Effects of Genistein and β-Sitosterol on Early Life of Caspian Kutum

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ABSTRACT

Aims The phytoestrogen, genistein and β-sitosterol, naturally occurring compounds found in soy products and pulp and paper mill effluent, respectively, could act as endocrine disrupting compounds (EDC) in the environment. The aim of this study was to evaluate the effects of β-sitosterol and genistein on the early life stages of Kutum (*Rutilus kutum*), specifically developing post-fertilized embryos.

Materials & Methods In this experimental study, Kutum's fertilized egg exposed to 3 different levels of genistein and β-sitosterol (10, 50, 500ng.l-1, respectively) up to 7 days post-fertilization (dpf). At the end of the research period, newly hatched larvae were sampled and testosterone (T), 17β-estradiol (E2), Aromatase and ethoxyresorufin-O-deethylase (EROD) were measured according to standard protocols. One-way analysis of variance (ANOVA), Duncan multiple range test and SPSS 17 software were used for data analyses.

Findings A high level of genistein lead to increased 17β-estradiol, testosterone concentration and aromatase activity. Also, β-sitosterol treated embryos (500ng.l-1) showed a high level of testosterone and EROD as compared to the control group. While other treatment had no significant effect.

Conclusion It seems that β-sitosterol and genistein could effect on the endocrine system of Kutum embryos by altering steroid biosynthesis and disturb enzyme activity. So it could lead to change the population structure and reduce reproduction performance of Kutum in the long period.

Keywords Phytoestrogens; 17β-Estradiol; Testosterone; EROD; Aromatase Activity

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CITATION LINKS

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Introduction

Endocrine disrupting compounds (EDCs) are chemicals with the potential to elicit negative effects on the endocrine systems of vertebrates. They include a broad class of compounds such as natural estrogens/androgens, synthetic estrogens/androgens, industrial chemicals as well as phytoestrogens [1]. Genistein and β-sitosterol are two of the most abundant and biologically active phytoestrogens. It has been reported that these compounds can compete with endogenous 17β-estradiol (E_2) for binding to the estrogen receptor (ER) [2].

Genistein is one of the phytoestrogen compounds which can mimic estradiol activity to increase the concentration of E_2 in fish.

In a range of fish species, phytoestrogens have a variety of hepatic, gonadal, hormonal, and gametic effects. Hepatic effects include the induction of vitellogenin in male fish and changes in 7-ethoxyresorufin-O-deethylase (EROD) activity. In an early life-stage exposure experiment, it was reported that the phytoestrogens induced gonadal intersex in medaka (*Oryzias latipes*) [3] and channel catfish (*Ictalurus punctatus*) [4]. Gonadal production of the sex-steroid hormones testosterone (T), 11-ketotestosterone (11-KT), and E_2, as well as circulating blood plasma levels, can be affected by phytoestrogens. Zhang *et al*. reported that exposure to genistein decreased production and circulating levels of T in medaka [5]. Similar suppressive effects on T, have been found since β-sitosterol decreasing T production by ex vivo tests in goldfish (*Carassius auratus*) [6, 7].

Both of these compounds naturally are found in aquatic ecosystems and their concentrations in natural waters in several European countries, are usually under 5ngl⁻¹ for the estrogens and progesterones [8]. The rivers located in the south of the Caspian Sea create an unsafe environment for natural reproduction and migration of fish due to receive of industrial effluent, agricultural and human wastewater annually. Caspian Kutum (*Rutilus kutum*), an economic species of the Caspian Sea [9], is an anadrom fish and annually migrate to some rivers of the south Caspian Sea during March-April for spawning. Based on the increase of water pollution in the northern rivers of Iran, especially those which is used for Kutum spawning, embryogenesis and also restocking program, it is necessary to determine, how these compounds can affect reproduction physiology. So, sexual maturity and embryogenesis disruption is very critical and sensitive and any naturally disruption can negatively affect reproduction performance of fish [10].

The aim of this study was to evaluate the effects of β-sitosterol and genistein on the early life stages of Kutum, specifically developing post-fertilized embryos.

Materials and Methods

In this experimental study adult Kutum (Rutilus kutum) was caught from the Shirood River inlet (E50.8002 N36.8559) during their spawning migration in March and April 2015.

