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Cryopreservation of spotted halibut (*Verasper variegatus*) sperm

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ABSTRACT

Spotted halibut (*Verasper variegatus*) is now regarded as being endangered in China because of its continuous decline in the last three decades. Preservation and protection of its genetic resources were demanded in order to protect this rare fish species. In this project, cryopreservation technique for sperm of spotted halibut was developed. The effects of various extenders and cryoprotectants on motility of frozen–thawed sperm were examined. The motility of frozen–thawed sperm in TS-2 was higher than that in ASW and MPRS ($p<0.05$) and was not significantly different from that of fresh sperm ($p>0.05$). While the motility of frozen–thawed sperm cryopreserved with 13.3% EG (ethylene glycol), 13.3% glycerol, 13.3% MeOH (methanol) and 13.3% DMF (dimethylformamide) was less than 5%, no significant differences were observed in the motility between fresh sperm and frozen–thawed sperm cryopreserved with 13.3% DMSO (dimethyl sulfoxide), 13.3% PG (propylene glycol) ($p>0.05$). Using the above method, we cryopreserved spotted halibut semen with extender TS-2 and 13.3% DMSO or 13.3% PG. As a result, the fertilization rate (34.52 ± 10.92%) and hatching rate (23.53 ± 11.80%) of frozen–thawed sperm were not significantly different from that of fresh sperm ($p>0.05$). Motility and time delay in the activation of frozen–thawed sperm activated by artificial seawater at different salinity were different. Low salinity (low osmolality) could delay the activation of frozen–thawed sperm. The highest motility was observed with artificial seawater of 30‰ ($p<0.05$). The most suitable temperature of seawater to activate spotted halibut frozen sperm was determined to be 18 °C ($p<0.05$). However, temperature of seawater has no significant effect on time delay in the activation of frozen–thawed sperm ($p>0.05$).

1. Introduction

Spotted halibut (*Verasper variegatus*) inhabits naturally in East China Sea, Yellow Sea and Bohai Bay, and coastal areas of Japan and Korea. It is a potential marine species for aquaculture because of its high market value. In the recent years, its stocks have been on decline and, therefore, it has been selected as a target species for stock enhancement in China. However, the halibut males frequently mature earlier than females, making propagation difficult.

The cryopreservation of fish sperm can improve efficiency in artificial propagation by increasing the total volume of available semen and avoiding aging of sperms. It is also an effective way for long-term preservation of germplasm (Blaxter et al., 1953). Fish sperm cryopreservation has been successfully carried out on more than 200 species such as Chinese carps (Chen et al., 1992a,b); turbot (Chen et al., 2004), sea perch (Ji et al., 2004), Summer flounder (Tian et al., 2006), and rainbow trout (Wheeler and Thorgaard, 1991). However, the procedure and conditions for fish sperm cryopreservation vary from species to species, and an applicable procedure for cryopreservation of spotted halibut sperm is not yet available (Sequet et al., 2000). The objective of this project is to establish a protocol for successful cryopreservation of the halibut sperm, and to determine the effect of extenders, cryoprotectants, and cooling and thawing conditions on fertilization rate for the development of an optimal protocol for cryopreservation of the halibut sperms.

2. Materials and methods

2.1. Gamete collection and motility assessment

Eleven (eight male and three female) spotted halibuts, bred in Mingbo aquatic Co., Ltd., were used in this study. Semen was collected by gently pressing the genital area of the fish and then was transferred into 25-ml bottles. Owing to sperm quality variation among different individuals (Withler, 1998), semen from 3–4 males was pooled and each experiment or treatment was carried out using three different pools. The time interval between milt collections for each fish was 5 days. Within 30 min after collection, percentage and duration of sperm motility were determined by microscopic observation immediately after mixing 5 μl semen with 50 μl seawater (11 °C). To validate

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the motility measurement, preliminary tests were carried out using three samples. The duration of sperm motility was assessed by recording the time after the addition of seawater to the sperm sample, but before 90% of the sperm stopped moving. Eggs were collected by abdominal pressure of the females. Eggs from three females were pooled and used for fertilization experiment.

