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CRYOPRESERVATION OF BLACK-LIP PEARL OYSTER (*PINCTADA MARGARITIFERA*, L.) SPERMATOZOA: EFFECTS OF CRYOPROTECTANTS ON SPERMATOZOA MOTILITY

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ABSTRACT Cryopreservation of sperm is seen as an important step in developing effective hatchery culture techniques for the black-lip pearl oyster, *Pinctada margaritifera*. As a preliminary investigation into cryopreservation of the gametes of this species we tested 5 cryoprotectant agent combinations for their ability to retain sperm motility: (1) 1 M trehalose and 5, 10 and 15% dimethyl sulphoxide (DMSO); (2) Hanks calcium-free balanced salt solution (C-F HBSS) and 5%, 10% and 15% DMSO; (3) C-F HBSS and 5, 10 and 15% propylene glycol (PG); (4) 1 M trehalose and an equal combination of DMSO and PG making up 5, 10, 15% total volume; and (5) C-F HBSS and an equal combination of DMSO and PG making up 5%, 10% and 15% total volume. Total, rapid and progressive sperm motilities were estimated through computer assisted sperm analysis (CASA). Sperm cryopreserved in 1 M trehalose and 5% DMSO retained the highest total (86.0 ± 1.2% SE), progressive (46.0 ± 1.2% SE) and rapid (25.1 ± 0.6% SE) motilities of all cryoprotectant solutions, whereas those cryopreserved with PG generally retained poor motility. Although the 1 M trehalose and 5% DMSO treatment compared favorably with that of fresh sperm for total motility ($P < 0.01$), all cryoprotectant treatments were poor at retaining the proportion of original rapid and progressively moving sperm. This study highlights the potential for cryopreservation of gametes from *P. margaritifera*, which will benefit selective breeding and conservation programs with this important commercial species.

KEY WORDS: pearl oyster, *Pinctada margaritifera*, cryopreservation, spermatozoa, motility, progressive motility, CASA

INTRODUCTION

The black-lip pearl oyster (*Pinctada margaritifera*, L.) supports well-established cultured "black" pearl industries in French Polynesia and the Cook Islands (Fassler 1995). The success of these industries has stimulated expansion of black-lip pearl oyster culture to the island nations of the western Pacific, Australia and the east coast of Africa. Black pearl production has traditionally relied on oyster spat (or juveniles) collected from the wild (e.g., Coeroli et al. 1984). Collection of wild *P. margaritifera* spat is, however, influenced by seasonal fluctuations in recruitment (Friedman et al. 1998), may be inappropriate in some environments (Friedman et al. 1998) and does not allow genetic manipulation of culture stock as would be possible in selective breeding programs. Hatchery production has consequently assumed greater significance to the pearling industry over recent years (Gervis & Sims 1992) and hatchery culture techniques for *P. margaritifera* are now well established (Southgate & Beer 1997, Doroudi & Southgate 2000, Pit & Southgate 2000).

An aspect of *P. margaritifera* hatchery production that has so far received little research attention is the potential for long-term storage of sperm through cryopreservation. Although previous studies with Pacific oysters and abalone have shown that cryopreservation of mollusc sperm is possible (Kurokura et al. 1990, Yankson & Moyse 1991, McFadzen 1995, Lin & Chao 2000, Chao & Liao 2001, Adams et al. 2004), no tested methodologies have been reported to reliably preserve pearl oyster sperm. The capability to preserve sperm will have major benefits for the future development of hatchery technology for *P. margaritifera*. For example, when genetic improvement programs are initiated for this species, sperm preservation will allow flexibility in spawning regimes and facilitate increasing availability, distribution and conservation of genetically selected lines. Long-term storage of sperm would also reduce the criticality of synchronizing spawning of males and females, as well as potentially reducing the number of broodstock that have to be maintained and conditioned in the hatchery (Adams et al. 2004).

