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Short-term Storage and Cryopreservation of Abalone (*Haliotis discus hannai*) Sperm

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ABSTRACT

In present study, attempts were made to preserve abalone (*Haliotis discus hannai*) sperm in liquid form at low temperature, to evaluate the effect of various diluents in short-term storage on sperm, and cryopreservation procedures were optimized for the cryoprotectants and freezing rates, as well as the motility, survival rate, and the ultrastructural changes of sperm after short-term storage and cryopreservation were observed. The abalone sperm reached maximum motility until about 4 min after activation. The motility was constant for about 16 min, after which it dropped gradually, and about 50 min later all motility ceased. In Hanks' balanced salt solution (HBSS, 300 and 400 mOsmol/kg) and 150, 250 and 350 mOsmol/k artificial seawater (ASW), the sperm was immotile. After 100% ASW was added, motility of those sperm, which are in 300, 400 mOsmol/kg HBSS, 250, 350 mOsmol/kg ASW, could be again restored incompletely. Sperm motility can be maintained for 20 days of cold storage only in ASW of 850 and 1200 mOsmol/kg. A high motility index of 3.5-4.5 was observed for the first 8 days in 850 and 950 mOsmol/kg ASW. In other diluents sperm motility was constant less than 10 days, and the motility index was obviously lower than that of sperm in 850 and 1200 mOsmol/kg ASW. After 20 days of cold storage, survival rates of 10.2%-20.7% were obtained in ASW and 300 mOsmol/kg HBSS, and that in 400 HBSS (65.3%) was significantly higher than others. The constant period of sperm motility stored in 850 mOsmol/kg ASW was obviously longer than that in 1200 mOsmol/kg ASW after 6 days of storage. The sperm plunged into liquid nitrogen all died except that sperm using 15% glycerol as cryoprotectant restored 10.4% of motility. The highest motility index (3.4) was obtained with 5% glycerol and freezing procedure: -50℃/min from 20℃ to -80℃.

Keywords: Abalone, *Haliotis discus hannai,* Sperm, Short-term storage, Cryopreservation.

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INTRODUCTION

Abalone is an economically important marine gastropod commanding moderate to high prices. The culture of abalone has been investigated for a number of decades in Japanese and Chinese (Chen *et al.*, 1977; Ino, 1951), and a rapid improvement in culture techniques in recent years has had a significant impact on abalone production, especially in Japan and Taiwan (Chen, 1989). Today, the techniques of abalone culture, especially the seed production, such as the induction of spawning, fertilization and hatch, larval rearing and induction to settle, have been well established (Fleming, 1996).

In seed production artificial insemination requires a large quantity of good quality semen available. Collection and storage of good quality semen for future use may improve the convenience of artificial insemination and reduces stress to male broodstock caused by repeated semen sampling, which reduces semen quality (Yao *et al.*, 2000). The short-term storage of sperm in liquid form at low temperature is applied mostly in hatcheries to overcome problems such as asynchrony in maturation, and difficulties in gamete transportation. The milt has been successfully preserved in some fish species, e.g., *Ctenopharyngodon*

idella, *Hypophthalmichthys molitrix*, and Atlantic sturgeon *Acipenser oxyrhynchus* (Di Lauro *et al.*, 1994; Jaehnichen, 1992). In most fish, however, milt may only be stored for 2-3 weeks. After that, sperm motility remarkably decreases, and currently there is no method other than cryopreservation that can prolong the duration of sperm storage (Chao and Liao, 2001). Cryopreservation is an effective method for long-term storage of viable sperm. It has long been 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 of sperm has been well established for many years 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). Since procedures for sperm cryopreservation are generally species specific, the present study selected appropriate diluents for cold storage, and optimized methods of cryopreservation of abalone (*Haliotis discus hannai*) sperm for the cryoprotectants and freezing rates, in terms of the motility, survival rate, and the ultrastructural changes of sperm after short-term storage and cryopreservation.

MATERIALS AND METHODS

1. Abalone

Male Abalones (shell length: 6.69-7.2cm; body weight: 41.13-48.09 g) were collected from a shellfish hatchery on Yosu city of Chonnam province, Korea, during the reproductive season.

