Animal Reproduction Science 137 (2013) 230–236

Contents lists available at SciVerse ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Sperm cryopreservation of sex-reversed seven-band grouper, *Epinephelus septemfasciatus*

Yongsheng Tian[a,*], Wenshan Qi[a,b], Jing Jiang[a,b], Na Wang[a], Di Wang[a,c], Jieming Zhai[d], Chao Chen[a], Songlin Chen[a,*]

[a] *Key Laboratory of Sustainable Development of Marine Fisheries, Ministry of Agriculture, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao 266071, China*
[b] *College of Fisheries and Life Science, Shanghai Ocean University, Shanghai 201306, China*
[c] *College of Life Sciences and Technology, Qingdao Agricultural University, 266109, China*
[d] *Ming Bo Aquatic Co. Ltd., Laizhou 261418, China*

ARTICLE INFO

Article history:
Received 16 July 2012
Received in revised form 14 January 2013
Accepted 27 January 2013
Available online 8 February 2013

Keywords:
Epinephelus septemfasciatus
Sex-reversal
Sperm
Cryopreservation

ABSTRACT

Seven-band grouper *Epinephelus septemfasciatus* is a protogynous hermaphrodite. The male individuals' number is more less than the female one. Thus, it is necessary for artificial reproduction and crossbreeding to research the sperm cryopreservation of sex-reversed seven-band grouper. In present study, the spermatozoa of sex-reversed immature fish were frozen using the different cryopreserving solutions for cryopreservation. The several factors that may affect the freezing survival rate of seven-band grouper spermatozoa such as the spermatozoa diluent, the concentration and composition of cryoprotective agent have been studied. The results showed that ES1-3 (60 g/L glucose + 10 g/L NaCl + 0.5 g/L $NaHCO_3$) was significantly better as a diluent compared with MPRS, TS-2 and other series of diluent ES1. The further experiment revealed that the optimal cryoprotectants were 10% dimethyl sulfoxide (DMSO) or 10% 1,2-propylene glycol (PG) with the post-thaw sperm motility was 76.67 ± 0.00% and 75.00 ± 5.00%, respectively. In addition, salinity of seawater is an important motility stimulator because that the highest motility of 96.00 ± 1.73% was obtained at salinity 30‰. In crossbreeding test with fresh unfertilized eggs of kelp grouper *Epinephelus moara*, the ratio of fertilization and hatchability had no significant differences between the cryopreserved sperm and fresh sperm of seven-band grouper. It is suggested that the frozen sperm of seven-band grouper could be applied in artificial reproduction and crossbreeding.

1. Introduction

Groupers are the important captured and cultured fish in Asia with great economic value for aquaculture. There are about 100 populations in the world distributed in the tropical, subtropical and temperate zone waters (Meng et al., 1995). In China, there are 36 populations mostly distributed in the South Sea and East Sea (Meng et al., 1995). Since the 1970s and 1980s, groupers were cultivated in China's Hong Kong, Taiwan, Philippines, Indonesia and Thailand (Wang, 1997). Owing to the high marker value of wild-caught fish (Kline et al., 2008), seven-band grouper is considered a potential candidate for aquaculture and sea ranching in Japan (Sabate et al., 2009). In China, seven-band grouper has also become a popular species for aquaculture in the north. For example, Shandong Laizhou MingBo Aquatic Products Limited Company in China has developed artificial reproduction and breeding.

* Corresponding authors. Tel.: +86 532 85844606; fax: +86 532 85844606.
E-mail addresses: tianys@ysfri.ac.cn (Y. Tian), chensl@ysfri.ac.cn (S. Chen).

0378-4320/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.anireprosci.2013.01.014

Seven-band grouper is a protogynous hermaphrodite species as other groupers. They mature only as females and have the ability to change sex after sexual maturity. It is speculated that male would become sexual mature when the weight is more than 6 kg (Liu et al., 2010). The number of male in the breeding group of seven-band grouper is extremely limited, which is the population characteristics of grouper (there are no relevant published articles). Thus the lack of males in broodstocks population has greatly limited the extension of artificial breeding, abundant fry culture and large-scale farming. In order to reverse the female individuals into functional males as early as possible, 17α-methyltestosterone (MT) and Human Chorionic Gonadotropins (HCG) were added into the feed or injected into the muscle (Glamuzina et al., 1998; Sarter et al., 2006). However, it is still unable to meet the fertilization requirement, because only a small amount of sperm (0.5–1 ml/tail, each time) was obtained at sexual maturity stage.

Cryopreservation of fish sperm is a germplasm conservation technology developed since 1970s and it can be applied to the artificial reproduction, crossbreeding between species of temporal isolation and geographical isolation, selection breeding based on the family establishment and gynogenesis induction. In recent years, successful cryopreservation studies have already been developed for sea perch *Lateolabrax japonicus* (Ji et al., 2004), turbot *Scophthalmus maximus* (Chen et al., 2004), Japanese flounder *Paralichthys olivaceus* (Zhang et al., 2003), spotted halibut *Verasper variegatus* (Tian et al., 2008a,b) and half-smooth tongue sole *Cynoglossus semilaevis* (Tian et al., 2009). Meanwhile, it is reported that the frozen sperms have been used to induce artificial gynogenesis in half-smooth tongue sole (Tian et al., 2008a,b; Chen et al., 2009) and interspecific hybridization in summer flounder *Paralichthys dentatus* (Tian et al., 2006). In groupers, sperm cryopreservation has also been developed in black grouper *Epinephelus malabaricus* (Chao et al., 1992; Gwo, 1993) and kelp grouper (Miyaki et al., 2005). In seven-band grouper, sperm cryopreservation has been studied, but the cryopreservation volume is too small to apply to practical production (Koh et al., 2010), and the sperm survival ratio was only about 10% (Ou et al., 2011). Thus, the cryopreservation of sperm from the sex-reversed seven-band grouper still needs to be studied in further. Hence, in order to solve the problem that the seven-band grouper sperm are seriously insufficient in the artificial breeding, the component of cryopreservation extender, the variety and concentration of cryoprotectants and salinity of seawater were evaluated in present study. The results made great improvement in volume of cryopreservation, the fertilization rate and hatchability rate of frozen-thawed sperm, which provides the basis for the application of seven-band grouper in artificial reproduction, crossbreeding and selection breeding.

