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Effects of the Aromatase Inhibitor, Letrozol, on Levels of Serum 17β-Estradiol (E_2) And Enzymes in Rainbow Trout (*Oncorhynchus Mykiss* Walbaum) Females

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Abstract

In the present study, changes of values of serum 17ß-estradiol (E2), Asparate amino teransferase (AST), Alanine amino transferase (ALT) and Lactate dehydrogenase (LDH) following intraperitoneal (i.p) injection with the aromatase inhibitor (AI) letrozole were investigated in rainbow trout (*Oncorhynchus mykiss*). Forty- eight apparently healthy rainbow trout (mean weight of 845 g) divided in four groups (twelve fish for each group). (1) Control vehicle injected, (2) group treated once with 1.0 mg AI kg^{-1}, (3) group treated once with 2.5 mg AI/ kg and (4) group treated with 2.5 mg AI/ kg weekly (3×2.5 mg AI/ kg. Results revealed that that, in the group injected weekly, serum E2 levels were significantly ($P<0.05$) lower than the other groups. Also levels of serum enzymes at days 14 and 28 of injection, significantly increased in the group injected weekly compared to the other groups ($P<0.05$). No significant differences have been observed between control and the groups injected once in respect to all parameters ($P>0.05$). This study showed that multiple injections with letrozole may decrease E_2 levels and may impact negatively on the physiology of the fish as manifested in changes in some of serum enzymes under laboratory conditions

Keywords: Non- steroid aromatase, Asparate amino teransferase (AST), Alanine amino transferase (ALT), Lactate dehydrogenase (LDH)

Introduction

The aquatic environment represents the ultimate sink for broad group of natural and synthetic chemicals. Increased attention is currently being paid to pharmaceutical substances as a class of environmental contaminants (Hilton and Thomas, 2003; Ashton *et al.*, 2004.). Letrozole (CGS 20264), with commonly used brand name Femara, is a non- steroidal trizole derivation and one of the most potent aromatase inhibitors yet developed as endocrine disrupter component (EDC)(Smith, 1999). Like other human pharmaceutical compounds, AI_S can enter aquatic systems and cause ecotoxicological effects (Haynes *et al.*, 2003). 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; Kitano *et al.*, 2000; Ankley *et al.*, 2002). Association between decreased brain aromatase activity, circulating E2 levels and ovarian somatic

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index in females of perch (*Perca fluviatilis*) was reported (Noaksson *et al.*, 2001). Also when fish are exposed to toxic chemicals in the water, at a dose that is not so high as to cause in a few hours or days, there can occur a wide variety of physiological effects. The sublethal physiological changes that fish exhibit have practical applications in water pollution control. Therefore the major physiological changes of the poisoned fish are yet to be examined (Çelik, 2004). Analysis of biochemical parameters in aquatic organisms is used to detection of status of stress, mode of action of toxicants and also monitoring of aquatic environment (Ra˚ berg and Lipsky, 1997. Sharma, 1990. ; Barron *et al.*, 1999. ; Basaglia, 2000.). Since there is a close association between the circulatory system of fish and external environment (12), estimation of enzymes likes alkaline and aspirate aminotransferase (ALT, AST) and lactate dehydrogenase (LDH) are considered useful biomarkers to determine pollution level during chronic exposure. Not many of the field and laboratory studies considered reported on the activities of the serum transaminases ALT and AST in fish (Bucher and Hofer, 1990. ; Balint *et al.*, 1995; Beyer *et al.*, 1997. ; Velisek *et al.*, 2006.).

Rainbow trout is recognized as an appropriate model for studying the inhibition of aromatase (CYP19) A crucial advantage is that in vivo studies involving rainbow trout for larger sample size, providing stronger statistical power at a lower cost than mammal in model(Shilling *et al.*, 1999).To our knowledge, neither the toxicity of letrozole (AI), nor its potential to disrupt reproductive function on fish has been studied in depth, the present study, was carried out to assess some biochemical blood parameters, such as serum E_2, ALT, AST and LDH of *O. mykiss* injected with different sublethal concentrations of letrozole (endocrine disruptor) under laboratory conditions

Materials and methods

Letrozole

The non- steroidal aromatase inhibitor Letrozole (CGS 20267) H-4- Androstron-3,17- dione, was obtained as a gift from Iran hormone venture pharmaceutical technology development Co., Ltd., Iran was dissolved in the vehicle ethanol (28). Stock solutions which contained 1.0, 2.5 mg of AI ml^{-1} were prepared.

