Ecotoxicology and Environmental Safety 161 (2018) 610–615

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Evaluation of acetamiprid-induced genotoxic and oxidative responses in *Eisenia fetida*

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ARTICLE INFO

Keywords:
Acetamiprid
Geno-toxicity
Oxidative stress
DNA damage
Earthworm

ABSTRACT

As a novel neonicotinoids insecticide, acetamiprid has been widely used worldwide. In this study, a laboratory test was conducted to expose earthworms (*Eisenia fetida*) to artificial soil spiked with various concentrations of acetamiprid (0, 0.05, 0.10, 0.25 and 0.50 mg/kg of soil) respectively after 7, 14, 21 and 28 d. Reactive oxygen species (ROS) generation, antioxidant enzymes activity including superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferases (GST), malondialdehyde (MDA) content, and DNA damage were determined in earthworms. The ROS level increased in varying degrees at most exposure concentrations. The SOD activity was not significantly affected. The CAT activity was increased in the beginning, then gradually suppressed and resumed to the control level at the end, with the maximum change (171%) occurred at 14 d for 0.05 mg/kg. The GST activity was induced at 7 d, and then inhibited, with the maximum change (67.6%) occurred at 14 d for 0.50 mg/kg. The MDA content had a tendency that increasing at the first and decreasing at the end. The olive tail moment (OTM) in comet assay reflected a dose-dependent relationship, and DNA damage initially increased and then decreased over time. The results suggest that the sub-chronic exposure of acetamiprid can cause oxidative stress and DNA damage of earthworm and change the activity of the anti-oxidant enzyme. In addition, ROS content and DNA damage can be good indicators for assessing environmental risks of acetamiprid in earthworms.

1. Introduction

Acetamiprid, (1*E*)-*N*-[(6-chloro-3-pyrinyle) methyl]-*N′*-cyano-*N*-methylethanimidamide, is a type of chlorinated neonicotinoids insecticide, the latest major class of insecticides (Saha et al., 2017). This systemic insecticide has a unique mode of action of nicotinic acetylcholine receptor agonists (n ACh R), which makes it highly efficient in combating insect pests such as aphids, whiteflies, and jassid (Renaud et al., 2018). Nowadays, acetamiprid has been commercialized and widely used in crop protection around the world (Murano et al., 2018). As a promising insecticide, acetamiprid is inevitably released into the environment due to its extensive use. Although acetamiprid shows relatively low acute and chronic toxicity for mammals, its impact on non-target organisms has received more and more concern. Wang et al. (2012) reported that the 24 h-LC_{50} from acetamiprid to earthworm (*Eisenia. fetida*) is 0.0088 μg cm^{-2} in a contact filter paper toxicity bioassay and 7 d-LC_{50} and 14 d-LC_{50} to earthworm are 1.72 and 1.52 mg/kg in a soil toxicity bioassay respectively. In addition, Wang et al. (2015) found that acetamiprid can significantly inhibit the fertility and cellulose activity of earthworm, and can also damage the epidermal and mid-gut cells of the earthworm.

Earthworms are the largest biomass invertebrates in the soil, which play a crucial part in maintaining the ecological function of soil (Datta et al., 2016). Moreover, earthworms are near the bottom of the food web, and closely contacted with types of pollutants in soil. Therefore, earthworms are often used as a priority test organism to monitor soil pollution and estimate the environmental toxicity of chemicals for soil invertebrates (Vasseur and Bonnard, 2014). In particular, at the macro level, the mortality, growth rate, avoidance behavior and reproduction of earthworms are used as common indicators (Han et al., 2014; Santadino et al., 2014; Travlos et al., 2017). Furthermore, on a micro level, the enzyme activity, DNA damage and sperm aberration are also used as common indicators (Han et al., 2014; Muangphra et al., 2016). The latter is more sensitive than the first, a dose below the acute toxicity test can also be monitored. It is therefore of great importance for the monitoring and early warning of pesticides and the protection of

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https://doi.org/10.1016/j.ecoenv.2018.06.022
Received 4 April 2018; Received in revised form 4 June 2018; Accepted 9 June 2018

the population and the ecosystem. However, only a limited data on subchronic oxidative stress and genotoxic effects of acetamiprid on earthworms are available.

The main objectives of this study were therefore to assess the subchronic effects of different concentrations and exposure times of acetamiprid on the reactive oxygen species (ROS), anti-oxidant enzyme system including superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferases (GST), lipid peroxidation (LPO) and DNA damage in earthworms.

2. Material and methods

2.1. Chemicals and reagents

Acetamiprid (purity ≥ 97%) was obtained from Shandong Pesticide Factory. Other organic and inorganic chemicals were purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China) and Sigma Chemical Co., Ltd. (St. Louis, Missouri, US).

2.2. Soil and earthworms

Artificial soil consists of 10% sphagnum peat moss (Premier), 20% kaolin clay (Fisher Scientific), 70% sand (grade 70, particle size 0.1 − 0.3 mm) was prepared according to the method described in OECD (2004). This soil was spiked with mixed solutions of different concentrations of acetamiprid to make final concentrations 0, 0.05, 0.10, 0.25 and 0.50 mg/kg of soil. Mature earthworms (*E. fetida*) weight 300 – 400 mg were purchased from earthworm culture farm in Taian, China. Before using, all earthworms were cultured in the laboratory for a month.

