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Survival of Pacific oyster, *Crassostrea gigas*, oocytes in relation to intracellular ice formation ☆

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Abstract

The effect of IIF in Pacific oyster oocytes was studied using cryo and transmission electron microscopy (TEM). The viability of oocytes at each step of a published cryopreservation protocol was assessed in an initial experiment. Two major viability losses were identified; one when oocytes were cooled to −35 °C and the other when oocytes were plunged in liquid nitrogen. Although the cryomicroscope showed no evidence of IIF in oocytes cooled with this protocol, TEM revealed that these oocytes contained ice crystals and were at two developmental stages when frozen, prophase and metaphase I. To reduce IIF, the effect of seven cooling programmes involving cooling to −35 or −60 °C at 0.1 or 0.3 °C min^{-1} and holding for 0 or 30 min at −35 or −60 °C was evaluated on post-thaw fertilization rate of oocytes. Regardless of the cooling rate or holding time, the fertilization rate of oocytes cooled to −60 °C was significantly lower than that of oocytes cooled to −35 °C. The overall results indicated that observations of IIF obtained from cryomicroscopy are limited to detection of larger amounts of ice within the cells. Although the amount of cellular ice may have been reduced by one of the programmes, fertilization was reduced significantly; suggesting that there is no correlation between the presence of intracellular ice and post-thaw fertilization rate. Therefore, oyster oocytes may be more susceptible to the effect of high solute concentrations and cell shrinkage than intracellular ice under the studied conditions.

Keywords: *Crassostrea gigas*; Intracellular ice formation; Cryomicroscopy; Transmission electron microscopy

Intracellular ice formation (IIF) is one of the most harmful factors that affect cell survival during cryopreservation; it occurs during rapid cooling when cells are no longer able to maintain equilibrium with their extracellular environment [29]. A number of factors influence the probability and temperature at which IIF will occur. These include the presence and concentration of a cryoprotective agent (CPA), rate of cooling and degree of supercooling inside cells, as well as the cell's membrane permeability characteristics and water volume [16,29]. Three mainstream hypotheses have been proposed to explain the mechanisms of IIF (for detailed review see [29]). In brief, intracellular ice could be seeded into the cytoplasm through protein pores that allow the growth and propagation of small ice crystals, or if the plasma membrane lacks integrity and is disrupted, or due to intracellular nucleation when the cell is super cooled.

Cryomicroscopy allows real time observation of events that take place during cooling and warming of cells. In oocytes and other cell types, IIF has been previously described as a "flashing" or "darkening" of cells [2,4,12,16,18,19,25,41]. It is presumed that this blackening is a result of the incidence of light from the microscope that is reflected and refracted by many small highly branched crystals [32,40].

Besides cryomicroscopy, other methods to assess IIF in the frozen state are available, such as freeze substitution

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[5,13,28,34]. When freeze substitution and electron microscope observations are combined, these two techniques may provide means to establish a relationship between structural cryoinjury due to IIF or dehydration during freezing and thawing [34]. It is necessary to clarify that, when employing the freeze substitution technique, "ice" refers to spaces that are left after substitution with organic solvent has occurred.

In the present investigation, the viability of oyster oocytes cryopreserved using a previously published protocol [39] was tested at each step of the protocol to determine the key steps at which oocytes were damaged. Cryomicroscopy and freeze substitution in combination with transmission electron microscopy helped to determine whether IIF had occurred in oocytes and the effect of cooling rates, holding times and plunging temperatures on IIF and post-thaw fertilization.

