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Molecular toxicity of earthworms induced by cadmium contaminated soil and biomarkers screening

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Abstract

Earthworms (*Eisenia fetida*) were used to study the impact of low-dose cadmium in treated artificial soil (0, 0.6, 3, 6, 15, 30 mg/kg) and contaminated natural soil (1.46 mg/kg). The changes of earthworms' physiological related gene expressions of metallothionein (MT), annetocin, calreticulin and antimicrobial peptides were detected using real-time PCR after a 70-day incubation period. The results showed that low doses of cadmium could up regulate earthworms' MT and down regulate annetocin gene expression and show a significant positive and negative correlation respectively. The expression of two other genes, calreticulin and anti-microbial peptides, was induced at low doses of cadmium (highest gene expression at 0.6 mg/kg for calreticulin and 6 mg/kg for anti-microbial peptides) and inhibited at high doses. No significant correlation was found for these two genes. This study shows that MT and annetocin genes expression found in earthworms in contaminated soil have the potential to be developed as biomarkers of soil cadmium pollution.

Key words: earthworm; cadmium; low-dose; genes expression; molecular toxicity
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Introduction

With the development of industry and agriculture, heavy metal pollution in soil and crops is becoming increasingly serious. Particularly, soil cadmium pollution is broader and more harmful than other contaminants (Yan et al., 2000). Anthropogenic activities such as disposal of industrial waste, application of fertilizer and disposal of sewage sludge on land have also led to the accumulation of cadmium in soil (Alloway, 1990; Naidu et al., 1997), which leads to the eventual increase of its concentration in food crops. Normally, the content of cadmium in arable soil is not very high; according to Holmgren et al. (1993), cadmium content ranges from 0.005 to 2.4 mg/kg, and the mean value is 0.27 mg/kg with a median value of 0.20 mg/kg in the US. The investigation of 16 study sites in China indicated that cadmium in contaminated soil ranges from 1.09 to 27.9 mg/kg (Wang, 1997).

Earthworms are key representatives of soil fauna, and are essential in maintaining soil fertility through their burrowing, ingestion, and excretion (Edwards, 2004). They are increasingly recognized as indicators of soil ecosystem health and eco-toxicological sentinel species which can be constantly exposed to soil contaminants (Pirooznia et al., 2007). At present, a large set of eco-toxicological data on contaminated soil has been collected for diagnosis and evaluation of ecological risk. Most studies on heavy metal toxicity of earthworms were carried out under high doses (Assensio et al., 2007; Li et al., 2005; Gultekin et al., 2000). However, this does not accurately reflect the actual exposure or the potential biological toxicity of heavy metals in contaminated soil.

It has been many years since eco-toxicologists began seeking more effective ways to detect the severity of heavy metal pollution in soil (McCarthy and Shugart, 1990; Spurgeon et al., 2005). With the application of molecular bio-techniques, it is possible to develop applied soil biomarkers to detect the ecological risks of heavy metal contaminated soil. Biomarkers play significant roles in eco-toxicology as they can be signal indicators when abnormal changes of organisms occur at different levels, such as at the molecular, cellular, individual, or population level, due to environmental pollution (Weeks, 1995; Walker, 1995). It is a significant practice to use molecular biology to find a rapid and effective way to analyze the harmful level of heavy metal contaminated soil, especially under low dose situations (Calisi et al., 2011; Santoyo et al., 2011).

The present study of earthworms using molecular biology has provided useful information, based on the fact that earthworms can tolerate and concentrate heavy metals in soil (Ramseier et al., 1989; Morgan et al., 1993). However, heavy metals such as cadmium may be a severe stress factor for the earthworms, and may disturb their physiological functions (Labrot et al., 1996). Therefore,

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we can use real-time PCR to analyze the expression of earthworm physiological genes affected by heavy metals in soil. In this study, we chose four physiologically related genes including MT gene, which is a family of cysteine-rich metal-binding proteins and is induced in the presence of heavy metal cadmium to enable heavy metal tolerance (Gruber et al., 2000); annetocin gene which could affect earthworms' reproductive system (Ricketts et al., 2004); calreticulin gene, which plays a key role in cell metabolism and development, and anti-microbial peptides genes which are generated by the immune system to defend against extraneous pathogens, which are also suppressed by heavy metal cadmium (Silerová et al., 2007). Both natural soil and artificial soil were introduced to incubate earthworms in this experiment.

