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Long-term cryopreservation of sperm from Mandarin fish *Siniperca chuatsi*

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ABSTRACT

In order to develop cryopreservation techniques for long-term preserving the sperm of Mandarin fish *Siniperca chuatsi*, we examined the effects of various extender and cryopreservation on post-thaw motility. We found the optimal freezing procedures for the Mandarin fish sperm is diluting the semen in D-15 extender, chilling it to 4 °C, adding ME_2SO to a final concentration of 10% (v/v), then transferring the semen in cryotubes, holding the cryotubes for 10 min at 6 cm (about −180 °C) above the surface of liquid nitrogen, for 5 min on the surface of liquid nitrogen, and finally plunged into liquid nitrogen. After thawed at 37 °C for 60 s, the sperm had the highest post-thaw motility (96.00 ± 1.73%). The optimal fertilization procedures for the frozen sperm is mixing the eggs with sperm, then adding 1 ml of swimming medium (SM = 45 mM NaCl + 5 mM KCl + 20 mM Tris–HCl, pH 8.0) immediately. At the sperm/egg ratio of 100,000:1, the fertilization rate and the hatching rate of the frozen sperm cryopreserved for 1 week or 1 year in liquid nitrogen (66.01 ± 5.14% and 54.76 ± 4.40% & 62.97 ± 14.28% and 52.58 ± 11.17%) were similar to that of fresh sperm (69.42 ± 8.11% and 59.82 ± 5.27%) ($p > 0.05$). This is the first report that the Mandarin fish (*S. chuatsi*) sperm can successfully fertilized eggs after long-term cryopreservation.

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1. Introduction

The Mandarin fish (*Siniperca chuatsi* Basilewsky) has been widely cultured in China, because it is one of the favorite fish consumed by most Chinese (Tao et al., 2007). However, outbreaks of diseases caused by parasites, bacteria and viruses have caused severe economic losses in the aquaculture industry. In some extreme cases, the mortality of the diseased fish reached as high as 100% (He et al., 2002). In addition, the wild stock of Mandarin fish is declining because of excessive exploitation and environmental pollution. Therefore, preserving fish germplasm and preventing fish species from extinction is becoming an urgent work in China.

Cryopreservation is a valuable technique for saving endangered species by facilitating the storage of their gametes in gene bank (Gausen, 1993). This technique is routinely used for sperm from large-bodied cultured and sport fishes, such as salmon and trout of the family salmonidae (Scott and Baynes, 1980), carps of the family cyprinidae (Horváth and Urbányi, 2001), and catfishes of the families claridae, ictaluridae, pangasiidae, and siluridae (Tiersch, 2000). But there are no reports on successful cryopreservation of Mandarin fish sperm until now. In this study, by comparing the effects of four extenders and three cryoprotectants on fertilizing ability and the hatch rates of frozen sperm, we report the successful cryopreservation of Mandarin fish *S. chuatsi* sperm for the first time.

2. Materials and methods

2.1. Animal and collection of germ cells

Mandarin fish *S. chuatsi* with 3 years of age and 750–900 g body weight were collected from Qinhuai River (Nanjing, China) and maintained in outdoor flow-through tanks (5 m × 2 m × 0.5 m) supplied with flow tap water at a rate of approximately 1 l/s at about 25 °C. The fish were fed with small carps (5–10 cm) three to four times a week. Females were injected intradorsaly 24 h before collection of egg with the powder of carp pituitary gland (PG) at a dosage of 6 mg/kg body weight and LHRH-A (des Gly10 (D-Ala6) LHRH ethylamide) at a dosage of 400 μg/kg body weight, and males were injected with PG at a dosage of 3 mg/kg body weight and LHRH-A at a dosage of 200 μg/kg body weights. As to collection of germ cells of the fish, semen was sequestered into dry glass tubes and eggs were stripped into dry plastic bowls.

