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Short Term Storage and Cryopreservation of Trumpet Shell *Charonia sauliae* Sperm

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ABSTRACT

With the purpose to estimate the possibility of short-term storage and cryopreservation for sperm of *Charonia sauliae*, which is a potential preparation for its artificial reproduction and further research, in this study, protocols for short-term storage and cryopreservation of trumpet shell sperm was optimized. The effects of different immobilizing solutions, dilution ratios were estimated for short-term storage. And the effects of different cryoprotectant extenders and freezing rates were estimated for cryopreservation in terms of motility and survival of sperm. The results indicated that the artificial sea water of 350 mOsmol/kg is a better immobilizing solution and sperm which was diluted at a ratio of 1:1 (v/v) had higher motility and survival rate during short-term storage. The effect of 5% dimethyl sulfoxide was significantly better than those of other cryoprotectant extenders. And a freezing rate of -20℃ min^{-1} showed better effect than other freezing rates. In conclusion, this study optimized some key factors of the short-term and cryopreservation of *C. sauliae* sperm, which can provide valuable data for germplasm conservation and artificial propagation of *C. sauliae*.

Key words : Cryopreservation, Short-term storage, Sperm, *Charonia sauliae*, Cryoprotectant, Freezing rate

INTRODUCTION

Since the discovery of cryoprotectants in the mid-1900s, the technique of cryopreservation has developed immensely, and become a widely applied method (Fahning and Garcia, 1992; Mazur, 1984). The cryopreservations of sperms, oocytes, embryos and larvae of marine animals have been proven effective for the preservation of strains and hatchery, and have also been proven useful as components of a variety of biological assays (Usuki *et al.*, 2002; Fabbrocini *et al.*, 2000). As far as now, the cryopreservations of sperms, eggs and embryos have provided several benefits to the aquaculture industry, including: reduced cost of brood stock; higher efficiency of resource use (overcoming seasonal limitations); reduced cost of spat production; selective breeding; and the maintenance of threatened genetic strains. For artificial propagation and further aquaculture research of

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endangered shellfish, cryopreservation techniques also play a key role (Smith *et al.*, 2001).

However, researches into cryopreservation of marine organisms have been focused on spermatozoa (Suquet, 2000; Liu et al., 2007), while the cryopreservations of ova, embryos, and larvae seem more difficult, because of their more complicated structures than those of spermatozoa. Although many species have been successfully cryopreserved, the cryopreservation protocol of sperm varies among species.

Charonia sauliae, which is an endangered and valuable species belongs to the Cymatiidae family, and is commonly referred to as trumpet shell, Saul s triton or the white triton shell. Its shell is white with a few brown spots, and the steepled spiral shell can reach lengths of 10-30 cm. This species is mainly distributed in tropical and semi-tropical areas of the Atlantic, Indian and Pacific Oceans, and normally inhabits the bottoms of coral reefs in shallow seas, at a depth of 10-20 m, but can also be detected in deeper seabeds, at a depth of 200-300 m. The trumpet shell performs an important function in the reef community, and it prefers to prey on star fish which threaten many aquatic economic animals, therefore it may be a means of biological control of predators of economically important shellfish (Kang and Kim, 2004). But it has a low yield and growth under natural environment, so the artificial propagation is necessary. As far as we know, the cryopreservation of sperm of *C. sauliae* has never been studied, so this is the first attempt.

This study examined some key factors affecting the motility and survival of sperm during short-term storage and in cryopreservation. The methods developed here could contribute to the germplasm conservation and artificial propagation of *C. sauliae*.

MATERIALS AND METHODS

Adult and sperm collection

The mature males (mean shell length was 30.34±3.24 cm, mean total weight was 870.53±5.70 g) and females (mean shell length was 33.74±4.35 cm, mean total weight was 960.30±7.33 g) of trumpet shells were collection by diving from Jeju Island, South Korea, and cultivated in the laboratory of Division of Marine Technology, Chonnam National University. Sperm were collected by dry abdominal stripping of the gonad. All sperm samples were collected with the same method and placed separately into a 15-mL polyethylene centrifuge tube on crushed ice.

