Available online at www.sciencedirect.com
SCIENCE DIRECT®
Aquaculture
ELSEVIER
Aquaculture 241 (2004) 517–528
www.elsevier.com/locate/aqua-online

Cryopreservation of sea perch (*Lateolabrax japonicus*) spermatozoa and feasibility for production-scale fertilization

X.S. Ji[a], S.L. Chen[a,b,*], Y.S. Tian[a], G.C. Yu[a], Z.X. Sha[a], M.Y. Xu[a], S.C. Zhang[c]

[a]*Yellow Sea Fisheries Research Institute, Chinese Academy of Fisheries Sciences, Qingdao 266071, China*
[b]*Aquaculture Division, E-Institute of Shanghai Universities, Shanghai Fisheries University, Shanghai 200090, China*
[c]*Ocean University of China, Qingdao 266003, China*

Received 19 April 2004; received in revised form 5 July 2004; accepted 12 July 2004

Abstract

With the objective of germplasm conservation and cryobank construction, a method for cryopreserving sea perch (*Lateolabrax japonicus*, Cuvier) semen in 1.8-ml cryovials was developed. The effects of various extenders, cryoprotectants, volume of diluted semen on the motility score of post-thaw spermatozoa were examined. Post-thaw motility of frozen sperm obtained with extender modified plaice Ringer solution (MPRS) was higher than those achieved with extenders D-15 and modified Mounib's medium (MMM). With MPRS, the most effective cryoprotectant was determined to 10% dimethyl sulphoxide (DMSO). Post-thaw motility of frozen semen was not significantly reduced when the volume of diluted semen in the cryovial was increased from 0.5 to 1.0 ml ($p>0.05$). When the sperm/egg ratios varied from 320,000:1 to 20,000:1, fertilization rates of frozen semen cryopreserved for 3 days or 1 year in liquid nitrogen were not significantly different from that of fresh sperm ($p>0.05$). In fertilization trials of 230-ml eggs with frozen semen cryopreserved for 3 days in liquid nitrogen, 84.8% fertilization rate and 70.1% hatching rate were obtained, which was similar to control (81.0±2.3% and 87.2±3.1%) ($p>0.05$). Insemination of large egg batches (440-ml eggs) with frozen sperm cryopreserved for 1 year in liquid nitrogen resulted in high fertilization rates

* Corresponding author. Yellow Sea Fisheries Research Institute, Chinese Academy of Fisheries Sciences, Nanjing Road 106, 266071 Qingdao, China. Tel.: +86 532 5844606; fax: +86 532 5811514.
E-mail address: chensl@ysfri.ac.cn (S.L. Chen).
[1] Present address: Shandong Agricultural University, Taian, 271018, China.

0044-8486/$ - see front matter
doi:10.1016/j.aquaculture.2004.07.012

(83.5%) and hatching rates (90.0%) that resembled rates obtained with fresh (control) semen ($p>0.05$). Scanning and transmission electron microscopic observation indicated that while most of frozen–thawed sperm remained morphologically normal, some exhibited more or less damage, which probably caused the decrease in motility and fertility of the post-thawed sperm.

Keywords: Cryopreservation; Sperm; Motility score; Fertilization; Sea perch; *Lateolabrax japonicus*

1. Introduction

Cryopreservation of fish spermatozoa is a powerful technique for preserving fish germplasm and preventing fish species from extinction (Chen et al., 1992a, 1993; Ohta et al., 2001; Robles et al., 2003). In addition, it is a prerequisite for establishing gene banks (Munkittrick and Moccia, 1984) and can provide a year-round supply of fish semen, which brings great convenience for breeding and genetic studies. But variable results are obtained among species and even between the ejaculates of the same male. Numerous experiments are therefore needed in order to cryopreserve fish spermatozoa successfully. Most studies on gamete preservation are conducted on a laboratory scale. Not much practical progress of fish spermatozoa cryopreservation in large volume cryovials for the construction of cryobanks or commercial purposes has yet been achieved (Wheeler and Thorgaard, 1991; Cabrita et al., 2001; Chen et al., 2004; Lahnsteiner et al., 2002).

Sea perch (*Lateolabrax japonicus*), which naturally inhabits in the coastal areas of China, Japan and the Korea peninsula, has been cultivated widely in China in recent years and a great deal of research has also been conducted on the control of reproduction (Sun et al., 1994). The long-term preservation of sea perch sperm will simplify the production of selected strains and facilitate genetic selection of beneficial traits for intensive commercial farming (Xu et al., 2001). In addition, the wild stock of sea perch is declining because of excessive exploitation and environmental pollution. Constructing a cryobank of sea perch semen is becoming more and more urgent. With the aim of establishing a simple and practical cryopreservation technique for sea perch semen, a series of experiments were performed to examine the effects of various factors on the motility, fertility and morphology of frozen–thawed semen.