2. Materials and methods

2.1. Sperm collection

Spermatozoa was obtained from male broodstocks of twenty individuals of seven-band grouper (3.4–4.8 kg, body weight) cultured at the Laizhou MingBo Aquatic Products Limited Company (lat: 37.117017, long: 119.942327). In October 2010, twenty male broodstocks were implanted with 17α-methyltestosterone (α-MT; at the dosage of 25 mg/tail) sustained-release capsules. Male broodstocks were cultured in the indoor tanks (6.8 m × 6.8 m × 1.8 m; DO, 7–8 mg/L) under conditions of 18–19 °C and intermediate day length (12 h:12 h, L:D) from April to June 2011. Male broodstocks were fed wild fish with 1.2% body weigh once a day. In late May of 2011, 16 seven-band groupers were successfully reversed to male individuals. Spermiation was induced by intramuscular injection of Human Chorionic Gonadotropins (HCG, 100 IU/kg) and Luteinizing Hormone Releasing Hormone (LHRH-A_3, 3 μg/kg) to all sex-reversed males. After hormone injection for two days, mature male were aestheticized with 10 mg/L MS-222. The sperm were collected by abdominal massage with a 5 ml straw for each respective male. The collected sperm were dispensed into 2 ml cryovials. Generally, 1–2 ml sperm was sucked up from each male individual each time. Finally, the total volume of sperm collected was 50 ml in this experiment.

2.2. Sperm diluent preparation

Sperm diluents were prepared with the basic liquid composed of glucose, Tris alkali (Beijing Solarbio Science and Technology Co., Ltd.), sucrose, $KHCO_3$, KCl, $NaHCO_3$, $MgCl_2{\cdot}6H_2O$ (Sinopharm Chemical Reagent Co., Ltd.), NaCl, $CaCl_2{\cdot}2H_2O$ (Tianjin Regent chemicals Co., Ltd.), NaH_2PO_4 (Tianjin BASF Chemical Co., Ltd.), Fetal Bovine Serum (FBS, USA Gibco Co., Ltd.). The pH and Osmolality of sperm dilutions were measured respectively by PHS-25 pH meter (Shanghai Electronics Scientific Instruments Co., Ltd) and The Fiske Micro-Osmometer Model 210 (Norwood, Massachusetts, USA, FISKE Associates Company) (Table 1).

2.3. Sperm cryopreserving solution selecting

Four kinds of sperm cryopreserving solutions, MPRS, TS-2, CS1 and ES1, were used with 20% dimethyl sulfoxide (DMSO). The sperms were packed into 2 ml cryovials and diluted 1:1(v/v) with dilution consisting of cryoprotectant and extender in different cryopreserving solutions (Ji et al., 2004). The concentration of cryoprotectant was 10% after diluted according to the ratio of 1:1 (v/v). After equilibrated for 1–2 min, the cryovials were packed into small cloth bags with 5–6 cryovials per bag. The 30 L liquid nitrogen (LN) tank filled with 20 L LN was used as experimental cryopreservation equipment. The cryovials were first held for 10 min at 10 cm above the surface of liquid nitrogen (LN) vapor in order to precooling, and then directly plunged into LN for 4–5 h. Prior to the application of the frozen sperm, cryovials were equilibrated for 1 min above the surface of liquid nitrogen vapor before removed from the LN tank, and immediately immersed in water bath at 38 °C for 70–80 s. In order to examine the sperm motility, 1 μl sample was placed on a slide and activated by 200 μl seawater. The sperm were immediately observed under the microscope at a total magnification of 200× for the motility, fast-moving time and longevity. After diluted with seawater, spermatozoa concentration was determined by

Table 1
Component of diluent of seven-band grouper *E. septemfasciatus* sperm.

Solution composition (g/L)	MPRS	TS-2	CS1	ES1	ES1-1	ES1-2	ES1-3	ES1-4	ES1-5	ES1-6
Glucose	11.01			60.00	60.00	60.00	60.00	60.00	60.00	60.00
Sucrose		37.65								
NaCl	3.53		7.50	30.00	3.00	7.00	10.00	14.00	14.00	20.00
$NaHCO_3$	0.25		0.20	0.50	0.50	0.50	0.50	0.05	0.50	0.50
KCl	0.39		0.20						0.20	
$CaCl_2{\cdot}2H_2O$	0.17		0.20							
NaH_2PO_4	0.22									
$MgCl_2{\cdot}6H_2O$	0.23									
$KHCO_3$		10.01								
FBS (%)		10								
Tris alkali		1.21								
Osmolality (mOsm/L)	202.00	335.00	271.11	1369.30	444.60	581.60	684.40	810.69	826.77	1026.80
pH	6.68	8.20	8.87	8.77	9.13	9.04	8.96	8.46	8.94	8.85
Remarks	Ji et al. (2004)	Chen et al. (2004)	Kurokura et al. (1984)							

Fig. 1. The seven-band grouper *E. septemfasciatus* frozen sperm observed using a hemocytomter at objective lens magnification of 20×. The picture showed the concentration of post-thaw spermatozoa.

a hemocytomter (Fig. 1). The sperm motility refers to percentages of the swimming spermatozoa. The fast-moving time refers to the time of sperm from strenuous exercise (the swirling movement) to track movement (s). The longevity means all the swimming time of spermatozoa (s) (Huang et al., 2007).

