Original Article

Single and Combined Effects of Estrone and 17β-Estradiol on Male Goldfish*

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Abstract

Objective To assess the single and combined effects of estrone (E_1) and 17β-estradiol (E_2) on goldfish (*Carassius auratus*).

Methods Batch tests were conducted. Serum levels of vitellogenin (VTG) and E_2, gonadosomatic indices (GSI), gonadal DNA damage and liver 7-ethoxyresorufin-O-deethylase (EROD) activity were measured after exposure for 14 days.

Results The VTG level increased significantly in a concentration-dependent manner. The serum E_2 level was significantly higher and the GSI level was significantly lower in goldfish after exposed to the 3 drugs. DNA damage occurred in treated samples and EROD activity was significantly suppressed 7 days after exposure. The joint effect of E_1 and E_2 was additive with regard to VTG induction.

Conclusion The results of our study highlight a series of effects of steroidal estrogens on goldfish. Further study is needed to confirm their effect as a whole.

Key words: Vitellogenin; DNA damage; EROD activity; *Carassius auratus*

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INTRODUCTION

Several studies demonstrated that environment estrogens (EE) can reduce sperm count, abnormal sexual organs, and feminization of males in wildlife[1-2], which have come into consideration and caused attention over the past decades. These substances, particularly natural estrogens, such as estrone (E_1) and 17β-estradiol (E_2) in aquatic environment from point (sewage treatment, pulp mill and industrial effluent) and non-point (urban and agricultural runoff) sources, have been identified in several studies[3-6]. EE can disrupt the endocrine system at multiple levels and exert its effects in organisms by mimicking the actions of endogenous estrogens and combine with estrogen reporter (ER), altering the lesion of reproductive systems and immune systems, and even leading to changes in biodiversity at the population level[7-8].

It was reported many biological effects of chemicals can be located on single estrogen[9]. Because chemical-related effects are likely the consequence of exposure to a mixture of EE rather than to a single chemical, recent studies are focused on the combined effects of multiple chemicals in aquatic organisms[10].

Vitellogenin (VTG) is an egg yolk precursor (lipophosphoprotein) normally produced in females. However, under estrogen or estrogen mimic

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exposure, VTG gene is expressed in males and synthesized. Abnormal VTG level in males can be used as a highly sensitive biomarker of estrogen disruption in aquatic environment[11-12]. In addition, abnormal serum E_2 concentration, gonadal somatic index (GSI) and gonadal DNA damage can be used as auxiliary biomarkers of estrogen contamination and reproductive function assessment in fish[13-15]. Moreover, it was reported that steroid can suppress cytochrome P4501A (CYP1A) and constitutive CYP1A-associated 7-ethoxyresorufin-O-deethylase (EROD) activity can respond to estrogens[16].

The aim of this study was to investigate the effect of E_1, E_2 and their mixture on male goldfish (*Carassius auratus*) and their dose-response and time-response relationship.

MATERIALS AND METHODS

Chemicals and Reagents

E_1 (≥99% purity), E_2 (≥98% purity), ethidum bromide, low melting temperature agarose and alkaline phosphatase (AP)-labeled goat anti-rabbit IgG were purchased from Sigma-Aldrich (St. Louis, MO, USA). Oasis hydrophile lipophile balance (HLB) solid phase extraction (SPE) cartridge (500 mg, 6 mL volume) was purchased from Waters (Shanghai, China). Phenylmethyl sulfonyl fluoride (PMSF) and heparin sodium were purchased from Nanjing Sunshine Biotechnology Co., Ltd. (Nanjing, China). Primary antibody (rabbit anti-goldfish VTG) and purified VTG were obtained from Ocean University of China (Qingdao, China). Coomassie brilliant blue G-250 (Ultra Pure Grade) and dimethylsulfoxide (DMSO) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Methanol (HPLC grade) and ethyl acetate (HPLC grade) were purchased from Merck Corporation (Darmstadt, Germany). Derivatization agent N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) was purchased from Regis Corporation (Massachusetts, USA).

Animals and Exposure

Male goldfish weighing 28.4±2.7 g, obtained from Nanjing Institute of Fishery Science (Nanjing, China), were fed daily with commercial fish food and acclimatized in dechlorinated municipal water for two weeks before tests. Fish were not fed 24 h before experiment.

