Effects of letrozole, non-steroidal aromatase inhibitor, on serum sex steroid levels, oocyte diameter and gonadosomatic index (GSI) in rainbow trout *(Oncorhynchus mykiss)*

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Abstract

The objective of this study was to contribute to investigate the efficacy of letrozole, a potent non-steroidal aromatase inhibitor, on serum sex steroid levels and oocyte growth in rainbow trout females. Serum steroid levels were measured using an indirect enzyme-linked immunosorbent assay (ELISA) before and 22 days after an initial injection with 1, 2.5 mg kg^{-1} and subsequent weekly injection with 2.5 mg kg^{-1}letrozole. After the last blood collection, gonad tissues were removed and oocyte diameter and GSI were determined. In all groups treated with letrozole, serum 17β- esteradiol levels (E2) decreased significantly and inversely serum 17α-20ß dihydroxy-4 pregnen- 3- one (17α-20ß-P), testosterone(T) levels increased significantly from 6 to 96 h after injection (p<0.05). The lowest E2 levels, oocyte diameter and GSI were measured in the group treated weekly with 2.5 mg kg^{-1} letrozole (p<0.05). This study demonstrates that letrozole has the potential to interfere in the synthesis of endogenous estrogens from androgens and delay gonadal development.

Keywords: Aromatase inhibitor, Sex steroid, GSI, *Oncorhynchus mykiss*

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Introduction

The gonads in teleost fishes are stimulated by gonadotropic (GTH), follicle stimulating (FSH) and luteinizing (LH) hormones to produce steroid hormones and germ cell (Yaron *et al*., 2003; Amini *et al*., 2012). Some androgens, as testosterone (T), can be aromatized to estrogens (Afonso *et al*., 2000; Ankley *et al*., 2002). Aromatase is a member of the P450 cytochrome super family of enzymes that catalyzes the formation of estrogen from androgen. Estrogens, particularly 17β-estradiol (E2), are sex steroids implicated in many crucial processes in vertebrates, including development, growth and reproduction (Haynes *et al*., 2003; Sun *et al*., 2007). As such, the first clinical aromatase inhibitor, aminoglutethimide, was developed for the treatment of advanced breast cancer around 25 years ago (Howell and Buzdar, 2005). Recently non- steroidal competitive inhibitors letrozole and anastrozole are many times more specific and potent than aminoglutethimide (Seralini and Moslemi, 2001; Haynes *et al*., 2003). The potential of Aromatase inhibitors (AIs) for blocking estrogen biosynthesis has been demonstrated in both invivo and invitro studies on mammals (Selvaraj *et al*., 1994; Bajetta *et al*., 2000). Letrozole (CGS 20267) is one of the most potent aromatase inhibitors yet developed (Smith, 1999).

It has potential for use both to prevent the conversion of androgenic steroids to estrogens and to prevent or diminish the side effects of androgenic steroid abuse (Haynes *et al*., 2003). Aromatase inhibitors can enter aquatic systems and cause ecotoxicological effects. The effects of aromatase inhibitors have been shown in aquaculture to sex differentiation and reproduction of fish (Piferrer *et al*., 1994; Afonso *et al*., 1999,2000; Ankley *et al*., 2002; LI *et al*., 2005).

The high activity of aromatase in teleost suggests that aromatase enzyme constitutes a potential target for endocrine disrupting chemicals (EDC_S) (Hinfray *et al*., 2006). Association between decreased brain aromatase activity, circulating E2 levels and ovarian somatic index in females of perch (*Perca fluviatilis*) was reported (Noaksson *et al*., 2001). Also the effects of letrozole on reproductive fitness and early life stages of Japanese medaka (*Oryzia latipes*) have been reported by Sun *et al*. (2007). They showed that inhibition of aromatase in fish could result in significant adverse effects on endocrine function and reproduction (Sun *et al*., 2007).

