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The developmental effect of difenoconazole on zebrafish embryos: A mechanism research☆

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ABSTRACT

Difenoconazole is a widely used triazole fungicide and has been reported to have negative impacts on zebrafish embryos. To investigate the mechanism of its developmental toxicity, zebrafish embryos were exposed to 0.5 and 2.0 mg/L difenoconazole for 96 h. The morphological and physiological indicators of embryo development were tested. The total cholesterol (TCHO) level, triglyceride (TG) level and malondialdehyde (MDA) content were measured at 96 hpf (hours post-fertilization). In addition, the transcription of genes related to embryo development, the antioxidant system, lipid synthesis and metabolism was quantified. Our results showed that a large suite of symptoms were induced by difenoconazole, including hatching regression, heart rate decrease, growth inhibition and teratogenic effects. 0.5 mg/L difenoconazole could significantly increase the TG content of zebrafish embryos at 96 hpf, while no apparent change in the TCHO and MDA level was observed post 96 h exposure. Q-PCR (quantitative real-time polymerase chain reaction) results showed that the transcription of genes related to embryonic development was decreased after exposure. Genes related to hatching, retinoic acid metabolism and lipid homeostasis were up-regulated by difenoconazole.

1. Introduction

Triazole fungicides are one of the main classes of pesticides that are widely used for the treatment and protection of corn, fruit, and other plants. These compounds can enter the water environment through spray drift or surface run-off after rainfall (Raudonis et al., 2004; Konwick et al., 2006), and they possess several characteristics, such as high chemical and photochemical stability, low biodegradability and easy transportation in the environment (Wang et al., 2011). Thus, the possible detrimental effects of triazole compounds on environmental organisms have received increasing attention. Difenoconazole (cis-trans-3-chloro-4-(4-methyl-2-(1H-1,2,4-triazol-yl methyl)-1,3-dioxolan-2-yl) phenyl 4-chlorophenyl ether) is a typical triazole fungicide used for the control of fungal disease in vegetables, cereals and other field crops (Vawdrey et al., 2008; Horsfield et al., 2010). It inhibits fungal lanosterol-14α-demethylase (Cyp51) activity and blocks ergosterol biosynthesis, thus resulting in the blocking of fungal cell wall chitin synthesis and the overspill of the cytoplasm (Buchenauer, 1995; Hamada et al., 2011; Ragsdale, 1977). In China, difenoconazole has been used as the main pesticide to combat rice diseases for many years (Wang and Zhang, 2012).

Since it is used extensively in fruits and cereals (Gopinath et al., 2006), especially in rice, difenoconazole has greater opportunity to come into contact with and contaminate water in the environment. Many studies have been published on the occurrence of difenoconazole in the environment (Table 1). Compared with other triazole fungicides, difenoconazole is reported to possess relatively high acute toxicity toward a wide range of aquatic organisms (Dong et al., 2013). According to a recent publication by the European Food Safety Authority (EFSA), it has been identified that difenoconazole is very toxic to aquatic organisms in view of its high toxicity toward *Daphnia magna* (chronic NOEC = 0.0056 mg active substance/L) (EFSA, 2011). It is therefore important to carry out further studies on its action mechanism in aquatic organisms.

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Table 1
The reported environmental difenoconazole dosage in water area.

Area	Data source	Reported value (ug/L)	Reference
China (Changsha, Changchun, Hangzhou)	Paddy water (spraying day)	1.98–2.91 mg/L	Zhang et al., 2011
Thailand (Salakru, Nong Sua)	PEC[a] (agricultural water area)	0.028 mg/L	Satapornvanit et al., 2004
Malaysia (Kedah)	Surface water (river near field)	0.30 mg/L	Latiff et al., 2010
China (Fujian)	Surface water (river)	0.0039–0.0061 μg/L	You, 2008
Italy (River Meolo)	PEC (river)	0.0095 μg/L	Verro et al., 2009
Australia (Victoria)	Surface water (river and stream)	0.15 μg/L	Schäfer et al., 2011

[a] PEC = Predicted Environmental Concentration.

At present, research focused on the evaluation of the negative effects of chemicals in water environments via fish bioassay, and developmental toxicity is a core topic of widespread concern (Barbee et al., 2013; Gongzález-Doncel et al., 2014; Zhu et al., 2015). There are several tools for monitoring developmental effects. Morphological indicators, such as development status, body length, and weight, could reflect the toxic effects of chemicals on animals obviously and directly; however, they provide little help in understanding the action mode of toxicants. Currently, molecular and biochemical parameters, which possess high sensitivity and could provide mechanistic information, are playing an important role in monitoring the responses of early life stage aquatic organisms toward xenobiotics (Zhu et al., 2014a,b; Kong et al., 2013).

