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Effect of acetochlor on transcription of genes associated with oxidative stress, apoptosis, immunotoxicity and endocrine disruption in the early life stage of zebrafish

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ABSTRACT

The study presented here aimed to characterize the effects of acetochlor on expression of genes related to endocrine disruption, oxidative stress, apoptosis and immune system in zebrafish during its embryo development. Different trends in gene expression were observed after exposure to 50, 100, 200 μg/L acetochlor for 96 h. Results demonstrated that the transcription patterns of many key genes involved in the hypothalamic-pituitary-gonadal/thyroid (HPG/HPT) axis (e.g., *VTG1*, *ERβ1*, *CYP19a* and *TRα*), cell apoptosis pathway (e.g., *Bcl2*, *Bax*, *P53* and *Cas8*), as well as innate immunity (e.g., *CXCL-C1C*, *IL-1β* and *TNFα*) were affected in newly hatched zebrafish after exposure to acetochlor. In addition, the up-regulation of *CAT, GPX, GPX1a, Cu/Zn-SOD* and *Ogg1* suggested acetochlor might trigger oxidative stress in zebrafish. These finding indicated that acetochlor could simultaneously induce multiple responses during zebrafish embryonic development, and bidirectional interactions among oxidative stress, apoptosis pathway, immune and endocrine systems might be present.

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1. Introduction

The endocrine disrupting chemicals (EDCs) contamination of aquatic ecosystems has become a concern worldwide because of their detrimental effects on the endocrine system of aquatic lives (Colborn et al., 1993; Mills and Chichester, 2005). Many pesticides have been identified as EDCs, could simultaneously induce endocrine disruption, oxidative stresses, cell apoptosis or immunotoxicity in aquatic organisms (Chang et al., 2012; Garanzini and Menone, 2015; Jin et al., 2013; Tangtian et al., 2012; Tu et al., 2013; Valavanidis et al., 2006). The bidirectional interactions between these pathways have been noted previously. Evidences showed that the free radicals induced by oxidative stress can act as signal molecules to trigger apoptotic cell death and inflammatory response, and also have a detrimental effect on steroidogenesis (Livingstone, 2001; Naik and Dixit, 2011; Murugesan et al., 2005; Stocco et al., 1993). Meanwhile, cell apoptosis could destroy immune cells and reduce immune defense ability, and some contaminants affecting the immune system could cause endocrine disruption in fish (Battaglia et al., 2010; Roh et al., 2011; Milla et al., 2011). However, the precise biochemical basis for the bidirectional interactions among oxidative stress, cell apoptosis, immune and endocrine systems has not been fully elucidated.

Acetochlor is used globally for pre-emergent control of grasses and broadleaf weeds in agricultural environments (Lengyel and Foldenyi, 2003; Ye, 2003). Although acetochlor has low acute toxicity (Ashby et al., 1996), the United States Environmental Protection Agency (USEPA) and European Union have declared it to be a probable human carcinogen and a suspected endocrine disruptor in a wide range of vertebrates. Studies have showed that the measurable concentration of acetochlor ranged from 0.05 μg/L to 2.5 μg/L in surface water (Kolpin et al., 1996; Boyd, 2000; Hladik et al., 2008). With the increasing use of acetochlor, its concentration in contaminating surface water exceeded the USEPA maximum contamination levels and EU allowed environmental levels (Konda and Pásztor, 2001). Moreover, acetochlor is high stability and persistence in aquatic environments (Foley et al., 2008; Zheng and Ye, 2003), thus, increasing concerns should been developed regarding the effect of a large dose of acetochlor and its possible toxic effects upon non-target organism.

Previous studies have given much attention to the effect of acetochlor on the thyroid system in aquatic organisms. It has been reported that acetochlor can alter thyroid hormone related gene

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expression or accelerate 3,5,3′-triiodothyronine (T3)-dependent metamorphosis in *Rana pipiens*, *R. catesbeiana*, *Gobiocypris rarus* and *Xenopus laevis* (Cheek et al., 1999a; Crump et al., 2002; Helbing et al., 2006; Li et al., 2009; Veldhoen and Helbing, 2001). Furthermore, acetochlor exposure induces oxidative stress and DNA damage in *Bufo raddei* tadpole liver (Liu et al., 2006), causes cytotoxicity, apoptosis in human alveolar A549 cells (Zerin et al., 2015). However, little is known about the effect of acetochlor on the immune system and hypothalamic-pituitary-gonadal (HPG) axis, and the bidirectional interactions of oxidative stress, cell apoptosis, immune and endocrine systems in aquatic organisms are largely unknow.

Fishes are directly exposed to toxic chemicals in aquatic environment, considerable amount of studies showed that zebrafish is a popular vertebrate model in the evaluation of environmental toxicology because of its short lifecycle, well established genetic and genomic tools and transparency in early life stages (Behra et al., 2004; Chakraborty et al., 2009; McGrath and Li, 2008; Peterson et al., 2008; Yang et al., 2009). In addition, the embryo developmental stage is considered to be sensitive to environmental contaminants, and also provide the possibility to perform small-scale, high-throughput analyses (Scholz et al., 2008). Therefore, the objective of this study was to characterize and quantify ontogenetic changes in the transcriptional level of genes related to endocrine disruption, oxidative stress, cell apoptosis and immune system during the early developmental stage of zebrafish. The results obtained from this study will enable a better understanding of bidirectional interactions of these pathways and the molecular mechanism of acetochlor-induced toxic response.

