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Sperm cryopreservation protocols for the large-scale fertilization of Japanese eel using a combination of large-volume straws and low sperm dilution ratio

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ABSTRACT

The aim of this study was to develop sperm cryopreservation protocols for large-scale fertilization in Japanese eel (*Anguilla japonica*), by testing the feasibility of combining large-volume straws and a low sperm dilution ratio. Experiments were conducted using both 0.25-ml straws and 2.5- and 5-ml straws (macrotubes) for sperm cryopreservation. Cooling rates in macrotubes were measured at three internal positions and at different heights above the surface of liquid nitrogen (LN). There was no significant difference in the cooling rate among internal positions in the straws. The cooling rate could be adjusted to the appropriate range for Japanese eel sperm (6.3 °C–8.6 °C min^{-1}) by positioning the 2.5- and 5.0-ml straw 0.5–5.0 cm above the surface of LN. We succeeded in scaling up the storage volume from 0.25 to 4.5 ml (18-fold) without decreasing the motility using a 5.0-ml straw. The number of storable motile spermatozoa was the highest at a range of dilution ratios (sperm to extender, *v*/v) from 1:1 to 1:5. Although frozen-thawed sperm showed a significant decrease in the proportion of motile sperm to egg compared with than fresh sperm, there was no significant difference in the fertilization rate, and fertilized eggs with cryopreserved sperm did not affect the hatching rate or survival rate at 7 days after hatching. Using a segmented linear analysis, we found that 10,180 ± 3077 motile spermatozoa per egg are required to maximize fertilization success. We estimated that spermatozoa stored in a 2.5- and 5.0-ml straw can fertilize approximately 184,185 and 331,532 eggs, respectively. Furthermore, our investigation of the effect of long-term storage in LN on the sperm quality showed that the storage period does not affect the post-thaw motility rate even after 2 years of storage. Our results demonstrate that cryopreservation techniques for Japanese eel sperm using 2.5- and 5.0-ml straws could be used for large-scale fertilization programs.

1. Introduction

Japanese eel (*Anguilla japonica*) is one of the most important aquaculture fish species in East Asia. Because seedlings for the eel culture trade completely depend on the capture of wild glass eels in estuaries, a global shortage and rising price of wild glass eel have been serious restricting factors in the eel culture industry. Artificial seedling production technology is required to sustainably develop a commercial-scale, eel culture industry. Because eels never mature naturally under ordinary rearing conditions, the induction of artificial maturation using hormone administration is essential for reproduction in captivity (Ohta et al., 1996; Kagawa et al., 2005; Unuma et al., 2012; Okamura et al., 2014; Tanaka, 2014).

Currently, two methods are used for obtaining fertilized eggs from Japanese eel (Ohta et al., 2017). One is the artificial insemination (AI) method and the other is the spontaneous spawning (SS) method. In the AI method, eggs are collected by gently stripping them from an ovulated female and immediately inseminating them with pre-diluted milt. Conversely, in the SS method, a female and 2–3 males are kept in the same tank after injecting 17α,20β-dihydroxy-4-pregnen-3-one (DHP: a maturation-inducing steroid in eels) or 17α-hydroxyprogesterone (17α-OHP: a precursor of DHP) to induce natural spawning behavior. Fertilization success using the SS method has been reported to be significantly higher than that using the AI method in both Japanese eels

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(Horie et al., 2008) and European eels (*A. anguilla*) (Di Biase et al., 2016). However, for effective induction of SS in eels, it is necessary to synchronize peak maturation in males with the timing of ovulation in females (Ohta et al., 2017); therefore, the SS method is less flexible than the AI method, which uses stored gametes that can be combined at any time. In addition, by combining the AI method with cryopreserved sperm, the potential success of the AI method dramatically increases, enabling a more systematic, efficient, and flexible mating. Therefore, the AI method has advantages over the SS method, particularly for selective breeding programs and inexpensive seeding production. Therefore, it is essential to establish practical sperm cryopreservation techniques and develop their use in fertilizing eggs via the AI method.

Recent advances in the artificial production of glass eels of the Japanese eel have made it possible to produce progeny for genetic studies (Kai et al., 2014; Nomura et al., 2011, Nomura et al., 2006, Nomura et al., 2004 and to apply a selective breeding program to improve production performance. Sperm cryopreservation and its use in fertilization protocols have been developed for both the Japanese eel (Koh et al., 2017; Tanaka et al., 2002) and European eel (Asturiano et al., 2016; Asturiano et al., 2007; Asturiano et al., 2004; Asturiano et al., 2003; Marco-Jiménez et al., 2006; Müller et al., 2004; Peñaranda et al., 2009; Pérez et al., 2009; Szabó et al., 2005). These techniques would help to investigate and improve breeding and culture techniques, promote the exchange of genetic material for use in breeding and genetic studies, and enhance the flexibility in broodstock management and breeding programs (Koh et al., 2017). However, most cryopreservation studies stored semen in small freezing containers, usually 0.25-ml straws [with the exception of a 2-ml plastic, cryogenic vial that was used by Tanaka et al., 2002]. This means that minimal quantities of semen could be stored. Such small quantities of semen are sometimes insufficient for many large-scale applications, such as selective breeding programs, commercial production, and large-scale experiments (Butts et al., 2011).

