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Short communication

Changes in milt volume and sperm quality with time after an injection of recombinant Japanese eel luteinizing hormone in male Japanese eels

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ABSTRACT

Fifteen males of the Japanese eel (9 as the Japanese eel recombinant LH (rLH) group and 6 as the 0.9% NaCl group), which had received 7 weekly injections of rLH (500 μg/kg BW/wk), were used in the experiment. Two days after the 7th injection, milt was collected until the males were spent just before an injection of rLH (500 μg/kg BW) or 0.9% NaCl solution. After the injection, the amount of expressible milt weight and its quality (percentage motility, the velocity of the sperm movement, and sperm density of the milt) were examined every 6 h until 48 h after injection (h AI). In the 0.9% NaCl group, the amount of expressible milt remained small throughout the experimental period. In the rLH group, however, expressible milt increased after 12 h AI and peaked at 18–24 h AI before decreasing thereafter. The total weight of obtainable milt from each male up to 48 h AI was 13.7 times more in the rLH group compared to that of the 0.9% NaCl group. The percentage motility of sperm increased after 6 h AI of rLH and was maintained at high values (> 60%) until 42 h AI. Thus, a large quantity of high-quality milt was transported at 18–24 h AI from the testes to the sperm duct for storage when treated with rLH. Because female eels that have completed egg yolk formation release eggs within 13 to 14 h after the administration of a maturation-inducing steroid (MIS), the administration of rLH as the final shot for males should be given at 6 h before the MIS injection to females for the effective induction of spontaneous spawning.

1. Introduction

The Japanese eel, *Anguilla japonica*, is one of the major aquaculture species in East Asia. Although research for the artificial production of the seedlings for eel aquaculture has been conducted for more than half a century in Japan, its production at commercial aquaculture scale has not yet been successful (Tanaka, 2015). As with other temperate eels, *i.e.* the European eel, *A. anguilla*, and the American eel, *A. rostrata*, the decline in the resource of the Japanese eel has been extreme. Therefore, the development of artificial seedling production technology of the species is an urgent issue.

Because eels do not mature under artificial rearing conditions, promotion of gametogenesis by hormone administration is essential to obtain their gametes (Ohta et al., 1997). Traditionally, salmon pituitary extract (Yamamoto and Yamauchi, 1974) or carp pituitary extract (Boëtius and Boëtius, 1980) has been used for stimulating vitellogenesis in females, whereas human chorionic gonadotropin (hCG) stimulates spermatogenesis and spermiation in males (Ohta et al., 1996a).

Despite numerous studies on the induction of ovulation followed by artificial fertilization, the quality of eggs greatly fluctuated resulting in a wide range of fertilization success (Kagawa et al., 1997; Kagawa, 2003; Unuma et al., 2004). Milt, which is obtained by weekly injections of hCG in males, also shows large individual differences in quality and quantity (Ohta et al., 1997). These fluctuations in gamete quality have been hypothesized to originate from the use of the heterologous gonadotropic hormones from humans, salmon, or carp. Therefore, several researchers considered that this problem could be solved by administration of the homologous gonadotropic hormone(s) to induce normal maturation. For this purpose, Japanese eel recombinant follicle stimulating hormone (rFSH) and luteinizing hormone (rLH) were produced using methylotrophic yeast (Kamei et al., 2003, 2006; Ohta et al., 2007), Drosophila S2 cells (Kazeto et al., 2008), or a baculovirus in silkworm larvae (Kobayashi et al., 2010). These recombinant hormones showed stimulation of GTH receptors and induced spermatogenesis *in vitro* (Kazeto et al., 2008) but had little effect *in vivo* compared to hCG. A possible reason for low biological activities *in vivo* is due to high metabolic clearance of non-sialylated glycoprotein produced by non-vertebrate hosts (see Mylonas et al. (2016) for a

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review).

Recently, Kazeto et al. (2014) succeeded in producing recombinant gonadotropic hormones of the Japanese eel, which were synthesized with cell lines of the Chinese hamster ovary. They showed that the rLH and rFSH provide more effective results than conventional salmon pituitary extract or hCG injections for the induction of oogenesis and spermatogenesis. Production of the recombinant hormones on a commercial basis can be practically achieved in the laboratory of ARK Resource Co. Ltd. (Kumamoto, Japan). The development of a method for inducing maturation using these recombinant GTHs would be important for obtaining good quality gametes.

Currently, two kinds of methods are used for obtaining fertilized eggs in the Japanese eel. One is an artificial insemination method (*e.g.* Ohta et al., 1996b; Unuma et al., 2004), and the other is a spontaneous spawning method in which a female and a few males that received the final shot of steroid hormone and/or hCG are placed in a tank for natural spawning (Dou et al., 2008). Horie et al. (2008) compared the fertilization success between the artificial insemination method and the spontaneous spawning method and reported that the fertilization and hatching in the spontaneous spawning method are significantly higher than those of the artificial insemination method. Recently, Biase et al. (2016) also reported that in the European eel, the fertilization success in the spontaneous spawning method was higher compared to that in the artificial insemination method.