1 Materials and methods

1.1 Experiment setup

Adult and healthy earthworms (*Eisenia fetida*), of around 300 mg each, were obtained from Shangzhuang Experiment Station of China Agricultural University in Haidian District, Beijing, China.

Artificial soil was prepared according to the guidelines described by OECD (1984). Cadmium-contaminated natural soil was taken from Zhangziying Village, Daxing District, Beijing, China, and an uncontaminated control sample was collected no more than 500 m away from the contaminated site. The basic chemical properties of contaminated and uncontaminated natural soil are shown in Table 1.

Three replicates were set for each treatment, in which 10 earthworms were incubated in 500 g soil. Nominal cadmium concentrations in the artificial soil were adjusted to 0 (CK), 0.6, 3, 6, 15 and 30 mg/kg respectively with $CdCl_2$. Soil pH was adjusted to 7 with $CaCO_3$. Earthworms were fed weekly with sterile cattle manure and purified water. All treatments were placed in an incubator with an ambient temperature of 23°C and moisture level of approximately 70% for 10 weeks of incubation.

Table 1 Basic chemical properties of experimental soil

Total	Cd (mg/kg)	Soil pH	CEC (mol/kg)	Soil organic matter (g/kg)
Contaminated soil	1.462	7.64	21.2	26.38
Natural soil	0.419	7.99	20.1	20.49

CEC: cation exchange capacity.

1.2 Designation of tested genes and primers used for real-time PCR

According to previous studies and the NCBI database (www.ncbi.nlm.nih.gov), four genes (MT, annetocin, calreticulin and antimicrobial peptide) were selected for real-time PCR. Beta-actin gene was selected as an internal reference gene to check for inter-individual differences in total gene expression. Primers used in this study were acquired from published studies or designed using Primer Express 3.0 according to the manufacturer's instruction (Applied Biosystems, USA). Primer sequences are shown in Table 2.

1.3 Isolation, purification and detection of RNA

Earthworms were kept in clean petri dishes covered with sterile filter paper for 24 hr to clear their intestines. Afterwards, earthworms were rinsed with distilled water, dried with sterile filter paper, and then encased in a sterile centrifuge tube to be weighed, in order to calculate the volume of Trizol reagent to be used (Invitrogen, USA). Earthworms were then ground with liquid nitrogen and RNA was extracted using the Trizol method. For each replicate, RNA was extracted individually from at least 2 earthworms in case of failure or RNA degradation.

RNA extraction was purified using DNase I (Takara, Japan). Purified RNA product was examined by 2% agarose gel electrophoresis and UV spectrophotometry. Subsequently the absorbance values A_{260} and A_{280} measured by a Nanodrop 2000 spectrophotometer (Thermo, USA) were checked to determine the concentration and purity of the RNA used for the downstream experiments. RNA concentration (C, ng/μL) was calculated by Eq. (1):

$$C = A_{260} \times 40 \times N \quad (1)$$

where, N is the RNA dilution factor. Only samples with A_{260}/A_{280} value of RNA between 1.9 and 2.1 could be used for the subsequent steps.

