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*

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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.

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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