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Effects of continuous administration of human chorionic gonadotropin, salmon pituitary extract, and gonadotropin-releasing hormone using osmotic pumps on induction of sexual maturation in male Japanese eel, *Anguilla japonica*

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ABSTRACT

The purpose of this study was to examine the effects of continuous administration of human chorionic gonadotropin (HCG), salmon pituitary extract (SPE), and gonadotropin-releasing hormone analogue (GnRHa) via an osmotic pump with a long-term sustained hormone-release system on the induction of sexual maturation in male Japanese eels (*Anguilla japonica*). Implantation of a single HCG-loaded osmotic pump induced spermatogenesis and increased the gonadosomatic index (GSI) values at 35–42 days postimplantation; GSI values were higher (10.7 ± 1.0) than those of the fish implanted with a cholesterol pellet containing HCG (2.4 ± 0.6). Implantation of an SPE-loaded osmotic pump also stimulated spermatogenesis, although the GSI values were lower than those of the fish implanted with the HCG-loaded osmotic pump. Implantation of a single osmotic pump loaded with various doses of GnRHa (0.94, 1.86, or 3.75 μg/day) did not stimulate spermatogenesis. The GSI values and milt weight of the HCG-administered eels significantly increased at a dosage of 5 IU/day in a dose-dependent manner up to 50 IU/day. Sperm motility was not significantly different among the eels treated with different doses of HCG. Therefore, implantation of a single osmotic pump loaded with HCG (50 IU/day) is a reliable method for inducing spermatogenesis and spermiation in sexually immature Japanese eels.

1. Introduction

Cultivated male eels are sexually immature in general and are never mature under commercial rearing conditions (Yamamoto et al., 1972). One exceptional report describes spermatogenesis and spermiation of two cultivated male eels which were maintained under natural environmental conditions (Matsubara et al., 2008). It has been observed that repeated injections of salmon pituitary extract (SPE) induce vitellogenesis in female eels (Kagawa, 2003; Kagawa et al., 2005; Ohta et al., 1996a; Yamamoto and Yamauchi, 1974; Yamamoto et al., 1974). Further, human chorionic gonadotropin (HCG) induces spermatogenesis and spermiation in male eels (Ohta et al., 1996b): Repeated injections of HCG at 250 IU/fish (1 IU/g body weight) over 10 weeks efficiently induced spermatogenesis and spermiation, and 1–2 g of approximately 70% motile spermatozoa was obtained. However, weekly injection of HCG requires repetitive handling of the broodstock and substantial labor, time, and monitoring, resulting in stress to and increased mortality of the fish. Miura et al. (1991) reported that a single injection of HCG can induce the complete process of spermatogenesis from the proliferation of spermatogonia to spermiogenesis. However, the males which are induced to maturity by a single injection of HCG produced little milt.

In recent studies on drug delivery systems in fish, a variety of gonadotropin-releasing hormone analogue (GnRHa) delivery systems have been developed for sustained hormonal release. These include cholesterol pellets (Weil and Crim, 1983; Zohar and Mylonas, 2001); microspheres prepared using copolymers of lactic and glycolic acids (LGA; Mylonas et al., 1993; Zohar et al., 1995) and nondegradable copolymers of ethylene and vinyl acetate (EVAc; Mylonas et al., 1998; Zohar, 1996); as well as other copolymer pellets (Hirose et al., 1990; Matsuyama et al., 1993). These hormone delivery systems effectively induce oocyte maturation and ovulation or spermiation in reproductively dysfunctional cultured fish (Zohar and Mylonas, 2001). However, no reports have investigated the effects of long-term (>1 month) sustained-release delivery systems of gonadotropins on sexual maturation in such fish.

An osmotic pump is a delivery device for long-term administration of drugs and hormones. Implantation of a single osmotic pump loaded with a gonadotropic hormone (e.g., pregnant mare serum gonadotropin or HCG) has been carried out experimentally in mammals

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(Gibson et al., 1994; Patton et al., 1990) and birds (Girling et al., 2002; Wakabayashi et al., 1996). However, the use and efficacy of osmotic pumps for inducing sexual maturation have not been studied in teleosts. Therefore, in this study, we examined the effects of HCG, SPE, and GnRHa administration via an osmotic pump which has a long-term sustained hormone release on the induction of sexual maturation in male Japanese eel *Anguilla japonica*.

2. Materials and methods

2.1. Fish and hormonal treatments

Cultured male Japanese eels were obtained from a fish farm or from the Shibushi Station, National Center for Stock Enhancement, Fisheries Research Agency, Japan. After acclimation to seawater, they were maintained without food in indoor 400-L circulating tanks under a natural photoperiod at a water temperature of 20 °C. After anesthetization with 2-phenoxyethanol (Nacali Tesque, Tokyo, Japan), the fish were weighed and then treated with the various hormones.

A newly introduced osmotic pump (Osmotic Pump Type 2002; Alzet Osmotic Pumps Co., Cupertino, CA; diameter = 7 mm, length = 30 mm, reservoir volume = approximately 200 µl) that can release constant amounts of hormones for a long period was loaded with various amounts of GnRHa, HCG, or SPE. According to the manufacturer's instruction manual, this osmotic pump can release 5 µl of a solution per day for approximately 45–50 days when the fish are maintained at a water temperature of 20 °C. We confirmed that the expected volume of hormone solutions is retained in the osmotic pump reservoir at the end of experiment. Moreover, constant HCG release from the osmotic pumps was confirmed by measuring serum levels of HCG during each experiment (data not shown). An osmotic pump was implanted into the peritoneal cavity of each eel after cutting the abdomen with a scalpel approximately 8 mm. The wound was not sutured, but it was healed in 2 weeks.