2. Materials and methods

2.1. Gamete collection

Sea perch broodstock with an average weight of 3–6 kg, purchased from Maidao Experimental Station (Qingdao, China), was raised in 36-m^3 concrete tanks supplied with flow-through seawater (11–14 °C). A total of 12 males were used for the study. The semen was collected by syringe after applying gentle abdominal pressure and then was transferred into 25-ml bottles. The semen concentration was determined using the equation $SC=(0.806OD-0.032)\times10^8$, where SC and OD are, respectively, semen concentration

(spz/ml^{-1}) and optical density at a 260-nm wavelength (Fauvel et al., 1998). Eggs were collected by abdominal pressure of the females and placed in a 500-ml beaker. About 5-ml eggs were added to a 250-ml beaker containing 200-ml fresh seawater to test egg quality. If the majority of the eggs were floatable, this batch of eggs was used in the experiment.

2.2. Sperm motility assessment

Sperm were activated by adding a drop of seawater on the sperm spread on a glass slide. After activation, sperm motility was immediately determined under light microscope (×100). The motility was expressed by values from 0 to 5, with 0 representing no motile spermatozoa and 5 from 80% to 100% sperm showing progressive movement. To validate the motility measurement, preliminary tests were carried out using three samples.

2.3. Cryopreservation of spermatozoa

Semen from each male was separately cryopreserved using the "three-step" method developed for Chinese carps (Chen et al., 1992a,b). In brief, within 10 min of harvesting, semen was mixed with precooled (4 °C) extenders containing cryoprotectants at a ratio of 1:1 (extender/semen) and then was equilibrated at 4 °C for 30 min. The equilibrated semen was transferred into 1.8-ml cryovials (Nalgen, USA). Then, the cryovials filled with diluted sperm were transferred into gauze pocket with a dimension of 6 cm in width and 9 cm in length and was equilibrated for 10 min in liquid nitrogen vapour 2, 6 or 13 cm above the liquid nitrogen surface, and then equilibrated for 5 min on the surface of liquid nitrogen, finally immersed in liquid nitrogen. Cooling rate of semen in the cryovial was measured using a programmable freezer (Shanghai University of Science and Technology, Shanghai, China). Effect of cooling rate on the motility score of frozen–thawed semen was also examined.

Composition of three extenders used in the study is shown in Table 1. Modified plaice Ringer solution (MPRS) was modified according to the plaice Ringer solution (Yao et al.,

Table 1
Composition of extenders for sea perch sperm

Components	MPRS	D-15	MMM
NaCl (mM)	60.35	136.75	–
NaH_2PO_4 (mM)	1.80	–	–
$NaHCO_3$ (mM)	3.00	–	–
KCl (mM)	5.23	6.71	–
$CaCl_2 \cdot 2H_2O$ (mM)	1.13	–	–
$MgCl_2 \cdot 6H_2O$ (mM)	1.13	–	–
D-Glucose (mM)	55.55	83.33	–
Sucrose (mM)	–	–	125.00
$KHCO_3$ (mM)	–	–	100.00
Reduced glutathione (mM)	–	–	6.50
BSA (mg/ml)	–	–	10.00
pH	6.68	6.50	7.80
Osmotic pressure (mosM/kg)	202	363	310

2000). D-15 was prepared as described (Chen et al., 1992a). In addition, modified Mounib medium (MMM) was also investigated in the experiment (Dreanno et al., 1997). Effects of type and concentration of cryoprotectants on motility of post-thaw semen were determined using dimethyl sulphoxide (DMSO), methanol, dimethylformamide (DMF) and glycerol (Amresco, USA).

2.4. Thawing of frozen sperm

After storage for 1 day to 1 year in liquid nitrogen, the gauze pocket containing cryovials was equilibrated for 5 min in liquid nitrogen vapors and then removed from the liquid nitrogen container. Semen was thawed in a water bath at 37 °C and thawing time for 0.5, 1 and 1.8 ml volume of diluted semen was 50 s, 1.5 min and 2.5 min, respectively. The motility score of frozen–thawed sperm was determined as described above.

