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Biomarker analysis of combined oxytetracycline and zinc pollution in earthworms (*Eisenia fetida*)

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HIGHLIGHTS

- Antagonistic effects of OTC and Zn^{2+} exposure were proofed compared to Zn^{2+} alone.
- A mechanism involving complexation of Zn^{2+} and OTC at alkaline pH is proposed.
- Lysosomal membrane stability and coelomocyte apoptosis are sensitive biomarkers.

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ABSTRACT

To determine the interactive action of antibiotics and heavy metals, this study assessed pollutant-induced responses of cellular biomarkers in earthworms (*Eisenia fetida*) exposed to zinc (Zn^{2+}) and oxytetracycline (OTC) in soil. Lysosomal membranes were damaged and coelomocyte apoptosis occurred with exposure to the individual and combined pollutants. Compared with Zn^{2+} alone, lysosomal membrane stability and coelomocyte apoptosis decreased in the Zn^{2+}–OTC combined treatment, possibly as a result of complexation of Zn^{2+} and OTC at alkaline pH. Such complexation could reduce the toxicity of the pollutants. Lysosomal membrane stability and coelomocyte apoptosis are sensitive biomarkers and could be economical and rapid tools for the monitoring and assessment of a variety of pollutants.

1. Introduction

The antibiotic oxytetracycline (OTC) is used extensively worldwide in veterinary drugs and feed additives, and is beginning to pollute soils. Most veterinary antibiotics cannot be completely absorbed by animals and are discharged through feces and urine as the parent compound or as metabolites (Halling-Søensen, 2001; Liao et al., 2001; Kong and Zhu, 2007). The impact of OTC on the environment is increasing with the development of intensive livestock and poultry breeding programs and the wide use of manure fertilizer. The average OTC content is as high as 9.1 mg kg^{-1} (1.1–135 mg kg^{-1}) in pig manure and 6.0 mg kg^{-1} (2.9–23 mg kg^{-1}) in chicken manure (Zhang et al., 2005). OTC concentrations averaged 5.2 mg kg^{-1} in surface soils (0–20 cm) from agricultural fields treated with animal manure in northern Zhejiang Province, China (Zhang et al., 2008). Hamscher et al. (2000) showed that contents of oxy- and chlortetracycline in surface soils (0–40 cm) were as high as 32 and 26 mg kg^{-1}, respectively, after application of manure fertilizer.

Tetracyclines, including OTC and other veterinary antibiotics, may lead to antibiotic resistance in microorganisms, which is potentially hazardous to non-target organisms in terrestrial environments. Various effects of OTC have been observed, including the inhibition of bacteria, actinomycetes, and total microorganism populations (Wang and Zhang, 2009); decreased urease, sucrose phosphatase, and hydrogen peroxidase activity (Yao et al., 2010); reduced root and shoot elongation in wheat and Chinese cabbage (Jin et al., 2009); and genotoxicity to earthworms (Dong et al., 2012).

Zinc (Zn) is a trace element that is required for biological growth. The content of Zn in soil generally ranges from 10 to 300 mg kg^{-1} (Li et al., 2006), but Zn concentrations exceeding 13,500 mg kg^{-1} have been reported for polluted soil in China (Li et al., 2006). Some plants can absorb and accumulate Zn, but excess zinc uptake can inhibit plant growth and development (Song et al.,

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2003; Lin et al., 2007). Zinc can also affect soil respiration (Vanhala and Ahtiainen, 2006), enzyme activity (Yang et al., 2006; Lin et al., 2007), and bacterial diversity and population size (Moffett et al., 2003; Lu et al., 2010), and can have negative effects on soil and human health (Lock and Janssen, 2001; Jia et al., 2005).

Antibiotics and heavy metals are often detected together in soils and organic fertilizers (Zhang et al., 2005). Combined antibiotic and heavy metal pollution has received increasing global attention. For example, metal- and antibiotic-resistant bacteria were found in Antarctic marine waters, and this was attributed to long-term exposure to low concentrations of these pollutants (De Souza et al., 2006).

Ecotoxic effects of antibiotics in soil have been reported (Thiele, 2005; Bao, 2008; Hammesfahr et al., 2008; Liu et al., 2009). However, little information is available on the potential biochemical and genetic effects of veterinary antibiotics and heavy metals in soils, although a few studies have indicated that the combination of antibiotics and metals can be harmful to environmental bacteria and earthworms (Kong et al., 2006; Gao et al., 2013, 2014). More information on the effects of these pollutants is needed for improved soil risk assessment.

Biomarkers in terrestrial invertebrates can be used to estimate the exposure to and effects of pollutants. Earthworms are sensitive indicators of soil quality and are widely used in terrestrial ecotoxicology (ISO, 1998; Lin et al., 2007). Many biomarkers have been utilized to profile the toxicological effects of pollutants, including lethality, LD_{50}, reproductive rate, enzymatic activity, lysosomal membrane stability, cell apoptosis, and DNA damage (Sforzini et al., 2012; Wu et al., 2012). However, mortality, growth rate, and reproduction are commonly considered inappropriate for evaluating chemicals of lower toxicity and concentration. Qu et al. (2005) reported that OTC does not induce earthworm mortality at a concentration of OTC 500 mg/L. However, using the comet assay, these authors showed that OTC induced significant DNA damage in earthworm coelomocytes ($P < 0.01$).

