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Evaluating estrogenic and anti-estrogenic effect of endocrine disrupting chemicals (EDCs) by zebrafish (*Danio rerio*) embryo-based vitellogenin 1 (*vtg1*) mRNA expression

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ABSTRACT

By measuring the vitellogenin 1 (*vtg1*) expression through quantitative PCR and *in situ* hybridization, we used the zebrafish embryo as an *in vivo* model to access the estrogenic or anti-estrogenic effects of several endocrine disrupting chemicals (EDCs), such as natural estrogen 17β-estradiol (E2), estriol (E3), synthetic hormones including diethylstilbestrol (DES), 4-octyl phenol (OP), bisphenol A (BPA), tamoxifen (TMX) and 3-(2,3-dibromopropyl) isocyanurate (TBC). According to our data, the estrogenic effect of the tested chemicals was ranked as: DES > E_2 > E_3 > OP > BPA, which is consistent with various *in vivo* and *in vitro* models. Therefore, the measurement of *vtg1* gene expression in zebrafish embryos would be a valuable method for screening EDCs including both environmental estrogens and anti-estrogens.

1. Introduction

Endocrine disrupting chemicals (EDCs) are a class of exogenous substance or mixture that alter the normal function of the endocrine system (Diamantikandarakis et al., 2009). Nowadays EDCs are of great concern to the health issue of human and wildlife. Be natural or man-made, increasing numbers of chemicals with estrogenic or anti-estrogenic effects have been defined as EDCs by using various *in vivo* or *in vitro* models. 17β-estradiol (E_2) is a natural steroid estrogen with strong estrogenic activity to male zebrafish and rainbow trout (*Oncorhynchus mykiss*) juveniles (Van den Belt et al., 2003; Pawlowski et al., 2000). It has been widely used as a positive control compound in evaluating the estrogenic effects of some new pollutants. Estriol (E_3), metabolite of estradiol and estrone, is also a natural estrogen in human body, which could bind to and acts as a weak agonist of estrogen receptor (ER) (Russo et al., 2001; Head, 1998). As a synthesized non-steroidal estrogenic substance, diethylstilbestrol (DES) has been proved to seriously threat human health through the food chain enrichment (Stefanick, 2005; Yin et al., 2006). With toxicity to development of fetus' reproductive organs, DES could increase the risk of reproductive system cancer during adulthood (Li et al., 2003; Hendry et al., 2006). 4-octylphenol (OP) as a representative EDCs with estrogenic activity was reported to stimulate the proliferation of MCF-7 cell (White et al., 1994). With both weak estrogenic activity and strong anti-androgenic activity (Sohoni and Sumpter, 1998), bisphenol A (BPA) was reported to cause metabolic disorders in various organisms, by interacting with ER (Richter et al., 2007), thyroid hormone receptor (Moriyama et al., 2002) and peroxisome proliferator-activated receptor (Riu et al., 2011). Additionally, BPA exposure was identified to alter the early dorsoventral patterning, segmentation, and brain development in zebrafish embryos (Tse et al., 2013). Tamoxifen (TMX) as a pharmaceutical antagonist of the estrogen could competitively bind to ER in breast cells, playing an important role in the treatment of certain breast cancer and ovarian cancer (Wallen et al., 2005). Tris-(2, 3-dibromopropyl) isocyanurate (TBC), which is released into the environment during the use of flame retardants, is a potential EDC with anti-estrogen activity (Ruan et al., 2009; Zhang et al., 2011). The toxicity of EDCs and related molecular mechanism have been explored in multiple studies. Cultured

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cell lines have been widely used, while *in vivo* models could better reflect the physiological and functional changes caused by EDCs. However, traditional animal models are costly, time-consuming, may have some ethic problems and individual differences (Kuhn et al., 2003; Legler et al., 2002). Therefore, it is necessary to develop a fast approach which could accurately identify EDCs and elucidate the potential molecular mechanism of these pollutants.

There are several advantages by using zebrafish (*Danio rerio*) embryos in scientific studies. Due to the embryo transparency, zebrafish embryos are easily visualized and operated. It is also convenient to utilize their comprehensive heredity feature and different development stages for biological research (Lam et al., 2005). Vitellogenin (VTG) is the yolk precursor protein of vitellin (vn) in oviparous animals like zebrafish, which is predominantly synthesized in liver of mature female fish. VTG expression is regulated by luteinizing hormone and follicle-stimulating hormone, also specifically by estrogens and anti-estrogens (Okuzawa, 2002). Previous studies have reported that VTG protein could be induced in male and larval fish in response to exogenous estrogen (Mitsui et al., 2003). Thus, VTG can be a good biomarker of estrogen exposure (Sumpter and Jobling, 1995). Additionally, vitellogenin 1 (*vtg1*) gene expression is the highest among the seven forms of *vtg* genes (*vtg1-7*) in zebrafish genome and has been proposed to be an indicating molecule for estrogenicity (Muncke and Eggen, 2006; Wang et al., 2005). The relative expression pattern of *vtg1* gene can be induced by exogenous estrogens in adult male and larval zebrafish (Henry et al., 2009). For zebrafish embryos, *vtg1* transcript usually occurs at 24 h post-fertilization (hpf) and at all later time points. It can be significantly up-regulated by 17α-ethinylestradiol, which also appears early at 24 hpf (Muncke and Eggen, 2006). Additionally, though *vtg1* mRNA expression in adult zebrafish and adult zebrafish embryos has been used to test the estrogenic effects of emerging chemicals (Chow et al., 2013; Muncke and Eggen, 2006), assessment of the anti-estrogenic effect of certain compound by this model is so far lacking.

