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Cryopreservation of sperm in farmed Australian greenlip abalone *Haliotis laevigata* ☆

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ABSTRACT

This study investigated factors important to the development of the liquid nitrogen (LN) vapor sperm cryopreservation technique in farmed greenlip abalone *Haliotis laevigata*, including (1) cryoprotectant agent (CPA) toxicity; (2) cooling temperature (height above LN surface); (3) thawing temperature; (4) sperm to egg ratio; and (5) sugar supplementation, using sperm motility, fertilization rate or integrity/potential of sperm components and organelles as quality assessment indicators. Results suggested that among the single CPAs evaluated 6% dimethyl sulfoxide (Me2SO) would be the most suitable for sperm cryopreservation in this species. The highest post-thaw sperm motility was achieved with the sperm that had been exposed to LN vapor for 10 min at 5.2 cm above the LN surface, thawed and recovered in 60 and 18 °C seawater bathes, respectively after at least 2 h storage in LN. The highest fertilization rates were achieved at a sperm to egg ratio of 10,000:1 or 15,000:1. Addition of 1% glucose or 2% sucrose produced significantly higher post-thaw sperm motility than 6% Me2SO alone. Among the three cryoprotectant solutions further trialled, 6% Me2SO + 1% glucose produced the highest fertilization rate of 83.6 ± 3.7%. Evaluation of sperm has shown that the addition of glucose could significantly improve the sperm plasma membrane integrity and mitochondrial membrane potential. These results demonstrated a positive role of glucose in the improvement of sperm cryopreservation in farmed greenlip abalone.

Introduction

The abalone aquaculture industry in Australia has developed rapidly in the last decade with production reaching about 681 metric tons worth about 23 million dollars in the financial year 2010–2011 [5]. Greenlip abalone (*Haliotis laevigata*) is the most valuable species in this sector, accounting for 70% of the total abalone production [36], with farms having been established in South Australia, Victoria, Tasmania and Western Australia. Research to improve abalone production has focused on system design [18], optimization of diet [10] and environmental parameters [23,24]. The opportunity now exists to make use of genetic improvement techniques to further advance the abalone aquaculture industry, and selective breeding programs have been initiated in this species [36,39,55]. However, due to restrictions on translocation of live abalone between states in Australia, each state needs to establish enough families to allow for the long-term maintenance of genetic diversity, avoid inbreeding and meet the demand on new traits in the future. The efficiency of these programs is also reduced by the following biological and technical limitations: (1) difficulty in achieving the desired mating as selected males and females cannot be induced to spawn synchronously; (2) short spawning window period in a breeding season; and (3) risks associated with keeping superior broodstock alive and healthy. These limitations are not unique to the abalone aquaculture industry, because they have been experienced in other species and mitigated by the development and application of sperm cryopreservation techniques [7,37,45,54,71].

Sperm cryopreservation has been extensively used in livestock animal breeding programs and for distributing genetically improved sperm for commercial production [45,54], and has become a near-billion-dollar business world-wide [64]. In aquatic species, sperm cryopreservation research has mainly been conducted in fish species [7,47,63]. Studies on marine molluscs have increased substantially over the last decade, especially in oysters [3,4,11–15]. In abalone, this technique has been investigated in small abalone (*Haliotis diversicolor*) [21,66], disc abalone (*Haliotis*

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discus hannai) [34], red abalone (*Haliotis rufescens*) [56], wild greenlip abalone and wild blacklip abalone (*Haliotis rubra*) [38], resulting in ~90% fertilization rate in both small and wild greenlip abalone [38,66]. In the study on wild greenlip abalone, two cryoprotectant agents (dimethyl sulfoxide and glycerol) were evaluated and a cryopreservation protocol was developed [38]. However, when this protocol was applied to farmed stocks, low and highly variable fertilization rates were experienced, hindering the adoption of this technique (Li, pers. comm.).

It has been shown in the published data that sperm quality, such as motility, viability and the integrity or potential of components and organelles, could be compromised by the cryopreservation processes, thus reducing sperm competency [2,50,56,61,75]. However, this reduction in sperm quality could be improved by the addition of cryoprotectant agents (CPAs). In some species, this could be further improved if permeable CPAs (e.g., dimethyl sulfoxide and propylene glycol) are used in combination with non-permeable CPAs (e.g., sugars) [32,48,54,58]. For example, the post-thaw sperm motility, viability and membrane integrity were significantly improved by the addition of trehalose in rams [32] and in some marine molluscan species such as blacklip pearl oysters (*Pinctada Marganitifera*) [41]. Furthermore, disaccharides gave better protection than monosaccharides in boars [20]. The aim of this study was to establish a sperm cryopreservation protocol for farmed greenlip abalone. We have evaluated most permeable CPAs and key factors affecting sperm cryopreservation in molluscan species, and assessed if the addition of sugars could further improve the post-thaw sperm quality.

