Theriogenology 83 (2015) 703–710

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Summer flounder (*Paralichthys dentatus*) sperm cryopreservation and application in interspecific hybridization with olive flounder (*P olivaceus*)

Q.H. Liu [a,b], D.Y. Ma [a], S.H. Xu [a], Z.Z. Xiao [a], Y.S. Xiao [a], Z.C. Song [c], J. Li [a,b,*]

[a] Center of Biotechnology R&D, Institute of Oceanology, Chinese Academy of Sciences, Qingdao, PR China
[b] National & Local Joint Engineering Laboratory of Ecological Mariculture, Chinese Academy of Sciences, Qingdao, PR China
[c] Weihai Shenghang Aquatic Product Science and Technology Co. Ltd., Weihai, PR China

ARTICLE INFO

Article history:
Received 22 April 2014
Received in revised form 3 November 2014
Accepted 3 November 2014

Keywords:
Summer flounder
Sperm
Cryopreservation
Interspecific hybridization
Olive flounder

ABSTRACT

The present study established an efficient technology for summer flounder (*Paralichthys dentatus*) sperm cryopreservation, and successfully applied the cryopreserved sperm into interspecific hybridization with olive flounder (*P olivaceus*). The best motility of postthaw sperm (78.00 ± 4.70% and 76.60 ± 7.90%), fertilization rates (95.70 ± 3.60% and 79.40 ± 5.20%), and hatching rates (93.10 ± 4.00% and 77.20 ± 2.90%) were achieved when using propylene glycol or DMSO as cryoprotectant. Furthermore, we have successfully improved the cryopreservation method from using 2-mL cryotube to 5-mL cryotube, and the dilution ratio has been increased to 4:1. By this method, the cryopreservation efficiency has been improved to 30 times of that with routine method. Computer-assisted sperm motion analysis showed the freezing–thawing process decreased the sperm speed but did not significantly change the sperm movement pattern, and the progressive linear motion still was the dominant movement pattern. The ultrastructural analysis showed 50% to 60% of the spermatozoa had normal morphology, 20% to 30% were slightly damaged, such as swelling or rupture of head, midpiece, and tail region, and 10% to 20% were severely damaged. In the artificial hybridization experiment, high fertilization rates and hatching rates were achieved when using 15% DMSO (95.7 ± 3.6% and 79.4 ± 5.2%, respectively) and 15% propylene glycol (93.1 ± 4.0% and 77.2 ± 2.9%, respectively), with no significant difference in comparison with control (94.7 ± 2.6% and 72.5 ± 6.5%, respectively). In addition, we found the embryos and larvae from postthaw sperm of *P dentatus* developed and grew normally. The results of the present study further validated the safety of the cryopreserved sperm in breeding production by assessing the fertilization capacity, embryo development, and larval growth.

1. Introduction

Cryopreservation is one of the most important methods for germplasm preservation. Sperm cryopreservation provides many benefits such as ease of global germplasm shipping and supply [1], selective breeding and hybridization with desirable characteristics [2,3], and conservation of genetic diversity [4–7]. Furthermore, a frozen sperm bank could maintain a continuous and stable supply of gametes for hatchery seed production or laboratory experimentation [8].

Olive flounder *Paralichthys olivaceus* (Temminck & Schlegel) is one of the most commercially important species along the coastal waters of China, southwest of the Korean Peninsula, and Japan. Over the past years, great progress has been made on the management and chromosome set manipulation of this fish species in

* Corresponding author. Tel./fax: +86 532 82898718.
E-mail address: junli@ms.qdio.ac.cn (J. Li).

0093-691X/$ – see front matter © 2015 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2014.11.004

aquaculture systems [9,10], but limited achievement has been obtained in selective breeding, hybridization, and genetic improvement. Summer flounder, *P dentatus* (Linneaus, 1766), a high-temperature tolerant species, is also a commercially important flatfish, which inhabits coastal waters from Maine to Florida (USA) in the North Atlantic [11,12]. In 2002, *P dentatus* was introduced into China for its advantages such as rapid growth rate, high disease resistance, and high-temperature tolerance in comparison with native cultured species, *P olivaceus* [13].

Interspecific hybridization is an efficient method for genetic improvement and has been widely used in aquaculture. From 2004, hybridization experiments have been performed in our laboratory, and the hybrids of *P dentatus* (♂) × *P olivaceus* (♀) showed improved survival rate, rapid growth, and high thermal tolerance in cage and pond culture, and led to commercial interests by the aquaculture industry [14–17].

However, one difficulty for interspecific hybridization is the synchronization of gamete availability of both sexes for *P olivaceus* (♀) and *P dentatus* (♂). Although off-season spawning can be induced by the manipulation of photoperiod and temperature cycles, the technique is cost intensive. If the cryopreserved sperm is available all year round, the manipulation of the spawning season could be restricted to females. This also enables the hybridization if the males and females are cultured in different locations. Banked sperm of *P dentatus* can be shipped and stored on site where *P olivaceus* females are environmentally conditioned to spawn year round in hatcheries.

