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Cryopreservation of sperm from turbot (*Scophthalmus maximus*) and application to large-scale fertilization

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Abstract

This paper reports on cryopreservation of sperm from turbot (*Scophthalmus maximus*). The effects of various extenders, cryoprotectants and sperm–egg insemination ratios on motility score and/or fertilization capacity of post-thaw turbot spermatozoa were examined to optimize cryopreservation procedures. Post-thaw motility of frozen sperm obtained with extender TS-2 was higher than those achieved with extenders D-15 and modified plaice Ringer solution (MPRS). The most effective cryoprotectant was determined to be 10% dimethyl sulfoxide (DMSO). Fertilization of small egg batches (2 ml eggs) with frozen sperm resulted in average fertilization rate (FR) ($70.1 \pm 8.9\%$) and hatching rates (HR) ($46.8 \pm 5.2\%$) similar to the fertilization rates ($74.7 \pm 8.0\%$) and hatching rates ($47.5 \pm 6.8\%$) of fresh sperm. The minimal density of frozen sperm required to obtain satisfactory fertilization rate was determined to be 2000:1 (sperm/egg). Fertilization of larger egg batches (40 ml eggs) with sperm frozen in 1.8-ml cryovials provided similar fertilization rates ($71.6 \pm 7.3\%$) to that of the fresh sperm ($76.2 \pm 5.7\%$), whereas the hatching rates ($34.7 \pm 3.6\%$) of eggs fertilized with frozen sperm were slightly lower than those of fresh sperm ($41.1 \pm 3.7\%$). Our results demonstrated that cryopreservation technique for turbot sperm in 1.8-ml cryovial could be used for hatchery purposes.

Keywords: Turbot; *Scophthalmus maximus*; Sperm; Cryopreservation; Motility; Fertility

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1. Introduction

Considerable progress has been made recently in the development of techniques for the cryopreservation of sperm of salmonids and cyprinids. High fertility of frozen sperm was reported in these species (Mounib, 1978; Stein and Bayrle, 1978; Erdahl and Graham, 1980; Baynes and Scott, 1987; Chen et al., 1992a; 1993). In spite of successful cryopreservation in laboratory conditions, there have been few examples of application to hatchery situations (Stoss and Refstie, 1983; Alderson and MacNeil, 1984; Cloud et al., 1990; Chao and Liao, 2001) because some problems remain to be solved: (1) High densities of frozen–thawed spermatozoa are required to achieve fertility close to fresh sperm (Kurokura et al., 1984; Legendre and Billard, 1980; Stoss and Holtz, 1981a,b); (2) The duration of motility in cryopreserved cells can be extremely short, significantly reducing fertility in rainbow trout sperm (Stoss and Holtz, 1981a,b); (3) Small-volume straws limit the widespread use of this technique in most fish hatcheries (Wheeler and Thorgaard, 1991), although large volume straws (5 and 10 ml) were successfully used for cryopreservation of salmonid sperm (Cloud et al., 2000; Harvey, 2000).

Few reports on successful cryopreservation of spermatozoa are available from marine fish, particularly from turbot (*Scophthalmus maximus*), although a large literature exists for salmonids and cyprinids (Chao and Liao, 2001). Turbot is an important cultured marine fish in Europe. Since its importation to China in the 1990s, turbot culture has been firmly established in China with a production of 3000 tons in 2001. Cryopreservation of turbot sperm could reduce the number of males needed in the hatchery and facilitate hybridization and genetic studies. Small scale cryopreservation of turbot sperm in 0.2-ml straws has been reported (Dreanno et al., 1997). This technique is useful for laboratory purposes such as gene banking. However, cryopreservaton of turbot sperm in large volumes is urgently required for hatchery use in order to reduce the time required for sperm packing and thawing and facilitate sperm handling and artificial insemination.

The aim of our work was to develop a feasible method for large volume cryopreservation of sperm from turbot and to check the viability of application of frozen sperm to large-scale fertilization in turbot hatchery.

