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Imazalil exposure induces gut microbiota dysbiosis and hepatic metabolism disorder in zebrafish

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ABSTRACT

The fungicide imazalil (IMZ) is used extensively to preserve freshness, prevent decay and control fungal infections in fruits, vegetables or other plants. Recently, some studies have reported that the real in aquatic systems have reached very high levels. Here, male adult zebrafish were exposed to 100 and 1000 μg/L IMZ for 1, 7, 21 days, and the gut microbiota and hepatic metabolism were evaluated. Exposure to a high concentration of IMZ for 21 days decreased mucin secretion in the gut. Sequencing of the V3–V4 region of the bacterial 16S rRNA gene revealed a significant increase in the diversity of gut microbiota in male zebrafish. At the phylum level, the composition of *Proteobacteria* and *Bacteroidetes* was decreased, while those *Fusobacteria* and *Firmicutes* increased in the gut after exposure to 1000 μg/L IMZ for 21 days. At the genus level, 29 species of microorganisms were significantly changed after IMZ exposure. Based on GC/MS metabolomics analysis, 101 metabolites were observably significantly altered in the 1000 μg/L IMZ-treatment group. These changed metabolites were mainly associated with the pathway of glycolysis, amino acid metabolism, and lipid metabolism. In addition, the transcription of some genes related to glycolysis and lipid metabolism, including *Aco*, *Cpt1*, *Acc1*, *Srebp1a* and *Fas*, was decreased significantly in the liver of zebrafish when exposed to 100 and 1000 μg/L IMZ for 7 or 21 days. These results indicated that exposure to IMZ could cause gut microbiota dysbiosis and metabolic disorders in adult zebrafish.

1. Introduction

In the past few decades, fungicides have been widely used in agriculture and industry for economic benefit. In agriculture, most fungicides are mainly used to preserve freshness, prevent decay and control fungal infections in fruits, vegetables or other plants. Imazalil (1-[2-(2,4-dichlorophenyl)-2(2-propenyloxy)ethyl]-1H-imidazole, IMZ) can inhibit fungal cell wall synthesis by interfering with a specific cytochrome P450 enzyme (Zega et al., 2009). As a highly effective fungicide, IMZ is widely used to protect vegetable and fruit plantations or post-harvest crops from rot and to increase the lifetime of produce on the market (Faniband et al., 2015). Because of its widespread use, IMZ could be detected in vegetables and fruits, as well as in soil, surface water and even aquatic organisms worldwide (Masiá et al., 2015; Ruiz-Rodríguez et al., 2015; Xu et al., 2015; Ccanccapa et al., 2016).

According to several previous studies, the toxicity of IMZ has been linked to embryo toxicity, malformation, endocrine disruption, neurotoxicity and genotoxicity in mice and other experimental models (Tanaka 1995; Tanaka et al., 2013; Jin et al., 2016a). The toxicity of IMZ in aquatic organisms is very limited, although the concentrations of IMZ in actual environmental aquatic systems have reached hundreds of ng/L and even 1 mg/L in seriously polluted agricultural areas (Castillo et al., 2006; Belenguer et al., 2014; Ccanccapa et al., 2016), indicating that residual IMZ in aquatic systems can adversely affect non-target aquatic organisms (Diaz et al., 2013).

Recently, zebrafish (*Danio rerio*) has emerged as an ideal experimental model to study the aquatic toxicology of environmental pesticides (Jin et al., 2010; Tran et al., 2014; Liu et al., 2016; Liu et al., 2017). Regarding the toxicity of IMZ to zebrafish, Şişman and Türkez (2010) reported that exposure to ≥ 10 μM IMZ decreased the embryonic survival rate and hatching success and induced malformations in larvae at 72 hpf. We recently observed that exposure to ≥ 300 μg/L IMZ could induce developmental toxicity and locomotor behavior abnormalities in the early developmental stage of zebrafish (Jin et al., 2016a). As a fungicide, IMZ may can influence the composition of gut microbiota; however, no related study was reported. In zebrafish, the gut microbiota plays some critical roles in the nutrition, development, metabolism and immunity of the host (Wang et al., 2017a; Wang et al.,