A certain amount of fertilized eggs was distributed in glass jars incubator (60gr per each Weiss). Seven Weiss was used to continuously expose fertilized eggs with three concentrations of β-sitosterol (10, 50 and 500ngl⁻¹; S1270; sigma; Germany) and three concentrations of genistein (10, 50 and 500ngl⁻¹; G6649; sigma; Germany) and one for control. The test solutions were refreshed every 2 days. Eggs were entered the test solutions, no later than 2h post-fertilization (hpf) and incubated for 7 days, at 17±1°C.

Steroid hormones assays: Whole-body sex steroids (T and E_2) were assayed by a modified radioimmunoassay [11]. Briefly, for each steroid, 1g of per hatched-eggs were first homogenized with 1ml of 50% ethanol and washed with 3ml of absolute ethanol. The extract was centrifuged at 4000g for 15min at 4°C, then the supernatant was collected and the residue was washed again with 1ml of 80% ethanol. After partial evaporation of all the supernatant, steroid was extracted three times with dichloromethane (v/v 1:5) and then stored in absolute ethanol. Two hundred microliter of this solution was analyzed for each sex steroid. The T and E_2 concentration were assayed by Enzyme-linked Immunosorbent Assay (ELISA) according to the kit protocol (MyBioSource; USA). MBS933475; MBS700179) respectively.

Aromatase and EROD activity assay: 1g of egg or embryo tissue was rinsed with ice-cold PBS (0.02mol.L⁻¹, pH=7.2-7.4). The tissues minced into small pieces and homogenized in a specific amount of PBS (Usually 10 mg tissue to 100μl PBS.) with a hand glass homogenizer (Glass Teflon Homogenizer; USA) on ice. Afterwards, homogenates samples were centrifuged for 20min at 3000rpm 4°C and the

supernatant was stored immediately at -20°C until use. Finally, 40μl of supernatant was used to assay aromatase activity by ELISA kit (Cat. No: E0034FI; Bioassay Technology Laboratory, China). At the end, samples were standardized based on the protein content of the tissue homogenate [12].

To assay EROD activity in the liver, 1g of tissue was homogenized with 5cc of 1.15% KCl buffer by a glass homogenizer on ice. The differential centrifugation was used in order to prepare microsomes from fish liver, then they were washed by suspending the 1.15% KCl buffer containing 1mM EDTA and collected by centrifugation. The pellet was re-suspended in 10% glycerol containing 1mM of EDTA and stored in liquid nitrogen until use. The enzyme concentration was assayed by, Enzyme-linked immunosorbent Assay (ELISA). Samples were then standardized based on the protein content of the tissue homogenate [12]. The protein concentration of each sample was determined by the Biuret method [13].

The results were examined in the tests of normality and homogeneity of variance by kolmogorov-smirnov test. One-way analysis of variance (ANOVA) was used to examine a significant difference between control and test group. Finally, a Duncan multiple range test was used to compare the means of different treatments. All analyzes were performed using SPSS 17 software.

Findings

The measurement of E_2 under different concentrations of two types of phytoestrogens showed the different effects in the Caspian Kutum embryos.

The exposure to the highest concentration of genistein (500ngl^{-1}) caused the highest levels of E_2 compared to control and other treatments. While the level of E_2 in embryos treated with 500ngl^{-1} of β-sitosterol show significant difference with the treatments of 10ngl^{-1} (Diagram 1; $p>0.05$). So, the lower E_2 level observed in embryos treated with the high concentrations of β-sitosterol. However, this group didn't have the significant difference with control.