HCG (Teikoku Zhoki Co. Ltd., Tokyo, Japan) was dissolved in 0.9% sterilized sodium chloride solution. GnRHa, des-Gly^{10}-[D-Ala^{6}]-LH-RH ethylamide (Sigma), was dissolved in 0.9% sodium chloride containing 0.1% bovine serum albumin (BSA), which was added to prevent the adhesion of GnRHa onto the reservoir wall of the osmotic pump. SPE was prepared by homogenizing salmon (*Oncorhynchus keta*) pituitary powder with 0.9% sodium chloride solution, followed by centrifugation at 9700*g* (Kagawa et al., 1995, 1997, 1998). The SPE was concentrated with a concentrator (Vivapore 10/20; Sartorius Stedim Lab, Ltd., Gloucestershire, UK) before loading into the osmotic pumps at a concentration of 22.5, 45, or 90 mg/200 µl. Cholesterol pellets were prepared according to the method applied in our previous study (Kumakura et al., 2003; Matsuyama et al., 1992). The pellets (diameter = 2 mm, length = 6 mm) containing HCG (1500 IU/pellet) were stored at −30 °C until use. Each cholesterol pellet was implanted into the peritoneal cavity by using a 2-mm trocar.

In experiment 1, twenty-four fish (mean body weight = 314.0 ± 3.1 g, range = 281.0–343.0 g) were randomly divided into four groups (n = 6 eels/group): the saline control group, the cholesterol pellet group, the osmotic pump group, and the injection group. HCG was administered by injection (330 IU/week), cholesterol pellets (1500 IU/pellet), and osmotic pump (50 IU/day). The injection group received intraperitoneal injections of HCG once a week (5 times totally). The fish in the cholesterol pellet group and osmotic pump group received a single pellet and a single osmotic pump, respectively, on the first experimental day.

In experiment 2, thirty fish (mean body weight = 232.7 ± 3.9 g, range = 201.3–277.7 g) were randomly divided into six groups (n = 5 eels/group). The eels were implanted with a single osmotic pump loaded with various doses of SPE (0.56, 1.12, or 2.24 mg/day) or with a single osmotic pump loaded with HCG (50 IU/day). The eels were implanted with a single osmotic pump loaded with various doses of SPE (0.56, 1.12, or 2.24 mg/day). The saline control group received a single osmotic pump containing 0.9% sodium chloride. The initial control fish were sampled on the first experimental day.

In experiment 3, thirty-five fish (mean body weight = 234.8 ± 9.2 g, range = 171.3–367.2 g) were randomly divided into seven groups (n = 5 eels/group). The eels were implanted with a single osmotic pump loaded with various doses of GnRHa (0.9, 1.8, or 3.6 µg/day), a single osmotic pump loaded with HCG (50 IU/day), or a single osmotic pump loaded with SPE (2.24 mg/day). The saline control group received a single osmotic pump containing 0.9% sodium chloride. The initial control fish were sampled on the first experimental day.

In experiment 4, forty-eight fish (mean body weight = 336.7 ± 10.3 g, range = 253.0–532.0 g) were randomly divided into six groups (n = 8 eels/group). The eels were implanted with a single osmotic pump loaded with various doses of HCG (1, 5, 10, 25, or 50 IU/day). The saline control group received a single osmotic pump containing 0.9% sodium chloride.

2.2. Sampling

At 35 days postimplantation in experiment 1, and at 42 days postimplantation in experiments 2, 3, and 4, the eels were terminally anesthetized with 2-phenoxyethanol and weighed. Their milt and gonads were sampled, and the gonadosomatic index (GSI), milt weight, and sperm motility were calculated. Survival rates of experimental fish in each experiment were 75% (experiment1), 90% (experiment 2), 100% (experiment 3), and 98% (experiment 4), respectively.

2.3. Measurement of milt weight and spermatocrit

The milt weight and spermatocrit were measured according to a previous report (Ohta et al., 1996b). Briefly, the area around the genital pore was rinsed with distilled water, dried with a soft towel, and milt was collected by using a preweighed Pasteur pipette and small tube. Care was taken to avoid contamination of the milt with seawater or urine. The milt from the treated fish was obtained by application of gentle pressure on the abdomen. The milt weight was calculated from the difference in weight of the Pasteur pipette and small tube before and after measurement. Spermatocrit ([packed cell volume / total milt volume] × 100) values were determined for each milt sample by centrifuging the capillary tubes (12 000 rpm for 15 min).

2.4. Sperm motility

The milt was maintained on crushed ice in a cool box until the assessment of sperm motility. According to the method used in a previous study (Ohta et al., 1996b), 1-µl milt was collected with a micropipette and diluted in 1 ml 450-mM sodium chloride buffered with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and sodium hydroxide (pH 7.6) in 24-well culture plates (Corning Inc., Corning, NY). Immediately after agitation of the diluted milt ~20 µl of the mixture was mounted onto a glass slide with a micropipette and sperm motility was observed under a light microscope. As reported in a previous study (Viveiros et al., 2003), sperm motility was subjectively classified according to the percentage of motile spermatozoa showing forward movement as follows: 0, no movement; 1, up to 25% of the cells are motile; 2, up to 50% of the cells are motile; 3, up to 75% of the cells are motile; and 4, more than 75% of the cells are motile. Measurement of each sample was repeated thrice by three persons and the data were averaged.

2.5. Histology

Parts of the testes obtained at the end of the experiments were fixed in Bouin's solution. Serial 6-µm-thick paraffin sections were stained with Mayer's hematoxylin and eosin.