2.2. Measurement of fresh sperm motility

The motility of sperm that obtained from each male fish was subjectively estimated at 200× magnification first using Nikon Eclipse 80i microscopy (Nikon, Japan). Sperm 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 freshwater (about 50 μl), placed on a glass slide that was prepositioned on the microscope stage for observation (Chen et al., 2004). The percentage of spermatozoa performing progressive forward movement (motility percent) was estimated immediately after being mixed. Sperm vibrating in place were not considered to be motile. Only the sperm showing high motility scores (>95%) after freshwater activation were used for cryopreservation. The spermatozoa concentration was determined using a hemocytometer.

2.3. Sperm cryopreservation

Semen pools were prepared by mixing 3 individual samples in equal ratios, depending on the required amount of semen. Then a series of experiments were designed to optimize the fish sperm conditions.

2.3.1. Effect of extenders

The effects of four extenders on the post-thaw motility of frozen sperm were studied. Semen was diluted in different types of extenders (Pan et al., 2008) at a ratio of 1:3 (semen:extender). After the

extended semen was cooled at 4 °C for 30 min, 10% ME_2SO was added. One milliliter of the mixture was transferred into a 2.0 ml cryotube, and each extender was in triplicate. The cryotubes were then put into liquid nitrogen to freeze using a three-step method. Briefly, the cryovials were first held for 10 min at 6 cm (about −180 °C) above the surface of liquid nitrogen, then for 5 min on the surface of liquid nitrogen, and finally plunged into liquid nitrogen. After storage for 2 h in liquid nitrogen, post-thaw motility of frozen sperm was measured.

2.3.2. Effect of cryoprotectants and concentrations

The effects of three cryoprotectants on the post-thaw motility of frozen sperm were studied. Semen was diluted in extender D-15 at a ratio of 1:3 (semen:extender). After the extended semen were cooled at 4 °C for 30 min, ME_2SO at final concentrations (v/v) of 8%, 10% and 12%, Gly at final concentrations (v/v) of 8%, 10% and 12%, or Meth at final concentrations (v/v) of 8%, 10% and 12% was added respectively. One milliliter of the mixture was transferred into a 2.0 ml cryotube, and each cryoprotectant at one concentration was in triplicate. The cryotubes were then put into liquid nitrogen to freeze using a three-step method as above description. After storage for 2 h in liquid nitrogen, post-thaw motility of frozen sperm was measured.

2.4. Measurement of post-thaw sperm motility

After the frozen sperm was equilibrated for 5 min in the vapour of liquid nitrogen in an open system (Lahnsteiner et al., 2000) and thawed in a water bath at 37 °C for 60 s, post-thaw motility of sperm was subjectively estimated using Nikon Eclipse 80i microscopy at 200× magnification. Swimming ability of sperm was estimated by mixing a minute volume of milt (about 0.5 μl) using the tip of a toothpick into a drop of swimming medium (SM = 45 mM NaCl + 5 mM KCl + 20 mM Tris–HCl, pH 8.0) (about 50 μl), placed on a glass slide that was prepositioned on the microscope stage. The percentage of spermatozoa performing progressive forward movement (motility percent) was estimated immediately after being mixed. Sperm vibrating in place were not considered to be motile.

2.5. Fertility test

Fertilization experiments were performed to compare the fertilization rate and the hatching rate of frozen sperm to those of fresh sperm. The eggs used for all experiments were from same fish.

The first experiment was to detect the fertilization rate (%) and the hatching rate (%) of frozen sperm and fresh sperm at various sperm:egg ratios. Two hundred microliters of eggs (about 200 eggs) were placed into a 15 cm-diameter-dish, and 2 μl, 4 μl, 10 μl, 20 μl frozen sperm (1×10^6 cells/ml) or 0.5 μl, 1 μl, 2.5 μl, 5 μl fresh sperm (4×10^6 cells/ml) was immediately dropped on them respectively. Gametes were activated by 1 ml of SM, leaning the dish and agitating to make eggs enough contact with sperm. Approximately 2 min after activation, the eggs were incubated at about 25 °C with 400 ml the pond water. Each trial was in triplicate. Dead eggs which became opaque were removed from each cage after fertilization.

