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# DNA damage and effects on antioxidative enzymes in earthworm (*Eisenia foetida*) induced by atrazine

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ABSTRACT

To evaluate atrazine (2-chloro-4-ethylamino-6-isopropylamino-1, 3, 5-triazine) ecotoxicology in soil, the effect of atrazine on the activity of antioxidative enzymes (superoxide dismutase, SOD; catalase, CAT; and guaiacol peroxidase, POD) and DNA damage induced by atrazine were investigated in earthworms. Atrazine was added to artificial soil at rates of 0, 2.5, 5 and 10 mg per kg of soil. Earthworm tissues exposed to each treatment were collected on the 7th, 14th, 21st, and 28th day of the treatment. Compared to the controls, the CAT activity was stimulated at 2.5 mg $kg^{-1}$ treatment except on the 14th day, and inhibited at 5, 10 mg $kg^{-1}$ atrazine except 5 mg $kg^{-1}$ on the 28th day and 10 mg $kg^{-1}$ on the 21st day; the overall SOD activity was inhibited, while the POD activities were stimulated by all atrazine concentrations in 28 days. The olive tail moments of single-cell gel electrophoresis of coelomocytes, as an indication of DNA damage, were increased after treatment with different doses of atrazine on the 7th, 14th, 21st, and 28th day, and significant differences were found compared to the controls. In conclusion, atrazine induces oxidative stress and DNA damage on earthworms, and the adverse effects may be the important mechanisms of its toxicity to earthworms.



## 1. Introduction

In agricultural areas worldwide, there is an increasing concern about soil contamination due to the widespread use of pesticides. Atrazine is one of the most widely used pesticides in the world, mainly due to its relatively low cost and ease of application. It is used as a selective pre- and post-emergent agent to control annual grasses and broad-leaved weeds in selected vegetable and cereal crops, maize, sugarcane, vines, fruit orchards, citrus groves, grassland, and forestry (Graymore et al., 2001). Atrazine is considered a moderately persistent chemical in the environment with a half-life ranging from a few days to months (Khan and Saidak, 1981; Jones et al., 1982). It has a greater mobility in soils than many other herbicides (Buhler et al., 1993) and is the most commonly detected herbicide in the groundwater of many countries. Besides other transformation, biotic transformations are considered a major route whereby atrazine is decomposed in most soils (Kaufman and Kearney, 1970; Barriuso and Houot, 1996). Atrazine has induced severe hormonal disturbances in amphibians (Hayes et al., 2002) and tumors in rats (Stevens et al., 1999). Atrazine has been classified as a possible human carcinogen by the International Agency for Research on Cancer (IARC, 1999). A study conducted in Kentucky suggests a modest association between triazine exposure and an increased risk of breast cancer (Kettles et al., 1997).

Soil contamination with pesticides can be detrimental to earthworm populations. Earthworms play an important role in the ecotoxicological studies as biomonitors to determine the ecological hazards of pesticide-contaminated soils (OECD, 1984; Stürzenbaum et al., 1998). Therefore, understanding the adverse effects of pesticides on earthworms is essential to predict the potential food-chain effects of soil contamination.

Biochemical responses in organisms against environmental stress are regarded as early warning indices of pollution in the environment. Many enzymatic activities have been considered as biomarkers of environmental pollution. These enzymes of living organisms possess antioxidant activities and can protect cells against adverse effects of reactive oxygen species (ROS). Saint-Denis et al. (1998) studied the activities of enzymes (catalase, glutathione peroxidase, glutathione *S*-transferase, and glutathione reductase) involved in antioxidant defense systems in *Eisenia fetida andrei*; these enzymes are mainly localized in the cytosolic fractions. However, there is lack of information on the effect of atrazine on antioxidative enzymes in earthworms.

Accumulation of ROS such as $H_2O_2$ and superoxide radical ($O_2\bullet^-$) does damage to cellular components such as DNA, proteins, and lipids (López et al., 2006). Atrazine exhibits genotoxicity by causing single- and double-strand breaks in DNA through the formation of ROS. Alkaline single-cell gel electrophoresis (SCGE,

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comet assay), developed by Singh et al. (1988), is a widely used technique to detect DNA damage due to environmental stress. Heavy metal toxicity was studied in detail in earthworms (Reinecke, 2004; Di Marzio et al., 2005), and genotoxicity of two novel pesticides in earthworms was investigated using the comet assay. However, there is lack of data on genotoxicity of atrazine in earthworm coelomocytes. Therefore, the aim of the present study was to assess induction of oxidative stress and genotoxicity of atrazine in earthworms.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Atrazine of greater than 95% purity was obtained from Shandong Jingbo Agricultural Chemical Co. Ltd. All other chemicals were of reagent grade and purchased from Sigma Chemical Co. and Beijing Chemical Co.

### 2.2. Toxicological tests

Earthworms used in this assay (*Eisenia foetida*) were purchased from an earthworm culturing farm in Tianjin. Toxicological tests on earthworms were conducted in OECD artificial soil (OECD, 2004), which is composed of 10% sphagnum peat moss (Premier), 20% kaolin clay (Fisher Scientific), and 70% sand (grade 70 particle size 0.1–0.3 mm). Atrazine treatment concentrations were established by preliminary tests following reported application rates of 1.12–2.24 kg $ha^{-1}$, which results in soil concentration range of 3–6 mg $kg^{-1}$ (Funari et al., 1998; Lesan and Bhanadari, 2003). Atrazine was applied to artificial soil in 500 ml glass bowls for final treatment concentrations of 0, 2.5, 5, 10 mg of atrazine $kg^{-1}$ of soil. Three bowls were used for each concentration and 10 worms were added to each bowl. Two earthworms, one each for enzyme and comet assays, were collected from each replicate bowl on the 7th, 14th, 21st, and 28th day following application of atrazine. During the experiment progress, the mortality was 0%.

