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Evaluation of genotoxicity of combined soil pollution by cadmium and phenanthrene on earthworm

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Abstract: The DNA-damaging effects of the combined pollution of heavy metal and organic contamination on earthworms were evaluated by single cell gel electrophoresis (SCGE) assay. Earthworms *Eisenia andrei* were exposed to single or combined test compounds in different doses of cadmium (Cd) 5, 10, 50 mg/kg and phenanthrene (Phe) 0.5, 2.5, 12.5 mg/kg with a treatment of 14 d. In SCGE assay, isolated coelomcytes and electrophoresis were employed to determine DNA damage degree after a 14-d treatment by test compounds. The results showed that there was a significant statistical difference between earthworms treated with Cd combined Phe with them treated alone with Cd or Phe. The Olive tail moment (OTM) of SCGE assay using earthworm coelomcytes appears to be a sensitive biomarker for evaluating exposure to genotoxic compounds. These tests also revealed that the interaction between Cd and Phe to DNA damaging effects was negative, and was strongly dependent on the concentration of pollution. This study corresponds to exploratory phase in order to reveal interaction effects of heavy metal and organic contamination on earthworms and then to set up more in-depth analysis to increase progressively the understanding of the genotoxicity mechanisms involved.
Keywords: Cd; Phe; earthworm; combined pollution; SCGE; DNA damage

Introduction

Soil pollution has become a serious problem in many countries. Heavy metals (HM) and polycyclic aromatic hydrocarbons (PAHs) are usually entering the soil simultaneously or successively, in many cases, HM and PAHs are frequently found together as contaminants in soils. The combined effect of HM and PAHs has attracted much attention in recent decades (Mielke *et al.*, 2001; Shen *et al.*, 2005a, b; Stalikas *et al.*, 1997). Besides their natural occurrence, heavy metals may enter the ecosystems through anthropogenic activities, such as mining, smelting, sewage sludge disposal, application of pesticides and inorganic fertilizers, and atmospheric deposition (Shen *et al.*, 2005b). Cadmium (Cd) is a widely occurring metal pollutant. Emissions of Cd from human activities have been estimated to be about 30000 t annually (Di Toppi *et al.*, 1998). Soil contamination with Cd due to irrigation with Cd-contaminated wastewater or fertilizing and mining/smelting activities poses a long-term risk to human and animals, which has received increasing attention worldwide. Cd is considered to be the metal having the most adverse effects on earthworm and activity in heavy metal contaminated soils (Nahmani and Lavelle, 2002; Spurgeon *et al.*, 2004; Stürzenbaum *et al.*, 1998). PAHs are a ubiquitous group of hazardous organic pollutants that exhibit strong carcinogenic and toxic properties. The main anthropogenic sources include industrial processing power and heat generation in waster incineration and traffic. PAHs can enter the soil via atmospheric deposition. It is estimated that more than 90% of total burden of PAHs resides in the surface soils. PAHs have often been found to co-exist with HM. Soil contaminated with PAHs often contain large amount of HM. There is a strong and significant association between HM and PAHs (Shen *et al.*, 2005b). Therefore, research on combined pollution is of great theoretical and practical significance.

Earthworm, as bio-indicators of soil pollution, had been long used as a key index of eco-toxicology diagnosis (Diao *et al.*, 2004; Song *et al.*, 2002, 2003; Spurgeon *et al.*, 2003). In the recent 20 years, with the broad application of chemical materials in agricultural ecosystem, the study in earthworm eco-toxicology has becoming increasingly widespread. Meantime, researchers around the world built up a lot of earthworm eco-toxicology methods to test the presence of the chemical materials, which have been nationally or internationally registered and tested (EPPO, 2002; European Commission, 2002; OECD, 1984, 2000a, b; ISO, 1998, 1999). Presently, many studies about effect of eco-toxicology on the earthworm, such as heavy metal (Nahmani and Lavelle, 2002; Stürzenbaum *et al.*, 1998), combined pollution of heavy metals (Spurgeon *et al.*, 2004), PAHs (Ma *et al.*, 1995; TANG *et al.*, 2002), pesticide (Kong *et al.*, 2002; Zang *et al.*, 2002; Zhong *et al.*, 1999; Zuo *et al.*, 2005), have been reported. However, reports of combined pollution of heavy metals and PAHs are scarcely seen. Only few studies have been published describing the toxicity of combined pollution of heavy metal and organic contamination on earthworms, and all focusing on combined pollution of heavy metals (Conder *et al.*, 2000; Khalil *et al.*, 1996a, b).

