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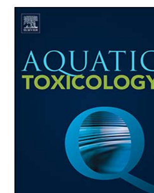

Short-term developmental effects and potential mechanisms of azoxystrobin in larval and adult zebrafish (*Danio rerio*)

Fangjie Cao, Peizhuo Wu, Lan Huang, Hui Li, Le Qian, Sen Pang, Lihong Qiu*

College of Sciences, China Agricultural University, Beijing, 100193, China

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ABSTRACT

Previous study indicated that azoxystrobin had high acute toxicity to zebrafish, and larval zebrafish were more sensitive to azoxystrobin than adult zebrafish. The objective of the present study was to investigate short-term developmental effects and potential mechanisms of azoxystrobin in larval and adult zebrafish. After zebrafish embryos and adults were exposed to 0.01, 0.05 and 0.20 mg/L azoxystrobin (equal to 25, 124 and 496 nM azoxystrobin, respectively) for 8 days, the lethal effect, physiological responses, liver histology, mitochondrial ultrastructure, and expression alteration of genes related to mitochondrial respiration, oxidative stress, cell apoptosis and innate immune response were determined. The results showed that there was no significant effect on larval and adult zebrafish after exposure to 0.01 mg/L azoxystrobin. However, increased ROS, MDA concentration and *il1b* in larval zebrafish, as well as increased *il1b*, *il8* and *cxcl-c1c* in adult zebrafish were induced after exposure to 0.05 mg/L azoxystrobin. Reduced mitochondrial complex III activity and ATP concentration, increased SOD activity, ROS and MDA concentration, decreased *cytb*, as well as increased *sod1*, *sod2*, *cat*, *il1b*, *il8* and *cxcl-c1c* were observed both in larval and adult zebrafish after exposure to 0.20 mg/L azoxystrobin; meanwhile, increased *p53*, *bax*, *apaf1* and *casp9*, alteration of liver histology and mitochondrial ultrastructure in larval zebrafish, and alteration of mitochondrial ultrastructure in adult zebrafish were also induced. The results demonstrated that azoxytrobin induced short-term developmental effects on larval zebrafish and adult zebrafish, including mitochondrial dysfunction, oxidative stress, cell apoptosis and innate immune response. Statistical analysis indicated that azoxystrobin induced more negative effects on larval zebrafish, which might be the reason for the differences of developmental toxicity between larval and adult zebrafish caused by azoxystrobin. These results provided a new insight into potential mechanisms of azoxystrobin in larval zebrafish and adult zebrafish.

1. Introduction

As a broad spectrum, highly effective and systemic strobilurin fungicide, azoxystrobin has been registered for use in soybeans, rice, cereals, vegetables and fruit trees around the world, including United States, Germany, France, Denmark and China, etc (Battaglin et al., 2011; Han et al., 2014; Rodrigues et al., 2013). Due to wide application of azoxystrobin, its environmental behaviour, fate and occurrence has caused great concerns around the world (Rodrigues et al., 2013).

Azoxystrobin has been detected in aquatic environment, which might pose hazards to aquatic organisms, e.g. the detected values of azoxystrobin were between 0.15 and 34 μg/L in streams, surface water and groundwater around the world (Battaglin et al., 2011; Jorgensen et al., 2012; Schriever et al., 2007; Wang et al., 2009); the mean detected concentration was 0.183 mg/L in the paddy water after 10 days of spraying with 506.25 g/ha of azoxystrobin in Hangzhou, China (Xie and Gong, 2013). Azoxystrobin is known as an inhibitor of mitochondrial complex III (mitochondrial cytochrome *bc1* complex) in the fungi, and it inhibits mitochondrial respiration by binding tightly to cytochrome b at so-called Q_o site of cytochrome *bc1* complex and blocking electron transfer between cytochrome b and cytochrome c1, which results in the disruption of ATP synthesis (Esser et al., 2004; Hnatova et al., 2003). Previous studies showed that azoxystrobin could also inhibit mitochondrial complex III activity and respiration rate in rat livers (Gao et al., 2014), and trigger the release of cytochrome c (Cyt c) in mitochondrial respiration pathways, which further induced cell

Abbreviations: CAT, catalase; Cyt c, cytochrome c; GPx, glutathione peroxidase; MD, membrane degradation; MDA, malondialdehyde; MR, membrane rupture; MS, mitochondrial swelling; OECD, Organization for Economic Co-operation and Development; PN, partial necrosis; ROS, reactive oxygen species; RT-qPCR, real-time quantitative polymerase chain reaction; SOD, superoxide dismutase

* Corresponding author.
E-mail address: lihongqiuyang@126.com (L. Qiu).