Goldfish were randomly divided into different chemical concentration exposure group (12 fish in each) and kept in 30 L glass tanks (40 cm × 25 cm × 30 cm) containing 20 L of experimental solution under constant aeration. Stock solution was prepared by dissolving estrogens in DMSO with its concentration controlled at 0.1 mL/L. According to the environmental concentrations[17-19] and the median effective concentrations for VTG-induced E_1 and E_2[20], the goldfish were exposed to nominal E_1 concentrations at 20, 40, 80, 160, 320 ng/L and E_2 concentrations at 10, 20, 40, 80, 160 ng/L. The concentrations of E_1 and E_2 mixture were 10+5, 20+10, 40+20, 80+40, 160+80 ng/L, respectively. A dechlorinated municipal water control (WC) and a solvent control (0.1 mL/L DMSO, SC) were included in the experimental design. The exposure time was 14 days as previously described[14,20]. A flow-through test was conducted with a daily renewal of 100% of the test volume. All experiments were performed in triplicate. Results from the 3 assays were combined for statistical analysis. Water temperature was 19-21°C (pH 7.0±0.2) and dissolved oxygen was 8.5±0.2 mg/L during the exposure.

Fish Sampling

Three fish were selected to observe the time-response relationship of estrogen after exposed to estrogen for 3, 7, 10, and 14 days. Blood samples (about 1 mL) were taken from caudal peduncle with heparinized syringe, immediately added into 0.1 mmol/L phenylmethanesulfonyl fluoride to inhibit proteolytic activity, and then centrifuged at 4000 ×g for 10 min at 4 °C. Serum fractions were removed and stored at -80 °C for VTG and E_2 analysis.

The sampled fish were weighed. Their gonads were removed and weighed after blood samples were taken. Gonad tissue was cut into sections which were frozen in liquid nitrogen for gonadal DNA damage analysis. Liver tissue was collected, washed with 0.15 mol/L KCl, weighed, frozen and stored at -80 °C for EROD analysis.

Biomarker Assay

Serum VTG levels were measured by ELISA as previously described[15]. Serum E_2 level was measured with ELISA kit (Nanjing Jiancheng Bioengineering Institute, China) according to its manufacturer's protocol. The absorbance was recorded with a microplate reader (Molecular Device VersaMax, USA) at a wavelength of 450 nm. GSI (%)

was expressed as 100 × gonad wet weight (g)/wet weight of fish (g). Gonadal DNA damage was detected by comet assay as previously described.[21] After staining, each slide was observed under epifluorescent microscope (Olympus BX51-RFA, Japan). About 5 nuclei in each sector were randomly counted and overlapping nuclei or tails were not counted. DNA damage was detected by tail moment, which was expressed as tail length × DNA % in the tail/100.

Liver tissues were homogenized in 9 volumes of cold buffer (0.15 mol/L KCl, 0.1 mol/L Tris-HCl, pH 7.4) and centrifuged at 9000 ×g for 15 min at 4 °C. The supernatants were removed for activity assay. EROD activity was quantified at 572 nm using a microplate reader (Molecular Device VersaMax, USA)[22]. Serum VTG and EROD activities were normalized to total protein per sample in order to control the potential individual difference among replicates or between treatments. Serum protein concentrations in liver were measured at 595 nm as previously described[23], with bovine serum albumin as a standard.

Chemical Analysis

Estrogen concentrations were monitored throughout the experiment. Water samples (500 mL) were collected from each glass tank on days 0, 7, and 14 after exposure. Methanol (5‰) was added into each water sample to prevent bacteria growth. After filtered through a 0.45 μm vacuum glass fiber filter, each water sample was passed onto Oasis HLB cartridge pre-conditioned with methanol (5 mL) followed by acidification in 1% acetic acid (5 mL), maintaining a consistent loading flow rate of equal or less than 5 mL/min. After rinsed in 10 mL ultrapure water and freeze-dried under vacuum, the cartridge was eluted with ethyl acetate (10 mL). After gently evaporated to dryness under nitrogen, 50 μL ethyl acetate and 100 μL BSTFA/TMCS reagent were added into the vials containing e dried residues. Each vial was well mixed and derivatized at 70 °C for 30 min. The derivatives were cooled to room temperature and analysis was performed using a gas chromatography-mass spectrometer (GC-MS, Thermo DSQП, USA)[24]. E_1 and E_2 recovery was 99.69% and 110.98% respectively, and the detection limits were 2 ng/L.