The single cell gel electrophoresis (SCGE) assay

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introduced by Ostling and Johanson (1984) and developed by Singh *et al.* (1988) now plays an important role in many areas of science. It is widely used to evaluate various physical and chemical genotoxic agents by detecting DNA single strain breaks and alkali-sensitive sites damage, and has been recognized as one of the most sensitive methods available for detecting strand breaks. Also known as the Comet assay, SCGE assays employing coelomcytes from earthworms have been used in several studies for assessing levels of genotoxic agents in contaminated soil (Salagovic *et al.*, 1996; Verschaeve and Gilles, 1995). However, the combined genotoxicity effect of heavy metals and PAHs on earthworms is scarce. Selecting Cd and Phe (phenanthrene), which are very common in the soil, as target pollutants, this study explored the genotoxicity effect of their combined pollution on earthworm by comet assay to measure DNA strand break. The results obtained in this work should lead to a better understanding of the combined toxicity of Cd and Phe. with the purpose of providing a technical basis for eco-toxicological soil risk assessment on combined pollution.

1 Materials and methods

1.1 Test animal

Eisenia andrei, is being preserved and propagated by College of Agriculture and Biology in Shanghai Jiao Tong University. After a small adaptive phase, healthy adult clitellum earthworms with similar size and weight were chosen with an average wet weight of 295 mg.

1.2 Reagents

$CdCl_2 \cdot 2.5H_2O$, DMSO (dimethyl sulphoxide), NaCl, $CaCO_3$, Phe were all analytic reagent, kaoline, industrial quartz sand (containing above 50% small particles in the size of 0.05—0.2 mm) were both chemical pure. Low-melting-temperature agarose (LMP), normal-melting-temperature agorose (NMP) were purchased from Gibco Co. (Los Angeles, USA). cyclophosphamide (CP), Typan Blue were from Sigma Co. (St. Louis, USA). Heparin sodium salt, Na_2-EDTA, GGE, Triton-X 100, Tris and DMSO were supplied by China National Medicine Group, Shanghai Chemical Reagent Company (Shanghai, China). SybrGreen was from Biotium Co. (Pittsburgh, USA).

1.3 Instrument

Constant temperature and humidity chambers: SPX-250C (Shanghai Bo Yuan Industrial Co., Ltd., China). Fluorescent microscope (Olympus BX60F-3, Olympus, Japan). Electrophoresis system was from Beijing Liuyi Instrument Factory (Beijing, China).

1.4 Experimental methods

1.4.1 Soil preparation

Each portion of artificial soil, with the total weight of 500 g, consisted of 10% paddy soil (the soil was taken from the experimental farm of Qibao District of Shanghai Jiao Tong University, in the depth of 0—20 cm). The main physical and chemical properties of soil are listed as: pH 8.18, organic matter 16.7%, cation exchange capacity (CEC) 15.6 cmol/kg, total N 1.14 g/kg, total P 1.36 g/kg, sand 51.49%, slit 28.37%, clay 20.14%, Fe_2O_3 5.53%, Al_2O_3 14.38%, SiO_2 58.7% , 20% kaolinfon (Containing >50% kaolin), 69% industrial quartz sands and 1% $CaCO_3$. Added various ingredients into the cultivating bottle of 1 L one by one and mixed evenly (Smit *et al.*, 1996; Spurgeon and Hopkin, 1996).

