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Practical Procedure of Sperm Cryopreservation of the Bar-tailed Flathead *Platycephalus indicus*

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ABSTRACT This study was conducted to investigate protocol standardization for cryopreservation spermatozoa of the bar-tailed flathead *Platycephalus indicus*. The suitability of the cryoprotectants, dimethyl sulphoxide (DMSO), glycerol and methanol were tested against three freezing rates and three thawing temperatures. DMSO and glycerol gave significantly higher motile index and survival rates than methanol. Among the freezing rates, freezing at a height of 2 cm above LN_2 surface for 10 min^{-1} gave higher motile index and survival rates. In terms of best thawing temperature, 20°C obtained the highest motility.

Key words : *Platycephalus indicus*, bar-tailed flathead, spermatozoa, cryopreservation, cryoprotectants

INTRODUCTION

Cryopreservation is a process where biological samples, such as cells or tissues, are preserved by cooling to sub-zero temperatures. It is an effective method for long-term storage of sperm and used in breeding strategies of numerous animal species (Gañán *et al.*, 2009; Anzar *et al.*, 2010; Hu *et al.*, 2010). Preservation of spermatozoa at low temperatures was initially reported by Polge *et al.* (1949), where in a brief article they stated that the addition of 20% glycerol to the freezing process achieved high survival rates for human and fowl sperm after thawing. Cryopreservation offers benefits in aquaculture and experimental studies related to protecting stocks from extinction due to sudden disease outbreaks, natural disasters, or anthropogenic factors by making top-quality gamete and larvae available year-round, and providing greater ease in conducting selective breeding for disease resistance, preserving desirable characteristics and establishing gene banks (Chang *et al.*, 1997; Chang, 1998; Bart, 2000; Chao and Liao, 2001). Cryopreservation techniques of fish sperm is well established in many species, including puffer, sea bass, mandarin fish, cod, zebrafish and carp (Mounib, 1978; Gwo *et al.*, 1993; Babiak *et al.*, 1995; Fauvel *et al.*, 1998; Linhart *et al.*, 2000; Ding *et al.*, 2009; Jinga *et al.*, 2009), but only in a limited number of shellfish such as several commercially important species, abalone, mud crab, *Mytilus galloprovincialis*, oyster, marine shrimp (Jeyalectumie and Subramonian, 1989; Tsai and Chao, 1994; McFadzen, 1995; Adams *et al.*, 2004; Kawamoto *et al.*, 2007; Vuthiphandchai *et al.*, 2007; Matteo *et al.*, 2009).

Bar-tailed is one of the most important commercial species in the Korean fisheries industry (Yoon, 2008). However, there remains a paucity of data on standardized procedure for cryopreservation of sperm. The present study attempted to identify an optimized method of sperm cryopreservation with various cryoprotectants at different freezing rates and thawing rates. Sperm motility and survival rates were assessed post-thawing in order to decide on the best procedure.

MATERIALS AND METHODS

Bar-tailed flatheads were captured from shallow areas near Yeosu city, Jeonnam province, Korea in July 2008

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

	Motility index										
	5.0	4.5	4.0	3.5	3.0	2.5	2.0	1.5	1.0	0.5	0.0
Forward sperm (%)	100	90	80	65	50	30	20	15	10	5	0

and transported to the laboratory. Semen was collected by abdominal stripping and stored in polyethylene tubes on crushed ice until use.

1. Motility estimation

Percentage of sperm exhibiting rapid, vigorous and forward movement was evaluated under a microscope by diluting sperm samples in Artificial SeaWater (ASW; 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) at a ratio of 1 : 1000 (Table 1). Samples with high motility were kept on crushed ice until use for the experiments outlined below.

2. Cryopreservation of sperm

DMSO (dimethyl sulfoxide), glycerol or Methanol was added to 36% ASW to formulate the extenders at concentrations between 5 and 15% of total volume. Sperm was diluted 1 : 3 with the extenders. The diluted sperm was inserted into 0.5 mL plastic straws and frozen at different freezing temperatures according to the method of Bouysson and Chupin (1982). The straws were then placed in a polystyrene box at 2 mm, 4 mm and 6 mm above liquid nitrogen. The straws were thawed in a 30°C water bath for 2 min. Survival rate was also estimated by the eosin-nigrosin staining technique.

To determine the optimum thawing temperature, frozen tubes were thawed in a water bath at different temperatures of 10°C, 20°C or 30°C.

