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Fipronil-induced toxic effects in zebrafish (*Danio rerio*) larvae by using digital gene expression profiling

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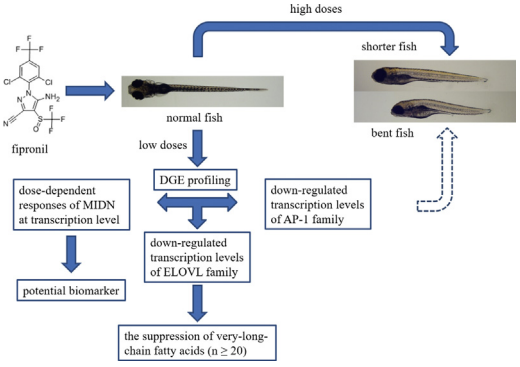
HIGHLIGHTS

- Zebrafish larva is sensitive to fipronil.
- Low-dose fipronil changed the transcriptome of zebrafish larvae.
- Low-dose fipronil affected the transcripts of AP-1 family in zebrafish larvae.
- MIDN showed dose-dependent responses to fipronil at the transcriptional level.

GRAPHICAL ABSTRACT



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ABSTRACT

Fipronil residue has caused widespread concern around the world, especially after the recent "toxic eggs" event in seven European countries. To evaluate the effects of fipronil on vertebrates, zebrafish larvae were used as an animal model to examine the lethal effect, developmental phenotypes at high doses, and possible mechanisms of toxicity by employing digital gene expression (DGE) profiling at environmentally relevant doses. The results of acute toxicity test indicated that treatment with fipronil from 75 h post-fertilization (hpf) led to the death of larvae with a 96-h LC50 value of 459 μg/L, as well as abnormal development including bent spine and shortened body length. Besides, we obtained high-quality-sequencing DGE profilings at fipronil concentrations of 0.5, 5, and 50 μg/L, respectively. The results revealed that 44 differentially expressed genes, 10 GO terms, and 3 KEGG pathways were overlapped among the three concentrations. MIDN, one of the 44 differentially expressed genes, showed dose-dependent responses at the transcriptional level, indicating that it was possibly a potential biomarker to reflect fipronil toxicity in zebrafish. Furthermore, we presumed that the changing transcriptional level of AP-1 family was possibly a reason for bent spine and shortened body length in larvae exposed to fipronil. Concurrently, altered abundance of transcripts of the ELOVL family in a key step of fatty acid elongation could possibly lead to the accumulation of long-chain fatty acids. Collectively, our results suggested that exposure to fipronil caused lethal and developmental toxicity in zebrafish larvae, and demonstrated the need for a comprehensive understanding of the potential mechanisms of fipronil toxicity due to fipronil's frequent presence in the environment and its potential threat to human health.

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1. Introduction

In recent decades, freshwater ecosystems throughout the world have been seriously threatened and destroyed by chemical pollution, leading to a large loss of species diversity (Beggel et al., 2012; Burkhardt-Holm et al., 2005; Geist, 2011). Pesticides, the main source of chemical contamination, are widely used and even abused in agricultural and urban areas, and are hazardous to aquatic organisms in freshwater ecosystems (Schafer et al., 2011). Internationally, there is a growing recognition of the effects of pesticides on non-target species, including humans.

Fipronil is the first phenylpyrazole insecticide, and widely known to target the gamma-aminobutyric acid type A receptors ($GABA_A$Rs) and block the chloride channel in the neurons of the central nervous system (CNS) of insects (Hainzl and Casida, 1996; Le Novere and Changeux, 2001). With high insecticidal activity and low mammalian toxicity, fipronil has replaced other traditional insecticides such as pyrethroids and organophosphates, and has been used extensively in crop protection (Cole et al., 1993; Tingle et al., 2003; Yan et al., 2016). Landscape maintenance and structural pest control are typical applications in urban areas (Beggel et al., 2012). However, repeated application and abuse of fipronil have been a disaster to many non-target organisms, such as aquatic vertebrates (Yan et al., 2016), as it significantly changes physiological and biochemical traits of fishes (Clasen et al., 2012; Hayasaka et al., 2012). A recent investigation into field spraying of fipronil showed a suppression of body growth in both adult and juvenile *Oryzias latipes* (Hayasaka et al., 2012). Other studies have shown that fipronil disturbs the redox system of *Cyprinus carpio* in rice fields (Clasen et al., 2012). A few lab studies concerning acute and sublethal doses of fipronil have found toxic effects, including developmental toxicity (Stehr et al., 2006), endocrine disruption (Sun et al., 2014), and behavioral deficiency (Beggel et al., 2012; Wang et al., 2016). Besides, enantioselective developmental toxicity induced by fipronil involves changes of DNA methylation in zebrafish embryos (Qian et al., 2017). In light of its high toxicity to aquatic non-target organisms, the use of fipronil has been forbidden or restricted, especially in rice fields. However, as the worldwide media reported, 920 μg/kg fipronil was recently detected in eggs, an incident that covered most European countries and regions. This "toxic eggs" event made fipronil residue become a hot topic again.