Statistical Analysis

Data were analyzed using SPSS 13.0, and expressed as mean±SD. All data from different treatments were examined for normality. Data from different treatments were compared by one-way analysis of variance (ANOVA) and statistically different treatments were identified by Dennett's test. $P<0.05$ was considered significant.

RESULTS

Estrogens in Water

The estrogen concentrations in each treatment were measured (Table 1). The E_1 and E_2 concentrations in the single treatment during the exposure period were 83.5%-90.6% and 88.9%-97.4% respectively, while those of their mixture were 85.7%-97.7% and 80.6%-93.7% respectively. The measured concentrations were obviously below the nominal concentrations and the subsequent bioassay analysis was performed based on the measured concentrations.

Table 1. Nominal and Measured Concentration of E_1 and E_2 at Different Time Points after Exposure

Estrogen	E_1		E_2		E_1/E_2	
	nominal	measured	nominal	measured	nominal	measured
Concentration (ng/L)	20	16.91±0.38	10	9.74±0.42	10/5	9.77±0.11/4.03±0.53
	40	36.25±0.81	20	18.62±0.78	10/20	18.28±0.48/9.05±0.82
	80	66.80±1.04	40	38.53±0.64	40/20	34.87±0.34/18.00±0.20
	160	141.39±0.77	80	71.12±0.58	80/40	68.53±0.52/37.73±0.49
	320	281.90±1.32	160	145.86±1.03	160/80	137.60±0.79/74.96±0.98

Biomarker Response

No death occurred during the exposure experiment, indicating that the tested compounds have no acute toxic effect and the sampled fish are not stressed unduly. No significant difference was observed in weight of goldfish before and after treatment and in VTG and E_2 levels, GSI value,

gonadal DNA damage and EROD activity between goldfish exposed to DMSO and water controls. The biomarker responses were also compared with those of solvent controls.

The serum VTG levels in male goldfish exposed to different estrogenic concentrations on days 3, 7, 10, and 14 are presented in Figure 1. The VTG concentrations were not detectable in control group. As seen in Figure 1A and 1B, the lowest E_1 and E_2 concentrations induced significant VTG expression on days 3-14 after exposure. The serum VTG level increased in concentration-dependent manner. The VTG was synthesized in a time-dependent manner. The VTG level was the highest on day 14 after exposed to E_1 at the concentration of 208.35 ng/mg and to E_2 at the concentration of 329.70 ng/mg . The VTG responses in male goldfish exposed to the E_1 and E_2 mixture are shown in Figure 1C. The mixture induced significant VTG expression in all cases and the VTG contents increased significantly at all concentrations. No significant change was observed in VTG level in regard of response time.

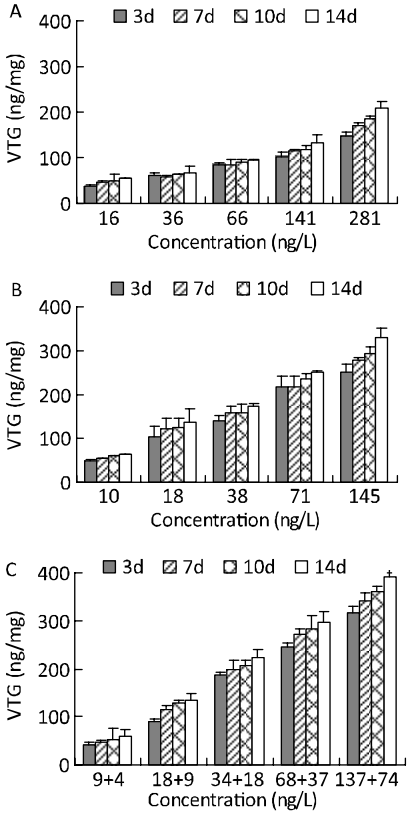

Figure 1. Serum VTG levels in male goldfish after exposure to E_1 (A), E_2 (B), and their mixture (C).