Assessment of sperm motility and survival

The assessment of sperm motility was conducted according to Ritar and Campet (2000) with slight modification. The sperm were activated by adding artificial sea water (ASW, pH 7.8, osmolality 1200 mOsmol/kg) in a ratio of 1:100 (v/v). And movements of these sperm were observed under a microscope. And the percentage of sperm exhibiting rapid, vigorous and forward movement in each samples were recorded for estimation of sperm activity. The samples with high motility were used in the following experiments. Sperm motility, defined as the mean percentage of forward-moving cells from at least 3 fields of view, was determined within 10 to 15 sec after activation.

Sperm survival was estimated with 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. After mixture smears were made, the survival rate was determined under a microscope in terms of the percentage of unstained sperm.

Experiments of short-term storage

1) Effects of different immobilizing solution

To select an appropriate immobilizing solution, effects of different ASW of 250, 350 and 450 mOsmol/kg were examined. The fresh sperm were suspended in these ASW at a ration of 1:10 (v/v). After dilution, the motility of sperm was estimated before and after activation with undiluted ASW according to the method described above.

2) Influence of dilution ratio on motility and survival of stored sperm

Sperm were diluted at different dilution ratios (1:1, 1:3, 1:6, 1:9 and 1:12 (v/v)) for short-term storage of sperm and their effects were estimated. The sperm were placed into 1.5-mL polyethylene tubes respectively, and preserved in a refrigerator at 4℃. The motility and survival rate of sperm were estimated during the storage of 6 days.

Experiments of sperm cryopreservation

1) Effect of different cryoprotectant extenders

Two common cryoprotectants, dimethyl sulfoxide (DMSO) and glycerol (Gly), were added to ASW to formulate extenders at concentrations of 5, 10, 15 and 20%. The sperm was diluted at a ration of 1:6 (v/v) with the extenders. After an equilibration for 10 min at room temperature (20℃), the diluted sperm were inserted into 0.5-ml plastic straws and frozen at a freezing rate of -30℃ min^{-1} to -100℃, and subsequently plunged into liquid nitrogen. After 60 days, the straws were thawed in a 30℃ water bath for 15 sec. After thawing, the motility and survival of frozen-thawed sperm was estimated according to the methods described above.

2) Effect of different freezing rates

To evaluate effects of different freezing rates, four freezing rates were estimated: a) -5℃ min^{-1} to -100℃; b) -10℃ min^{-1} to -100℃; c) -20℃ min^{-1} to -100℃; d) -30℃ min^{-1} to -100℃. The sperm was diluted at a ration of 1:6 (v/v) with 5% DMSO. After an equilibration for 10 min at room temperature (20℃), the diluted sperm were inserted into 0.5-ml plastic straws and frozen at the freezing rates described above respectively, and subsequently plunged into liquid nitrogen. After 60 days, the straws were thawed in a 30℃ water bath for 15 sec. After thawing, the motility and survival of frozen- thawed sperm was estimated according to the methods described above.

Statistical analysis

Data from the experiment were subjected to one-way analysis of variance and Duncan s multiple-range tests. Statistical comparison was based on 5 parallels for each treatment. Significance of differences was defined as $p<0.05$ in all cases. Statistics were performed using the statistical software SPSS for Windows.

RESULTS

After dilution with ASW of 150, 250, 350, 450, 550 and 650 mOsmol/kg, motility of all sperm samples was significantly inhibited immediately. When 100% ASW (1200 mOsmol/kg) was added, the motility of sperm which were diluted with ASW of 350 mOsmol/kg was restored once again (Fig. 1).