2.2. Sperm cryopreservation and thawing of frozen semen

Within 1 h after sperm collection, pooled semen was mixed with extender TS-2 containing cryoprotectants at a ratio of 1:2 (semen: extender), and 1 ml of diluted semen was aspirated into 1.8 ml cryovials (Nalgen, USA). Then the cryovials were transferred into gauze pocket with a dimension of 6 cm in width and 9 cm in length, and was suspended for 10 min at 6 cm above the liquid nitrogen surface (about −180 °C), and then suspended for 5 min on the surface of liquid nitrogen, finally plunged into liquid nitrogen (Chen et al., 2004; Chen, 2007). According to previous studies, the freezing rate was 18.6 °C/min (Ji et al., 2004). Cryovials containing diluted semen were thawed at 37 °C water bath for 1.5 min. Immediately after thawing, percentage and duration of sperm motility were determined as described above.

2.3. Determination for the effect of extenders and cryoprotectants

Three extenders were evaluated in this study: TS-2 (110 mM Sucrose, 100 mM $KHCO_3$, 10 mM Tris–Cl, pH 8.2, osmotic pressure 335 mOsm/kg) (Chen et al., 2004), MPRS (Ji et al., 2004) and ASW seawater (Zhang et al., 2003). The extender and cryoprotective agents were precooled in 4 °C before use. Semen was separately mixed with extender containing DMSO. After cryopreservation storage for 24 h in liquid nitrogen, cryovials were thawed. Sperm motility was determined as described above.

Six cryomedia were evaluated for their effects on post-thaw motility of spotted halibut sperm: DMSO, PG, EG, Glycerol, MeOH and DMF (Amresco, USA). These cryoprotectants were each used at a final concentration of 13.3% in a mixture of TS-2 and semen. The diluted semen was cryopreserved and thawed as described above.

2.4. Sperm activation using artificial seawater

Artificial seawater was prepared by adding NaCl or ddH_2O in seawater to obtain salinity of 20, 25, 30, 35 and 40‰, as determined by using a salinometer. After the semen was cryopreserved and thawed, post-thaw semen was mixed with artificial seawater, and observed under a microscope for the determination of sperm motility and time delay in the activation of sperm. Time delay in the activation of sperm was assessed with the period beginning with the addition of artificial water to the sperm sample and ending when 50% sperm in the field of view began to move.

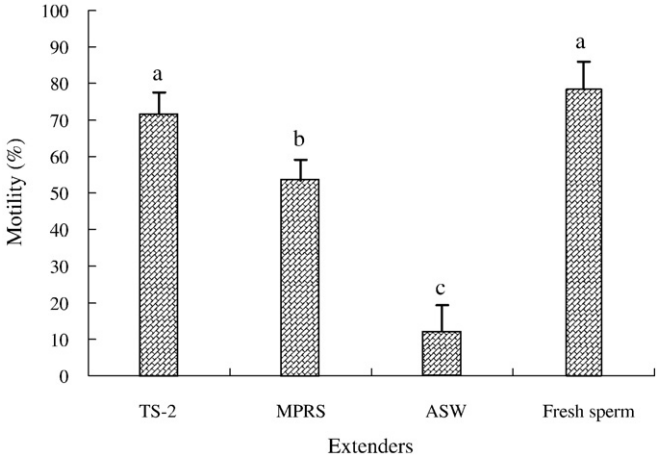

Fig. 1. Effect of extenders on motility score of frozen–thawed sperm ($n=3$). Semen was mixed with TS-2 containing DMSO and the volume of diluted semen in the cryovials was 1 ml. The values having different letters above the column were significantly different ($p<0.05$).

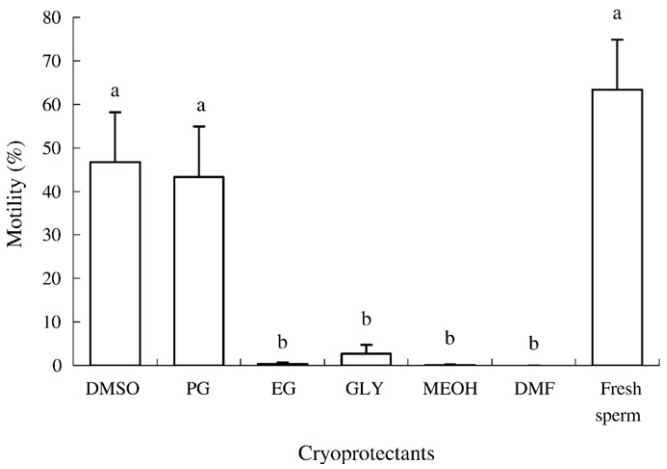

Fig. 2. Effect of cryoprotectants on the motility score of frozen–thawed sperm ($n=3$). Semen was diluted in TS-2 containing cryoprotectants at a ratio of 1:2. Different letters indicate that motility is significantly different ($p<0.05$).

