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Effects of *p,p′*-DDE exposure on gonadal development and gene expression in Japanese medaka (*Oryzias latipes*)

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Abstract

Although 1,1-dichloro-2,2-bis(*p*-chlorophenyl)-ethylene (*p,p′*-DDE), the major and most persistent metabolite of dichlorodiphenyltrichloroethane (DDT), was continually detected in wild fishes that showed abnormal gonad development such as intersex, little is known about the impact of *p,p′*-DDE exposure on gonad development in fishes. To survey the effects of *p,p′*-DDE on gonadal development and gene expressions, male juvenile (20-d post hatch) Japanese medaka (*Oryzias latipes*) was exposed to 1, 5, 20, and 100 μg/L *p,p′*-DDE for two months. Increased hepatosomatic index (HSI) and decreased gonadosomatic index (GSI) were found in the *p,p′*-DDE-treated groups. Intersex was found in 100 μg/L *p,p′*-DDE exposure group, as well as 100 ng/L 17α-ethynylestradiol (EE2) group. By quantitative real-time RT-PCR, it was found that gene expressions of vitellogenins (*VTG-1*, *VTG-2*), choriogenins (*CHG-H*, *CHG-L*), and estrogen receptor α (*ER-α*) in the liver of the fish were significantly up-regulated by *p,p′*-DDE exposure. *VTG-1* and *VTG-2* were recommended as the preferred biomarker for assessing anti-androgenic *p,p′*-DDE because they were the highest up-regulated among the genes and showed good dose-response relationship. The up-regulated *ER-α* suggested that a potential synergetic effect would occur when *p,p′*-DDE coexists with other *ER-α*-binding endocrine-disrupting chemicals (EDCs).

Key words: *p,p′*-DDE; intersex; vitellogenin; quantitative real-time RT-PCR; *Oryzias latipes*

Introduction

In the past decade, endocrine-disrupting chemicals (EDCs) were of great public and scientific concern since these chemicals can mimic, block, or interfere with hormones in the body and can subsequently induce detrimental effects on reproductive processes in wildlife and in humans (Vos *et al.*, 2000; Fox, 2004). In recent years, abnormal gonadal development such as intersex has been increasingly observed in the wild fishes and has been commonly linked to estrogenic contaminants such as nonylphenol and estrogens, based on the fact that these chemicals had the ability to induce intersex when they were tested in the laboratory (Gray and Metcalfe, 1997; Nash *et al.*, 2004; Allen *et al.*, 1999).

1,1-Dichloro-2,2-bis(*p*-chlorophenyl)-ethylene (*p,p′*-DDE), the major metabolite of insecticide dichlorodiphenyltrichloroethane (DDT), is frequently detected in many fish populations. Wan *et al.* (2007) have recently reported that the concentration of DDTs (*p,p′*-DDE accounting for 54%–100% of the total DDTs) in muscle of wild Chinese sturgeon (*Acipenser sinensis*) in the Yangtze River was up to 15900 ng/g lipid weight, and the intersex has been reported in the population (Wei *et al.*, 1997). It was reported that the *p,p′*-DDE concentration was up to 780 ng/g ww (wet weight) in roe of shovelnose sturgeon in the Mississippi River, where intersexual characteristics were also observed in shovelnose sturgeon (Harshbarger *et al.*, 2000). Similarly, high concentrations of *p,p′*-DDE and intersexual phenomenon have been found in flounder (*Platichthys flesus*) in the estuary of the River Mersey (Scott *et al.*, 2006; Leah *et al.*, 1997), flatfish (*Limanda limanda*) in Dogger Bank, North Sea (Stentiford and Feist, 2005), and largemouth bass (*Micropterus salmoides*) in the rivers of the Columbia River Basin (Schmitt *et al.*, 2005). The concentrations of *p,p′*-DDE detected in wild fishes are almost higher than 63.6 μg/L, which is the concentration of *p,p′*-DDE required to inhibit the transcriptional activity of the androgen receptor in monkey kidney CV-1 cell (Kelce *et al.*, 1995), implying that there is a potential risk to induce intersex due to exposure of wild fish to *p,p′*-DDE. Unfortunately, to the best of our knowledge, no laboratory study was reported to demonstrate the possibility of intersex inducement by *p,p′*-DDE.