In the second experiment, the fertilization rate (%) and the hatching rate (%) of frozen sperm with different storage time (1 week and 1 year) was examined. The process of in vitro fertilization was the same as above description, the sperm:egg ratio was 100,000:1. The fresh sperm was used as control.

2.6. Statistical analysis

All data were shown in mean ± S.D. The motility score of sperm was analyzed with one-way analysis of variance (ANOVA). SNK (Student–Neuman–Keuls) was applied to compare the significant differences. The fertilization rate and hatching rate were analyzed with Duncan's multiple-range tests. A value of $p < 0.05$ was considered as statistically significant. The statistical analysis was computed with SAS software.

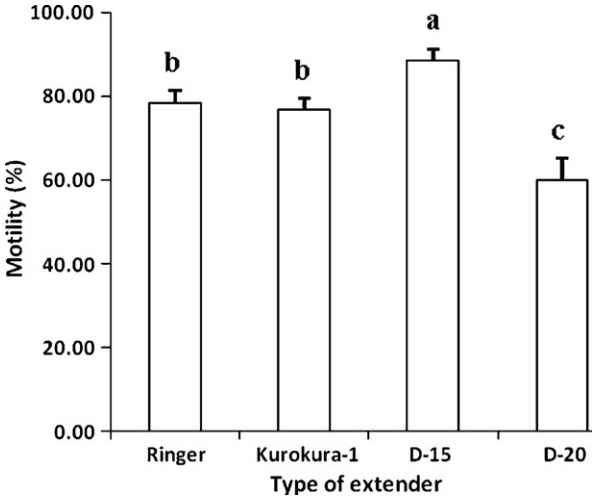

Fig. 1. Effect of extenders on motility of frozen sperm. The motility was determined after activation. Each experiment was repeated three times. The values were expressed as mean ± S.D. The values having different letters above the column were significantly different ($p < 0.05$).

3. Results

3.1. Effect of extenders on post-thaw sperm motility

Effect of extenders on the motility score of frozen sperm was shown in Fig. 1. The motility of sperm frozen with extender D-15 (88.33 ± 2.89%) was higher than those frozen with extender Ringer (78.33 ± 2.89%), extender Kurokura-1 (76.67 ± 2.89%) and extender D-20 (60.00 ± 5.00%) ($p < 0.05$).

3.2. Effect of cryoprotectants and concentrations on post-thaw sperm motility

Protection for sperm provided by different concentrations of different cryoprotectants was shown in Table 1. ME_2SO used as cryoprotectant in a concentration range of 8–12% resulted in higher post-thaw motility score than other cryoprotectants ($p < 0.05$). No significant differences were observed from 8% to 12% ME_2SO ($p > 0.05$). The use of methanol and glycerol resulted in low motility.

Table 1
Effect of cryoprotectants on motility of frozen sperm

Cryoprotectants	Concentration (%)	Motility score post-activation (%)
ME_2SO	8	83.33 ± 2.89^a
	10	91.00 ± 1.73^a
	12	83.33 ± 2.89^a
Gly	8	3.33 ± 2.89^d
	10	31.67 ± 5.77^c
	12	8.33 ± 5.77^d
Meth	8	61.67 ± 5.77^b
	10	65.00 ± 10.00^b
	12	55.00 ± 10.00^b

Extender D-15 was used for all cryoprotectants. Each experiment was repeated three times for each treatment ($n = 3$). Values within column followed by different superscript letters are significantly different ($p < 0.05$).

Table 2
The fertilization rate (%) and the hatching rate (%) of frozen sperm and fresh sperm at various sperm/egg ratios