The zebrafish (*Danio rerio*) is a typical small tropical aquarium fish well known to most people. The reason it has a long history of general use in toxicology research is that it is inexpensive, hardy, small, and easy to care for in large numbers (Lele and Krone, 1996). In addition, the zebrafish embryo has some characteristics, such as in vitro fertilization, high fecundity, rapid embryonic development and optical transparency, which make it easy to detect morphological endpoints or to observe the development process in the early life stages (Yang et al., 2009). Our previous study indicated that difenoconazole could induce a series of abnormalities during zebrafish embryonic development, such as hatching inhibition, pericardial edema and yolk sac edema (Mu et al., 2013); however, the mechanism involved still remains unknown. In this study, the developmental effects of difenoconazole on zebrafish embryos were assayed and the related mechanism was investigated through biochemical and molecular pathways. Our research is intended to reflect the eco-toxic effects of difenoconazole, and to provide information on the toxicity mechanism of triazole fungicides.

2. Materials and methods

2.1. Zebrafish maintenance and embryo collection

Wild-type zebrafish were purchased from a local fish shop. All adult zebrafish were maintained in flow-through feeding equipment (made by Esen Corp.) at 26 °C with a photoperiod of 14/10 (light/dark), and fed with live brine shrimp daily. The preparation and collection of zebrafish embryos followed the procedure described in our previous work (Mu et al., 2013).

2.2. Chemicals and reagents

The standard water was prepared in the lab with the formula of iso-7346-3, which contained 2 mmol L^{-1} Ca^{2+}, 0.5 mmol L^{-1} Mg^{2+}, 0.77 mmol L^{-1} Na^{+}, and 0.08 mmol L^{-1} K^{+} (ISO, 1996). 96% difenoconazole (CAS: 119446-68-3) was obtained from China Ministry of Agriculture, and the stock solution used for drug exposure was prepared using acetone AR. All of the other reagents utilized were of analytical grade.

2.3. Exposure and sample collection

Experiments were performed in accordance with current Chinese legislation and were approved by the independent animal ethics committee at China Agricultural University.

2.3.1. Exposure for morphological endpoints

Test solutions with a difenoconazole concentration of 0 (control), 0.5, and 2.0 mg/L were made up using standard water, designed on the basis of pre-experiment data. Embryos at about 1 h post-fertilization (hpf) were randomly transferred into test solutions in 24-well plates. Twenty wells were used in each plate, and each well contained 2 mL of exposure solution and one embryo. Both blank control and solvent control were set up. The exposure solutions for the solvent control and all treatment groups contained the same concentration of acetone (0.05 mL/L). Each test concentration and control was replicated 3 times. The exposure lasted 4 days, and embryos were transferred to freshly dosed plates every 24 h. The external conditions during exposure, including the temperature, humidity and light cycle, were the same as in the culture environment. The number of dead individuals and the stage of embryonic development were determined daily. For embryos, death was judged using the lethal toxicological endpoints proposed by Nagel (2002). Larvae that had no heartbeat under microobservation were considered to be dead. Dead individuals were removed in a timely manner. The hatching and malformation of embryos were checked daily. Heart rate (48 and 96 hpf) and 24 hpf spontaneous movement were observed using a microscope. Teratogenic effects were recorded, and the body lengths were measured using an Aigo GE-5 digital microscope (Aigo, Beijing, China).

2.3.2. Exposure for biomarkers and gene expression tests

Embryos were randomly transferred into test solutions in 1 L beakers at 2 hpf. Each beaker contained 500 mL of exposure solution and about 150 embryos, and there were 3 beakers in each treatment group. At 48 hpf, 25 embryos from each replicate were collected and washed twice with standard water (for RNA extraction). At 96 hpf, 75 hatched (or decorticated) larvae from each replicate were collected and washed twice with standard water (50 for biomarker measurement; 25 for RNA extraction). The embryo samples were stored at −80 °C until analysis.

2.4. Difenoconazole in water analysis

Exposure solutions in each replicate for all treatments were analyzed three times: at the beginning of exposure, and at 48 and 96 hpf. Sample preparation and chromatographic conditions were conducted via the method described previously (Mu et al., 2013). GC ChemStation Rev.A.10.02 software was used for chromatographic data processing.