2. Materials and methods

2.1. Fish and gamete collection

Turbot was imported to China from England between 1993 and 1995. Broodstock were cultured and managed in Haiyang High-tech Experimental Station and Laizhou Mingbo Aquatic of China. Two months before use, 3-year-old turbot males and females with a size of 2–3 kg were subjected to a constant photoperiod of 16 h of light/8 h of darkness and to a constant water temperature of 14–15 °C. Eggs were collected daily by abdominal massage of females, whereas sperm was collected by stripping ripe males. Sperm containing urine and faeces was discarded. Generally, 4–5 ml of sperm from two to three males was pooled and used for each experiment. Eggs from one female were

used for small volume fertilization experiments, and eggs from three females were pooled and used for large volume fertilization experiments.

2.2. Assessment of sperm motility

Sperm obtained from each male were examined for motility under the microscope. Spermatozoa were activated by mixing a minute volume of milt (about 0.5 μl) by means of the tip of a toothpick into a drop of seawater (about 50 μl). The motility of the spermatozoa was observed under a microscope (× 100) before and after freezing and recorded as percentage of motile spermatozoa. Only the sperm that showed high motility scores (>80%) after seawater activation were used. Spermatozoa concentration was determined after dilution using a hemocytometer.

2.3. Extender composition and cryoprotectant concentration

Three extenders were tested for cryopreservation of turbot sperm. Extender D-15 was developed for cryopreservation of sperm from Chinese carps (Chen et al., 1992a). Extender modified plaice Ringer solution (MPRS) was prepared according to the plaice Ringer solution (Yao et al., 2000). Extender TS-2 was modified according to the components of the medium described by Mounib (Mounib, 1978). The compositions of the three extenders are shown in Table 1. Three cryoprotectants, dimethyl sulfoxide (DMSO), propylene glycol (PG) and dimethylformamide (DMF), were tested. TS-2 was selected to test dilution ratio and the effect of cryoprotectant concentration (6%, 10% and 14%). In addition, the effect of semen volume (0.2, 1 and 1.8 ml) on motility of frozen–thawed sperm was tested in order to develop a large-scale cryopreservation method for turbot sperm. All experiments were repeated three times ($n = 3$).

2.4. Dilution and freezing of sperm

Sperm from two to three males were pooled and diluted 1:3 in precooled (4 °C) TS-2 containing 10% DMSO as soon as possible after collection. The effect of

Table 1
Compositions of three extenders

Compositions	D-15	MPRS	TS-2
NaCl (mM)	136.75	60.35	–
$NaHCO_3$ (mM)	–	3.0	–
KCl (mM)	6.71	5.23	–
D-Glucose (mM)	83.33	55.55	–
Sucrose (mM)	–	–	110
$KHCO_3$ (mM)	–	–	100
Reduced gluthation (mM)	–	1.8	–
BSA (mg/ml)	–	1.13	–
Tris–Cl (mM)	–	1.13	10
pH	6.50	6.68-	8.20
Osmotic pressure (mosM/kg)	363	202	335

equilibration time at 4 °C before freezing on motility score was studied. After prechilling, the diluted sperm was loaded into 1.8-ml cryovials. The cryovials filled with diluted sperm were cryopreserved without equilibration, using a "three-step" method developed for Chinese carps (Chen et al., 1992b). Briefly, the cryovials were first equilibrated for 10 min at 6 cm (about − 180 °C) above the surface of liquid nitrogen, then equilibrated for 5 min on the surface of liquid nitrogen, finally plunged into liquid nitrogen. The cooling rate was determined by using programmable freezing (University of Technology and Sciences of Shanghai, China) to be 31 °C/min from 16 (room temperature) to − 15 °C, and 18.6 °C/min from − 12 to − 180 °C (Fig. 1). After storage for 1 to 3 days in liquid nitrogen, the frozen sperm was thawed in a water bath at 37 °C for 60–90 s for motility observation or fertilization trials.

