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Metabolomics and transcriptomics reveal the toxicity of difenoconazole to the early life stages of zebrafish (*Danio rerio*)

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ABSTRACT

Difenoconazole is widely used to inhibit the growth of fungi, but its residue in the water environment may threaten ecosystem and human health. Here, ^{1}H nuclear magnetic resonance (NMR) and LC–MS/MS based metabolomics and transcriptomics approaches were used to assess the response of zebrafish to difenoconazole exposure. Early life stages of zebrafish were exposed to difenoconazole at environmentally relevant concentrations for 168 h. Their comparison with the control group suggested an adverse development and disturbance of steroid hormones and VTG. KEGG pathway analysis identified five biological processes on the basis of differentially expressed genes (DEGs), as well as altered metabolites and amino acids in zebrafish following difenoconazole exposure. These affected processes included energy metabolism, amino acids metabolism, lipid metabolism, nucleotide metabolism, and an immune-related pathway. Collectively, these results bring us closer to an incremental understanding of the toxic effects of difenoconazole on zebrafish in its early development, and lend support to the continued use of the early life stages of zebrafish as a classical model to evaluate underlying environmental risks of xenobiotics in aquatic organisms.

1. Introduction

Difenoconazole, as a lanosterol-14α-demethylase (a cytochrome P-450 enzyme CYP51) inhibitor, is widely used in agriculture and horticulture because of its broad-spectrum, high efficiency, and long-lasting effects at inhibiting fungal growth (Fletcher et al., 2000; Pavanello et al., 2015; Vanden Bossche et al., 1990). However, with greater application of this fungicide, the concentrations of difenoconazole in the aquatic environment can exceed those found in the farmed crops (Zhang et al., 2011). For example, the residue level of difenoconazole has been detected at 1.98 mg/L in paddy water (Zhang et al., 2011), at 0.028 mg/L in an agriculutral water area (Satapornvanit et al., 2004), and at 0.15 μg/L in surface water (Schäfer et al., 2011), drawing considerable attention to its possible impact on aquatic organisms.

Previous studies have reported that difenoconazole inhibited the growth and weight of adult zebrafish and induced mortality, teratogenic effects and behavior abnormity in embryos (Mu et al., 2013). Then the significant increased triglyceride content and altered expression of genes related to hatching, retinoic acid metabolism and lipid homeostasis in zebrafish embryos were induced by difenoconazole (Mu et al., 2016). Researches indicated that difenoconazole can influence the cholesterol synthesis of zebrafish and that it can disturb the balance of lipid metabolism in fish (Dong et al., 2016; Mu et al., 2015b). Mu et al. also demonstrated that the oxidative stress, cell apoptisis, and inflammatory reactions were induced in zebrafish adults and embryos following exposure to difeonconazole(Mu et al., 2015a). Additionally, an *in vitro* experiment revealed that difenoconazole – an endocrine-disruption chemical – is capable of inhibiting aromatase activity in H295R human adrenocortical carcinoma cells (Sanderson, 2006; Sanderson et al., 2004). In vivo, difenoconazole influnced the homeostasis of sex steroid hormones in parental zebrafish and caused deterimental response in its offspring (Teng et al., 2017a). Nevertheless, few studies have tried to explain the integrated toxicity of difenoconazole on aquatic organisms, or sought to comprehensively evaluate its adverse health risks, through the application of biological system approaches.

Omics techniques are now a powerful tool for unravelling the complex toxic effects of chemical contaminants on the health of organisms as based on different levels of biological profling; the tool also provides integrated information for the purposes of environmental risk

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assessment and chemical toxicity evaluation (Beger et al., 2009; Meyer et al., 2013; Santos et al., 2010). Specifically, a high-throughput dataset is generated by the combined practical approaches of metabolomics and transcriptomics. Hence, significant variations of endogenous metabolites and differentially expressed genes (DEGs) in particular tissues (or in the whole organism) are detected, and then used to identify potential biomarkers to discover relevant biological pathways of interest (Zhang et al., 2013b). Furthermore, by using both metabolomics and transcriptomics, we could better understand the complex toxicology mechanisms of environmental pollutants (Santos et al., 2010).

In many studies, the early life stages of zebrafish were used as a chemical mode, because this species not only has rapid life cycle and high fecundity, but also is quite sensitive to environmental toxicants (Sipes et al., 2011; Volz et al., 2011). Further, the transparent embryos of zebrafish allow for greater insight into the physiological and biochemical processes of development. According to current legislation, the zebrafish embryo reduce the stress of the animal and minimize its suffering (Parliament et al., 2010; Strähle et al., 2012). It is not surprising that, as a classical model organism, the zebrafish embryo could be used for investigating in detail the alterations of biological processes following continous exposure to environmental contaminants and evaluating their potential health and environmental risks (Hill et al., 2005; Kimmel et al., 1995; Osterauer and Köhler, 2008).

The purpose of this study was to reveal, through transcriptomics and metabolomics, the complex toxicity of difenoconazole and its underlying toxicology mechanism in the early life stages of zebrafish. To our best knowledge, this is the first study to investigate the toxic effects of difenoconazole via a systematic 'omics' approach in the early life stages of zebrafish.

2. Materials and methods

2.1. Reagents

The *cis*-trans-3-chloro-4-(4-methyl-2-(1H-1,2,4-triazol-yl methyl)-1,3-dioxolan-2-yl) phenyl 4-chlorophenyl ether (difenoconazole, CAS: 119446-68-3; 96% purity) was purchased from the China Ministry of Agriculture. Difenoconazole was dissolved in acetone at 1×10^4 mg/L and stored at 4 °C. Other reagents used in this study were of analytical grade.