Testosterone (T), the other steroid hormones, showed the same trend in exposed embryos at different treatments (Diagram 2). The highest concentration of T was measured in egg treated with 500ngl^{-1} genistein. The same peak

obtained at egg-exposed to 500ngl^{-1} β-sitosterol but with lower intensity. Both compounds showed a significant difference with control and low concentration of them (10ngl^{-1}). However, the level of T raised by the increasing of genistein and β-sitosterol concentration (Diagram 2).
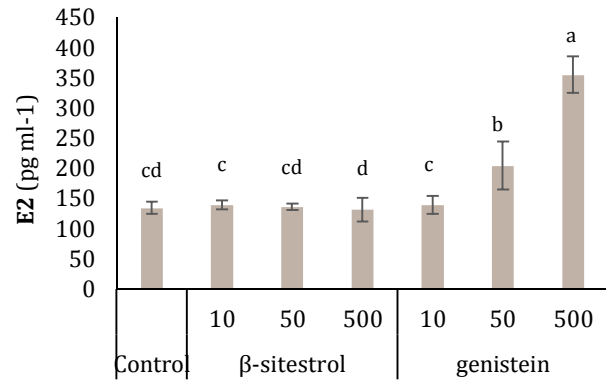
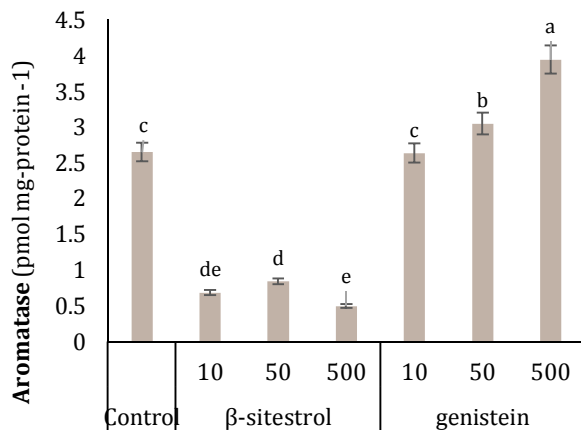**Diagram 1)** Effect of different levels of genistein and β-sitosterol (ng.l^{-1}) on E_2 concenterations in 7 day post-fertilization (dpf) of kutum eggs. Significant differences are indicated with letters ($p<0.05$). The data are presented as mean±SEM (n=3).
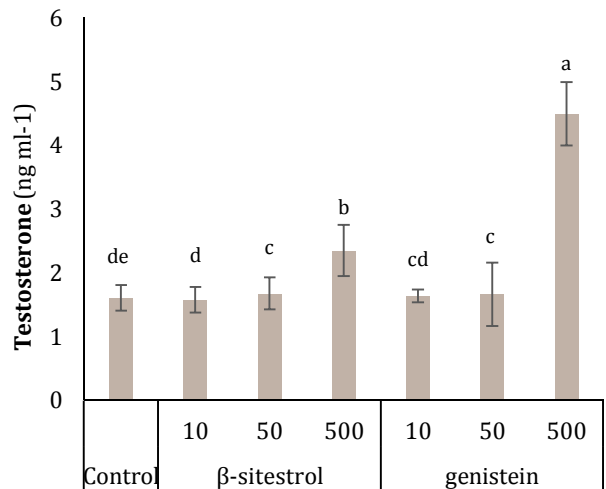
Diagram 2) Effect of different levels of genistein and β-sitosterol (ng.l^{-1}) on T concenterations in 7 day post-fertilization (dpf) of kutum eggs. Significant differences are indicated with letters ($p<0.05$). The data are presented as mean±SEM (n=3).
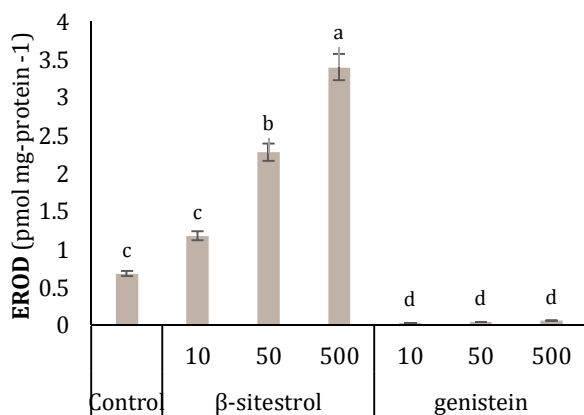
After 7 days of exposure, the genistein and β-sitosterol in compared with control could affect the aromatase activity in Kutum embryos. Although, increasing genistein up to 500ngl^{-1} could elevate it, but the lower level of it has not caused any significant differences with unexposed egg (Diagram 3). The treated egg by β-sitosterol showed the lower activity compare to other ones. The lowest activity was obtained

in the exposed egg to 500ngl⁻¹ β-sitosterol.

other hand, the E_2 concentration decreased by EROD induction (Diagram 5).