2.5. Morphology observation

Firstly, the semen was cryopreserved using MPRS supplemented with 10% DMSO. The volume of diluted semen in the cryovial was 1 ml. The frozen semen was thawed in a water bath at 37 °C. And then, fresh and frozen–thawed semen were firstly fixed in 3% glutaraldehyde in seawater. After 15 min, the supernatant was removed and the pellet was resuspended in fresh 3% glutaraldehyde in seawater. For scanning electron microscopy, they were then fixed in 1% osmium tetroxide, dehydrated with graded ethanol and dried by dry ice. Observation was carried out under a JEOL JOM 7000 scanning electron microscope. For transmission electron microscopy, they were dehydrated with graded ethanol, embedded with Epon812 and cut with LKB slicer. After staining, observation was carried out under a JEOL transmission electron microscope.

2.6. Fertilization trials

Two fertilization trials were conducted to compare the fertility and hatching rates of fresh and frozen semen. Experiment 1: 1-ml eggs (about 1000) were inseminated with different volume of frozen–thawed and fresh sperm to give four sperm/egg ratios (20,000:1, 40,000:1, 80,000:1 and 320,000:1). Experiment 2: After semen was conserved for 1 year in liquid nitrogen, 1-ml eggs (about 1000) were inseminated with different volume of frozen–thawed sperm to give four sperm/egg ratios (20,000:1, 40,000:1, 80,000:1 and 320,000:1) . The fresh control was done with a sperm/egg ratios of 80,000:1. After mixing of both semen and egg, 2-ml seawater was then added to the mixture of semen and eggs to activate semen. After 5 min, about 100 eggs were transferred to 100ml beaker for incubation. The eggs were incubated at 11–14 °C and dead eggs were removed in a timely fashion. When the eggs developed to gastrula stage, the fertilization rate (fertilization rate=number of gastrula stage eggs/number of eggs) was determined. The hatching rate was expressed as the percentage of hatched larvae in the fertilized eggs. The eggs were from only one female for every specific experiment and every experiment was replicated three times with semen from same male.

To test application potential of frozen semen in hatchery, two large-scale fertilization experiments were done. Experiment 3: 230-ml (about 230,000) eggs were inseminated with 1.75-ml frozen semen cryopreserved for 3 days in liquid nitrogen. The control study was done with 1-ml eggs and 0.05-ml fresh semen and replicated three times. Experiment 4: 440-ml eggs were inseminated with 3.5-ml frozen semen conserved for 1 year in liquid nitrogen. The 1-ml eggs were inseminated with 0.05-ml fresh semen as control. The eggs fertilized with frozen semen were incubated in big tanks (0.5 m^3) simulating production conditions.

2.7. Statistical analysis

Data are expressed as mean±S.E.M. Motility score of semen after activation was analyzed using one-way analysis of variance (ANOVA). When differences were significant, least-significant-difference (LSD) and Turkey was used for comparison. A value of $p<0.05$ was considered as being statistically significant. The fertilization rate and hatching rate in Experiment 1 were tested by independent-samples *t*-test. The statistical analysis was computed using SPSS software.

3. Results

3.1. Effect of extenders and cryoprotectants

Effects of extenders on the motility score of frozen–thawed sperm are shown in Fig. 1. The highest motility score was observed with MPRS and the lowest with MMM ($p<0.05$). DMSO used as cryoprotectant in a concentration range of 6–14% resulted in higher post-thaw motility score than other cryoprotectants ($p<0.05$) (Table 2). No significant differences were observed between 6% and 10% DMSO ($p>0.05$), while 14% DMSO resulted in a lower motility score ($p<0.05$). The use of DMF resulted in low motility and glycerol and methanol were completely ineffective for sea perch sperm cryopreservation.

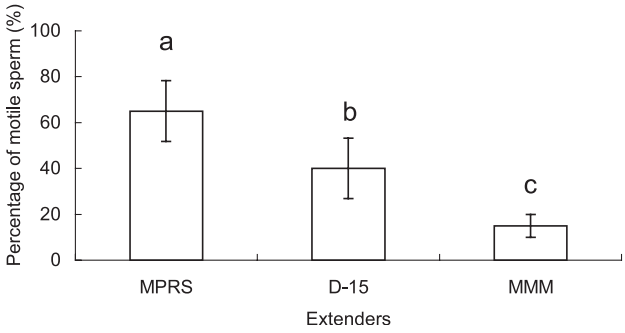

Fig. 1. Effect of extenders on motility score of frozen–thawed sperm ($n=3$). The semen was mixed at a 1:1 dilution ratio with the extenders supplemented with 10% DMSO and equilibrated at 6 cm above the surface of the liquid nitrogen. The volume of diluted-semen in the cryovials was 1 ml. Different letters indicate significant differences among treatments ($p<0.05$).

