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Oxidative stress and gene expression of earthworm (*Eisenia fetida*) to clothianidin

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ABSTRACT

Neonicotinoid insecticides have become the most widely used pesticides in the world. Clothianidin is a novel neonicotinoid insecticide with a thiazolyl ring that exhibits excellent biological efficacy against a variety of pests. In the present study, the oxidative stress and genotoxicity of clothianidin on earthworms were evaluated. Moreover, the effective concentrations of clothianidin in artificial soil were monitored during the whole exposure period. The results showed that clothianidin was stable in artificial soil and that the residue concentrations were 0.094, 0.476, and 0.941 mg/kg after 28 d of exposure, which represented changes no more than 10% compared to the concentrations on the 0th day. Additionally, both the concentration of and exposure time to clothianidin had a substantial influence on biomarkers in earthworms. At 0.5 mg/kg and 1.0 mg/kg, the reactive oxygen species (ROS) levels were greatly enhanced, causing changes in antioxidant enzyme activities, damage to biological macromolecules and abnormal expression of functional genes. Additionally, the present results showed that superoxide dismutase (SOD), DNA damage and heat shock protein 70 (HSP70) may be good indicators for environmental risk assessment of clothianidin to earthworms.

1. Introduction

Neonicotinoid insecticides have become the most widely used pesticides in the world (Goulson and Kleijn, 2013; Sparks and Nauen, 2014). Neonicotinoid insecticides are acetylcholine receptor agonists that have high insecticidal activities against sucking insect pests, such as *Thysanoptera* and *Hemiptera* (Jeschke et al., 2011; Sparks and Nauen, 2014). Clothianidin [(E)-1-(2-chloro-1,3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine] is a novel neonicotinoid insecticide with a thiazolyl ring that exhibits excellent biological efficacy against varieties of pests, such as weevils, leafminers, whiteflies, beetles and various species of jassids (Uneme et al., 2006; Zhang et al., 2015). To date, clothianidin has been commercialized and applied by a variety of methods, such as seed dressing, root watering and syringing (Xu et al., 2016).

However, the abuse of pesticides is a serious problem worldwide (Li et al., 2014; Rasmussen et al., 2015). As a type of pesticide, clothianidin, will inevitably be released into the environment. Although studies have shown that clothianidin has little effect on birds, mammals and aquatic species, it is very persistent in soil with a half-life of 13–1386 d (Morrissey et al., 2015; Perre et al., 2015). Therefore, clothianidin is a potential soil contaminant, and we must consider the effect of clothianidin on soil environment. However, the current emphasis of research on clothianidin is its residue and dissipation in soil (Bo et al., 2012; Li et al., 2012; Ramasubramanian, 2013). The study of the ecotoxicological effects of clothianidin on soil organisms is still in its infancy. Therefore, it is necessary to obtain a series of data about the influence of clothianidin on soil organisms.

Earthworm, an important soil organism, can convert organic waste and biodegradable material into nutrients, thus playing an important role in the maintenance and development of the nutrient content of soil (Datta et al., 2016). Moreover, earthworms are sensitive to environmental pollution and used as model organisms for assessing the environmental impact of pollutants (Pelosi et al., 2013; Vasseur and Bonnard, 2014).

In addition, studies have shown that the concentrations that induced toxic effects on organisms are not the nominal concentrations due to some pollutants being able to combine with environmental media or be degraded (Bondarenko and Gan, 2009; Avio et al., 2015). The freely dissolved concentrations of pollutants are the main constituent inducing toxic effects on organisms (Bondarenko and Gan, 2009; Oleszczuk et al., 2016). Consequently, using an efficient analytical method to determine the effective concentrations of pollutants is essential to evaluate their toxicity.

Therefore, the influence of a series of background concentrations of clothianidin on earthworms was evaluated. Additionally, the effective concentrations of clothianidin during the whole exposure period were

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monitored using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). The purpose of the present research was to evaluate the induction of oxidative stress and genotoxicity by clothianidin in earthworm. Moreover, it may provide a theoretical basis for the environmental risk posed by clothianidin to soil organisms.

2. Materials and methods

2.1. Materials

Clothianidin (98.0% purity, CAS NO. 210880-92-5) was obtained from Dr. Ehrenstorfer GmbH (Germany).

Earthworms (*Eisenia fetida*) were supplied by an earthworm breeding base (Qingdao, China). Before the experiments, the earthworms were cultured in the laboratory at 20 ± 1°C for 2 weeks. Subsequently, healthy earthworms with adult clitellum (300–350 mg) were used for experiment.

Artificial soil, containing 10% sphagnum peat moss, 20% kaolin clay and 70% sand, was prepared according to the method described by the Organization for Economic Co-operation and Development (OECD, 2004).

2.2. Bioassays

Studies have shown that the initial residual concentration of clothianidin in different types of soil ranged from 0.14 mg/kg to 0.44 mg/kg (Uneme et al., 2006; Chowdhury et al., 2012; Ramasubramanian, 2013). Therefore, the concentrations of clothianidin used in the present experiment were 0, 0.1, 0.5 and 1.0 mg/kg. The appropriate amounts of clothianidin in methanol were added to the artificial soil. For the control group, the same amount of methanol without clothianidin was added to the artificial soil. The soil was sufficiently mixed until all the methanol had evaporated and transferred into a glass beaker. Subsequently, twenty earthworms were put into the beaker and cultured in a greenhouse at 20 ± 1°C with a 12/12 h light/dark cycle. All treatments were replicated five times. On the 2nd, 7th, 14th, 21st and 28th days, 20 g of soil and four earthworms were randomly sampled from each glass beaker for the various analyses.