Before cryopreservation can be realized, evidence from numerous studies on fish and other species of bivalve mollusc has highlighted the importance of identifying the type and concentration of appropriate sperm cryoprotective agents (CPAs) for the species of interest, as many CPAs show varying degrees of toxicity to sperm either in isolation, or when applied in different combinations (Chao et al. 1994, Tsai & Chao 1994, Gwo 2000, Paniagua-Chavez & Tiersch 2001, Gwo et al. 2002, Sansone et al. 2002, Adams et al. 2004). In an initial effort to develop reliable methodologies for the long-term storage of *P. margaritifera* sperm, this study tested the effects of various CPAs on preservation of sperm motility.

MATERIALS AND METHODS

Source of Oysters

Adult male *P. margaritifera* were collected from pocket (panel) nets suspended from a long-line at Magnetic Island, north Queensland (19°12′S, 146°52′E) Australia and transported to aquarium facilities at James Cook University. Oysters were then cleaned of fouling, washed with chlorinated water and rinsed in freshwater before being placed upright in plastic aquaria with oxygenated 1-μm filtered seawater (FSW). Oysters were held overnight at 19°C to prevent them from spawning.

Extraction of Spermatozoa

To avoid the possible confounding effect of mixing seawater with CPAs, sperm from three males were manually stripped by cutting a small incision in the gonad and removing gametes with a Pasteur pipette. The concentrated sperm from each individual were then combined in equal proportions and evenly distributed with the appropriate CPAs on a 1:12.5 (by volume) basis at ambient temperature (ca. 22°C). Enough sperm was extracted from the three

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males to fill 12 straws per treatment. Spermatozoa concentration in the pooled semen sample was 6.1×10^7 cells/mL.

Cryopreservation of Sperm

We evaluated 5 CPA combinations at each of three concentrations: (1) 1 M trehalose and 5, 10 and 15% dimethyl sulphoxide (DMSO); (2) Hanks Calcium-free Balanced Salt Solution (C-F HBSS) (Paniagua-Chavez et al. 1998) and 5%, 10% and 15% DMSO; (3) C-F HBSS and 5%, 10% and 15% propylene glycol (PG); (4) 1 M trehalose and an equal combination of DMSO and PG made up to 5%, 10% and 15% total volume and (5) C-F HBSS and an equal combination of DMSO and PG making up 5%, 10% and 15% total volume. Twelve straws were prepared for each CPA concentration and 36 straws for each combination of CPAs. Prior to addition of sperm, compressed air was passed through the CPAs for 15 min to oxygen-saturate the solutions.

Sperm and CPAs were thoroughly mixed by gentle agitation and then immediately drawn into 0.25 mL "Cassou" semen straws (IMV France) by suction. The straws were then placed horizontally for 15 min on a precooled tray situated above a Styrofoam box where the liquid nitrogen steam was at −120°C. Straws were left in steam for 10 min before being transferred into a liquid nitrogen Dewar where they were stored until further analysis. At the same time samples were cryopreserved in the various CPAs, motility statistics were collected from the remaining unfrozen sperm to act as a control group. Sperm in this fresh sample were quantified in the same manner as the cryopreserved treatments (outlined below).

Estimates of Motility

Cryoprotectant effectiveness was assessed by examining sperm motility after 2 wk postpreservation. Straws were thawed in a 25°C water bath for 30 sec before the contents of the straws were rinsed in an equal amount of FSW. Motility of sperm was assessed objectively using a Hamilton Thorne computer-aided semen analyzer (CASA) (HTR Ceros 12.1, Orange Medical, Brussels, Belgium) by placing 5-μl aliquots into a Leja counting chamber (Orange Medical, Brussels, Belgium) at 28°C and examining sperm in the field of view for percent total motility (number of sperm with average path velocity (VAP) >5 μm/sec), rapid motility (number of sperm with VAP >20 μm/sec) and progressive motility (number of sperm with VAP >5 μm/sec and straightness threshold of pathway >75%). Sperm with VAP <5 μm/sec were considered nonmotile. Four fields of view for each of two repeat aliquots were examined per straw to obtain motility and within-treatment variability statistics. Instrument settings for the HTR Ceros 12.1 were 100 frames at 60 frames/sec, analysis duration 1.7 sec, minimum contrast 30, minimum cell size (pixels) 1 and magnification 3.87.