Table 2
Effect of various permeating cryoprotectants on frozen–thawed sperm motility (n=3)

Cryoprotectants	Concentration (%)	Percentage of motile perm 10 s after activation (mean±S.E.M.)	Percentage of motile sperm 300 s after activation (mean±S.E.M.)
DMSO	6	60.0±5.7^{a}	16.6±3.3^{ab}
	10	68.3±4.4^{a}	21.6±1.6^{a}
	14	36.6±3.3^{b}	8.3±4.4^{bc}
DMF	6	6.6±1.6^{c}	0.0±0.0^{c}
	10	11.6±2.8^{c}	0.0±0.0^{c}
	14	11.6±1.6^{c}	0.0±0.0^{c}
Glycerol	6	0.0±0.0^{c}	0.0±0.0^{c}
	10	0.0±0.0^{c}	0.0±0.0^{c}
	14	0.0±0.0^{c}	0.0±0.0^{c}
Methanol	6	5.0 ±0.0^{c}	0.0±0.0^{c}
	10	0.0±0.0^{c}	0.0±0.0^{c}
	14	0.0±0.0^{c}	0.0±0.0^{c}

The semen diluted in MPRS was equilibrated at 6 cm above the liquid nitrogen. The volume of diluted semen in the cryovials was 1 ml. The values within column followed by different superscripts are different (p<0.05).

3.2. Determination of freezing rate

When the 1.8-ml cryovials containing 1 ml diluted semen were equilibrated at 6 cm above the surface of liquid nitrogen for 10 min, a linear decrease of temperature from 16 to −15 °C at a cooling rate of −31 °C/min was obtained. Freezing point occurred after 65 s at−15 °C and the temperature surged to −12 °C. The freezing rate from −12 to −180 °C was determined to be −18.6 °C/min. Similar temperature profiles were obtained when the cryovials containing 1-ml diluted semen were equilibrated at 2 or 13 cm above the surface of liquid nitrogen. However, their freezing points and temperature surges were different. When the cryovials were equilibrated at 2 cm above the surface of liquid nitrogen, the freezing point occurred after 55 s at −25 °C and the temperature surges were up to 9 °C (from −25 to −16 °C), which was equal to those equilibrating at 13 cm above the surface of liquid nitrogen except their freezing time occurred after 70 s. Equilibrating at 6 cm above the surface of the liquid nitrogen for 10 min resulted in 73.3±5.7% motility score, which was higher than that equilibrating at 2 cm (41.7±10.6%) and 13 cm (48.3±2.9%) (p<0.05) .

3.3. Effect of semen volume in cryovials

The post-thaw motility was not reduced significantly when the volume of diluted semen in the cryovials was increased from 0.5 to 1.0 ml (Fig. 2) (p>0.05). However, the post-thaw motility of 1.8-ml diluted semen in the cryovials was significantly lower than that of 0.5 ml after 30 s (Fig. 2) (p<0.05). In addition, the increases of volume of diluted semen in the cryovials also prolonged the thawing time. The thawing time was 50 s, 1.5 min and 2.5 min for 0.5-, 1- and 1.8-ml diluted semen volume, respectively.

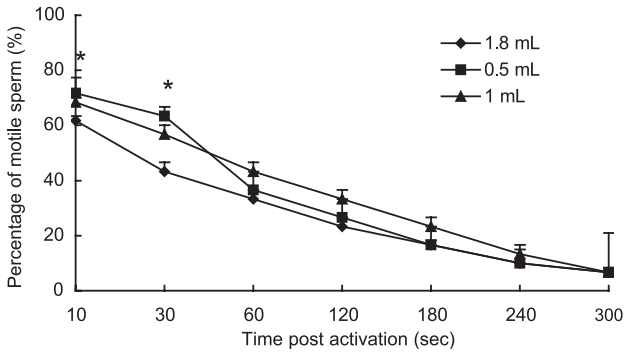

Fig. 2. Effect of semen volume in cryovial on post-thaw motility score (n=3). Semen was mixed with MPRS supplemented with 10% DMSO at a ratio of 1:1 and the diluted semen was equilibrated at 6 cm above the surface of the liquid nitrogen. Asterisks indicate a percentage of motile spermatozoa significantly different between 0.5- and 1.8-ml diluted semen in the cryovials (p<0.05).