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apoptosis in human esophageal squamous cell carcinoma KYSE-150 cells (Shi et al., 2017). Presently, studies about effects and mechanisms of azoxystrobin on mitochondrial respiration chain of aquatic organisms, especially fish, were very limited.

Mitochondrial cytochrome *bc1* complex was identified as the main producer of reactive oxygen species (ROS) (Bleier and Drose, 2013; Dröse and Brandt, 2012). ROS could activate antioxidant enzymes activities, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) to eliminate ROS in aquatic organisms (Valavanidis et al., 2006; Winston and Di Giulio, 1991). As the major superoxide scavengers in the mitochondria, both Cu/Zn-SOD and Mn-SOD could catalyze superoxide ($O_2 \cdot^-$) to H_2O_2, which would further be converted by GPx and CAT into O_2 and H_2O (McCord and Fridovich, 1969; Muller et al., 2007; Weisiger and Fridovich, 1973). Excessive ROS was believed to be associated with oxidative stress (Lin and Beal, 2006; Shigenaga et al., 1994), cell apoptosis (Dada and Sznajder, 2011; Green and Reed, 1998), and immune response (Lopez-Armada et al., 2013; van Horssen et al., 2017). The pathways mentioned above and bidirectional interactions between them have been the main topics in aquatic toxicology (Jiang et al., 2015; Jin et al., 2013; Mu et al., 2015).

So far, studies about effects of azoxystrobin on aquatic organisms were mainly about lethal effects, physiological responses and possible mechanisms involved with oxidative stress, cell apoptosis or growth-related metabolism (Liu et al., 2013; Olsvik et al., 2010). Zebrafish (*Danio rerio*) is a powerful vertebrate model organism that has been extensively used to study mitochondrial dysfunction, oxidative stress, cell apoptosis and innate immune response (Eimon and Ashkenazi, 2010; Novoa and Figueras, 2012; Steele et al., 2014). Mu et al. (2017) reported that hatching inhibition, pericardial edema and reduced body length were induced after zebrafish embryos were exposed to 0.25 mg/L azoxystrobin. However, developmental effects and potential mechanisms of azoxystrobin in different life stages of zebrafish were unclear.

To elucidate short-term developmental effects and potential mechanisms of azoxystrobin in larval and adult zebrafish, mitochondrial complex III and antioxidant enzyme activities, ROS production, malondialdehyde (MDA) and ATP concentrations, liver histology, liver mitochondrial ultrastructure, as well as expression alteration of genes related to mitochondrial respiration, oxidative stress, cell apoptosis and innate immune response were measured after 4 and 8 days of exposure to azoxystrobin. The results of the present study would provide a better understanding on toxicity and potential mechanisms of azoxystrobin in larval and adult zebrafish.

2. Materials and methods

2.1. Chemicals and reagents

Azoxystrobin (CAS: 131860-33-8) with a purity of 98% was obtained from Shenyang Chemical Industry Research Institute, China. Stock solution of 10000 mg/L azoxystrobin was prepared by dissolving azoxystrobin in acetone (analytical grade) with 5% Tween 80 and was stored at 4 °C for further dilution

2.2. Zebrafish maintenance and egg production

Parental zebrafish (six months old) and adult zebrafish (four months old) of wild type (AB-strain) were purchased from Beijing Hongdagaofeng Aquarium Department, China. The procedure for zebrafish maintenance and egg production, and culturing condition was the same as that in a published protocol (Cao et al., 2016a). The experiment was approved by the guidelines of Institutional Animal Care and Use Committee (IACUC) of China Agricultural University.