In rainbow trout, morphological and physiological changes occur during growth to sexual maturity, and sexual maturation (gonadal maturity and reproductive activities) can potentially affect both the innate and adaptive immune responses (Slater and Schreck, 1993; Suzuki *et al*., 1997; Harris and Bird, 2000). Therefore the development of techniques, which could retard sexual maturation in fishes are important. The objective of this study was to evaluate the effects of letrozole,

on serum sex steroid secretion and gonadal development in females of rainbow trout.

Materials and methods

Forty eight female rainbow trout fishes *(O. mykiss)* with an average weight of 854 g, were obtained from a coldwater fish farm (Shiraz, Iran) in mid-September 2011. A seven day acclimation period preceded the exposure period. During acclimation, the fish were maintained outdoors in a 10 m^2 concrete pond supplied with through- flowing river water. The fish were kept in well- aerated water (pH 7.2-7.8, dissolved oxygen 5.5-6 ppm) at a constant temperature (15.8-16°C). Then the fishes were randomly divided into experimental groups and each group was in a 2 m^2 concrete pond supplied with through- flowing river. During the experiment, the fish were fed with commercial salmon food (Beyza Feed Mill (BFM) Co., Ltd., Iran). The non- steroidal aromatase inhibitor Letrozole (CGS 20267) [1, 2, 6,7^{3}H]-4- Androstron-3,17- dione, was obtained as a gift from Iran hormone venture pharmaceutical technology development Co., Ltd. Iran, dissolved in the vehicle ethanol (Shilling *et al*., 1999). Stock solutions which contained 1.0, 2.5 mg of AI ml^{-1} were prepared. The fish were anesthetized (150 ppm clove oil), then injected intraperitonially with letrozole. Fishes were divided in four groups, (1) Control group injected with the vehicle ethanol only (1.0 ml kg^{-1}body weight (n=12), (2) group injected once with 1.0 mg kg^{-1} letrozole (n=12), (3) group injected once with 2.5 mg kg^{-1} letrozole (n=12) and (4) group injected weekly with 2.5 mg AI kg^{-1} letrozole (3×2.5 mg AI kg^{-1}) (n=12).

Sampling

Blood samples (3ml) were taken and collected from the caudal vein at 0 h (just before injection with letrozole), 6, 24, 48, 96, 144,192, 288,384,482,528 h after injection with letrozole. Serum samples were collected from six fishes of each group. The serum was aliquoted into 1.5 ml plastic tubes and frozen on dry ice and stored at -20°C until assay (Salamat *et al*., 2012). After the last blood collection, three fish of each group, were dissected and ovaries were excised and weighted in order to determine the gonado-somatic index [GSI=100× (gonad weight / total body weight)] and oocyte diameter (Afonso *et al*. , 1999).

Steroid ELISAs

For steroid analysis, serum samples first extracted with alcohol ice-cold methanol was added to the serum (6:1 v/v), shaken and centrifuged (3000g, 15 min, 4°C). The pellet was re-extracted twice with 200 µl of methanol. Supernatants were pooled, dried and reconstituted in 120 µl of potassium phosphate buffer (0.1 M, pH 7.4), then stored at -20°C for analysis. Serum E2, 17α-20ß-P and T levels were measured by Enzyme linked immunosorbent assays (ELISA) described by Navas and Segner (2000) and Guzmán, *et al* (2008) with slight modification. In the

steroid ELISA, the microtiter plate provided in this kit has been pre coated with a goat-anti- rabbit antibody (Cusabio Biotech Co.,Ltd). 50 µl standards or samples were added to the appropriate microtiter plate wells with a HRP-conjugated (E2, T,17α-20ß-p) and antibody preparation specific for steroid and incubated. Then substrate solutions were added to each well. The enzyme-substrate reaction was terminated by the addition of a sulphuric acid solution (H_2SO_4) and the color change was measured spectrophotometrically at a wavelength of 450 nm± 2nm. The concentration of steroid in the samples was then determined by comparing the O.D of the samples to the standard curve. All samples were placed in triplicate on the plates. Data were expressed as ng ml^{-1} for serum steroid levels (Navas and Segner, 2000; Guzmán *et al*. , 2008).