In toxicological processes, alterations of gene expression are responsible for the conventional histological responses (Moggs, 2005; Fielden and Zacharewski, 2001). Some genes such as vitellogenin (*VTG*), choriogenin (*CHG*), and steroid-hormone receptors have been widely used as marker genes to evaluate the estrogenic EDCs (Sugiyama *et al.*, 1999; Rankouhi *et al.*, 2004; Zhang *et al.*, 2005). *p,p′*-DDE is commonly considered a potent androgen-receptor antagonist but xenoestrogens because

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p,p′-DDE inhibit androgen binding to androgen receptors of fish, rat, and human and has little ability to bind to the estrogen receptor (Bayley *et al.*, 2002; Kelce *et al.*, 1995; Wells and Van Der Kraak, 2000). Some studies have found that *VTG* and *CHG* were up-regulated by *p,p′*-DDE in largemouth bass (Larkin *et al.*, 2002), whereas several papers reported that *VTG* and *CHG* were not induced by *p,p′*-DDE treatment in flounder *Paralichthys dentatus* (Zaroogian *et al.*, 2001; Mills *et al.*, 2001). The estrogenic effect of *p,p′*-DDE due to its anti-androgenic activity is still not very clear, and further study is needed in this direction.

In this study, the most popular model organism species, Japanese medaka (*Oryzias latipes*), in EDC studies (Gray and Metcalfe, 1997; Patyna *et al.*, 1999; Shima and Mitani, 2004), was used to investigate the effect of *p,p′*-DDE on gonadal development and gene expressions of *VTG-1*, *VTG-2*, *CHG-H*, *CHG-L*, estrogen receptor α (*ER*-α), *ER*-β, and androgen receptor (AR) to clarify the effects of *p,p′*-DDE on gonadal development and the reliability marker genes in assessing anti-androgenic *p,p′*-DDE.

1 Materials and methods

1.1 Chemicals

The 17α-ethynylestradiol (EE2) was purchased from Fluka Chemie AG (Buchs, Switzerland); *p,p′*-DDE from Chem Service (West Chester, PA, USA); and dimethyl sulfoxide (DMSO) from Sigma (St Louis, MO, USA).

1.2 Fish and experimental design

Juvenile Japanese medaka (*Oryzias latipes*), orange red strain, male, 20-d post-hatch (dph) old, was selected from brood stock maintained for several years in the laboratory, the Toxicology Laboratory in Peking University. Each of twelve fishes was separately exposed to 1, 5, 20, and 100 μg/L *p,p′*-DDE, with a final volume of 10 L in glass tanks, in a flow-through system with four-fold water flowing through every 24 h. Chemical-stock solutions were prepared in DMSO and the ratio of the chemical-stock solution/water was 0.005% (v/v). A 0.005% (v/v) DMSO group was used as negative control and a 100 ng/L EE2 was used as positive control in the experiment. Water used in the experiment was active carbon-treated with hardness 8.1 ± 0.1 mg/L, pH 7.7 ± 0.2, dissolved oxygen 7.8 ± 0.3 mg/L, at 25 ± 1°C. The fishes were kept under a constant 16-h light/8-h dark photoperiod and fed with live brine shrimp (*Artemia nauplii*) twice a day. After 2 months of exposure, the fishes were sampled. The weights of whole body and isolated liver and gonad were measured. The hepatosomatic index (HSI) and gonadosomatic index (GSI) were evaluated by: liver (gonad) weight/body weight × 100%. Livers were frozen immediately in liquid nitrogen until the RNA was isolated, and gonads were fixed in 10% neutral buffered formalin for histological analysis. No fish died during the course of the exposure.

1.3 Histological examination

The fixed gonad samples were dehydrated and embedded in paraffin blocks, sectioned at 5–10 μm, stained with hematoxylin and eosin (H&E), and examined by light microscopy for routine histological and morphometrical analyses. Histological measurements were taken using an ocular micrometer.

1.4 Total RNA preparation

Total RNA from 10 μg of frozen liver sample was isolated using 1 ml of Trizol reagent (Gibco BRL, Life Technologies, USA), according to the manufacture's instructions. To prevent genomic DNA contamination, the total RNA was digested by DNase I (TaKaRa Biotechnology, China) and then purified. The total RNA recovered from DNase I digestion was measured at 260 and 280 nm using a spectrophotometer. The 260 nm reading was used to estimate the concentration of total RNA. The 260-nm/280-nm ratios, as well as a 1% agarose-formaldehyde gel stained with ethidium bromide, were used to verify the RNA quality in each sample.

