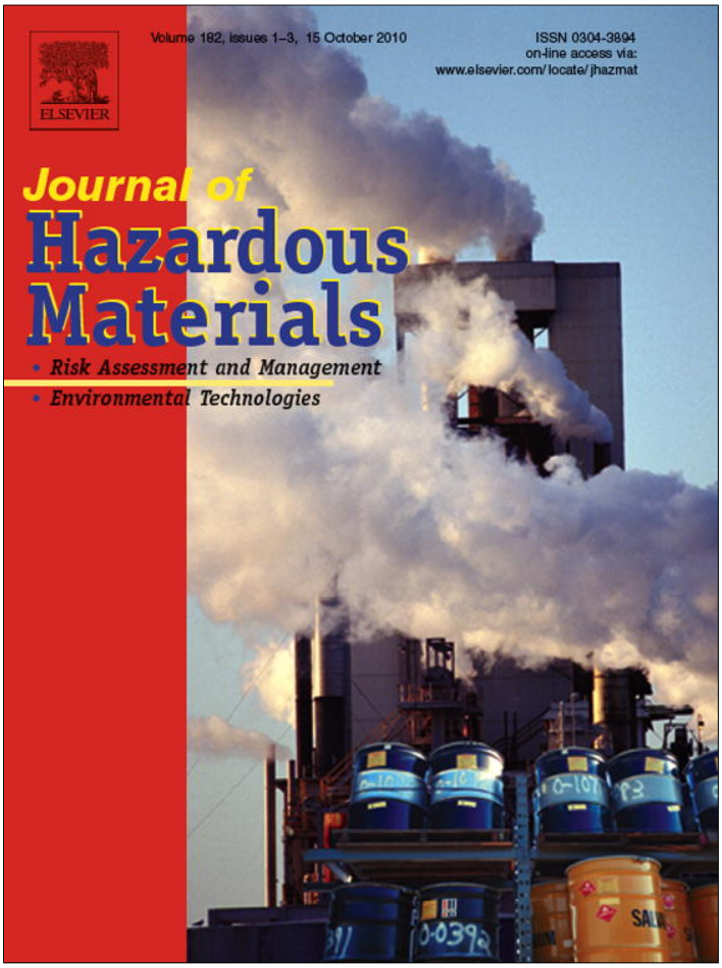
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Effects of sulfathiazole, oxytetracycline and chlortetracycline on steroidogenesis in the human adrenocarcinoma (H295R) cell line and freshwater fish *Oryzias latipes*

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ABSTRACT

Pharmaceuticals in the environment are of growing concern for their potential consequences on human and ecosystem health. Alterations in the endocrine system in humans or wildlife are of special interest because these alterations could eventually lead to changes in reproductive fitness. Using the H295R cell line, the potential endocrine disrupting effects of six pharmaceuticals including diclofenac, erythromycin, sulfamethazine, sulfathiazole, oxytetracycline, and chlortetracycline were investigated. After exposure to each target pharmaceutical for 48 h, production of 17β-estradiol (E2) and testosterone (T), aromatase (CYP19) enzyme activity, or expression of steroidogenic genes were measured. Concentrations of E2 in blood plasma were determined in male Japanese medaka fish after 14 d exposure to sulfathiazole, oxytetracycline, or chlortetracycline. Among the pharmaceuticals studied, sulfathiazole, oxytetracycline and chlortetracycline all significantly affected E2 production by H295R cells. This mechanism of the effect was enhanced aromatase activity and up-regulation of mRNAs for *CYP17*, *CYP19*, and *3βHSD*, all of which are important components of steroidogenic pathways. Sulfathiazole was the most potent compound affecting steroidogenesis in H295R cells, followed by chlortetracycline and oxytetracycline. Sulfathiazole significantly increased aromatase activity at 0.2 mg/l. In medaka fish, concentrations of E2 in plasma increased significantly during 14-d exposure to 50 or 500 mg/l sulfathiazole, or 40 mg/l chlortetracycline. Based on the results of this study, certain pharmaceuticals could affect steroidogenic pathway and alter sex hormone balance. Concentrations of the pharmaceuticals studied that have been reported to occur in rivers of Korea are much less than the thresholds for effects on the endpoints studied here. Thus, it is unlikely that these pharmaceuticals are causing adverse effects on fish in those rivers.

1. Introduction

Pharmaceuticals are used in both human and veterinary medicine, to treat and prevent diseases, and to promote growth of some livestock [1]. Widespread use of pharmaceuticals eventually leads to the contamination of surface waters environment [2]. Approximately 80–100 pharmaceuticals and their metabolites have been detected in sewage, surface water, groundwater, and drinking water worldwide [3–10].

Although pharmaceuticals are designed for specific physiological functions, they could cause unintended adverse effects on non-target organisms even at relatively small concentrations [11]. However, toxicological studies on pharmaceuticals in the environment are mostly limited to the lethal effects during acute exposures. There are information gaps that need to be filled in order to fully understand the consequences of pharmaceutical contamination in the environment and to develop appropriate

Abbreviations: CYP19, aromatase; E2, 17β-estradiol; ELISA, enzyme-linked immunosorbent assay; DMSO, dimethyl sulfoxide; H295R, human adrenocarcinoma; T, testosterone; E1, estrone.

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management plans. One such gap is effects on during chronic, sublethal exposures and potential mechanisms of effect. Effects of pharmaceuticals on steroidogenesis of non-target aquatic organisms are of interest because such disruption of endocrine function could result in effects on fecundity [12].

