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The developmental effects of low-level procymidone towards zebrafish embryos and involved mechanism

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HIGHLIGHTS

- Exposure to PCM increased pericardial edema rate and spine curvature rate.
- PCM exposure resulted in cardiac and skeletal defects.
- PCM exposure altered the transcription of genes related to cardiac and skeletal development.
- The activities of Na^+, K^+-ATPase and Ca^{2+}-ATPase were inhibited.

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ABSTRACT

Procymidone (PCM), a dicarboximide fungicide, is widely used in agriculture to control plant diseases. In the present study, zebrafish embryos were exposed to PCM at 0, 10, 100 and 1000 ng/L for 72 h, the development and cardiac functioning of larvae were observed and determined. The results showed that hatching rate was significantly decreased in the 1000 ng/L treatment, and pericardial edema rate and spine curvature rate were significantly increased in the 100 and 1000 ng/L groups. The PCM-treated larvae exhibited an increased heart rate as well as arrhythmia, and shortened low jaw. The transcription levels of cardiac development-related genes *tbx5, nkx2.5, tnnt2, gata4, myh6, myl7, cdh2, ryr2* were 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 *ihh, shh, bmp2b, bmp4, gh, igf1, sox9, gli2*. The activities of Na^+/K^+-ATPase and Ca^{2+}-ATPase were significantly inhibited by 100 ng/L and 1000 ng/L PCM exposure, which might be a cause for the occurrence of pericardial edema and skeletal deformation. The results of this study will be helpful in evaluating the potential threat of PCM to fish population in the aquatic environment.

1. Introduction

Procymidone (PCM), an amide-type fungicide, is used to control plant diseases such as gray mold on fruits, grapes and vegetables, and sclerotinia rot of beans, stone fruit and vegetable crops (Tomigahara et al., 2014). The dose of PCM used is 0.5–1 kg/ha (Tomlin, 1994). Since PCM is stable to light, temperature and moisture, it would persist in soil for several weeks (Tomlin, 1994). Thus, PCM may contaminate aquatic environments through run-off. PCM concentrations in water samples draining from a cherry orchard in the Mt. Lofty Ranges, South Australia are 0.02–0.41 μg/L (Oliver et al., 2012). It is reported that PCM concentrations range from 0.05 to 9.06 μg/L in a river near an agricultural region in South Africa (Dabrowski et al., 2002). Among the 82 pesticides detected in water from the Jiulong River, China, that with the highest concentration is PCM (3904 ng/L) (Zheng et al., 2016), suggesting that the toxic effects of PCM on fishes need to be paid greatly attention to.

PCM displays an androgen receptor (AR) antagonist activity both in vitro and in vivo (Hosokawa et al., 1993; Nellemann et al., 2003; Vinggaard et al., 1999), it competitively antagonizes the binding of androgens to AR, and mainly affects the reproductive development in male offspring (Ostby et al., 1999). A few studies were conducted

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to evaluate the toxicity of PCM to fishes. The 96 h-LC_{50} of PCM is 22.9 mg/L for bluegill (*Lepomis macrochirus*) and 3.6 mg/L for rainbow trout (*Oncorhynchus mykiss*) (Tomlin, 1994). The 96 h-LC_{50} of vinclozolin, another dicarboxamide fungicide, is 2.8 mg/L for rainbow trout and about 50 mg/L for bluegill and pumpkinseed (*Lepomis gibbosus*) (Makynen et al., 2000). In fathead minnow (*Pimephales promelas*), mortality is not significantly increased in embryonic/larval fish exposed to 75–1200 μg/L vinclozolin for 34 days (Makynen et al., 2000). PCM can inhibit AR activation induced by 11-ketotestosterone in stickleback (*Gasterosteus aculeatus*) (Lange et al., 2015). Exposure of mullet fish (*Mugil dussumier*) to PCM at environmental concentrations (5.0, 50.0, 500.0 μg/L) for 14 days not only significantly increased the mRNA levels of vitellogenin (VTG) in female, also significantly decreased the transcription of *vtg* in male (Zheng et al., 2016). PCM induces VTG production in rainbow trout hepatocytes via a pathway involving the activation of estrogen receptor (Radice et al., 2004). Exposure to 0.2 or 0.4 mg/L PCM for 4 days increased hepatic metallothionein content in Roach (*Rutilus rutilus*) (Paris-Palacios et al., 2003). These adverse impacts of PCM on the reproduction and health of fishes would lead a reduction of fish population. However, up to date, there is not a report concerning the influence of PCM on the embryos development of fishes.