2.3. Short-term developmental toxicity assays of azoxystrobin to larval and adult zebrafish

To determine short-term developmental effects and potential mechanisms of azoxystrobin in larval and adult zebrafish, zebrafish embryos at 2 h of post fertilization (hpf) and adult zebrafish (four months old) were randomly exposed to water control, solvent control (0.002% acetone and 0.0001% Tween 80, *v/v*), 0.01, 0.05 and 0.20 mg/L azoxystrobin (equal to 25, 124 and 496 nM azoxystrobin, respectively). Exposure concentrations were chosen based on previous studies: (1) 0.01 mg/L was close to the maximum detected concentration in streams in Germany (Less and von der ohe, 2005); (2) 0.05 mg/L was close to the maximum detected concentration in river water and paddy water in China (Wang et al., 2009; Xie and Gong, 2013); (3) 0.20 mg/L was close to the concentration of 0.25 mg/L, which caused adverse developmental effects on larval zebrafish (Mu et al., 2017) and would help to elucidate potential mechanisms of azoxystrobin. Exposure period was 8 days and exposure condition was the same as the culturing condition. The graph for experimental setup was provided in supplementary material (Fig. S1). Abnormal behavior and appearance were recorded, and dead individuals were recorded daily and removed timely. Exposure solutions were renewed daily. Larval zebrafish and adult zebrafish were not fed during 8 days of exposure.

Short-term developmental toxicity of azoxystrobin was tested according to Organization for Economic Co-operation and Development (OECD) Guideline 212 for Short-term Toxicity Test on Embryo and Sac-fry Stages (OECD 212., 1998). Each 1 L beaker contained 600 mL of exposure solutions and 200 embryos (Fig. S1A). Each treatment contained 6 beakers. Three beakers were randomly selected for sample collection after 4 days of exposure and the rest were used for sample collection after 8 days of exposure. Almost all embryos hatched to larvae after 4 days of exposure, so larval zebrafish were used in the following test. At termination of exposure, body lengths of 10 surviving larval zebrafish from each beaker were measured using an Aigo GE-5 digital microscope (Aigo Digital Technology Co. Ltd, China). After 4 and 8 days of exposure, 40 larval zebrafish were pooled for mitochondrial complex III activity and ROS production analysis, 40 larval zebrafish were pooled for ATP concentration analysis, 40 larval zebrafish were pooled for antioxidant enzyme activities and MDA concentration analysis, and 30 larval zebrafish were pooled for gene expression analysis. All samples were frozen in liquid nitrogen and stored at −80 °C for further analysis. After 8 days of exposure, two larval zebrafish were randomly selected from one beaker for liver histological analysis and two individuals were randomly selected for liver mitochondrial ultrastructure analysis. Each treatment contained three replicates (N = 3).

For adult zebrafish exposure, each 25 L tanks contained 20 L of exposure solutions and 40 individuals (Fig. S1B). Each treatment contained 6 tanks. Three tanks were randomly selected for sample collection after 4 days of exposure and the rest were used for sample collection after 8 days of exposure. Before exposure, 10 adult zebrafish in each replicate were randomly selected, euthanized with anaesthetic buffer, which contained 100 mg/L tricaine and 300 mg/L $NaHCO_3$, and measured using digital caliper (Mitutoyo Corp., Japan). At termination of exposure, body lengths of 10 surviving adult zebrafish in each replicate were randomly selected and measured using the same method mentioned above. After 4 and 8 days of exposure, the livers of 4 adult zebrafish were pooled for mitochondrial complex III activity and ROS production analysis, the livers of 4 adult zebrafish were pooled for ATP concentration analysis, the livers of 4 adult zebrafish were pooled for antioxidant enzyme activities and MDA concentration analysis, and the livers of 3 adult zebrafish were pooled for gene expression analysis. All samples were frozen in liquid nitrogen and stored at −80 °C for further analysis. After 8 days of exposure, two adult zebrafish were randomly selected from one tank for liver histological analysis and two adult zebrafish were randomly selected from one tank for liver mitochondrial analysis. Each treatment contained three replicates (N = 3).

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