1.5 Quantitative real-time RT-PCR assay

Syntheses of first-strand cDNA were performed according to the methods described previously (Zhang and Hu, 2007), after the RNA was prepared. TaqMan® Reverse Transcription Reagents purchased from Applied Biosystems (USA) was used. Primers for quantification of mRNA of each gene were designed using Primer Express 2.0 (Applied Biosystems, USA), validated by Premier Primer 5.0 (PREMIER biosoft international, USA), and are shown in Table 1. To minimize the problems associated with DNA contamination, most pairs of primers were designed to span at least one intron of the genomic sequence. Quantitative real-time PCR with SYBR Green detection was performed using an ABI Prism® 7000 Sequence Detection System (Applied Biosystems, USA) according to protocols established by the manufacturer. PCR reaction mixture contained 12.5 μl of 2×SYBR® Green PCR master

Table 1 Sequence of primer for quantitative real-time PCR

Gene symbol	Full name of gene	Accession number	Sequences (up, forward primer; down, reverse primer; from 5′ to 3′)	Amplicon size (bp)
RPL-7	Ribosomal protein L7	DQ118296	AGCTCTCGGCAAATATGGCATGCAGGCTTGAAGTTCTTTCCAA	81
VTG-1	Vitellogenin 1	AB064320	ACGCAAAGAACGAACTCCTTGTCCTGGATCTTCCTTATTGGCT	86
VTG-2	Vitellogenin 2	AB074891	TGCTCAGTGCATCTTGGTCAATCTCGTTCTGTTCTGCAGTTTGG	163
AR	Androgen receptor	AB076399	ACTGCACGATCGACAAGCTCATAGTTTGCGTGCTCCGAGAGTC	95
CHG-H	Choriogenin H	AF500195	TCGGCACTGTTGTCATGGAAGAATAGCTCCAAGCGGTCCAACTC	93
CHG-L	Choriogenin L	AF500194	TCGACCAGCTGAAAACAAGCTTGTGATGCATACCATTCCACTG	82
ER-α	Estrogen receptor α	D28954	TGTAAGGTTGCGTCCCATGAGGCCCCTTTAATCCAAGAGGAAG	105
ER-β	Estrogen receptor β	AB070901	TATAGACAAGAACCGGCGCAACGAACACCGCATTTGGTCAT	84

mix (Applied Biosystems), 250 nmol/L each of forward and reverse primers, 3 μl of cDNA template, and nuclease-free water in a total volume of 25 μl. The reactants were incubated at 50°C for 2 min to activate the uracil N9-glycosylase (in SYBR® Green PCR master mix) and then for 10 min at 95°C to inactivate the uracil N9-glycosylase and activate the Amplitaq Gold® DNA polymerase (in SYBR® Green PCR master mix), followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Following the final cycle of the PCR, the reactions were denatured over a 35°C temperature gradient at 0.03°C/s from 60 to 95°C to determine the quality of PCR products. Ribosomal protein L7 (*RPL-7*) was used as the internal control (Zhang and Hu, 2007), and relative expression was evaluated by the methods described by Applied Biosystems (Foster City, USA).

1.6 Statistical analysis

The statistical program SPSS (Version 11.5; Chicago, IL, USA) was used to collate and to analyze all the collected data. Differences were evaluated by ANOVA followed by Tukey's test. Differences with $p < 0.05$ were considered to be significant.

2 Results and discussion

2.1 Hepatosomatic index and gonadosomatic index

p,p'-DDE-treated male medaka exhibited increased hepatosomatic index (HSI) and decreased gonadosomatic index (GSI). The HSIs of groups exposed to 100 ng/L EE2, 20 and 100 μg/L *p,p'*-DDE were significantly ($p < 0.05$) higher than those of control (Fig.1). The GSIs in 100 ng/L EE2, 20 and 100 μg/L *p,p'*-DDE groups showed a significant decrease ($p < 0.05$).