1.4 cDNA synthesis and detection

To reduce experimental error, all RNA used in this experiment was diluted to 100 ng/μL using Easy Dilution (Takara, Japan). RT Enzyme Mix (Takara, Japan) was used following the instructions of the reverse transcription system. Then, the PCR reaction was carried out at 37°C for 5 min of reverse transcription and 85°C for 5 sec to inactivate

Table 2 Detailed information of tested genes and primers used in the Real-Time PCR

Serial No.	Gene name	Primer	Genbank No.
0	Beta-actin	F: 5′- CGCCTCTTCATCGTCCCTC-3′ R: 5′- GAACATGGTCGTGCCTCCG-3′	Y09623
1	Metallothionein	F: 5′-CGCAAGAGAGGGATCAACTTG-3′ R: 5′-AGCGTCAGCACAGCAAAGC-3′	AJ236886.1
2	Annetocin	F: 5′-TTCCATGGCTTGCACTAAGAAGTCG-3′ R: 5′-TCAGCATTGAGCGTCGTAAC-3′	AB164320.1
3	Calreticulin	F: 5′- ACACTCTTATCGTCCGTCCTG-3′ R: 5′- CCTCTGGCTTCTTCGCTTC-3′	DQ887090.1
4	Anti-microbial peptide	F: 5′- CATACTCGGAACGCAAGAACC-3′ R: 5′- TTTGATGACCTTCTGCGGTG-3′	AF060552

the reverse transcriptase. The cDNA concentration and purity were evaluated using the same methods as used for RNA. The cDNA product was diluted by DEPC (Sigma, USA) treated RNase-free water. DNA concentration (D, ng/μL) was calculated by Eq. (2):

$$D = A_{260} \times 50 \times N \quad (2)$$

Only qualified DNA whose A_{260}/A_{280} value is between 1.8 and 2.0 can be used for real-time PCR. Sometimes RNase should be used in cases of RNA interference. Chloroform and isoamyl alcohol could be used if protein contamination was somewhat severe.

1.5 Quantitative detection of genes expression using real-time PCR

1.5.1 Detection of genes amplification efficiency

cDNA was gradient-diluted from 10^{-1} to 10^{-5} using Easy Dilution with a range of 6 orders of magnitude. The real-time PCR reaction system was set according to the Takara SYBR Premix Ex Taq TM II protocol, and equivalent volumes of specific primers were also used to amplify the target sequences. The standard procedures of PCR reaction were as follows: Stage 1: 94°C, 30 sec for 1 cycle as pre-denaturation; Stage 2: 95°C, 5 sec; 60°C, 30 sec for 40 cycles as exponential amplification.

After the reaction, amplification curves, melting curves and standard curve slope were checked to ensure that no non-specific amplification was detected and the dilution was proper. The C_t value, which is the amplification cycle number of each reaction to reach the fluorescence signal threshold, was kept between 20 and 30 to make it more accurate.

1.5.2 Quantitative detection of the genes expression in all treatments

For each gene to be detected, the earthworm cDNA of each treatment was diluted according to the experimental results of Section 1.5.1. The reaction and PCR system was set as described in Section 1.5.1. The internal reference gene beta-actin was applied accompanied with the 4 genes and a relative quantification C_t value was calculated according to the C_t values of beta-actin and the detected genes.

$$\Delta C_{t(s)} = C_{t(s)} - C_{t(ir)} \quad (3)$$

$$\Delta\Delta C_{t(s)} = \Delta C_{t(s)} - \Delta C_{t(ck)} \quad (4)$$

where, $C_{t(s)}$ and $C_{t(ir)}$ are the C_t values of each treatment and beta-actin respectively. $\Delta C_{t(ck)}$ for each control treatment was calculated by Eq. (3).

The gene expression ratio (Q) was calculated by Eq. (5)

$$Q = 100 \times 2^{-\Delta\Delta C_{t(s)}} \quad (5)$$

where, $\Delta\Delta C_{t(s)}$ was calculated by Eq. (4) with the control treatment $\Delta\Delta C_{t(ck)} = 0$.

SAS software was used to analyze variance of the data and LSD t-test were used to perform all pair-wise comparisons between group means and the control.

2 Results

2.1 RNA extraction, cDNA synthesis and gene amplification efficiency using real-time PCR

Cox (1977) proposed that there are gaps in the large subunit rRNA of the earthworms, and as a result, the brightness of the 18S band is higher than that of 28S, which is different from other species. As shown in Fig. 1a, bands representing 28S and 18S rRNA can be clearly seen, and the band for low molecular weight 5S rRNA was weak, which indicated the high integrity of the RNA extracts. The quality of synthesized cDNA was perfect as well (Fig. 1b).

