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Effects of Manchurian Trout Gonadotropins on Sexual Maturation in Female Rainbow Trout

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Manchurian trout (*Brachymystax lenok*) is an endangered fish species in East Asia including the Korean peninsula. To establish a method for artificial propagation and to improve our understanding of the reproduction in the species, we have produced recombinant gonadotropins, follicle-stimulating hormone (r-mtFSH) and luteinizing hormone (r-mtLH), which may play central roles in reproductive activities. In the present study, the biological activities of the recombinant hormones were analyzed by gonadosomatic index (GSI), ovarian follicle diameter, and sex steroid levels in mature rainbow trout (*Oncorhynchus mykiss*). In the 6th day post-injection, FSH-injected fish were slightly decreased in the GSI value, although there were no significant differences among those of control, r-mtFSH, and r-mtLH treatments. Injection of the r-mtFSH increased follicle diameters significantly as compared with those of control- and r-mtLH-injected fish. The plasma steroid levels showed wide differences in the groups at 1, 3, or 6th day post-injection. Despite the variable steroid levels, three individuals receiving either r-mtFSH or r-mtLH showed a great increase in a maturation-inducing steroid, 17α,20β-dihydroxy-4-pregnen-3-one, at 3 and 6 days. Taken together, these results suggest that biological efficacies of the recombinant FSH and LH should be further studied in the Manchurian trout.

Keywords: Gonadotropin, Maturation, Sex steroid, Rainbow trout, *Oncorhynchus mykiss*

Introduction

Manchurian trout (*Brachymystax lenok*) is a cold water fish classified in Salmonidae family. This species lives in fresh water of clean upper streams in the East Asia, mainly Korea, North China, and Siberia (Chyung et al., 1977; Reshetnikov et al., 1997). In Korea, populations of this species are dramatically declining and the habitat of Manchurian trout was designated to Natural Monument by government. Therefore, in order to increase the population resources it is essential to develop methods for an artificial propagation of Manchurian trout seed.

A control of gonadal maturity of broodstock fish is an important process in aquaculture as well as in artificial propagation of endangered fish species, and a stable supply of seedling is critical in fish culture (Sullivan et al., 2003). However, in some species, gonadal maturation, ovulation or spermiation does not occur in in-door state because of an inappropriate environment for reproductive progress. For the artificial control of reproductive activity of fish, manipulation of environmental factors, such as photoperiod and water temperature and/or hormonal treatments have been employed depending on the reproductive stages of many species (Zohar and Mylonas, 2001; Pankhurst and Porter, 2003). As in other vertebrates, the reproductive activity of fish is regulated by the brain-pituitary-gonad axis. Gonadotropin-releasing hormone (GnRH) produced in the brain stimulates secretion of two types of gonadotropins (GTHs), follicle-stimulating hormone (FSH; formerly termed GTH-I) and luteinizing hormone (LH; formerly GTH-II) in the pituitary gland, and FSH and LH act on the gonads to produce sex steroids that regulate gonadal development (Suzuki et al., 1988a,b; Swanson et al., 2003). In general, FSH is considered to regulate early phases of gametogenesis, such as vitellogenesis and spermatogenesis, whereas LH is responsible for the final maturation processes, such as oocyte maturation, ovulation and spermiation (Yaron et al., 2003). To mimic the effects of these endogenous hormones, synthetic GnRH peptide or extract of