2.5. Evaluation of fertility

Fertility trials were performed with cryopreserved and fresh sperm. Three kinds of fertilization trial were carried out. (1) For small volume fertilization trials, 2 ml of eggs (about 1800 eggs) was fertilized with 0.15 ml thawed sperm or 0.04 ml fresh sperm and gently mixed before activation with 2 ml of sea water. The experiment was repeated five times. (2) Fertilization trials were done using three different sperm/egg ratios (1000:1, 2000:1 and 5000:1) to determine the minimal sperm/egg ratio required for satisfactory fertilization. The experiment was repeated three times. (3) For large volume fertilization trials, 40 ml of eggs was fertilized with 1 ml frozen–thawed sperm or 0.25 ml fresh sperm. The experiment was repeated four times. The fertilized eggs were then transferred into incubation tanks and incubated at 13–15 °C. Fertilization

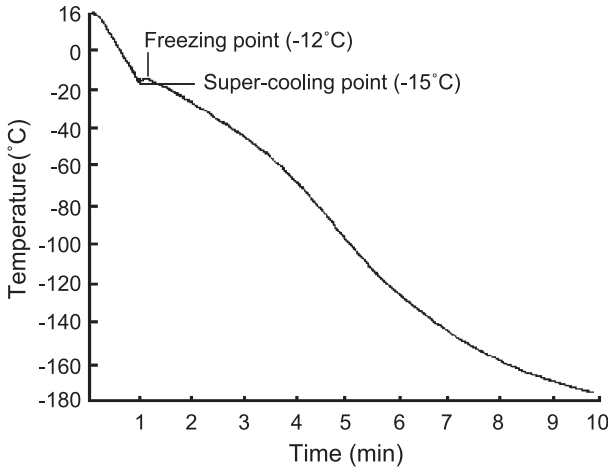

Fig. 1. Freezing curve when cryovial containing 1 ml sperm was equilibrated at 6 cm (− 180 °C) above liquid nitrogen. The supercooling point is − 15 °C, and the freezing point is − 12 °C.

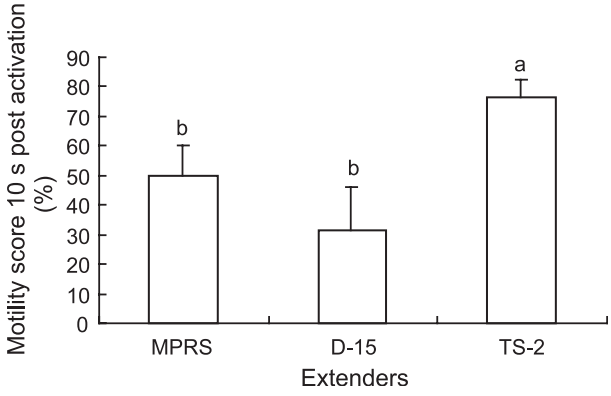

Fig. 2. Effect of extenders on motility of frozen–thawed sperm. The motility was determined 10 s after activation. The experiment was repeated three times. The values were expressed as mean ± S.D. The values having different letters above the column were significantly different ($p < 0.05$).

rate (FR) was evaluated at the early gastrula stage and defined as percentage of gastrula-stage embryos in initial viable eggs (FR = number of gastrula-stage embryos/number of initial viable eggs). Hatching rate (HR) was defined as percentage of hatched fry in initial viable eggs (HR = number of hatched fry/number of initial viable eggs).

2.6. Statistical analysis

Data were expressed as mean ± S.D. The motility score was analyzed with one-way ANOVA. When the differences were significant, SNK (Student–Neuman–Keuls) was used for comparison. The fertilization rate and hatching rate were analyzed with

Table 2
The effect of cryoprotectants on motility of frozen–thawed sperm

Cryoprotectants	Concentration (%)	Motility score 10-s post-activation	Motility score 60-s post-activation
DMSO	6	50.0 ± 5.0^{a}	40.0 ± 5.0^{a}
	10	78.3 ± 7.6^{b}	73.3 ± 2.9^{b}
	14	76.6 ± 5.8^{b}	71.7 ± 2.9^{b}
DMF	6	0 ± 0.0^{c}	0 ± 0.0^{c}
	10	0 ± 0.0^{c}	0 ± 0.0^{c}
	14	0 ± 0.0^{c}	0 ± 0.0^{c}
PG	6	1.7 ± 2.9^{c}	1.7 ± 2.9^{c}
	10	51.7 ± 2.9^{b}	38.3 ± 2.9^{a}
	14	1.7 ± 2.9^{c}	1.7 ± 2.9^{c}

Extender TS-2 was used for all cryoprotectants. The experiment was repeated three times for each treatment ($n = 3$). Values within column followed by different superscripts are significantly different ($p < 0.05$).

