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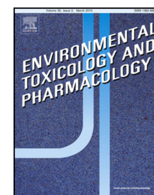

Perturbation of metabonome of embryo/larvae zebrafish after exposure to fipronil

Lu Yan [b], Chenxue Gong [a], Xiaofeng Zhang [b], Quan Zhang [b], Meirong Zhao [b], Cui Wang [a,*]

[a] *College of Life Science, Zhejiang Chinese Medical University, Hangzhou 310053, China*
[b] *College of Environment, Zhejiang University of Technology, Hangzhou 310032, China*

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ABSTRACT

The escalating demand for fipronil by the increasing insects' resistance to synthetic pyrethroids placed a burden on aquatic vertebrates. Although awareness regarding the toxicity of fipronil to fish is arising, the integral alteration caused by fipronil remains unexplored. Here, we investigated on the development toxicity of fipronil and the metabolic physiology perturbation at 120 h post fertilization through GC–MS metabolomics on zebrafish embryo. We observed that fipronil dose-dependently induced malformations including uninflated swim bladder and bent spine. Further, the "omic" technique hit 26 differential metabolites after exposure to fipronil and five significant signaling pathways. We speculated that changes in primary bile acid synthesis pathway and the content of saturated fatty acid in the chemical-related group indicated the liver toxicity. Pathway of Aminoacyl-tRNA biosynthesis changed by fipronil may relate to the macromolecular synthesis. Concurrently, methane metabolism pathway was also identified while the role in zebrafish needs further determination. Overall, this study revealed several new signaling pathways in fipronil-treated zebrafish embryo/larval.

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1. Introduction

Usage of the phenylpyrazole insecticide fipronil is expected to continuously grow worldwide with increasing resistance to synthetic pyrethroids (SPs) and organophosphates (OPs) in insects. Statistically, the consumption of fipronil reached to 10,000 tons in China alone in 2009 (Bayer Cropscience, China, personal communication). In 2011, the global sales figures of fipronil grew to six billion dollars. The large demand for fipronil led to the increased risk to non-targets such as aquatic in/vertebrates. Although strongly adsorbed by organic matter, fipronil can be frequently detected in surface water within the range of ng/L to μg/L (Nillos et al., 2009; Xu et al., 2011). The potential threat of fipronil to aquatic invertebrate has long been recognized, yet there remains substantial uncertainty regarding the risk on aquatic vertebrate such as fish.

Although the affinity of GABA receptor in vertebrate is less sensitive than that in insects, toxic potential of fipronil to vertebrate cannot be ruled out. Recent studies on field investigation have observed a body growth retardant both in adult and juvenile medaka after two-years spraying (Hayasaka et al., 2012). Also, field spraying of fipronil was proven to disturb the balance of the oxidative stress/antioxidant profile in *Cyprinus carpio* after 90 days of exposure in a rice field (Clasen et al., 2012). Similarly, laboratory research demonstrated a sub-organism effect (genetic transcription, erythrocyte damge, etc.), developmental toxicity (Stehr et al., 2006), behavior deficiency (Beggel et al., 2012; Wang et al., 2016), and endocrine disruption (Sun et al., 2014) under the sublethal and acute dose. As is well known, early life stage of vertebrates is extremely vulnerable to exogenous damage and can be fatal to descendent. Researchers observed the malformation phenotype in zebrafish embryo (96 h post fertilization (hpf)) including notochord degradation and rostral-caudal body shorten, which subsequently lead to the ineffective in touch response (Stehr et al., 2006). Mechanisms associated with fipronil-caused toxicity referred to erythrocytes DNA damage (Ghisi et al., 2011), mRNA transcription (Beggel et al., 2012), miRNA transcription (Wang et al., 2010), and antagonist to glycine receptor in embryo/larval (Stehr et al., 2006). However, genes are not the be-all and end-all of investigations into the workings of life. The holistic risk assessment on fish response to fipronil at embryo stage still remains on its paucity.

Environmental metabolomics was still considered an emerging field. It can be an ideal tool to scrutinize the slight perturbation on the metabolic physiology of embryo development and adult animals when exposure to environmental contaminations. Toxicological effects induced by several pollutants may be concurrent at the metabolic level. For example, researchers have used -omics

* Corresponding author.
E-mail address: wangcui198506@163.com (C. Wang).

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tests to identify the early biomarker of Cd-related nephrotoxicity (Gao et al., 2014). Others have revealed that metabolites with low molecular weight changed the response to aquatic contaminants in vertebrates or invertebrates (Zhang et al., 2013b). Studies concerning on the metabolomics after exposure to metals or organic chemicals have also been explored (Bundy et al., 2009; Gao et al., 2014). Thus, being successfully applied in the field of environmental science, metabolomics would pave a new way for biomarkers or biological pathways exploring in exposure science.

The aim of the current study was to reveal the potential molecular pathways induced by fipronil to zebrafish embryo. In addition to the morphological observation, we investigated the inner environmental change after exposure to fipronil. This study tried to relate the adverse effects to the pathways induced by fipronil.

2. Experimental procedures

2.1. Chemicals

Fipronil (purity > 97.6%) and MS222 (purity > 97%) were purchased from Sigma Chem. Co. (St. Louis, MO). The live Artemia was brought from Jiahong Feed Co., Tianjin, China. Chemicals used for the culture medium were analytical grade. Other regents were high performance liquid chromatography grade.