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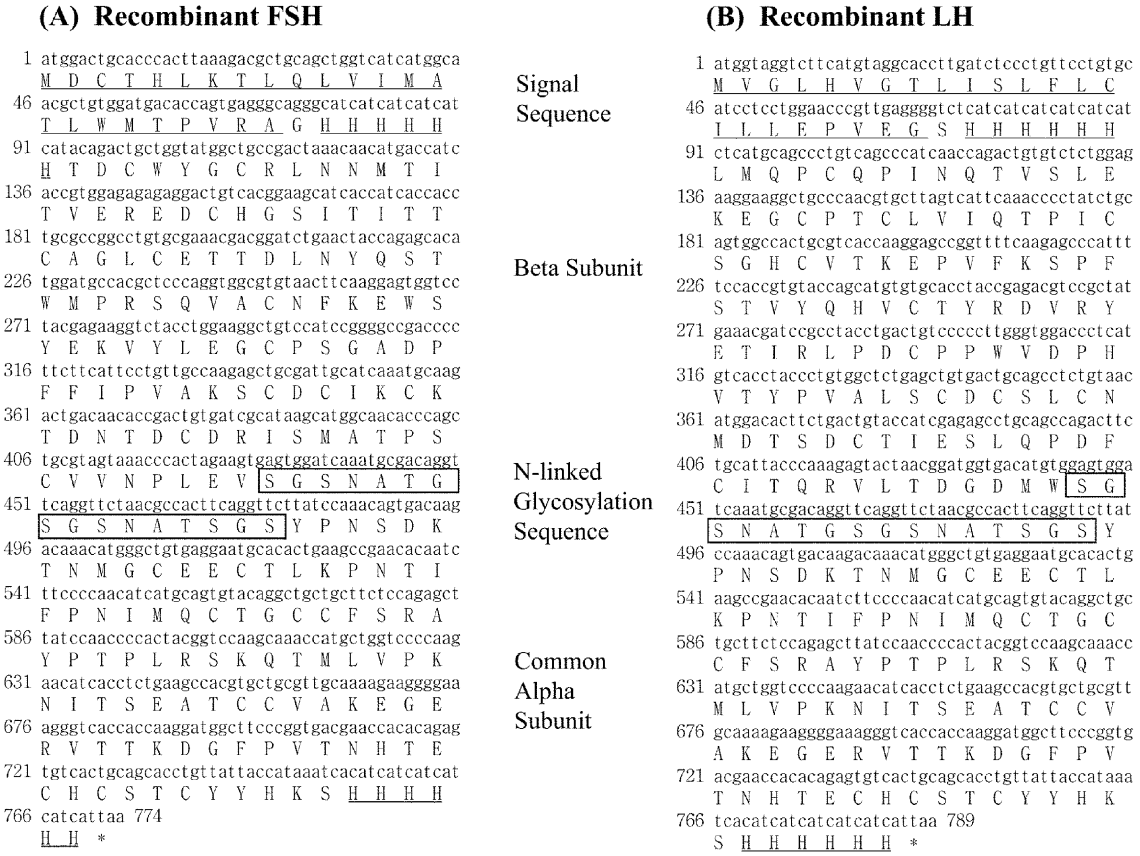

Fig. 1. Nucleotide and deduced amino acid sequences of the recombinant Manchurian trout follicle-stimulating hormone (r-mtFSH) (A) and Luteinizing hormone (r-mtLH) (B). Amino acids are given in single letters under the cDNA sequences, and signal peptides and histidineX6 sequences are underlined. The putative N-linked glycosylation site and the stop codon are indicated by the box and the asterisk.

pituitary gland has been administered to fish to accelerate gonadal development and sexual maturation (Patino, 1997; Zohar and Mylonas, 2001). In spite of some differences depending on fish species, both FSH and LH stimulated *in vitro* production of estradiol-17β by vitellogenic ovarian tissues from chum salmon (*Oncorhynchus keta*), coho salmon (*O. kisutch*), goldfish (*Carassius auratus*), tuna (*Thunnus obesus*) and red seabream (*Pagrus major*) (Suzuki et al., 1988a,b; Van Der Kraak et al., 1992; Tanaka et al., 1993; Okada et al., 1994; Planas et al., 2000). In contrast, LH stimulates *in vitro* production of the maturation-inducing steroid, 17α,20β-dihydroxy-4-pregnen-3-one, by the mature follicles of salmonids (Suzuki et al., 1988b; Planas et al., 2000). However, the uses of purified FSH and LH are limited to assess unique biological function of FSH and LH due to contamination of either FSH or LH in hormone preparation from the pituitaries, although administration of pituitary extract has been successfully conducted for a long time for the induction of gonadal maturation and spawning in some species. Human chorionic GTH (hCG) also has been used as a hormone expecting LH effect in aquaculture species (Patino, 1997; Zohar and Mylonas, 2001), but a useful counterpart for fish FSH is not available in present.

As a first step toward an establishment of artificial propagation method for the Manchurian trout, we produced recombinant Manchurian trout FSH and LH from Chinese hamster ovary (CHO) cells (Choi et al., 2005) and a baculovirus-silkworm larvae system (Ko et al., 2007). However, the amount of GTHs produced in the CHO cell culture system is small, and the produced GTHs were basically for *in vitro* physiological studies. In the present study, the FSH and LH produced by the silkworm larvae system were analyzed with respect to their effects on gonadal weight gain, follicle diameter, and sex steroid production in mature rainbow trout *in vivo*.