The aims of the present study were to (1) establish an efficient technology for *P dentatus* sperm cryopreservation and improve the cryopreservation method in large-volume cryotube and high sperm concentration for commercial application in aquaculture; (2) analyze the damage of freezing and thawing to the spermatozoa during the cryopreservation process by assessing the postthaw sperm physiological characteristics, fertilization capacity, and fine structure changes; and (3) apply the postthaw sperm into hybridization with *P olivaceus* eggs and analyze the embryo development and larvae growth performance.

2. Materials and methods

2.1. Gametes collection

The *P olivaceus* (2.3–4.1 kg) was a local population and cultured in Qingdao hatchery for years. The *P olivaceus* and *P dentatus* (1.3–2.4 kg) were housed in Qingdao and Yantai hatchery at 14 °C to 16 °C and 17 °C to 19 °C, respectively. For sperm cryopreservation, 42 males and 11 females *P dentatus* were cultivated in a 30-m^3 concrete rearing pond with flow-through seawater. Sperm of *P dentatus* were collected into Petri dishes by gently hand stripping the abdomen of the ripe males. Extreme care was taken to avoid contamination of sperm with seawater, blood, urine, and feces. The fresh sperm was activated at a ratio of 1:1000 (v:v) with natural seawater, and the percentage of motile spermatozoa was checked using Nikon-YS-100 light microscope (Nikon Corporation, Tokyo, Japan) at × 250 magnification. Sperm with motility greater than 85% were kept on crushed ice and transported to the laboratory for further use (2–3 hours). The eggs of *P olivaceus* were collected by abdominal massage of the females at the time of ovulation. Good eggs were slightly yellowish, translucent, and round. The collected eggs were stored at room temperature (19 °C ± 1 °C). The artificial insemination experiments were performed as soon as the eggs were obtained, and the mixture of eggs and sperm were finished in 5 to 10 minutes.

2.2. Sperm cryopreservation

The collected sperm was cryopreserved following the method as described by Liu et al. [18]. Experiment 1, for optimizing cryoprotectant, five cryoprotectants, DMSO, propylene glycol (PG), ethylene glycol (EG), glycerol (GLY), methanol (METH) in different concentrations (5%–20%) in combination with the Hanker extender (NaCl, 7.25 g/L; KCl, 0.38 g/L; $CaCl_2$, 0.18 g/L; $NaHCO_3$, 1.00 g/L; $MgSO_4 \cdot 7H_2O$, 0.23 g/L; $NaH_2PO_4 \cdot H_2O$, 0.41 g/L; and glucose, 1.00 g/L) were used to test their protection effect on the postthaw sperm motility. The sperm were diluted in extender containing cryoprotectant in various concentrations at a 1:3 (v:v) dilution ratio (sperm: [extender + cryoprotectant]). The samples were mixed thoroughly and placed into a 2-mL cryotube. Experiment 2, for optimizing the preservation efficiency, sperm was diluted with 15% PG in various dilution ratio (1:3–4:1), separated loading in a 0.5-mL straw, 2- or 5-mL cryotube for the following freezing experiment (Table 1). Each time of sample collection, sperm from 12 to 20 males were collected and transported to laboratory for cryopreservation experiments.

In all experiments, the samples were frozen and thawed according to the same method: equilibrate for 5 minutes at 0 °C, freeze from 0 °C to −150 °C at a cooling rate of 20 °C/min with a Kryo-360-1.7 programmable freezer (Planer

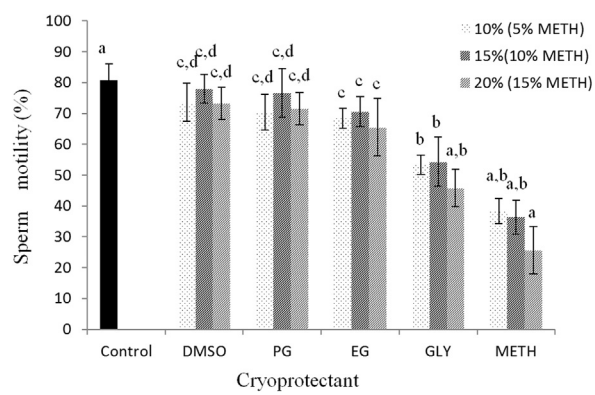

Fig. 1. Effect of various cryoprotectants on postthaw sperm motility in summer flounder (*Paralichthys dentatus*). Sperm were diluted with various cryoprotectant with different concentration at a ratio of 1:3, sucked into a 2-mL cryotube, equilibrated for 5 minutes at 0 °C, frozen from 0 °C to −150 °C at a cooling rate of 20 °C/min, and plunged into liquid nitrogen. Values are mean ± standard deviation. Columns marked with the same superscript letter are not significantly different (n = 3). EG, ethylene glycol; GLY, glycerol; METH, methanol; PG, propylene glycol.