Serum E_2 concentrations in sampled fish exposed to estrogens at different time points are shown in Figure 2. No significant difference was found in serum E_2 and E_1 at different exposure time points except for on day 14 (Figure 2A). The higher concentrations of E_1 increased the serum E_2 concentrations in the goldfish at different exposure time points while the lower concentrations of E_2 (≤18 ng/L) did not significantly increase the serum E_2 concentrations at different exposure time points except for on day 14 (Figure 2B). The serum E_2 concentrations were significantly higher at higher exposure concentrations of E_2 (≥38 ng/L) than at lower exposure concentrations of E_2. The lowest mixture concentrations on day 3 and/or on day 7 after exposure did not significantly increase the E_2 concentrations in control group while significantly increased the E_2 concentrations in the other cases (Figure 2C). The highest serum E_2 concentration was

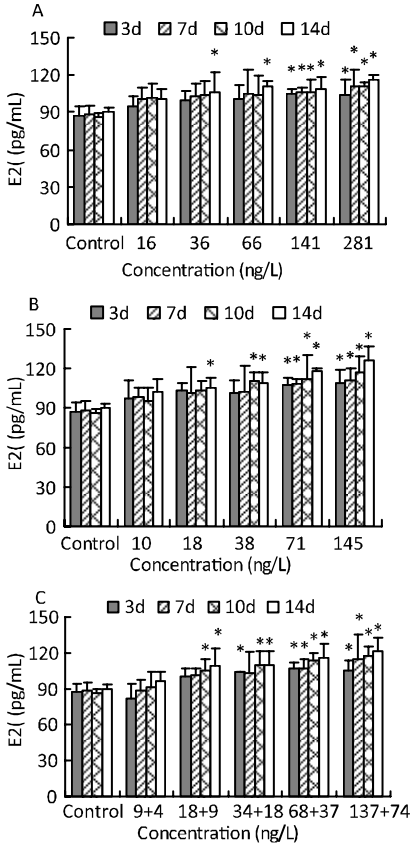

Figure 2. Serum E_2 concentrations in male goldfish after exposed to E_1 (A), E_2 (B), and their mixture (C). Asterisks indicate values significantly different from those in controls ($^*P<0.05$).

observed in response to treatment with E_1 and E_2 at the highest concentrations (alone and in combination) on day 14 after exposure.

The effect of estrogens on GSI is shown in Figure 3. No gonad atrophy was observed in the controls, while different degrees of gonad atrophy occurred in sampled fish treated with E_1 and E_2. GSI decreased significantly on days 7, 10, and 14 after exposed to E_1 at the concentration of equal to or higher than 66 ng/L. The 3 highest concentrations of E_2 and the mixture decreased the GSI value on days 7, 10 and 14 except for on day 10.

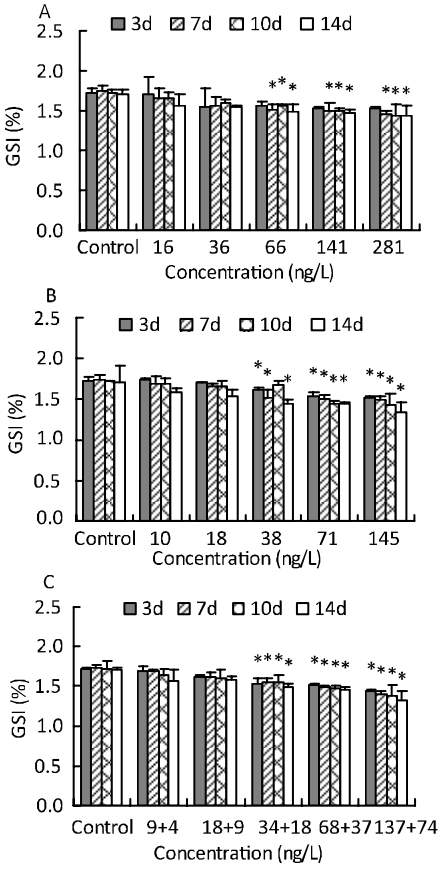

Figure 3. GSI in male goldfish after exposure to E_1 (A), E_2 (B), and their mixture (C). Asterisks indicate values significantly different from those in controls ($^*P<0.05$).

