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Effects of Cryoprotectants and Freezing Rates on Cryopreservationof Sea Urchin, *Anthocidaris crassispina* Sperm

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In the present study, attempts were made to cryopreserve sea urchin, *Anthocidaris crassispina* sperm in liquid nitrogen, to evaluate the effects of various cryoprotectants and freezing rates on motility, survival rate and fertilization rate of the post-thawing sperm, and the ultrastructural changes of sperm after cryopreservation were observed. The highest values of sperm motility (motility index: 3.3±0.37) and survival rate (72±3.5%) were obtained with 15% dimethyl sulfoxide (DMSO), and these values were significantly higher than those of sperm preserved with glycerol. Comparisons of motilities and survival rates between treatments of difference freezing rates showed that there was no difference between procedures (a) 50°C/min to -80°C (motility index: 3.3±0.31; survival rate 70±2.7%) and (b) 30°C/min to -80°C (motility index: 3.1±0.29; survival rate 69±3.7%), while the results of (c) 10°C/min to -80°C were significantly lower than the others (motility index: 2.2±0.33; survival rate 42±4.6%). There was no significant difference in fertilization rate between fresh sperm and sperm preserved with 15% DMSO as cryoprotectant and freezing rate (30°C/min to -80°C). Some ultrastructural changes of sperm, such as the detachment of plasma membrane, the destruction of mitochondria, and the flagellum rolling up head, were observed after cryopreservation. Morphological normality of the sperm in 15% DMSO frozen at the ratio of 50°C/min to -80°C was better than the others.

Keywords: Sea Urchin, *Anthocidaris crassispina*, Sperm, Cryopreservation

Introduction

Cryopreservation is an effective method for long-term storage of viab used in breeding many species of animals since Polge et al. (1949) found that the addition of glycerol allowed survival of human and fowl sperm after thawing. Cryopreservation offers some benefits in aquaculture and experimental studies, such as protecting stocks from being totally eliminated due to sudden disease outbreak, natural disasters, or accidents such as oil spills, making top-quality gamete and larvae available year-round, providing greater ease in conducting selective breeding for disease resistance, preserving desirable characteristics and establishing gene banks (Bart, 2000; Chao and Liao, 2001). At present cryopreservation technique of sperm has been well established in many fish species, such as salmonids, tilapias, carps and catfishes (Rana, 1995; Tiersch, 2000), but only in a limited number of shellfish, e.g., several commercially important species, hard clam, oyster, small abalone (Chao, 1996; Chao and Liao, 2001; Paniagua-Chavez and Tiersch, 2001). Sea urchin is an important species in experimental biology and aquaculture industry, but an available procedure for cryopreservation of sea urchin sperm is limited. Therefore, the present study optimized methods in sea urchin (*Anthocidaris crassispina*) for the cryoprotectants and freezing rates, in terms of the motility, survival rate and fertilization rate of post-thawed sperm, and the ultrastructural changes of sperm after cryopreservation were observed.

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Materials and Methods

Animals and sperm collection

Sea urchins were collected from a seafood market in Yeosu, Korea, during the reproductive season. One ml of 0.5 M KCl was injected into the coelom per sea urchin to induce the release of sperm. Sperm was placed in 1.5 ml plastic centrifuge tube on ice.

Motility Estimation

The motility of sperm from each male was examined immediately after collection. The sperm exhibiting rapid, vigorous and forward movement was counted under a microscope after diluting the sperm in artificial seawater (ASW; 423.00 mM NaCl, 9.00 mM KCl, 9.27 mM $CaCl_2$, 22.94 mM

Table 1. Motility index[1] in Relation to Percentage of Sperm with Rapid, Vigorous and Forward Movement

	Motility index										
	5	4.5	4	3.5	3	2.5	2	1.5	1	0.5	0
Forward sperm[2] (%)	100	90	80	65	50	30	20	15	10	5	0

1: Numerical index according to percentage of motile sperm
2: Sperm exhibiting rapid, vigorous and forward movement

$MgCl_2$, 2.114 mM $NaHCO_3$, 10 mM HEPES-pH 7.8) in a ratio of 1:1000 (Table 1). The samples with high motility were kept on crushed ice until use in the following experiments.

