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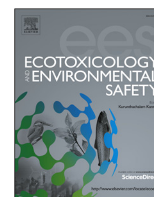

Fenbuconazole exposure impacts the development of zebrafish embryos

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ABSTRACT

Fenbuconazole (FBZ), a triazole-containing fungicide, is widely used in agriculture and horticulture. In the present study, the development and cardiac functioning were observed and determined in zebrafish embryos exposed to FBZ at 5, 50 and 500 ng/L nominal concentrations for 72 h. The results showed that 500 ng/L FBZ significantly increased pericardial edema rate, spine curvature rate, disturbed cardiac function, and led a shortened lower jaw. The transcription of genes such as *tbx5*, *nkx2.5*, *tnnt2*, *gata4*, *bmp2b*, *myl7* was altered, which might be responsible for the cardiac developmental and functioning defects in the larvae. The deformation in bone development might be related with the impaired transcription levels of *shh* and *bmp2b*. The transcription of *cyp26a1* (encoding retinoic acid metabolism enzyme) was significantly up-regulated in the 500 ng/L group, which might be a reason causing the teratogenic effect of FBZ. These results suggest that FBZ could have toxic effects on embryonic development, which should be considered in the risk evaluation of FBZ application.

1. Introduction

Among pesticides currently in use, triazole-containing fungicides are one of the most widely applied (Konwick et al., 2006; Goetz et al., 2007). Fenbuconazole (FBZ) is used in agriculture and horticulture against powdery mildew of vegetables, cereal and fruits (Holb and Schnabel, 2007; Sobeiha et al., 2009). Since its long biodegradation half-life with 61-day under field soil under anaerobic conditions (Pesticide Properties Data Base), FBZ could persist in the environment for a long period. After 190 d of incubation with soil, only a small amount of total FBZ (~46.8% or ~36.4% for Langfang or Changsha soil, respectively) disappeared (Li et al., 2012). The detected enantiomers of FBZ range from 10.49 to 23.54 μg/kg in soil samples collected from Langfang, China (Li et al., 2012). Thus, FBZ may contaminate aquatic environments through run-off. FBZ concentration detected in two waterworks of Xiamen city, China ranged from 0.22 to 6.98 ng/L (our unpublished data), indicating that the Jiulong river, from which these two waterworks got water source, was contaminated by FBZ. There is concern regarding potential exposure of fishes from its residues in aquatic environment (Li et al., 2012). The detected residues of FBZ in fruits such as oranges, pears, peaches and grapes range from 0.11 to 0.34 mg/kg (EFSA, 2015). People are also probably exposed to FBZ via food uptake. The WHO (1997) established an Acceptable Daily Intake for humans of 0.03 mg/kg/day, and maximum admissible concentration in drinking water is 0.1 μg/L (EFSA, 2015). Due to its wildly use, the toxic effects need to be adequately researched.

Existing reports show that FBZ seems to be low toxicity to animals. Acute 96 h LC_{50} of FBZ to fish is 1.5 mg/L; acute oral LD_{50} to mammals is > 2000 mg/kg (Pesticide Properties Data Base: Fenbuconazole). FBZ is not genotoxic (Federal Register, 2002), studies in rodent show that it does not induce liver tumor but is associated with a slight increase in the incidence of liver adenomas (Lassalle et al., 2015). Mice treated with FBZ exhibit a reversible change of liver including increased liver weight and histological damages (Juberg et al., 2006). FBZ perturbs thyroid-pituitary functioning and induces thyroid tumors in rodent (Hurley et al., 1998). However, there are few studies concerning the developmental effects of FBZ on fishes.

As a vertebrate model, zebrafish (*Danio rerio*) has numerous advantages such as small size, ease of culture, high fecundity, morphological and physiological similarities to mammals. The optical clarity of the chorion is in favor of the observation of embryogenesis and the assessment of endpoints of toxicity (Yang et al., 2009; He et al., 2014). The molecular weight (336.82 Da) of FBZ much lower than the threshold (3000 Da) (Braunbeck et al., 2015) allows its passage across the chorion. The rapid development of zebrafish involves all organic effects, and the genetic basis of development has been extensively studied, providing many benefits to mechanism assay (Fernández et al., 2018). These characteristics suggest that zebrafish embryos/larvae are an ideal research model for investigating the developmental effects of chemicals. In the present study, the influence of FBZ on the

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development of zebrafish embryos was researched, and the mechanisms involved were investigated.

2. Materials and methods

2.1. Zebrafish maintenance and embryos collection

All fish experiments followed the ethical guidelines of Xiamen University. Twenty wild-type TU zebrafish were housed in 20 L tanks with dechlorinated and aerated freshwater in a recirculation system at 28 ± 1 °C, under a normal photoperiod at 14:10 h light: dark. The pH and dissolved oxygen were 7.2–7.3 and 7–8 mg/L respectively. Fish were fed with live brine shrimp and commercial fish diet twice daily. Sexually mature fish without any signs of disease were selected as breeders.

Adult fish were mated at a ratio of 1:2 (female: male). Spawned eggs were collected within 0.5 h. Fertilized eggs were washed with zebrafish facility water and randomly distributed to multiple petri dishes for exposure experiments.