2.2 Intersex and gonad histology

After two-month exposure, individuals with intersex (presence of immature oocytes within the testis) gonadal morphology were observed in 100 ng/L EE2 and 100 μg/L *p,p'*-DDE groups. A typical intersex testis observed in 100 μg/L *p,p'*-DDE group is shown in Fig.2. Several oocytes are scattered in the testicular tissue, spermatozoa, spermatogonia, spermatocyte, spermatid and spermatozoa are also observed in the testicular tissue with thickened tubule walls. Most of the oocytes that occurred in the testis were primary oocytes with diameters less than 200 μm. The frequencies of intersex testis in 100 ng/L EE2 and 100 μg/L *p,p'*-DDE groups were 58.3% and 25.0%, respectively. No intersex testes were observed in fishes from the control group. In the previous studies, intersex testes in fishes have been reported to be caused by estrogenic chemicals such as EE2 and 4-nonylphenol (Nash *et al.*, 2004; Gray and Metcalfe, 1997). Our result clearly demonstrated that anti-androgenic *p,p'*-DDE can also induce intersex in medaka. This should be one of the clues in explaining the intersex observed in wild fishes (Scott *et al.*, 2006; Jobling *et al.*, 2006; Jackson *et al.*, 2006; Stentiford and Feist, 2005). In addition, thickening of tubule wall and reduction of the sperm were also observed in the *p,p'*-DDE-treated groups with a dose-dependent relationship, for which the phenomena is similar to those reported in the previous study (Bayley *et al.*, 2002; Baatrup and Junge, 2001).

2.3 Gene expression

In this study, the newly developed and validated quantitative real-time RT-PCR assays, SYBR Green detection, for *VTG-1*, *VTG-2*, *CHG-H*, *CHG-L*, *ER-α*, *ER-β*, and *AR* were used to study their expression in the livers

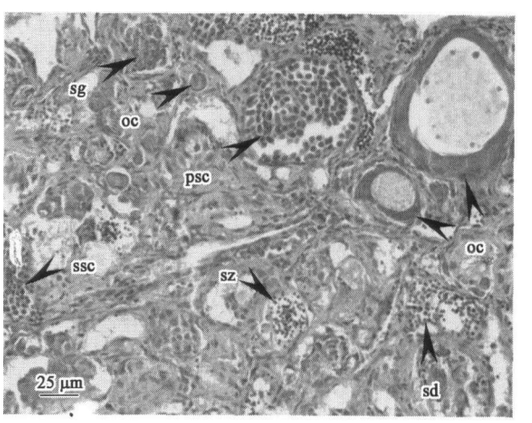

Fig. 2 Light micrograph of intersex testis in 100 μg/L *p,p'*-DDE-treated group, by paraffin slice. oc: oocytes; sz: spermatozoa; sg: spermatogonia; psc: primary spermatocyte; ssc: secondary spermatocyte; sd: spermatid; sz: spermatozoa.

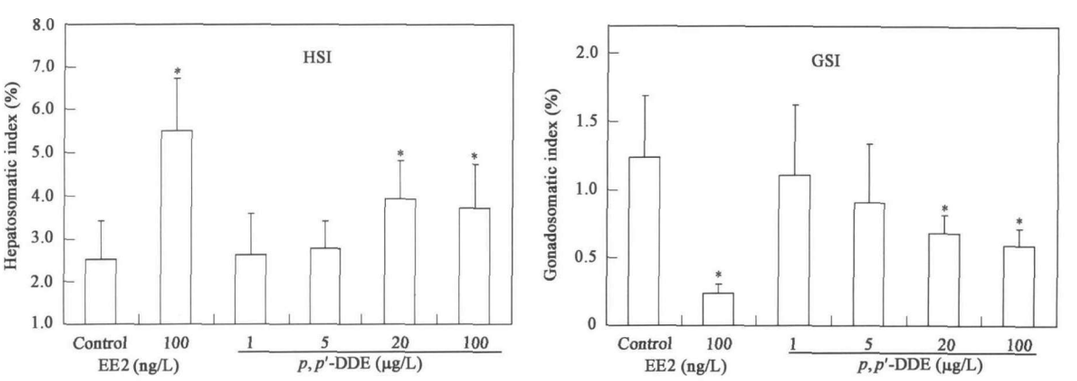

Fig. 1 Hepatosomatic index (HSI) and gonadosomatic index (GSI) of fish in control (0.005% DMSO), positive control (100 ng/L EE2), and *p,p'*-DDE (1, 5, 20, 100 μg/L) exposure groups ($n = 12$, mean ± SD). * Significance at $p < 0.05$.