The changes in motility and survival rate of sperm during short-term storage at different dilution ratio were showed in Fig. 2. The motility of the sperm which was diluted at a ratio of 1:1 (v/v) was maintained relative higher during the

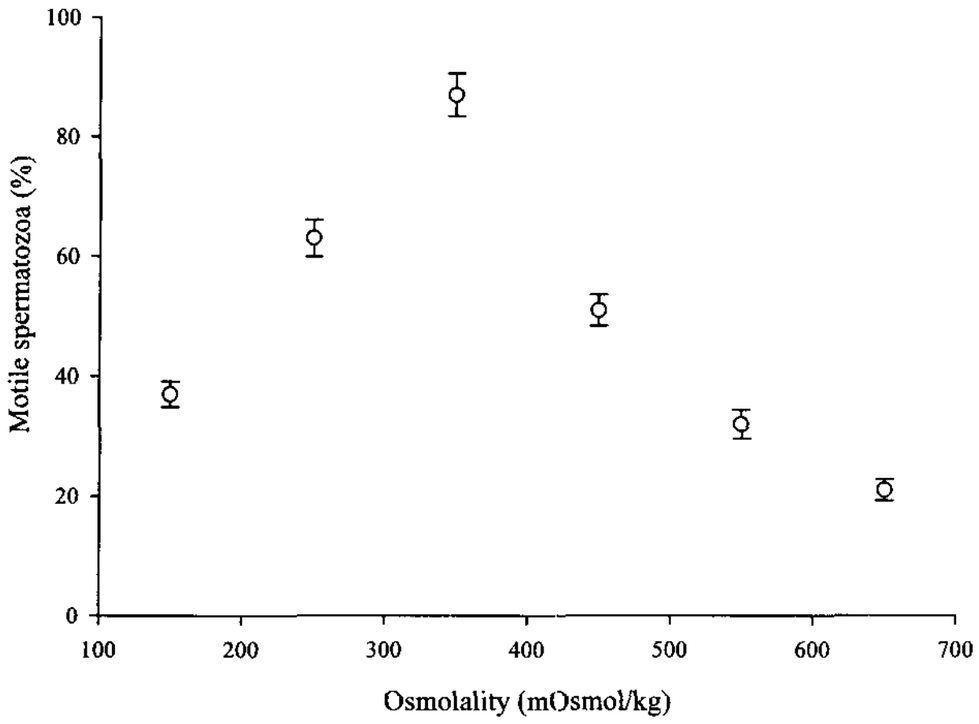


Fig. 1. Motility (mean±SD) of restored spermatozoa which were diluted with different artificial sea waters of 150, 250, 350, 450, 550 and 650 mOsmol/kg.