This study primarily aimed to assess both the estrogenic or anti-estrogenic effect of certain compounds by measuring zebrafish embryo-based *vtg1* mRNA levels. Multiple EDCs including E_2, E_3, DES, OP, BPA, TMX and TBC were investigated by single exposure as well as co-exposure experiment using zebrafish embryo. *Vtg1* mRNA expression measurement assay clearly indicate an estrogenic response and anti-estrogenic response by the testing chemicals. The analysis was furthermore verified through a comparison among different *in vitro* and *in vivo* experiment models. Therefore, our approach could be considered as an economic and promising evaluation for rapid testing and screening of multiple EDCs.

2. Materials and methods

2.1. Test compounds

E_2 (≥ 98% purity), E_3 (≥ 97% purity), DES (≥ 99% purity), OP (99% purity), BPA (≥ 99% purity), TMX and TBC (97% purity) were purchased from Sigma-Aldrich (St Louis, MO, USA). All chemicals were dissolved in dimethyl sulfoxide (DMSO, Amresco, Solon, OH, USA) and diluted into fish water.

2.2. Zebrafish embryo and exposure experiment

Zebrafish (wild-type, AB strain) were obtained from China Zebrafish Resource Center (http://zfish.cn; Wuhan, China). The fish were raised in a closed flow-through system with fresh water at 26 ± 1.5 °C under a 14 h light/10 h dark cycle, maintained according to the Zebrafish Book (Westerfield, 2000).

Zebrafish embryos were exposed to E_2, E_3, DES, OP and BPA since 4 hpf for 7 days at series of concentrations as following: 0.05, 0.1, 0.25, 0.5 and 1 μM of E_2; 0.1, 0.25, 0.5, 1 and 2.5 μM of E_3; 0.05, 0.1, 0.25, 0.5 and 1 μM of DES, 0.1, 0.25, 0.5, 1, 2.5 and 5 μM of OP; 0.5, 1, 2.5, 5 and 10 μM of BPA. Co-exposure of TMX (0.5 and 1 μM) and E_2 (0.5 μM) as well as TBC (1 and 5 μM) and E_2 (0.5 μM) were also performed at the same time point. Additionally, zebrafish embryos were exposed to 0.5 μM E_2 at several exposure window including 4 to 28 hpf (1d), 4 to 52 hpf (2d), 4 to 76 hpf (3d), 4 to 100 hpf (4d), 4 to 124 hpf (5d), 4 to 148 hpf (6d), 4 to 172 hpf (7d). 0.5% DMSO was used as vehicle control in each exposure experiment.

2.3. RNA extraction and real-time PCR (RT-PCR)

Total RNA was extracted from forty pooled zebrafish embryos in each group by using Trizol reagent (Invitrogen), following the manufacturer's instructions. The yield and purity of extracted total RNA was determined by UV spectrophotometry (A260 and A260/A280 ratio). 2 μg of total RNA was used to synthesize cDNA by M-MLV (Moloney murine leukemia virus) RNase H reverse transcriptase (Promega, Fitchburg, WI, USA). Each PCR reaction mixture contained 1 μL cDNA template, 0.1 μmol/L primer, 7 μL water, and 10 μL 2 × SYBR QPCR Master Mix (Toyobo, Osaka, Japan). Reactions were performed in the Bio-Rad iQ5 thermal cycler equipped with a real-time fluorescence detector. The thermal cycling program consisted of a denaturing step (95 °C, 3 min) followed by 40 cycles of denaturation (95 °C for 10 s), annealing (55 °C for 10 s), and extension (72 °C for 20 s). The PCR primers were designed with Primer Premier 5.0 (Premier, Palo Alto, CA, USA): *vtg1* forward 5′-AGCTGCTGAGAGGCTTGTTA-3′; vtg1 reverse 5′-GTCCAGGATTTCCCTC AGT-3′; β-actin forward 5′-GTCACACCATCA CCAGAGTCCATCAC-3′; β-actin reverse 5′-CAACAGAGAGAAGATGAC ACAGATCA-3′. β-actin was used as the internal standard. The target gene (*vtg1*) expression level was calculated by $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and normalized by β-actin.

2.4. In situ hybridization

Whole-mount *in situ* hybridization was performed using digoxigenin (DIG)-labeled antisense RNA probes and an anti-DIG alkaline phosphatase-conjugated antibody as described (Thisse et al., 2004). The *vtg1* probe was synthesized by T3 polymerase (Promega, Fitchburg, WI, USA) using *vtg1* (GenBank Accession Number of NM_001044897) PCR product as the templates, which were amplified from cDNA of 500 nM E_2 exposed zebrafish embryo using a set of primers 5′-CAGCAGTCG TAACAGTCGC-3′ and 5′-GATCCATTAACCCTCACTAAAGGGAACTCCG CACC CCAAGAAA-3′, which contained T3 promoter sequence (underlined).

2.5. Statistical analyses

Statistical analyses were performed in SPSS 16.0 for Windows (IBM Corp. (2006), Somers, NY, USA). Values are presented as group means ± standard error (SE). One-way analysis of variance (ANOVA) was used to analyze the significant difference between the control and treatment groups, with *post hoc* analysis by Fisher's least significant difference (LSD) test. P values < 0.05 were considered statistically significant, P values < 0.01 were considered very significant.

3. Results

3.1. Dose-dependent expression of endogenous vtg1 gene after E_2 exposure in the zebrafish embryos

To determine the response of the *vtg1* gene to E_2 exposure at the zebrafish embryos levels, we primarily detected the transcriptionally expression levels of *vtg1* by RT-PCR analysis. By referring to the data from a previous study (Wang et al., 2011), the zebrafish embryos were exposed to various concentrations of E_2 for 7 days. A dose-dependent increase of *vtg1* mRNA-expression occurred in zebrafish embryos following E_2 exposure (Fig. 1A). The relative mRNA levels of *vtg1* in the