Because estrogenic effects of pharmaceuticals has been observed *in vitro*, which suggests the need for further *in vivo* studies on the estrogenic potential of environmental pharmaceuticals. Using a recombinant yeast system expressing human estrogen receptor α, six pharmaceuticals including cimetidine, fenofibrate, furosemide, paracetamol, phenazone and tomoxifen out of 37 pharmaceuticals tested, were found to be estrogenic [12]. Based on the E-screen assay, 11 pharmaceuticals including atenolol, erythromycin, gemfibrozil, and paracetamol of 14 that were tested were found to have the potential to interfere with the endocrine system [13]. Antibiotics, such as amoxicillin, tylosin, and oxytetracycline, can modulate gene expression and hormone production related to steroidogenesis [14]. In male Japanese medaka fish (*Oryzias latipes*), oxytetracycline and chlortetracycline induced production of vitellogenin [15,16]. There is still a lack of understanding of the mechanisms by which such alterations could be manifested. Information on the mechanisms of such effects is necessary to be able to aggregate chemicals into groups of similar mechanisms so that assessments of the mixtures can be made. In addition to acting through hormone receptor-mediated pathways, environmental chemicals can alter endocrine function by modulating production or breakdown of steroid hormones [17,18].

We chose diclofenac, erythromycin, sulfamethazine, sulfathiazole, oxytetracycline, and chlortetracycline as model chemicals based on the frequency of detection in Korean waterways [19,20], and tested them for their effects on steroidogenesis *in vitro* with human adrenocarcinoma (H295R) cells and *in vivo* with male medaka fish.

The H295R steroidogenesis assay was developed for the quantitative evaluation of xenobiotic effects on transcription of genes involved in steroidogenesis [17,18,21]. H295R cells express major key enzymes involved in the synthesis of steroid hormones, and the assay has been successfully used for the characterization of effects of chemicals on steroidogenesis. For compounds that were determined to affect steroid hormone production (testosterone (T) and 17β-estradiol (E2)), the mechanisms of steroidogenic effect were investigated by measuring changes in aromatase (CYP19) enzyme activity and expression of genes (*3βHSD2*, *CYP11β2*, *CYP17*, *CYP19*, and *17βHSD*) in the steroidogenic pathways in H295R cells. Fish were exposed to target pharmaceuticals and concentrations of E2 in blood plasma (plasma).

2. Materials and methods

2.1. Test chemicals

Test pharmaceuticals included diclofenac sodium salt (CAS No. 15307-79-6), erythromycin (CAS No. 114-07-8), sulfamethazine (CAS No. 1981-58-4), sulfathiazole sodium salt (CAS No. 144-74-1), oxytetracycline hydrochloride (CAS No. 2058-46-0), and chlortetracycline hydrochloride (CAS No. 57-62-5). All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). Each compound was dissolved in dimethyl sulfoxide (DMSO) and the final DMSO concentration in the exposure medium was less than 0.1% (v/v).

2.2. H295R cell culture

H295R cells (CRL-2128) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured at 37 °C in a 5% CO_2 atmosphere. H295R cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 Nutrient mixture (DMEM/F12) (Sigma D-2906; Sigma–Aldrich) supplemented with 1% ITS + Premix (BD Biosciences, San Jose, CA, USA), 2.5% Nu-Serum (BD Biosciences), and 1.2 g/l Na_2CO_3 (Sigma–Aldrich). Medium was changed every 4 d, and cell subculture was performed every 7 d.

2.3. Hormone measurement of H295R cell

T and E2 were measured following methods described by Hecker et al. [21] and He et al. [22]. H295R cells were seeded into 24-well plates at a concentration of 3×10^5 cells/ml in 1 ml of medium per well. After 24 h, cells were exposed to pharmaceuticals at concentrations ranging from 0.02 to 20 mg/l for 48 h. Cells were inspected under a microscope for viability and cell number. In instances where exposure resulted in cell viability less than 85%, the cells were not used for assays that evaluated hormone production, aromatase activity and gene expression [14,23]. The culture medium was collected and kept frozen at −80 °C. Frozen medium was thawed on ice, and 500 μl medium was extracted twice with 2.5 ml diethyl ether. The solvent phase containing target hormones was evaporated under a stream of nitrogen, and the residue was reconstituted in 300 μl enzyme-linked immunosorbent assay (ELISA) buffer (Cayman Chemical, Ann Arbor, MI, USA) and frozen at −80 °C for subsequent analysis. Medium extracts were diluted 1:75 and 1:1 for T and E2, respectively. Hormones were measured by competitive ELISA following the manufacturer's recommendations (Cayman Chemical; Testosterone [Cat # 582701], 17β-Estradiol [Cat # 582251]).

2.4. Aromatase activity assay of H295R cell

Aromatase enzyme activity was measured by use of the method described by He et al. [22] with some modifications. Direct and indirect effects on aromatase activity were evaluated for pharmaceuticals that caused significant changes in hormone production [23]. To measure direct effects of chemicals on aromatase activity, cells were treated with a range of concentrations (0.02–2 mg/l) of each target pharmaceutical in the medium that contained 54 nM 1β-^{3}[H]-androstenedione (PerkinElmer, Boston, MA, USA) with no pre-exposure to a target pharmaceutical. In order to measure indirect effects of chemicals on aromatase activity, H295R cells were initially exposed to various concentrations of each pharmaceutical for 48 h, after which the cells were washed twice and incubated with 0.25 ml of serum-free medium that contained 54 nM 1β-^{3}[H]-androstenedione. After 1.5 h incubation at 37 °C and 5% CO_2, the cells were placed on ice to stop the reaction. A 200 μl aliquot of medium was removed and added to chloroform and dextran-coated charcoal to remove all remaining 1β-^{3}[H]-androstenedione. Aromatase activity was determined by the rate of conversion of 1β-^{3}[H]-androstenedione to estrone. The quantity of ^{3}H in extracts of medium was determined by liquid scintillation counter (Beckmann LS6500, Beckmann Coulter Inc., Fullerton, CA, USA). Forskolin (0.1, 1, and 10 μM) was used as a positive control for aromatase induction, while prochloraz (0.1, 1, and 10 μM) was used as a negative control for aromatase catalytic inhibition.