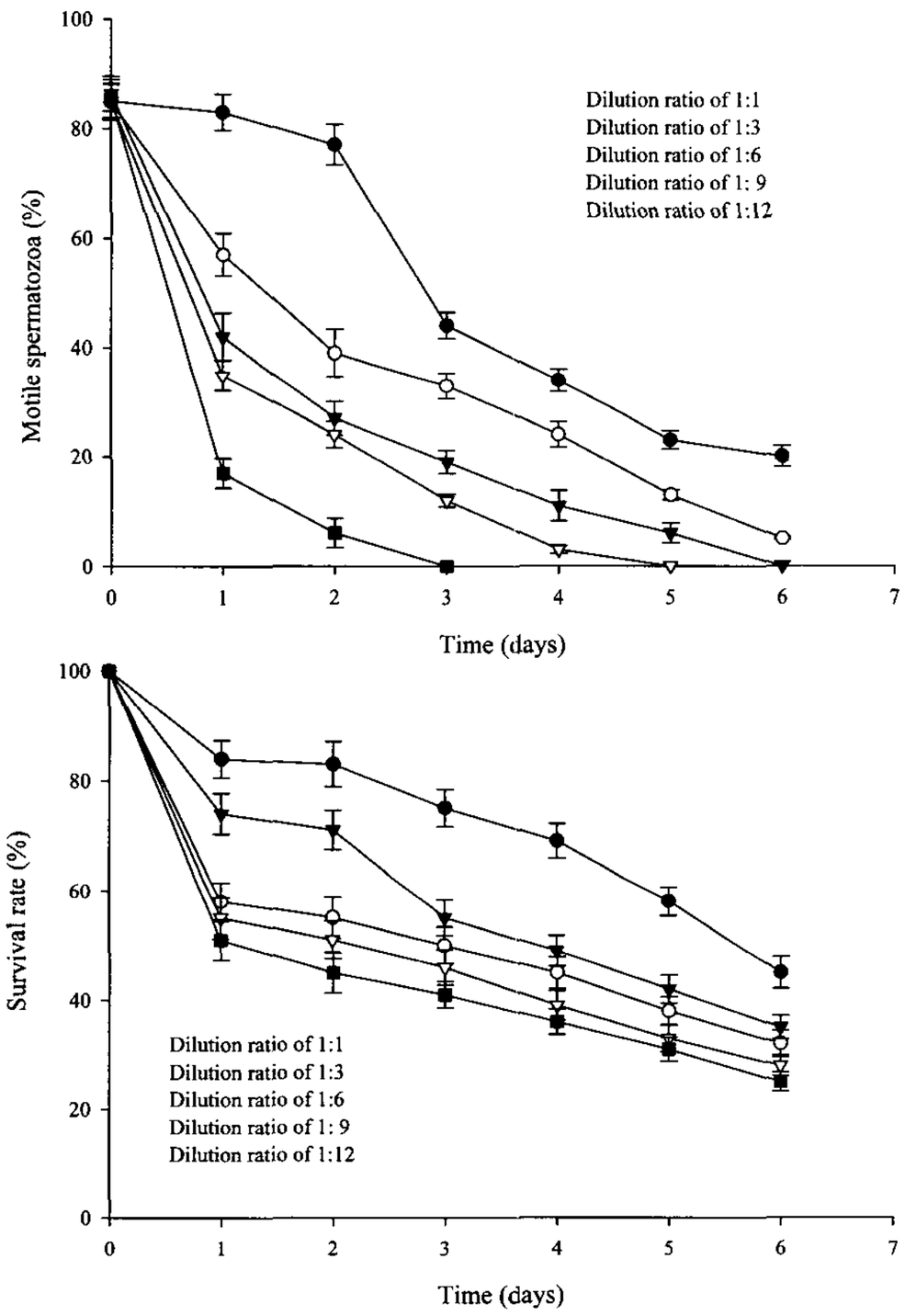
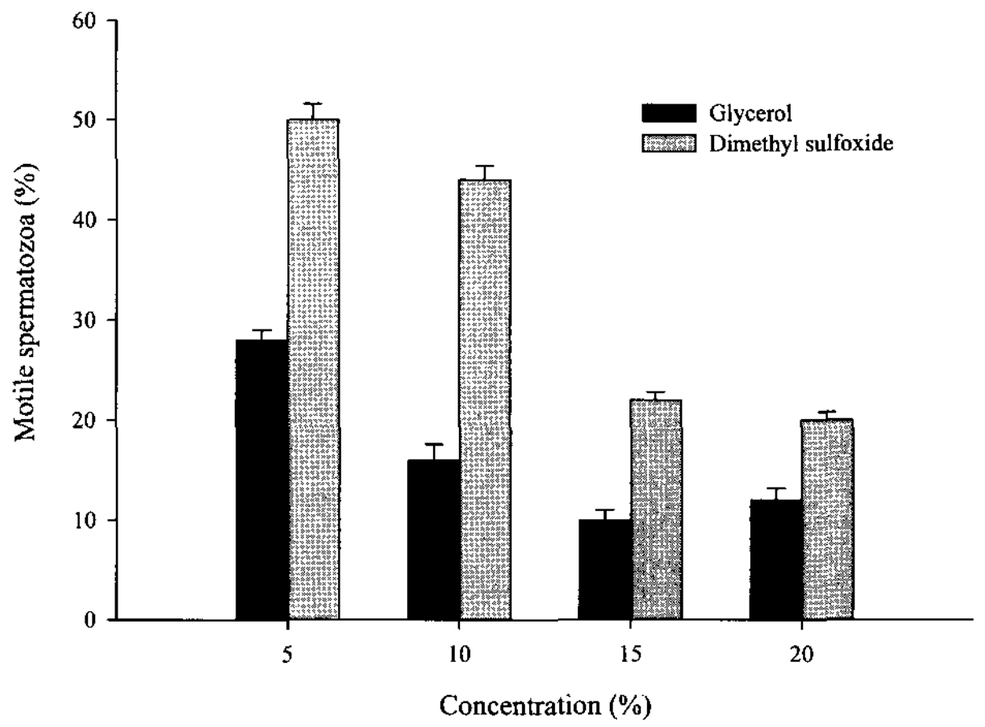