2. Motility Estimation

Sperm was obtained from dissected testes. The percentage of sperm exhibiting rapid, vigorous and forward movement was classified under a microscope by diluting the sperm in artificial seawater (ASW;

Table 1. Classes of motility in relation to percentage of sperm with rapid, vigorous and forward movement.

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

Table 2. Concentration of ingredients (g/L) for Hanks' balanced salt solution prepared at various osmolalities using distilled water (pH 7.8, 10 mM HEPES solution as a buffer).

Ingredient	Osmolality		
	200 mOsmol/kg	300 mOsmol/kg	400 mOsmol/kg
NaCl	5.26	8.00	10.53
KCl	0.26	0.40	0.53
$MgSO_4.7H_2O$	0.13	0.20	0.26
$NaHCO_3$	0.23	0.35	0.46
$C_6H_{12}O_6$	0.66	1.00	1.32
$CaCl_2$	0.09	0.14	0.19

423.00 mM NaCl, 9.00 mM KCl, 9.27 mM $CaCl_2$, 22.94 mM $MgCl_2$, 2.114 mM $NaHCO_3$, 10 mM HEPES; pH 7.8; osmolality 1200 mOsmol/kg) in a ratio of 1:1000 (Table 1), and the constant time of vigorous forward movement of sperm was estimated. The samples with high motility were kept on crushed ice until use in the following experiments.

In order to assess the relationship between osmotic pressure and sperm activation, the effect of various media, diluted ASW with distilled water at different osmolalities and Hanks' balanced salt solution (HBSS, pH 7.8) at different osmolalities (200, 300, and 400 mOsmol/kg, see Table 2), on sperm motility were estimated as above method. The osmolality of different media was tested using osmometer (OSMOMAT 030-D). The motility of sperm in an immobilizing solution were examined after activation again with 100% ASW.

3. Short-term storage

According to results of above trials, diluted ASW (250, 350, 850, 1200 mOsmol/kg) and HBSS (300 and 400 mOsmol/kg) were prepared as diluents in cold storage trials. The sperm was suspended in diluents at a 1:1 ratio, placed in 1.5-ml plastic centrifuge tube, and preserved in a refrigeratory at 4℃.

The effect of short-term storage was evaluated by daily estimates of sperm motility and the survival rate of preserved semen estimated by an eosin-nigrosin staining technique (Bllom, 1950; Fribourgh, 1966) that continued until sperm no longer exhibited motility. The semen was stained with a drop of 5% eosin and two drops of 10% nigrosin. After mixture smears were made, the survival rate was determined under a microscope in terms of the percentage of unstained sperm. The ultrastructural examination also was performed following the method described bellow.

4. Cryopreservation of Sperm

Glycerol was used as a cryoprotectant in cryopreservation trial. It was added to 100% ASW to formulate the extenders at the concentrations of 5%, 10%, 15% and 20% by volume for freezing. The sperm was diluted 1:9 with the extenders. The diluted sperm was inserted into 0.5-ml plastic straws, equilibrated for 10 min at room temperature (20℃), and then the straws were plunged into liquid nitrogen directly or cooled as followed freezing procedures before transfer into liquid nitrogen: (a) -50℃/min to -80℃, (b) -20℃ /min to -80℃, (c) -10℃/min to -80℃. The straws were thawed in a 30℃ water bath for 15 sec. On thawing, the motility of frozen-thawing 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. The survival rate was also estimated by the eosin-nigrosin staining technique.

5. Ultrastructural change

The fresh and frozen-thawing 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-Ⅱtransmission electron microscope.

6. 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 SigmaPlot 8.0 (SPSS Inc., 2002).

RESULTS

1. Motility characteristic

In order to characterize each sample, motility was separated into three periods: time to reach maximum motility, duration of motility, and the time until all motility ceased (Fig. 1). On contact with 100% ASW, the sperm was immediately activated, but did not reach maximum motility until about 4min after activation. This motility was constant for about 16min, after which it dropped gradually, and about 50min later all motility ceased.