Diagram 3) Effect of different level of genistein nd β-sitosterol (ng.l⁻¹) on aromatase activity in day post-fertilization (dpf) of kutum eggs. Significant differences are indicated with letters (p<0.05). The data are presented as mean±SEM (n=3)

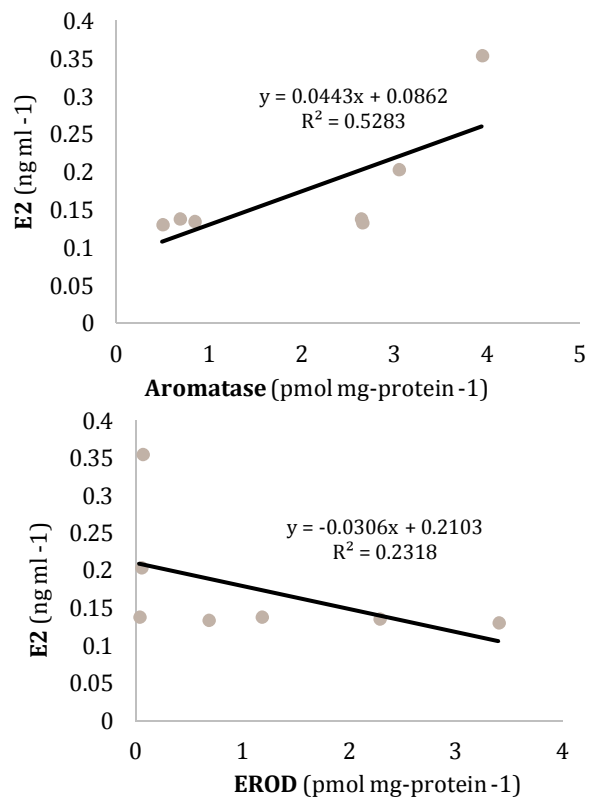EROD induction was different based on the type of compound. EROD induction increased with elevating β-sitosterol concentration. Therefore, the high EROD induction was observed in the treated egg by 500ngl⁻¹ β-sitosterol. However, it was no significant difference with the control group at the low level. The exposed embryos to genistein showed no significant differences but there were significant differences in control group at all levels (Diagram 4).

Diagram 4) Effect of different level of genistein nd β-sitosterol (ng.l⁻¹) on EROD induction in 7 day post-fertilization (dpf) of kutum eggs. Significant differences are indicated with letters (p<0.05). The data are presented as mean±SEM (n=3)

Aromatase activity had a positive correlation with 17β-estradiol concentration, while EROD induction showed a negative correlation. On the

$$y = 0.0443x + 0.0862$$
$$R^2 = 0.5283$$

$$y = -0.0306x + 0.2103$$
$$R^2 = 0.2318$$

Diagram 5) Corrolation between E_2 concenteration and aromatase activity (left), and EROD induction (right)

Discussion

The aim of this study was to evaluate the effects of β-sitosterol and genistein on the early life stages of Kutum, specifically developing post-fertilized embryos.

Kutum embryos were exposed to 3 levels of β-sitosterol and genistein during early life stage until egg hatch. The result showed low concentrations of genistein had no significant effect on steroid hormones and aromatase activity, but in high concentration (in 500ngl⁻¹) it could change hormone level and increase aromatase activity.

In this study, β-sitosterol and genistein elicited different estrogenic responses in Kutum embryos. Other studies have shown that in vivo or short-term β-sitosterol or Genistein exposure could have estrogenic effects and increase sex steroids [14]. The high E_2 concentration was in embryos, which treated with high levels of genistein. It confirms how this compound can change reproduction performance, especially at high concentrations. It is believed that phytoestrogens like genistein, act as natural selective estrogen receptor

modulators (SERMs) that elicit distinct clinical effects of estrogens by selectively recruiting co-regulatory proteins to ER_b triggering specific transcriptional pathways [15].