2.5. Quantitative PCR assay of H295R cell

Transcription of mRNA of five steroidogenic genes plus one housekeeping gene (*β-actin*) were measured in cells exposed to pharmaceuticals that caused significant changes in hormone production by the method outlined by Hilscherova et al. [17]. Briefly, 1 ml cell suspension (initial cell density of 3×10^5 cells/ml) was transferred to each well of a 24-well culture plate. After 24 h, cells were exposed to chemicals for another 48 h, and then total RNA

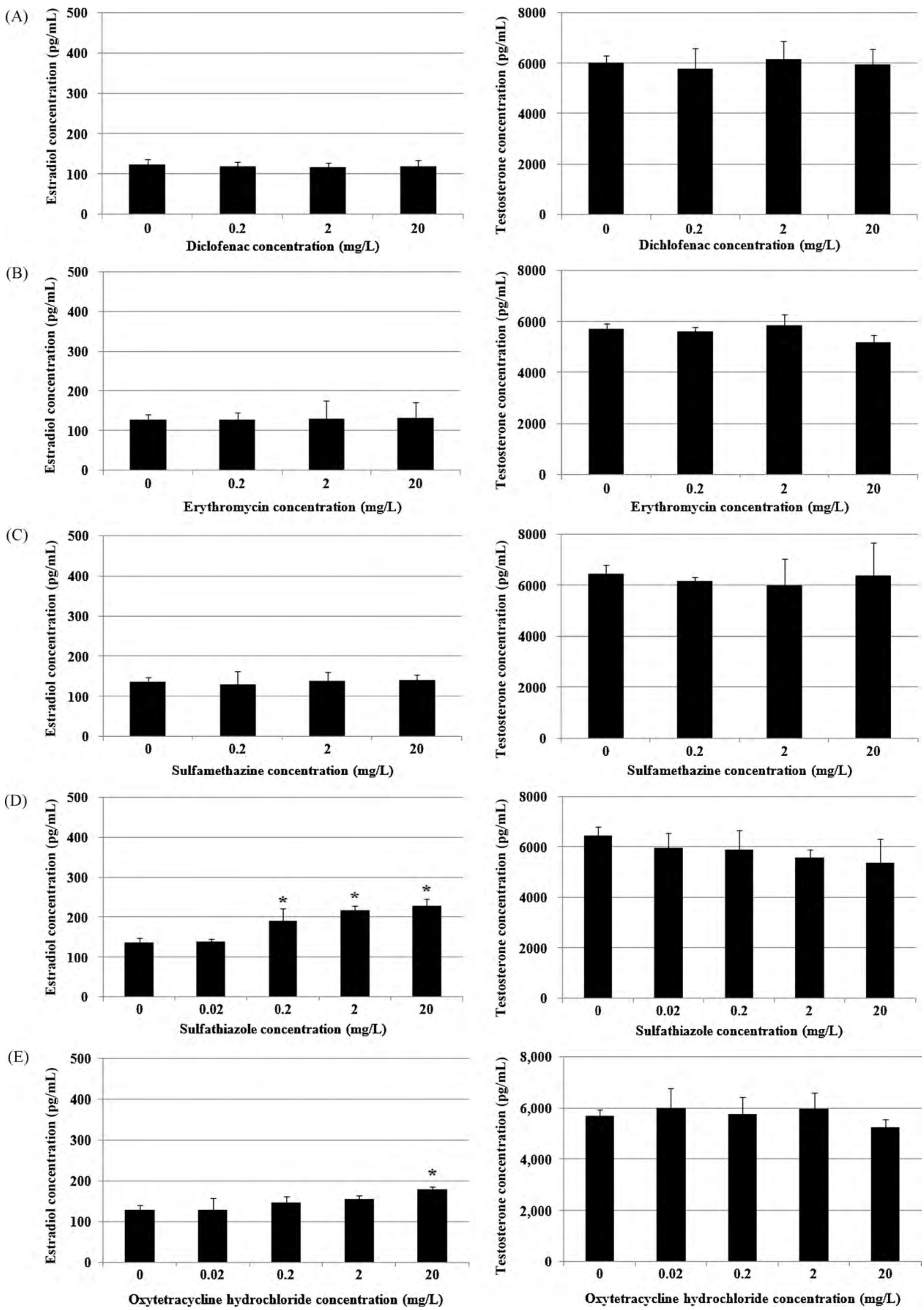

Fig. 1. Effect of diclofenac (A), erythromycin (B), sulfamethazine (C), sulfathiazole (D), oxytetracycline hydrochloride (E), and chlortetracycline (F) on hormone release by H295R cells. Cells were treated for 48 h with the indicated concentrations of pharmaceuticals. Hormone values represent the mean ± standard deviation. Significant differences for estradiol and testosterone (*) are reported relative to the solvent control.