Fish and rearing conditions

Forty eight adult rainbow trout *(Oncorhynchus mykiss)* females were obtained in mid- September 2011 from Dalkhan rainbow trout hatchery farm, Sepidan, west of Shiraz, Iran and held outdoors in a 10 m^2 concrete pond in a flow-through water system. The fish were kept in well-aerated water at 15.8±0.5°C, dissolved oxygen 5.5±0.1 ppm and pH 7.8±0.18. Fish were initially weighted (854±0.1g) under anesthesia (150 ppm clove oil). After 7 days of acclimation to the condition, they were randomly divided in four groups and each group was kept in a 2 m^2 concrete pond with a water depth of 50 cm in the same flow-through water system. During the experiment, the fish were fed with commercial salmon food (Beyza 121 Feed Mill (BFM) Co., Ltd., Iran).

Letrozole injection of brood fish

These procedures were carried out at least 3 days after females had been transferred to $2m^2$ concrete ponds. In mid August 2011, the fish were injected intraperitonially with letrozole at the base of the right ventral fin using individual 4 ml syringes fitted with an 18.5 gauge needle. Control group was injected with the vehicle ethanol only (1.0 ml/kgbody weight). Fish were divided in four groups, (1) control vehicle injected (n=12), (2) group treated once with 1.0 mg AI/kg (n=12) , (3) group treated once with 2.5 mg AI/kg (n=12) and (4) group treated with 2.5 mg AI/kg weekly (3×2.5 mg AI/kg)(n=12). Before any

handling procedure, fish were anaesthetized a solution of freshly powdered clove oil with concentration of 150ppm.

Serum sampling

After injection of letrozole, blood samples (3ml) were collected from the caudal vein at 1, 2, 4, 6, 8, 12, 16, 20, 22 day after injection with AI (for determining serum E_2 levels) and at days 14 and 28 after injection (for evaluating the levels of serum enzymes), and allowed to clot at room temperature for 1-2 h and then at 4° C overnight. Serum, collected after it, was divided into ependroff and stored at -20° C (Salamat *et al.*, 2012).

Biochemical analyses

For serum E_2 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 E_2, levels were measured by Enzyme linked imunosorbent antibody assay described by Navas and Segner(2000) and Guzmán *et al*. (2008).
Separated serum samples were analyzed for aspartate amino transferase (AST), alanine amino transferase (ALT), and lactic acid dehydrogenase (LDH) byCobas Mira 27-6764 auto-analyzer using enzymatic procedures with a diagnostic kit (Pars Azmoon Chemical Co.). Biochemical measurements were carried out 1 h after sample collection. Sample collection and biochemical analyses were performed using the methods derived from several different researchers (Cech *et al.*, 2000; Anderson and Anderson, 2002; Çelik, 2004; Shalaby, 2005).

Statistical Analysis

Data were evaluated using one- way analysis of variance (ANOVA).Groups were considered to be significantly different if $P < 0.05$ .When a significant *F* value was obtained for ANOVA the differences between all groups were tested by using Duncan multiple comparisons test. All statistics were performed using SPSS for windows version 16.

Results

In the control group injected with the ethanol vehicle, serum E2 levels 22 day after injection increased (2.12±0.08 ng/ml) significantly (P<0.05) in relation to the first blood collection and In the group injected with 1mg AI/kg, decreased significantly (P<0.05) at 6 h up to day 4 after injection (Table 1). After 4 days, E2 levels rebounded significantly (P<0.05) in the group injected with 1mg AI / kg. In the group injected with 2.5 mg AI/kg, E2 levels decreased significantly (P<0.05) at 6h up to day 4 after injection. From day2 up to 4, serum E2 levels had not significant different (P>0.05). After 4 days, serum E2 levels increased significantly (P<0.05) up to day 22 after injection. At day 22, serum E2 levels were similar (P>0.05) to the levels observed before injection (Table 1). In the group injected weekly with 2.5 mg AI/kg, from 6 h up to day 4, E2 levels declined significantly (P<0.05). Between 0 and 6 h and between 6 and day 2, serum E2 levels decreased significantly (P<0.05), but there was no significant difference (P>0.05) from day2 up to 4 (Table 1). Injection weekly with 2.5 mg AI/kg was effective in remaining low serum E2 levels up to day22 after injection (0.07±0 ng/ml). Comparison among the groups showed that at 6 h after injection, the groups injected with Letrozole showed significantly (P<0.05) lower serum E2 levels than the control group which injected with ethanol vehicle, but there were no significant difference (P>0.05) among the groups injected with Letrozole in the same time. At day 22, serum E2 levels in the group injected weekly with 2.5 mg AI/kg were significantly lower (P<0.05) than the other groups (Table 1).