Statistical analysis

Normality of variance was tested by the Kolmogorov-Smirnov and all data were shown as mean ±standard error (SEM). One-way ANOVA were used to detect variation among experimental groups. Group dependent variation in serum concentrations for each hormone were detected by analysis of variance (ANOVA) followed by all pairwise multiple comparison by Duncan test (Sokal and Rohlf, 1969). Time-dependent variation within each group were studied by repeated measures analysis of variance (General linear Model) followed by a Student-Newman-Keuls test, and were considered significantly at $p<0.05$. Statistical analysis was conducted using SPSS software version 16.0.

Results

In the control group injected with the ethanol vehicle, serum E_2 levels 528 h after injection increased (2.12±0.08 ng ml^{-1}) significantly in relation to the first blood collection ($p<0.05$). In the group treated with 1mg kg^{-1} letrozole, decreased significantly from 6 h to 96 h after injection, after 96h, rebounded significantly ($p<0.05$) (Table 1). In the group injected with 2.5 mg kg^{-1} letrozole, from 6h to 96h after injection, decreased significantly ($p<0.05$). But serum E_2 levels didn't show significant difference from 24 h to 96h ($p>0.05$). After 96 h, serum E2 levels increased significantly ($p<0.05$) up to 528 h after injection. At 528 h after injection, serum E_2 levels were similar to the levels observed before injection (Table 1) ($p>0.05$). In the group injected weekly with 2.5 mg kg^{-1} letrozole, declined significantly between 0 and 6 h and between 6 and 24 h ($p<0.05$), and remained low throughout the experimental period (0.07±0 ng ml^{-1}).

At 6 h after injection, the groups injected with letrozole showed significantly ($p<0.05$) lower serum E2 levels than the control group ($p<0.05$), but there were not significantly different among the groups injected with letrozole at the same time ($p>0.05$). At 192 h, serum E_2 levels in the group injected weekly with 2.5 mg kg^{-1} letrozole were significantly lower

(p<0.05) than the other groups (Table 1).

Table 1: Serum 17ß-estradiol level in pre-spawning female rainbow trout injected or not with Letrozole (mg AI kg^{-1}).

Treatment	17ß-esradiol (ng ml^{-1}) hours after injection										
	0	**6**	**24**	**48**	**96**	**144**	**192**	**288**	**384**	**480**	**528**
C n=6 ♦	1.48 ± 0.15b/a	0.92 ± 0.04a/b	1.09 ± 0.04a/c	1.06 ± 0.04a/c	1.08 ± 0.04 a/c	1.09 ± 0.03a	1.06 ± 0.02a	1.15 ± 0.02a/b	1.61 ± 0.15bc	1.82 ± 0.10c	2.12 ± 0.08 d
1.0 n=6 ■	1.39 ± 0.17c/a	0.60 ± 0.04a/a	0.36 ± 0.04a/b	0.43 ± 0.03a/b	0.50 ± 0.03a/b	1.04 ± 0.03b	1.19 ± 0.05bc	1.16 ± 0.04c/b	1.43 ± 0.10c	1.74 ± 0.12d	1.84 ± 0.04d
2.5 n=6 ▲	1.58 ± 0.19d/a	0.48 ± 0.03b/a	0.23 ± 0.02a/a	0.13 ± 0.01a/a	0.20 ± 0.02a/a	1.06 ± 0.05c	1.08 ± 0.03c	1.09 ± 0.02c/b	1.16 ± 0.06c	1.13 ± 0.06c	1.59 ± 0.03d
2.5×3 n=6 ×	1.44 ± 0.15c/a	0.49 ± 0.04c/a	0.19 ± 0.02ab/a	0.12 ± 0ab/a	0.23 ± 0.02ab/a	1.04 ± 0.06d	0.04 ± 0.01 a	0.32 ± 0.03bc/a	0.13 ± 0ab	0.45 ± 0.02c	0.07 ± 0a

Each value represents the mean ± standard error. Serum 17ß-esradiol (E2) levels which are similar (p>0.05) within each group are identified by the same letter before slash. Serum 17ß-esradiol (E2) levels which are similar (p>0.05) among the groups are identified by the same letter before slash. n= number of fish in each group. C= control .The ♦, ■, ▲and× indicate the different treatments