From Table 3, we can see the melting temperature was around 80–89°C. Specific amplification could be clearly seen. The slope of the standard curve was about –3.0 and $\Delta K < 0.3$, which demonstrated that the gene amplification efficiency was about the same. The R^2 value of all the tested groups basically reached 0.9, which showed a good linear relationship between amplification results and template concentrations. As a result, the C_t method of relative quantification can be rationally used in the detection of changes in the expression of the following genes.

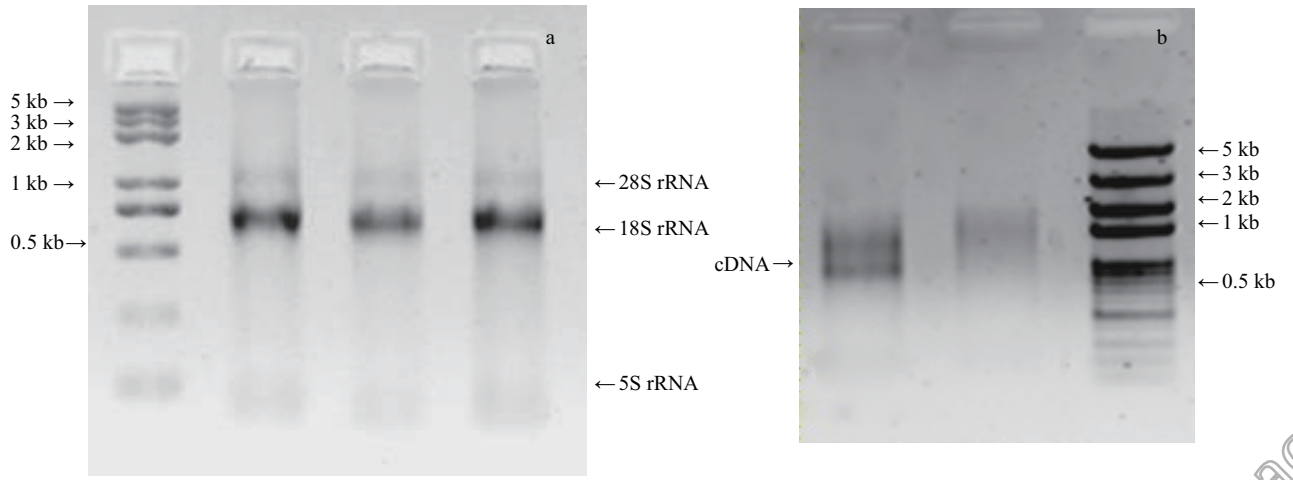

Fig. 1 Earthworms total RNA extraction results (a) and cDNA transcribed by total RNA (b).

Table 3 Amplification efficiency and melting temperature of genes tested

Tested gene	Melting temperature (°C)	R^2	Slope K	ΔK
β-Actin (internal reference gene)	87.5	0.902	–3.000	0.066
Metallothionein	83.5	0.899	–2.934	0.000
Annetocin	86.0	0.951	–3.148	0.214
Anti-microbial peptide	84.5	0.995	–2.959	0.026
Calreticulin	84.5	0.993	–3.089	0.155

R^2: correlation coefficient.
$\delta K_{(i)} = K_{(i)} - K_{(min)}$, $K_{(i)}$ represents the standard curve slope of each tested gene (i), including the internal reference gene. $K_{(min)}$ is the standard curve slope of MT gene whose slope K is the lowest of all the tested genes.