In order to select out more effective sperm dilution, we further optimized and reformed to ES1 dilution, and then prepared a series of ES1 dilutions named ES1-1, ES1-2, ES1-3, ES1-4, ES1-5, and ES1-6. Then sperm were cryopreserved with six dilutions with 20% DMSO respectively, observed the movement after post-thaw, and recorded the motility.

2.4. Sperm cryoprotectants selecting

To obtain an appropriate sperm cryoprotective agent, such as 20% dimethyl sulfoxide (DMSO), dimethyl acetylamide (DMAC), 1,2-propylene glycol (PG) and glycerol (Gly) were prepared with ES1-3 as base fluid, The spermatozoa were frozen, thawed and observed as above methods after diluted with those cryoprotective agents in proportion of 1:1.

2.5. Optimal concentration of dimethyl sulfoxide and 1,2-propylene glycol selecting

In order to select an optimal concentration of dimethyl sulfoxide and 1,2-propylene glycol, sperm diluent, ES1-3, was used to prepare different concentration the DMSO (10%, 15% and 20%) and 1,2-propylene glycol (10%, 15% and 20%). Spermatozoa were diluted (1:1, v/v) and frozen for 9 h according to the above method. The spermatozoa were thawed in a water bath at 38 °C, and observed under a microscope at a total magnification of 200×.

2.6. The seawater salinity selecting

Seawater of salinity 30‰ was diluted with distilled water in accordance with the proportion of 100%, 66.7%, 60%, 55%, 50%, 45%, 40%, 33.3%, 25% and 20% to compound diluted seawater of salinity 30, 20, 18, 16, 14, 13, 10, 9, 7 and 5. Measured by the Fiske Micro-Osmometer Model 210, the osmotic pressure for salinity of seawater were 901, 588, 512, 444, 359, 350, 255, 210, 187 and 121 mOsm/L, respectively. 1 μl sperm was placed on glasses slide and stimulated with 200 μl different salinity of seawater. The spermatozoa were immediately observed under a microscope at a total magnification of 200× to record the motility to select out the optimal seawater salinity.

2.7. Hybridization fertilization test

Seven-band grouper spermatozoa were frozen for 120 h with the ES1-3 + 20%DMSO. Unfertilized eggs (400 ml) collected from kelp grouper, were averagely held in two beakers. The unfertilized eggs in one beaker were inseminated by adding post-thaw cryopreserved sperm (4.6×10^9 cells/ml, 1 ml), with seawater at 20 °C as activating solution. Simultaneously, the unfertilized eggs in the other beaker were inseminated by the fresh sperm (9.2×10^9 cells/ml, 0.5 ml) of seven-band grouper by the same method. After 5 min of fertilization, fertilized eggs were poured into nylon net and swashed repeatedly with seawater in order to wash the dead eggs and the mucus from eggs and sperm. The fertilized eggs were respectively incubated with uninterrupted tiny flow at 20 °C in 0.5 m^3

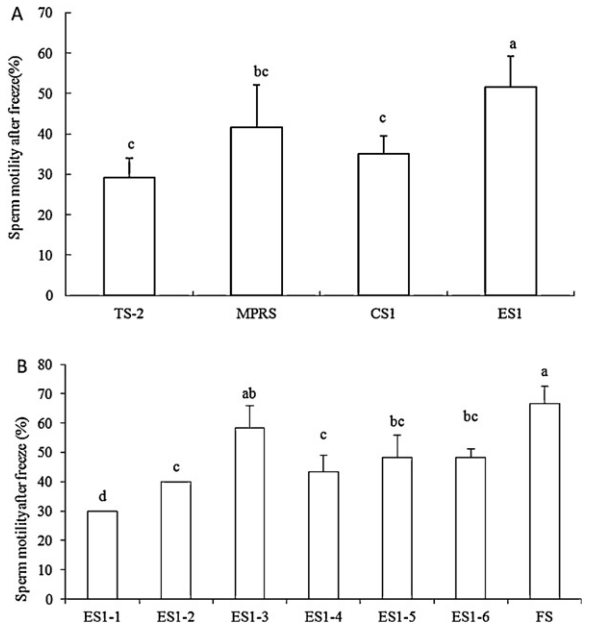

Fig. 2. Cryopreservation effect of seven-band grouper *E. septemfasciatus* spermatozoa with four kinds of diluents: MPRS, TS-2, CS1, ES1. ES1 produced the highest post-thaw motility (A). The sperm motility was the highest in the ES1 ($P < 0.05$). Motility of fresh and frozen-thawed spermatozoa diluted with different ES1 diluents (ES1-1–ES1-6). There were no significant differences between values of sperm diluted with ES1-3 and fresh sperm (FS) ($P > 0.05$) (B), means with different letters are significantly different ($n = 3$, $P < 0.05$). The values were expressed as mean ± S.D.