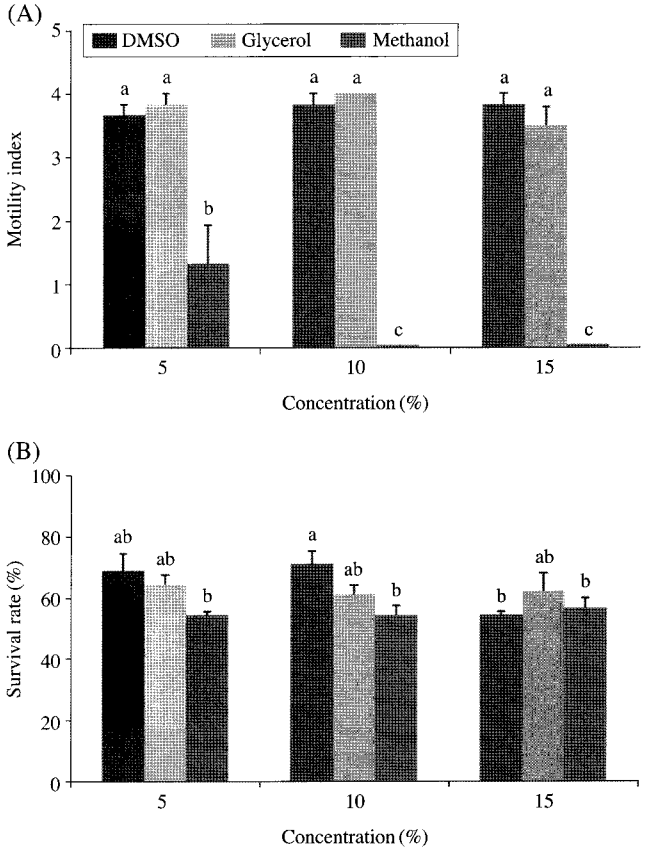

Fig. 1. Motility and survival rate (mean ± SE) of *Platycephalus indicus* sperm after cryopreservation with DMSO, glycerol and methanol at the indicated concentrations. Difference letters on the bars are significantly difference ($p < 0.05$).

RESULTS AND DISCUSSION

On contact with ASW, sperm became immediately activated and reached maximum motility. Vigorous forward movement was constant for about 10 sec, after which sperm lost total motility.

1. Cryoprotectant trial

Motility and survival rate using different cryoprotectants are shown in Fig. 1. The highest post-thawed sperm motile index 4. and survival rate (70%) were obtained with glycerol and DMSO, respectively, while sperm motility with methanol was below motile index 1.5.

2. Freezing trial

Results of cryoprotectants trial indicated that 10% glycerol was most effective, hence it was selected for the freezing trials. The best result (motility: motile index 4; survival rate: 75%) was achieved when freezing depth (the 2 cm above the surface of liquid nitrogen) was used (Fig. 2).

3. Thawing trial

The survival rates and motile index of sperm cells thawed at different temperatures are shown in Fig. 3. Motility at 20°C was significantly higher than those of other treatments ($p < 0.05$).

Spermatozoa from teleost fish may be stored successfully in liquid form for short periods, or by cryopreservation for longer periods (Chang *et al.*, 1997; Chang *et al.*, 1999; Daly *et al.*, 2008; Yasui *et al.*, 2009; Cabrita *et al.*,

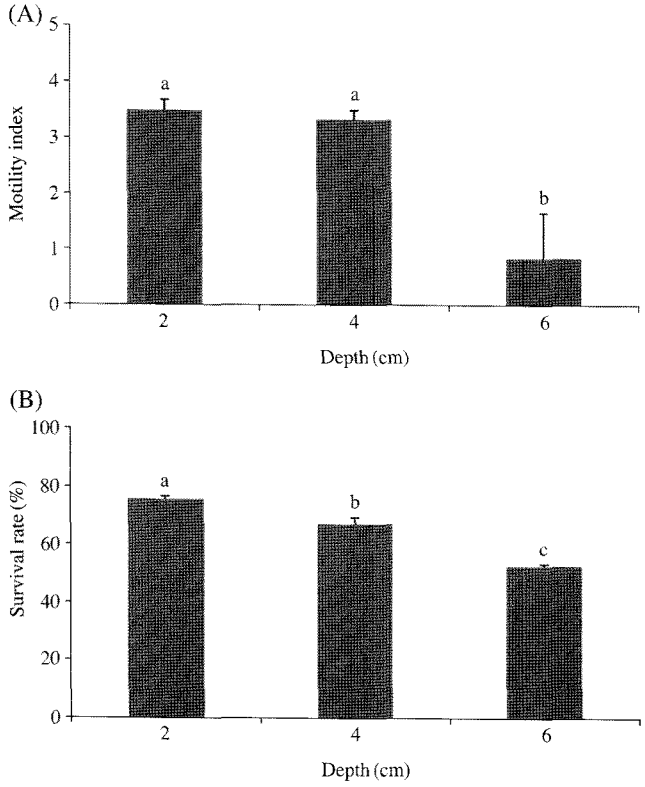

Fig. 2. Motility and survival rate (mean ± SE) of *Platycephalus indicus* sperm after cryopreservation at three depths. Difference letters on the bars are significantly difference ($p < 0.05$).