Sperm/egg ratio	Fresh sperm		Frozen sperm	
	Fertilization rate (%)	Hatching rate (%)	Fertilization rate (%)	Hatching rate (%)
10,000: 1	$54.91 \pm 4.95^{a,A}$	$44.18 \pm 5.13^{a,A}$	$38.76 \pm 4.25^{c,B}$	$32.16 \pm 7.14^{c,A}$
20,000:1	$59.39 \pm 8.16^{a,A}$	$48.94 \pm 8.79^{a,A}$	$42.81 \pm 2.23^{c,B}$	$36.15 \pm 3.14^{c,A}$
50,000: 1	$69.06 \pm 15.70^{a,A}$	$59.40 \pm 17.26^{a,A}$	$56.50 \pm 2.10^{b,A}$	$45.50 \pm 2.31^{b,A}$
100,000:1	$69.42 \pm 8.11^{a,A}$	$59.82 \pm 5.27^{a,A}$	$66.01 \pm 5.14^{a,A}$	$54.76 \pm 4.40^{a,A}$

The sperm was cryopreserved using extender D-15 supplemented with 10% ME_2SO. Each experiment was repeated three times. The values were expressed as means ± S.D. Groups with a common superscript (small type for columns and high type for rows) for each parameter did not differ significantly ($p > 0.05$).

3.3. Effect of sperm to egg ratio on post-thaw sperm fertility

Fertility experiments showed that frozen sperm could fertilize fresh eggs (Table 2). When the sperm/egg ratio decreased from 100,000:1 to 20,000:1, the fertilization rate and the hatching rate of frozen sperm dropped ($p < 0.05$), while the fertilization rates and the hatching rate of fresh sperm at various sperm/egg ratios changed very little ($p > 0.05$). Under various sperm/egg ratios, the hatching rate of frozen sperm were not significantly different from that of fresh sperm ($p > 0.05$), while the fertilization rate of frozen sperm were lower than that of fresh sperm at the sperm/egg ratio (10,000:1 and 20,000:1) ($p < 0.05$) and were similar to that of fresh sperm at the sperm/egg ratio (50,000:1 and 100,000:1) ($p > 0.05$).

3.4. Effect of storage time on post-thaw sperm fertility

The highest fertilization rate and hatching rate of fresh sperm ($69.42 \pm 8.11\%$ and $59.82 \pm 5.27\%$) were achieved at a sperm/egg ratio of 100,000:1. At this ratio, the fertilization rate and the hatching rate of frozen sperm cryopreserved for 1 week in liquid nitrogen ($66.01 \pm 5.14\%$ and $54.76 \pm 4.40\%$) were similar to that of fresh sperm ($p > 0.05$). After being cryopreserved for 1 year in liquid nitrogen, the fertilization rate and the hatching rate of frozen sperm ($62.97 \pm 14.28\%$ and $52.58 \pm 11.17\%$) were still similar to that of fresh sperm ($p > 0.05$) (Fig. 2).

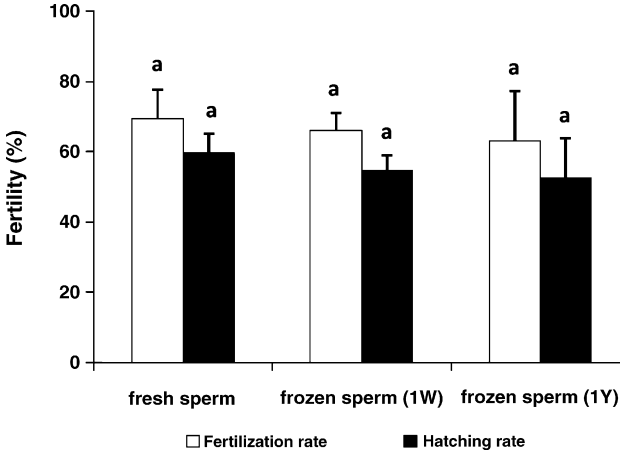

Fig. 2. Fertility of frozen sperm with different storage time. The sperm was cryopreserved using extender D-15 supplemented with 10% DMSO. Two hundred microliters of eggs was fertilized with 20 μl frozen sperm (1W), 20 μl frozen sperm (1Y) or 5 μl fresh sperm. The ratio of sperm to egg was 100,000:1. The experiment was repeated three times. The values were expressed as means ± S.D. The values having common letters above the column do not differ significantly ($p > 0.05$).