2.5. Sperm activation using seawater at different temperature

Semen was diluted with TS-2 containing DMSO and then was cryopreserved as described above. Samples of frozen semen were thawed at 37 °C in a water bath. The thawed semen was mixed with seawater at various temperatures ranging from 6 °C to 24 °C with 2 °C intervals. Percentage and duration of sperm motility and time delay in the activation of sperm were determined as described above.

2.6. Fertilization trial

60 000 eggs from three females were divided into three 500-ml beakers. The eggs in each beaker were fertilized with 0.6 ml of frozen–thawed semen and sperm to egg ratios were 10 000:1. The fertilization solution was natural seawater (30‰) and the temperature of seawater was 11 °C. Three positive controls, in which 0.2 ml of fresh semen was respectively fertilized with 20 000 eggs from the same three females, were conducted. The fresh and frozen semen used in fertilization experiment was collected from the same males. The fertilized eggs were incubated in seawater at 12 °C and the dead eggs were removed. After 24 h, when the fertilized eggs developed to embryos at the gastrula stage, the fertilization rate (number of gastrula stage embryos/numbers of eggs) was calculated. The hatching rate was expressed as the percentage of hatched fry from the fertilized eggs.

2.7. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA), and pairwise contrasts were used to identify significant differences at a 5% level between the means.

3. Results

3.1. Effects of extenders and cryoprotectants

Effects of different extenders on motility of frozen–thawed semen was shown in Fig. 1. The highest motility of frozen–thawed sperm was

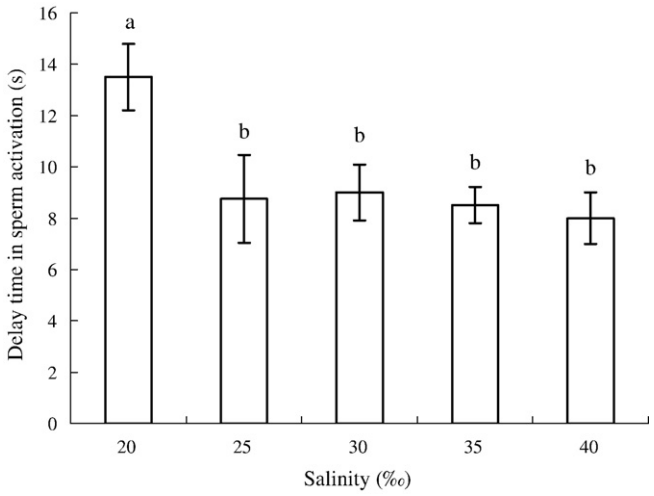

Fig. 3. Effect of artificial seawater at different salinity on delay time in sperm activation of frozen–thawed sperm (n=3). The values having different letters above the column were significantly different (p<0.05).

achieved with TS-2, which was not significantly different from that of fresh sperm (p>0.05), but significantly higher than that in ASW or MPRS (p<0.05) because TS-2 could inhibit sperm motility in spotted halibut. The lowest motility was achieved with ASW at a level significantly lower than that of MPRS. The duration of frozen–thawed sperm motility in TS-2, MPRS, ASW and fresh sperm was 456±104.17 s, 476.33±33.84 s, 260.67±200.00 s and 455.33±120.27 s, respectively. The duration of sperm motility with TS-2, MPRS was not statistically different from that of the fresh sperm, but from that of frozen–thawed sperm in ASW (p<0.05).

DMSO and PG appeared to be effective cryoprotectants as no significant differences were observed in motility between fresh and frozen–thawed sperm cryopreserved with DMSO or PG at 13.3% each (Fig. 2). In contrast, EG, glycerol, methanol, and DMF were all poor cryoprotectants as they allowed less than 5% motility after cryopreservation (Fig. 2).