In the control group, serum 17α-20β-P levels did not show significant difference (p>0.05) between 0 h up to 480 h (Table 2). But 524 h after injection with vehicle (1 ml kg^{-1} ethanol), serum 17α- 20β-P levels (2.15±0.24 ng ml^{-1}) increased significantly (p<0.05). 6h after injection, serum 17α- 20β-P levels increased significantly in the group injected with 1 mg kg^{-1} letrozole, then declined significantly from 96h to 480h (p<0.05). At 192 and 384 h after injection, serum 17α-20β-P levels were similar to the levels observed before injection (p>0.05). In the group treated with 2.5 mg kg^{-1} letrozole, increased significantly (p<0.05) at 6 h after injection, but there was not significant differences between 24 and 48h (p>0.05). From 144 h to 480 h after injection, serum 17α-20β-P levels declined significantly (p<0.05). But 17α-20β-P levels at 480 and 288 h were similar to which observed before injection (p>0.05). In the group injected weekly with 2.5 mg kg^{-1} letrozole, increased significantly from 6 h to 96 h (p<0.05). At 528 h, serum 17α-20β-P levels were higher than the levels observed at 0 h (Table 2).

At 6 h after injection, in the groups injected with Letrozole, 17α-20β-P levels were higher than the control group injected with ethanol vehicle (p<0.05). In the group injected weekly with letrozole, 17α-20β-P levels were higher than those the other groups (p<0.05) at 192 h after injection (2.60±0.04 ng ml^{-1}).

Table 2: Serum 17α-20ß-P level in pre-spawning female rainbow trout injected or not with Letrozole (mg AI kg^{-1}).

Treatment	17α-20ß-P (ng ml^{-1})/ hours after injection										
	0	**6**	**24**	**48**	**96**	**144**	**192**	**288**	**384**	**480**	**528**
C	0.29	0.20	0.27	0.31	0.10	0.17	0.25	0.18	0.23	0.16	2.15
n=6	±	±	±	±	±	±	±	±	±	±	±
♦	0.03a/a	0.04a	0.03a/a	0.04a/a	0.04 a	0.03a/a	0.06a/a	0.06a	0.01a/a	0.05a/a	0.24 b
1.0	0.21	1.16	0.6	1.69	0.91	0.57	0.38	0.58	0.33	0.39	1.52
n=6	±	±	±	±	±	±	±	±	±	±	±
■	0.04a/a	0.06d	0.08b/b	0.08f/b	0.04c	0.04b/b	0.03a/a	0.03b	0.04a/b	0.03b/c	0.05e
2.5	0.26	3.19	2.36	2.34	3.47	0.69	0.36	0.30	0.48	0.27	2.14
n=6	±	±	±	±	±	±	±	±	±	±	±
▲	0.02a/a	0.19d	0.07c/c	0.07c/c	0.11c	0.07b/b	0.02c/a	0.03a	0.05ab/c	0.03a/ bc	0.09c
2.5×3	0.25	3.08	2.29	2.33	3.56	0.66	2.60	3.15	2.65	2.57	2.89
n=6	±	±	±	±	±	±	±	±	±	±	±
×	0.01a/a	0.19c	0.05c/c	0.06c/c	0.12f	0.06b/b	0.04cd/b	0.17e	0.04cd/d	0.04cd/d	0.05de

Each value represents the mean ± standard error. Serum 17α-20ß-P levels which are similar (p>0.05) within each group are identified by the same letter before slash. Serum 17α-20ß-P levels which are similar (p>0.05) among the groups are identified by the same letter before slash. n=number of fish in each group. C= control. The symbols ♦, ■, ▲and× indicate the different treatments.