The high concentrations of T in treated embryos by the high level of genistein and β-sitosterol suggested, T synthesis was impaired at the early life stage in the present study. In short-term tests, β-sitosterol has been shown to alter the endocrine status of fish [6, 16-18]. Structurally, β-sitosterol closely resembles cholesterol [18], which is a precursor to the reproductive sex steroid hormones. It has been shown that β-sitosterol reduces the plasma cholesterol concentration, which leads to a decrease in plasma sex steroids. [19]. Even though, in the present study T concentration of the exposed embryos was increased. It may be referred to decrease steroidogenic biosynthetic capacity and disrupting plasma cholesterol concentrations and mitochondrial translocation to the steroidogenic pathway [20].

The same result has been reported by Flinders *et al.* and Nakari *et al.* [21, 22]. However, the wide range of responses across species is suggestive of a species-specific sensitivity to β-sitosterol, and there are documented differences in the relative sensitivities to other substances among fish models [23]. These differences were typically smaller across species within a taxa group (i.e., Species of algae, insect, fish, etc.) than the differences in fish species' responses to β-sitosterol exposure [24].

In the present study increasing of E_2 was, according to the high activity of aromatase. Aromatase CYP19 is the key enzyme regulating local and systemic levels of estrogens in the body [25]. It seems that increasing aromatase activity in egg-exposed to phytoestrogens elevated E_2 concentrations. Embryos presumably possess the ability for steroidogenesis and estrogen signaling, as aromatase CYP19 and ERs also undergo maternal transfer in the egg. The high expression of cyp19b was observed already in the early developmental stages [26, 27]. However, endogenous E_2 and T levels in medaka (Oryzias latipes) eggs decrease to a low basal level by 2 dpf, and no increase of these steroids was observed during early development [28]. Similar results are reported for tilapia and coho salmon (*Oncorhynchus kisutch*), in which endogenous E_2 and T levels in developing embryos decline sharply after fertilization [28]. Even though, our result showed significant increasing of E_2 in 7dpf egg. The elevation of cyp19b expression, either naturally or by exogenous estrogen, might have the same effect on ovarian ontogeny as the endogenous high cyp19a expression [29].

The ethoxyresorufin-O-deethlyase (EROD) activity changing in fish suggested as well in vivo biomarker of exposure to aromatic hydrocarbons and xenoestrogen [30, 31]. Hepatic EROD induction has been consistently demonstrated in fish sampled in the vicinity of pulp and paper mills [32]. The present study showed EROD induction by β-sitosterol and genistein in early life stage of Kutum. Although the induction was statistically significant, it was depended on the type of compound and dose of treatment. Significant EROD induction was seen in treated embryos by β-sitosterol. Even though other studies didn't report it in rainbow trout after a 2-week in vivo exposure to β-sitosterol [16] or in β-sitosterol-injected goldfish [17].

Results showed a correlation between E_2 concentration, EROD and aromatase activity. Uncertainties exist about the physiologic significance of elevated EROD activity and the identity of the compounds present in pulp and paper mill effluents responsible for this induction [33]. In fish, a negative correlation has been found between the elevated activity of EROD and the depressions in plasma sex steroids [4]. In the present study, the reduction of the sex steroid paralleled the increases in EROD activity. Evidence exists that wood extract may contribute to the EROD induction [32].

The limitations of this research include lack of access to eggs and embryos in the rivers polluted by pulp and paper mills effluent, absent of accurate data about the amount of phytoestrogenic compounds in Iran's water resources and the suggestions are investigation of the adverse effects of these compounds in the natural environment on the eggs and embryos of Kutum and other economic fishes of the Caspian Sea and finally study effects of other phytoestrogenic compounds or other endocrine disrupting compounds.

Conclusion

The endocrine system of Kutum embryos can be affected by the high concentration of genistein (500ngl^{-1}) according to steroid hormone levels and aromatase activities. Although, the high

concentration of β-sitosterol can alter the EROD activity and raise testosterone levels. Genistein and β-sitosterol can modify biosynthesis of sex hormones and disrupt the function of an endocrine system of Kutum embryo, which may lead to the changes of population structure and reduce reproduction ability of Kutum in future.

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