2.2. Zebrafish maintenance and difenoconazole exposure

Four-month-old zebrafish (AB strain, *Danio rerio*) were purchased from the Beijing Hongdagaofeng Aquarium Department. The zebrafish were cultured at 28 °C under a photoperiod of 14 h:10 h light/dark in a flow-through system (Esen Corp, Beijing, China) for two weeks, during which time they were fed hatched *Artemia nauplii* (Snoweagle, Tianjin, China) twice daily. All embryos for study were obtained following a previously established method (Mu et al., 2015a). Experiments were according to current Chinese legislation and were approved by the independent animal ethics committee at China Agricultural University. Three-hundred fertilized embryos were randomly placed into beakers containing 1 L of 0 (control; CK), 0.5, 5, 50, and 500 μg/L (0, 1.23×10^{-3} μM, 1.23×10^{-2} μM, 1.23×10^{-1} μM, 1.23 μM) difenoconazole solutions with six replicates: 100 for metabolomics analysis, 100 for amino acids determination, 30 for hormones assay, 30 for transcriptomic analysis, 30 for RNA extraction, 10 for observing daily and recording the mortality, hatchability, heartbeat, and malformation by light micropy and Aigo GE-5 digital microscope (Aigo, Beijing, China), respectively. The final content of acetone was 0.005% in all five groups. During the 168-h exposure period, the exposure media were renewed every day. After the exposure period, all samples were frozen in liquid nitrogen and stored at −80 °C until they were analyzed.

2.3. Determination of difenoconazole in exposure solutions

The water samples for each replicate for difenoconazole-treatments were analyzed at 0 h, 24 h, respectively. The samples preparation and analytical method of difenoconazole were performed with previous study (Teng et al., 2017b).

2.4. Determination of vitellogenin (VTG), 17β - estradiol (E2), and testosterone (T) levels in zebrafish embryo

After the 168-h exposure period, 30 zebrafish embryos for each treatment (n = 3 replicates) were homogenized in 0.25 mL 0.9% physiological saline solution for the hormones assay. Samples were centrifuged at 3000 g, 4 °C for 30 min, and the supernatant was stored at −80 °C for further determination of VTG (Trans Genic Inc, Cat NO. KH075), E2 (USA, Abcam, Cat NO.ab108667), T (USA, Abcam, Cat NO.ab108666), via an enzyme-linked immunosorbent assay (ELISA) kit. All quantification procedures followed the manufacturer's protocols.

2.5. Transcriptomic analysis

Transcriptomic analysis was done in the embryo groups that were exposed to 0, 50 and 500 μg/L difenoconazole, because 50 and 500 μg/L difenoconazole concentrations elicted an adverse effect on the zebrafish heartbeat at 48 and 72 hpf (hours post-fertilization), versus the control group. Thirty embryos (three replicates) were homogenated and the total RNA was isolated by using a TRIzol regent (Tiangen, Beijing, China) following the manufacturer's instructions. The purity and concentrations of the total RNA were measured by a NanoDrop 2000c spectrophotometer (Thermo Scientific Wilmington, DE). Only when the A260/A280 ratio of the RNA samples was in the 1.9 ~ 2.1 range they could be used for further analysis.

The length of total RNA fragment were detected by Agilent 2100 (California, USA). When the RNA samples met the criteria, they would be pooled, added to fragmentation buffer, synthesized cDNA and then sequenced. Lower quality reads were removed after passage through the Illumina (HiSeqTM2500) sequencer. The pass-filter reads were aligned against the zebrafish genome assembly with the Danio rerio GRCz10.84 by using the Tophat2 software (Cunningham et al., 2015). The FastQC, as a tool, was evaluated the sequencing quality control. Our results indicated that single base position of the sequencing error rate was less than 0.1%. The total mapped reads exceed 70% and multiple mapped reads were lower than 10%, which were used to aligned RNA-seq data. Reads were operated through a gene structure and gene expression-level analysis (FPKM > 1) that used the DESeq (Anders and Huber, 2010). Hence, differentially expressed genes (DEGs) were identified between control and treated groups for using in the further analyses ($|\log2(\text{FoldChange})| > 1$ and q-value < 0.05, hypergeometric test). All reads data were publicly deposited in NCBI Sequence Read Archive (SRA) database (Acession Number: SRP115388).

2.6. Quantitative real-time polymerase chain reaction assay (qRT-PCR)

To verify the transcriptomic results, quantitative real-time polymerase chains reaction (qRT-PCR) assays were applied to six target genes (Table S1). These genes are known for their involvement in lipid metabolism (*pla2 g*, phosphatidylcholine 2-acylhydrolase), energy metabolism (*egfra*, epidermal growth factor receptor alpha; *galβ1*, ganglioside sialidase beta-1), amino acid metabolism (*mlck*, myosin light chain kinase), an immune- related pathway (*il22ra2*, interleukin-22 receptor subunit alpha-2), and cytochrome P450 (*cyp2r1*, cytochrome P450 2R1). The synthesis of first strand cDNA was performed with FastQuant RT kits (with gDNase) following the manufacturer's instructions (Tiangen Biotech, Beijing, China). The qRT-PCR assay was followed the SYBR Green PCR Master Mix reagent kits protocol