Zebrafish (*Danio rerio*) is a good model for ecotoxicological research, due to its characteristics such as high fecundity, rapid embryonic development, short generational cycle and easy to raise in large numbers. The optical transparency of the chorion is in favor of the observation of development process and the detection of morphological endpoints in early life stages (Yang et al., 2009). The development of fish includes all organic effects and militates in favor of mechanism research. In the present study, the effects of PCM at environmental levels on the development of zebrafish embryos were investigated, and the mechanisms involved would be assayed.

2. Materials and methods

2.1. Zebrafish maintenance and embryos collection

Wild-type TU zebrafish were maintained in a flow-through aquatic habitat system, under a normal photoperiod at 14:10 h light: dark. The water temperature was 28 ± 1 °C, the pH and dissolved oxygen were 7.2–7.3 and 7–8 mg/L respectively. Fish were fed with live brine shrimp twice daily. All fish experiments followed the ethical guidelines of Xiamen University. Sexually mature fish without any signs of disease were selected as breeders. Adult fish were mated at a ratio of 1:2 (female: male). After acclimatization overnight (for 12 h) in a spawning tank, in the beginning of the light period of the following day, the breeders were removed after egg-laying. Spawned eggs were collected within 0.5 h. Fertilized eggs were washed with zebrafish facility water and were used for experiment.

2.2. Embryonic exposure and sampling

PCM (purity > 98%) was purchased from Dr. Ehrenstorfer GmbH, Germany. It was dissolved in acetone at analytic grade to reach stock concentrations of 1, 10 and 100 μg/mL. PCM exposure solutions were obtained by adding the appropriate volume of the stock concentration to zebrafish culture medium (3.5 g/L NaCl, 0.05 g/L $NaHCO_3$, 0.05 g/LKCl, 0.05 g/L $CaCl_2$). Fertilized eggs were exposed to PCM at concentrations of 0, 10, 100 and 1000 ng/L. Approximately 150 embryos were cultured in 40 mL exposure solution in petri dish at 28 ± 1 °C, and there were five replicates for each treatment. Similar criteria were applied to solvent control group, which received an equal volume of acetone (10 μl/L). The exposure solutions were changed twice daily. The development of the embryos and mortality were monitored with an Olympus SZ51 stereo microscope. Pericardial edema is recognized as any appearance of coelomic cavity that separates visceral organs from the body wall (Westerfield, 2000). 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%. The embryos exposed to PCM for 72 h were collected for multiple analysis.

2.3. Cardiac function analysis

The heart rate and quantitative analysis of cardiac arrhythmia were performed with 20-s video segments collected from the exposed larvae 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 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). The number of frames between cardiac contraction initiations was calculated using NIS-Elements Imaging Software (Nikon, Tokyo, Japan). The mean and standard deviations (SD) were analyzed for each larva. This SD is a measure of heart rate irregularity, since a regular rhythm would have essentially the same number of frames between beats and therefore a low SD. Three larvae from each replicate were assessed to get a mean for this replicate. There were five replicates in each treatment.

2.4. Whole mount Alcian-blue staining

Exposed larvae were at random chosen in each treatment for the analysis of craniofacial cartilage development. Larvae were stained with 0.1% Alcian-blue 8GX in 80% ethanol/20% acetic acid solution based on the method described by He et al. (2011). Stained larvae were observed using a Leica M165FC stereo fluorescence microscope, and the lower jaw length as well as width was measured following the method of Hu et al. (2009). Three larvae from each replicate were assessed to get a mean for each replicate.

2.5. ATPase activity analysis

Approximately 40 larvae from each replicate were pooled, homogenized to obtain a supernatant, which was used as the source of enzyme. The ATPase activities were measured (by) using an ultramicro Ca^{2+}-ATPase kit and Na^{+}/K^{+}-ATPase kit from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturer's instructions. Protein content was measured by a coomassie blue protein assay kit; ATPase activities were expressed as mol Pi liberated per mg protein per hour (mol Pi/mg prot/h).

2.6. Real-time quantitative PCR (qPCR)

Fifty larvae from each replicate were pooled into a subsample. The method of RNA extraction and reverse transcription has been described previously (Huang et al., 2012). Total RNA was extracted from the whole embryos using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. First-strand cDNA was synthesized from 1 μg of total RNA using a Revert Aid Mu-MLV