Fig. 2. Motility (mean±SD) and survival rate (mean±SD) of spermatozoa which were diluted at different ratios during short-term storage.



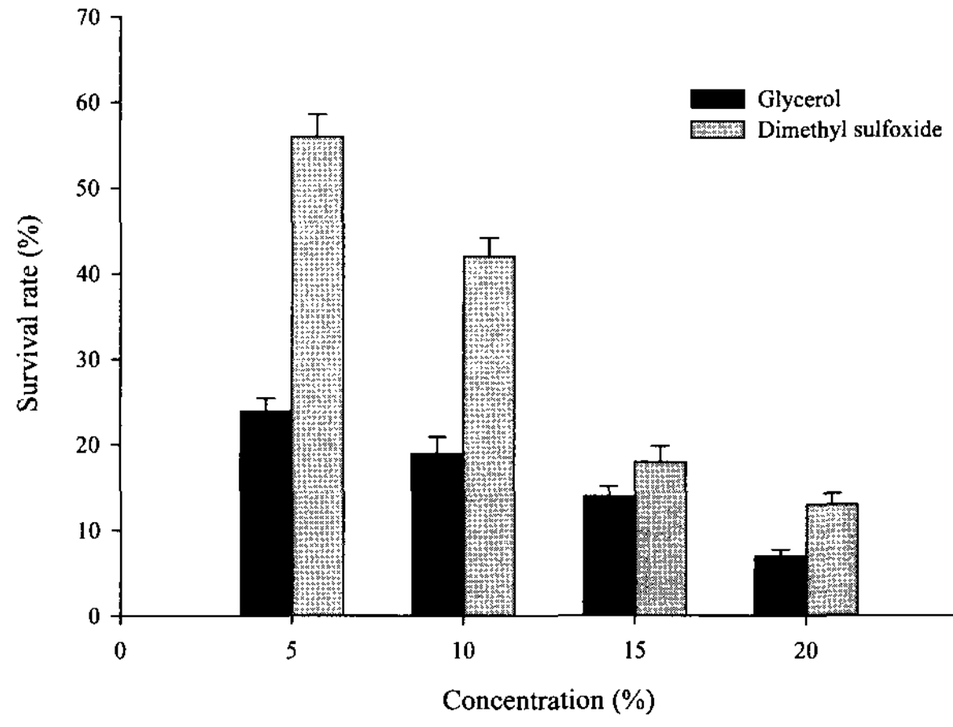


Fig. 3. Motility (mean+SD) and survival rate (mean+SD) of frozen-thawed spermatozoa which were cryopreserved with different cryoprotectant extenders.

storage. But the motilities of sperm which were diluted at the ratio of 1:9 and 1:12 (v/v) only persisted for 2 and 4 days respectively. The survival rate of sperm which was diluted at a ratio of 1:1 (v/v) was significantly higher than those of the sperm which were diluted at other ratios ($P<0.05$).

