the test tubes and the abnormal and normal D-shaped larvae were counted under the microscope. Response to the different treatments were recorded as the percentage of embryos failing to develop (or developing abnormally), in relation to the maximum number of D larvae expected ($n = 100$), in the absence of any treatment [6,7].

Bivalve embryos are defined as the stage between fertilized egg and the ciliated trochophore. Normal larvae are defined as being perfectly D-shaped, at the Prodissoconch I stage. Abnormal larvae had irregular or misshapen shells, completely or incompletely formed. In the statistical analysis those embryos remaining at the end of the 24 h test were also counted as abnormal, since they did not develop to D larvae, as would normally be expected in 24 h [12]. The percentage abnormalities were calculated as the % net risk, according to Finney [2]. Critical values, represent-

ing the estimated chronically safe concentrations (EC15–24 h) for each cryoprotectant were determined by the statistical method ICp (inhibition concentration for a percent effect), version 2.0, edited by Norberg-King [8]. These critical values are considered as the chronic effects concentrations, determined as point estimate interpolated from cryoprotectant concentrations, at which chronic effects are first observed. Those critical values for each cryoprotectant, and for different exposure periods (10, 20, and 30 min), were arc-sin transformed and compared by ANOVA (SPSS). Multiple range tests (SNK) have permitted comparison of cryoprotectants, which could indicate the most or the least toxic products to *C. rhizophorae* gametes and trochophores.

The obtained results for *C. rhizophorae* support data from previous authors [1,4] who reported that the trochophore stage was more resistant to cryoprotectants than the earlier embryos stages or gametes. In fact, in this study, the EC15–24 h for trochophores treated with Me_2SO, PG or MET was found to range from 7.34 to 16.58% (Fig. 3), values which are comparatively higher than for gametes (Figs. 1 and 2). This helps to explain why cryopreservation of oysters embryos is generally more successful than oyster gametes, whose oocytes present a high level of difficulty to be cryopreserved [1]. The present study also shows that trochophores had a relative different pattern of sensitivity than the gametes. Propylene glycol (PG) was the most toxic to the trochophores at 30 min of exposure (EC15–24 h = 7.34). Its chronic toxic value was significantly different ($p < 0.05$) from all the other values obtained for the different cryoprotectants and exposure (Fig. 3), showing a very clear increase in toxicity with exposure time. The EC15–24 h values for MET were not significantly different ($p > 0.05$) from each other when different exposure times have been compared. Both MET and Me_2SO were less toxic for the trochophore stage (Fig. 3).

These results agree with previous findings that cryopreservation methods cannot necessarily be applied intra or inter-specifically between embryos and larvae [10]. In relation to cryopreserved spermatozoa of various oysters species, Yankson and Moyse [13] found evidence of interspecific differences in the retention of viability, related not only to the concentration of the cryoprotectant, but also to the exposure time.

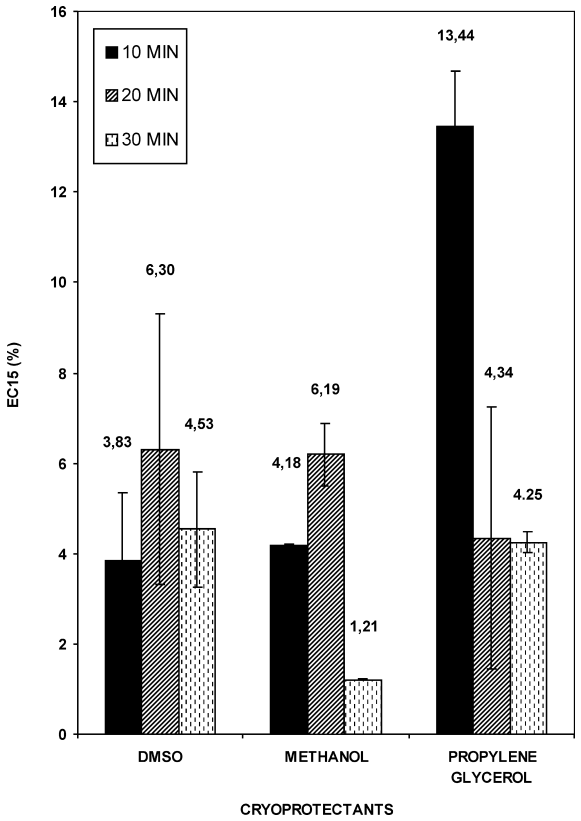

Fig. 1. Chronic effect levels (EC15–24 h values expressed as percentage), for *C. rhizophorae* embryos originated from unexposed oocytes and spermatozoa exposed for 10, 20, and 30 min to dimethyl sulfoxide (Me_2SO), methanol (MET), and propylene glycol (PG).

In terms of exposure time the present research has shown varying statistical differences in effects within the range of 10–30 min for the same cryoprotectant, dependent on the used biological material (spermatozoa, oocytes or trochophores). The variation of exposure time in cryopreservation protocols is mostly related to the nature of the cryoprotectant and the test species. However, in most of these protocols a time of 10–30 min is considered favourable [5].