2.3. Exposure experimental design

The experiment was carried out in 1-L glass beakers, each contained 500 g spiked soil. The water content of the artificial soil was adjusted to 35% (w/w). Ten *E. fetida* acclimatized in the laboratory (each wet weight was approximately 350 ± 5 mg) were put on the surface of soil. Perforated aluminum foils were covered on the beakers to prevent the earthworms from escaping and reduce the water loss. The treatments were performed in triplicate. Exposures were conducted at 20 ± 1 °C under a photoperiod of 12:12 light: dark. All beakers were checked daily for the soil moisture content and the earthworm mortality. Earthworms were sampled on 7, 14, 21, and 28 days respectively. At each sampling, three earthworms were removed from the beaker in each dose treatment, washed with distilled water and transferred to clean wet filter paper to purge for 12 h.

2.4. ROS measurement

The ROS level was determined using the DCFH-DA method according to Wang et al. (2016). Briefly, gut-cleaned earthworms were homogenized in 100 mM ice-cold PBS buffer (pH 7.4). The homogenate was centrifuged at 1000*g* for 10 min at 4 °C. The supernatants were re-centrifuged at 20,000*g* for 20 min at 4 °C. Then the pellet was re-suspended in 1 mL of 100 mM PBS buffer (pH 7.4). The DCFH-DA solution (10 mM) was mixed with the re-suspended solution, and reacted at 30 °C for 30 min. Finally, a fluorescent spectrophotometer was used to measure the absorbance of the mixture at 485 and 538 nm respectively (Shimadzu, RF-530PC).

2.5. Measurement of enzyme activities (SOD, CAT, and GST) and MDA content

Gut-cleaned earthworms were homogenized in a 50 mM ice-cold PBS buffer (pH 7.8). The homogenate was centrifuged at 13,000*g* for 10 min at 4 °C. The supernatants were used to determine the earthworm enzymatic activity and MDA content.

The inhibition of nitro blue tetrazolium (NBT) reduction was measured to determine the SOD activity according to the method described by Song et al. (2009). The amount of enzyme that inhibited the NBT reduction by 50% was considered as one unit (U) of SOD activity. The consumption of H_2O_2 at 250 nm for 1 min was monitoring to determine the CAT activity according to the method described by Wang et al. (2016). The amount of enzyme that decomposed half of H2O2 in 100 s at 250 °C was considered as one unit (U) of CAT activity. The GST activity was determined using the 1-chloro-2,4-dinitrobenzene (CDNB) method according to Liu et al. (2016). The CDNB solution (15 mM) was mixed with the supernatants and the PBS buffer (100 mM, pH 7.4), and incubated at 30 °C for 3 min. Then, the GSH solution (15 mM) was added in the mixture and reacted at 25 °C for 3 min. During the reaction, the absorbance of the mixture was recorded every 30 s at 34 nm. The thibabituric acid (TBA) colorimetric method according to the method described by Han et al. (2014) was slightly modified to determined the MDA content. The supernatants was mixted with SDS solution (8.1%, w/v), acetate buffer (20%, w/v), TBA solution (1%, w/v) and distilled water and incubated at 95 °C for 1 h. The mixture was centrifuged at 1000*g* for 15 min. The absoebance of the supernatants was determined at 532 nm.

2.6. Measurement of DNA damage

The method described by Wang et al. (2016) was used to obtain the coelomocytes of earthworm. Briefly, gut-cleaned earthworms were placed in 1 mL physiological saline solution to clean up the residual soil, then to a 2 mL extraction solution that contained ethylenediaminetetraacetic acid (0.25%, w/v), ethanol (5%), physiological saline (95%) and guaiacol glyceryl (1%, w/v) to conceal spontaneous coelomocytes. Coelomocytes extraction was centrifuged at 3000*g* for 10 min at 4 °C. The pellet was re-suspended by PBS buffer and re-centrifuged at 3000 *g* for 10 min at 4 °C. Then re-suspend the pellet by PBS buffer to recover the coelomocytes extraction. The comet assay was performed according to the method described by Song et al. (2009). The magnitude of damage caused by DNA could be reflected efficiently and susceptibly by the parameters of OTM.

2.7. Statistical analysis

The images of the comet were first analyzed by the Comet Assay Software Project (CASP). The statistical analysis was performed using the SPSS19.0 and Origin 8.5. One-way analysis of variance (one-way ANOVA) were used to examined the differences between the control and treatments ($p < 0.05$ of significance). The two-way analysis variance (two-way ANOVA) was used to examined the biochemical responses of the concentration, exposure time and their interaction.

3. Results

3.1. Effects of Acetamiprid on ROS levels in E. fetida

As shown in Fig. 1, there was no significant difference in ROS levels between the low concentration treatments (0.05 and 0.10 mg/kg) and control after exposure to acetamiprid for 7 days, while the ROS levels were significantly higher than the control where high concentrations (0.25 and 0.50 mg/kg) of acetamiprid treatments were applied. On day 14, the values of ROS in earthworms exposed to acetamiprid were significantly higher than the control, except for treatment where acetamiprid was applied in concentration 0.05 mg/kg ($p < 0.05$). On the 21st and 28th days, the ROS values rose significantly in various treatments ($p < 0.05$). Overall, the ROS levels in earthworms increased significantly after exposure to acetamiprid.