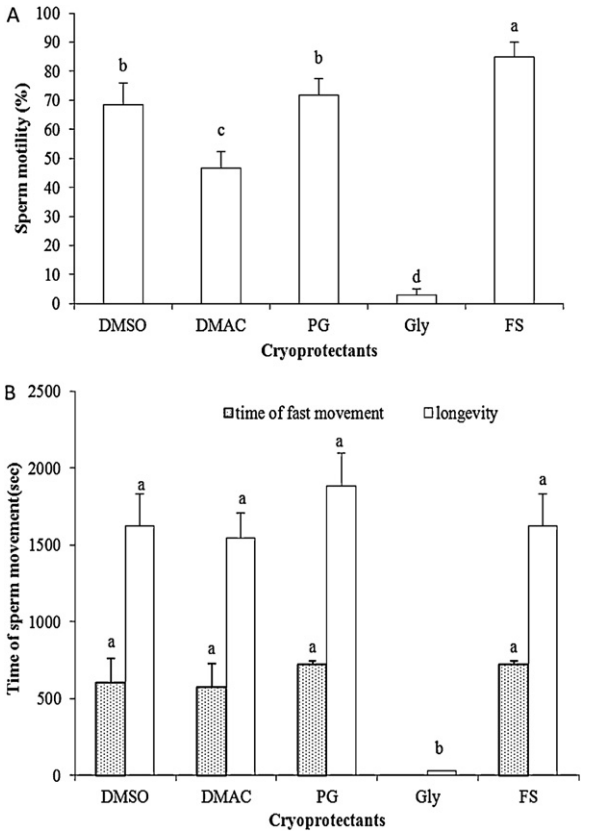

Fig. 3. Motilities of post-thaw sperm and fresh sperm (FS) of seven-band grouper *E. septemfasciatus* in different cryoprotectants (A) and fast-moving time and longevity (B). Motilities of sperm in PG and DMSO were higher than DMAC and Gly ($n = 3$, $P < 0.05$). And Gly produced almost no post-thaw motility. There was no significant differences between post-thaw sperm in PG, DMSO, DMAC and fresh sperm in the fast-moving time and longevity ($n = 3$, $P > 0.05$). The values were expressed as mean ± S.D.

net cage. In order to evaluate the rate of fertilization, the fertilized eggs at blastula stage were observed under a microscope after 3 h of fertilization. In addition, in order to record the rate of hatchability, 100 eggs fertilized with fresh sperm and frozen-thawed sperm, respectively collected in beakers, were incubated in the same condition. The hatchability rates were estimated by the percentage of embryos, with three replications per group.

2.8. Statistical analyses

The results were analyzed by a one-way analysis of variance (ANOVA). Differences between treatments were detected with the Student–Newman–Keuls (SNK) generated by SPSS software. The significance level was set at $P < 0.05$.

3. Results

3.1. Effect of different sperm diluent

Four diluents such as TS-2, MPRS, CS1 and ES1 were used for the sperm cryopreservation, respectively. As the Fig. 2 showed, the motility of the frozen-thawed sperm was 29.17 ± 4.94%, 41.67 ± 10.41%, 35.00 ± 4.47% and 51.67 ± 7.53%, respectively. The results showed that the effect of sperm cryoprotectant ES1 was significantly higher than the other cryoprotectants (Fig. 2A).

Accordingly, a series of dilution ES1 named ES1-1, ES1-2, ES1-3, ES1-4, ES1-5, and ES1-6 prepared with the ES1 diluent were used to freeze the sperm. The results showed that the motility of spermatozoa diluted with ES1-3 was 58.33 ± 7.64%, there was not significantly different from that of the fresh spermatozoa (66.67 ± 5.77%). The motility of spermatozoa preserved in ES1-1 was a minimum of 30.00%. And there was no significant difference from the motility with other diluents (40.00–48.33 ± 2.89%) (Fig. 2B).

3.2. Effect of different cryoprotective agent

The motility percentages of the post-thaw spermatozoa in PG and DMSO were 71.67 ± 5.77% and 68.33 ± 7.64%, respectively, which were significantly higher than dimethylacetylamide (DMAC) and glycerine (Gly). Especially, the sperm in Gly were almost immobile with only 3% motility percentage. However, compared with fresh spermatozoa (85.00 ± 5.00%), the motility of post-thaw spermatozoa in PG and DMSO decreased significantly (Fig. 3A).

The fast-moving time of spermatozoa in DMSO, DMAC and PG were 597.67 ± 164.66 s, 569.67 ± 157.14 s and 720.33 ± 24.54 s, respectively. And their longevities