2.2 Gene expression of earthworms in the artificial and natural soil

2.2.1 MT gene

As shown in Fig. 2a, the earthworms' MT gene expression increased dramatically with increasing cadmium concentration: MT gene expression was 2.1 fold higher in 0.6 mg/kg treatment soil than that of CK (0 mg/kg). With soil cadmium content increasing from 3 to 30 mg/kg, MT gene expression was higher. When soil cadmium content grew to 30 mg/kg, MT gene expression was 12.68 fold that of CK. The MT gene expression was significantly different between the cadmium-treated earthworms and the CK ones in artificial soil ($p < 0.01$). The results show a strong positive correlation between cadmium doses and MT expression.

The results shown in Fig. 2b indicate that MT expression was twice as high (2.4 fold) for the earthworms that survived in natural contaminated soil compared with natural control farmland soil. It was significantly different ($p < 0.01$) between the two treatments. This indicates that MT gene expression can be sensitively induced under low cadmium content, and the results from natural soil were consistent with the artificial soil treated groups.

2.2.2 Annetocin gene

The results in Fig. 3a show that the earthworms' annetocin gene expression decreased with increasing content of heavy metal cadmium. Annetocin gene expression of 0.6 mg/kg (artificial soil cadmium content) treated earthworms was 0.71 fold that of CK earthworms. With soil cadmium content increasing from 3 to 30 mg/kg, annetocin gene expression decreased. Annetocin gene expression of 30 mg/kg treated earthworms was only 0.24 fold that of CK ($p < 0.01$). The annetocin gene expression of artificial soil cadmium-treated earthworms was severely inhibited and significantly different compared with the CK earthworms ($p < 0.01$).

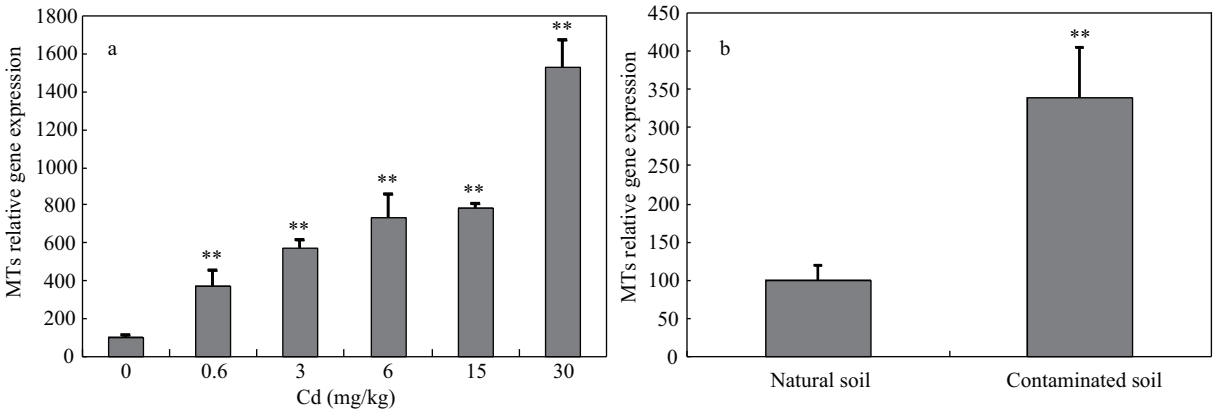

Fig. 2 Earthworms relative gene expression of metallothionein (MT) induced by contaminated artificial soil (a) and contaminated natural soil (b) (*$p < 0.05$; **$p < 0.01$ compared with CK).

