Cryopreservation of the sperm of spotted halibut *Verasper variegates* (Pleuronectiformes, Pleuronectidae)

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The sperm of spotted halibut *Verasper variegates* was cryopreserved by a simple and convenient protocol. The cryoprotectants used were dimethyl sulfoxide (Me2SO), glycerol (Gly), methanol (MeOH), Me2SO + egg yolk, Gly + egg yolk and MeOH + egg yolk. The percentage of forward motile sperm in semen cryopreserved in the different extenders with Me2SO, Gly, MeOH, Me2SO + egg yolk, Gly + egg yolk and MeOH + egg yolk was 40.5 ± 4.2%, 49.5 ± 4.2%, 38.3 ± 2.4%, 65.0 ± 4.0%, 67.5 ± 2.9% and 49.5 ± 8.4%, respectively. The fertilization and hatching rates of cryopreserved sperm were from 59 ± 2.4% to 70 ± 3.0%, and from 11 ± 3.0% to 18 ± 2.6%, respectively. These tended to be highest when Gly + egg yolk or Me2SO + egg yolk were used as cryoprotectants. Under SEM, it could be seen that while the majority of the freeze-thawed sperm remained morphologically normal, some exhibited damaged or lost mitochondria, which possibly caused the decrease in motility and fertility of the freeze-thawed sperm.

[Key words: Cryopreservation, sperm, spotted halibut, *Verasper variegates*]

1. Introduction

Cryopreservation is an effective method for long-term storage of viable sperm, and can provide a year-round supply of male gametes. The technique is useful for the preservation of sperm from genetically superior males for later use, in the transportation of sperm, and for use in breeding programs such as cross breeding, genetic studies or conservation of endangered species1. Since Blaxter’s2 pioneering work on the cryopreservation of herring sperm, sperm cryopreservation has been established in over 200 fish species mostly for the salmonids, tilapias and carp3, but little study has been performed in marine fish, especially marine flatfish. Reports of flatfish sperm cryopreservation are limited to only a few species including halibut (*Hippoglossus hippoglossus*)4,5, plaine (*Pleuronectes platessa*)6, yellow flounder (*P. ferrugineus*)7,8 and olive flounder (*Paralichthys olivaceus*)9.

The spotted halibut *Verasper variegates* (*Pleuronectiformes*, *Pleuronectidae*, Verasper, T.&S.) naturally inhabits the seas of China, Korea, and Japan. Its flesh is tasty and nutritious, and this species has a great potential for farming in China. However, the male and female fish do not usually mature synchronously in captivity10. In addition, their numbers in the wild are diminishing because of over-fishing and marine pollution. To preserve this potentially important commercial fish and to breed it artificially, the cryopreservation of its sperm is of great importance. The purpose of this study is to examine the changes in motility, fertility and morphology of the sperm before and after cryopreservation and to determine the hatching rate of the larvae from the eggs fertilized by the freeze-thawed sperm, and to provide cryopreserved sperm for utilization in artificial fertilization.

2. Materials and Methods
2.1. Fish and semen

Twenty-five sexually mature spotted halibuts *Verasper variegates* were cultured in a 50 m2×1.4 m tank with flow-through seawater with an ambient temperature of 12 to 15°C (in the spring) and aeration. They were fed once a day with chopped white Chinese croaker. Semen was collected from the sperm ducts by gently compressing the genital area of the fish, pipetted into 1.5-ml Eppendorf tubes and kept at

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room temperature (13 to 16°C) until used in the following experiments, which were always conducted within 30 min after semen collection.

The percentage of motile sperm was measured by thoroughly mixing 5 µl semen with 50 µl seawater on a slide, and immediately observing under a microscope. The concentration of sperm in the semen was estimated by counting sperm diluted 1:2000 in a Burker cell counter. Only semen with over 70% forward motile sperm was pooled and used in the following experiments. Usually, semen from at least three fish were pooled for each freezing experiment. A total of seven adult males were used for this study.