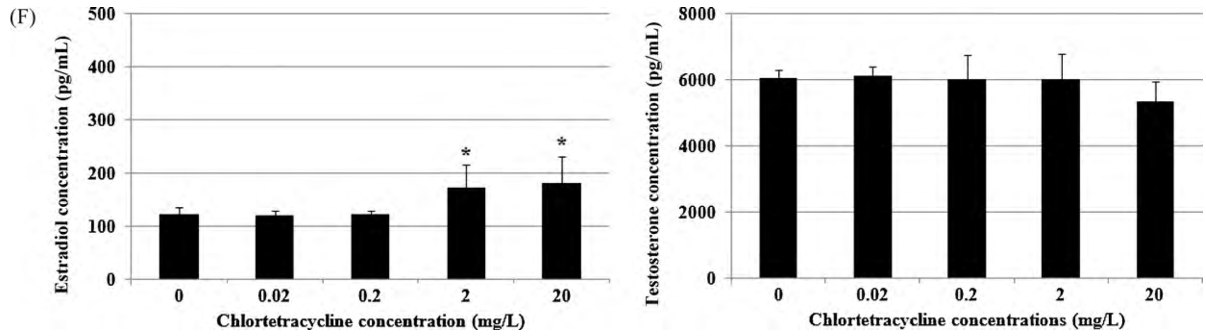

Fig. 1. (*Continued*).

was isolated from each well using a total RNA isolation kit (Agilent Technologies, Ontario, CA, USA). Two micrograms of cellular total RNA for each sample was used for reverse transcription using the iScript™ cDNA Synthesis Kit (BioRad, Hercules, CA, USA). The ABI 7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) was used to perform quantitative real-time PCR. PCR reaction mixtures (15 μl) contained 0.3 μl (0.2 μM) of forward and reverse primers [18], 3 μl of cDNA sample, and 7.5 μl of 2X SYBR Green™ PCR Master Mix (Applied Biosystems). The thermal cycle profile was: denaturing at 95 °C for 10 min, followed by 40 cycles of denaturing for 15 s at 95 °C, annealing with extension for 1 min at 60 °C; and a final cycle of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. Melting curve analyses were performed during the 60 °C stage of the final cycle to differentiate between desired PCR products and primer–dimers or DNA contaminants.

Expression of mRNA was quantified by use of the threshold cycle, *C*t method. *C*t values for each gene of interest were normalized to β-actin. Normalized values were used to calculate the degree of induction or inhibition and expressed as a “fold difference” compared to normalized control values. Therefore, all data were statistically analyzed as “fold induction” between the exposed and control cultures. Gene expression was measured in triplicate for each treatment and control, and each exposure was repeated at least three times.

2.6. Fish culture and exposure

Japanese medaka were maintained at 25 ± 1 °C in the Environmental Toxicology Laboratory at Seoul National University (Korea) since 2003. The fish were maintained under a 16:8 h light:dark photoperiod and fed with *Artemia* nauplii (<24 h after hatching) twice daily.

To investigate the effects on concentrations of E2 in plasma, three groups of three male medaka fish (3–4 mo old) were exposed to pharmaceuticals that were identified to alter steroidogenesis in H295R cells. The chosen pharmaceuticals included chlortetracycline (0, 4, and 40 mg/l), oxytetracycline (0, 5, and 50 mg/l), and sulfathiazole (0, 50, and 500 mg/l). The exposure duration was 14 d, during which the fish were fed *Artemia* nauplii (<24 h after hatching) *ad libitum* twice daily. Exposure medium was renewed at least three times per week. After 14 d, all surviving fish were anesthetized with ice-cold water. The tail of each medaka was transected, and blood was collected in a glass capillary tube. Five microliter bloods was pooled from three fish, and placed in a 1.5 ml microcentrifuge tube for hormone analysis.

2.7. E2 measurement in fish blood

Concentrations of E2 in plasma were determined in male medaka using a commercially available assay kit. Briefly, 5 μl of plasma was extracted with 1 ml diethyl ether, and the solvent phase containing target hormone was evaporated under a stream of nitrogen. The residue of the organic phase was dissolved in 150 μl of extraction buffer and blood E2 concentration was measured by ELISA kit (Cayman Chemical, Ann Arbor, MI, USA). Absorbance of each sample at 415 nm was measured using Tecan Infinite 200 (Tecan, Männedorf, Switzerland). The blood E2 concentration of each sample was determined by comparing with standard curve.

2.8. Statistical analysis

One-way analysis of variance (ANOVA) with Dunnett’s test was performed using SPSS 15.0 for Windows® (SPSS, Chicago, IL, USA) to test for differences among all treatments. Differences with $p < 0.05$ were considered significant.

3. Results

3.1. Hormone production of H295R cell

Significantly greater concentrations of E2 relative to the controls were observed when H295R cells were exposed to 0.2 mg/l sulfathiazole, 20 mg/l oxytetracycline, or 2 mg/l chlortetracycline (Fig. 1). The rest of pharmaceuticals did not cause any significant effects on E2 concentrations compared to that of control. None of the pharmaceuticals caused any significant effect on T production in the range of concentrations tested.

3.2. Aromatase activity of H295R cell

A 48 h exposure to sulfathiazole, oxytetracycline, or chlortetracycline resulted in significant increases of aromatase activity compared to that of the control (Fig. 2). Significantly greater activities of aromatase were observed when H295R cells were exposed to 0.02 mg/l sulfathiazole, 2 mg/l oxytetracycline, or 0.2 mg/l chlortetracycline. However, in the direct aromatase assay, the enzyme activity did not change.

3.3. mRNA expression assay of H295R cell

Treatment of H295R cells with each of the three pharmaceuticals resulted in significant changes in the expression of several mRNAs (Fig. 3). In cells exposed to sulfathiazole, transcript for *CYP17* and *CYP19* did significantly increase. Exposure to oxytetracycline or chlortetracycline resulted in up-regulation of expression of both *CYP19* and *3βHSD2* mRNAs compared to control.

3.4. Effect on blood E2 concentration of male medaka fish

Concentrations of E2 in plasma were significantly greater (1.56, 2.06, and 1.55 ng/ml) after the exposure to 50 or 500 mg/l of sulfathiazole, or 40 mg/l of chlortetracycline, respectively (Fig. 4).