2.3. Determination of effective concentrations

Clothianidin in soil (5.0 g) was extracted using 5 mL of deionized water and 10 mL of acetonitrile. Subsequently, 4*g* of anhydrous magnesium sulfate and 1*g* of sodium chloride were added to make the sample layered. The sample was vibrated for 3 min and then centrifuged at 4000 rpm for 10 min. The supernatant (2 mL) was added to a tube that contained N-propyl ethylenediamine-bonded solid adsorbent (25 mg) and anhydrous magnesium sulfate (150 mg). The sample was vibrated for 3 min and then centrifuged at 5000 rpm for 5 min. Subsequently, the supernatant was filtrated through a 0.22 μm syringe filter.

The separation of clothianidin was performed on an HPLC system (Accela, Thermo Fisher Scientific, San José, CA, USA). Clothianidin was separated by injecting a 10 μL sample onto a Thermo Hypersil GOLD C_{18} column (2.1 × 100 mm, 3.0 μm). The mobile phase used in the present study consisted of acetonitrile (A) and 1% formic acid in water (v/v, B). The gradient elution procedure was as follows: 0–0.5 min, 90–10% A; 0.5–6.0 min, 10–90% A; and 6.0–10.0 min, 90–10% A. The column temperature was maintained at 25°C, and the flow rate was controlled at 0.25 mL/min.

The determination of clothianidin was performed on a triple-quadrupole mass spectrometer (Thermo TSQ Quantum Ultra, Thermo Fisher Scientific Inc., San José, CA, USA). The capillary voltage of the positive electrospray ionization (ESI^+) source was 3.0 kV. The capillary temperature was 350 °C, and scans were obtained in multiple reaction monitoring (MRM) mode. The qualitative ion pair was 250.0/131.9 (*m/z*) with a collision energy of 18 eV. The quantitative ion pair was 250.0/169.0 (*m/z*) with a collision energy of 13 eV.

2.4. Determination of the reactive oxygen species (ROS) level

The ROS level was determined using the 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) method described by Liu et al. (2014). One earthworm from each beaker (five for each treatment) was randomly selected to determine the ROS level and homogenized in 100 mM phosphate buffer (1:9, w/v, pH 7.4). The samples were centrifuged at 4000 rpm for 5 min, and the supernatants were collected. Subsequently, the supernatants were centrifuged at 20,000 × *g* for 20 min, and the precipitates were resuspended in 1 mL of 100 mM phosphate buffer (pH 7.4). DCFH-DA (5 μM) was added to the sample, and the mixture was reacted at 37°C for 15 min. The fluorescence intensity of the mixture was determined using a fluorescence spectrophotometer (Shimadzu, RF-5301PC).

2.5. Determination of enzyme activities

One earthworm from each beaker (five for each treatment) was randomly selected to determine the enzyme activities and homogenized in 50 mM phosphate buffer (pH 7.8). The samples were centrifuged at 10,000 rpm for 15 min, and then the supernatant was used to measure the content of protein and activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and glutathione S-transferase (GST).

The protein content was measured using bovine serum albumin (BSA) as the standard according to the method of Liu et al. (2016).

SOD activity was measured using the method described by Song et al. (2009). The sample solution contained 750 μM nitroblue tetrazolium, 130 mM methionine, 100 μM ethylenediamine tetraacetic acid disodium salt, 50 mM phosphate buffer (pH 7.8), 20 μM riboflavin, deionized water and enzyme extracting solution. The sample was illuminated at 4000 Lx for 20 min, and then the absorbance was recorded at 560 nm. One unit (U) of SOD activity was defined as the quantity of SOD causing the photoreduction of NBT by half and expressed as U mg^{-1} protein.

CAT activity was determined as described by Liu et al. (2016). The sample solution consisted of 10 mM hydrogen peroxide (H_2O_2), 100 mM phosphate buffer (pH 7.0) and enzyme extracting solution. The H_2O_2 was decomposed by CAT, and the decrease in ultraviolet absorption was monitored at 250 nm for 1 min. One unit (U) of CAT activity refers to the amount of CAT inducing a decrease in H_2O_2 concentration in 1 min at 250 nm and expressed as U mg^{-1} protein.

POD activity was measured as described by Xu et al. (2013). The sample consisted of 10 mM H_2O_2, 20 mM guaiacol, 100 mM phosphate buffer (pH 7.0) and enzyme extracting solution. The guaiacol was oxidized to a dark brown species, and the change in absorbance was monitored at 470 nm for 1 min. One unit of POD activity refers to the amount causing a 0.01 increment of Δ_{470} in 1 min and was expressed as U mg^{-1} protein.

GST activity was determined as described by Liu et al. (2016). The sample contained of 10% glycerinum, 15 mM 1-chloro-2,4-dinitrobenzene (CDNB), 15 mM reduced glutathione (GSH), 0.1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 1 mM ethylenediaminetetraacetic acid (EDTA) and enzyme extracting solution. The increase in absorbance was measured at 340 nm for 3 min. One unit of GST activity was defined as the quantity of GST leading to combination of GSH and CDNB in 3 min at 340 nm and expressed as U mg^{-1} protein.

2.6. Determination of malondialdehyde (MDA) content

MDA content was determined using the method of Han et al. (2014). The sample contained 200 μL of enzyme extracting solution, 200 μL of sodium dodecyl sulfate solution, 1 mL of deionized water, 1.5 mL of 1%