Although they strongly bind to soil organic substances, insecticides can still be transported through the processes of eluviation, migration, and accumulation into urban freshwater systems, even if it was not applied nearby surface water (Gunasekara et al., 2007; Jiang et al., 2010). This is the main reason that fipronil has been detected frequently in surface waters and sediments (Nillos et al., 2009; Sprague and Nowell, 2008; Xu et al., 2011); the residue concentrations of fipronil in surface water ranged from 1 to 70 μg/L (Bedient et al., 2005), and measured concentrations in irrigation runoff from residential areas were in a range of 0.13 to 12.6 μg/L (Gan et al., 2012). This small environmentally relevant dose of fipronil can also disturb zebrafish larvae metabolism *in vivo* (Wang et al., 2016), so these levels pose risks to individual and population-wide fish health (Little and Finger, 1990). In addition, aquatic invertebrates and early life stages of vertebrates like fish are generally extremely sensitive to the toxicity of fipronil (Beggel et al., 2010; Floyd et al., 2008; Jin et al., 2010). On the basis of these findings, more consideration should be given to the potential risks of fipronil exposure on aquatic organisms. Developmental toxicity of fipronil in aquatic organisms has been reported in a number of previous studies, but the detailed molecular mechanisms involved remain largely unknown.

Digital gene expression (DGE) profiling is a high-throughput transcriptome sequencing method based on next-generation sequencing technologies and used for measuring gene expression (Guo et al., 2016; Liu et al., 2014; Yu et al., 2014). In recent years, the DGE profiling has become a popular and effective approach that allows direct quantification of transcription levels (Hong et al., 2011; Ren and Pan, 2014). Compared with traditional transcriptomes, DGE profiling exhibits considerable superiority, with high sensitivity, low cost, and no need for genomic information (Zhang et al., 2013). DGE profiling has been widely used to detect differences in stress responses among different tissues in many species (Guo et al., 2016). However, few reports have used DGE profiling to analyze the toxic effects of fipronil on transcription levels in zebrafish.

In the present study, DGE profiling of zebrafish larvae, as a vertebrate model, was used to evaluate the toxic effects of fipronil and to explore the potential molecular mechanisms of toxic effects under environmentally relevant exposure levels. Moreover, we investigated the concentration-dependent response between differential transcription levels of genes and concentrations of fipronil with the goal of finding a potential marker gene that can reflect fipronil contaminations in its transcriptional level.

2. Materials and methods

2.1. Zebrafish husbandry

Adult zebrafish (*Danio rerio*, wild-type AB strain) were purchased from the China Zebrafish Resource Center and maintained in an ESEN-AW-SS1 system (ESEN Science & Technology Development Co., LTD., Beijing, China). The fish were fed artemia and maintained at 28 °C with a 14: 10 h light: dark cycle, water conductivity range of 500–550 μS, and pH range of 7.2–7.6. The female and male zebrafish were mated in spawning tanks, then embryos were collected, rinsed with system water, and cultivated in illumination incubators until 75 h post-fertilization (hpf), when they were used in the experiment (Peterson et al., 2011).

2.2. Acute toxicity test and morphological assessment following fipronil exposure

Standard stock solutions of fipronil (98.0% purity) were prepared in methanol (AR) at concentrations of 100 mg/L and 1000 mg/L. Working solutions at concentrations of 100, 200, 300, 400, 500, 600, 750, and 1000 μg/L were prepared by dilution of standard stock solutions with system water. Methanol (0.1% (v/v)) was used as a carrier solvent to dissolve fipronil in water and also added to a control without fipronil, which was considered the solvent control (SC). Both standard stock solutions and working solutions were stored at 4 °C until use. Following OECD guidelines No. 236, an acute toxicity assay was performed in 24-well plates at 75 hpf with 20 larvae per concentration per plate (one larva per well and one concentration per plate). All larvae were exposed to fipronil for a period of 96 h in an illumination incubator as described above. A manageable semi-static system was applied, and the renewal interval was every 24 h. There were three biological replicates in the experiment. Upon completion of exposure, at 96 h, the larvae from each treatment were analyzed with an Olympus BX63 light microscope with cellSens Standard imaging software to determine mortality (lack of heartbeat) rate, spine deformity (cyrtosis) rate and total larval length (measured from snout to tail).

2.3. Digital gene expression profiling analysis following fipronil exposure

2.3.1. Fipronil exposure

75-hpf larvae were exposed to working solutions of 0, 0.5, 5, and 50 μg/L fipronil in 9-cm plates with 50 larvae per plate and 3 plates per concentration. After exposing 72 h (at 147 hpf), 50 larvae were chosen randomly from 3 plates per concentration and collected in 1.5-mL centrifuge tubes cleaning with ultrapure water as one sample per concentration, then flash frozen in liquid nitrogen. Three biological replicates were completed and a total of 12 samples were obtained (3