2.2. Embryonic exposure and sampling

FBZ (purity > 98%) was purchased from Agro-Environmental Protection Institute, Ministry of Agriculture, China. It was dissolved in acetone at analytic grade to reach stock concentrations of 5, 50 and 500 μg/mL. FBZ exposure solutions were obtained by adding 1 μL the stock concentration to 1 L zebrafish culture medium (3.5 g/L NaCl, 0.05 g/L $NaHCO_3$, 0.05 g/LKCl, 0.05 g/L $CaCl_2$). Eggs were collected after oviposition and remove coagulated eggs and debris. Eggs at 0.5–1 h post-fertilization (hpf) were exposed to nominal concentrations of FBZ (0, 5, 50 and 500 ng/L). One hundred fertilized eggs were cultured in 30 mL exposure solution in petri dish at 28 ± 1 °C. Similar criterion was applied to solvent control group, which received an equal volume of acetone (1 μl/L). There were five replicates for each treatment. The test solutions were renewed twice daily, and dead embryos were removed. The development of the embryos and mortality was monitored with an Olympus SZ51 stereo microscope every 24 h. The rates of malformation, such as dorsal curvature, pericardial edema were calculated as: malformation rate = malformed embryos number / survival embryos number × 100%; the hatching success (embryonic membrane opening and larva swimming up) of the embryo were assessed: hatched rate = hatched embryos number / total embryos number × 100%. After exposure for 72 h, the embryos were collected for analysis.

The remainder larvae after exposure for 72 h were transferred to clean water. Since the remaining amount of fish is small, they were put together from the five replicates and cultured in an aquatic system. Larvae within 7-day post-fertilization (dpf) occupied 10 mL water per fish in beak; and at 30 dpf, 400 mL in transparent fish tank. Fish at 60 dpf were maintained in the Aquatic Habitats Zebrafish System up to 90 days and occupied 1 L water per fish. The culture conditions were the same as those mentioned above. The numbers of remainder adult fish at 90 d were 28–47 in each treatment, whose morphological changes were observed and the occurrence rates of deformation were recorded.

2.3. Cardiac function analysis

The heart rate and cardiac arrhythmia of the exposed larvae were assayed based on the method of Incardona et al. (2009). The end-diastolic volume (EDV), end-systolic volume (ESV), stroke volume (SV) and cardiac output (CO) in the larvae were measured and assessed from 20-s video segments collected from individual embryos following the method of Chen et al. (2008). SV was calculated by using the equations SV = EDV - ESV, and CO was calculated as: CO = SV × HR.

Cardiac arrhythmia was obtained by determining the interbeat variability (Incardona et al., 2009). Using NIS-Elements Imaging Software (Nikon, Tokyo, Japan), the number of frames between cardiac contraction initiations was calculated to obtain a mean and standard deviations (SD) for each larva. Since a regular rhythm would have a low SD, this SD is a measure of heart rate irregularity. Three larvae from each replicate were assessed to get a mean for this replicate. The means from five replicates were used for analysis.

2.4. Whole mount Alcian-blue staining

Exposed larvae were randomly chosen in each treatment for Alcian-blue staining to observe the craniofacial cartilage development. Alcian-blue staining was followed the method described by Walker and Kimmel (2007). Stained larvae were observed using a Leica M165FC stereo fluorescence microscope, and the morphology of the craniofacial cartilage was analyzed following the method of Carvan et al. (2004). Three larvae from each replicate were assessed to get a mean for each replicate.

2.5. Real-time quantitative PCR (qPCR)

Fifty larvae from each replicate were pooled into a subsample. Total RNA was isolated by a Trizol Kit (TaKaRa, Dalian, China). The mRNA expression was determined based on the previously described method (Huang et al., 2012). QPCR analysis was performed on an Mx3000P Real-Time PCR system (Stratagene, La Jolla, CA, USA) using the Brilliant SYBR Green QPCR reagent kit (Stratagene) following the manufacturer's protocol. Gene expression levels were normalized to zebrafish *gapdh*. The real time quantitative PCR primers (Table S1) were designed using Primer Premier 5.0. Threshold cycles (Ct) and dissociation curves analysis of the amplification products were determined with MxPro software (Stratagene), to confirm that only one PCR product was amplified and detected. The Relative Expression Software Tool (REST-MCS_-version 2) was used to calculate the relative expression of target gene mRNA (Pfaffl et al., 2002). Three housekeeping genes (*gapdh*, *α-tubulin*, *β-actin*) were analyzed for suitable reference gene, the results showed that FBZ exposure did not alter the expression of *gapdh* (supplemental material). Therefore, gene expression levels were normalized to zebrafish *gapdh*.

2.6. Determination of FBZ in exposure solutions

Exposure solutions, freshly made up with the stock solutions, were collected three times at random for determination. The FBZ concentrations were measured based on the method of Zhang et al. (2017) with slight modification. Briefly, 1 L exposure solution was added with simeconazole (purity > 97%) (Witega Laboratories Berlin-Adlershof GmbH, Berlin, Germany) as a surrogate, and was extracted using a liquid-liquid extraction method with 50 mL CH_2Cl_2 in a separatory funnel. The organic phase was collected and dried with anhydrous sodium sulfate, and the extracts were concentrated to dryness under a rotary evaporator, and then were diluted with acetone/n-hexane (1:1) solution. FBZ concentration was detected using a GC/MS/MS system (Agilent Technology, USA) following the description of Zhang et al. (2017).

The recoveries of FBZ were 94% ± 0.6% (n = 3) and the limit of detection was 3.0 ng/L. The detected concentration of FBZ in the exposure medium was 0, 4.96 ± 0.09, 48.29 ± 0.41 and 473.58 ± 9.13 ng/L in the each group.

2.7. Data processing

Results are reported as means ± SE (standard error). After the homogeneity of variances being determinated, the data were statistically analyzed with one-way analysis of variance (ANOVA) followed by the Duncan test via SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). A value of $P < 0.05$ was used to indicate significant difference.

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