Threshold activation of sperm was found in 450 mOsmol/kg ASW, and motility increased as the concentration of ASW increased (Fig. 2). In HBSS

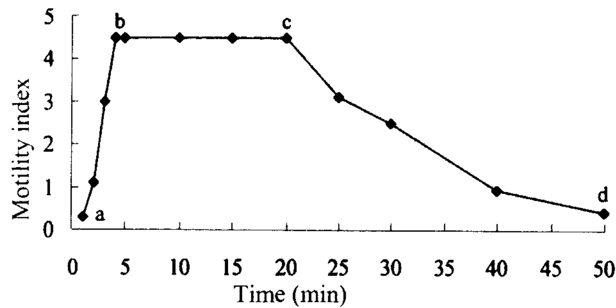

Fig. 1. Motility characterization of abalone sperm activated with 100% artificial seawater. Motility was characterized in three intervals: 1) time to reach maximum motility (segment a-b), 2) duration of maximum motility (b-c), and 3) time until all motility ceased (c-d).

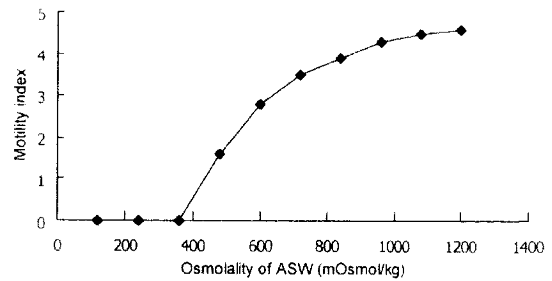

Fig. 2. Motility (mean ± SD) of abalone sperm in various osmolalities of artificial seawater.

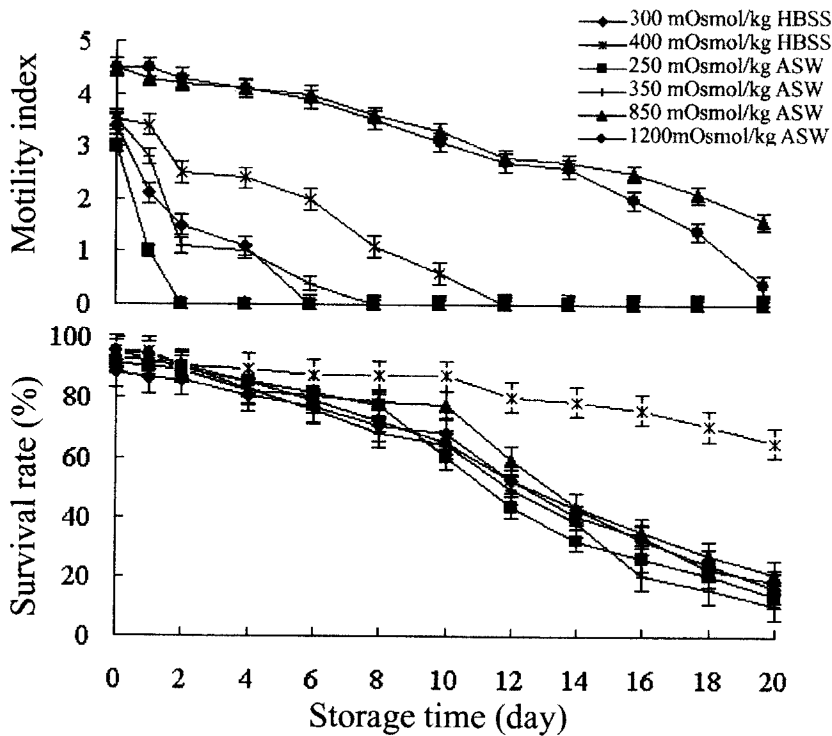

Fig. 3. Changes in motility and survival rate (mean ± SD) of abalone sperm stored with 6 kinds of diluents at 4℃.HBSS: Hanks' balanced salt solution; ASW: artificial seawater.

(200, 300 and 400 mOsmol/kg) and 150 mOsmol/kg ASW, the sperm was immotile. After 100% ASW was added, motility of those sperm, which are in 300, 400 mOsmol/kg HBSS, 250, 350 mOsmol/kg ASW, could be again restored incompletely, while all those treatments were significantly lower than that of fresh sperm (Table 3).