1.4.2 Concentration-response relationship of single Phe or Cd on DNA damage

The choice of concentration was based on the environmental quality standards for soils of China and Canada. The standard of Phe is less than 0.1 mg/kg in Canada (Cerniglia, 1984). There was no evident DNA damage of earthworm treated with Phe less than 0.1 mg/kg. The DNA damage of nuclei increased with the increasing Phe concentrations, especially at concentrations more than 0.1 mg/kg. The concentration-response relationship of Phe is shown in Fig.1a.

In China, the soil environmental quality

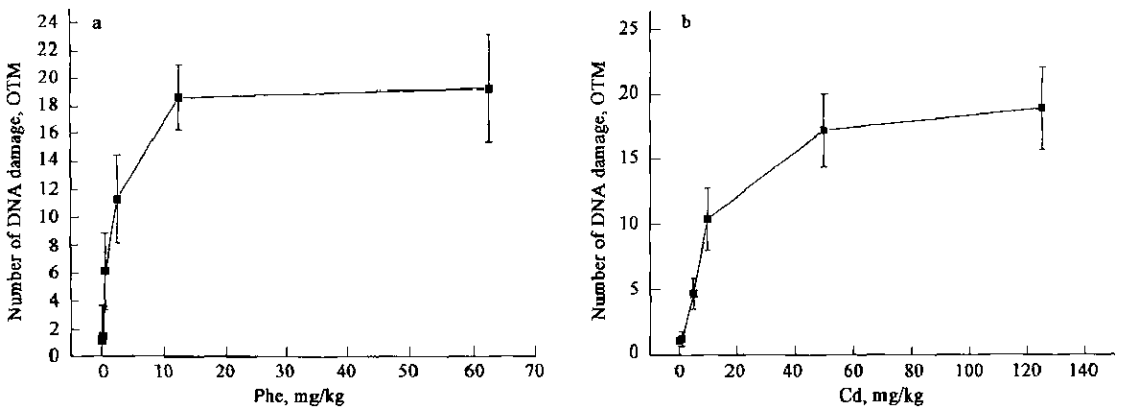

Fig.1 Dose-response curve of the DNA damage caused by Phe (a) and Cd (b) to the coelomcytes of earthworm *Eisenia andrei*
Temperature: (20±1)℃; humidity: (75±7)%; intermittent 12 h light, 12 h darkness; n=3

standards (GB15618-1995) for Cd is 0.2 mg/kg. The DNA damage of nuclei increased with the increasing Cd concentrations especially at concentrations more than 1 mg/kg. The concentration-response relationship of Cd is shown in Fig.1b.

1.4.3 Soil treatment of combined Phe and Cd

According to the concentration-response relationship of single Phe or Cd on earthworm *Eisenia andrei*, the highest treatment concentration as the high-test dose was selected only when the cell livability was more than 80%, serried of formal single toxicity test concentrations were determined. The dosages of the Cd and Phe were calculated in dry soils of 500 g, Cd was added to the soil by adding Cd stock solutions to give the concentration of 5, 10, 50 mg/kg in air-dried soil. For the combined pollution treatment Phe was added to the soil by adding acetone stock solutions to give the concentration of 0.5, 2.5, 12.5 mg/kg in air-dried soil. The soil was thoroughly mixed and allowed to be air-dried to remove the acetone. The soil was adjusted to humidity of 75% using deionized water before filling into nine cultivating bottles averagely. One control group was set and each experiment was repeated 3 times.

1.4.4 Culture earthworms

The healthy clitellum earthworms were put in a constant temperature and humidity chambers (temperature: (20 ±1)℃; humidity: 75%) for the intestines to be cleaned. 10 earthworms of similar weight were picked into one cultivating bottle after 24 h and put them into the climate chamber with the illumination intensity of 1333 Lx (intermittent light, namely 12 h light, 12 h darkness). Coelomocytes were collected to SCGE assay on the day 14.