At the subcellular level, the stability of lysosomal membranes in coelomocytes can be used as a biomarker for the toxicological effects of contamination. One method that can be used to determine lysosomal membrane stability is the neutral-red retention assay. Neutral red retention time (NRRT) is a sensitive marker in earthworms that meets soil pollution risk assessment requirements (Svendsen et al., 2004; Jia et al., 2005). Flow cytometry accurately detects cell survival and apoptosis rates and reflects damage to cell membranes. This technique has been widely used to evaluate the toxicological effects of pollutants. Gao et al. (2014) reported a synergistic effect of 5 mg kg^{-1} OTC + 50 mg kg^{-1} lead (Pb) on earthworm lysosomes and they found antagonistic effects at higher concentrations (10–20 mg kg^{-1} OTC + 50 mg kg^{-1} Pb). In addition, coelomocyte apoptosis decreased significantly with combined OTC and Pb treatment compared with OTC alone, indicating an antagonistic reaction.

In general, the modes of interactive effects between chemicals include antagonism, addition, and synergism. However, there is little information available on the interactive action of antibiotics and heavy metals. Knowledge of such interactions is important for assessing the toxicity, migration and transformation of pollutants, particularly compound pollution. Therefore, the objectives of this study were to (1) determine the interactive action of OTC and Zn^{2+} on earthworms, and (2) investigate the use of cellular biomarkers in earthworms (*Eisenia fetida*) exposed to zinc alone and in combination with OTC. We accordingly investigated lysosomal membrane stability and coelomocyte apoptosis for their potential use as sensitive, simple, and rapid biomarkers for soil monitoring and assessment.

2. Materials and methods

2.1. Soils and chemicals

Surface soils (0–30 cm) were sampled from suburban farmland in Tianjin, China. The samples were air-dried, crushed to pass through a 10-mm-mesh screen, and stored in containers at 4 °C. The background contents of OTC and Zn were 0.0 and 8.3 mg kg^{-1}, respectively. The soil had a medium loam texture with a density of 1.3 g cm^{-3}; a pH of 8.2; an organic matter content of 1.6%; a water-holding capacity of 42%; a composition of 21% sand, 50% silt, and 29% clay; and a cation exchange capacity of 11 cmol kg^{-1}.

Oxytetracycline hydrochloride (95%–105% purity), EDTA, and dimethyl sulfoxide (DMSO, >99% purity) were obtained from Amresco (Ohio, USA). Guaiacol glyceryl ether (>98% purity) was purchased from Sigma (Beijing, China). Zinc nitrate (>99% purity) was obtained from Bodi Chemical Co. Ltd. (Tianjin, China). *Lumbricus* balanced salt solution (LBSS) consisted of 71.5 mM NaCl, 4.8 mM KCl, 3.8 mM $CaCl_2$, 1.1 mM $MgSO_4{\cdot}7H_2O$, 0.4 mM KH_2PO_4, 0.3 mM $Na_2HPO_4{\cdot}7H_2O$, and 4.2 mM $NaHCO_3$.

2.2. Earthworms

Earthworms (*E. fetida*) were purchased from Tianjin Chenggong Earthworm Farm Company, China. The worms were preincubated in clean soil for 1 week under controlled conditions (22 ± 2 °C, 12 h light:12 h dark, 75% humidity) before the experiment. Adult earthworms with a developed clitellum were collected for testing; the individual wet weight of adults ranged from 300 to 500 mg.

2.3. Experimental design

Zinc nitrate at a concentration of 0, 50, 100, or 200 mg kg^{-1} was added to beakers containing 500 g soil. Oxytetracycline hydrochloride at a concentration of 5, 10, or 20 mg kg^{-1} was added to beakers containing 500 g soil and 50 mg kg^{-1} Zn. These dosages were chosen based on reported concentrations of OTC and Zn in soil. Moisture content was adjusted to 60% of the water-holding capacity. All tests were performed in triplicate. The soil was allowed to stabilize for 24 h prior to earthworm introduction. Ten worms were placed in each beaker and the beakers were enclosed with plastic film perforated with small ventilation holes. The test was conducted under a photoperiod of 12 h light:12 h dark at 20 ± 2 °C for 28 days. Humidity was maintained at 75% ± 5% by an artificial climate chamber. Worms were fed rewetted oven-dried cow manure (0.5 g per worm) every 7 days thereafter over the 28-day period. In order to maintain the original moisture percentage, distilled water was added gravimetrically to the replicates every week. Two earthworms were collected from each beaker without replacement at 7, 14, and 28 days. Although there was no earthworm death during the experiment, there was a significant difference in body weight loss among the different groups of earthworms.

2.4. Harvesting of coelomocytes

Earthworms were allowed to empty their guts for 18 h in Petri dishes on filter paper moistened with distilled water. Each worm was rinsed with distilled water and saline (6.5 mg mL^{-1} NaCl, 4 °C) before being placed into a 1.5-mL Eppendorf tube containing 1 mL cold extrusion medium for 2 min. The extrusion solution contained 5% ethanol, 95% saline, 2.5 mg mL^{-1} EDTA, and 10 mg mL^{-1} guaiacol glyceryl ether and was adjusted to pH 7.3 with 1 M NaOH. Earthworms were removed from the tubes and the cell suspension