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Developmental toxicity of penconazole in Zebrfish (*Danio rerio*) embryos

Feyza Icoglu Aksakal*, Abdulkadir Ciltas

Department of Agricultural Biotechnology, Faculty of Agriculture, Atatürk University, 25240, Erzurum, Turkey

HIGHLIGHTS

- Penconazole induced developmental toxicity in zebrafish embryos.
- Penconazole inhibited the survival rate and hatching rate of zebrafish embryos.
- Exposure to penconazole caused several developmental abnormalities in zebrafish embryos.
- Penconazole at 0.8–2.4 mg/L altered the mRNA levels of some oxidative stress and immune related genes.

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ABSTRACT

Penconazole is a widely used fungicide that is toxic to a variety of organisms including fish. In the present study, we investigated the developmental toxicity of penconazole on zebrafish embryos by exposing to different concentrations of penconazole (0.8, 1.6 and 2.4 mg/L) from 4-h post-fertilization (hpf). Hatching, survival, and heart rates, body length, malformation and expression of several genes were detected. The results showed that penconazole exposure induced developmental toxicity, including delayed hatching, reduced survival, and heart rate. In addition to this, exposure to penconazole caused malformations, including pericardial edema, yolk sac edema, axial malformation, tail malformation and spinal curvature. Furthermore, RT-PCR results showed that mRNA levels of antioxidant genes were down-regulated after penconazole exposure. On the other hand, mRNA levels of *interleukin 1 beta* and *interferon* in embryos were up-regulated after exposure to penconazole. In summary, our data indicated that penconazole cause embryonic development toxicity on zebrafish embryos.

1. Introduction

Triazole fungicides are widely used throughout the world to control fungus and enhance agricultural production (Lv et al., 2017). As a triazole fungicide, penconazole [1-(2,4-dichloro-β-propyl-phenethyl)-1H-1,2,4-tri-azole] controls powder mildew, pome fruit scab, and other pathogenic ascomycete and basidiomycete diseases by inhibiting the cytochrome P450-dependent 14α-demethylase activities that is the key enzyme for ergosterol biosynthesis in fungi (Mercadante et al., 2016; Husak et al., 2017). Penconazole is normally sprayed directly on plants and is rapidly absorbed and distributed to the interior of the leaves (Chaabane et al., 2016). Due to drifting during application, rain-washing the pesticides off the foliage, and plant leaves falling onto the soil, penconazole can reach the soil and water (Husak et al., 2017). Its residues might affect the environmental safety and aquatic life. Therefore, the possible detrimental effects of penconazole on living organisms have received increasing attention. For example, Agirman et al. (2015) revealed that penconazole negatively effects cell number and protein amount in microalgae *Scenedesmus acutus*. The toxic effects on antioxidant enzymes and AChE activity induced by penconazole have been observed in adult rats (Chaabane et al., 2017). Husak et al. (2017) reported that antioxidant enzymes including CAT, GR, G6PDH, and SOD were affected in goldfish (*Carassius auratus* L.) tissues after exposure to 15–25 mg L^{-1} of penconazole. It has also been reported that the mRNA expression levels of *cat*, *gpx*, *mn-sod*, *cu/zn-sod* genes encoding antioxidant enzymes are increase or decrease under conazole treatment (Mu et al., 2015, 2016). In

* Corresponding author.
E-mail address: ficoglu@atauni.edu.trm (F. Icoglu Aksakal).

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addition, Mu et al. (2015) shown that conazole fungicides could affect the innate immune system that occurs early period in embryonic development in fish, altering the expression of genes (eg, *il-1β* and *ifn*) related to this system. Although toxic effects of penconazole on fish and other aquatic invertebrates are known, limited studies have been conducted on the negative effects of penconazole treatment of fish especially in the early life stages (Perdichizzi et al., 2014; Chaabane et al., 2016; Mercadante et al., 2016; Husak et al., 2017). It is therefore necessary to perform toxicological studies of penconazole to reflect its environmental risk during the early life stages of fish.

Small freshwater fish species have been generally used to examine the toxic properties of chemicals in terms of their chronic and acute effects especially in their role in ecotoxicological regulatory purposes. Among these fish species, the zebrafish is a well-known and typical small tropical aquarium fish. Many studies have proved that embryonic and larval zebrafish are useful research model to explore the environmental toxicity by using survivorship and development, as well as gene expression as endpoints because this model is small in size and transparent, and easy to maintain and handle, and it has high fecundity, rapid embryogenesis and a high degree of genomic homology to mammals (Jin et al., 2010a, 2015; Yang et al., 2012; Abe et al., 2017). Moreover, it has an advantage for immuno-toxicological studies since they depend only on the innate immune system for the first 30 days (Rojo et al., 2007).