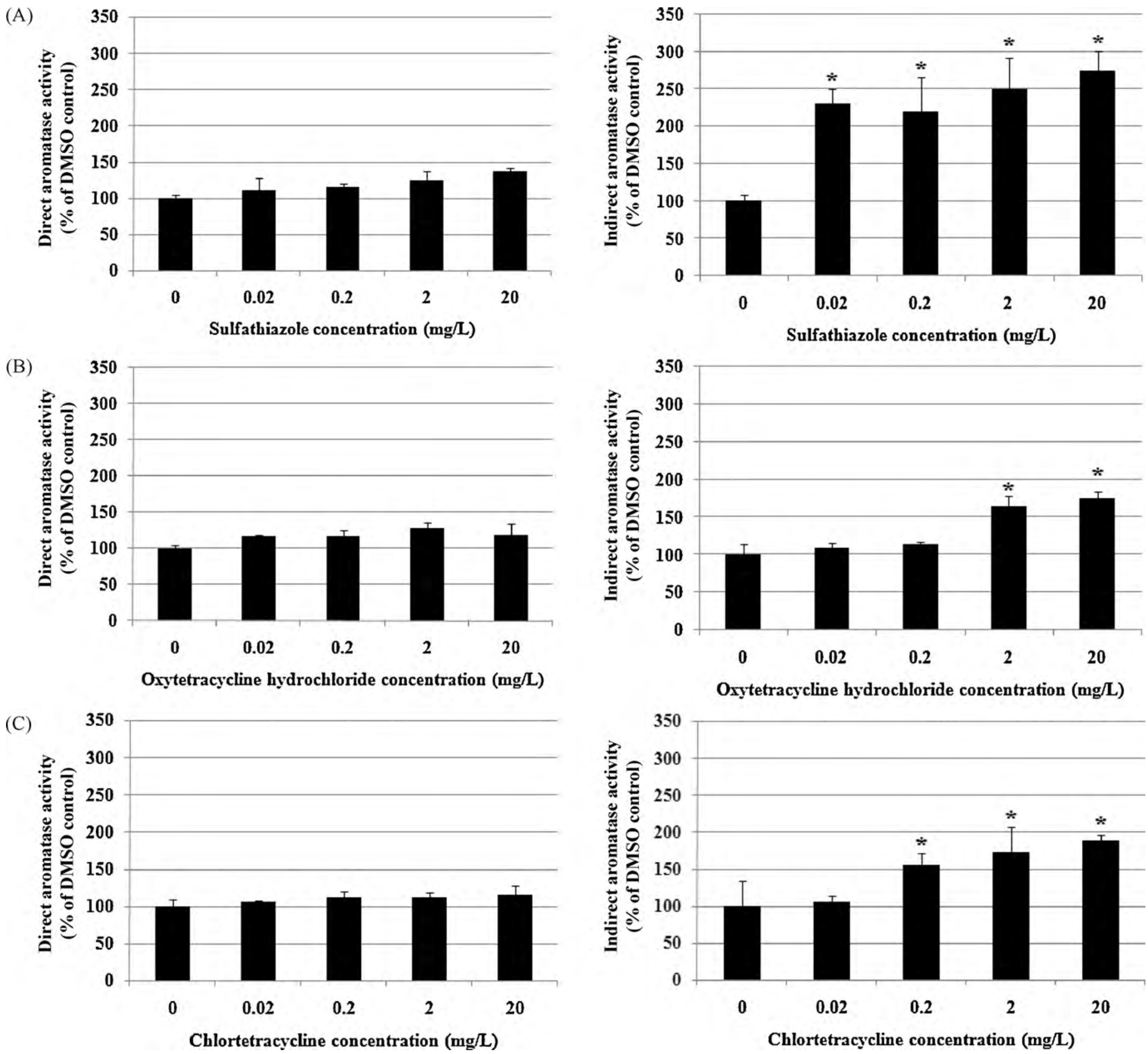

Fig. 2. Induction of aromatase activity by sulfathiazole (A), oxytetracycline (B), and chlortetracycline (C). The induction level represents the aromatase activity with respect to the 0.1% DMSO solvent control. Values presented are the means of triplicate measurements, and asterisks indicate activities that are statistically different from the DMSO solvent control ($p < 0.05$).

These values were 1.32, 1.75, and 1.31 times greater than that of control (1.18 ng/ml).

4. Discussion

Among the six pharmaceuticals, sulfathiazole, oxytetracycline, or chlortetracycline enhanced E2 production *in vitro* in H295R cells and *in vivo* in male medaka fish. This observation was consistent with increased aromatase activity and enhanced expression of *CYP17*, *CYP19*, and *3βHSD2* mRNAs of H295R cells. Aromatase (CYP19) is a cytochrome P450 enzyme that converts androstenedione into estrone (E1), and T into E2, which are two major estrogens in humans. In this study, 48 h pre-exposure to sulfathiazole, oxytetracycline, and chlortetracycline increased aromatase activity. This observation suggests that exposure to sulfathiazole, oxytetracycline, or chlortetracycline might increase the catalytic activity of aromatase indirectly, potentially through transcriptional level modulation [23]. Even though the significant dose-dependent pattern of decrease was not observed for T in this study, a decreasing trend was observed in cells exposed to the greatest concentrations of sulfathiazole, oxytetracycline, or chlortetracycline. Changes in expression of mRNA were consistent with this proposed mechanism of a transcriptional level modulation (Fig. 3 and Fig. 5). Exposure to sulfathiazole, oxytetracycline, or chlortetracycline resulted in greater expression of *CYP17*, *CYP19*, or *3βHSD2*, which play crucial role in steroidogenic pathways. *CYP17* is responsible for conversion of pregnenolone to 17α-OH-prognenolone to dihydroepiandrosterone (Fig. 5). This enzyme is also involved in conversion of progesterone to 17α-OH-progesterone to androstenedione (Fig. 5). Up-regulation of the *CYP17* gene expression may shift the steroidogenic process to the production of androgenic substrates leading to the formation of T and E2 [24]. *CYP19* catalyzes both the conversion of androstenedione into E1, and T into E2. *3βHSD2* is responsible for conversion of pregnenolone to progesterone, of 17α-OH-pregnenolone to 17α-OH-progesterone, and of dihydroepiandrosterone to androstenedione.