3.2. Sperm activation using artificial seawater at different salinity

Time delay in the activation of frozen–thawed sperm with artificial seawater at salinity of 20‰ (13.5±1.29 s) was significantly longer than

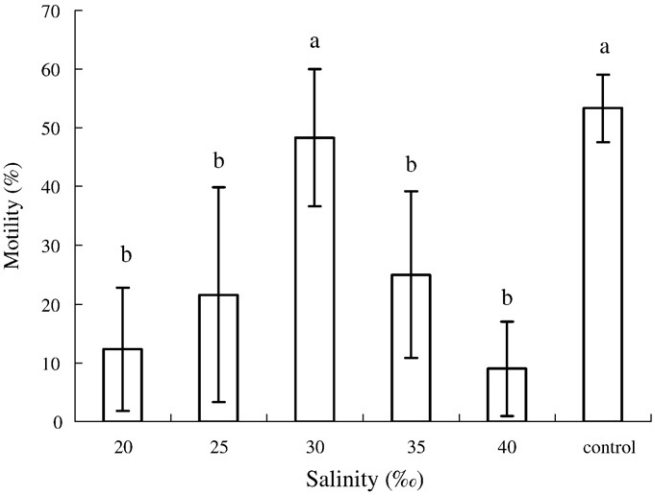

Fig. 4. Effect of artificial seawater at different salinity on motility of frozen–thawed sperm (n=3). The values having different letters above the column were significantly different (p<0.05).

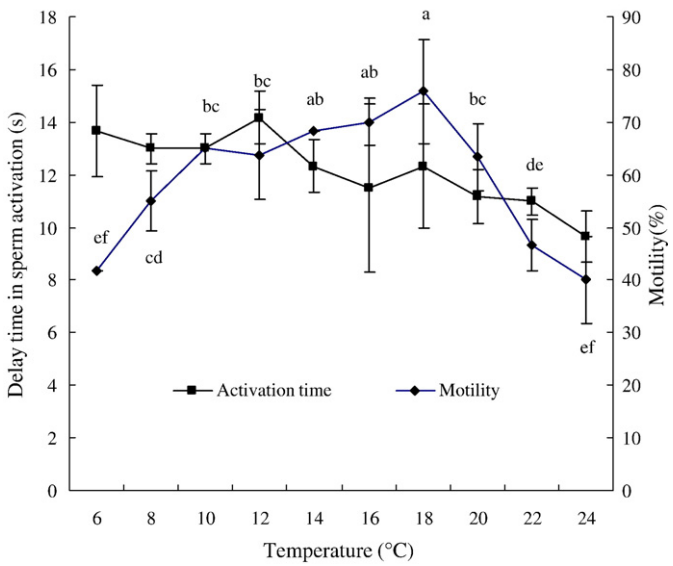

Fig. 5. Effect of seawater at different temperature on motility and delay time in sperm activation of frozen–thawed sperm (n=3). Different letters indicate significant differences among treatments (p<0.05).

that at salinity of 25‰ (8.75±1.71 s), 30‰ (9.00±1.10 s), 35‰ (8.50±0.71 s) and 40‰ (8.00±1.00 s) (p<0.05) (Fig. 3), suggesting that low salinity could slow down the activation of frozen–thawed sperms.

Artificial seawater of 30‰ appeared to provide the highest motility of frozen–thawed sperms activated by artificial seawater, at a rate comparable to that of fresh sperms, but significantly higher than that of frozen–thawed sperms activated by artificial seawater at salinities of 20‰ (12.33±10.52%), 25‰ (21.6±18.24%), 35‰ (25.00±14.14%), and 40‰ (9.00±8.04%) (Fig. 4).

3.3. Sperm activation using seawater at different temperature

Temperature significantly affected the motility rate of frozen–thawed sperms activated by seawater (Fig. 5). Arise in temperature from 6 °C to 18 °C correlated with an increase in motility. The motility rate was highest at 75.83±4.91% when the frozen–thawed sperms were activated by seawater at 18 °C. Further temperature increases above 18 °C caused a decrease of motility. Temperature of seawater had no significant effects on time delay in the activation of frozen–thawed sperms (Fig. 5).