2.2. Sperm cryopreservation

A total of 6 cryopreservation extenders were used in the experiments. They were 12% dimethyl sulphoxide (Me₂SO), 12% glycerol (Gly), 12% methanol (MeOH), 12% Me₂SO + 10% egg yolk, 12% Gly + 10% egg yolk and 12% MeOH + 10% egg yolk, respectively, in the base solution, which is artificial seawater, containing 24.72 mg/ml NaCl, 0.67 mg/ml KCl, 1.36 mg/ml CaCl₂·2H₂O, 4.66 mg/ml MgCl₂·6H₂O, 6.29 mg/ml MgSO₄·7H₂O and 0.18 mg/ml NaHCO₃ (pH 8.2). Pooled semen was diluted with the cryopreservation solutions at the ratio of 1:3 (semen : extender), and 1 ml of the diluted semen was pipetted into 2-ml cryo-vials (Sigma, 2000-µl, Cat. No. V4631), that were then placed in cylindrical storage canister in a single layer. The canister was inserted into the neck of a liquid nitrogen container (10-L portable refrigerator, Chengdu, China), and lowered step-wise into the container at 3-cm intervals holding for 2 min at each step. The first step began with the top of the canister at the mouth level, and after 4 steps, the bottom of the canister reached about 4 cm above liquid nitrogen surface, and the canister was kept there for 5 min, and then immersed into liquid nitrogen, where it was stored until thawed. Each trial was performed in three replicates. Generally, three cryo-vials in each trial were frozen/thawed and used in the fertilization experiments.

2.3. Thawing of the frozen sperm and motility assessment

Seven to twenty days after freezing, the canisters containing cryo-vials were removed from the liquid nitrogen container (10-L portable refrigerator, Chengdu, China), and lowered step-wise into the container at 3-cm intervals holding for 2 min at each step. The first step began with the top of the canister at the mouth level, and after 4 steps, the bottom of the canister reached about 4 cm above liquid nitrogen surface, and the canister was kept there for 5 min, and then immersed into liquid nitrogen, where it was stored until thawed. Each trial was performed in three replicates. Generally, three cryo-vials in each trial were frozen/thawed and used in the fertilization experiments.

2.4. Assessing of fertilization and hatching rates

Eggs were removed from mature female halibut and placed in a 500-ml beaker. These were then divided into seven groups with each containing 10 ml of eggs, and placed in a 250-ml beaker. An aliquot of 1 ml of the frozen-thawed semen or fresh semen (as control) was diluted with 10 ml seawater. The diluted sperm was immediately added onto the eggs in the beakers, well mixed, and 100 ml of fresh seawater then added to each beaker.

Halibut eggs vary in quality, and only those that suspend in seawater are fertilizable. After 10 min post-fertilization, the fertilized eggs were washed three times with fresh seawater, and then placed into a 250-ml beaker. From each beaker, 500 suspended eggs were randomly picked up, and transferred into a 15 liter glass tank with 10 liter fresh seawater. The fertilized eggs were incubated at 13 ± 0.5°C with gentle aeration. After 8 h post-fertilization, the fertilized eggs developed into 8- to 16-cell stage embryos, at which point the embryos were counted. The fertilization rate was calculated as the percentage of 8- to 16-cell embryos in relation to the total number of suspended eggs. Larvae hatched at about 110 h post-fertilization and they were counted. The hatching rate was expressed as the percentage of hatched larvae in relation to the initial number of suspended eggs. Eggs from six females were used randomly for different fertilization trials, yet for a specific fertilization trial the eggs were from only one female. All experiments were performed in triplicates.

2.5. Morphological observation

An aliquot of 1 µl of sample of both fresh semen and cryopreserved semen were stained with fluorescent dye as described by Pope et al. For scanning electron microscopy (SEM), the fresh and freeze-thawed semen were placed onto cover glasses and fixed in 2.5% glutaraldehyde in seawater at 4°C for 24 h. They were then washed in cacodylate buffer (pH 7.2) and post-fixed in 1% osium tetroxide for 1 h, dehydrated with graded ethanol and dried by the
critical point method\textsuperscript{9,12}. Observation was made under a JSM-840 scanning electron microscope.

2.6. Statistics

Analysis of variance (ANOVA) was performed to determine significant differences between treatment groups, and Student-Newman-Keul’s multiple range test for pair-wise comparisons was also performed. Data are expressed as mean ± SD.

3. Results

3.1. Semen characteristics

A total of 1 to 3 ml of semen was usually collected at one time from a male halibut. The concentration of spermatozoa in normal semen samples ranged from $3.5 \times 10^9$ to $7.9 \times 10^9$ cells/ml ($6.28 \pm 1.64 \times 10^9$), and the motile sperm in fresh semen was from 70% to 90% with an average motility of $77 \pm 2.45\%$.

