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Short communication

Successful oocyte cryopreservation in the blue mussel *Mytilus galloprovincialis*

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ABSTRACT

The development of oocyte cryopreservation techniques is widely acknowledged being very difficult in aquatic species and has only been successfully achieved in Pacific oysters *Crassostrea gigas*. Results from published data have shown that oocyte cryopreservation in other bivalve species (including blue mussels *Mytilus galloprovincialis*) is challenging with, if any, very limited D-larvae having been produced. In this study, a technique to cryopreserve blue mussel oocytes has been developed through investigating the effects of polysaccharide and sugar at cryopreservation and post-thaw cryoprotectant agent (CPA) removal, respectively. The highest post-thaw D-larval rate of 14% was achieved when mussel oocytes were cryopreserved in the CPA consisting of 10% ethylene glycol + 7.5% Ficoll PM 70 and post-thaw CPA was removed by 9% sucrose. Over the subsequent development to spat stage, the performances (survival and growth rates) of the progenies produced with cryopreserved oocytes were similar to controls.

1. Introduction

Gamete cryopreservation is an effective and reliable technique to facilitate the reproduction, biodiversity preservation, endangered species conservation and genetic improvement programs (Tervit et al., 2005; Zhang, 2004). In comparison, oocytes are more challenging to cryopreserve than sperm as they are much larger in size and therefore have a smaller surface area to volume ratio and a lower rate of water and cryoprotectant movement into and out of cells during cryopreservation, thus creating permissive conditions for detrimental ice formation at freezing temperatures (Anchamparuthy et al., 2009; Checura and Seidel, 2007; Isayeva et al., 2004). Successful oocyte cryopreservation has been mainly reported in mammalian species, such as humans (Chen et al., 2003; Jain and Paulson, 2006; Santis et al., 2007), mice (Kohaya et al., 2013), rabbits (Trigos, 2014) and pigs (Somfai et al., 2014). In aquatic species, only oocytes from Pacific oysters *Crassostrea gigas* have been successfully cryopreserved (Tervit et al., 2005). However, when the same technique was applied in other bivalves, such as greenshell mussels *Perna canaliculus* (Adams et al., 2009) and blue mussels *Mytilus galloprovincialis* (Wang et al., 2014), limited D-larvae were produced. When mussel oocytes are frozen to a critical subzero temperature they are irretrievably damaged (Adams et al., 2009; Wang et al., 2014), suggesting that the sensitivity of bivalve oocytes to cryoinjuries might be species specific.

Oocyte cryopreservation in marine bivalves involves the following key processes: 1) oocytes collection and preparation, 2) cryoprotectant agent (CPA) stock solution preparation, 3) preparation of CPA and oocyte suspension and equilibration, 4) transferring CPA and oocyte suspension into cryo-containers, 5) freezing in liquid nitrogen vapor, 6) storage in liquid nitrogen, 7) thawing and CPA removal, and 8) post-thaw oocyte quality evaluation. Most cryoinjuries occur at the processes 5 (freezing) and 7 (thawing and CPA removal) and could be minimized through investigation on CPAs and development of CPA removal method (Fabbri et al., 2001). Normally, CPAs can be divided into permeable and non-permeable agents according to their ability to penetrate the cell membrane, with the former playing cryoprotective functions both intra- and extracellularly, whereas the latter theoretically functioning extracellularly only (Adams et al., 2009; Zhou and Li, 2009). Therefore, an effective CPA solution is essential for the success of oocyte cryopreservation (Zhou and Li, 2009). In human and livestock species the solutions comprised of permeable and non-permeable CPAs are often used (Jain and Paulson, 2006; Kohaya et al., 2013; Santis et al., 2007; Somfai et al., 2014). Among the non-permeable CPAs, disaccharide and polysaccharide sugars are frequently applied to improve the success of oocyte cryopreservation (Hurtt et al., 2000; Kohaya et al., 2013; Santis et al., 2007; Somfai et al., 2014). In marine bivalves, on the other hand, only disaccharides have been investigated in mussels with limited success (Adams et al., 2009; Wang et al., 2014). Ficoll is a polysaccharide and has been used as an essential CPA component for oocyte cryopreservation in mice (Seki and Mazur, 2012), bovines (Anchamparuthy et al., 2009; Hurtt et al., 2000) and horses (Hurtt et al., 2000).