After the second injection of AI, serum AST (Fig 1a), ALT (Fig 1b) and LDH (Fig 1c) levels in the group injected weekly were significantly (P<0.05) higher than other groups at day 14. Also comparison among the groups at day 28 showed that the vehicle injected group and groups injected once with AI presented significantly (P<0.05) lower levels than the group injected weekly with AI, which at this point had received 3 injections of 2.5 The increase was not dose- dependent, since the serum enzyme levels were not significantly (P>0.05) higher in the group injected once with 2.5 mg AI kg^{-1} than the group injected once with 1 mg AI kg^{-1}.Also there were no significant difference (P>0.05) between the groups injected once with letrozole and the control group injected with the ethanol vehicle

Discussion

Since there is a close association between the circulatory system of fish and external environment (Haynes *et al.*, 2003) measurement of biochemical parameters is a commonly used diagnostic tool in aquatic toxicology and biomonitoring (Harikrishnan *et al.*, 2003. ; Çelik, 2004).E2 levels were twice as high in untreated females compared to treated females from day2 up to day 4 after injection with letrozole and serum E2 levels were significantly (P<0.05) lower in females injected with 2.5 mg AI/ kg weekly. Also decreases in E2 levels (6 h after injection) in the groups injected with letrozole suggest that this anti-steroid inhibit aromatase, the enzyme that converts androgens to estrogens. Synthetic estrogen level possibly through two mechanisms, one mechanism is through aromatization of testosterone and the other is a decrease in converting steroid in to estrogen. Our result shows that a carful time and dose – response study has to be performed to determine dosages and times that will influence on changes of steroid hormones. There are no previous studies of the effects of letrozole on serum steroid levels in females of different species of fish before spawning but 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 invitro for activity in trout ovarian microsomes and 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 (28) . Also similar E2 levels have been exhibited in vitellogenic Coho salmon. Afonso *et al*. (2000) demonstrated that the aromatase inhibitor fadrozole was capable of reducing E2 biosynthesis in female Coho salmon This observation suggests that aromatase inhibition could be an important mechanism of action for environmental contaminants in fish(Ankley *et al.*, 2002).

In the group injected weekly with letrozole, the values of serum enzymes at days 14 and 28 were AST, 387.86± 10.24 and 410±17.32; ALT, 12.60± 0.20 and 14.33±0.88; LDH,1173.3±93.33 and 1366.7±88.19 IU/ L.Mean values of serum AST,ALT,LDH were were significantly (P<0.05) higher than other groups. It shows that the multiple injections of non- steroid chemicals like letrozole cause the liver cells to synthesize more of these enzymes in an attempt to metabolize the contaminants. The leakage of specific enzymes (e.g.transaminases) into the blood may be indicative of the disruption of cellular membranes in certain. Therefore LDH, ALT and AST have been employed for diagnosing liver, muscle and gill damages caused by pollutants in fish organs (Moss *et al.*, 1986.). The increase was not dose- dependent, since the serum enzyme levels were not significantly (P>0.05) higher in the group injected once with 2.5 mg AI kg^{-1} than the group injected once with 1 mg AI kg^{-1}.Also there were no significant difference (P>0.05) between the groups injected once with letrozole and the control group injected with the ethanol vehicle. This observation suggests that long term exposure with aromatase inhibitor letrozole as a toxic agent or factor which leads to chronic impairment of animals´ metabolism will be caused changes usually, increases of the activities of some serum enzymes. However here is not any report on the effects of aromatase inhibitors in point of biochemical analysis on fish. The values observed by us were in

agreement with those reported by other authors who have determined the toxicity of deltamethrin for various species of fish. Velisek *et al*. (2006.)observed a significant increase ($p < 0.05$) in AST and ALT levels in carp after acute exposure to deltamethrin in concentration of 3.25_g/l.
Balint *et al* observed an increase of lactated ehydrogenase and glucose in common carp (*Cyprinus carpio*) after exposure to deltamethrin. Thus there is a close association between the circulatory system of fish and external environment(Cech *et al.,* 1996).

Conclusion

In conclusion, our results demonstrated that the aromatase inhibitor letrozole is capable of reducing E_2 levels in female rainbow trout .Also the data showed that the effects of letrozole were dose- and time-dependent, and the higher the dose used the longer and stronger was the reduction in serum E_2 levels. The study also showed that multiple injections with letrozole increased levels of serum enzymes. The 28 day study period demonstrated that estimation of enzymes likes ALT, AST and LDH may be useful biomarkers to determine pollution level of aromatase inhibitors such as letrozole in rainbow trout under laboratory conditions. All these have led to the concern that aromatase inhibition could be an important mechanism of action for environmental contaminants, disrupting endocrine system in fish.

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Table 1. Serum 17ß-estradiol levels in rainbow trout females injected or not with letrozole (mg / kg).