2. Short-term storage

The changes in motility and survival rate of sperm after 20 days of storage at 4℃were showed in Fig. 3. Sperm motility was extended following 20 days of cold

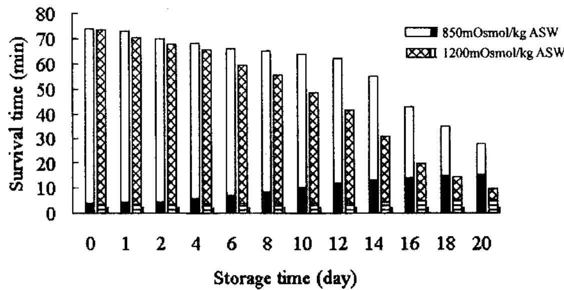

Fig. 4. Changes in constant period of sperm motility and time to reach maximum motility after cold storage in 850 mOsmol/kg and 1200 mOsmol/kg ASW.

storage only in 850 and 1200 mOsmol/kg ASW. A high motility index of 3.5-4.5 was observed for the first 8 day in 850 and 1200 mOsmol/kg ASW, and a motility index of 2.5-3.5 was found for following 4 days in 850 mOsmol/kg ASW and 3 days in 1200 mOsmol/kg ASW. In other diluents, sperm motility was constant less than 10 days: 10 days in 400 mOsmol/kg HBSS, 4 days in 300 mOsmol/kg HBSS, 6 days in 350 mOsmol/kg ASW, and 1day in 250 mOsmol/kg ASW, and the motility index was obviously lower than that of sperm in 850 and 1200 mOsmol/kg ASW. The changes in survival rate of stored sperm were not similar with that in motility. After 20 days of cold storage survival rates of 10.2%~20.7% were obtained in ASW and 300 mOsmol/kg HBSS, and that in 400 HBSS (65.3%) was significantly higher than others, which was held at a high level all along the cold storage.

Fig. 4 showed the changes in constant period of sperm motility and time to reach maximum motility after cold storage in 850 and 1200 mOsmol/kg ASW. The constant period of sperm motility in 850 mOsmol/kg ASW was obviously longer than that in 1200 mOsmol/kg ASW after 6 days of storage, and the time to maximum motility of sperm stored in 850 mOsmol/kg increased gradually, while the difference in which of sperm in 1200 mOsmol/kg ASW was not significant.

The sperm of *Haliotis discus hannai* belonged to primitive type, consisting of a bullet-shaped acrosome, a nucleus, a midpiece and a flagellum (Fig. 5A). The ultrastructural changes after 6 days of short-term storage in different diluents were showed in Fig. 5B-I. Sperm stored in 850 and 1200 mOsmol/kg ASW presented detachment of acorsome and plasmatic membrane. The flagellum rolling up the head appeared in sperm preserved in 250 and 350 mOsmol/kg ASW, 300 mOsmol/kg HBSS. Furthermore, the swell of plasmatic membrane covering the acrosome was found in sperm stored in 350 mOsmol/kg ASW and 300 and 400 mOsmol/kg HBSS. The mitochondria also were damaged at various degrees.

3. Cryopreservation of sperm

The motility, survival rate, constant period of motility and time to reach maximum motility of frozen-thawing sperm after cryopreservation were showed in Fig. 6. The sperm plunged into liquid nitrogen all died except that sperm using 15% glycerol as cryoprotectant, which restored 10.4% of motility. The highest motility index (3.4) was obtained with 5% glycerol and freezing procedure (a): -50℃/min to -80℃.

Table 3. Motility of abalone sperm diluted in various media before and after activation with 100% ASW.

Diluents (Osmolality, mOsmol/kg)		Motility index	
		Before adding 100% AS	After adding 100% AS
ASW	150	0	0
	250	0	3 ± 0.23
	350	0	3.5 ± 0.16
HBSS	200	0	0.2 ± 0.11
	300	0	3.4 ± 0.25
	400	0	3.5 ± 0.13

The relatively high motility index was found with 5% glycerol using procedure (b), and 10% glycerol using procedure (a). The changes in survival rate, constant period of motility and time to reach maximum motility were similar with that of motility.