Methods to remove CPA after thawing are another pivotal procedure in oocyte cryopreservation as inappropriate treatments can cause oocyte swelling beyond their physiological limitation, or even rupture

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due to the excessive influx of free water (Jain and Paulson, 2006). In some studies in mammalian species, these adverse osmotic effects are mitigated by using sugar solutions for post-thaw CPA removal (Cao et al., 2009; Huang et al., 2008; Santis et al., 2007). Among them, sucrose is the most commonly used agent (Cao et al., 2009; Huang et al., 2008; Jain and Paulson, 2006). However, the use of sugar solution to remove CPA has not been investigated in marine bivalve oocyte cryopreservation.

The blue mussel *M. galloprovincialis* is one of the most important aquaculture bivalve species in the world. Mussel embryo/larvae have also been extensively utilized as a biological indicator in pollution monitoring programs (Beiras et al., 2003; Geffard et al., 2002; Jha et al., 2000). Therefore, the development of mussel oocyte cryopreservation technique could not only provide progenies without seasonal limitations for the environmental monitoring programs and aquaculture productions and genetic improvement programs, but also maintain the long-term sustainable development and competitive advantages of mussel aquaculture industry (Pettersen et al., 2010; Sánchez-Lazo and Martínez-Pita, 2012a,b). In our previous study on the oocyte cryopreservation in this species, although ethylene glycol produced the best post-thaw fertilization rate in the permeable CPAs evaluated, limited oocytes have developed into D-larvae (Wang et al., 2014). In this study, the combination effect of ethylene glycol and Ficoll on mussel oocyte cryopreservation and the effect of sugar solutions in post-thaw CPA removal were investigated, with an aim to develop an oocyte cryopreservation technique in the blue mussel *M. galloprovincialis*.

2. Materials and methods

2.1. Gamete collection

The mature blue mussels were supplied by Kinkawooka Mussels in Port Lincoln, South Australia (SA) and transported in a refrigerated container overnight to Aquatic Sciences Centre, South Australian Research and Development Institute (SARDI), Adelaide, SA. Upon arrival, the animals were washed with 25 μm filtered seawater (FSW) prior to spawning induction. Mussels were induced to spawn individually in 500 mL containers by thermal shock (Wang et al., 2014). The oocytes from at least five broodstock were gently poured into a 90 μm sieve to remove large debris and retained on a 35 μm sieve. They were then gently rinsed by 5 μm FSW, washed into a settlement beaker and stored in ice. At the same time, a subsample of 1 mL oocyte suspension was taken and diluted 100 times before being counted under a binocular microscope. The oocyte density was then standardized to 4×10^5 mL^{-1}. Sperm from individual males were filtered through a 25 μm sieve to remove the debris and their quality was examined under a light microscope. Sperm with motility above 80% were pooled from at least 3 individuals and then used in the subsequent experiments.

2.2. Chemicals and equipment

All chemicals (ethylene glycol (EG), sucrose and Ficoll PM 70 (FIC)) used in this study were purchased from Sigma-Aldrich Pty Ltd (St. Louis, MO, USA). The stock solution was prepared in Milli-Q water at a concentration two times as high as that required in the experiments. Therefore, when the same volume of stock solution and oocyte suspension was mixed, the required final chemical concentration was produced.

In this study the oocytes were cryopreserved with a CL863 programmable freezer (Cryologic, Mulgrave, Victoria, Australia) and 0.25 mL straws (Minitube, Germany). The freezer was driven by the CryoGenesis software (V5) provided by Cryologic.