Statistical Analyses

Statistical analyses for all comparisons were performed using SPSS 12.0.1 software (SPSS Inc). Residuals were checked to ensure that assumptions of normality and homogeneity of variance were not violated and the effects of CPAs analyzed using a two-factor ANOVA. CPA's and CPA concentration were treated as fixed effects. Tukey multiple comparison tests were applied to identify significant differences among CPA treatments.

RESULTS

Total Sperm Motility

Mean (± SE) total sperm motility from the three male oysters was very high (96.9 ± 0.6%) (Fig. 1), indicating that the stripping procedure we used to obtain the gametes was an effective technique to obtain motile sperm in this species. The success of the various CPA treatments on cryopreserving sperm were variable ($F_{15,\ 128} = 56.3$, $P < 0.001$), with the Treatment 1 sample (1 M trehalose and 5% DMSO) showing the highest total sperm motility. A decline in motility of 11.2% was recorded in Treatment 1 (86.0 ± 1.2%) from that of the fresh sample (Tukey HSD, $P < 0.01$). There was no significant effect of DMSO concentration on total motility in this treatment. Sperm motility in Treatment 2 (CF-HBSS and DMSO) was also moderately high in the sample tested (46.7 ± 0.6%), but was significantly lower than that of Treatment 1 and the control (Tukey HSD, $P < 0.001$). The addition of PG to cryopreservatives impacted dramatically on total motility, with sperm frozen in Treatments 3 (C-F HBSS and PG), 4 (trehalose, DMSO and PG) and 5 (C-F HBSS, DMSO and PG) agglutinated upon thawing. Total motility in these treatments was very low (Fig. 1). No significant effects of CPA concentration were observed in these later treatments.

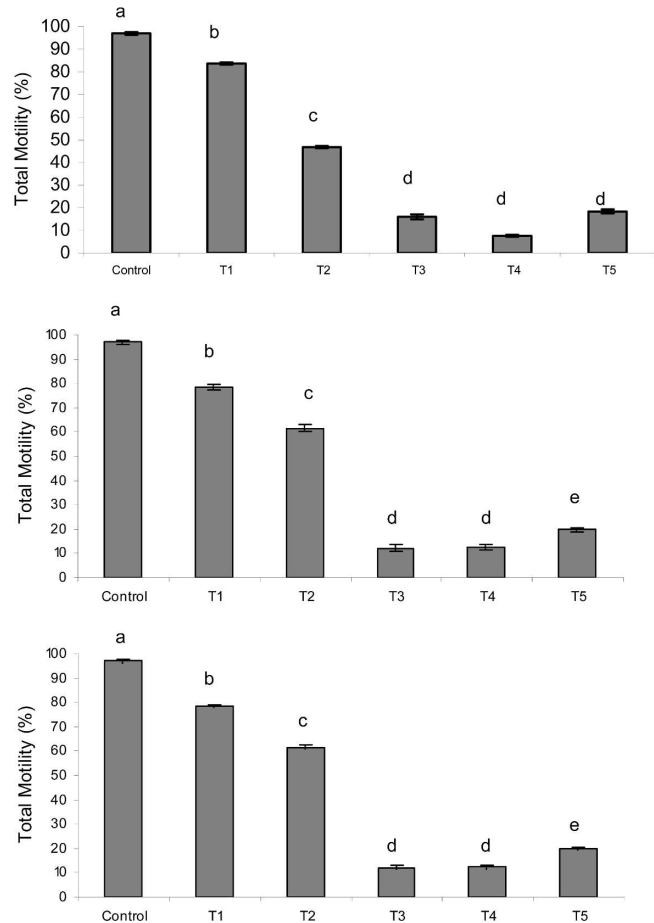

Figure 1. Mean (± SE) total motility rates at 2 wk postpreservation of cryopreserved *Pinctada margitifera* sperm frozen in 5% (upper panel), 10% (middle panel) and 15% (bottom panel) concentrations of CPAs. T1 = 1 M trehalose and DMSO; T2 = C-F HBSS and DMSO; T3 = C-F HBSS and PG. T4 = 1 M trehalose, DMSO and PG and T5 = C-F HBSS, DMSO and PG. Means with the same superscript are not significantly different ($P > 0.05$).