The ultrastructure of sperm plunged into liquid nitrogen directly was damaged badly, such as the absence of acrosome, and granular nucleus (Fig. 7A, B). Sperm preserved in 15% glycerol using procedure (a) showed distortion of acrosome and mitochondria, and detachment of plasmatic membrane (Fig. 7C).

Sperm preserved in 5% glycerol using procedure (a) showed integrated plasmatic membrane, but the acrosome was detached from nucleus (Fig. 7D), while sperm using procedure (c) also presented the flagellum rolling up the head (Fig. 7E).

DISCUSSION

Sperm are typically not motile in the testes or seminal fluid of fish, and during reproduction motility is induced after sperm are released into the water (Grier, 1981). Sperm motility is stimulated by the ionic composition, pH, or the osmolality of water (Stoss, 1983). In freshwater fish sperm are activated by suspension in hypotonic solution (Christensen and Tiersch, 2001; Morisawa and Suzuki, 1980), while sperm of marine fish are activated by suspension in hypertonic solution greater than 400 mOsmol/kg (Gwo *et al.*, 1991; Morisawa and Suzuki, 1980; Wayman *et al.*, 1998). This study showed that the osmolality also is the pivotal factor for determining the sperm motility, and the increase of osmolality can activate sperm motility. In this study, the motility of abalone sperm could be activated at the osmolality of about 450 mOsmol/kg.

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 expenditure of energy by sperm movement and optimizing storage time. 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).

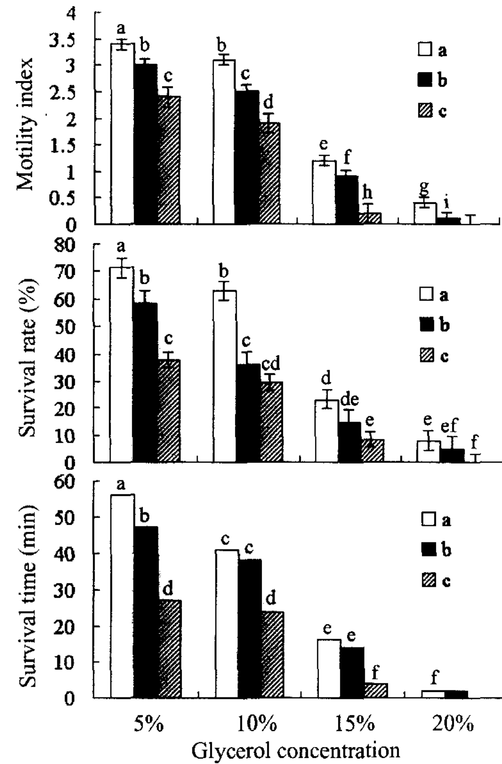

Fig. 6. Motility, survival rate (mean ± SD) of abalone sperm, and constant period of sperm motility and time to reach maximum motility after cryopreservation using various cryoprotectants at different freezing rates. The trail (a) of 5% glycerol had a significantly better result than others ($p < 0.01$).

In the present study, abalone sperm reached maximum motility at about 4min after activation, and the motility was constant for about 16min, after which it dropped gradually, and about 50min later all motility ceased. This result 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 100% ASW as diluents in this study. Furthermore, the motility of sperm diluted in immobilizing solutions (200, 300 and 400 mOsmol/kg HBSS, 150, 250, 350

Fig. 5. The ultrastructural changes in *Haliotis discus hannai* sperm after 6 days of short-term storage in different diluents. **A**: Longitudinal section through the head and midpiece of fresh sperm showing integrated plasmatic membrane, nucleus, acrosome, mitochondria, and flagellum. **B**: Sperm stored in 250 mOsmol/kg artificial seawater (ASW) with abnormal acrosome and mitochondria, and flagellum rolling up the head (arrow). **C**: Sperm in 850 mOsmol/kg ASW showing detachment of plasmatic membrane. **D**: Sperm in 850 mOsmol/kgASW showing acrosome detached from nucleus. **E**: Sperm in 1200 mOsmol/kgASW showing normal acrosome and detached plasmatic membrane. **F**: Sperm in 1200 mOsmol/kg ASW containing detached acrosome. **G**: Sperm in 350 mOsmol/kg ASW showing rupture (asterisk) and swell (plus) of plasmatic membrane, abnormal mitochondria, and flagellum rolling up the head (arrow). **H**: Sperm in Hanks' balanced salt solution (HBSS) of 300 mOsmol/kg showing swollen plasmatic membrane (plus) and flagellum rolling up the head (arrow). **I**: Sperm in 400 mOsmol/kg HBSS showing swollen plasmatic membrane (plus) and plasmatic membrane detached slightly. a: acrosome; f: flagellum. m: mitochondria. n: nucleus. scale bar = 0.5 μm.