In the control group, serum testosterone levels increased significantly (p<0.05) at 48 h after injection (2.60± 0.04 ng ml^{-1}). Up to 528 h after injection, increased significantly (p<0.05). There was no significant difference (p>0.05) between 192 and 288 h after injection (Table 3). In the group injected with 1 mg kg^{-1} letrozole, testosterone levels were similar to the levels observed before injection (p>0.05). At 48h after injection, increased significantly (p<0.05) whereas E_2 levels decreased significantly (p<0.05), and declined significantly from 144h to 528h after injection (p<0.05). In the group injected with 2.5 mg kg^{-1} letrozole, increased significantly from 6h up to 96 h after injection (p<0.05). From 144h to 528h declined significantly (p<0.05) when compared with 96 h after injection. But up to 528h, serum testosterone levels were higher than the levels observed at 0 h to 24 h after injection (p<0.05). In the group injected weekly with 2.5 mg kg^{-1} letrozole, increased significantly from 28 h up to 96 h, and serum testosterone levels declined significantly from 144 h up to 288 h after injection (p<0.05).

In the groups injected with letrozole, serum testosterone levels increased significantly (p<0.05) at 24h after injection when compared with the control group (Table 3). Comparison among the groups showed that the group injected weekly indicated significantly (p<0.05) lower serum testosterone levels than the other three groups at 528h (2.77±0.12 ng ml^{-1}).

Table 3: Serum testosterone level in pre-spawning female rainbow trout injected or not with Letrozole (mg AI kg^{-1}).

Treatment	testosterone (ng ml^{-1})/ hours after injection										
	0	**6**	**24**	**48**	**96**	**144**	**192**	**288**	**384**	**480**	**528**
C	2.01	1.80	1.60	2.60	3.04	3.54	4.47	4.96	5.73	6.07	6.81
n=6	±	±	±	±	±	±	±	±	±	±	±
♦	0.07b	0.07ab	0.03a/a	0.04c/a	0.11d	0.06e/c	0.10f/c	0.0.19f/b	0.07g/c	0.16h/c	0.08i/c
1.0	3.18	3.39	2.61	2.64	3.17	2.97	3.29	3.40	3.39	3.47	3.57
n=6	±	±	±	±	±	±	±	±	±	±	±
■	0.19bc	0.24bc	0.06a/b	0.06c/b	0.04bc	0.04b/a	0.05bc/a	0.09bc/a	0.07bc/a	0.04c/a	0.05c/b
2.5	2.22	2.15	2.92	4.46	4.87	3.24	3.67	3.63	3.53	3.81	3.68
n=6	±	±	±	±	±	±	±	±	±	±	±
▲	0.03a	0.01a	0.05b/c	0.04e/c	0.18f	0.06c/b	0.07d/b	0.06d/a	0.07d/a	0.07d/a	0.06d/b
2.5×3	2.29	2.11	2.96	4.42	5.24	3.25	3.41	3.69	5.47	4.80	2.77
n=6	±	±	±	±	±	±	±	±	±	±	±
×	0.04a	0.04a	0.04bc/c	0.07e/c	0.18g	0.04cd/b	0.16cd/ ab	0.27d/a	0.11g/b	0.14f/b	0.12b/a

Each value represents the mean ± standard error. Serum testosterone levels which are similar (p>0.05) within each group are identified by the same letter before slash. Serum testosterone levels which are similar (p>0.05) among the groups are identified by the same letter before slash. n= number of fish in each group. C= control . The symbols ♦, ■, ▲and× indicate the different treatments

In this paper, the injection procedure of letrozole was started in mid- September 2011 while all females were in prepubertal stage (2 months before spawning). At the end of the experiment, gonads were weighed (three fish per group) and oocyte diameter and gonadosomatic index were checked to evaluate the oocyte maturation. The oocyte diameter and GSI in all groups except the group received multiple injection of 2.5 mg kg^{-1} letrozole (3× 2.5) showed that during the 22 days trial period, there were a change in the developmental stages of the oocytes when compared with the developmental stage of the oocytes of the fish examined and killed at the beginning of the experiment (Fig.1). Oocyte diameter in the group injected weekly was similar to the group examined and killed at the beginning of the experiment (p>0.05).