of medaka. *VTG* is the most frequently used biomarker for assessing estrogenic chemicals (Zhang *et al.*, 2005; Scott *et al.*, 2006). Expressions of *VTG-1* and *VTG-2* in livers of the fish exposed to EE2 and *p,p'*-DDE are shown in Fig.3. It was found that *VTG-1* and *VTG-2* were both significantly up-regulated by *p,p'*-DDE exposure with good dose-response relationships. The mRNA levels of *VTG-1* reached 99.5 ± 86.2, 263 ± 33.6, 11,426 ± 12,107, and 96,409 ± 55,029 folds of those of control in 1, 5, 20, and 100 μg/L *p,p'*-DDE exposure groups, respectively. And those of *VTG-2* were 64.4 ± 110, 319 ± 222, 7,975 ± 10,879, and 64,692 ± 47,625 folds in 1, 5, 20, and 100 μg/L *p,p'*-DDE exposure groups, respectively. In addition, the fold changes of *VTG-1* and *VTG-2* in 100 ng/L EE2 exposure group were 220,088 ± 59,926 and 36,103 ± 8,497 folds, respectively. These results clearly demonstrated that *VTG-1* and *VTG-2* are also useful in assessing anti-androgenic chemicals by *in vivo* experiment using medaka after long-term exposure.

Choriogenins (*CHG-H*, *CHG-L*) are precursor proteins of egg envelope of medaka and are synthesized in the spawning female liver in response to estrogen, which have been also proved as biomarkers for monitoring estrogenic EDCs in the aquatic environment (Sugiyama *et al.*, 1999). We found that *CHG-H* was up-regulated by *p,p'*-DDE with a dose-dependant relationship (Fig.4). The relative expressions of of *CHG-H* in 1, 5, 20, and 100 μg/L *p,p'*-DDE exposure groups were 4.76 ± 3.71, 11.9 ±14.1, 20.4 ± 11.7, and 61.6 ± 22.4 folds to those of control, respectively. *CHG-L* was also found to be up-regulated, but a "U" shaped dose-response curve was found as shown in Fig.4. The relative expressions of *CHG-L* in 1, 5, 20, and 100 μg/L *p,p'*-DDE exposure groups were 20.4 ± 6.16, 6.99 ± 3.05, 2.09 ± 1.54, and 6.60 ± 3.89 folds, respectively. The up-regulation of *CHG-H* and *CHG-L* were consistent with the result that has been reported in largemouth bass that received a single intraperitoneal injection of 100 mg/kg *p,p'*-DDE (Larkin *et al.*, 2002). Although *CHG-H* and *CHG-L* were significantly up-regulated by by *p,p'*-DDE, the expression changes were much lower than those of and *VTG-2*, suggesting that *VTG* should be the preferred marker gene for evaluating the endocrine disruption by *p,p'*-DDE.

The expressions of *ER*-α, *ER*-β, and *AR* genes were also investigated. It was found that *ER*-α was significantly up-regulated in all the *p,p'*-DDE exposed groups with a good dose-response relationship (Fig.5). The relative mRNA levels of *ER*-α were 13.3 ± 10.3, 61.9 ± 25.4, 203 ± 117, and 808 ± 670 folds in 1, 5, 20, and 100 μg/L *p,p'*-DDE exposed groups, respectively. It is interesting that the mRNA expression levels of *ER*-α in 20 and 100 μg/L *p,p'*-DDE groups were both higher than those in the 100 ng/L EE2 group (197 ± 57.9). *ER*-α is known as the dominant estrogen receptor present in the liver of medaka and is involved in activating estrogen-responsive genes, such as *VTG* and *CHG* (Green and Chambon, 1996; Scholz *et*