Materials and methods

Sources of reagents

Unless otherwise stated, all reagents used for cryopreservation and electron microscopy were analar grade or higher. Ethylene glycol (EG) was obtained from Sigma–Aldrich (St. Louis, MO, USA). Glutaraldehyde (electron microscopy grade) was sourced from Electron Microscopy Sciences (Hatfield, PA, USA); osmium tetroxide, Quetol-615 resin and nonenyl succinic anhydride (NSA) were sourced from Pro. Sci. Tech. (Qld., Australia); methyl nadic anhydride (MNA) and 2,4,6-tris dimethylaminomethyl phenol (DMP-30) were sourced from Agar Scientific Ltd. (Essex, UK); uranyl acetate was sourced from Agar Scientific Ltd. (Stansted, Essex, UK) and lead citrate was sourced from Laurylab, Leica (Austria); solvents were sourced from BDH Ltd. (Poole, UK). Filtered seawater (FSW) was obtained by passing previously sand filtered (1 μm) seawater through a 0.22 μm Millipore membrane filter.

Collection of gametes

Ripe Pacific oysters were obtained from marine farms throughout New Zealand and maintained at the Glenhaven Aquaculture Centre in Nelson, New Zealand. For experimental work, oysters were transported overnight to the Department of Food Science, University of Otago in Dunedin, New Zealand. Gamete collection was performed upon arrival of the oysters. Oocyte recovery was performed as described by Tervit et al. [39] with some modifications. Briefly, oocytes were obtained by stripping the gonad. Oyster sex and gamete quality were assessed under the light microscope. Recovered oocytes were transferred into 250 ml glass beakers, re-suspended in 100 ml of FSW at ambient temperature (~23 °C) and allowed to settle for approximately 30 min in a refrigerated water bath at 5 °C. The settled oocyte suspension was filtered through a 74-μm mesh and then placed in 50 ml Falcon tubes (Becton Dickinson, Franklin Lakes, NJ) at 5 °C. Depending on the experiment, oocytes from individual females were kept separately or pooled. For fertilization assays, sperm was collected by "dry" stripping of the gonad and kept at 5 °C until use. In most cases, sperm from at least two males was pooled and used for fertilization assays.

Assessment of oocyte damage in the cryopreservation process

In the present study, an initial experiment was designed to assess the steps in a published cryopreservation protocol [39] (for convenience, this published protocol will be referred to as the standard protocol) at which the oocytes lose their viability. Oocytes were collected as described previously and were concentrated to a density of 2×10^6 ml^{-1}. Oocyte viability (% fertilized) was assessed at each of the following steps of the cryopreservation process: (a) after loading with CPA and equilibration for 10 min at 20 °C, (b) after cooling to −10 °C at 1 °C min^{-1} and before seeding, (c) after a 5 min hold at −10 °C and seeding, (d) after cooling to −35 °C at 0.3 °C min^{-1}, and (e) after plunging into liquid nitrogen. A control treatment was included by fertilizing fresh oocytes. For this experiment, three pools containing oocytes from three different females per pool were tested.

To assess the effect of loading and unloading CPA (step a), an aliquot of oocyte suspension was diluted 1:1 with 20% EG in deionized water in 10 steps of equal volume each minute for 10 min at ambient temperature (~21 °C). The final concentration of EG in the equilibration medium was 10%. The solution was left to equilibrate for 10 min. After equilibration, the CPA was removed by diluting the incubated solution 1:1 with filtered seawater (FSW), and left for 30 min, following a further dilution (1:9) with FSW for another 30 min prior to fertilization [39].

To assess the effect of the remaining steps of the standard protocol (steps b–e) on oocyte viability, an aliquot of oocyte suspension was loaded with CPA as previously described, aspirated into 0.25 ml cryopreservation straws (IMV, France) and sealed with PVC powder. Four straws were frozen for each female pool and assessment step of the protocol. Straws were placed into a programmable freezer (Cryologic Pty Ltd, Mt. Waverly, Australia) and held at 0 °C for 5 min. After holding, samples were cooled to −10 °C, where the straws corresponding to step b were removed from the freezer and thawed by placing in a water bath at 28 °C for ~10 s. The warming rate using this procedure is estimated to be ~1300 °C min^{-1}. Cryoprotectant removal was performed as described previously. The same procedure was followed for the remaining assessment steps (c–e).

Fertilization assays

The ability of oocytes to fertilize after each of the steps of the cryopreservation protocol was carried out as