1.4.5 SCGE assay

Earthworm coelomcytes were collected using a noninvasive method (Eyambe *et al.*, 1991; Brousseau *et al.*, 1997a, b, 1999). Briefly, each worm was rinsed in cold lumbricus balanced salt solution (LBSS) and placed on a paper towel. One-fourth of the posterior part was massaged to expel the content of the lower gut. Then each worm was palced for 3 min in a 15-ml polypropylene tube containing 3.0 ml of cold extrusion medium. The extrusion medium contained 5% ethanol, 2.5 mg/ml Na_2-EDTA, 10 mg/ml of the mucolytic agent GGE, and was adjusted to pH 7.3 with 1 mol/L NaOH. After 3 min, the worm was removed and the volume was made up by adding 12 ml iced-cold Ca-free LBSS containing 1.5 mmol/L NaCl, 4.8 mmol/L KCl, 1.1 mmol/L $MgSO_4 \cdot 7H_2O$, and 4.2 mmol/L $NaHCO_3$ adjusted to pH 7.3, and osmolarity adjusted to 300 mmos (Brousseau *et al.*, 1997a, b). The cells were washed twice with the same medium and recovered by centrifugation at 150 g for 15 min at 4℃ (Eyambe *et al.*, 1991). Finally, the cells were resuspended in Ca-LBSS (containing 3.8 mmol/L $CaCl_2$). Following their isolation, the cells at a density of 1×10^6 cells ml^{-1} were cultivated in Ca-LBSS, cell viability was measured immediately after treatment using the Trypan Blue exclusion method (Eyambe *et al.*, 1991; Verschaeve and Gilles, 1995).

The assay was basically performed in accordance with Singh *et al.* (1988). Two solutions, one containing 0.6% NMA and the other containing a 0.5% LMA dissolved in Ca-LBSS, were prepared. The microscope slides were firstly covered with 110 μl of 0.6% NMP, NMP was transferred onto the slide, spread evenly, covered with a cover ship and placed at room temperature for 2 min to solidify the agarose. After removal of the cover ship, 85 μl LMA mixed with 10^4 cells (10 μl cell suspension +75 μl LMA) was applied, after this layer had solidified, a third layer of 75 μl LMA was added. Slides were immersed in fresh prepared ice-cold lysing solution (2.5 mol/L NaCl, 100 mmol/L Na_2EDTA, 10 mmol/L Tris, with 1% Triton-X 100 and 10% DMSO added fresh, pH 8.2—8.4) to lyse the cells overnight and to allow DNA unfolding. Then the slides were placed on a horizontal electrophoresis unit filled with alkaline fresh buffer (1 mmol/L Na_2-EDTA, 300 mmol/L NaOH, pH 8.2—8.4) for 30 min to allow DNA unwinding and expression of alkali-labile sites. Electrophoresis was conducted for 20 min at 20 V adjusted to 300 mA by raising or lowering the buffer level in the tank. After that, the slides was washed gently twice, each for 5 min in a neutralization buffer (0.4 mol/L Tris-HCl, pH 7.5). The slides were dried and 50 μl 0.01% SybrGreen were added. Analyses were performed immediately after a 30-min staining. To prevent additional DNA damage, all the steps described above were run under weak light.

Each slide was viewed with a fluorescent microscope (Olympus BX60F-3) with an excitation filter of 510—560 nm and a barrier of 590 nm. The stained DNA gives a red emission. Three slides were analyzed for each treatment and control group. A Nikon digital camera captured images of the comets.

An image analysis system CASP developed by Koňca *et al.* (2003) was employed to measure various comet parameters. The percentage of nuclei with tails was calculated. The head DNA, tail DNA, tail moment (TM) (integrated value of DNA density multiplied by the migration distance), Olive tail moment (OTM) (the product of the distance between the center of gravity of the head and the center of the gravity of the tail and percent tail DNA) were used as the primary measurement of DNA damage. Statistical differences between controls and treated samples were determined by least significant difference. Data are subjected to the analysis of variance using SPSS14.0 software.