3.4. Observation of sperm morphology

Scanning electron microscopic observation showed that most of the frozen–thawed sperm remained morphologically normal (Fig. 3B) in comparison with the fresh sperm (Fig. 3A). The major damage of frozen–thawed sperm occurred in mitochondria and cell membrane of sperm head. For example, some of them had lost all or partial mitochondria and burst cell membrane (Fig. 3C). Transmission electron microscopic observation showed that sea perch sperm had 4–6 round mitochondria surrounding the mid-piece sleeve (Fig. 4D). Some of the frozen–thawed sperm had burst cell membrane (Fig. 4B) and even lost all the cytoplasm (Fig. 4C) compared with fresh sperm (Fig. 4A).

3.5. Fertilization trials

Under various sperm/egg ratios, the fertilization and hatching rates of frozen semen cryopreserved for 3 days in liquid nitrogen were not significantly different from that of fresh sperm (p>0.05) (Table 3). After being cryopreserved for 1 year in liquid nitrogen,

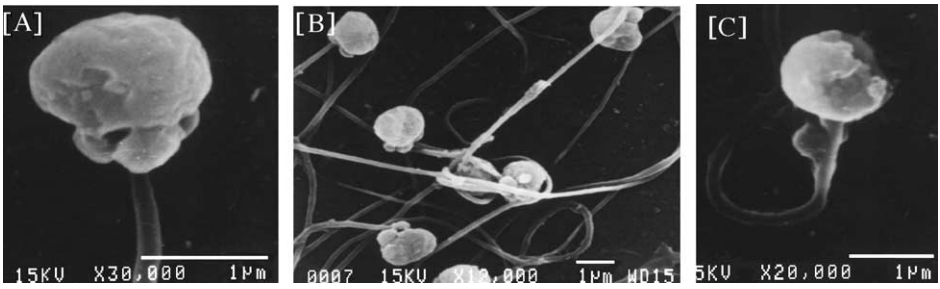

Fig. 3. Scanning electron microscopic photographs of fresh sperm (A) and frozen–thawed sperm (B, C). Note normal sperm morphology of fresh sperm (A), the normal morphology of most frozen–thawed sperm (B), membrane damage in the head and loss of mitochondria of frozen–thawed sperm (C).

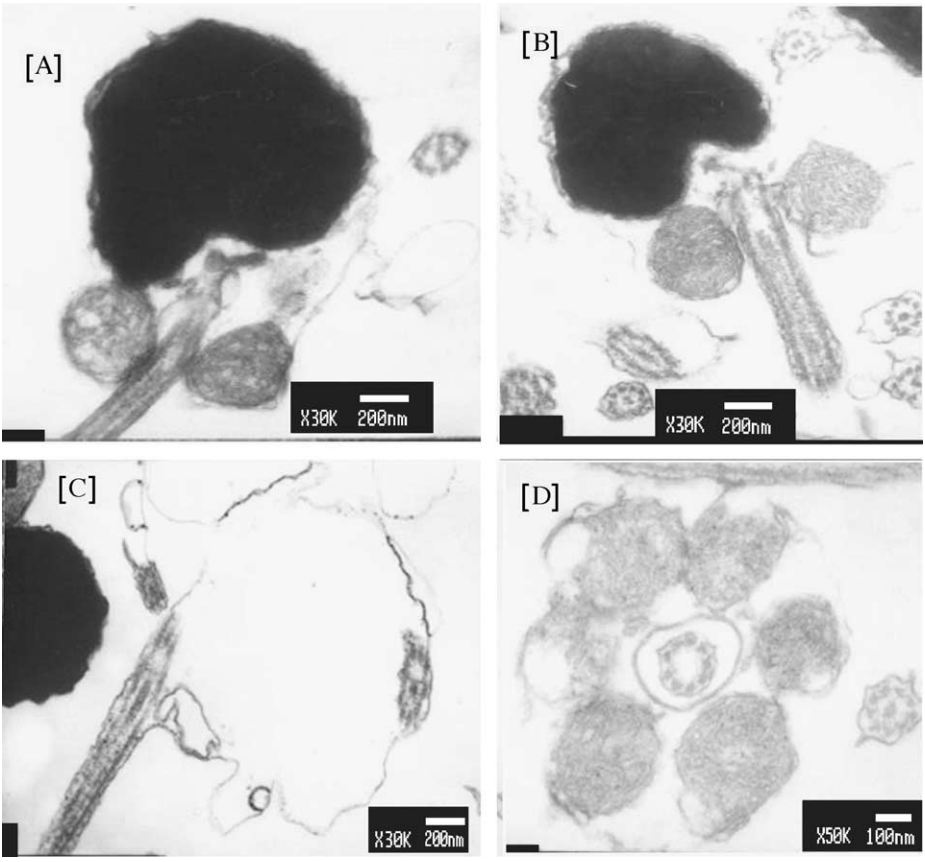

Fig. 4. Transmission electron microscopic photographs of fresh sperm (A) and frozen–thawed sperm (B–D). Note the membrane damage (B), the loss of cytoplasm (C) and six mitochondria surrounding the mid-piece sleeve (D).