Estrogens-induced gonadal DNA damage is shown in Figure 4. Significant DNA damage occurred after exposed to E_1 at the 3 higher concentrations. Meanwhile, severe DNA damage occurred after exposed to E_1 at the concentration of 36 ng/L and the mixture at the concentration of 18+9 ng/L on day 14. The DNA was damaged in a concentration- and time-dependent manner.

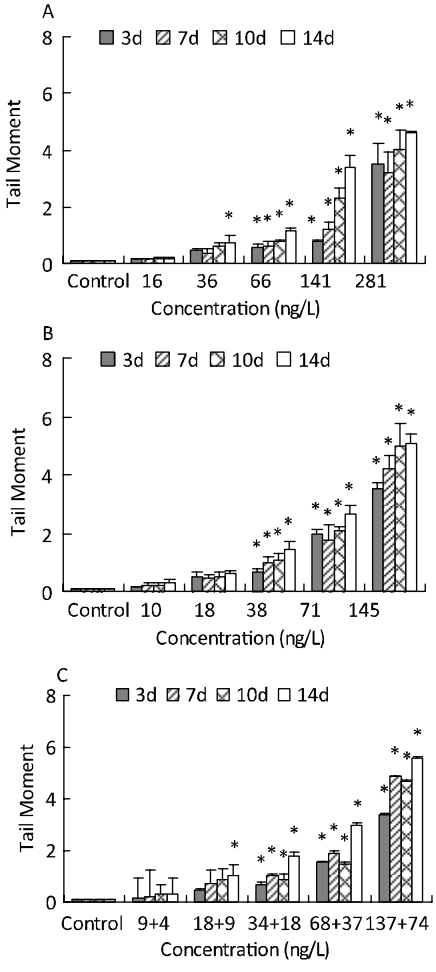

Figure 4. Gonadal DNA damage in male goldfish after exposure to E_1 (A), E_2 (B), and their mixture (C). Asterisks indicate values significantly different from those in controls ($^*P<0.05$).

The effect of estrogens on liver EROD activity at different exposure concentrations and time points are shown in Figure 5. The EROD activity was obviously lower in E_1-treated fish than in controls on day 14 (Figure 5A). E_2 significantly inhibited the EROD activity except for at its lowest concentration. The mixture significantly inhibited the EROD activity on days 7, 10, and 14 (Figure 5C).

In order to estimate the combined effect of E_1 and E_2 on VTG, VTG responses were compared the effect of individual E_1 and E_2 using the simple addition method (Figure 6). The levels of VTG were highly consistent after exposed to E_1 and E_2 mixture with those after exposed to E_1 or E_2 alone. Such a phenomenon was also observed in gonadal DNA damage.

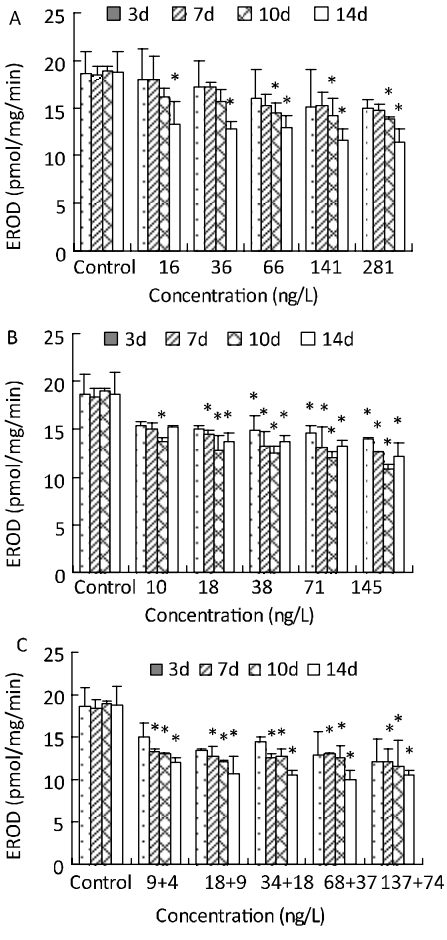

Figure 5. EROD activities in liver of male goldfish after exposure to E_1 (A), E_2 (B), and their mixture (C). Asterisks indicate values significantly different from those in control ($^*P<0.05$).