2 Results and discussion

2.1 DNA damage by single Phe pollution

The DNA-migration was expressed by head DNA, tail DNA (relative percent of DNA in the comet tail), TL, TM and OTM as shown in Table 1. It was seen that with increasing Phe concentration the OTM increased ($p<0.05$, $p<0.01$) by showing a clear concentration-response relationship. OTM of negative the highest concentration Phe is 6.286 times of negative control. Similarly, tail length, tail DNA and TM also increased with the increasing Phe concentration.

Table 1 Index of the DNA damage caused by Phe to the coelomcytes of earthworm *Eisenia andrei* (mean±*SD*, n=150)

Phe, mg/kg	Head DNA, %	Tail DNA, %	TL	TM	OTM
Control	89.12±5.23	10.88±5.23	18.32±8.12	2.49±1.89	2.48±1.32
0.5	79.78±7.18*	20.22±7.18*	36.95±17.01*	8.32±7.03**	5.71±2.85**
2.5	70.01±9.26*	29.99±9.26**	60.23±17.95**	17.74±9.97**	9.86±4.52**
12.5	65.75±6.96**	34.25±6.96**	82.98±16.55**	27.82±9.62**	15.59±3.86**

Notes: *$P<0.05$; ** $P<0.01$; TL. tail length; TM. tail moment. OTM. Olive tail moment

2.2 DNA damage by single Cd pollution

Table 2 shows that with increasing Cd concentration the OTM increased ($p<0.05$), showing a clear concentration-response relationship. OTM of the highest concentration Cd is 5.792 times of negative control. Similarly, tail length, tail DNA, TM, OTM also increased with the increasing Cd concentration.

Table 2 Index of the DNA damage caused by Cd to the coelomcytes of earthworm *Eisenia andrei* (mean±*SD*, n=150)

Cd, mg/kg	Head DNA, %	Tail DNA, %	TL	TM	OTM
0	90.14±5.22	9.86±5.22	18.24±7.23	2.67±2.13	2.36±1.23
5	82.72±6.10	17.28±6.10	36.45±15.51	8.24±6.81	5.41±2.94
10	71.21±8.15	28.79±8.15	59.23±17.32	17.78±11.07	9.01±3.94*
50	69.05±6.03	30.25±6.03*	79.97±17.23*	25.37±9.01*	13.67±3.52*

Notes: * $P<0.05$; ** $P<0.01$; TL, TM and OTM are the same as Table 1

2.3 DNA damage by Cd combined Phe

The combined effects of Cd and Phe on DNA damage are shown in Table 3. OTM in coelomcytes of earthworm treated with Phe at all of dosages increased compared with control ($p<0.05$, $p<0.01$). OTM in coelomcytes of earthworm treated with Cd (5, 10, 50 mg/kg) combined Phe (12.5 mg/kg) for 14 d increased significantly compared with control ($p<0.01$). There was a significant statistical difference when earthworm treated with the same concentration Cd combining with different concentration Phe (0.5, 2.5, 12.5 mg/kg). There was no statistical difference between 0.5 and 2.5 mg/kg ($p>0.05$), which suggested a great DNA damage to the cells with a higher dose (12.5 mg/kg) of Phe in the same experiment condition. The coelomcytes of earthworm exposed to a high dose (12.5 mg/kg) of Phe also manifested a higher amount of DNA damage than that exposed to a lower dose (0.5, 2.5 mg/kg). The average percentages of nuclei increased with increasing Phe concentration.