Cryopreservation of Sperm

DMSO or glycerol was added to ASW as cryoprotectants to formulate the extenders at the concentrations between 5 and 20% of total volume. The sperm was diluted 1:5 with the extenders. The diluted sperm was inserted into 0.5 ml plastic straws and frozen at a freezing rate of 30°C/min to -80°C in a programmable freezer (Kryosave integra, Rovers Polska, UK) after equilibration for 10 min at room temperature, and subsequently plunged into liquid nitrogen. The straws were thawed in a 30°C water bath for 15 sec. On thawing, the motility of frozen-thawed sperm was evaluated and subsequently the sperm was fixed by 5% glutaraldehyde in phosphate buffer (pH 7.2) for ultrastructural examination following the method described bellow. Survival rate was also estimated by an eosin-nigrosin staining technique (Bllom, 1950; Fribourgh, 1966). The sperm was stained with a drop of 5% eosin and two drops of 10% nigrosin. Survival rate (the percentage of unstained sperm) was determined under a microscope.

To evaluate the effect of different freezing rates, 15% DMSO was used as a cryoprotectant according to results of above experiments. Three freezing rates were tested: (a) 50°C/min to -80°C (b) 30°C/min to -80°C (c) 10°C/min to -80°C.

Ultrastructural change

The fresh and frozen-thawed sperm fixed in 5% glutaraldehyde were washed with phosphate buffer, post-fixed for 2 hr in 1% osmium tetroxide. After dehydration through an ascending ethanol series, the samples were embedded in Spurr resin. Ultrathin sections were contrasted with alcoholic uranyl acetate and lead citrate, and examined with a JEM 1200 EX-II transmission electron microscope.

Fertility of cryopreserved sperm

1 ml of 0.5 M KCl was injected into the coelom of sea urchin to induce spawning. About 300 eggs in each plastic dish were mixed with 50 ul fresh sperm or 100 ul post-thawed sperm preserved with 15% DMSO as a cryoprotectant and freezing rate (a) followed by adding 10 ml of filtered seawater to activate the sperm. After 1 hr the fertilized eggs were incubated in 1 L glass beaker at room temperature. The fertilization rate was determined by the percentage of blastula stage.

Data Analysis

Each trial of this study was carried out in triplicate. Differences in the means between treatments were tested by one way analysis of variance (ANOVA) and Student's *t*-test. All statistical procedures were run using Sigma- Plot 8.0 (SPSS Inc., 2002).

Results

Cryopreservation of Sperm

The motilities and survival rates of post- thawed sperm preserved using two cryoprotectants at different concentrations declined significantly after cryopreservation (Fig. 1). The highest sperm motility (motility index: 3.3±0.37) and survival rate (72±3.5%) were obtained with 15% DMSO ($P<0.05$), and those of sperm preserved with glycerol were significantly lower than the former ($P<0.01$).

Comparisons of motility and survival rate between treatments of different freezing rate showed that there was no difference between procedures (a) (motility index: 3.3±0.31; survival rate 70±2.7%) and (b) (motility index: 3.1±0.29; survival rate 69±3.7%), while the results of (c) were significantly worse (motility index: 2.2±0.33; survival rate 42±4.6%; Fig. 2).

Ultrastructural change

The sperm of sea urchin belonged to primitive type, consisting of a small acrosome, a nucleus, a midpiece and a flagellum (Fig. 3A). Some ultrastructural changes of sperm, such as the detachment of plasma membrane, destruction of mitochondria, and flagellum rolling up the head, were observed after cryopreservation (Fig. 3B). Morphological normality of the sperm in 15% DMSO frozen at the ratio of 50°C/min to -80°C was better than that of others (Fig. 3C).