In the spontaneous spawning method, an injection of 17α-OHP or DHP into females is essential for the induction of ovulation in the eel, whereas the necessity of administration of 17α-OHP (Miura et al., 2013) or 17α-OHP plus hCG (Dou et al., 2008; Horie et al., 2008) as a final shot to males is not sufficiently verified. In addition, Miura et al. (2013) reported that when 17α-OHP was administered to male eels as a final shot, the K^+ concentration in the seminal plasma decreased within 24 h, which in turn caused a drastic decrease in the percentage of sperm motility. Although it is not clear at present whether an injection of 17α-OHP as the final shot is necessary or not in male eels, the results suggest that an adequate consideration is needed for the injection in males.

In this paper, we thus examined the effectiveness of the rLH injection as a final shot for males instead of progestins. Furthermore, to clarify the optimum timing for rLH administration to the males, changes in the expressible milt weight and its quality were examined every 6 h until 48 h after injection (h AI).

2. Materials and methods

2.1. Fish and hormone treatment

All the experiments were conducted at the Shibushi Laboratory, National Research Institute of Aquaculture, Japan Fisheries Research Agency and Education. Twenty-nine eels of about 300–400 g body weight were purchased from a commercial eel supplier in Kagoshima Prefecture, Japan. The eels were acclimated to sea water (20 °C) for a period of 5 days. Injection of Japanese eel recombinant luteinizing hormone (rLH) was started on the 13th July 2015. During the hormone treatments, the eels were held in a 400 l flow through the seawater indoor tank under a natural photoperiod. The fish were marked individually by freeze branding (Sorensen et al., 1983). Before each injection and collection of the milt, eels were anesthetized with 2-phenoxyethanol so as not to distress the fish.

Eels were injected intraperitoneally with rLH (Ark Resource Co. Ltd., Kumamoto, Japan; http://www.ark-resource.co.jp/english/) once a week at a dose of 500 μg/kg BW, and their body weight was measured at about 9 a.m. each Monday morning. We confirmed that 0.3 ml of milt could be obtained from 28 out of 29 of the eels just prior to the 7th weekly injection. After the experiments, we verified that the eel from which milt could not be obtained at the 7th injection was female by dissection. Two days after the 7th injection (on Wednesday), we randomly selected 15 males among the 28 spermiated males. The mean body weight of the 15 eels was 365.4 ± 6.8 g ranging from 338 to 411 g. At 11 a.m. (50 h after the 7th injection), the eels were randomly divided into two groups: 9 eels as the rLH injection group and 6 eels as the 0.9% NaCl injection group. Nine male eels of the rLH group received an intraperitoneal injection of rLH at 500 μg/kg BW, while the six male eels of the 0.9% NaCl group received an injection of 0.15 ml 0.9% NaCl. After these injections, males from both groups were immediately transferred to a separate aquarium in which the rearing seawater temperature was set at 22 °C. This temperature was adjusted to the rearing water temperature for males and females that received a final shot for inducing spontaneous spawning (Miura et al., 2013).

2.2. Collection of milt

Just before the injection on Wednesday (initial) and at every 6 h up to 48 h AI, expressible milt was obtained by the application of gentle pressure on the abdomen. Each milt collection was done together by two skilled researchers. The abdomen was pressed using both hands along just below the lateral midline, where the sperm ducts of the male eel exist. Repeated pressure from the front of the sperm duct towards the genital pore and from the back of the duct towards the genital pore was applied so that all milt existing in the duct was obtained. Another researcher put a pre-weighed plastic cup near the genital pore to receive the milt. Care was taken to avoid contamination of the milt with urine. Expressible milt volume was measured by the weight of the plastic cup using an electronic balance.

2.3. Measurement of sperm density

Sperm density of the milt was estimated using a spectrophotometric technique (Ciereszko and Dabrowski, 1993; Butts et al., 2014). By the repeated preliminary experiments, we confirmed that the measured absorbance at 490 nm and the eel sperm density measured on the hemocytometer showed a statistically significant and strong correlation ($r = 0.953$, $P < 3.45 \times 10^{-18}$; Ohta et al., unpublished). Milt was diluted with artificial seminal plasma for eels (Ohta et al., 2001) 400-fold. Absorbance at 490 nm was measured in each sample using a spectrophotometer (a CO7500B Photoelectric colorimeter; Tech-Jam Co., Ltd. Osaka, Japan). Then, the measured absorbance was substituted into the following equation, and sperm density was estimated.

Sperm density (cells/ml) = $(6.637 \times \text{observed value} - 0.602) \times 400 \times 10^7$.

2.4. Measurement of sperm motility

We quantified the percentage motility of the spermatozoa and the velocity of the sperm motility using computer-assisted sperm analysis (CASA) by following the method described by Koh et al. (2017). Milt was diluted 1000-fold with the activating solution (450 mM NaCl and 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 each dilution were performed in duplicate, and the average result was used in the data analyses.

2.5. Statistics

All data are presented as the mean ± SEM. Statistical analysis was performed using Statcel 3 software (The Publisher OMS Ltd., Saitama, Japan). The significance level was set at 0.05. Percentage data were transformed using an arcsine square root transformation before analysis. The normality of the distribution of data was evaluated using the Chi-square test for goodness of fit. The equality of the standard deviation of the groups was assessed using an F-test for equality of variance. Changes in the experimental groups (0.9% NaCl group and rLH group) with elapsed time after injection were tested using repeated