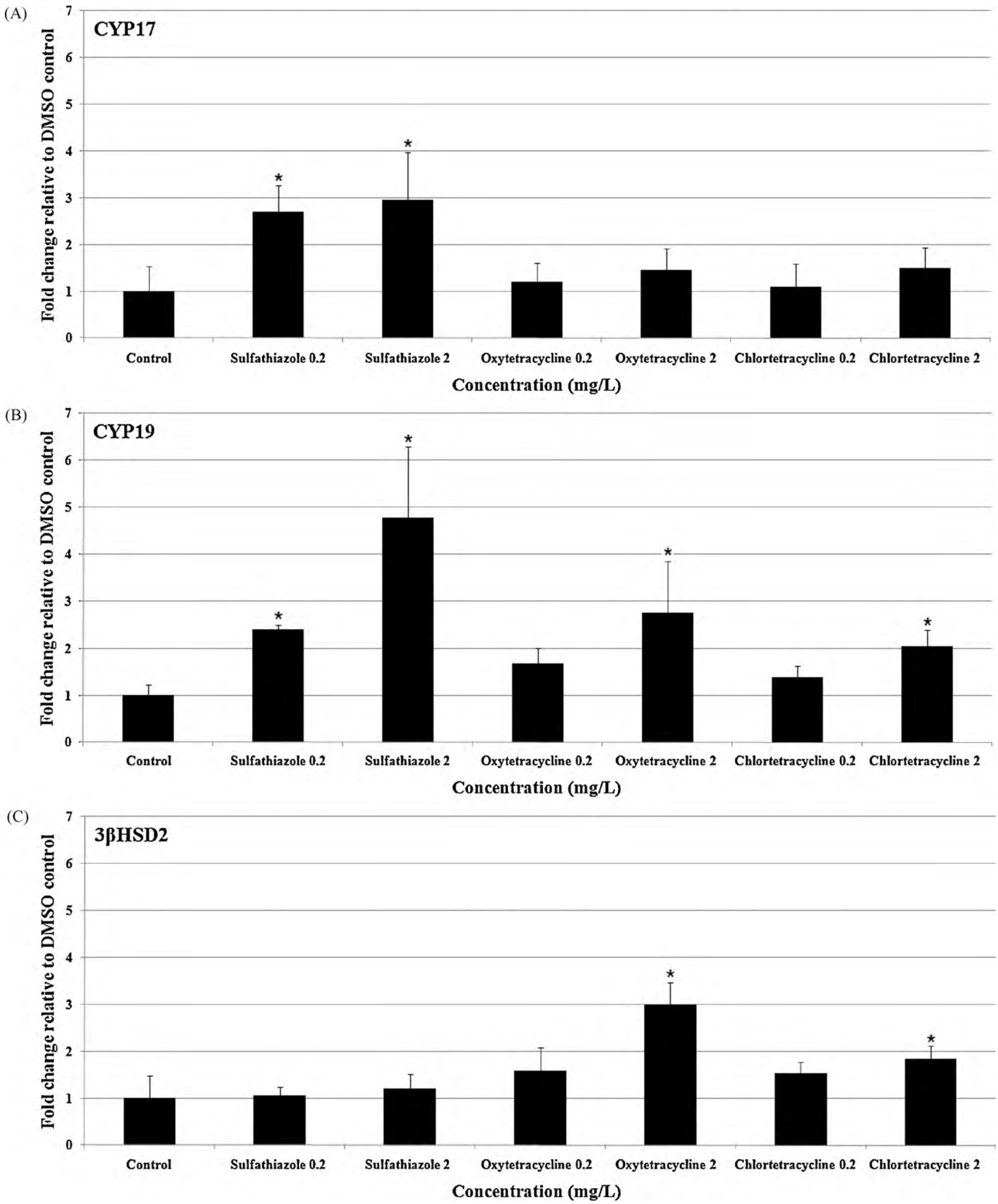

Fig. 3. Expression of *CYP17* (A), *CYP19* (B), *3βHSD2* (C), *CYP11β2* (D), and *17βHSD* (E) mRNAs in steroidogenic pathways of H295R cells which were exposed to various concentrations of sulfathiazole, oxytetracycline, and chlortetracycline. Fold-changes relative to DMSO control are shown as mean ± standard deviation. * indicates a statistically significant difference at $p < 0.05$.

Among the pharmaceuticals tested, sulfathiazole was the most potent in terms of altering hormone production, aromatase activity, and expression of steroidogenic mRNAs in H295R cells. The least concentration of sulfathiazole that caused effects in H295R cells was 0.2 mg/l, while those for oxytetracycline and chlortetracycline were 2 mg/l (Fig. 1). In the fish test, sulfathiazole also exhibited the greatest potency in terms of changes in concentrations of E2 in plasma of male medaka (Fig. 4).