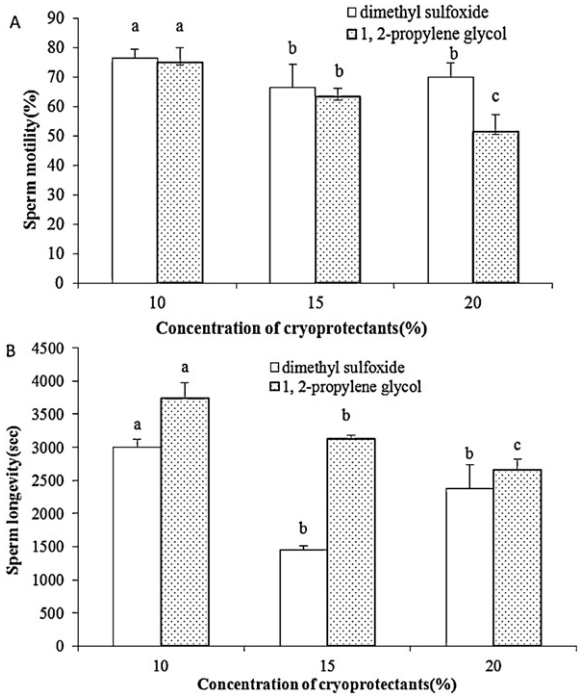

Fig. 4. Post-thaw motilities (A) and longevity (B) of seven-band grouper *E. septemfasciatus* spermatozoa froze with different concentrations of dimethyl sulfoxide (DMSO) and 1, 2-propylene glycol (PG). 10%PG and 10%DMSO produced the highest motility and longevity. Means with different letters are significantly different ($n = 3$, $P < 0.05$). The values were expressed as mean ± S.D.

were 1624.33 ± 206.00 s, 1543.00 ± 166.68 s and 1884.33 ± 212.03 s, respectively (Fig. 3B). There is no significant difference compared them with fresh sperm ($P > 0.05$) due to that the fast-moving time and longevity of fresh sperm was respectively 720.33 ± 24.54 s and 1624.33 ± 206.00 s. But there was significant difference in Gly compared with the fresh sperm ($P < 0.05$) because that no fast-moving phenomenon in Gly was observed, and the longevity of spermatozoa was only 30 s (Fig. 3B).

3.3. Optimal concentration of dimethyl sulfoxide and 1,2-propylene glycol selecting

The motilities of the spermatozoa in 10%, 15% and 20% DMSO were 76.67 ± 2.89%, 66.67 ± 7.64% and 70 ± 5.00%, respectively. The motilities of the spermatozoa in 10% DMSO was higher than in 15% and 20% DMSO ($P < 0.05$) (Fig. 4A). The longevity in 10%, 15% and 20% DMSO were 3003.67 ± 122.86 s, 1455.00 ± 59.57 s and 2376.33 ± 357.68 s, respectively, the motilities in 10% was significantly higher than those in 15% and 20% DMSO ($P < 0.05$) (Fig. 4B).

The motilities of the spermatozoa in 10%, 15% and 20% PG were 75 ± 5.00%, 63.33 ± 2.89% and 51.67 ± 5.77%, respectively. There were significant differences between values ($P < 0.05$) (Fig. 4A). The longevity of spermatozoa in 10%, 15% and 20%PG were 3739.67 ± 232.39 s, 3128 ± 232.39 s and 2668.32 ± 152.73 s, respectively. The longevity of spermatozoa in 10%PG was higher than the

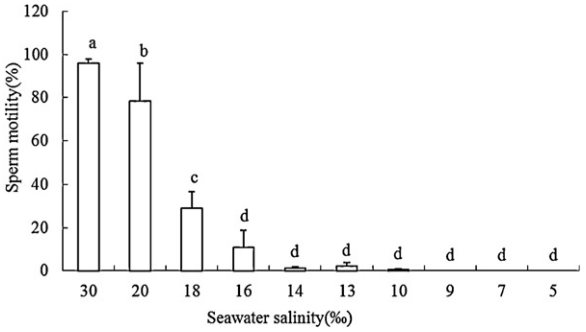

Fig. 5. Motility of seven-band grouper *E. septemfasciatus* sperm in different seawater salinity as activating solution. Salinity 30 produced highest motility. And the motility was 0 when the salinity was 9. Means with different letters are significantly different ($n = 3$–4, $P < 0.05$). The values were expressed as mean ± S.D.

one in 15% and 20% PG ($P < 0.05$) (Fig. 4B). The motility of the spermatozoa in DMSO was higher than in PG, but longevity of spermatozoa in different concentrations of PG was higher than in DMSO.

3.4. Sperm motility of different seawater salinity

The activation effects of different salinity seawater from 5‰ to 30‰ were shown in Fig. 5. The motility increased with the rise of salinity. The motility of frozen-thaw sperm activated by seawater of salinity 30‰ was 96.00%, which was significantly higher than all the other salinities. When the salinity was lower than 9‰, the motility was 0 (Fig. 5).

3.5. Fertilization and hatchability

The frozen-thawed and fresh sperm of seven-band grouper were used to hybridize with kelp grouper eggs, and the sperm: egg ratio was 10,000:1. The results (Fig. 6) revealed that the fertilization rate and hatchability rate of eggs inseminated by the frozen-thawed sperm (68.08 ± 22.46% and 76.83 ± 18.31%) were not significantly different with those inseminated by the fresh sperm (69.87 ± 6.05% and 64.33 ± 4.04%). Simultaneously, it also

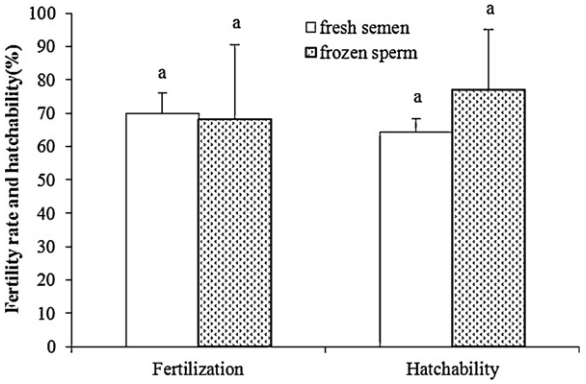

Fig. 6. The fertilization rate and hatchability rate comparison of hybridization fertilization by frozen-thawed sperm and fresh sperm of seven-band grouper *E. septemfasciatus*. There was no significant difference with different letters ($P > 0.05$). The values were expressed as mean ± S.D.

suggested that the seven-band grouper frozen spermatozoa can be applied successfully into the crossbreeding of the seven-band grouper and kelp grouper.