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2017b). Generally, the composition of gut microbiota is relative stable, but it could be influenced by diet, medicine/antibiotics, age and even pesticide and other environmental pollutants (Jin et al., 2016b; Dehler et al., 2017; Wang et al., 2017b). In the present study, male adult zebrafish were exposed to environmental-related concentrations of IMZ for 1, 7, 21 days, and the gut microbiota and hepatic metabolism were evaluated. It is believed that the results could provide some new information on IMZ-induced toxicity in fish.

2. Materials and methods

2.1. Chemicals

IMZ (CAS No: 35554-44-0; purity: > 97%) was purchased from Adamas-beta and was dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions. TRIzol reagent for isolating RNA was purchased from Takara Biochemicals (Dalian, China). The reverse transcription kit and SYBR green PCR kit were purchased from Toyobo (Tokyo, Japan). Magnetic Beads DNA Extraction Kit for isolating DNA was purchased from Hangzhou Foreal Nanotechnology (Hangzhou, China).

2.2. Animals and experimental design

Because egg protein transcripts are extremely abundant in the livers of breeding females and might confound the metabolic analysis, male fish were selected in the present study (Wang et al., 2005). Mature male adult (6 months of age) AB-PAS strain zebrafish (*Danio rerio*) were exposed to IMZ at the concentrations of 100 and 1000 μg/L (experimental groups) or 0.003% DMSO (control group) in water. Before IMZ exposure, the fish were maintained for > 1 week with tap water pH 7.0 to 7.5, hardness between 39.9 and 61.0 mg $CaCO_3$/L in glass tanks at 28 °C with a dark/light photoperiod of 10:14 h (Westerfield 1995). During the exposure, the fish were feed twice daily, and the water was changed every other day.

Because the toxicity of IMZ to zebrafish is very low and the concentrations observed in the water (Diaz et al., 2013; Ccanccapa et al., 2016), the exposure concentrations in this study was determined as 100 and 1000 μg/L. The selected male adult fish were exposed to 100 and 1000 μg/L IMZ for 1, 7 and 21 days, respectively. In each group, 6 male adult fish were reared in 3 L of each solution in a glass tank, for a total of 12 fish in each group. The livers excised from 2 fish were collected as 1 sample, resulting in 6 pooled samples for mRNA transcription analysis. Additionally, 6 guts selected randomly (3 samples in each glass tank) were used for DNA extraction, and each gut excised from each fish was collected as one sample. Additionally, 3 guts selected randomly in the control and group treated with 1000 μg/L IMZ for 21 days were used for histopathological analysis. During the exposure, the growth and survival were not affected.

For the hepatic metabolomic analysis, 84 male adult fish (in 12 separate glass tanks, and 7 fish in each tank) were exposed to 1000 μg/L IMZ for 21 days. The same number of fish were reared in water contain 0.003% DMSO as a control. After exposure, the livers excised from 14 fish (2 tanks) were collected from each group as one sample, and 6 pooled samples were collected in control and 1000 μg/L IMZ treated groups. The livers were kept on dry ice during preparation and then were stored at − 80 °C until they were further analyzed. In all experiments, the fish were anesthetized on ice before dissection.

2.3. Histopathological observation

After 21 days of exposure with 1000 μg/L IMZ, a portion of the middle gut was fixed in 10% formalin at 4 °C for 24 h. Subsequently, the fixed gut tissues were dehydrated in gradient ethanol, hyalinized in xylene, and embedded in paraffin wax at 56 °C. Next, the paraffin blocks were sectioned at 5-μm thickness. The sections were collected on glass slides and were stained with hematoxylin and eosin (H & E) or Alcian Blue-Periodic Acid Schiff (AB-PAS) before examination with a microscope (Olympus).