the fertilization rates of frozen–thawed sperm was still not significantly different from that of fresh sperm ($p>0.05$) (Fig. 5). Insemination of 230-ml eggs with 1.75-ml frozen semen cryopreserved for 3 days in liquid nitrogen resulted in a 84.8% fertilization rate and 70.1%

Table 3
Fertilization rate and hatching rate of fresh and frozen–thawed semen using various sperm/egg ratios (n=3)

Ratios (sperm/egg)	Fresh sperm		Frozen–thawed sperm	
	Fertilization rate (%)	Hatching rate (%)	Fertilization rate (%)	Hatching rate (%)
20,000:1	67.2 ± 4.2^{a}	79.9 ± 8.3^{b}	61.3 ± 5.6^{c}	91.5 ± 1.3^{d}
40,000:1	67.7 ± 0.9^{a}	79.9 ± 12.5^{b}	61.5 ± 1.5^{c}	87.2 ± 10.2^{d}
80,000:1	65.0 ± 3.3^{a}	81.8 ± 9.3^{b}	64.1 ± 0.7^{c}	91.7 ± 8.3^{d}
320,000:1	66.9 ± 5.4^{a}	85.8 ± 2.0^{b}	63.2 ± 1.9^{c}	89.8 ± 0.4^{d}

The semen was cryopreserved using MPRS supplemented with 10% DMSO. The volume of diluted semen in the cryovials was 1 ml. The frozen semen was thawed in a water bath at 37 °C. The values within column followed by same superscripts are same ($p>0.05$).

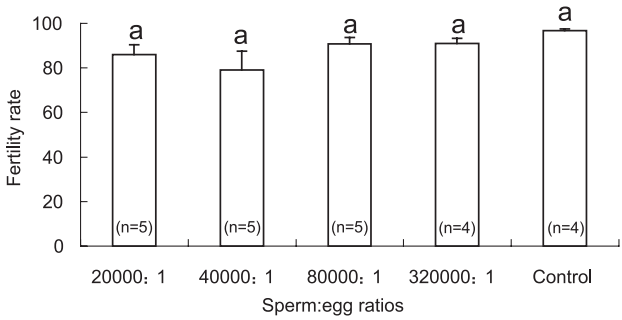

Fig. 5. Fertilization rates of fresh sperm and frozen semen stored for 1 year in liquid nitrogen using various sperm/egg ratios. The semen was cryopreserved using MPRS supplemented with 10% DMSO. The volume of diluted semen in cryovials was 1 ml. Different letters indicate significant differences among treatments ($p<0.05$).

hatching rate, which resembled the rates obtained with fresh sperm (81.0±4.6% and 87.2±3.2%) ($p>0.05$). In fertilization trials of 440-ml eggs with 3.5-ml frozen semen cryopreserved for 1 year in liquid nitrogen, a 83.5% fertilization rate and 90.0% hatching rate were obtained, which was similar to the control (96.8±2.3% and 87.2±3.1%) ($p>0.05$). As a result, about 300,000 fry hatched out.

4. Discussion

Sea perch is an important cultured marine fish. Cryopreservation of sea perch semen will provide a means for its germplasm conservation and convenience for breeding programs. Selecting a suitable extender is the key factor in successfully cryopreserving fish sperm. In the present study, we cryopreserved the sea perch sperm successfully using modified plaice Ringer solution supplemented with 10% DMSO. The extender D-15 is an efficient extender for cryopreservation of Chinese carp sperm (Chen et al., 1992a); however, when the sea perch semen was diluted in D-15 supplemented with 10% DMSO, the sperm were activated. The addition of $NaHCO_3$ only partly inhibited the movement of sperm before freezing, which could be due to the high osmotic pressure resulting from the presence of NaCl and glucose at high concentration in D-15. In addition, the cryoprotectants also enhance the osmotic pressure of the extender (Chen et al., 1987). The excessive osmotic pressure induces sperm to move and the inhibitors of sperm motility such as $NaHCO_3$ and $KHCO_3$ become inefficient.