4. Discussion

Many species of the *Epinephelus* are protogynous hermaphrodites, in which a female could transform into a male only when it grows big enough. So the male individual number is a few relatively in a same group. This peculiarity of grouper makes male become quite valuable in cultured populations. Furthermore, the less of male and sperm have limited the artificial insemination to fertilize plentiful eggs of grouper and cultivation. In order to increase the number of male in cultured population, the sustained-release capsules made of methyltestosterone were injected into the fish to reverse the immature individuals into functional males. The measures, for instance, 2.5 mg/kg BW 17α-methyltestosterone (α-MT) in silastic implants injected into immature female in dusky grouper *Epinephelus marginatus* (Cabrita et al., 2009), and 4 mg/kg BW 17α-methyltestosterone (α-MT) injected into immature female in orange-spotted grouper *Epinephelus coioides* (Peatpisut and Bart, 2010), contributed to obtain sex-reversed males and mature sperm, except for the sex instability of the male. The sex control experiments were taken in the Laizhou MingBo Aquatic Products Limited Company with the parallel approach, and eight functional males were acquired. However, sperm was only a few volumes (0.5–1 ml/tail, each time) at sexual maturity stage. Thereby, it is necessary to study further the cryopreservation protocol of seven-band grouper for the sperm utilization.

Miyak et al. has reported that the cryopreservation of kelp grouper sperm with extender consisting of 13–15% trehalose. The 67–94.6% fertilization rate was obtained in 0.5 ml polyethylene straws (Miyaki et al., 2005). The sperm of black sea bass was diluted 1:9 (sperm: extender) in 1% NaCl + 10% DMSO + 10 mg/ml BSA and cryopreserved in 0.5 ml straws (Cabrita et al., 2009). In this study, the sperm from seven-band grouper were cryopreserved in extender, such as MPRS (Ji et al., 2004), TS-2 (Chen et al., 2004) and the extender used to cryopreserve the carp sperm (Kurokura et al., 1984), whereas the survival rates of frozen-thawed sperm were too low to be applied. After the gradient experiment, the extender ES1-3 (60 g/L glucose + 10 g/L NaCl + 0.5 g/L $NaHCO_3$) was chosen due to high motility of frozen-thawed sperm (66.67–76.67%). Furthermore, the cryopreserved sperm volume can reach 1 ml in 2 ml plastic tube, which could apply to abundant cryopreservation with two times of the other *Epinephelus*.

Cryoprotective agent is an indispensible component in sperm cryopreservation solution, and the adaptability of sperm in various fish to different cryoprotective agent is also different. DMSO, Gly (Imaizumi et al., 2005), DMAC (Aoki et al., 1997), high concentration trehalose (Miyaki et al., 2005; Peatpisut and Bart, 2010) and fetal calf serum were used in the other fishes. In this study, DMSO and PG were fit for cryopreservation of seven-band grouper with the survival rate of 68.33–71.67%.

A suitable concentration of cryoprotective agent plays an important part in the cell dehydration, the sustainment sperm osmosis pressure, the protection the membrane structure, and the prevention the cells frostbite. Too high concentration of cryoprotective agent will kill mass sperm, and too low one could not protect sperm at low temperature. The appropriate concentration for sperm cryoprotective agent were screened out in turbot and sea perch with 10% DMSO (Ji et al., 2004; Chen et al., 2004), in spotted halibut with 13.3% DMSO or 13.3%PG (Tian et al., 2008a,b), in kelp grouper with 5% DMSO (Imaizumi et al., 2005), in black grouper with 20% DMSO, and in medaka with 10% DMF (Gwo, 1993). In this study, the optimal concentration of 10%DMSO and 10%PG were fit to cryopreserve the seven-band grouper spermatozoa.

Most of the teleost fish are in vitro fertilization. Many factors of osmotic pressure, ion, CO_2, temperature, salinity, pH value in the external environment will have some impacts on the biological characteristics of sperm such as motility, longevity and fertilization ability (Deng and Lin, 1999). Bull sperm were immotile when the sperm were exposed to hypo-osmotic (<35 mOsm) or hyper-osmotic (>2400 mOsm) TL-Hepes dilution. And the sperm motility reached 90% in 270 to 360 mOsm/L TL-Hepes dilution (Guthrie et al., 2002). No difference was found in the motility of thawed sperm cryopreserved with 14% glycerol and extended in 310 and 500 mOsmol/kg HBSS of green swordtail *Xiphophorus helleri* (Yang et al., 2006). The sperm of seven-band grouper made wider range of adaptation to osmotic pressure solution. The sperm had a certain motility in cryopreservation solution prepared with a series of dilution that osmolality is from 444.60 to 1369.30 mOsm/L. While the motility of cryopreservation and frozen-thawed sperm was highest in ES1-3 (684.40 mOsm/L, 60 g/L glucose + 10 g/L NaCl + 0.5 g/L $NaHCO_3$). ES1-3 dilution was able to activate sperm in a way before freezing, but immediately inhibit sperm movement after freezing. Therefore, the energy of sperm movement will not consume too much. In addition, high concentration glucose can provide certain energy to the sperm movement. Therefore, the sperm motility after cryopreservation has not significantly decreased (Fig. 2). At the same time, it entirely meets the level of application. Sugar, as the impervious antifreeze, is applied to sea perch (Ji et al., 2004), spotted halibut (Tian et al., 2008a,b) and other mammalian sperm cryopreservation, dog spermatozoa with 10 mM glucose or fructose rapidly increased the intracellular content of glucose 6-phosphate and fructose 6-phosphate and intracellular ATP content (Rigau et al., 2002), At the same time, it also can improve sperm motility (Ponglowhapan et al., 2004). The concentration of glucose in ES1-3 reached up to 60 g/L (333.04 mM), and it was the main ingredient in sperm dilution. Although the osmotic pressure of the diluent was greatly improved, the high concentration of the solution was able to reduce the freezing ice point. Thereby, this effectively protected the sperm cells and got the success of cryopreservation of seven-band grouper sperm.