Progressive Sperm Motility

Progressive mean (± SE) sperm motility for the fresh samples were high, with 75.5 ± 1.2% of cells having a VAP >5 μm/sec and

straightness threshold of pathway >75% during analysis (Fig. 2). This parameter, however, was observed to decline quite dramatically in all cryopreserved treatments, with Treatments 3, 4 and 5 having very few sperm undergoing progressive cell movement. Progressive cell motility in Treatment 1 (1 M trehalose and 5% DMSO) was around half that of the fresh sample (46.0 ± 1.2%) but was significantly better than that in all other treatments (Tukey HSD, P < 0.001). A significant reduction was also seen in this treatment as the CPA DMSO percentage increased (i.e., progressive motility for 1 M trehalose and 10% DMSO = 29.8 ± 1.2%, 1 M trehalose and 15% DMSO = 27.6 ± 1.1% – data not shown) (Tukey HSD, P < 0.001). Besides the effects of DMSO percentage observed in Treatment 1, there were no significant effects of CPA concentration on this motility parameter in other treatments.

Rapid Sperm Motility

In the fresh samples, half the sperm (50.0 ± 1.1%) were determined by CASA to be extremely active and showing rapid cell motility (Fig. 3). Of the three parameters tested in this study, analysis of rapid cell movement was the most adversely influenced by cryopreservation ($F_{15, 256}$ = 113.2, P < 0.001), with no appreciable rapid movement seen in replicate samples from Treatments 2, 3, 4 and 5 at any concentration of CPAs. Rapid sperm movement was still present in Treatment 1; however, there was a significant concentration effect on this parameter with post thaw rapid cell movement dropping from an average of 25.1 ± 0.6% of sperm in the 1 M trehalose and 5% DMSO samples, to a low of 8.4 ± 0.8% in 1 M trehalose and 15% DMSO samples (Tukey HSD, P < 0.001).

DISCUSSION

Our results showed that sperm preservation for *P. margaritifera* was best when a cryoprotectant solution containing 1 M trehalose and 5% DMSO was used. With this treatment, around 89% of the original total motility of fresh sperm was maintained, although there was an obvious preservation effect on estimates of both progressive and rapid sperm motilities. Despite reductions in these two motility statistics, however, our study clearly showed that it is possible to store *P. margaritifera* sperm for long-periods in liquid nitrogen or ultra-cold freezers. These results can be used as a precursor to further refine preservation techniques and develop methodologies applicable to large-scale sperm storage in commercial hatcheries.

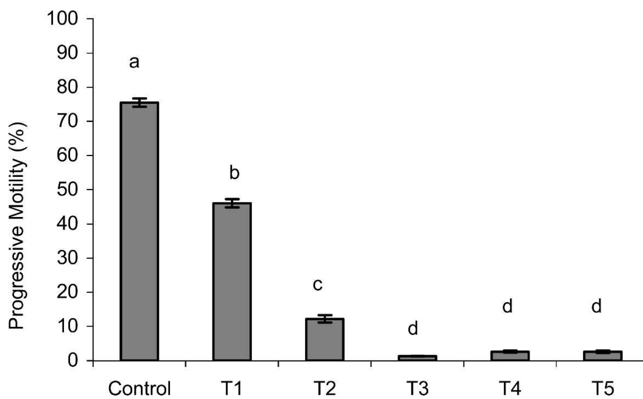

Figure 2. Mean (± SE) progressive cell motility rates at 2 wk postpreservation of cryopreserved *Pinctada margitifera* sperm frozen in 5% concentrations of CPAs. T1 = 1 M trehalose and DMSO; T2 = C-F HBSS and DMSO; T3 = C-F HBSS and PG. T4 = 1 M trehalose, DMSO and PG and T5 = C-F HBSS, DMSO and PG. Means with the same superscript are not significantly different (*P* > 0.05).