The effects of the three cryoprotectants used in this experiment on *C. rhizophorae* spermatozoa showed that MET was significantly ($p < 0.05$) the most toxic

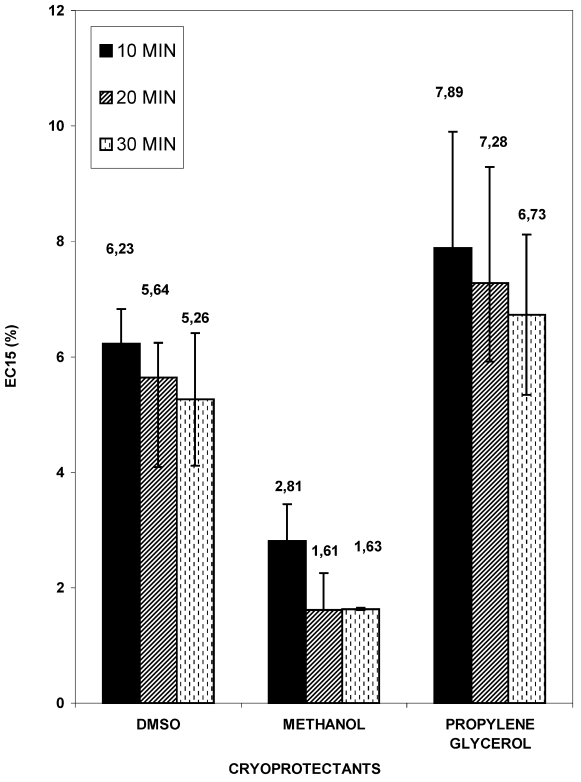

Fig. 2. Chronic effect levels (EC15–24 h values expressed as percentage), for *C. rhizophorae* embryos originated from unexposed spermatozoa and oocytes exposed for 10, 20, and 30 min to dimethyl sulfoxide (Me_2SO), methanol (MET), and propylene glycol (PG).

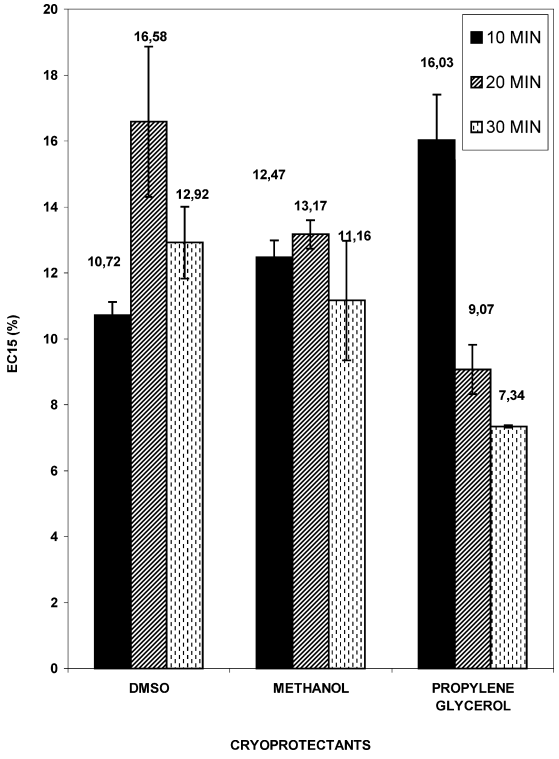

Fig. 3. Chronic effect levels (EC15–24 h values expressed as percentage), for *C. rhizophorae* larvae originated from trochophores exposed for 10, 20, and 30 min to dimethyl sulfoxide (Me_2SO), methanol (MET), and propylene glycol (PG).

after 30 min exposure (EC15–24 h = 1.21%) while Me_2SO and PG showed similar EC15 values (Fig. 1). The chronic values (EC15–24 h) for the three cryoprotectants obtained under exposure of 20 min did not differed significantly ($p > 0.05$). At 10 min of exposure, PG was the least toxic cryoprotectant, EC15 = 13.44%, significantly ($p < 0.05$) differing from the ones obtained for Me_2SO (EC15 = 3.83%) and MET (EC15 = 4.18%).

Similar response trend was obtained for oocytes (Fig. 2). Methanol was the most toxic among the three cryoprotectants, showing values of EC15–24 h equal to 2.81, 1.61, and 1.63, respectively, under exposures of 10, 20, and 30 min, not differing significantly ($p > 0.05$) from each other. However, there were significant differences ($p < 0.05$) in toxicity between MET, Me_2SO and PG. The higher values of EC15 found for Me_2SO (6.23, 5.64, and 5.26) and for PG (7.89, 7.28, and 6.73) under exposure of 10, 20, and 30 min respectively did not differ significantly from each other, but were significantly different ($p < 0.05$) from the obtained values for MET, indicating their lower toxicity to oocytes.

The results found here provide not only the chronic toxic levels (EC15–24 h values) for Me_2SO, MET, and PG, but also permitted a comparison of effects between three of the most used commonly cryoprotectants. The chemical and physical properties of most cryoprotectants are listed by Nash, 1966 (cited in [5]). Of these properties, water solubility and low toxicity are the most important for cryopreservation purposes. Dimethyl sulfoxide (Me_2SO) inhibits catalase and peroxide activity. However, its permeability is not markedly affected by low temperature and is, therefore, the most widely used per-

meating cryoprotectant. Propylene glycol decreases the polarity of the aqueous phase and change the partition of hydrophobic molecules between the cell membrane and the external phase, causing dehydration of the phospholipid bilayer and possible membrane damage [5]. The cell membrane is generally highly permeable to methanol, but it is generally considered the most toxic cryoprotectant. In the present research, MET was the most toxic for gametes among the used cryoprotectants, while for trochophores, a different toxicity trend was shown where PG was more toxic than Me_2SO or MET when the exposure time was 20 or 30 min.

Cryoprotectants can suppress most cryoinjuries but, when used at higher concentrations, most of them become toxic to biological material [5]. According to Freshney [3] the cryoprotectants can be effective when used at concentrations between 5 and 15%. In the present research the concentrations that caused a chronic effect to the exposed gametes varied from 1.21 to 13.44%, while for trochophores the variation was in a range of 7.34–16.58 according to a combination of concentration and exposure time.

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