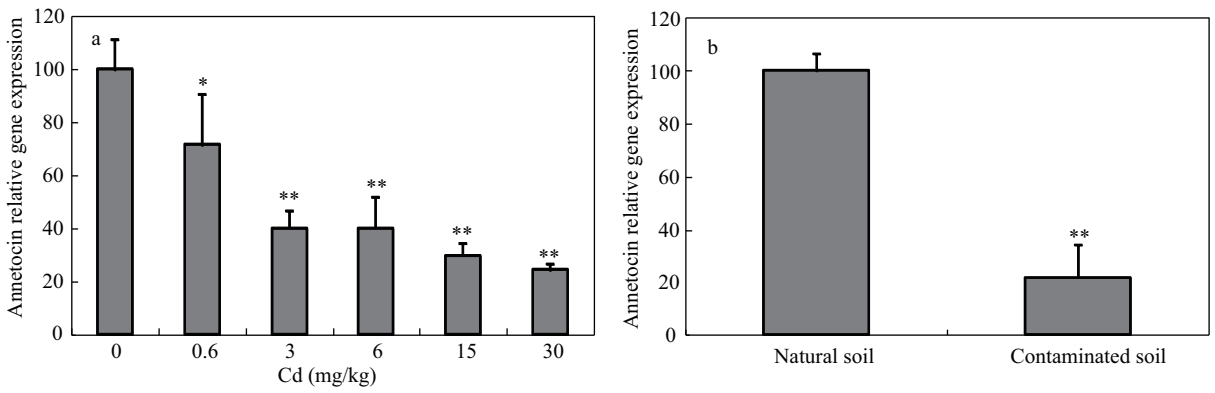

Fig. 3 Earthworms relative gene expression of annetocin induced by artificial soil (a) and natural soil (b) (*$p < 0.05$; **$p < 0.01$ compared with CK).

The results in Fig. 3b show that the natural polluted soil inhibition effect was even more severe than that of the artificial soil treated groups. Annetocin expression in earthworms exposed to the natural contaminated soil was only about 0.214 fold that of the uncontaminated ones and was significantly different ($p < 0.01$). The above results indicate as well that annetocin gene expression can be inhibited by low cadmium content in the soil.

2.2.3 Calreticulin gene

From Fig. 4a, the results showed that the calreticulin gene expression of 0.6 mg Cd/kg soil treated earthworms was the highest, 2.09 fold that of CK. However, when the heavy metal cadmium content increased to 3 mg/kg, the expression level dropped to 1.07 fold that of CK. There was no significant difference ($p > 0.05$) among the treatments of soil cadmium content 3, 6, 15, 30 mg/kg and the CK. However, when soil cadmium content reached 30 mg/kg cadmium, the expression of calreticulin was only 0.59 fold that of CK on average.

There was no significant correlation between the soil cadmium concentration and the calreticulin expression of the treated earthworms ($p > 0.05$). The results in Fig. 4b show that the calreticulin gene expression of the natural contaminated soil treated groups increased to 1.62 fold that of the control ones, and the difference was significant ($p < 0.01$). This result is nearly identical to that for 0.6 mg/kg artificial soil cadmium treated earthworms shown in Fig. 4a.

2.2.4 Anti-microbial peptide gene

From Fig. 5a, we can conclude that gene expression for the anti-microbial peptides did not change for earthworms treated in artificial soil under low-dose cadmium, as previously introduced that anti-microbial peptides of the earthworms showed constitutive expression (Ganz, 2004). A higher dose of cadmium (6 mg/kg) resulted in an increased expression level of the anti-microbial peptide gene. However, if the concentration was too high (30 mg/kg), cadmium showed an inhibiting effect on the anti-microbial peptide gene expression of the earthworms.

In Fig. 5b, the anti-microbial peptides gene expression showed little change for earthworms treated in the natural contaminated soil compared with the control ones due to the low cadmium content; the difference is not significant ($p > 0.05$).

3 Discussion

MT is a family of cysteine-rich metal-binding proteins that widely exist *in vivo* for mammals and invertebrates (Kägi, 1991). As a coping mechanism, MT expression was induced in the presence of cadmium to allow relief of heavy metal poisoning. In this study, a strong positive dose-response correlation between the cadmium and MT expression was observed. MT expression seems to be sensitive to cadmium: it was strongly induced even at low cadmium concentration (0.6 mg/kg). A similar conclusion was reached by Bernard et al. (2010). MT expression and