The group injected weekly with 2.5 mg kg^{-1} letrozole had significantly lower (p<0.05) GSI (6.95±0.06%) in comparison with the other groups (p<0.05). There was a significant increase in the GSI during the experimental period in all groups except the group injected weekly (Fig. 2). There were no significant differences (p>0.05) between the group injected with ethanol and the groups received a single injection of letrozole (p>0.05)

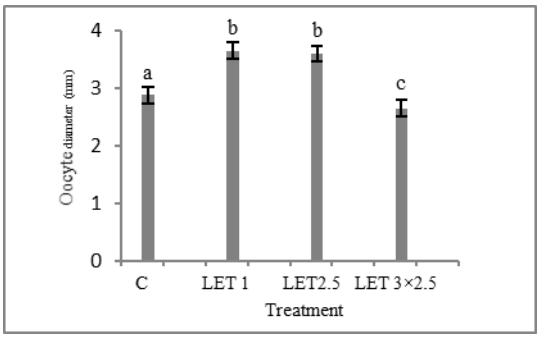

Figure 1: Effects of Letrozole on the oocyte diameter from pre- spawning female rainbow trout.Each bar represents the mean±SEM from females each treatment group.Oocyte diameters which are similar (*p*>0.05), as determined by Duncan's test among groups (C=control, LET1=1 mg Letrozole kg^{-1}, LET2.5= 2.5 mg Letrozole kg^{-1} and LET 3×2.5= 2.5 mg Letrozole kg^{-1} weekly) are identified by the same superscript letter. Number of fish used in each group is three.

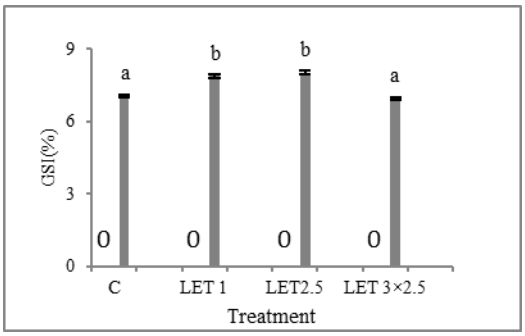

Figure 2: Effects of Letrozole on the gonadosomatic index(GSI) from pre-spawning female rainbow trout. Each bar represents the mean± SEM from females each treatment group.GSI which are similar (*p*>0.05), as determined by Duncan's test among groups (C= control, LET1= 1 mg Letrozole kg^{-1}, LET2.5= 2.5 mg Letrozole kg^{-1} and LET 3×2.5= 2.5 mg Letrozole kg^{-1} weekly) are identified by the same superscript letter. Number of fish used in each group is three. Gonadosomatic index= ovary weight /body weight ×100.

Discussion

In several teleosts, reduced growth and lower flesh quality coincides with sexual maturation and precocious maturation is a problem in commercial fish farming (Razmi *et al*., 2011). Therefore, reliable methods to control the onset of puberty are required (Afonso *et al*., 1999). Different strategies have been developed in order to circumvent this drawback (Lee *et al*., 2004). Our aim of studying the effects of letrozole was to assess an approach as a possible method of controlling oocyte maturation by a sharp decrease in aromatase activity in rainbow trout. Starting injection of the aromatase inhibitors may be imported on their efficiency in blocking pubertal maturation (Afonso *et al*., 1999; Ankley *et al*., 2002).

In our study the basal E_2 levels were twice as high in non-injected females compared to injected females from 24 h to 96 h after injection with letrozole, and serum E_2 levels were significantly lower in females injected weekly with 2.5 mg kg^{-1}letrozole. This suggests that letrozole, a non-steroidal inhibitor, inhibits armatase which catalyzes the conversion of androgens, androstendione and testosterone via three hydroxylation steps to estrone and estradiol (Sun *et al*., 2007). Similar results were obtained by Kelloff *et al.* (1998) that a dose responsive increase in blood serum steroid was observed in trout injected with 50 mg Fadrozole kg^{-1} per day. Shilling *et al* (1999) used letrozole (CGS20267) and aminoglotethimide (AG) as non-steroid and examined in vitro for activity in trout ovarian microsomes. They showed that letrozole reduced aromatase activity a maximum of 90% in dose-dependent manner. But letrozole and clorimazole fed to juvenile rainbow