mOsmol/kg ASW,) did not restore completely, even entirely lost in 150 mOsmol/kg ASW, after activation again. This phenomenon may be due to the osmotic shock caused by the immobilizing solutions, which might also be the reason for rapid loss of sperm motility in short-term storage using immobilizing solution as diluents, and increase of time to reach maximum motility for sperm stored in 850 mOsmol/kg ASW. The successful cold storage of sperm in 850 and 1200 mOsmol/kg ASW indicated that the excessive expenditure of energy did not appear. During storage the sperm all deposited at the bottom of centrifuge tube. The high sperm density in diluents may lead to the effective inhibition of sperm movement. The constant period of sperm motility in 1200 mOsmol/kg ASW, however, still was shorter than that in 850 mOsmol/kg ASW. In addition, the hypotonic diluents resulted in the loss of sperm motility, but did not arouse a great deal of death of sperm.

Generally, the cryopreservation of sperm results in considerable damages to cellular structure such as plasma membrane, nucleus, mitochondria, and flagellum, due to the formation of ice crystals, stress caused by heat shock and excessive dehydration

Fig. 7. The ultrastructural changes in *Haliotis discus hannai* sperm after cryopreservation. **A**, and **B**: Sperm preserved in 15% glycerol and plunged into LN_2 directly; A showed damages to nucleus, absence of acrosome, rupture of nuclear membrane, and detachment of plasmatic membrane. B showed normal nucleus, absence of acrosome and nuclear and plasmatic membranes, and distortion of mitochondria, **C**: Sperm preserved in 15% glycerol using procedure (a) showing distortion of acrosome and mitochondria, and detachment of plasmatic membrane, **D**: Sperm preserved in 5% glycerol using procedure (a) showing detachment of acrosome, **E**: Sperm preserved in 5% glycerol using procedure (c) showing abnormal acrosome and mitochondria, and flagellum rolling up the head (arrow). a: acrosome; f: flagellum. m: mitochondria. n: nucleus. scale bar = 0.5 μm.

during freezing (Lahnsteiner *et al.*, 1996a; Watson, 1995). This may lead to the decline of sperm motility and survival rate (Lahnsteiner *et al.*, 1996b), 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 sperm motilities, survival rates, and constant period of sperm motility were all less than those of fresh sperm, and best frozen-thawing motility (3.4) and survival rate (71.4%) were obtained by using 5% glycerol as a cryoprotectant and freezing at the rate of -50℃/min to -80℃. It is unbefitting to plunge sperm into liquid nitrogen directly in cryopreservation of abalone sperm using glycerol as cryoprotectant.

During cold storage some changes in sperm ultrastructure were found. The stored sperm holding high motility was obtained with 850 and 1200 mOsmol/kg ASW, accompanied by detachment of acorsome and plasmatic membrane. Sperm without motility, such as in 250 and 350 mOsmol/kg ASW, and 300 mOsmol/kg HBSS, all presented the flagellum rolling up the head. The better ultrastructure of sperm in 400 mOsmol/kg HBSS was accordant with high survival rate, although the motility was lower than that of sperm in 850 and 1200 mOsmol/kg ASW. After cryopreservation the ultrastructure of sperm plunged into liquid nitrogen directly was damaged badly. Various degrees of abnormalities, such as the detachment and rupture of plasmatic membrane, destruction of mitochondria, and flagellum rolling up the head, which may result in the decrease of sperm motility, whereas the nuclear chromatin was well preserved in this study. The higher integration of plasmatic and nuclear membrane, and the normality of mitochondria in sperm preserved in 5% glycerol using procedure (a) were accordant with its higher post-thawed motility.

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