2.4. DNA extraction, PCR amplification, and 16S rRNA gene sequencing

The microbial genomic DNA (gDNA) was extracted from each gut using a commercial magnetic bead DNA isolation kit provided by Hangzhou Foreal Nanotechnology (Hangzhou, China) following the manufacturer's instructions. All the extracted gDNA was quantified by ultraviolet spectroscopy for further analysis. Next, the microbial gDNA was amplified by specific primers (Forward primer: 5′-ACTCCTACG GGAGGCAGCAG-3′; Reverse primer: 5′-GGACTA CHVGGGTWTCTAAT-3′) targeting the V3 and V4 regions of the bacterial 16S rRNA gene. Furthermore, the composition of the gut microbiota was detected using dual-indexing amplification and sequencing on the Illumina MiSeq platform followed by QIIME (vision 1.9.0) bioinformatics analysis. In addition, some of the microbial gDNA in each sample was amplified by real-time qPCR with bacterial phyla-specific primers (Table S1) according to our previous study (Jin et al., 2015b). The following protocol was used for PCR amplification: 50 °C for 2 min, 95 °C for 10 min, 95 °C for 15 s, 56 °C for 30 s, and 72 °C for 1 min, repeated for 40 cycles, followed by 72 °C for 10 min. The relative abundance of each microbiota was analyzed according to a previous study (Jin et al., 2016c).

2.5. GC/MS-based metabolomic analysis

As an internal standard, 20 μL of 2-chloro-phenylalanine (0.3 mg/mL, methanol) was added to 30 mg of liver tissue, and then the tissue solution was mixed with 400 μL of methanol-water solution (4/1, v/v) and homogenized at low temperature using Tissuelyser-192 (Shanghai Jing Technology Companies). The mixture was kept in an ice bath for 10 min using ultrasonic extraction, followed by centrifugation at 14,000*g* for 10 min at 4 °C. Next, 300 μL of the supernatant was transferred to a GC vial and evaporated to dryness. Oximation was carried out in an oscillation incubator at 37 °C for 90 min after 50 μL of methylhydroxylamine hydrochloride (15 mg/mL in pyridine) was added. Next, 80 μL of BSTFA (with 1% TMCS as the catalyst) was added, and the mixture was incubated at 70 °C for 60 min, followed by removal of the samples and incubation for 30 min at room temperature before GC/MS metabolomics analysis.

Next, 1.0 μL samples of the control group and test group (after 1000 μg/L IMZ exposure for 21 d) were injected into the Thermo Focus DSQ GC/MS system equipped with a DB-5MS (30 m × 0.25 mm, 0.25 μm) column using the splitless mode. We set the injection temperature, ion source temperature and transmission line temperature at 260 °C. The helium carrier gas flow was set at a constant rate of 1 mL/min. The initial temperature was isothermally held at 80 °C for 2 min and then was raised to 300 °C at a rate of 15 °C/min and was maintained for 5 min. The electron impact mode and full-scan monitoring were recorded over the mass slope of *m/z* 50–600. Six parallel samples (14 fish were sacrificed for each sample) were prepared for each treatment/control group.

The GC/MS data were analyzed using ChromaTOF software (v 4.34, LECO, St Joseph, MI). The resulting data were processed using principal component analysis (PCA) and partial least squares discriminate analysis (PLS-DA) using SIMCA-P + 14.0 software (Umetrics, Umea, Sweden).

2.6. RNA extraction and real-time quantitative polymerase chain reaction (RT-qPCR)

The livers from two zebrafish were dissected as one sample for total RNA extraction using TRIzol reagent (Takara, China). Six parallel total RNA samples were prepared for each group. Next, cDNA was synthesized using a reverse transcription kit (Toyobo, Japan). RT-qPCR analysis was performed using the SYBR Green system (Toyobo, Japan) and