Treatment	17ß-esradiol (ng/ ml) days after injection										
	0	6	24	48	96	144	192	288	384	480	528
C n=6	1.48 ± 0.15 b/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 ± 0.15 bc	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 ± 0.03 b	1.19 ± 0.05 bc	1.16 ± 0.04c/ b	1.43 ± 0.10c	1.74 ± 0.12 d	1.84 ± 0.04 d
2.5 n=6	1.58 ± 0.19 d/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 ± 0.03 d
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 ± 0.06 d	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 ± S.E. 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 after slash. n= number of fish in each group. C= control group injected with 1 ml kg^{-1} letrozole.

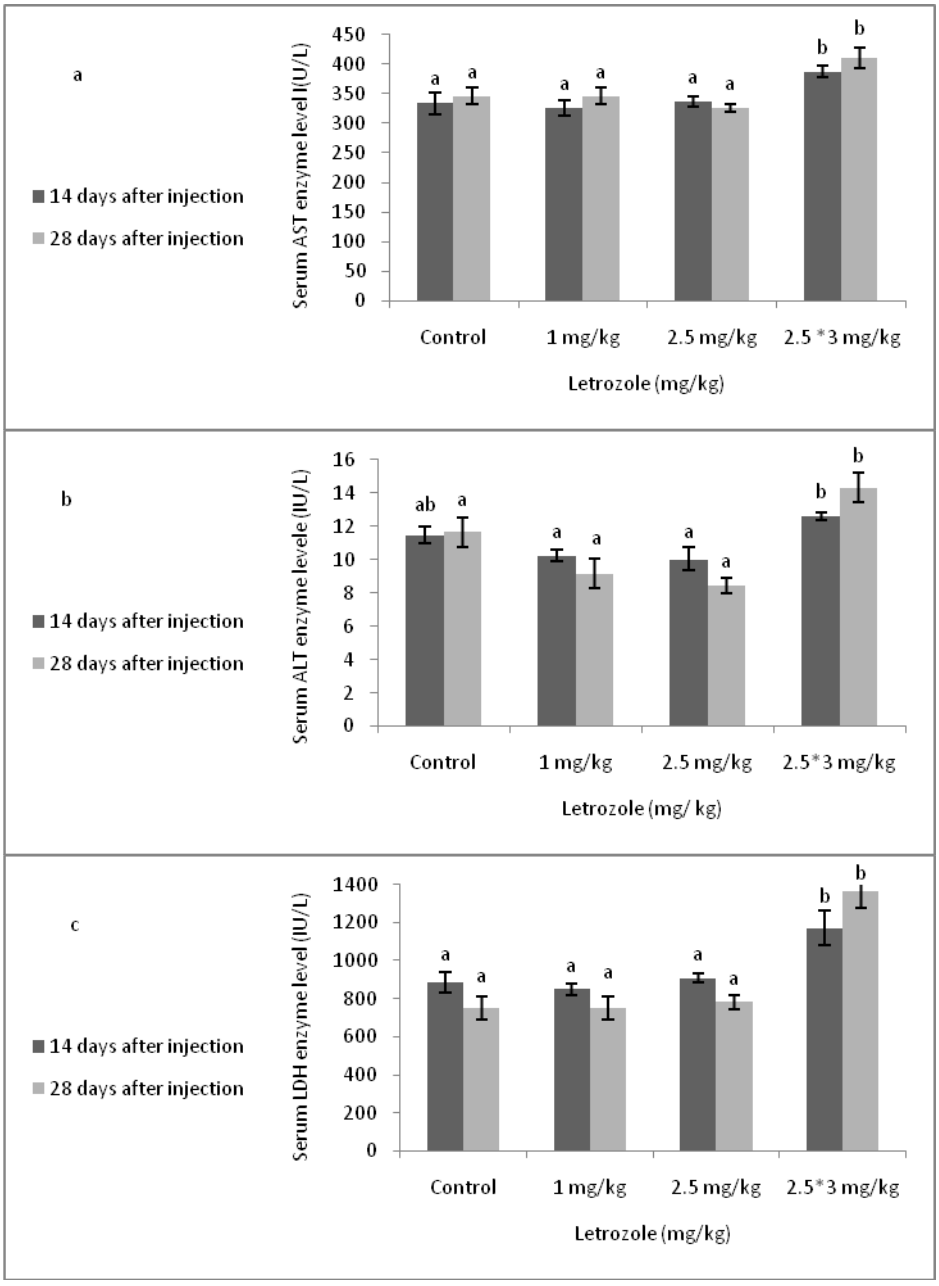

Fig. 1 Effects of different sublethal letrozole (AI_s) concentrations on (a) Asparate amino teransferase (AST), (b) Alanine amino transferase (ALT) , (c) Lactate dehydrogenase (LDH) activities (IU/L) in serum of rainbow trout (*O.mykiss*). Each data point represents the mean (± S.E.) of triplicates. Similar values among the groups are identified by the same superscript letter (P>0.05).