Cryobiology 58 (2009) 69–74

Contents lists available at ScienceDirect

Cryobiology

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

Towards cryopreservation of Greenshell™ mussel (*Perna canaliculus*) oocytes ☆

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ARTICLE INFO

Article history:
Received 3 July 2008
Accepted 30 October 2008
Available online 6 November 2008

Keywords:
Mussel
Perna canaliculus
Oocyte
Egg
Cryopreservation
Shellfish

ABSTRACT

Cryopreservation is a powerful tool for selective breeding in aquaculture as it enables genetic material from selected stock to be stored and crossed at will. The aim of this study was to develop a method for cryopreserving oocytes of the Greenshell™ mussel (*Perna canaliculus*), New Zealand's main aquaculture species. The ability of oocytes to be fertilized post-thawing was used as the criterion for success in initial experiments and then subsequently, the ability of frozen oocytes to develop further to D-stage larvae was assessed. Ethylene glycol, propylene glycol, dimethyl sulphoxide and glycerol were evaluated at a range of concentrations with and without the addition of 0.2 M trehalose using post-thaw fertilization as the endpoint. Ethylene glycol was most effective, particularly when used in combination with trehalose. A more detailed investigation revealed that ethylene glycol at 9% or 10% in the presence of 0.2–0.4 M trehalose afforded the best protection. In experiments varying sperm to egg ratio and egg density in post-thaw fertilization procedures, D-larval yield averaged less than 1%. Following these results, a detailed experiment was conducted to determine the damaging steps in the cryopreservation process. Fertilization losses occurred at each step whereas D-larval yield approximately halved following CPA addition and was almost zero following cooling to −10 °C. Cryomicroscopy studies and fertilization results suggest that the inability of oocytes to develop to D-larvae stage after cooling to −10 °C and beyond are most likely related to some form of chilling injury rather than extracellular ice triggering intracellular ice formation. Further research is needed to determine the causes of this injury and to reduce CPA toxicity and/or osmotic effects.

1. Introduction

The Greenshell™ mussel (*Perna canaliculus*) is the main shellfish species farmed in New Zealand [17]. The mussel industry currently obtains spat (juveniles) to on-grow from two wild populations. However, there is increased interest in moving to hatchery spat production to ensure a reliable supply for on-growing and to capture the benefits of selective breeding.

Cryopreservation can be a powerful tool in selective breeding and hatchery production. In selective breeding, it enables breeders to make parental crosses on demand: for example, crosses out of season or between stock types with different breeding seasons, between generations and even beyond the lifespan of the donors, giving breeders much more flexibility and enabling faster genetic gains. It also manages the biological and commercial risk that is associated with selective breeding: for example, animal loss due to disease, or economic and market changes [2,3,24]. In hatcheries, cryopreservation allows year-round spat supply without the need to condition broodstock for out of season production.

Towards this goal, we are developing methods to cryopreserve the oocytes of the Greenshell™ mussel. We evaluated the effects of different CPAs and concentrations, fertilization strategies and the ability of frozen mussel oocytes to develop to viable D-larvae. An experiment investigating the effect of each step in the cryopreservation protocol on fertilization and D-yield was used to determine the critical cryopreservation steps for this species and to speculate on potential mechanisms and aspects of cryopreservation damage relating to Greenshell™ mussel oocytes. Studies of intracellular ice formation (IIF) using cryomicroscopy formed part of this study.

2. Methods

2.1. Gamete collection and handling

Mature Greenshell™ (*P. canaliculus*) mussels in reproductive condition were obtained from farms in the Marlborough Sounds,

☆ Funding for this research project was provided by the New Zealand Foundation for Research, Science and Technology through contracts CAWX0304 and CAWX0702. This research is also covered under New Zealand patent 538902.

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0011-2240/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.cryobiol.2008.10.130

New Zealand, and maintained at the Glenhaven Aquaculture Centre in Nelson before being used in experiments.

Thermal cycling was used to induce mussels to spawn. Mussels were cleaned to remove external debris and placed in a shallow spawning tray containing 1 μm filtered seawater at ~18 °C. The tray was covered to exclude light and left for 2 h. After this time, the water was partially replaced with cold seawater to depress the temperature to ~14 °C. The tank was covered for 10 min followed by a cold seawater rinse and covered again for a further 10 min at ~14 °C. The cold water was then replaced with 18 °C seawater, the mussels covered and left for 1 h undisturbed. The seawater was then partially replaced with cold seawater to depress the temperature to 10 °C. Mussels were then rinsed twice as above, but in 10 °C water. The water was then changed, returning the temperature to ~18 °C for an hour, during which time the mussels were again covered. Replacement with cold water took place as above and the temperature cycle continued with warm water being replaced by cold after every hour. Most spawning took place when the water was dropped to 10 °C, commonly after 2–4 temperature cycles.

Spawning mussels were removed once gamete emission was seen to be strong and sustained. To minimize sperm contamination of females, any incipient male spawners were moved to the drain end of the tank, or in some cases, were placed in a separate smaller tank that could be covered at the same time as the main tank.

Spawning females were rinsed in freshwater and placed in individual containers with ~500 mL of 10 °C seawater. Females were allowed to spawn for ~10–20 min before the oocytes were discarded (to avoid collecting already fertilized eggs). The container was rinsed and refilled with clean 10 °C seawater. Oocytes were then collected every 30 min and stored at 5 °C. A sample of oocytes from each collection was examined for sperm contamination (indicated by the presence of polar bodies or cleavage) before oocytes were pooled. Oocyte density was determined by counting 20 μL aliquots from a known dilution of pooled concentrated oocytes in seawater.