According to current protocols for inducing maturation in Japanese eel, ripe eggs of up to 300–400 g (approximately 2000 eggs/g) can be obtained from one female. For this many eggs, approximately 1.5–2.0 ml of fresh semen is typically needed for fertilization with AI method. Moreover, an average of > 20 g of semen can be obtained from one rLH-injected male (Ohta et al., 2017). Therefore, developing a cryopreservation protocol for large-volume straws could reduce the time required for packaging sperm and would facilitate handling of gametes during the fertilization process (Cabrita et al., 2001).

Large-volume sperm cryopreservation has been successfully used for several fish species, including yellowtail flounder (Richardson et al., 1999), rainbow trout (Cabrita et al., 2001), turbot (Chen et al., 2004), red seabream (Liu et al., 2006), common carp (Miskolczi and Miha, 2007), wels catfish (Bokor et al., 2009), Atlantic cod (Butts et al., 2011), and Atlantic halibut (Ding et al., 2011). Such techniques used 2- or 5.0-ml freezing containers to increase the number of storable spermatozoa, which has proven to be acceptable and profitable in commercial-scale fertilization. Alternatively, the volume of preserved sperm could be improved by adopting lower dilution ratio standards (Ding et al., 2011).

The aim of this study was to develop sperm cryopreservation protocols for the large-scale fertilization of Japanese eel, using a combination of large-volume straws (2.5 and 5.0 ml) and a low dilution ratio of sperm. In addition, we also investigated the influence of the ratio of eggs to motile spermatozoa on the fertilization success rate and the effect of long-term cryopreservation on sperm quality.

2. Materials and methods

2.1. Ethics statement

This project was conducted in accordance with the Guidelines for Animal Experimentation of the National Research Institute of Aquaculture (NRIA). All animal procedures were approved by the Institutional Animal Care and Use Committee of NRIA.

2.2. Broodstock and gamete collection

Sperm samples were obtained from farmed Japanese eel (n = 35, approximately 200–300 g in BW), which were purchased from 2013 to 2015 from a commercial supplier in Shizuoka Prefecture, Japan. The eels were acclimated to seawater (20 °C) at the NRIA, Mie Prefecture, Japan. Spermiation was induced by weekly injections of rLH (Ark Resource Co. Ltd., Kumamoto, Japan) at a dose of 500 μg/kg BW for 8–14 weeks (Ohta et al., 2017). Milt was collected from each male by applying gentle pressure on the abdomen at 18–24 h after rLH injection. Each milt collection was dispensed into 15 ml tubes and immediately placed on crushed ice until dilution with K30 artificial seminal plasma (K30 ASP) consisting of 134.3 mM NaCl, 30 mM KCl, 20 mM $NaHCO_3$, 1.6 mM $MgCl_2$, and 1.3 mM $CaCl_2$ buffered at pH 8.1 with 20 mM TAPS-NaOH (Ohta et al., 2001).

Egg samples were obtained from a single female (298 g in BW), purchased from a commercial dealer as a wild-caught glass eel. The glass eel was feminized by treatment with estradiol-17β for the first 6 months (Tachiki et al., 1997), thereafter reared for 2 years on normal commercial feed under routine culture conditions in a freshwater tank at the Shibushi laboratory, NRIA, Japan. The female eel was transferred to the NRIA, Mie Prefecture, Japan and acclimated to seawater at 20 °C before being used for the maturation induction experiment. Maturation induction for the female was performed according to Unuma et al. (2012) with slight modification. Briefly, the female was stocked in a circulating tank holding 1000 L of sand-filtered seawater maintained at 17.5 °C and intraperitoneally injected with SPE (20 mg/kg BW) every Friday for 10 weeks. At 9 a.m. on the Monday of the 11th week (3 days after the 10th SPE injection), we assessed the maturational status of the female based on the morphology of lipid droplets in oocytes [lipid droplet stage: Unuma et al., 2011]. When the female was judged to be at maturation stage 4, she was intraperitoneally injected for priming with SPE (20 mg/kg BW) and transferred to a circulating tank holding 1000 L of sand-filtered seawater maintained at 21 °C. At 7 p.m. (10 h after SPE priming), the female was intraperitoneally injected with 17α-OHP (2 mg/kg BW) to induce completion of oocyte maturation and ovulation. The eggs were obtained by gently pressing the abdomen of the ovulated female at 14 h after the 17α-OHP injection. The collected eggs were immediately used for our artificial fertilization experiment.

2.3. Evaluation of sperm motility

We quantified percent motility of the spermatozoa using computer-assisted analysis following the method described by Koh et al. (2017). Milt was diluted 1000-fold with an activating solution (450 mM NaCl, 0.5% BSA, buffered with 20 mM HEPES-NaOH at pH 7.5). Sperm motility was recorded over a 15 ± 5 s period after dilution, using a digital, mini-DV tape recorder (GV-HD700: Sony, Tokyo, Japan). Measurements of sperm motility at 15 s after each dilution were performed in duplicate, and the average result was used in data analysis. Sperm motility (%) was evaluated by analyzing the video images using the CASA system with Image Pro Plus 5.3 (Media Cybernatics Inc., Bethseda, USA) software. To classify a sperm as motile, the CASA settings were as follows: 10 frames s^{-1} for acquisition, 0.3 s of acquisition time, and distance traveled of at least 7 μm.

Results evaluating percent motility were adjusted to the fresh sperm control and presented as comparative post-thaw motility (CPM) (Koh et al., 2017):

$$\mathrm{CPM} = [\text{sperm motility after cryopreservation} / \text{sperm motility of fresh sperm before cryopreservation}] \times 100(\%) \quad (1)$$