Materials and Methods

Production of recombinant FSH and LH

Open reading frames (ORF) of the Manchurian trout FSH and LH were amplified by polymerase chain reaction (PCR)

using previously cloned cDNAs for the Manchurian trout FSH and LH subunits (Choi et al., 2005). The strategy for constructions of the tethered single-chain mtFSHβ/α and mtLHβ/α was reported in a previous paper (Ko et al., 2007). Briefly, the ORF regions of mature β subunits of the hormones and common GTH were generated by overlapping PCR method. Both constructs include hexahistidinyl peptides (His-Tag) in the N- and C-terminals, and a synthetic *N*-linked glycosylation sequence (Fig. 1). Recombinant Manchurian trout FSH (rmtFSH) and LH (r-mtLH) were produced in a silkworm system as previously described (Kobayashi et al., 2006; Ko et al., 2007).

Protein purification

Histidine-tagged recombinant hormones were purified by Ni-NTA agarose beads (Qiagen, Valencia, CA, USA) as described in previous reports (Choi et al., 2005; Ko et al., 2007). Briefly, hemolymph supernatant was incubated with the Ni-NTA beads on a shaker at 4°C for 2-3 h. The beads were packaged and washed with 10 mL of a washing buffer twice. The Ni-affinity proteins were then eluted with an elution buffer including 500 mM imidazole. To remove excessive salt and imidazole, the eluted samples were centrifuged at 4°C for 30 min with a centrifugal filter device (Amicon 10,000 MWCO, Millipore Corp. Bedford, MA, USA). Protein concentration was measured by a spectrophotometer (Ultrospec3100pro™, Amersham Biosciences, Piscataway, NJ, USA) at 595 nm using a Bradford reagent (Sigma-Aldrich, St. Louis, MO, USA).

Effects of recombinant FSH and LH on rainbow trout

In November 2005, 2-year-old mature female rainbow trout (average body weight (BW), 337±31 g; average gonadosomatic index (GSI: gonad weight X 100/BW), 15.62±0.69), which were reared in fresh water of a race-way tank (Yeongdong Inland Fisheries Research Institute, Yang-yang), were anesthetized with 2-phenoxyethanol (0.5 mL/L) and randomly assigned to one of three treatment groups (N=13-14 per group): hemolymph-derived Ni-affinity proteins (negative control), r-mtFSH, or r-mtLH. All hormones were diluted to 80 μg/ml using PBS containing bovine serum albumin (1 mg/mL). Hormone was administered as a single intraperitoneal (i.p.) injection at a dose of 10 μg/100 g BW. Blood samples were collected from the fish after the injection 1, 3, or 6 day, taken from the caudal vasculature with a heparinized syringe and needle after anesthetization with 2-phenoxyethanol. Blood samples were centrifuged 4 at 4000 *g* and plasma was stored at -80°C until radioimmunoassay (RIA). The fish were sacrificed 6 days post-injection by decapitation and body as well as gonad weights were recorded. In addition, ovarian follicles from each fish were selected on a microscope in order of larger size (N=10) and the diameter was measured.

Radioimmunoassay (RIA)

Concentrations of testosterone, estradiol-17β and 17α,20β-dihydroxy-4-pregnen-3-one in blood plasma were measured by RIA as previously described (Aida et al., 1984; Kobayashi et al., 1988; Kim et al., 2006; Ko et al., 2007).

Statistics

All data were expressed as the mean ± SEM. Statistical significance was determined by one way ANOVA followed by Duncan's multiple-range test ($P<0.05$).

Results and Discussion

Production of recombinant Manchurian trout GTHs

To obtain the Manchurian trout GTHs stably without sacrificing of the endangered fish, we produced recombinant Manchurian trout GTHs using a baculovirus-silkworm larvae system. The sequence analysis of the FSH and LH constructs and deduced amino acid sequences are shown in Fig. 1. Between the hormone-specific β subunit and common GTHα subunit, 16 amino acids (*N*-linked glycosylation sequence) were introduced as a spacer to mimic natural β-α complex (Swanson et al., 2003). Also, the *N*-linked glycosylation sequence includes two additional glycosylation sites which may play as a long-acting agonist than native FSH (Klein et al., 2003). In an examination on the characteristics of the two recombinant hormones, the proteins were heavily glycosylated (Ko et al., 2007). In the present study, specific bands corresponding to molecular sizes of approximately 35 kDa for r-mtFSH and 38 kDa for r-mtLH were shown in the Ni-affinity fraction from hemolymph of infected silkworm larvae. Previously, we have confirmed that the 35 and 38 kDa molecules are the Manchurian trout FSH and LH respectively by Western blot analyses using GTH and His-Tag antibodies (Ko et al., 2007). Using the purified recombinant FSH and LH, we studied on gonad weight gain, sex steroid levels in mature rainbow trout.