On the other hand, the appropriate sea water temperature and salinity play important roles in improvement of cryopreserved sperm fertilization rate. The suitable salinities were ranging from 19.61 to 24.87 in large Yellow

Croaker (*Pseudosiaena crocea*) (Zhu et al., 2005) and 3 in *Megalobrama hoffmanni* (Pan et al., 1999). In this study, the appropriate salinity was screened from 5 to 30. The results showed that the spermatozoa motility were 96% in salinity 30 and reduced as the salinity decreased.

Kelp grouper distributed in East China sea and South China sea with karyotype 2n = 2st + 46t (Guo et al., 2006) and seven-band grouper distributed in Yellow China sea and East China sea coast with karyotype 2n = 48t (Zhong et al., 2010) both belong to the same genus. The spawning temperature of kelp grouper and seven-band grouper is 20–23 °C and 19–20 °C, respectively. In addition, kelp grouper grows faster than seven-band grouper. Due to the reproductive isolation stemming from different geographical distribution and breeding time between the two kinds of groupers, natural hybrid phenomenon cannot occur in the natural environment. In this study, we have successfully inseminated kelp grouper by the frozen-thawed seven-band grouper sperm with the same fertilization and hatchability rate as fresh sperm. This proves that the cryopreservation technique of seven-band grouper has not only reached the application level, but also explored a new way to break through the reproductive isolation between species.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (30972244) and State 863 High-Technology R&D Project of China (2012AA10A402) and Project of Shan-dong (2009ZHZX1A1201) and Taishan scholar project Fund of Shan-dong province.

References

Aoki, K., Okamoto, M., Tatsumi, K., Ishikawa, Y., 1997. Cryopreservation of medaka spermatozoa. Zoolog. Sci. 14 (4), 641–644.

Cabrita, E., Engrola, S., Conceição, L.E.C., Pousão-Ferreira, P., Dinis, M.T., 2009. Successful cryopreservation of sperm from sex-reversed dusky grouper *Epinephelus marginatus*. Aquaculture 287, 152–157.

Chao, N.H., Tsai, H.P., Liao, I.C., 1992. Short and long-term cryopreservation and sperm suspension of the grouper, *Epinephelus malabaricus*. Asian Fish. Sci. 5, 103–116.

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.

Chen, S.L., Tian, Y.S., Yang, J.F., Shao, C.W., Ji, X.S., Zhai, J.M., Liao, X.L., Zhuang, Z.M., Su, P.Z., Xu, J.Y., Sha, Z.X., Wu, P.F., Wang, N., 2009. Artificial gynogenesis and sex determination in half-smooth tongue sole (*Cynoglossus semilaevis*). Mar. Biotechnol. 11, 243–251.

Deng, Y.S., Lin, H.R., 1999. Advance in fishes sperm motility. Life Sci. Res. 3 (4), 271–278.

Glamuzina, B., Glavic, V., Kozul, V., Skaramuca, B., 1998. Induced sex reversal of the dusky grouper, *Epinephelus marginatus* (Lowe, 1834). Aquacult. Res. 29 (8), 563–568.

Guo, F., Wang, J., Su, Y.Q., Wang, D.X., Xu, L.N., 2006. Study on the karpyotype of *Epinephelus moara*. Mar. Sci. 30 (8), 1–3.

Guthrie, H.D., Liu, J., Critser, J.K., 2002. Osmotic tolerance limits and effects of cryoprotectants on motility of bovine spermatozoa. Biol. Reprod. 67, 1811–1816.

Gwo, J.C., 1993. Cryopreservation of black grouper *Epinephelus malabaricus* spermatozoa. Theriogenology 39, 1331–1342.

Huang, X.R., Zhang, L.Z., Qiao, Z.G., Zhuang, P., Zhang, T., Feng, G.P., Zhao, F., 2007. The effects of different cryoprotectants on the sperm motility and themovement time of Japanese eel (*Anguilla japonica*). Mar. Fish. 29 (3), 193–200.

Imaizumi, H., Hotta, T., Ohta, H., 2005. Cryopreservation of kelp grouper *Epinephelus bruneus* sperm and comparison of fertility of fresh and cryopreserved sperm. Aquacult. Sci. 53 (4), 405–411.

Ji, X.S., Chen, S.L., Tian, Y.S., Yu, G.C., Sha, Z.X., Xu, M.Y., Zhang, S.C., 2004. Cryopreservation of sea perch (*Lateolabrax japonicus*) spermatozoa and feasibility for production-scale fertilization. Aquaculture 241, 517–528.