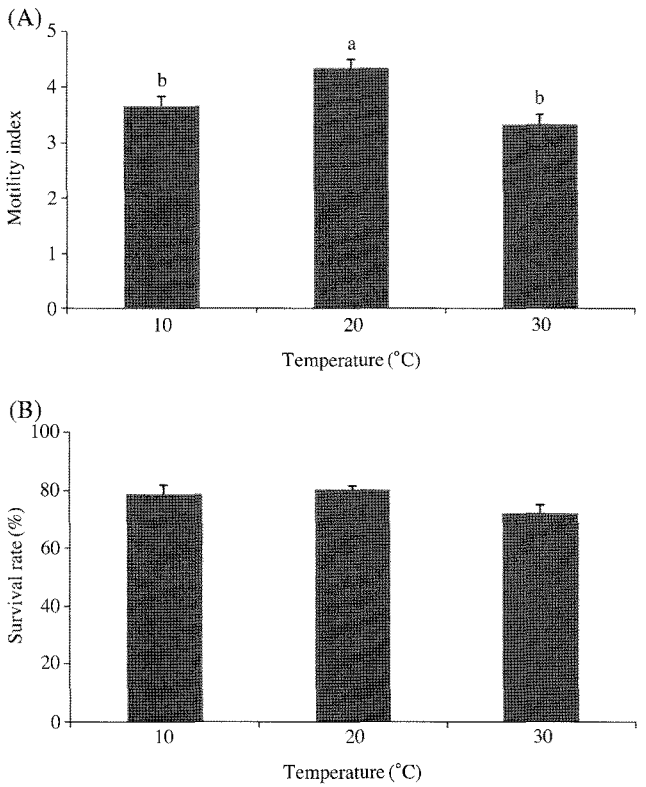

Fig. 3. Effects of thawing temperature on motility and survival rate (mean ± SE) of *Platycephalus indicus* sperm. Difference letters on the bars are significantly difference ($p < 0.05$).

2010). Overall, spermatozoa storage in marine species has received considerably less attention than freshwater species. This study provides detailed information on cryopreservation of semen in the bar-tailed flathead.

The application of sperm cryopreservation in aquatic animals is a possible tool in conservation and management of endangered species. Initiation of sperm motility is triggered primarily by environmental signals. Motility of spermatozoa usually occurs immediately after release into the environment, where chemical conditions are different to seminal plasma (Kho *et al.*, 2001; Wojtczak *et al.*, 2003; Lahnsteiner, 2007).

Immobilizing solutions, such as 1% NaCl, 0.3 M glucose, diluted seawater, and other solutions whose composition are similar with seminal plasma, are often used as diluents in short-term preservation and cryopreservation of fish sperm (Blaxter, 1953; Chang *et al.* 1999; Fabbrocini *et al.*, 2000; Yao *et al.*, 2000). Motility of bar-tailed flathead sperm was short-lived and only constant for about 10 sec after activation with ASW. An immobilization solution is necessary prior to cryopreservation so as to avoid excessive expenditure of energy by sperm through movement (Gwo, 1994). In this study, results of motility estimation showed that 36% diluted ASW was enough to inhibit sperm movement and ensure restoration of motility after re-activation.

Generally, Freezing and thawing of sperm caused structural damage to spermatozoan organelles. Post-thaw spermatozoa appeared to have undergone considerable damage to cellular structures such as plasma membrane, nucleus, mitochondria, and flagellum, due to stress caused by heat shock, ice crystals, and excessive dehydration during freezing (Gwo and Arnold, 1992; Watson, 1995). This damage appears to correspond well to reduced spermatozoan motility and fertility (Gwo and Arnold, 1992), which are used as evaluation parameters of sperm cryopreservation. In fact, many authors have linked the percentage of motile sperm and quality of movement to fertilization success rate (Lahnsteiner *et al.*, 1996a,b,c). In the present study, results of sperm motilities and survival rates were little less than those of fresh sperm, while the best post-thawing motility and survival rate was obtained by using 5% and 10% DMSO and glycerol, respectively, and freezing at 2 cm above liquid nitrogen. In addition, comparisons of motility between treatments showed that thawing at 20°C obtained the best results.

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양태, *Platycephalus indicus*의 정자의 냉동보존

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요 약 : 양태, *Platycephalus indicus* 정자의 동결보존을 위해 적정 동해방지제와 동결높이에 따른 예비동결(0, 2, 4, 6 cm), 그리고 해동온도에 따른 정자의 운동성과 생존율로 비교하였다. 적정 동해방지제로 dimethylsulphoxide (DMSO), glycerol, methanol에 대해 실험하였으며, DMSO와 glycerol에서 methanol에 비하여 높은 생존율과 운동성을 나타내었다. 또한, 동결보존에 있어 예비동결은 액체질소표면으로부터 2 cm 높이에서 높은 생존율과 운동성을 보였고, 해동온도로는 20°C가 가장 적합하였다.

찾아보기 낱말 : *Platycephalus indicus*, 양태, 정자, 동결보존