Effects of FSH and LH on GSI and follicle diameter

Average gonadosomatic indexes (GSI) and follicle diame-

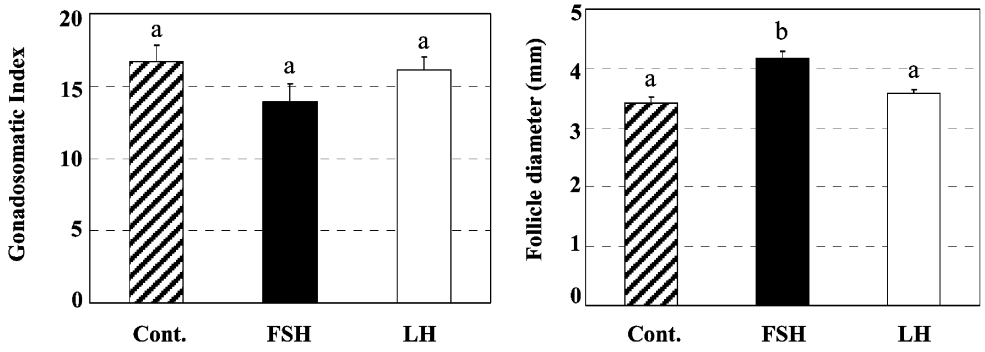

Fig. 2. *In vivo* effects of the Manchurian trout FSH (r-mtFSH) and LH (r-mtLH) on gonadosomatic index (GSI) and follicle diameter in mature female rainbow trout. The r-mtFSH or r-mtLH was administered as a single intraperitoneal (i.p.) injection at a does of 10 μg/100 g body weight. After 6 days post-injection, the ovaries were weight for GSI calculation and follicle diameters were measured by a light microscope. Values sharing the same letter do not differ significantly ($p<0.05$).

ter following a single injection of control protein, r-mtFSH, or r-mtLH in mature female rainbow trout are shown in Fig. 2. We have injected r-mtFSH or r-mtLH intraperitoneally and measured GSI and ovarian follicles at 6 days post-injection. There were no significant changes in GSI values among the three groups. In addition, no ovulated females were observed during the experiment. Whereas it showed no significant effects of recombinant FSH and LH on ovarian weight gain and ovulation, the r-mtFSH significantly increased follicular diameters in mature rainbow trout ovary ($P<0.05$). In female fishes, FSH may control early phases of gametogenesis, i.e., vitellogenesis, whereas LH of fish is responsible for the final maturation processes, such as oocyte maturation and ovulation (Swanson et al., 2003; Yaron et al. 2003). In the present study, it is unclear whether the recombinant hormones are effective for the ovary of the mature fish examined, since the FSH increased in follicle diameters in the fish. In a previous study, we observed that same dose of the r-mtFSH increases both GSI and follicle diameters in immature rainbow trout ovary and these phenomena are mainly due to uptake of vitellogenin to ovarian follicles at three days after injection (Ko et al., 2007). These results suggested that FSH is still involved in vitellogenin uptake in the rainbow trout used. Meanwhile, it is also speculated that one dose of the r-mtLH treatment was not enough to induce final maturation *in vivo*, since the recombinant LH specifically recognized LH receptor and stimulated sex steroid production from full-grown follicles *in vitro* (Ko et al., 2007).