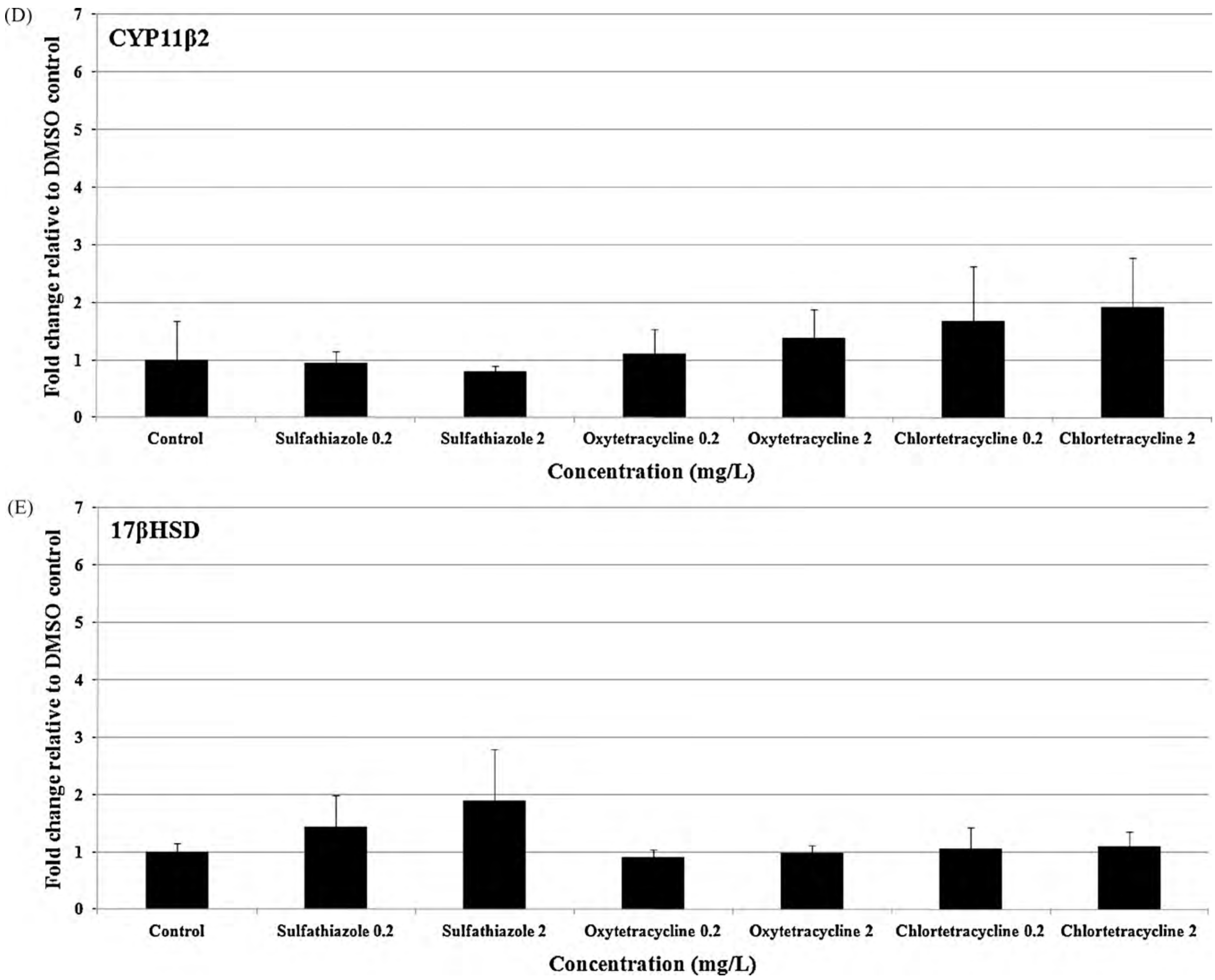

Fig. 3. (*Continued*).

The greater production of E2 by H295R cells (Fig. 1D) is consistent with up-regulation of aromatase activity (Fig. 2A) and changes in expression of *CYP17* and *CYP19* mRNAs (Fig. 3A and B). Changes in mRNA expression caused by sulfathiazole might eventually lead to the increased production of E1 and possibly E2.

Greater production of E2 by H295R cells after the exposure to oxytetracycline or chlortetracycline could be caused by

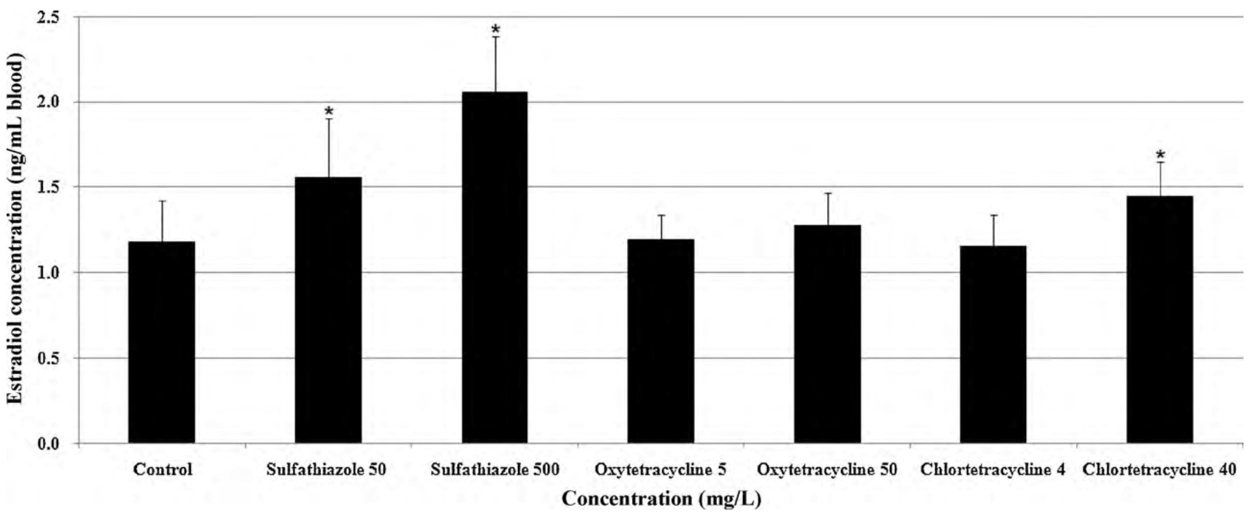

Fig. 4. Increase of blood E2 concentrations in male Japanese medaka after the 14-d exposure to sulfathiazole, oxytetracycline, and chlortetracycline. Values presented are the means of triplicate measurements, and asterisks indicate statistically significant difference from the control ($p < 0.05$).

