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Comparative effects of Cd and Pb on biochemical response and DNA damage in the earthworm *Eisenia fetida* (Annelida, Oligochaeta)

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ABSTRACT

There are rising concerns about the hazardous effects of cadmium (Cd) and lead (Pb) on the environment in China. Biochemical and comet assays were conducted on the earthworm *Eisenia fetida*, a suitable bio-indicator organism for evaluating soil pollution after exposure to two heavy metals, Cd and Pb. Protein content increased at low Cd concentrations ($p < 0.05$) and decreased at the highest concentration of 10 mg kg^{-1}, compared to control ($p < 0.05$). Pb showed an inhibitory effect on protein content at low concentrations but demonstrated no significant effect at higher concentrations. There were no significant differences between control and treated groups at the doses of 1 and 10 mg kg^{-1} Cd while at a dose of 0.1 mg kg^{-1} Cd the cellulase activity was significantly increased compared to control. Cellulase activities of Pb-treated *E. fetida* increased in a dose dependent fashion. Results of the comet assay indicated toxicant induced DNA damage. Cd exposure caused significant differences between control and treatment groups (ANOVA, $p < 0.05$, $p < 0.01$) and a positive dose-response profile. As for Pb treatment, there were no significant differences between the groups treated with 50 and 500 mg kg^{-1} of Pb and the control. Results showed that DNA damage from Cd was more serious than that from Pb. And this indicated that the earthworm was more sensitive to the effects of Cd.

1. Introduction

Increased pollution with heavy metals such as lead, zinc and cadmium has become a widespread problem in the environment (Bailey et al., 1999; Babel and del Mundo Dacera, 2006). Cadmium is a toxic element which is relatively rare and scarcely distributed. The background concentration of cadmium in soil is 0.1–0.2 mg kg^{-1} (Sauve et al., 2000) and that in China stands at only 0.097 mg kg^{-1} (Wei et al., 1991). In soil from sites where metallogenesis of cadmium and industrial pollution is prevalent, there is a hundred to thousand-time higher concentration of cadmium than the background value (Zheng et al., 2002). The soil dwelling nature of earthworms makes them ideal indicators of adverse effects from toxicants (Nahmani et al., 2007; Suthar et al., 2008). Cadmium accumulates in granules in the chloragogenous tissue surrounding the earthworm digestive tract (Morgan et al., 2002), which induces the formation of metallothioneins that bind the cation and allow it to be sequestrated (Dabrio et al., 2002). Mechanisms of Cd genotoxicity and mutagenicity in organisms are poorly understood, but recent advances suggest that Cd has a high mutagenic activity (Filipic and Hei, 2004) and it interferes with DNA repair, rather than causing damage directly (Waisberg et al., 2003).

Lead is one of the most studied toxic elements. It is widely distributed in the environment. It causes a range of adverse effects in animals, resulting in learning disability and retarded growth in human beings (IPCS, 1989; Campbell, 2001). Lead concentrations usually vary from 2 to 200 mg kg^{-1}, with the mean range from 13 to 42 mg kg^{-1}. A survey of soils from China determined that background Pb concentrations ranged from 0.68 to 1143 mg kg^{-1}, with a general average of 26 mg kg^{-1} (Chen, 1996).

Earthworms are common in a wide range of soil and may represent 60–80% of the total soil biomass and appeared to be the best organism for use in soil toxicity evaluation (Bouché, 1992). In addition, earthworms are readily available, sensitive, easy to handle and use in toxicity tests. It is possible to culture *Eisenia fetida* in the laboratory (Fourie et al., 2007), rendering them suitable test organisms for ecological risk assessment in terrestrial ecosystems (OECD, 1984).

Damage to DNA may lead to mutations, strand breaks, altered bases (Shugart, 2000) and eventually carcinogenesis, teratogenesis and health disorders such as the genotoxic disease syndrome (Kurelec, 1993). Therefore, the assessment of DNA damage is considered important in toxicity testing. Among numerous assays used to detect DNA damage, the comet assay is used widely in ecotoxicology to detect DNA single-strand breaks and has various advantages such as its sensitivity for detecting low levels of DNA damage in single cells and the relative ease of application (Tice et al., 2000).

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Several earthworm protocols have been developed to assess the effects of chemicals on earthworms (*E. fetida*), such as ISO (1993) and EPA (1991) and the OECD guideline 207 (OECD, 1984). Although these tests are useful in the assessment toxicant effects on earthworms, they have limited ecological significance (Reinecke, 1992) because many sub-lethal effects such as changes in behavior, reproduction and inhibition on enzyme activities are not addressed in standard acute toxicity tests (Kokta, 1992). In this paper, we used the comet assay to assess the DNA damage in coelomocytes of earthworms being directly exposed to the two heavy metals. We also report the biochemical toxicity in terms of effects on activities of cellulase. Our objective is to develop a more comprehensive understanding on the effects of heavy metals on earthworms and to provide more information about the potential ecological risk of metals in soil ecosystems.

2. Materials and methods

2.1. Test organism and test chemicals

E. fetida was adopted as the test species because it is widely available, easily reared in laboratory culture and reproduces rapidly and steadily relative to other earthworm species. Earthworms were purchased from a local market in Nanjing, China. Healthy adult earthworms of about 60 d old, weighing about 200–300 mg and having a well-developed clitellum, were used for all experiments.