Materials and methods

Broodstock

Farmed mature greenlip abalone were supplied by SAM Abalone in Port Lincoln, South Australia and transported in a foam box by air to the Aquatic Sciences Centre, South Australian Research and Development Institute (SARDI), Adelaide, South Australia. The animals were about 3 years old and 122.0 ± 8.9 g in total body weight. Upon arrival they were cleaned with 5 μm filtered seawater (FS) and their gender and gonad condition checked. The color of the male gonad is milky white while that of the female is dark green. Animals with a large and swollen gonad were selected to acclimatize in tanks for a week prior to spawning [26]. The tanks were on a flow through system with 5 μm FS at 15.0–16.0 °C. Over this period, the abalone were fed twice a week with the artificial diet provided by EP Aquafeeds (Adelaide, South Australia) and the system was cleaned when needed.

Gamete collection

Five to ten male (and female if needed) abalone were used in each experiment. The abalone were induced to spawn by raising the seawater temperature by 2–3 °C for about 2 h before the ultraviolet (UV) light connected to the water supply system was turned on. Immediately upon the commencement of spawning, the water supply to the tank was changed from UV irradiated seawater to 5 μm FS of the same temperature. To collect concentrated sperm, the water supply to the tank was turned off approximately 5–10 min after the commencement of spawning. The tank was then emptied and the abalone were left undisturbed in the tank for sperm collection. If abalone were dislodged from the tank wall, they were dried with paper towel and put on a rack with their shell facing down. Sperm were then collected by placing a shallow container underneath. Sperm collected from abalone that spawned earlier were stored on ice until sperm from at least 3 individuals were collected. An equal volume of sperm from each male was then pooled. Three sperm pools were established using different males, respectively. The sperm concentration was determined using 3 subsamples per pool. Each subsample was diluted 100 times and counted under a light microscope with a haemocytometer. The sperm concentration was then standardized to 1.6×10^8 mL^{-1}. Sperm samples with initial motility above 80% were used in the subsequent experiments. The time interval from when the sperm from the first male was collected to when the pooled sperm was used in the subsequent experiments was kept as short as possible and was never longer than 2 h.

Eggs in spawning tanks were gently poured into a sieve set consisting of a 300 μm upper sieve to remove large debris and a 90 μm lower sieve partly immersed in 5 μm FS to retain the eggs. The eggs were gently rinsed and then washed into a settlement beaker. After 15 min the eggs on the bottom were transferred into another container and mixed gently by hand. One milliliter egg suspension was taken and diluted 100 times before a 1 mL subsample was used to account the egg density under a binocular microscope. This estimation was repeated three times per collection. The eggs used for the fertilization rate evaluation were never older than 2 h post spawning.

Chemical solution preparation

Cryoprotectant agent (CPA)

Dimethyl sulfoxide (Me2SO), propylene glycol (PG), ethylene glycol (EG), glycerol, sucrose, glucose and trehalose used in this study were AR grade and purchased from Sigma–Aldrich Pty Ltd. Stock solutions were prepared in 5 μm FS at a concentration twice that of the final concentration required. When the stock solution was mixed with sperm at a 1:1 ratio in the experiments, the required final concentration was produced.

Fluorescent agent

A LIVE/DEAD sperm viability kit (L-7011) for plasma membrane integrity (PMI) evaluation and LysoTrack green DD-26 (LYSO-G) kit (L-7526) for acrosome integrity (AI) evaluation were purchased from Invitrogen Australia. Rhodamine 123 (Rh 123) for mitochondrial membrane potential (MMP) evaluation and propidium iodide (PI) used for AI and MMP evaluation were purchased from Sigma–Aldrich Pty Ltd. The working solution of 2 μM SYBR14 and 200 μM PI in the LIVE/DEAD sperm viability kit was prepared by diluting with 5 μm FS. LYSO-G in LysoTrack green DD-26 kit is 1000 μM and used directly. Rh123 was prepared in 5 μm FS at a concentration of 10 μM. PI was prepared in 5 μm FS at a concentration of 3000 or 130 μM for AI and MMP evaluations, respectively [25,56,61,65].

Equipment setup

In this study, a styrofoam box (39.0 × 24.5 × 35.5 cm), a foam rack with different heights (1.3, 2.6, 3.9, 5.2 and 6.5 cm) and a digital thermometer (Thermo Scan, Eutech Instruments, Singapore) with a low temperature probe were used. Liquid nitrogen (LN) was added into the styrofoam box with a LN withdrawal device to a depth of about 2 cm. The rack was then placed into the styrofoam box, floating on the LN surface. After 0.25 mL straws (Minitube, Germany) containing the sperm + cryoprotectant solution mixture were placed on the rack the lid of styrofoam box was closed partially to maintain the constant flow of vaporized LN during freezing [17,53]. The temperatures at different heights were measured by the digital thermometer.

Two seawater baths were used in the experiments to thaw and recover sperm at different temperatures. The required temperature (40–80 °C) in the thawing bath was achieved by mixing ambient