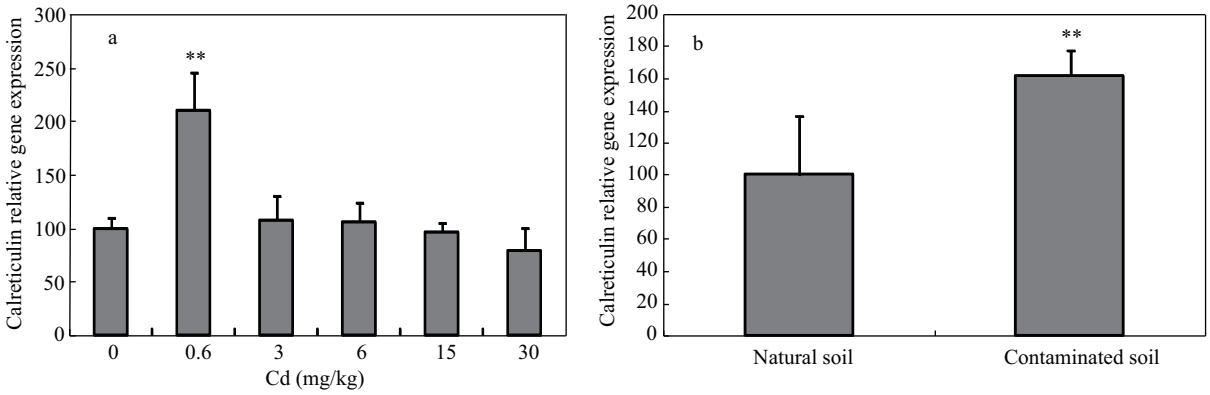

Fig. 4 Earthworms relative gene expression of calreticulin induced by artificial soil (a) and natural soil (b) (*$p < 0.05$; **$p < 0.01$ compared with CK).

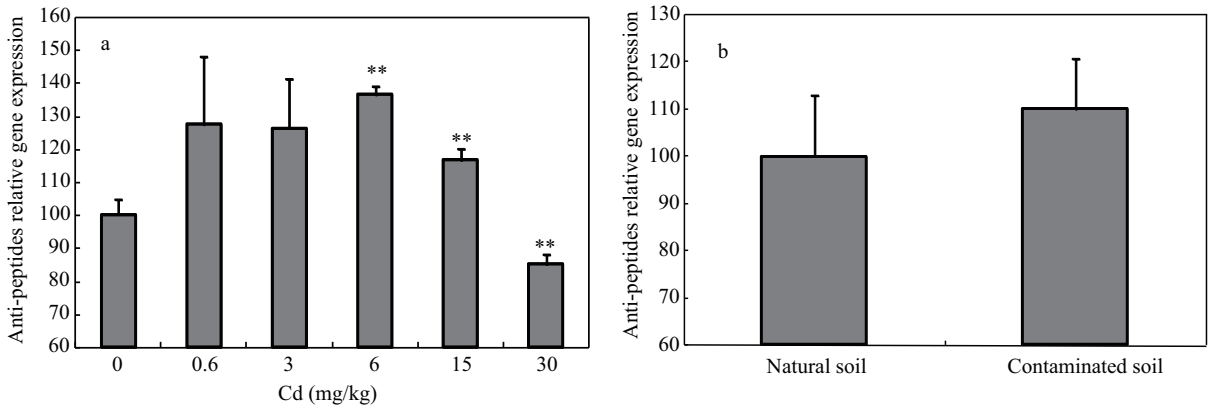

Fig. 5 Earthworms relative gene expression of anti-microbial peptides induced by artificial soil (a) and natural soil (b) (*$p < 0.05$; **$p < 0.01$ compared with CK).

Neutral Red uptake as biomarkers of *Eisenia fetida* and *Lumbricus terrestris* exposed to cadmium (100 mg/kg) was studied by Assensio et al. (2007). The study also showed a significant up-regulation of MT in both species. All the studies including our results suggest that MT could be developed as a more effective biomarker for determining soil cadmium contamination levels.