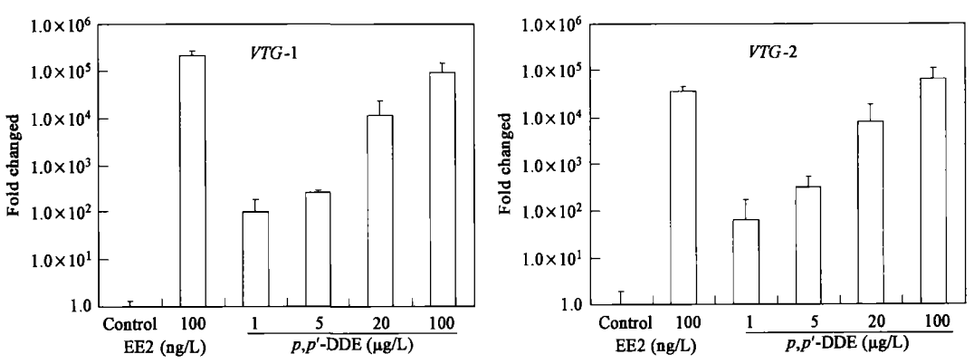

Fig. 3 Relative gene expressions of *VTG-1* and *VTG-2* in livers of fishes from control (0.005% DMSO), positive control (100 ng/L EE2), and *p,p'*-DDE (1, 5, 20, 100 μg/L)-treated groups by quantitative real-time RT-PCR, SYBR Green detection. (n = 6, mean ± SD).

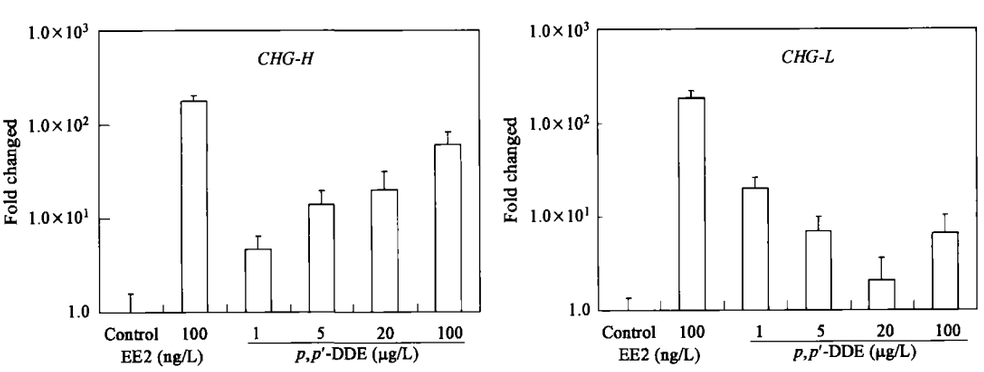

Fig. 4 *CHG-H* and *CHG-L* gene expressions in livers of fishes from control (0.005% DMSO), positive control (100 ng/L EE2), and *p,p'*-DDE (1, 5, 20, 100 μg/L)-treated groups by quantitative real-time RT-PCR, SYBR Green detection. (n = 6, mean ± SD).

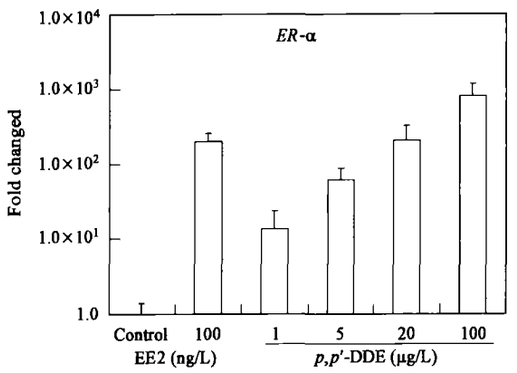

Fig. 5 *ER*-α gene expression in livers of fishes from control (0.005% DMSO), positive control (100 ng/L EE2), and *p,p'*-DDE (1, 5, 20, 100 μg/L)-treated groups by quantitative real-time RT-PCR, SYBR Green detection (n = 6, mean ± SD).

al., 2004). The largely up-regulated *ER*-α observed in the *p,p'*-DDE exposure groups suggested that there would be more *ER*-α in the liver, which could be bound by internal or external estrogenic chemicals, thereby resulting in an increase in the feminization effect. *ER*-β was found to be expressed at relatively lower levels compared with *ER*-α, but no obvious change was found after *p,p'*-DDE exposure. And *AR* was found show low expression in the liver of medaka, but was not affected by the *p,p'*-DDE exposure.

3 Conclusions

This study clearly demonstrated that anti-androgenic *p,p'*-DDE could induce intersex testis in Japanese medaka at 100 μg/L level after two-month exposure. *VTG-1* and *VTG-2* were both found to be significantly up-regulated by *p,p'*-DDE with dose-dependant relationships, suggesting they can also be used as biomarkers for anti-androgenic *p,p'*-DDE. *ER*-α was found to be significantly up-regulated by *p,p'*-DDE exposure, suggesting that there would be a would be a synergetic effect when *p,p'*-DDE coexists with other *ER*-α binding EDCs such as NP and estrogen.

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