Cadmium (Cd) and lead (Pb) over 99% pure were obtained from Sinopharm Chemical Reagent Co., Ltd. and Beijing Yili Fine Chemicals Co., Ltd. Individual metals were dissolved in distilled water to different concentrations. All glass vessels were treated with the corresponding concentrations of Pb and Cd solutions separately, in case the metals adsorbed to the active surfaces.

2.2. Earthworms exposures (7-d artificial soil method)

The earthworms were acclimated to the laboratory environment for at least one week before the test. Artificial soil which contained 10% finely ground sphagnum peat, 20% kaolin clay, 70% industrial sand, was adjusted to pH of 6.0 ± 0.5 by adding calcium carbonate. A water content of 35% was used in these experiments according to OECD (1996). Cd and Pb were introduced into soil by mixing solutions (35 mL) of different solutions by hand about 10 min to make final concentrations of 0.1, 1 and 10 mg kg^{-1} dry soil for Cd and 50, 500 and 5000 mg kg^{-1} dry soil for Pb.

After incubation in uncontaminated artificial soil for 24 h, the surface of the earthworms was washed briefly with distilled water, dried with filter paper and weighed. Five of *E. fetida* were added to contaminated soil of different concentrations, contained in wide-mouth bottles (1 L) supplied with continuous light source (to ensure that worms remained in the test medium throughout the duration of the test) at 20 ± 1 °C for 7 d. Water was added regularly to keep the moisture at 80%, and the bottles were covered with plastic film that had been punched with small holes using needles.

2.3. Comet assay

After exposure of the earthworms, their coelomocytes were obtained according to the non-invasive extrusion method described by Eyambe et al. (1991). Individual earthworms were rinsed in the extrusion medium which consisted of 5% ethanol, 95% saline, 2.5 mg mL^{-1} Na_2-EDTA and 10 mg mL^{-1} guaiacol glyceryl ether (pH 7.3). Coelomocytes were spontaneously secreted in the medium and washed twice with phosphate-buffered saline (PBS) prior to the comet assay. The cells were collected by centrifugation (3000 rpm, 3 min) and kept on ice in 4° C before the comet assay.

The comet assay was performed as described by Singh et al. (1988) and Tice et al. (2000) with some modifications. Frosted microscope slides, on which cells were embedded in an agarose sandwich, were submerged in cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% N-Lauroyl Sarcosine Na (NLSN), adjusted to pH 10.0 performed in the dark for 1 h to remove cellular proteins. Slides were placed on a horizontal electrophoresis unit filled with fresh alkaline electrophoresis solution (300 mM NaOH and 1 mM Na_2EDTA, pH 13.0) to a level approximately 0.2 cm above the sides at 20° C for 30 min to allow DNA unwinding before electrophoresis. Electrophoresis was conducted at 20° C using 25 V and 300 mA for 30 min. The above steps were conducted in red light to avoid DNA damage. After electrophoresis, slides were washed three times with a neutralizing buffer (0.5 M Tris, pH 7.5), the DNA were stained with ethidium bromide (EB) (2 μg mL^{-1}), and the slides were examined with a fluorescent microscope (BX41, Olympus, Japan). Five slides per treatment were prepared and at least 50 cells were analyzed from each slide. Photos were taken with a digital camera (C-5050ZOOM, Olympus). Images were analyzed according to the method of Collins et al. (1995) using the comet assay software project (CASP 1.2.2).

2.4. Biochemical assay

Enzyme activity of cellulase and protein content were determined for worms exposed to three different concentrations of each compound. Five earthworms were used for each concentration. Enzymes were extracted following the method of Mishra and Dash (1980) with some modifications. Earthworms were rinsed with distilled water and dried on filter paper. Earthworms were homogenized for 2 min in cold distilled water in a 1/4 w/v ratio using JY92-II homogenizer (Ningbo, China). Homogenates were centrifuged at 2500 rpm for 10 min. The supernatant fluid was removed into another centrifuge tube and centrifuged at 3000 rpm for 5 min. The supernatant fluid was collected and used to test enzyme activity. The protein content was determined as the method of Li (1996), using bovine serum albumin as standard. 0.5 mL enzyme preparation was added to 0.5 mL distilled water and 4 mL biuret solution. The reaction system was incubated at 20–25° C for 30 min. The A_{540} was tested and the content of protein was determined according to the standard curve.

The method of Zhang (1991) was followed to determine cellulase activity. A small volume (0.5 mL) of enzyme preparation was added into test-tube containing 1.0 mL preheated CMC-Na and the mixture was incubated at 50° C for 30 min. The concentration of glucose was determined by adding DNS and measured spectrophotometrically (VIS-7220, Rayleigh Analytical Instrument Corp., Beijing) at a reference wavelength of 530 nm in a cuvette with a 1 cm light path. Enzyme activities are expressed in mg of glucose per mg of protein per hour.

2.5. Statistical analysis

Differences between control and treated samples were analyzed by one-way ANOVA using SPSS 10.0 statistical software, taking $p < 0.05$ as significant according to the Mann–Whitney Test.

3. Results

3.1. Comet assay

No mortality of the earthworms was observed for the exposure to the different tested concentrations of Cd and Pb. Although the software reports several parameters, data for percentage tail