DMSO has been widely used in sperm cryopreservation of marine 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-thawed motility score and demonstrated that DMSO was more efficient for sea perch semen than DMF, methanol and glycerol. Although methanol is almost completely ineffective for sea perch semen, methanol is the most effective and generally applicable cryoprotectant for salmonid semen (Lahnsteiner et al., 1997). Glycerol is considered to be less toxic than DMSO for

most types of cells, but it appeared to be toxic to sperm of sea perch. To minimize the toxicity of glycerol, the equilibration time should be kept to a minimum. But glycerol is a slow permeating cryoprotectant (Ponniah et al., 1999) and it takes time to penetrate the sperm completely. DMF was once used as cryoprotectant for medaka sperm cryopreservation (Aoki et al., 1997), but it did not appear to be suitable for sea perch semen, as shown by our results.

Optimal cooling rates are different among species and change from 8 to 99 °C/min for sperm in marine species (Steyn, 1993; Sansone et al., 2002). In the present work, the sea perch semen was cryopreserved at a cooling rate of 31 °C/min from 16 to −15 °C and 18.6 °C/min from −12 to −180 °C. In addition, the temperature surges during freezing are harmful to sperm because the cooling rate is faster after the release of the heat latent of fusion. In the present study, equilibrating at 6 cm above the surface of the liquid nitrogen achieved the highest motility score, which could be due to the lower temperature surges during seeding.

Cryopreservation of sperm in large volume straws or cryovials could save time and facilitate handling, making it feasible for production and cryobank purposes. Cabrita et al. (2001) successfully cryopreserved rainbow trout sperm in 5-ml straws. Furthermore, straw shape has a great influence on cryopreservation because the release of the latent heat of fusion is affected by the surface/volume ratio. The 1.8-ml cryovial was used in the present study for the cryopreservation of sea perch sperm and similar post-thaw fertility was obtained to that of the fresh sperm. Most fertilization trials were performed in petri dishes in the cryopreservation of teleost sperm (Babiak et al., 1995). In the present study, high fertilization rates were gained when large batches of eggs (230 and 440 ml) were fertilized with frozen sperm in liquid nitrogen.

Because sperm head and mitochondria are relatively large, they are vulnerable during cryopreservation. The scanning and transmission electron microscopic observation confirmed that although most of the frozen–thawed sperm were morphologically normal, some of them had lost all or part of their mitochondria, had a cell membrane and had even lost cytoplasm. This damage might reduce the sperm motility score and the degree of sperm flagellar movement. Similar results have been reported in the cryopreservation of the flounder *Paralichthys olivaceus* semen (Zhang et al., 2003) and ocean pout *Macrozoarces americanus* (Yao et al., 2000).

In summary, a method for cryopreservation of sea perch sperm in 1.8-ml cryovials was developed. Fertility rates obtained in large-scale fertilization trials with frozen–thawed sperm were similar to those obtained with fresh sperm. After the sperm were cryopreserved for 1 year in liquid nitrogen, the fertilization and fertility rates were not significantly reduced. These results demonstrate the potential commercial application of the cryopreservation technique in sea perch hatcheries.

Acknowledgements

This work was supported by a grant from the State 863 High-Technology R&D Project of China (2001AA621100) and supported in part by E-Institute of Shanghai Municipal Education Commission.

References

Aoki, K., Okamoto, M., Tatsuimi, K., Ishikawa, T., 1997. Cryopreservation of medaka spermatozoa. Zoological Science 14, 641–644.

Babiak, I., Glogowski, J., Luczynski, M.J., Kucharczyk, D., Luczynski, M., 1995. Cryopreservation of the milt of the northern pike. Journal of Fish Biology 46, 819–828.

Cabrita, E., Robles, V., Alvarez, R., Herráez, P., 2001. Cryopreservation of rainbow trout sperm in large volume straws: application to large scale fertilization. Aquaculture 201, 301–314.

Chen, S.L., Zhang, L.Z., Guo, F., 1987. Effect of cryoprotectant DMSO on osmotic pressure and motility of fish spermatozoa. Freshwater Fisheries 17, 17–21 (in Chinese with English abstract).

Chen, S.L., Liu, X.T., Lu, D.C., Zhang, L.Z., Fu, C.J., Fang, J.P., 1992a. Cryopreservation of spermatozoa of silver carp, common carp, blunt snout bream and grass carp. Acta Zoologica Sinica 38, 413–424 (in Chinese with English abstract).

Chen, S.L., Liu, X.T., Lu, D.C., Fu, C.J., Zhang, L.Z., Fang, J.P., 1992b. Activation and insemination methods of cryopreserved sperm from several Chinese carps. Journal of Fisheries of China 16, 337–346. in Chinese with English abstract.