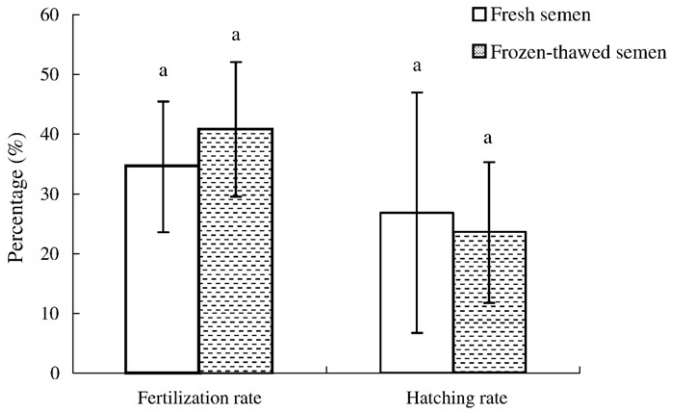

Fig. 6. Fertilization and hatching rate of fresh semen and frozen semen (n=3). 20 000 eggs were fertilized with 0.6 ml frozen–thawed sperm or 0.2 ml fresh sperm. The ratio of sperm to egg was 10 000:1. Same letters indicate no significant differences among treatments (p>0.05).

3.4. Fertilization trial

The fertilization rate (34.52±10.92%) and hatching rate (23.53±11.80%) of the frozen–thawed sperms were similar to those of the fresh sperms (40.86±20.15%, and 26.83±11.27%, respectively) (Fig. 6), suggesting that the cryopreservation protocols developed from this study are effective.

4. Discussion

Selecting a suitable extender is a key factor in successful cryopreservation of fish semen. In this work, three extenders (TS-2, MPRS, ASW), which had been used in cryopreserving semen of marine fish (turbot, sea perch, flounder) (Chen et al., 2004; Ji et al., 2004; Zhang et al., 2003; Chen, 2007), were tested for cryopreservation of spotted halibut sperms. While two of the three extenders work effectively for the halibut sperms, ASW (suitable extender for Japanese flounder semen) resulted in low motility (11.67±7.64%), suggesting the highly specific nature of extenders for various species. In this case, even though the spotted halibut and Japanese flounder are both flatfishes, they differ drastically in proper extenders.

The six tested cryoprotectants in this work: DMSO, PG, EG, Glycerol, MeOH and DMF, have been widely used in cryopreservation of fish semen and embryos (Chen et al., 1993; Ji et al., 2004; Kopeika et al., 2000; Brian, 1982). Chen and Tian (2005) reported that the toxicity of PG and MeOH was the lowest among six cryoprotectants. Because of small volume of fish sperm (Sequet et al., 1993), semen can be cryopreserved successfully using cryoprotectants at low concentration. Therefore, the toxicity of the cryoprotectants is not considered as an important factor in selecting a suitable cryoprotectant for the cryopreservation of fish sperms. In fact, permeability of the cryoprotectants may be a more important factor than toxicity in selecting a suitable cryoprotectant for the cryopreservation of fish sperm.

The characteristics (motility, duration of sperm motility, fertilization rate and hatching rate) of fish sperm can be affected by many factors such as osmolality, ionic composition, CO_2, temperature, salinity, and pH (Billard et al., 1992; Alavi et al., 2006). The frozen–thawed sperms should be more fragile to environmental conditions as they have experienced the process of cryopreservation. In the present study, the effects of salinity and temperature of seawater on motility and time delay in the activation of frozen sperms were studied. It was clear that motility was induced by hyperosmotic pressure in marine fishes (Suquet et al., 1993; Linhart et al., 1999). It was worth mentioning that activation could be an immediate response to the osmotic signal, acting via the stretch-activated channels of the sperm membrane (Alavi et al., 2006). However, motility occurs over a wide range of osmolalities (below or above that of seawater) in marine fishes than in freshwater fishes (Chauvaud et al., 1995). The optimal osmolality for motility was 300–1100 mOsm/kg with turbot (Chauvaud et al., 1995) and 333–645 mOsm/kg with tilapia (for fish raised in seawater) (Linhart et al., 1999). After the cryoprotectants are added to the extenders, cryoprotectants penetrate into sperm rapidly, resulting in an increase of the osmolality of sperms (Alavi et al., 2006). Low salinity (low osmolality) can not activate the frozen–thawed sperms and possibly reduces the fertilization rate. In this paper, when the frozen–thawed sperm were mixed with 20‰ seawater, the time delay in the activation of sperm was prolonged. The highest motility was observed with artificial seawater of 30‰ ($p<0.05$), which was similar to the salinity of the water in which spotted halibut was collected.

An increase in velocity caused by a temperature rise of the activation solution leads to shortened duration of motility, and conversely, lowering of temperature of activation solution results in a prolonged duration of motility (Perchec et al., 1995). With the result studied in the present work, temperature of activation solution did not affect the activation time, but significantly affected motility.

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