DISCUSSION

E_1 and E_2 have been detected in aquatic environment. Their concentrations are always low (ng/L) and have biological effects[25]. As showed in Figure 1, E_1 at the concentration of 16 ng/L and E_2 at the concentration of 10 ng/L significantly induced the production of VTG in male goldfish on day 3, suggesting that natural estrogens can induce VTG expression. Van den Belt et al.[26] reported that E_2 at the concentration of 20 ng/L significantly increases the VTG expression in male rainbow trout (*Oncorhynchus mykiss*) and zebrafish (*Danio rerio*) on day 21. Furthermore, it has been shown that E_1 at the concentration of 31.8 ng/L induces VTG expression in male fat head minnow (*Pimephales promelas*) after 3 weeks[27].

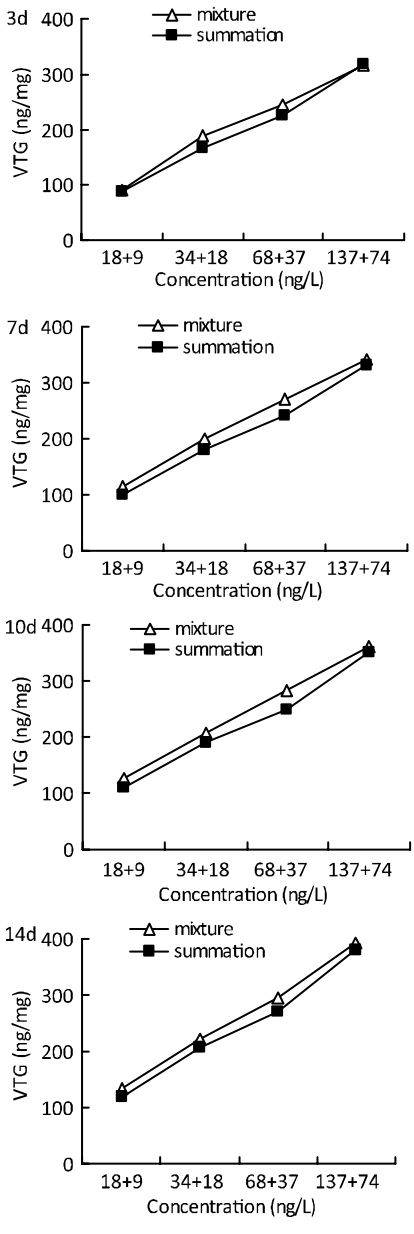

Figure 6. Levels of VTG after exposed to E_1 and E_2 mixture or after exposed to E_1 and E_2 alone.

The VTG level in goldfish is a useful biomarker for estrogenic compounds. In this study, the estrogenic potency of E_1 was lower than that of E_2 in inducing VTG. It was reported that the estrogenic potency of E_2 is 2.3-3.2-fold higher than that of E_1 in inducing VTG on day 14[28]. Moreover, MVLN-assay, yeast estrogen screen *in vitro* and ovarian somatic index *in vivo* displayed that the estrogenic potency of E_1 is 2.5-, 5-,and 2-fold lower than that of E_2[29].

As shown in Figure 6, E_1 and E_2 play an additive

effect on VTG induction in male goldfish. Zhang et al.[30] showed that E_2 and EE_2 act together in an additive manner and their combined effect can be accurately predicted by the model of concentration addition (CA). Similarly, Brian et al.[31] also demonstrated that 5 common estrogenic chemicals including E_2, EE_2, 4-tert-nonylphenol, 4-tert-octylphenol and bisphenol A can induce VTG in fathead minnows and their combined effects are consistent with the predictions of CA model.

After exposure to rainbow trout for 21 days, 25 ng/L E_1, or 25 ng/L E_2 cannot significantly elevate VTG contents, but exposure to combined E_1 and E_2 significantly increases serum VTG level[32]. Thorpe et al.[28] emphasized that it is necessary to regard the total estrogenic load of estrogenic chemicals after the potency of each estrogen is confirmed to contribute to the overall effect of a mixture, even below their individual effect concentration. That means any assessment of the estrogenic activity of mixed estrogens in aquatic environment should be considered as a whole rather than as an individual potency.