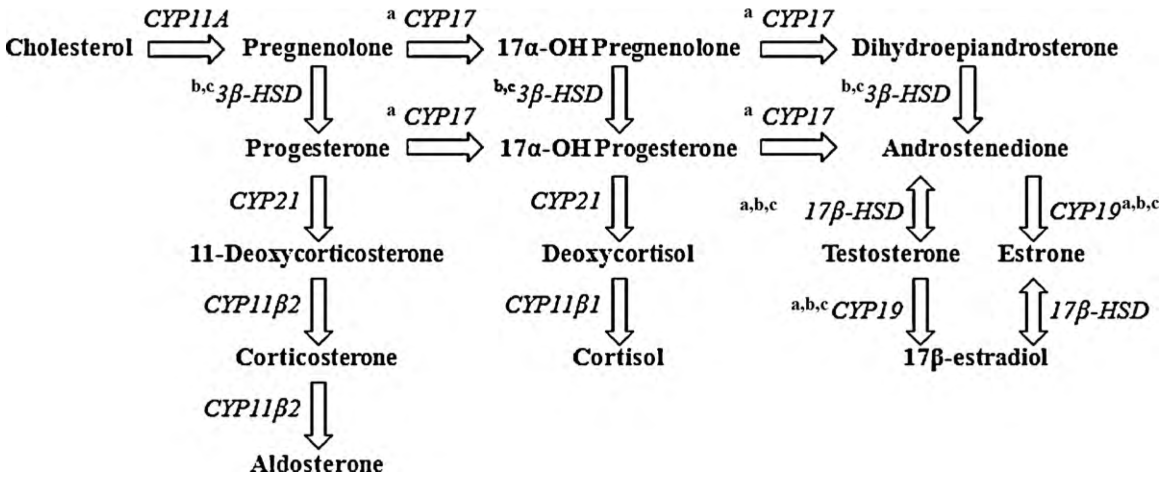

Fig. 5. Steroidogenic pathways in H295R cells that were altered by test pharmaceuticals. Enzymes are in *italics*, hormones are in **bold**, and *arrows* indicate the direction of synthesis. a, b, and c demonstrate points in the steroidogenic pathway where sulfathiazole, oxytetracycline, and chlortetracycline, respectively, have been shown to interfere with normal progression of the steroidogenesis pathway. 3β-HSD-Δ5-Δ4-isomerase = 3β-HSD. C21hydroxylase = CYP21. P450-aromatase = CYP19. 17α-hydroxylase and C17, 20-lyase = CYP17. 11β-hydroxylase = CYP11B. Hydroxysteroiddehydrogenase = HSD.

up-regulation of *CYP19* and *3βHSD2* expression. In addition, concentrations of E2 in plasma of male Japanese medaka greater when they were exposed to 40 mg/l chlortetracycline (Fig. 4). The greater E2 production in H295R cells (Fig. 1E and F) was consistent with greater aromatase enzyme activity (Fig. 2B and C) and changes in expression of *CYP19* and *3βHSD2* mRNAs (Fig. 3B and C).

Alteration in steroidogenesis could lead to changes in fecundity of aquatic species [12,25]. Reproduction-related effects of pharmaceuticals have been reported for *Daphnia magna* and Japanese medaka: oxytetracycline and chlortetracycline appeared to have an estrogenic potential that could eventually cause disruption of the endocrine system in medaka fish [15,16,26]. In addition, sulfathiazole, oxytetracycline, and chlortetracycline affected several reproduction-related endpoints such as the first day of reproduction, number of young per female, and population growth rates in water flea [27]. The results of our 14-d fish exposure correspond well with the previous reports. Further investigation is needed to whether the hormonal level changes in fish could be explained by altered steroidogenesis as suggested in the human adrenal cell line.

The antibiotics studied here have been frequently detected in ambient water worldwide, but the concentrations are small relative to the concentrations associated with the effects measured in this study. In a survey for antibiotic residues in six rivers of Australia, sulfathiazole, oxytetracycline and chlortetracycline were detected up to 0.04, 0.10, and 0.60 μg/l, respectively [28]. In Korea, nationwide monitoring for four major rivers during 2006–2008 period showed that sulfathiazole, oxytetracycline, and chlortetracycline were detected on average 0.10, 0.0039, and 0.13 μg/l, respectively [19,20,29]. These ambient concentrations are much less than the effect concentrations which were identified in the present study. Hence immediate impact on endocrine function is not likely.

Since many xenobiotic estrogen receptor agonists could be present in aquatic environment [30], however, the presence of estrogenic pharmaceuticals, even though at trace levels, should receive proper attention. Some weak estrogenic compounds were reported to produce significant mixture effects in a recombinant yeast estrogen screen even when they were combined at concentrations below no observed effect concentrations [31]. As an example, certain pharmaceuticals like ketoconazole are reported to increase the estrogenicity of 17α-ethylnylestradiol by inhibiting metabolic clearance of xenobiotics and steroids [32].

Because the steroidogenic pathways are regulated by multiple steroids and enzymes, the genes and enzymes that were measured in this study may not provide complete understanding for mechanisms of endocrine disruption due to the exposure to the target pharmaceuticals. However, the results of the present study showed that sulfathiazole, oxytetracycline, and chlortetracycline could affect several points of steroidogenic pathways at transcriptional level, and influence also enzyme activity and eventually the balance of sex hormones both in H295R cells and in male medaka fish. Since these pharmaceuticals are detected in relatively low levels but consistently, and given the importance of sex steroid hormones in fertility and reproduction, the consequences of steroidogenic disturbance due to the pharmaceutical contamination in the environment deserves further investigation.

Acknowledgments

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