Chen, S.L., Liu, X.T., Fu, C.J., 1993. Cryopreservation of Chinese carp spermatozoa. Aquaculture 111, 324–325.

Chen, S.L., Ji, X.S., Yu, G.C., Tian, Y.S., Sha, Z.X., 2004. Cryopreservation of sperm from turbot (*Scophthalmus maximus*) and application to large-scale fertilization. Aquaculture 236, 547–556.

Dreanno, C., Sequet, M., Quemener, L., Cosson, J., Fierville, F., Normant, Y., Billard, R., 1997. Cryopreservation of turbot (*Scophthalmus maximus*) spermatozoa. Theriogenology 48, 589–603.

Fauvel, C., Suquet, M., Dreanno, C., Zonno, V., Menu, B., 1998. Cryopreservation of sea bass (*Dicentrarchus labrax*) spermatozoa in experimental and production simulating conditions. Aquatic Living Resources 11, 387–394.

Lahnsteiner, F., Weismann, T., Patzner, R.A., 1997. Methanol as cryoprotectants and the suitability of 1.2 ml and 1.5 ml straws for cryopreservation of semen from salmonid fishes. Aquaculture Research 28, 471–479.

Lahnsteiner, F., Mansour, N., Weismann, T., 2002. A new technique for insemination of large egg batches with cryopreserved semen in the rainbow trout. Aquaculture 209, 359–367.

Munkittrick, K.R., Moccia, R.D., 1984. Advances in the cryopreservation of salmonid semen and suitability for a production-scale artificial fertilization program. Theriogenology 21, 645–659.

Ohta, H., Kawamura, K., Unuma, T., Takegoshi, Y., 2001. Cryopreservation of the sperm of the Japanese bitterling. Journal of Fish Biology 58, 670–681.

Palmer, P.J., Black, A.W., Garrett, R.N., 1993. Successful fertility experiments with cryopreserved spermatozoa of Barramundi, *Lates calcarifer* (Bloch) using dimethyl sulfoxide and glycerol as cryoprotectants. Reproduction, Fertility and Development 5, 285–294.

Ponniah, A.G., Lakra, W.S., Ogale, S.N., 1999. Effects of cryoprotectant and fertilization protocol on viability of mahseer Tor khudree cryopreserved spermatozoa. Journal of Aquaculture in the Tropics 14, 153–158.

Richardson, G.F., Wilson, C.E., Crim, L.W., Yao, Z., 1999. Cryopreservation of yellowtail flounder (*Pleuronectes ferrugineus*) semen in large straws. Aquaculture 174, 89–94.

Robles, V., Cabrita, E., Cuñado, S., Herráez, M.P., 2003. Sperm cryopreservation of sex-reversed rainbow trout (*Oncorhynchus myksis*): parameters that affect its ability for freezing. Aquaculture 224, 203–212.

Sansone, G., Fabbrocini, A., Leropoli, S., Langellotti, A., Occidente, M., Matassino, D., 2002. Effects of extender composition, cooling rate and freezing on the motility of sea bass spermatozoa after thawing. Cryobiology 44, 229–239.

Steyn, G.J., 1993. The effect of freezing rate on the survival of cryopreserved sharptooth catfish (*Clarias gariepinus*) sperm. Cryobiology 30, 581–590.

Sun, G.Y., Zhu, Y.Y., Zhou, Z.L., Chen, J.G., 1994. The reproductive biology of *Lateolabrax japonicus* in the Yangze river estuary and Zhejiang offshore waters. Journal of Fisheries of China 18, 18–23 (in Chinese).

Wheeler, P.A., Thorgaard, G.H., 1991. Cryopreservation of rainbow trout semen in large straws. Aquaculture 93, 95–100.

Xu, C., Wang, K.L., Zhang, P.J., 2001. Biochemical genitics of *Lateolabrax japonicus* population: II. Biochemical genetic structure and variation. Oceanologia et Limnologia Sinica 32, 248–254 (in Chinese).

Yao, Z., Crim, L.W., Richardson, G.F., Emerson, C.J., 2000. Motility, fertility and ultrastructural changes of ocean pout (*Macrozoarces americanus* L.) sperm after cryopreservation. Aquaculture 181, 361–375.

Zhang, Y.Z., Zhang, S.C., Liu, X.Z., Xu, Y.Y., Wang, C.L., Sawant, M.S., Li, J., Chen, S.L., 2003. Cryopreservation of flounder (*Paralichthus olivaceus*) sperm with a practical methodology. Theriogenology 60, 989–996.