Spawning males were rinsed, placed anterior-up in 70 mL plastic containers and left to spawn "dry" (i.e. no seawater was added). Concentrated sperm dripped from each male into the container and was collected every 30 min and stored at 5 °C until ready to be used in experiments. Sperm concentration was determined using a Neubauer haemocytometer.

For each experiment, oocytes and sperm were pooled from at least three individuals per pool and at least three female pools were used for each experiment.

Overall spawning success was 97% (max 100%, min 93%) over 8 spawnings involving a total of 198 mussels.

2.2. Cryopreservation experiments

Cryoprotectants were prepared by adding a known volume of CPA to a known volume of Milli-Q water at double the final concentration required during cryopreservation. All chemicals were sourced from Sigma (St. Louis, MO). Oocytes were standardized to a density of 2×10^6 mL^{-1} in seawater and stored at 5 °C until use. Aliquots (either 0.5 or 1 mL) were then added to 5 mL glass test tubes (Kimble Glass Inc., Vineland, NJ) at ambient temperature (~18–21 °C) for the various cryopreservation experiments.

Experiment 1 investigated the effect of CPA type, CPA concentration and diluent type on the ability of oocytes to be fertilized post-thawing. Dimethyl sulphoxide, propylene glycol, glycerol and ethylene glycol (EG) were made up using either Milli-Q water or Milli-Q water plus trehalose to give final concentrations of 5%, 10% and 15% permeating CPA after 1:1 dilution with oocyte suspension and 0.2 M trehalose in trehalose treatments. The CPA solutions were added 1:1 in a single step at ambient temperature and the diluted oocytes then loaded into straws (0.25cc, IMV, France) and sealed with PVC powder. Straws were placed into Cryogenesis freezers holding at 0 °C (Cryologic Pty Ltd., Mt. Waverley, Australia) 15 min after the CPA was added. The freezers were programmed to hold at 0 °C for 5 min, then cool at 1 °C min^{-1} to −10 °C and hold for a further 10 min, during which time ice formation in the straws had either already occurred or was seeded by touching the straws with a liquid nitrogen cooled cotton bud. Following the hold, the freezers cooled at 0.5 °C min^{-1} to −35 °C at which temperature the straws were plunged into liquid nitrogen and stored. Straws were thawed in a 28 °C water bath until the ice had melted and the contents then diluted with 1 mL of filtered seawater in multiwell plates. The thawed oocytes were left for a further 15 min before being used in fertilization assays.

Based on the results of Experiment 1, a more detailed investigation of EG and trehalose concentration on oocyte post-thaw fertilization was undertaken [Experiment 2]. Ethylene glycol was evaluated at final concentrations of 6%, 8%, 9%, 10%, 11%, 12% and 14% and trehalose at final concentrations of 0.2, 0.3, 0.4 and 0.5 M.[1] The solutions were added 1:1 to the oocyte suspension in a single step and the same cooling and warming procedures were used as outlined for the previous experiment.

Subsequent experiments investigating post-thaw fertilization methods [Experiment 3] and the effects of cryopreservation on subsequent development of fertilized oocytes to D-larvae [Experiment 4] used either 9% or 10% EG in 0.2 M trehalose (final) and the same cooling and warming regime as outlined above.

2.3. Fertilization assays

Oocytes were fertilized using the miniaturized fertilization assay described previously with some modifications [2,24]. Unfrozen oocytes were fertilized at the time of cryopreservation at an oocyte concentration of 200 oocytes mL^{-1} and at sperm concentrations of 10^2, 10^3, 10^4, 10^5 and 10^6 mL^{-1} in seawater containing 0.1% bovine serum albumin (Albumax I Lipid Rich BSA, Gibco™ Invitrogen Corporation, New Zealand). The unfrozen assays were used to verify sperm and oocyte fertility. Thawed oocytes were fertilized at a target concentration of 1000 oocytes per well (333 oocytes mL^{-1}) and at a single sperm concentration of 10^6 mL^{-1}. Duplicate assays were conducted on each straw and assays included a negative control where no sperm were added to check for parthenogenetic activation. Development was stopped after cleavage (2–8 cells) by addition of formalin (~0.5% final) and the percentage of oocytes fertilized was determined using cleavage as the criterion for fertilization success ($n \sim 100$ per replicate).

2.4. Bulk fertilization and larval rearing

In Experiments 3 and 4 investigating the effect of cryopreservation on subsequent development, thawed oocytes were fertilized and reared through to D-larval stage (36–48 h post-fertilization). In the first of these experiments, the effect of sperm to oocyte ratio and oocyte density was assessed. Straws were thawed and diluted individually before being pooled for fertilization. For each treatment, approximately 1 million thawed oocytes from each pool were fertilized at sperm: oocyte ratios of 500, 1000 and 5000 sperm per oocyte and oocyte densities of 200, 500, 1000 and 2000 oocytes mL^{-1}. After a 20 min contact time, the oocytes were transferred to tanks containing ~150 L of filtered seawater and 1 mg L^{-1} EDTA at ~18 °C. After at least 36 h, the tanks were drained through a 45 μm mesh. The resulting D-larvae were col-

[1] At the highest concentrations of EG and trehalose (11%, 12% and 14% and 0.5 M final, respectively), trehalose crystallized from solution.