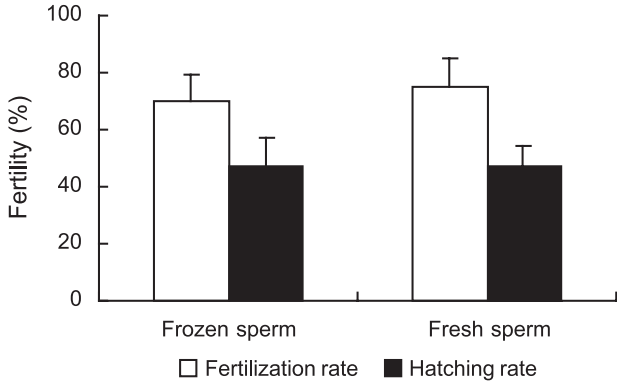

Fig. 3. Fertility of frozen–thawed sperm obtained in small volume fertilization trials. 2 ml of eggs were fertilized with 0.15 ml frozen–thawed sperm or 0.04 ml fresh sperm. The ratio of sperm to egg was 10000:1. The experiment was repeated five times. The values were expressed as mean ± S.D.

Independent-Samples Tests. A value of $p < 0.05$ was considered as statistically significant. The statistical analysis was computed using SPSS software.

3. Results

3.1. Effect of extenders and dilution ratio on post-thaw sperm motility

The motility of sperm frozen with TS-2 (76.7%) was higher than those frozen with D-15 (31.6%) and MPRS (50%) ($p < 0.05$) (Fig. 2). No significant difference was found for dilution ratios tested (1:1 to 1:9) ($p > 0.05$).

3.2. Effect of cryoprotectant on post-thaw sperm motility

Different cryoprotectants provided different protection for turbot sperm. DMSO provided better cryopretection than other cryoprotectants. The highest post-thaw motility (78.3% ± 7.6) was obtained when 10% DMSO was added to the TS-2 (Table 2). DMF and PG were inappropriate for cryopreservation of turbot sperm, as post-thaw motility was absent or low (Table 2).

Table 3
The fertilization rate (%) of frozen–thawed sperm and fresh sperm at various sperm/egg ratios

Sperm/egg ratio	Frozen–thawed sperm	Fresh sperm
1000:1	47.9 ± 0.35*	56.4 ± 2.33
2000:1	50.5 ± 0.21	56.8 ± 0.63
5000:1	52.1 ± 0.84	58.9 ± 16.0

The semen volume in the cryovial was 1 ml. The fertilization experiment was repeated three times for each treatment ($n = 3$). Asterisks indicate the significant difference of fertilization rate in the column ($p < 0.05$).

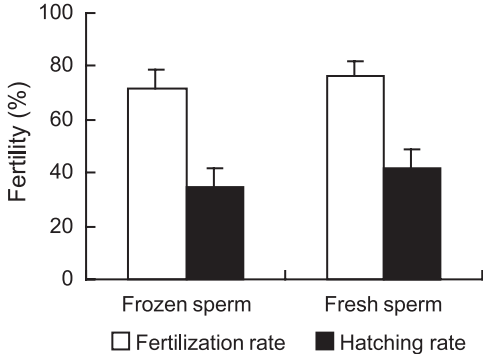

Fig. 4. Fertility of frozen–thawed sperm obtained in large volume fertilization trials. Forty milliliters of eggs was fertilized with 1 ml frozen–thawed sperm or 0.25 ml fresh sperm. The experiment was repeated four times. The values were expressed as mean ± S.D.

3.3. Effect of equilibration time and sperm volume

No significant difference was observed among the three different equilibration times (0, 10 and 30 min) ($p>0.05$). However, zero equilibration resulted in the highest motility score (71.7% ± 2.9). The motility of frozen–thawed sperm did not significantly change when semen volume was increased from 0.2 to 1.8 ml ($p>0.05$).