2.2. Experimental animals

Adult AB strain zebrafish (*Danio rerio*) were stocked in a flow-through tank at 28 °C with a dark/light photoperiod of 10:14 h (Westerfield, 1995). The maintain water was kept at pH 7.0 to 7.5 and conductivity between 450 and 1500 μs/cm. Fish were fed thrice daily with live Artemia. The day before spawning, male and female fish at ratio of 1:2 were maintained at a spawning box with a separated net overnight. In the next morning, the light was turned on to induce spawn. Within 1 h of spawning, fertilized embryos were siphoned, washed with essential medium (EM), staged and incubated in Petri dishes at 28 ± 1 °C with EM. Normal embryos were collected under a stereomicroscope (Nikon, Japan) at 6 hpf for use.

2.3. Embryo exposure assays

Embryo acute exposure assay was performed according to our previous study (Jin et al., 2010, 2009). Zebrafish embryos were exposed to control, negative control (0.1% ethanol), 100, 200, 400, 800 μg/L or 1000 μg/L fipronil (The stock solution of fipronil was dissolved in DMSO) at 6 to 120 hpf. Embryos were randomly placed in 96-well plates (Costar) with 200 μL of test or control solutions with one embryo/well and ten embryos for each dose. Three replicates were performed with 30 embryos in each treatment group. Test solutions were renewed by half per day. Plates were placed in a temperature-controlled incubator at 28 ± 1 °C with a 10:14 h dark/light photoperiod. The hatching and malformation of embryos/larvae were recorded every 12 h during exposure under an inverted dissecting microscope (Leica Microsystems). Dead embryos/larvae were immediately removed. At the end of the exposure (120 hpf), larvae were anesthetized with 0.02 g/ml MS222 for imaging. The length of larvae was marked as the distance from the anterior end of the mouth to the end of the caudal peduncle along the notochord using digital micrographs. The experiment was performed in triplicate. Finally, all larval were collected and stored at −80 °C for the metabolomic profiling.

2.4. GC–MS performance and metabolite analysis

The metabolite from whole larval body was extracted by a solution of CH_3Cl: MeOH: H_2O = 20:50:20. The samples were derivative prior to analysis. A 1.0 μL aliquot sample of the control group and test group (400 μg/L) were injected onto Thermo Focus DSQ GC/MS equipped with a DB-5MS (30 m × 0.25 mm, 0.25 um) column by the splitless mode. We set the injection temperature, ion source temperature and transmission lines temperature at 250 °C. The helium carrier gas flow was set at a constant rate of 1 mL/min. The initial temperature was isothermally held at 80 °C for 2 min and then raised to 300 °C at a rate of 15 °C/min and maintained for 6 min. Electron impact mode and full scan monitoring were recorded over the mass slope of *m*/*z* 50–650. A pooled samples consisted of pretreated samples from the two group were used as the quality control samples. Briefly, after every 4 samples injected, quality control samples were injected to the system to ensure the consistent of the method of performance and analysis.

2.5. Data processing and multivariate analysis

The raw mass spectrometric data was extracted on R-software. After peaks picking and integration, retention time correction, peak alignment and deconvolution, a two-dimensional data matrix including variables (RT_mz), observation samples and normalized peak area was generated. The multivariable analysis including unsupervised principal component analysis (PCA), the supervised recognition pattern population to partial least-square-discriminate analysis (PLS-DA) and supervised recognition pattern orthogonal projection to latent structures analysis (OPLS) was performed on SIMCA-P 11.0 software (Umetrics, Sweden) under unit variance scaling and mean-centered process. PCA was used to measure the multivariable to be differences or similarity. Then the PLS-DA was performed to determine the association between the two sets of variables. The OPLS was applied to determine the separation between two sets of variables and avoid the noise of unrelated classification. One primary component (t[1]P) and one orthogonal component (t[1]O) were fitted in OPLS. And then metabolites with significant difference between fipronil exposure and control group were identified by the criteria of variable importance in the projection (VIP >1) and student *t*-test ($p < 0.05$). Also, they were matched with the certified reference material database and NIST commercial database comparison.

2.6. Metabolic pathway analysis

Metabolic pathway analysis (MetPA) derived the results from both pathway enrichment and the topology analysis. Totally, the high quality Kyoto Encyclopedia of Gene and Genomes (KEGG), many well-established method, as well as novel algorithms and concepts are involved into pathway analysis. The biological function for the identified metabolites was sought via enrichment analysis using MetaboAnalyst 2.0 software (www.metaboanalyst.ca). The signaling pathway that was potentially involved in the treatment group was determined by comparing and merging. In the context of pathway analysis, we tested if compounds involved in a particular pathway was enriched compared by random hits with one-tailed Fisher's exact test. A −log(p)value for each assigned network reflecting the probability of the pathway was generated at random. Since many pathways were tested at the same time, the statistical *p* values from enrichment analysis are further adjusted for multiple testing.

2.7. Statistical analysis

The statistical significance between control and fipronil group for the endpoints of mortality, curved body axis and the body length was performed using one-way ANOVA. The probability (*p*) value set at 0.05 was termed as significance. Data analyses were performed

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