3.2. Sperm morphology

Spermatozoon of spotted halibut has a round head, which is about 1.3 $\mu$m in diameter, and a tail of about 40 $\mu$m in the length. Fluorescent staining revealed that there was no acrosome in the sperm head (Fig. 1A). Scanning electronic microscopic (SEM) observation showed that the cell membrane of fresh sperm was smooth, and there were usually a dozen round mitochondria assembled around the neck (Fig. 1B). The majority of the freeze-thawed sperm remained morphologically normal, but some of the spermatozoa had mitochondria lost or partly detached, a swollen mid-piece and the cell membrane became rough, depressed or damaged (Fig. 1C, D).

3.3. Motility, fertility and hatching rates

The spotted halibut sperm was capable of swimming in seawater for 5 min after stimulation at 13 ± 0.5°C. The average motility of the fresh sperm was $77\pm2.45\%$. In contrast, the percentage of forward motile sperm in semen cryopreserved in the different extenders with Me$_2$SO, Gly, MeOH, Me$_2$SO + egg yolk, Gly + egg yolk and MeOH + egg yolk were 40.5 ± 4.2%, 49.5 ± 4.2%, 38.3 ± 2.4%, 65.0 ± 4.0%, 67.5 ± 2.9% and 49.5 ± 8.4%, respectively (Fig. 2). The fertility of the fresh sperm was 78 ± 3.0%, while the fertility of the frozen-thawed sperm ranged from 59 ± 2.4% to 70 ± 3.0% with the sperm cryopreserved in the extender containing glycerol + egg yolk producing the highest fertility (Fig. 2). However, the motility, fertilizing rates and the hatching rates of the frozen-thawed sperm were significantly lower than those of the fresh semen ($P < 0.05$).

Fig. 1 — Micrographs of fresh (A, B) and freeze-thawed (C, D) sperm. A, a fresh sperm under light microscope; B), a fresh sperm under scanning electron microscope; C), a freeze-thawed sperm with mitochondria partially detached; D), a freeze-thawed sperm with mitochondria lost and apical plasma membrane depressed.

Fig. 2 — Motility, fertility and hatching rates of fresh and frozen-thawed sperm cryopreserved in different extenders. The eggs fertilized with the frozen-thawed sperm were able to develop to hatching stage and healthy larvae, and the hatching rates were from 11 ± 3.0% to 18 ± 2.6% with the eggs fertilized by the semen cryopreserved in glycerol + egg yolk producing the highest hatching rate (Fig. 2). However, the motility, fertilizing rates and the hatching rates of the frozen-thawed sperm were significantly lower than those of the fresh semen ($P < 0.05$).

4. Discussion

The effectiveness of the chemicals such as Me$_2$SO, Gly and MeOH as cryoprotectants varies with different fish species\textsuperscript{12-14}. Me$_2$SO, Gly and MeOH were tested for the cryopreservation of spotted halibut...
sperm in this study. The motility and fertility of sperm cryopreserved with these three cryoprotectants were all lower than those of fresh sperm. Among them, Gly gave the statistically significant motility and fertility followed by \( \text{Me}_2\text{SO} \) and MeOH. Interestingly, the motility of the cryopreserved sperm increased by addition of 10% egg yolk to all three cryoprotectants.

Although a sperm motility of 67.5 ± 2.9% was achieved with the sperm frozen in the extender with Gly + egg yolk, the motility level was still lower than that of the fresh sperm (77±2.45%). The decrease in motility upon cryopreservation may be related to changes in sperm morphology. The sperm has a long tail and a mid-piece usually containing a dozen mitochondria. After freezing and thawing, damage or loss of mitochondria in the mid-piece often occurred in the post-thawed sperm. Similar changes have also been reported in the post-thawed sperm of *Paralichthys olivaceus*\(^9\), *Thymallus thymallus*\(^12\), *Macrozoarces americanus*\(^14\) and *Micropogonias undulates*\(^15\). These changes may adversely affect the function of mitochondria, thus reducing sperm flagellate movement and fertilizing rate.

The farming of spotted halibut is only partially successful to date because of the difficulty in artificial reproduction. It is of interest to note that the motility of fresh sperm of the halibut was only 77±2.45%, which is markedly lower than that (94±1.9%) of the olive flounder\(^9\). Whether the lower motility level of the sperm is the main factor of the reproduction difficulty needs further study.

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**References**

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