In the present study, the potential developmental toxicity of penconazole to zebrafish was investigated for the first time by measuring survival, hatching and heartbeat rate, body length and malformations. The expression of several genes (*cat*, *gpx*, *mn-sod*, *cu/zn-sod*, *il-1β* and *ifn*) was also evaluated. Our results obtained here may provide useful information for better understanding the developmental toxicity of penconazole to aquatic organisms and contribute to a greater understanding of overall fungicide toxicity.

2. Materials and methods

2.1. Chemicals

A commercial formulation of penconazole (Topas 100 EC, 100 g L^{-1}) was purchased from a distribution company (Turkey). The stock solutions of penconazole were prepared by dissolving it in double-distilled water. All other chemicals and reagents utilized in this study were of analytical grade.

2.2. Fish maintenance and embryo treatments

Wild-type (AB strain) zebrafish were provided from Fisheries Faculty of the Ataturk University which were previously provided by Oregon State University (US) and maintained Aquatic Biotechnology Lab. Healthy 5 month old adult fish were selected and acclimatized separately in glass tanks (10 L) at constant temperature of 28 °C for at least 7 days with 14:10 h light-dark photoperiod. The fish were feed with *Artemia salina* twice a day. In the preparation and collection of zebrafish embryos, the procedure described in our previous work was followed (Icoglu Aksakal and Ciltas, 2018). The experimental concentrations of Penconazole (0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 mg/L) were determined with preliminary test. The 96 h LC_{50} values were determined by probit analysis. Calculated 96 h LC_{50} value by probit 95% Confidence Interval (CI) for Penconazole was 3.73 mg/L in zebrafish embryos. Sublethal concentrations (0.8, 1.6, 2.4 mg/L) were selected for detecting the potential developmental toxicity of penconazole to the embryos. Test solutions with a penconazole concentration of 0 (control), 0.8, 1.6 and 2.4 mg/L were made up using standard double-distilled water. Embryos were placed at temperature (28 °C) in Petri dishes containing various concentrations of penconazole in embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, %0,01 methylene blue), and the exposure time was up to 96 hpf. The solutions were changed once every 24 h at which time any dead embryos were discarded. In each experimental group, 150 embryos were used and each treatment was performed in triplicates. At 96 hpf, 90 larvae from each replicate were collected on dry ice and washed twice with UltraPure water, and then stored at −80 °C until analyzed.

2.3. Mortality, hatching, heart rate and malformation

The survival and hatching rate of zebrafish embryos/larvae were examined and recorded at 24, 48, 72 and 96 hpf by using a standard stereomicroscope (Imager. A2 Zeiss, Germany). During exposure, dead embryos/larvae were immediately removed. Mortality was identified by coagulation of the embryos, missing heartbeat, failure to develop somite, and a non-detached tail. Hatching rate was determined by the rate of successful hatching embryos divide totality embryos in each replicate. Malformations including pericardial edema, yolk sac edema, axial malformation, tail deformity, spinal curvature were noted under 24, 48, 72, and 96 hpf after exposure to penconazole. Malformation rate was determined from the percentage of the total number of malformed larvae to the total number of larvae hatched during the test. The heartbeat rate of zebrafish larvae was recorded at 48 hpf. For heartbeat counting, 24 embryos were randomly selected (for each treatment) and counted for 20s per embryo after being stabilized at room temperature for 10 min until the embryonic heartbeat reaching steady state. After 96 hpf of exposure, the length of 8 larvae from each treatment was measured with a digital microscope.

2.4. RNA isolation and reverse transcription real-time PCR

At the end of the exposure experiment, total RNA was isolated from zebrafish larvae (60 larvae in each sample) using RNeasy mini kit for animal tissue (Qiagen, Basel, Switzerland) according to the manufacturer's protocol. A DNase treatment (Qiagen) was used to remove sample contamination by genomic DNA, and 30 μL of water (RNase/DNase free) was used for elution of RNA. RNA concentrations and purity were determined using a Nanodrop ND-1000 spectrophotometer. The cDNA synthesis was performed by using RT^2 First Strand cDNA Synthesis Kit (Qiagen, Basel, Switzerland). Real-time PCR was conducted using Rotor-gene Q instrument using SYBR Green (RT^2 SYBR Green master mix, Qiagen, Cat. No: 330500) mixed with cDNA and gene-specific primer pairs (Table 1). The PCRs were repeated three times. The mRNA expression level of each target gene was normalized to the content of the reference gene, and changes of the relevant genes were calculated by using $2^{-\Delta\Delta Ct}$ method and reported as relative mRNA level over the control. Analysis of the relative gene expression was done based on Qiagen Data Analysis Center. *β-actin* was used as housekeeping gene and suitable negative controls were maintained for all the primes.

2.5. Statistical analysis

The findings of the survival, hatching, heartbeat, and malformation rate and body length were given as the mean ± standard error (SEM) while the gene expression data were shown as mean + standard deviation (SD). Two-way ANOVA followed by the Tukey's multiple comparison tests was performed to assess statistically the differences in the studied parameters among studied groups (SPSS statistical software package for Windows 20.0 (SPSS