Fertility of cryopreserved sperm

There was no significant difference in fertilization rate

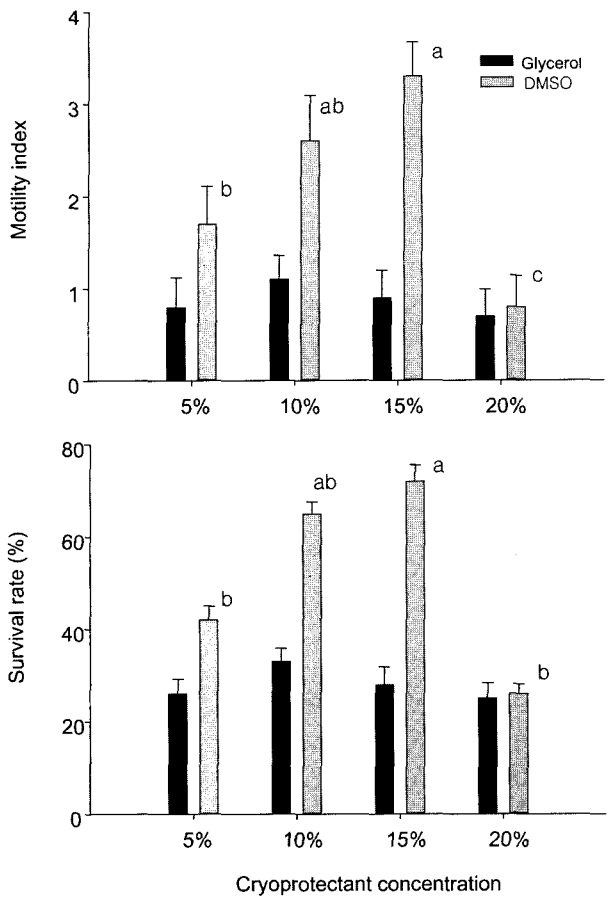

Fig. 1. Motility and survival rate (mean±SE) of sea urchin sperm after cryopreservation using two cryoprotectants at different concentrations. The data are expressed as mean SE (n=3). Difference letters on the bars are significantly difference (P<0.05).

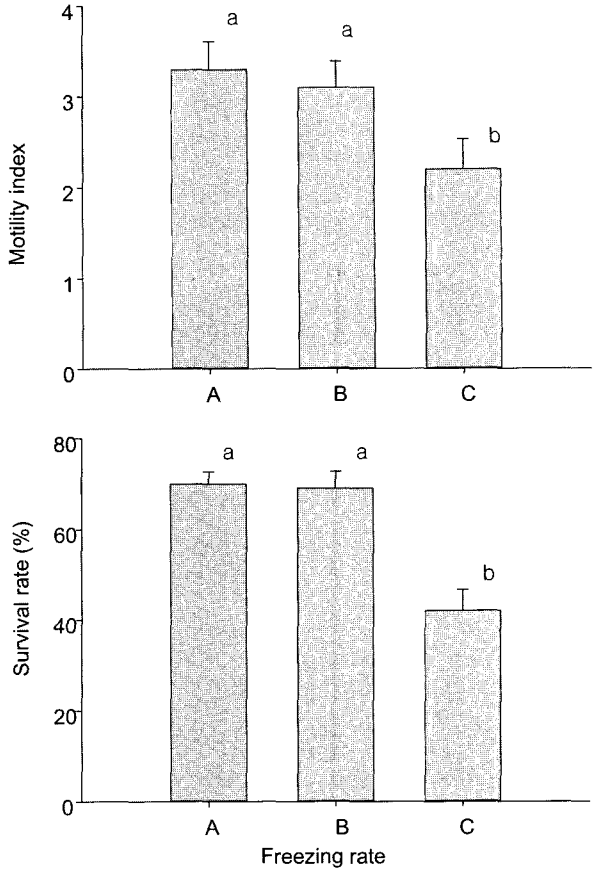

Fig. 2. Motility and survival rate of sea urchin sperm after cryopreservation using three freezing rates A: 50°C/min to -80°C, B: 30°C/min to -80°C, C: 50°C/min to -80°C. The data are expressed as mean±SE (n=3). Difference letters on the bars are significantly difference (P<0.05).

between fresh sperm (64±4.2%) and sperm preserved with 15% DMSO as cryoprotectant (59±3.9%) and freezing procedure (a) (P>0.05).

Discussion

Because fish sperm are quiescent while in the seminal plasma within the testes, preparation and use of extender solutions that are similar in chemical concentration and osmolality are essential in storage of sperm to avoid an excessive consumption of energy by sperm movement and optimizing storage time (Baynes et al., 1981). The immobilizing solutions, such as 1% NaCl, 0.3 M glucose, diluted seawater, and other solutions whose compositions were similar with seminal plasma, were often used as a diluent in the short-term preservation and cryopreservation of fish sperm (Fabbrocini et al., 2000; Yao et al., 2000). However, sperm of sea urchin can maintain motility for a long period in seawater. This would seem to render unnecessary the precaution of inhibiting motility in the phases prior to freezing in sperm cryopreservation, as has been suggested in fish, which was also proved by successful cryopreservation using ASW as diluents in this study.