trout at doses up to 1000 ppm for 2 weeks were not effective in suppressing 17 β- estradiol levels (Shilling *et al*., 1999). Our in vivo data showed that the mechanism and efficiency of inhibition of letrozole are different. Afonso *et al*. (1999) provided the first evidence that injection of the fadrozole was effective in vivo in lowering plasma E_2 levels, and demonstrated a shift from E_2 to 17α-20β-P biosynthesis in fadrozole injected female coho salmon (*O. kisutch*). In the adult female rat, letrozole (0.3-1mg kg^{-1} daily) completely interrupted ovarian and reduced uterine weight and serum estradiol (E_2) concentration (Bhatnagar *et al*., 1990, 1993; Schieweck *et al*., 1993). Sun *et al*. (2007) showed that the application of letrozole for 21 days affected the reproductive, gonadal development and vitellogenin production of Japanese medaka (*Oryzias latipes*) females

It took a 4×concentrated (10 mg AI kg^{-1}) sample of Fadrozole (Afonso *et al*. 1999) to inhibit aromatase to the same levels as the Letrozole at 2.5 mg AI kg^{-1} which suggest that differences in concentration of the compounds due to the anti-aromatase effect of Letrozole is very potent (Séralini and Moslemi, 2001; Sun *et al*., 2010).

Our results indicate that groups injected with letrozole contain potent component that can suppress activity of aromatase enzyme (Sioufi *et al.*, 1997; Haynes *et al*., 2003).

Our results showed that serum 17α-20β-P levels significantly increased at 6 h after injection in the groups injected with letrozole, and in the group treated weekly with 2.5 mg kg^{-1} letrozole, serum E_2 levels was declined up to 528h after injection, and serum 17α-20β-P levels significantly increased and remained high from 192 to 528 h after injection. But in the other two groups injected with letrozole, the increase of serum 17α-20β-P levels was transient. Similar results were obtained by Afonso *et al* (1999).

Letrozole had a global inhibitory effect on serum testosterone and estrogen in female trout. This is in accordance with the fact that in fish aromatase inhibitor plays a prominent role in the regulation of gametogenesis (Piferrer *et al*., 1994; Uchida *et al*. 2004; Suzuki *et al*., 2004;).

In this experiment, letrozole effects on ovarian development were assessed by oocyte diameter and GSI. We found that multiple injection of letrozole resulted in inhibition/ delay of the gonadal development. This was confirmed by comparing the egg diameter and GSI in the group examined at the beginning of the experiment with the multiple injected group after 22 days. These two groups had similar egg diameters. Other investigations have reported that EDC_S can specially affect the oocyte maturation stages. For example Afonso *et al*. (1999) reported that multiple injections of Fadrozole retarded the development of the oocytes in Coho salmon (*O. kisutch*). Bhatnagar *et al*. (1993) reported that letrozole inhibits the conversion of androstenedione to estrone in human and blocks estrogen-

dependent uterine growth and negative feedback on FSH secretion that is responsible for gonadal growth. Estrogens are responsible for growth and oocyte maturation, maturation and ovulation are also regulated by estrogens and progesterone (Peter and Yu, 1997; Lahnsteiner *et al*., 2006; Yelghi *et al*., 2012). It would be expected that multiple injection of letrozole would cause retardation in oocyte growth. Due to transitory effect of Letrozole on Serum E_2 secretion in the other two groups the GSI and oocyte diameter had not been affected.

In conclusion, we showed that multiple injection of letrozole displays marked inhibitory effect on gonadal development and serum sex steroid in rainbow trout. Letrozole is a triazole derivative, and is a competitive and reversible aromatase inhibitor which is highly potent and selective for aromatase. This study showed that letrozole effects are dose and time-depended dependent. The aromatase inhibitor technique, might also be a promising method for controlling the onset of puberty in aquaculture species.

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