2. Materials and methods

2.1. Chemicals

Acetochlor (technical grade AI: 93%) was purchased from Shandong Qiaochang Chemical Co., Ltd. Acetochlor was dissolved in N,N-Dimethylformamide (DMF). The stock solution (100,000 mg/L) was stored in the dark at 4 °C. Trizol reagent, reverse transcriptase kit and the SYBR green system were obtained from Takara (Dalian, China). All other chemicals used in this study were analytical grade.

2.2. Experimental fish

Adult wild type zebrafish (AB strain) were introduced from China Zebrafish Resource Center (Wuhan, China) and acclimatized at a constant temperature (27 ± 1 °C.) with a 14 h:10 h light/dark cycle in a flow-through system containing charcoal-filtered water. Zebrafish were fed twice daily with freshly hatched brine shrimp. Embryos were selected and staged using standard procedures as previous described by Westerfield (1993) for the subsequent experiments.

2.3. Exposure experiments and sample collection

Embryos were collected and exposed to various concentrations (50, 100, 200 μg/L) of acetochlor for 96 h. The control embryos were exposed to water with only solvent, both the exposure and control group received 0.001% (v/v) DMF. Thirty fertilized embryos were randomly placed in 6-well plates using a pipette, each 6-well plate was considered as one replicate for each exposure and three replicates were performed for each treatment. During the experiment, the plates were incubated under the ambient temperature (28 ± 1 °C) with a photoperiod of 14 h light and 10 h dark. The embryos were observed twice daily, and dead embryos were immediately removed. At least three-quarters of the exposure solutions were renewed daily to maintain the appropriate concentrations of acetochlor and water quality. After 96 h exposure to different concentrations of acetochlor, about fifteen newly hatched zebrafish were randomly sampled per treatment for gene expression analysis.

2.4. Gene expression analysis

Total RNA from each treatment was extracted from the fifteen homogenized zebrafish larvae using Trizol reagent (Takara, Dalian, China). The RNA samples were dissolved in ribonuclease-free water, the optical density OD_{260}/OD_{280} ratio and banding patterns on 1% agarose gel were used to evaluate the quality of RNA samples. Reverse-transcription (RT) reactions to synthesize cDNA were carried out using a reverse transcriptase kit (Takara, Dalian, China). Quantitative real-time PCR amplifications were performed on a Real-time PCR system (Biorad, CA, USA) using the SYBR green system (Takara, Dalian, China).

In order to evaluate the effect of acetochlor on endocrine disruption, oxidative stress, cell apoptosis, and immune system, the mRNA level of genes involved in the hypothalamic-pituitary-gonadal/thyroid (HPG/HPT) axis, such as VTGs (*VTG1*, *VTG2*), estrogen receptors (*ERα*, *ERβ1* and *ERβ2*), P450 aromatase (*CYP19a*), thyroid hormone receptors (*TRα* and *TRβ*), deiodinases (*Dio1* and *Dio2*), and thyroid stimulating hormone (*Tshb*), were determined in the present study. In addition, the transcription of genes encoding antioxidant proteins, including Cu/Zn-superoxide dismutase (Cu/Zn-SOD), manganese superoxide dismutase (Mn-SOD), catalase (CAT), peroxidases (GPX, GPX1a) and DNA repair gene *Ogg1*, as well as *P53*, murine double minute 2 (*Mdm2*), apoptotic protease activating factor-1 (*Apaf1*), B-cell lymphoma/leukaemia-2 gene (*Bcl2*), Bcl2 associated X protein (*Bax*), Bcl2 binding component-3 (*Bbc3*), Caspase 8 (*Cas8*) and Caspase 9 (*Cas9*) in the apoptosis pathway were investigated. Moreover, the dose-related changes in the expression of several cytokine and chemokine genes related to the innate immune system, such as tumor necrosis factor α (*TNFα*), interleukin-1 beta (*IL-1β*), interferon 1 (*IFN*), interleukin-8 (*IL-8*), *CXCL-C1C* and CC chemokine (*CCL1*), were also examined. The corresponding primer sequences are listed in Table 1. The quantitative RT-PCR amplifications were performed with the following parameters: denaturation for 3 min at 95 °C, followed by 40 cycles at 95 °C for 15 s, 57 °C for 15 s and 72 °C for 20 s to quantify the fluorescence at a temperature above the denaturation of primer dimers. Once the amplifications were completed, melting curves were obtained to identify the PCR products. The transcription of β-actin was considered as the housekeeping gene, each mRNA level was expressed according to its ratio to β-actin mRNA. The experiment was repeated thrice, and the relative quantification of gene expression among the treatment groups was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.5. Statistical analysis

Significant differences between the gene expressions for the control and experimental groups were assessed by the one-way analysis of variance (ANOVA) and Tukey's test using SPSS 16.0 (SPSS, Chicago, USA). All values were expressed as the mean ± standard error (SE). Values were considered statistically significant when p was less than 0.05 or 0.01.

3. Result

3.1. Effect on the transcription of genes involved in the HPG and HPT axis

The mRNA level of the VTG genes (*VTG1*, *VTG2*), estrogen receptors (*ERα*, *ERβ1* and *ERβ2*) and P450 aromatase gene (*CYP19a*)

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