The earthworm reproductive system is vulnerable to harmful substances and as a result the entire community may be adversely affected (Spurgeon et al., 2000). Siekierska (2007) found that farm pesticides may damage earthworm ovarian cells. As cells organelles and oocytes numbers and nutrition increased, the cells nuclear degeneration and egg formation processes were inhibited as well. The theory was proposed very early that earthworms' reproductive system could be used to assess soil contamination (Ferraguti, 1983; Olive and Clark, 1978). Ricketts et al. (2004) found that expression of reproductive-related genes of earthworms coming from an obsolete zinc-lead mine in Wales, UK was inhibited. He also found that annetocin expression was tissue-specific: the highest expression was near the reproductive pore but virtually none near the tail. In our study, the same tendency was also found as Ricketts et al. (2004) as well. The annetocin gene expression had a negative correlation to cadmium content in soil. It was sensitively induced at low doses. This suggests that annetocin could also be a sensitive biomarker for low-level heavy metal cadmium in contaminated soil.

Calreticulin is a Ca^{2+} combined molecular chaperone, which mostly exists in the endoplasmic reticulum (Silerová et al., 2007). A calreticulin cDNA full sequence was acquired through RACE and RT-PCR in 2007 by Silerová et al. (2007). Calreticulin protein has also been detected by Western blot. In this experiment, when soil cadmium content was about 0.6 mg/kg, calreticulin gene expression was the highest. It seems that low doses of cadmium acted as a stimulus, which led to the over-expression of gene and more calreticulin protein was generated. As a result, this may accelerate the protein synthesis, folding and processing processes of earthworms' endoplasmic reticulum (Silerová et al., 2007). The earthworms' ability to cope with heavy metal stress was therefore strengthened. However, when cadmium was 30 mg/kg, cell function including the endoplasmic reticulum might be harmed and the gene expression was therefore affected and decreased.

Anti-microbial peptides are generated by the immune system to defend against extraneous pathogens. Research by Sun (2001) confirmed that the expression of anti-microbial peptides could be induced sensitively by microbial injection. Earthworms' anti-microbial peptides expression was also somewhat increased when treated by mechanical injury or heavy metals like cadmium. The full sequence of Lumbricin I was acquired using RACE and it was found that anti-microbial peptides were only expressed in adult earthworms, using Northern blot analysis (Cho et al., 1998). In this study, we found that certain doses of cadmium could induce the gene expression of anti-microbial peptides, though not as sensitively as by microbial stimulus. However, at high doses, the expression would be inhibited to some extent. This may be attributed to a stimulus effect at low doses of cadmium while damaging cell functions at high doses.

4 Conclusions

In this study, changes of expression levels of four genes (MT, annetocin, calreticulin, and anti-microbial peptides) related to earthworm physiology were detected using real-time PCR through a 70-day incubation period with different doses of cadmium treatment in soil. MT expression could be induced by low doses of cadmium and showed a significant positive ($p < 0.01$) correlation to cadmium doses. Annetocin gene expression decreased with increasing soil cadmium concentration and had a significant negative correlation ($p < 0.01$) to cadmium doses. These two genes had the potential to be developed as biomarkers for cadmium-contaminated soil. The expression of the other two genes, calreticulin and anti-microbial peptides, was induced at lower doses of cadmium (the highest gene expression at 0.6 mg/kg for calreticulin and 6 mg/kg for anti-microbial peptides) and inhibited at high doses. No significant correlation ($p > 0.05$) was found to soil cadmium concentrations for these two genes, thereby they may not be suitable candidates as biomarkers in cadmium-contaminated soil.

The results also show that earthworms treated with both artificial and natural soil under approximately the same cadmium doses exhibited similar levels of gene expression, which indicated that artificial soil can be a substitute for natural soil in the study of eco-toxicity of earthworms. However, more research is needed for better understanding and application of results from artificial soil, such as how heavy metals interact with different soil types. Also, more works are needed to better understand the relationships between gene expression and toxic effects such as annectocin gene expression and cocoon reproduction, MT gene expression and heavy metal tolerance, etc.

Acknowledgments

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