Under normal circumstances, serum E_2 levels are extremely low in male fish, but the aromatase activity (an invertase responsible for the conversion of male hormones to female hormones) is up-regulated after exposure to estrogens, elevating the serum E_2 level both in males and in females[33]. In the present study, the tested estrogens at a higher concentration significant elevated the serum E_2 level in goldfish, which is consistent with that in male channel catfish (*Ictalurus punctatus*) after 7 days of injection with E_1 and E_2 as reported by Tilton et al.[34] However, not all treatments significantly increased E_2 and VTG expression in the present study. It was reported that exposure to estrogen is not as sensitive to increase serum E_2 as to induce VTG[14]. As shown in Figures 2 and 1, E_2 elevation is correlated with VTG expression with a correlation coefficient of 0.804, 0.978, and 0.903, respectively, as previously described[35-36].

GSI is often used as a biomarker for evaluation of the feminization in male fish exposed to estrogens. Reduced GSI may be caused by the inhibition of testicular growth or atrophy of the testis[37]. GSI was reduced nearly in all treatment groups, especially at the highest three concentrations. It has been shown that GSI reduction seems related to VTG elevation. Male goldfish sampled from estrogen polluted water exhibit that the lower GSI corresponds to the higher VTG level although they do not show an inverse correlation[38]. Meanwhile, after exposure to EE_2, GSI significantly decreases in male fathead minnow (*Pimephales promelas*) expressing the highest VTG level[13].

Estrogens are associated with different types of DNA damage or chromosomal variation and their genotoxicity a low concentration (10 nmol/L)[39-41]. Further studies of estrogenic genotoxicity indicate that E_2 can aggravate gonadal DNA damage in male hornyhead turbot (*Pleuronichthys verticalis*) and decrease DNA integrity in juvenile sea bass (*Dicentrarchus labrax L.*)[42-43]. The estrogenic genotoxicity exhibited in fish may be related with the presence of free radicals generated during redox cycling of estrogens and can damage cellular macromolecules, including DNA protein and lipids[44]. In addition, it was reported that oxidative metabolites of E_2, capable of regulating metabolic redox cycling and generating mutagenic free radicals and oxidative stress, may play an important role in estrogen carcinogenicity[45-46]. In this study, gonadal DNA damage was observed in goldfish after exposure either to E_1 or E_2 or their combination. The E_2 genotoxicity, expressed as the erythrocytic nuclear abnormality (ENA) frequency, has not been confirmed after exposed for 24 h[43]. However, the ENA increases significantly after exposed for 10 days[47]. Thus, the effect of exposure time on estrogenic genotoxicity should be emphasized, especially at a low concentration.

CYP1A is of critical importance in metabolism of many xenobiotics. Induction of hepatic mixed-function oxidase enzymes at phase I, especially CYP1A and associated EROD activity, is considered as a common indicator for fish exposed to environmental pollutants, such as polychlorinated biphenyls[48] and polycyclic aromatic hydrocarbons[49]. However, it has been demonstrated that estrogenic chemicals are able to down-regulate EROD activity[16,50]. Furthermore, Elskus[51] reported that the EROD activity is 15- and 13-fold lower in primary hepatocytes isolated from juvenile rainbow trout individually treated with E_2 and estriol than in the vehicle-treated controls. Exposure to E_1 and E_2 alone or in combination inhibited 30% EROD activity in the present study. However, the mechanism by which natural steroid hormones suppress CYP1A expression is not well understood. EROD activity can be achieved can be suppressed by direct or indirect competitive interaction between chemicals[52]. In the present study, the EROD activity was only significantly inhibited after exposed for 7 days in

most cases. Nevertheless, VTG was obviously induced on day 3, suggesting that E_1 and E_2 may indirectly suppress CYP1A by binding to ER[50,53], which is further supported by the findings in a previous study[51].

E_1 and E_2 could significantly express VTG in male goldfish and their mixture could produce an additive effect in the present study. Changes in serum E_2 concentrations and GSI corresponding to estrogenic concentrations showed that they were useful indicators for the assessment of estrogenic contamination. The genotoxicity of E_1 and E_2 was demonstrated in male goldfish with DNA damage. Inhibition of liver EROD activity indicated that steroidal estrogens might decrease the ability of mixed function oxygenase system to excrete xenobiotics. However, further study is needed to investigate the interacting mechanism underlying the induced VTG and inhibited CYP1A expression caused by steroidal estrogens.

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