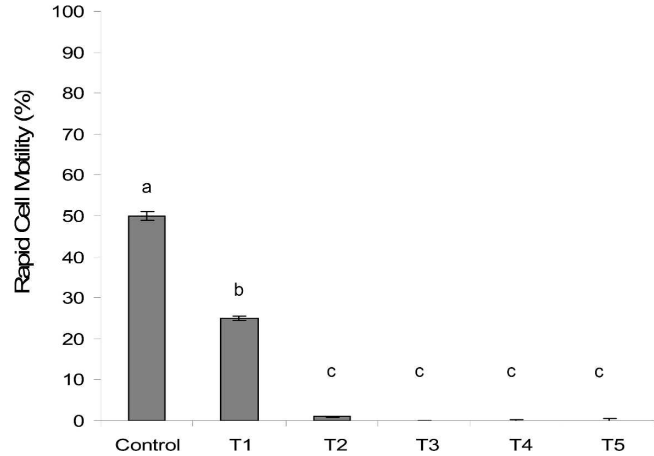

Figure 3. Mean (± SE) rapid cell motility rates at 2 wk postpreservation of cryopreserved *Pinctada margitifera* sperm frozen in 5% concentrations of CPAs. T1 = 1 M trehalose and DMSO; T2 = C-F HBSS and DMSO; T3 = C-F HBSS and PG. T4 = 1 M Trehalose, DMSO and PG and T5 = C-F HBSS, DMSO and PG. Means with the same superscript are not significantly different (*P* > 0.05).

Sperm quality is ultimately determined by its ability to fertilize oocytes (Aas et al. 1991). Quality can be influenced by factors such as condition of the broodstock, water temperature, dissolved oxygen, bacterial contamination and pH (Paniagua-Chavez et al. 1998). For most bivalves examined to date, sperm motility has generally been used as a predictor of sperm quality. For instance in the eastern oyster, *Crassostrea virginica*, treatments showing highest motility also had the highest "fertility" as determined by the number of developing oocytes at 12 h postfertilization (Paniagua-Chavez et al. 1998). The effects that reductions in more specific motility statistics (e.g., progressive and rapid motilities) have on the capability of sperm to fertilize oyster oocytes, however, has not been examined. This is because CASA technologies have not routinely been applied to the analysis of oyster sperm (or bivalve sperm in general) and therefore the correlation between these parameters and fertility has not been established. Unfortunately, because of seasonal difficulties we were unable to obtain mature oocytes and could not directly assess the effect these motility parameters had on the overall "fertility" of cryopreserved *P. margaritifera* sperm. Evidence from mammals and fishes, however, suggests that the higher the progressive and rapid motility, the higher the fertility of sperm. In humans and cattle for example, progressive and rapid cell motilities have been cited as being positively correlated with "fertility" (Kolb 1999, Tanghe et al. 2004). Similarly in fish, sperm motility has a critical influence on fertility (Kime et al. 2001, Rurangwa et al. 2004). If these relationships hold true for pearl oyster sperm it is likely that the sperm in Treatment 1 (1 M trehalose and 5% DMSO) retained a capability, although reduced from that of fresh sperm, to fertilize oocytes. On-going research in our laboratory will determine if this relationship holds true for pearl oysters.

Use of PG and/or C-F HBSS was not effective in maintaining motility of cryopreserved pearl oyster sperm. This finding has been reported in the past for other shellfish species (Chao et al. 1994, Lin et al. 1999, Sansone et al. 2002, Gwo et al. 2002). Post-thawed

samples containing PG showed agglutination (Treatments 3, 4 and 5), which causes a massive reduction in sperm motility (Tsai & Chao 1994, Dunn & McLachlan 1973). Agglutination of sperm has been previously reported using glycerol, but has not been described when PG is used. In this study, PG was seen to dissociate from the water column quickly, which may have influenced its effectiveness as a CPA. Future experiments should concentrate on refining cryopreservation through varying the molar concentration of trehalose and DMSO.

In summary, this preliminary investigation into cryopreservation of *P. margaritifera* sperm showed that total motility of sperm was retained at around 89% when cryopreserved in 1 M trehalose and 5% DMSO. Progressive and rapid sperm motilities, however, were dramatically reduced in all CPA treatments and the effects that reductions in these parameters have on fertility of sperm will need to be further examined before cryopreservation techniques can be routinely applied to this species in commercial hatcheries.

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