The motility and survival rate of frozen-thawed sperm with different cryoprotectant extenders were showed in Fig. 3. After cryopreservation, motilities of all sperm samples were significantly lower than those of sperm before cryopreservation. According to the results, the highest motility and

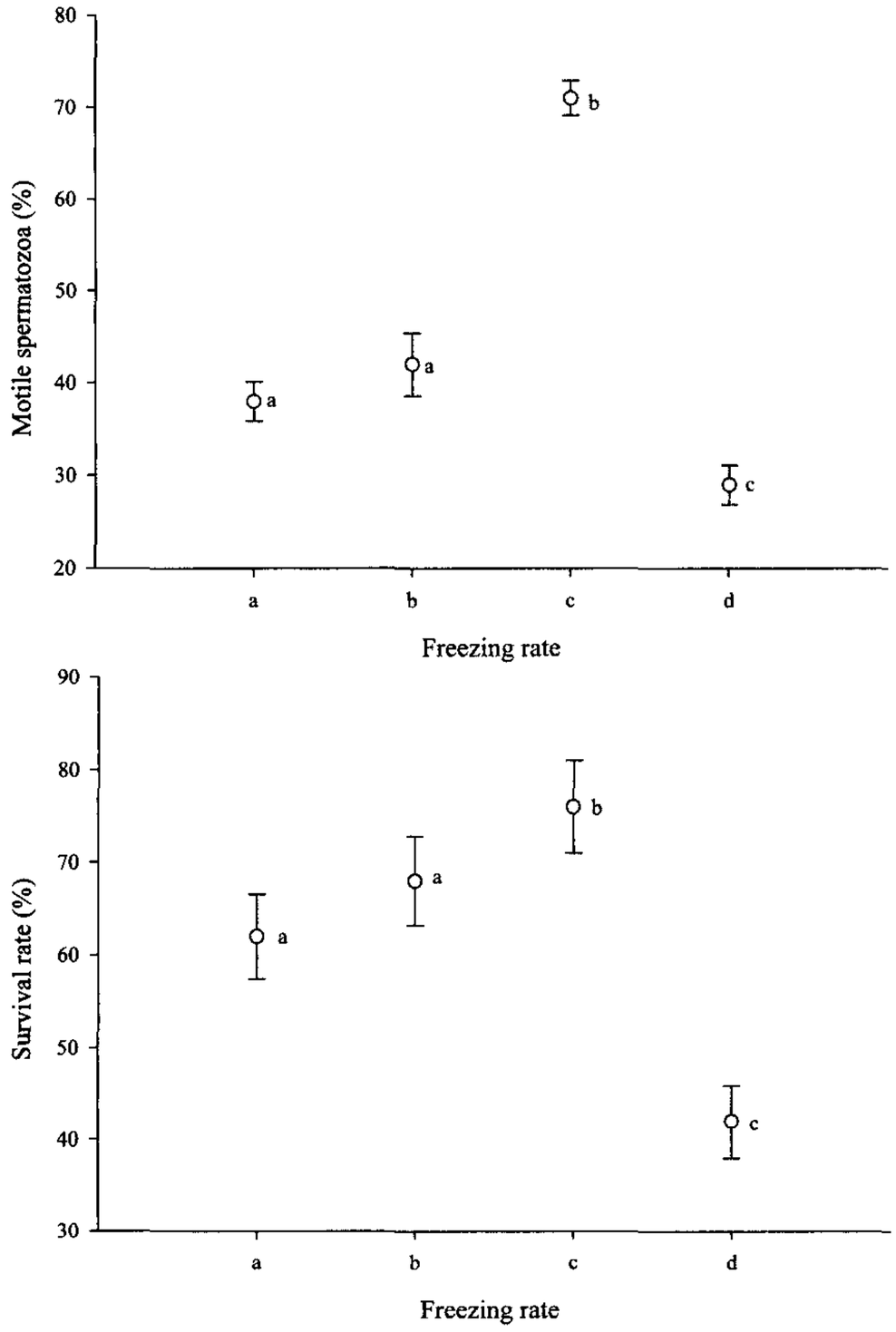


Fig. 4. Motility (mean±SD) and survival rate (mean±SD) of frozen-thawed spermatozoa which were cryopreserved at different freezing rates: a) -5℃ min-1 to -100℃; b) -10℃ min-1 to -100℃; c) -20℃ min-1 to -100℃; d) -30℃ min-1 to -100℃. Means with different letters are significant different (P〈0.05).

survival rates were observed in samples that were cryopreserved with 5% DMSO, which were significant higher than those of other samples (P〈0.05).