4. Discussion

The Mandarin fish (*S. chuatsi* Basilewsky) is a fresh water fish with economic importance (Liu et al., 1998). Cryopreservation is a valuable ways for preserving fish germplasm and preventing fish species from extinction (Ohta et al., 2000; Robles et al., 2003). Selecting a suitable extender is the key factor in successfully cryopreserving fish sperm. In the present study, four extenders were examined for their suitabilities for cryopreservation of Mandarin fish sperm. D-15 extender is known to be very efficient for freezing sperm of Grass carp *Cicnopharyngodon idellus* and Sliver carp *Hypophthalmichihys molitrix* (Chen et al., 1992a), D-20 extender is better for sperm of common carp *Cyprinus carpio* (Chen et al., 1992b), Ringer extender is used for diluting sperm of freshwater fish (Li et al., 1994), and Kurokura-1 extender is also used for sperm of Chinese carps (Linhart et al., 2000). The motility of sperm frozen with extender D-15 ($88.33 \pm 2.89\%$) was higher than those frozen with extender Ringer ($78.33 \pm 2.89\%$), extender Kurokura-1 ($76.67 \pm 2.89\%$) and extender D-20 ($60.00 \pm 5.00\%$) ($p < 0.05$) which demonstrated that D-15 extender was more efficient for Mandarin fish sperm than the other three extenders.

Various cryoprotectants have been successfully used for cryopreservation of different fish sperm. Dimethyl sulfoxide is the most commonly cryoprotectant used in sperm cryopreservation of fish such as turbot (Dreanno et al., 1997; Chen et al., 2004), sea bass (Fauvel et al., 1998), black grouper (Palmer et al., 1993) and yellow flounder (Richardson et al., 1999). We examined the effect of various cryoprotectants on the post-thaw motility. Our results revealed that ME_2SO was more efficient for Mandarin fish sperm cryopreservation than glycerol and methanol. Although glycerol is almost completely ineffective for Mandarin fish sperm, it is the most effective and generally applicable cryoprotectant for the summer whiting (*Sillago ciliata*) (Young et al., 1992). Ten percent methanol was a far more efficient cryoprotectant for common carp sperm than 10% ME_2SO (Horváth et al., 2003), but it was less suitable for Mandarin fish sperm than ME_2SO.

The success of fish sperm cryopreservation is usually assessed by the yield of fertilization. Although in external fertilizing fish natural fertilization occurs in water, it is generally not the optimal medium for the activation of frozen sperm (Lahnsteiner et al., 2002). In this study, SM was chosen as activation medium to improve motility and fertility of frozen sperm. The dead eggs must be removed from each cage after fertilization because they possibly stunted other eggs' development. As shown in Fig. 2, although the motility rates of cryopreserved Mandarin fish sperm were reduced, the fertilization rates were in a similar range as well as fresh sperm. The lower motility rates of cryopreserved semen are compensated by higher sperm to egg ratios (Lahnsteiner, 2000; Lahnsteiner et al., 1996). In our study, the fertilization rate of frozen sperm were lower than that of fresh sperm at the lower sperm/egg ratio of 10,000:1 and 20,000:1 and were similar to that of fresh sperm at the higher sperm/egg ratio of 50,000:1 and 100,000:1. Cryopreservation is an effective method for long-term storage of viable sperm (Yao et al., 2000). The fertilization rate and the hatching rate of frozen sperm that were cryopreserved for 1 year in liquid nitrogen ($62.97 \pm 14.28\%$ and $52.58 \pm 11.17\%$) were not significantly different from that of frozen sperm that were cryopreserved for 1 week in liquid nitrogen ($66.01 \pm 5.14\%$ and $54.76 \pm 4.40\%$) at the sperm/egg ratio of 100,000:1 ($p > 0.05$).

In summary, we cryopreserved Mandarin fish sperm successfully using D-15 supplemented with 10% ME_2SO. And the fertilization rates and the hatching rates of frozen sperm were similar to those of their fresh sperm control at the higher sperm to egg ratios. This is the first report on successful cryopreservation of Mandarin fish *S. chuatsi* sperm. These results imply the potential commercial application of the cryopreservation technique in Mandarin fish hatcheries.

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