### 2.3. Enzyme extraction

Earthworms were placed into a prechilled mortar and pestled under ice-cold conditions in 50 mM potassium phosphate buffer (1:8, w/v), pH 7.0. The homogenate was centrifuged at 10, 000 rev $min^{-1}$ at 4 °C for 10 min. The supernatant was used for the assay of enzyme activity and protein determination.

### 2.4. Enzyme assays

Total superoxide dismutase (SOD) activity was determined by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium chloride (NBT), as described by Giannopolitis and Ries (1977) with slight modification. The reaction mixture (3 ml) contained 50 mM phosphate buffer (pH 7.8), 100 μM ethylenediaminetetraacetic acid (EDTA), 130 mM methionine, 750 μM NBT, 20 μM riboflavin, and 50 μl enzyme extract. Riboflavin was added last, and the tubes were shaken and illuminated with 4000 lx fluorescent tubes. The reaction was allowed to proceed for 20 min, following which the lights were switched off, and the tubes were covered with a black cloth. Absorbance of the reaction mixture was read at 560 nm. One unit of SOD activity (U) was defined as the amount of enzyme required to cause 50% inhibition of the NBT photoreduction rate, and the result was expressed as U $mg^{-1}$ of fresh mass (FM).

Catalase (CAT) activity was determined as described by Jingbo et al. (1997). The reference cell contained 10 μl enzyme extract and 3 ml $H_2O_2$-phosphate buffer i (pH 7.0), and the sample cell contained 10 μl enzyme extract and 3 ml $H_2O_2$-phosphate buffer ii (absorbency of $H_2O_2$-phosphate buffer ii was approximately 0.500 and read at 240 nm). The $H_2O_2$-phosphate buffer ii was $H_2O_2$ (30%, w/v) diluted by $H_2O_2$-phosphate buffer i. In this assay, a solution of $H_2O_2$ was used as substrate for the enzyme. The enzyme activity was calculated from the decrease in ultraviolet absorption with time, following degradation of $H_2O_2$ by CAT present in the sample. One unit of CAT activity was defined as the enzyme quantity required to consume half of $H_2O_2$ in 100 s at 25 °C.

Guaiacol peroxidase (POD) activity was determined according to the method of Kochba et al. (1977) with slight modification. The reaction process was measured by recording absorbance at 470 nm as soon as the supernatant was added to 3 ml of reaction mixture containing 50 ml potassium phosphate buffer (100 mM, pH 6.0), 19 μl 30% $H_2O_2$ and 28 μl guaiacol.

### 2.5. Protein content

Measurement of protein content was necessary to calculate SOD, CAT, and POD activities. The protein content was determined using the Sigma Bradford method (Bradford, 1976) according to the manufacturer's instructions.

### 2.6. DNA damage in earthworm coelomocytes

After exposure of the earthworms to the pesticide, their coelomocytes were obtained using the non-invasive extrusion method described by Eyambe et al. (1991). Individual earthworms were rinsed in the extrusion medium composed of 5% ethanol, 95% saline, 2.5 mg $ml^{-1}$ EDTA, and 10 mg $ml^{-1}$ guaiacol glyceryl ether (pH 7.3). Coelomocytes were spontaneously secreted in the medium and washed with phosphate-buffered saline (PBS) prior to the comet assay. The cells were collected by centrifugation (3000 × *g*, 10 min) and placed on ice prior to the comet assay.

The comet assay was performed according to Singh et al. (1988), with slight modifications. The cell suspension was mixed with 100 μl of 0.7% low melting agar (LMA) in PBS at 37 °C and pipetted onto fully frosted slides precoated with a layer of 100 μl 0.8% normal melting agar (NMA). After solidification on ice, another layer of 85 μl LMA was added, and the slides were immersed into a lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM $Na_2EDTA$ (pH 10.0), 1% Na-sarcosinate, 10% dimethyl sulfoxide (DMSO), and 1% Triton X-100). Slides were then incubated in an electrophoresis tank containing 300 mM NaOH with 1 mM $Na_2EDTA$ for 20 min prior to electrophoresis for 15 min at 25 V (300 mA). The slides were then neutralized (0.4 M Tris, pH 7.5) thrice at 5 min intervals and stained with 40 μl ethidium bromide (13 μg $ml^{-1}$) for fluorescence microscopy analysis (Olympus BX51 fluorescence microscope) using a digital imaging system. The images of the SCGE were analyzed last using CASP (Końca et al., 2003). One-hundred cell cores on each slide were counted. The parameter used to quantify the extent of DNA damage was the olive tail moment (OTM). OTM is the product of the distance between the center of gravity of the head and the center of gravity of the tail and percent tail DNA.

### 2.7. Statistics

Each treatment was performed in triplicate, and a standard deviation (SD) was calculated. Independent-samples *t* tests were performed to evaluate the statistical significance of the results by using SPSS 11.5.