Kline, R.J., Khan, I.A., Soyano, K., Takushima, M., 2008. Role of follicle-stimulating hormone and androgens on the sexual inversion of seven-band grouper *Epinephelus septemfasciatus*. North Am. J. Aquacult. 70, 266–272.

Koh, I.C.C., Yokoi, K., Tsuji, M., Tsuchihashi, Y., Ohta, H., 2010. Cryopreservation of sperm from seven-band grouper, *Epinephelus septemfasciatus*. Cryobiology 61, 263–267.

Kurokura, H., Hirano, R., Tomita, M., Iwahashi, M., 1984. Cryopreservation of carp sperm. Aquaculture 37, 267–273.

Liu, X.F., Zhuang, Z.M., Meng, Z., Lei, J.L., Yang, Z., 2010. Progress of artificial breeding technique for seven-band grouper *Epinephelus septemfasciatus*. J. Fish. Sci. China 17 (5), 1128–1137.

Meng, Q.W., Su, J.X., Miao, X.Z., 1995. Systema Tics of Fish. China Agriculture Press, Beijing, China, pp. 606–622.

Miyaki, K., Nakano, S., Ohta, H., Kurokura, H., 2005. Cryopreservation of kelp grouper *Epinephelus moara* sperm using only a trehalose solution. Fish. Sci. 71, 457–458.

Ou, Y.J., Liao, G.Y., Chen, C., Li, J.E., Zhuang, Z.M., Liu, X.Z., Yang, Z., Sun, T., 2011. Relation of environmental factor and spermatozoa vitality of *Epinephelus septemfasciatus*. Mar. Environ. Sci. 30 (4), 516–519.

Pan, D.B., Xu, S.Y., Ye, X., Xie, G., Pang, S.X., Qi, B.L., Su, Z.F., 1999. Study on the main biological characteristics of sperm of *Megalobrama hoffmann*. J. Fish. Sci. China 6 (4), 111–113.

Peatpisut, T., Bart, A.N., 2010. Cryopreservation of sperm from natural and sex-reversed orange-spotted grouper (*Epinephelus coioides*). Aquacult. Res. 42, 22–30.

Ponglowhapan, S., Esse'n-Gustavsson, B., Forsberg, C.L., 2004. Influence of glucose and fructose in the extender during long-term storage of chilled canine semen. Theriogenology 62, 1498–1517.

Rigau, T., Rivera, M., Palomo, M.J., Fernández-Novell, J.M., Mogas, T., Ballester, J., Peña, A., Otaegui, P.J., Guinovart, J.J., Rodríguez-Gil, J.E., 2002. Differential effects of glucose and fructose on hexose metabolism in dog spermatozoa. Reproduction 123 (4), 579–591.

Sabate, S., Sakakura, Y., Shiozaki, M., Hagiwara, A., 2009. Onset and development of aggressive behaviour in the early life stages of the seven-band grouper *Epinephelus septemfasciatus*. Aquaculture 290, 97–103.

Sarter, K., Papadakim, M., Zanuy, S., Mylonas, C.C., 2006. Permanent sex inversion in 1-year-old juveniles of the protogynous dusky grouper (*Epinephelus marginatus*) using controlled-release 17α-methyltestosterone implants. Aquaculture 256, 443–456.

Tian, Y.S., Chen, S.L., Ji, X.S., Sun, L.J., Zhai, J.M., 2009. Cryopreservation of tongue sole *Cynoglossus semilaevis* sperm. Prog. Fish. Sci. 30 (6), 96–102.

Tian, Y.S., Chen, S.L., Ji, X.S., Zhai, J.M., Sun, L.J., Chen, C., Su, P.Z., 2008a. Cryopreservation of spotted halibut (*Verasper variegatus*) sperm. Aquaculture 284, 268–271.

Tian, Y.S., Chen, S.L., Liu, B.W., Wang, B., 2006. Embryonic and postembryonic development of hybrid produced with frozen sperms of *Paralichthys dentatus* and eggs of *Paralichthys olivaceus*. J. Fish. China 30 (4), 433–444.

Tian, Y.S., Chen, S.L., Shao, C.W., Liu, B.W., Zhuang, Z.M., 2008b. Embryonic development of *Cynoglossus semilaevis* induced by frozen sperm of *Lateolabrax japonicas*. Mar. Fish. Res. 29 (2), 1–9.

Wang, H.S., 1997. The problems and situation in the artificial propagation of Grouper, *Epinephelus*. J. Dalian Fish. Univ. 12 (3), 44–51.

Yang, H., Hazlewood, L., Walter, R.B., Tiersch, T.R., 2006. Effect of osmotic immobilization on refrigerated storage and cryopreservation of sperm from a viviparous fish, the green swordtail *Xiphophorus helleri*. Cryobiology 52 (2), 209–218.

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 (*Paralichthys olivaceus*) sperm with a practical methodology. Theriogenology 60, 989–996.

Zhong, S.P., Chen, C., Wang, J., Liu, Z.H., Liu, S.F., Zhu, L., Zhuang, Z.M., 2010. Chromosome karyotype of seven-band grouper *Epinephelus septemfasciatus* (Thunberg, 1793). J. Fish. Sci. China 17 (1), 150–156.

Zhu, D.F., Cheng, Y.X., Wang, C.L., Xue, L.Y., 2005. Effects of environmental factors on the vitality of sperm in large Yellow Croaker *Pseudosiaena crocea*. Fish. Sci. 24 (12), 4–6.