3.4. Fertility of frozen–thawed sperm

Fertility trials showed that frozen–thawed sperm could fertilize eggs. The fertilization rate (70.1 ± 8.9%) of frozen sperm obtained with small volumes of eggs was similar to that of fresh sperm (74.7 ± 8.0%) and the hatching rate (46.8 ± 5.2%) of fertilized eggs obtained with frozen–thawed sperm was close to that of fresh sperm (47.5 ± 6.8%) (Fig. 3). In another small volume fertilization trial, a fertilization rate of 47.8 ± 6.1% obtained with frozen sperm was also close to that of fresh sperm (51.1 ± 2.1%). When the sperm/egg ratio decreased from 5000:1 to 1000:1, the fertilization rate obtained with frozen sperm dropped ($p<0.05$), while the fertilization rates of fresh sperm at various sperm/egg ratios changed very little (Table 3). In large volume fertilization trials, the fertilization rate of frozen sperm obtained with 40 ml eggs was 71.6 ± 7.3%, similar to that of fresh sperm (76.2 ± 5.7%) (Fig. 3). The hatching rates obtained with frozen–thawed sperm and fresh sperm were 34.7 ± 3.6% and 41.1 ± 3.7%, respectively (Fig. 4).

4. Discussion

Cryopreservation of fish sperm has been studied since the first report on herring spermatozoa cryopreservation (Blaxter, 1953). Most work has focused on salmonids and cyprinids, using the pellet method or 0.25-ml straws (Holtz, 1993; Babiak et al., 1997;

Dreanno et al., 1997). This is useful for laboratory purposes such as gene banking. Sperm freezing in large volume is very interesting technique for cryopreservation of fish sperm from a practical point of view. In order to facilitate fertilization of large eggs batches with frozen–thaw sperm and transfer of this technique to commercial hatcheries, cryopreservation of turbot sperm in large volume cryovial was required. The present work demonstrates that turbot sperm could be successfully cryopreserved in 1.8-ml cryovials with high post-thaw motility and that frozen–thawed sperm can effectively fertilize large batches of eggs.

Various extenders have been successfully used for cryopreservation of fish sperm (Chao et al., 1987; Gwo et al., 1991; Chen et al., 1993; Palmer et al., 1993). In the present study, three extenders were examined for their suitability for cryopreservation of turbot sperm. Although being very efficient for sperm of Chinese carps, extender D-15 is not appropriate for turbot sperm because spermatozoa are activated in extender D-15 supplemented with 10% DMSO. Although slightly basic extender (TS-2) showed better results than the slightly acidic extenders (D-15 and MPRS) for cryopreservation of turbot spermatozoa, precise effect of pH value of extenders on post-thaw sperm motility remains to be studied.

In the cryopreservation of marine fish sperm, best results have been obtained with 10–20% DMSO (Wayman et al., 1997). DMSO can quickly penetrate into spermatozoa, which makes equilibration unnecessary or even disadvantageous. For example, an equilibration time of 60 min for Atlantic croaker sperm in the extender prior to freezing resulted in a significantly lower post-thaw motility and fertility (Gwo et al., 1991). In the present study, equilibration for 30 min for turbot sperm produced a slightly lower post-thaw motility. Similar observations were made in the cryopreservation of yellowfin sea bream sperm (Gwo, 1994).

Few reports are available on the cryopreservation of turbot sperm. Dreanno et al. (1997) cryopreserved turbot sperm in 0.20-ml straws and obtained post-thaw fertility of 58.5%, however, in which the fertilization rate was estimated when the fertilized eggs developed to four-cell stage. Moreover, hatching rate of eggs fertilized with frozen–thawed turbot sperm was not described in the paper (Dreanno et al., 1997). In the present study, 1.8-ml cryovials were used for cryopreservation of turbot sperm and gave similar post-thaw fertility to that of fresh sperm control. In addition, high fertilization rates were obtained when large batch of eggs (40 ml) were fertilized using 1 ml of frozen–thawed sperm.

In summary, cryopreservation of turbot sperm in 1.8-ml cryovials was developed. Fertility rates obtained in large volume fertilization trials with frozen–thawed sperm are similar to those obtained with fresh sperm. These results demonstrate the potential application of the cryopreservation technique in turbot hatcheries.

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