2.3. Experiments

2.3.1. Effects of Ficoll PM 70 (FIC) on blue mussel oocyte quality

In this experiment, the effects of the addition of FIC, a non-permeable CPA at 5, 7.5 or 10% concentration in 10% EG were evaluated without freezing. After a 15 min equilibration on ice, a 0.25 mL of oocyte + CPA mixture was transferred into a 4 mL tube and diluted with 5 μm FSW at a 1:1 (v:v) ratio for 3 times at a 10 min interval. The oocytes were then fertilized with fresh sperm at a sperm to oocyte ratio of 20:1. After a 15 min fertilization, the oocytes were washed gently on a 35 μm sieve with 5 μm FSW and then into a 500 mL container. The fertilization rate was evaluated microscopically 4 h post-fertilization. Oocytes with cleavages were considered as being fertilized. The D-larval rate was determined 40 – 48 h post-fertilization and calculated as the percentage of oocytes that develop into D-larvae. The controls were established using newly collected oocytes.

2.3.2. Effects of FIC on post-thaw oocyte quality

In this experiment, the effects of 10% EG + 5, 7.5 or 10% FIC on post-thaw oocytes quality were assessed. After a 15 min equilibration on ice, the oocyte-CPA mixture was transferred into 0.25 mL straws and maintained at 0 °C for 5 min in the CL863 programmable freezer. The straws were then frozen at a rate of −1 °C/min from 0 to −10 °C and at −0.3 °C/min from −10 to −34 °C before being plunged into the liquid nitrogen (LN; Cao et al., 2009). After at least 12 h of storage in LN, the straws were thawed in a 28 °C water bath until the ice in the straw melted. The content in each straw was then expelled into a 4 mL tube and diluted for 10 min using a 0.25 mL medium consisting of 9% sucrose and 5% FIC. The CPA concentration was further diluted twice by adding the same volume of FSW as that in the tube at 10 min intervals. The methods used for fertilization and assessment of fertilization and D-larval rates were the same as those applied in the previous experiment.

2.3.3. Effects of post-thaw CPA removal medium on oocyte quality

In this experiment, different post-thaw CPA removal mediums (5 μm FSW, 9% sucrose or its combination with 2.5, 5, 7.5 or 10% FIC) on post-thaw oocyte quality were evaluated after cryopreservation in 10% EG + 7.5% FIC. The other experimental procedures were the same as in the previous experiment.

2.3.4. Performance comparison between progenies produced with cryopreserved and fresh oocytes

The optimal cryopreservation technique (CPA: 10% EG + 7.5% FIC; post-thaw CPA removal medium: 9% sucrose) developed in this study was applied to compare the performances between progenies produced with fresh and cryopreserved oocytes. After the assessment of D-larval rate, larvae originally from two straws were transferred into a 10 L tank and stocked at a density of ~10 individuals mL^{-1} in both treated (cryopreserved oocytes) and control (fresh oocytes) groups, respectively. Methods for larval culture and settlement were the same as those used by Wang et al. (2011) and Pettersen et al. (2010). The eyed larval (25 days post-fertilization) or spat (32 days post-fertilization) rate was calculated as a percentage of D-larvae initially stocked in a 10 L tank that have developed in the eyed larvae or spat. After the estimation of spat rate, the spat shell lengths were measured from 50 spats randomly selected from each replicate in both control and treated groups.

2.4. Statistical analysis

The data were presented as mean ± standard error (SE) and were arcsine or log transformed for statistical analyses using SPSS 20. One-way analysis of variance (ANOVA) was applied to analyze the data on the effects of FIC or CPA removal medium on oocyte or post-thaw oocyte qualities (fertilization rate and D-larval rate). The Least-Significant Difference (LSD) comparison test was used when significance was observed. A paired sample t-test was applied to compare the performances (survival rate and spat sizes) between progenies produced with fresh and cryopreserved oocytes. Differences were considered statistically significant at $P < 0.05$.