Generally, the cryopreservation of sperm results in considerable damages to cellular structures such as plasma membrane, nucleus, mitochondria, and flagellum, due to the formation of ice crystals, stress caused by heat shock and excessive dehydration during freezing (Lahnsteiner et al., 1996b; Watson, 1995). This may lead to the decline of sperm motility and survival rate (Lahnsteiner et al., 1996c), which often used as the evaluation parameters of sperm cryopreservation. In fact, many authors have linked the percentage of motile sperm and the quality of the movement to the fertilization ability (Lahnsteiner et al., 1996a, b, c). In the present study, the motility and survival rate of post-thawed sperm were all lower than those of fresh sperm, and best result was

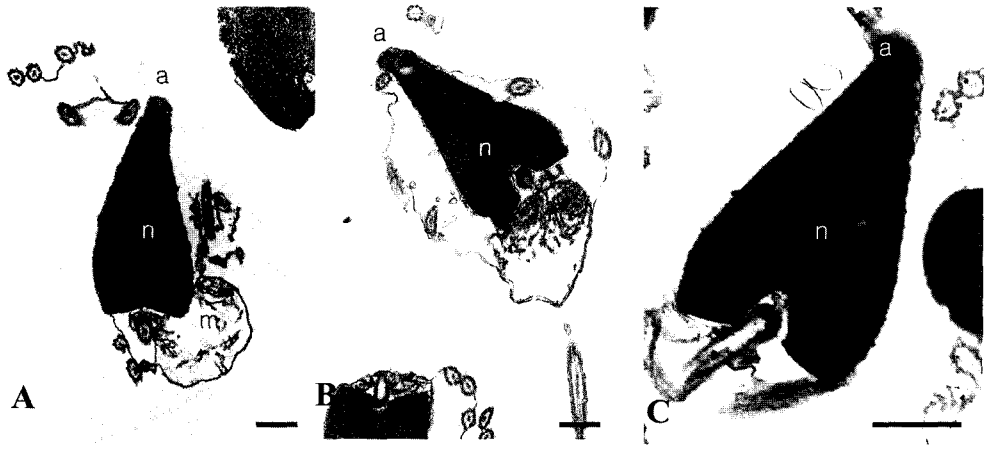

Fig. 3. The ultrastructural changes in sea urchin sperm after cryopreservation. A: Fresh sperm showing integrated plasma membrane, nucleus, acrosome, flagellum, and abnormal mitochondria, B: Sperm preserved in 15% glycerol showing normal nucleus, detachment of plasma membranes, distortion of mitochondria, and flagellum rolling up the head. C: Sperm preserved in 15% DMSO showing normal plasma membrane. a: acrosome; m: mitochondria. n: nucleus. Bar, 0.5 um.

obtained by using 15% Me_2SO as a cryoprotectant and freezing at the rate of 30 or 50°C/min to -80°C.

Of basic importance for sperm viability is the integrity of the cellular membrane. It is well known that the plasma membrane is the primary site of cooling and warming injury (Morris, 1981) and the resistance of spermatozoa to cooling is influenced by the biochemical composition of their membranes (Darin-Bennet and White, 1977). Cooling and warming induce phase transitions of the lipids in the sperm cell membranes, influencing the spatial redistribution of their components (Darin-Bennet and White, 1977; De Leeuw et al., 1990; Quinn, 1985). Consequent to these structural changes, membranes destabilize, modify their permeability, and impair the membrane transporting enzymes (Holt and North, 1984) thus, spermatozoa are more liable to suffer damages during freezing and thawing (De Leeuw et al., 1993). In addition to membrane destabilization, physical damage to the plasma and nuclear membranes can result from the formation of ice crystals within the cell. Cellular osmoregulation also could be compromised, resulting in swelling of the head and tail, and secondary injuries to other intracellular organelles can determine displacement and loss of functionality of mitochondria and outflow of nuclear chromatin (Taddei et al., 2001).

The ultrastructural analysis revealed varying degrees of aberrations, such as the detachment of plasma membrane, destruction of mitochondria, which may result in the decrease of sperm motility after cryopreservation, whereas the nuclear chromatin was well preserved in this study. The better integration of plasma, and the normality of mitochondria in sperm preserved using 15% DMSO were accordant with its higher post-thawed motility. In addition, it should be noticed that the fresh sperm of sea urchin also showed anomalies in mitochondria may be due to a combination of vulnerable or ageing cells and fixation procedures for microscopic observations (Taddei et al., 2001).

Although cryopreservation can cause damages to cellular structures, the fertilization rate of post-thawed sperm may be improved by increasing the volume of sperm in artificial insemination, as suggested by some authors (Gwo, 1994). This study also showed the fertilization rate with double volume of cryopreserved sperm did not decline significantly compared to fresh sperm.

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