The motility and survival rate of frozen-thawed sperm at different freezing rates were showed in Fig. 4. The results showed that the effect of freezing rate (c) was significantly better than those of other freezing rates (P〈0.05).

DISCUSSION

This study has examined two key factors (immobilizing solution and dilution ratio) during short-term storage of *C. sauliae* sperm. The results indicated that ASW of 350 mOsmol/kg was better to inhibit the sperm motility and furthermore ensure the restoration of sperm motility when ASW of 1200 mOsmol/kg was added. The decease of motility of sperm which were diluted with ASW of 150 and 550 mOsmol/kg may be caused by osmotic shock. In marine teleosts, puffers, flounders and cod, the osmolalities of their seminal plasma suppress the sperm motility, and the motility will be initiated when osmolality increases (Morisawa & Morisawa, 1990). The immobilizing solutions, such as 1% NaCl, 0.3 M glucose, diluted seawater, and other solutions which had a similar composition with seminal plasma, were often used as a diluent in the short-term storage and cryopreservation of fish sperm (Blaxer, 1953; Fabbrocini *et al.*, 2000; Yao *et al.*, 2000). In this study, the motility of trumpet shell sperm was short-lived, which maintained their movement for about 10 sec after activation with ASW of 1200 mOsmol/kg. Therefore, an immobilizing solution is necessary for short-term storage and cryopreservation as the diluent in the phases prior to freezing in order to avoid an excessive expenditure of energy by sperm movement (Gwo, 1994).

The dilution ratio of 1:9 and 1:12 resulted in a rapid loss of sperm motility, but did not cause a great deal of death of sperm. The loss of motility may be due to the excessive consumption of energy during storage. This result was different from the authors previous study on abalone sperm (unpublished), in which, abalone (*Haliotis discus hannai*) sperm were successfully stored at a ratio

of 1:9 (v/v) at 4℃. This may be attributed to differences in sperm characteristic and effective dilution ratios. The dilution ratio has been considered to be a key during storage of abalone sperm with activation solutions, because high sperm density in diluents may lead to the effective inhibition of sperm movement to avoid excessive consumption of energy. This hypothesis should be confirmed in other species, e.g., *C. sauliae*.

In this study DMSO presents better protective effects than Gly at each concentration. The result could be explained by the penetration speed result from their molecular weights (Renard and Cochard, 1989) which influenced the protective effect. The rapid penetration speeds of DMSO resulted in good protection. And the best result was obtained using 5% DMSO as the cryoprotectant. The results confirmed the viewpoint that although the cryoprotectants at high concentration could reduce the cryoinjuries, their toxicity to cell couldn 't be neglected (Nascimento *et al.*, 2005).

Many researches have demonstrated the importance of freezing rate in the process of cryopreservation (Adams *et al.*, 2004; Dong *et al.*, 2005; Acosta-Salmóna *et al.*, 2007), but the optimum freezing rate varied among different species. In present study, the freezing rate of -20℃ min^{-1} was better than other protocols. The sperm from *C. Sauliae* seemed to be tolerant to a medium-rapid freezing rate.

The structural damage and functional loss of spermatozoa may lead to the decline of sperm motility and survival rate (Lahnsteiner *et al.*, 1996), which often used as the evaluation parameters of sperm cryopreservation. In the present study, the sperm motilities and survival rates were all less than those of fresh sperm, and best frozen-thawed motility (71±3.4%) and survival rate (76±4.8%) were obtained by using 5% DMSO as a cryoprotectant extender and freezing at the rate of -20℃ /min to -100℃.

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