Effects of FSH and LH on plasma sex steroid levels

Figure 3 shows changes in plasma sex steroid levels in the control-, r-mtFSH-, or r-mtLH-injected fish at 1, 3, or 6th day post-injection. Since the plasma steroid levels of testosterone (T), estradiol-17β (E_2), and 17α,20β-dihydroxy-4-pregnen-3-one (DHP) greatly fluctuated in each individual throughout all sampling points, it made difficult to analyze effects of the r-mtFSH and r-mtLH on sex steroid production *in vivo*. On the other hand, two individuals in the r-mtFSH-injected group (labeled with (1) and (2) on the lines) and one fish receiving r-mtLH (labeled with (3) on the line) showed great increases in a maturation-inducing steroid, DHP, at 3 or 6th days post-injection. While DHP was increased in the samples, E2 showed a tendency of decreasing in the same individuals. These results suggest that the fish showing increase of DHP and decrease of E2 levels were entering into final maturation stages of ovaries, since a distinct shift in the steroidogenesis from E2 to DHP is well documented in sexual maturation process of salmonids and it occurs in the ovarian follicles immediately prior to oocyte maturation by dramatic changes in various steroidogenic enzymes (Nagahama et al., 1993). In our previous report, we observed that both FSH and LH have biological potencies to produce steroid hormones in rainbow trout maturing follicles *in vitro*; in full-grown follicles from a mature female (GSI=17.9), the production of E2 and T were significantly stimulated by the Manchurian trout FSH and LH, respectively at lower doses, whereas E2 and T increased by maturing follicles (GSI=12.6) in response to both recombinant hormones at higher doses (Ko et al., 2007). Thus, this discrepancy between *in vitro* and *in vivo* may be due to different stages of sexual maturation of fish used in the experiments, doses of hormones, and/or duration of hormonal treatment. In a subsequent work, it may be necessary to study the biological activities of the recombinant FSH and LH in synchronized mature individuals. In summary, the

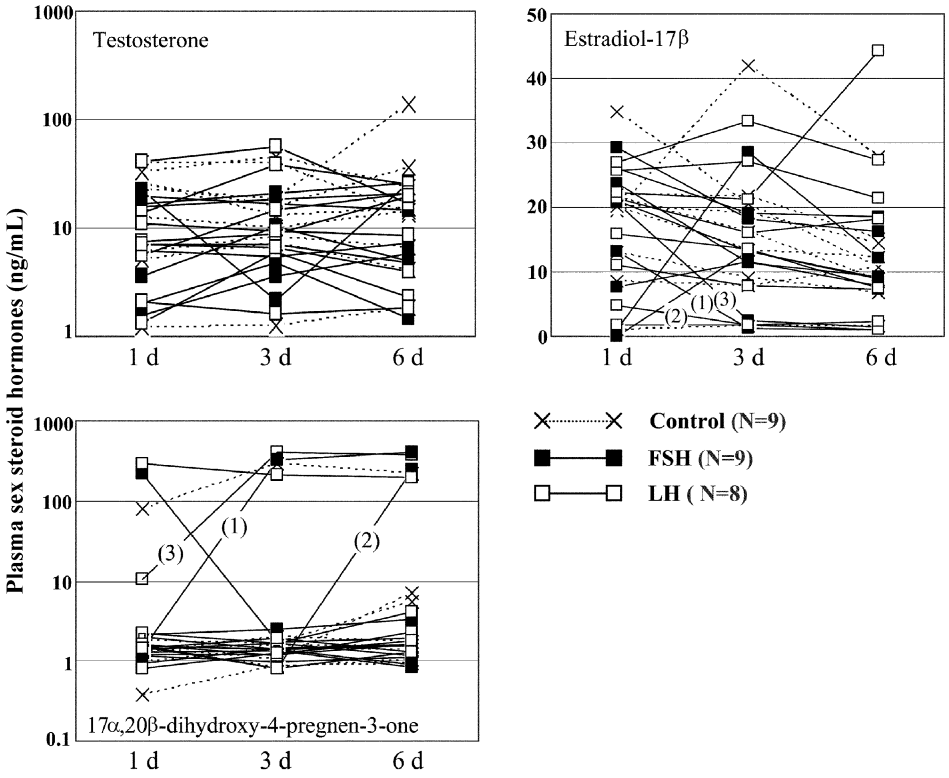

Fig. 3. *In vivo* effects of the Manchurian trout FSH (r-mtFSH) and LH (r-mtLH) on plasma sex steroid levels in mature female rainbow trout. Blood samples of each individual were collected at 1, 3, and 6 days post-injection. Plasma sex steroid levels were measured by radioimmunoassay (RIA) as described in Material and Methods.

recombinant Manchurian trout FSH-injected fish showed slightly decrease of gonadosomatic index and increase of follicle diameters as compared with those of control- and LH-injected fish. Despite of the variable plasma steroid levels, three individuals receiving r-mtFSH and r-mtLH showed a great increase of a maturation-inducing steroid, 17α,20β-dihydroxy-4-pregnen-3-one, whereas estradiol-17β levels decreased in the same samples. In order to establish a method for artificial propagation of the Manchurian trout, effects of gonadotropic hormones on sexual maturation in the salmonids should be further investigated.

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