2.4 Interaction between Cd and Phe

Cd was found to be genotoxic to cells of earthworm and Phe was demonstrated to enhance the genotoxicity of Cd by inducing more DNA strand breaks of the damaged nuclei. Cd caused damage to earthworm nucleic acid directly or indirectly. Cd not only increased the level of DNA breakage induced by oxidative stress but also impaired the ability of the cells to recover. These indirect effect may be more relevant to the toxicity of Cd combined Phe. Therefore, it was essential to understand the interaction between different pollutants. The combined genotoxicity of heavy mentals and polycyclic acromatic hydrocarbon should be taken into account when evaluating the potential genotoxic impact of pollution on organisms. Joint effects of pollutants may be similar (additive) or stronger (synergistic, more than additive) or weaker (antagonistic, less than additive) than expected effects from separate exposures. The effect of combined pollution depends on the constituents of the mixture and may vary significantly (Jensen, 2002).

As shown in Table 3, Cd and Phe applied together exhibited a significantly greater DNA damage effect on earthworm than either Cd or Phe applied alone. Lower OTM was observed in single Cd treatment than in Cd combined Phe. The OTM was increased with addition of Phe in Cd. This suggests an

Table 3 The index of the DNA damage caused by Phe combined Cd to the coelomcytes of earthworm *Eisenia andrei* (mean±*SD*, *n*=150)

Cd, mg/kg	Phe, mg/kg	Head DNA, %	Tail DNA, %	TL	TM	OTM
0	0	90.56±4.14	9.44±4.14	17.04±5.23	2.72±2.13	2.03±1.23
5	0.5	81.81±6.35*	18.19±6.35*	41.41±11.30*	7.23±4.58*	5.31±2.74*
5	2.5	80.79±6.50*	19.21±6.50*	37.55±16.81*	8.28±6.91*	5.64±2.76*
5	12.5	66.21±7.12*	33.79±7.12*	59.48±17.57*	19.36±8.55*	10.35±3.69*
10	0.5	74.11±8.26*	25.89±8.26*	59.85±14.55*	16.54±9.47*	11.21±4.53*
10	2.5	70.46±8.56*	29.54±8.56*	55.59±16.94*	17.64±9.61*	12.13±4.67*
10	12.5	65.57±9.31**	34.43±9.31**	72.91±25.73**	25.85±16.73**	13.86±7.81**
50	0.5	67.16±8.12*	32.84±8.12*	78.97±19.36*	24.47±9.43*	13.54±3.89*
50	2.5	65.05±7.05*	34.95±7.06*	80.37±17.45*	25.79±9.83*	12.53±3.33*
50	12.5	57.47±6.97**	42.53±6.97**	108.59±32.75**	45.34±16.85**	21.44±7.01**

Notes: * $P<0.05$; ** $P<0.01$; TL, TM and OTM are the same as Table 1

additive effect on each other, Such additive effects may be caused by the total concentration of two pollutants in the soil. Since the total concentration of the pollutants in the soil was higher in the cases of a combined application of pollutants, this might have resulted in a greater DNA damage effect on earthworm.

Interaction between Cd and Phe was basically performed in accordance with Zhou (1999). Main genotoxicity effect of Cd or Phe on earthworm *Eisenia andrei* was subjected to the analysis of variance using SPSS14.0 software. The concentration-response relationship between toxicological index of earthworm (Y) and the concentration of Cd (X_{Cd}) and Phe (X_{Phe}) fit the regression linear equation $Y=5.925+0.560X_{Cd}+0.153X_{Phe}$. For each of the variables, the beta Cd was higher than Phe, $0.153/0.560<1$, indicating Cd was the main factor of contribution of DNA damage. The order of combined genotoxicity on earthworms was Cd+Phe>Cd combined Phe>single Cd or single Phe. It indicated the Phe antagonized the genotoxicity of Cd.

3 Conclusions

The test revealed that the interaction between Cd and Phe to DNA damaging effects was negative. There was a positive correlation between effect of Cd genotoxicity and Cd concentration. The effect of Phe genotoxicity increased with the increasing Phe concentration especially at higher Phe concentrations. The results showed the ingredients and concentration combinations of a combined contamination were the key factors in determining the genotoxicity of a combined contamination.

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