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Effects of Uranium on the Antioxidant Responses of Chinese Oak Silkworm, *Antheraea pernyi*

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ABSTRACT

Uranium (U) is one of the heavest metals in nature, which has been mainly used in atomic energy industrial system, but its toxic effects on most organisms are largely remained unknown especially in insects. *Antheraea pernyi* pupae were injected with uranyl acetate, which concentrations were 6.25mM, 12.5mM, 25mM. Changes in malondialdehyde (MDA) and reduced glutathione (GSH) and activities of superoxide dismutase (SOD), and catalase (CAT) as well as their corresponding mRNA levels in the fat bodies of the pupae were evaluated. As a result, we found that the contents of MDA were increased at 4 h and then decreased compared with control. And, the contents of GSH were decreased gradually. However, the activities of catalase were inhibited and the trend of SOD activity changes was down-regulation in a time-dependent manner with higher concentration treatments. The changing trends in the mRNA levels of these enzymes were not always consistent with those of enzymatic activities. The fat body of *A. pernyi*, as an important antioxidant tissue, can increase the expression of oxido-reductas system enzymes by promoting the expression of SOD, CAT, GST and glutathione peroxidase (GSH-Px) to improve the body's response to heavy metal stimulation injury. Our results collectively support a relationship between uranyl acetate and alterations in the levels of antioxidant enzymes in *A. pernyi* fatbodies. The activities and expression of reactive oxygen species (ROS) enzymes can be used as a biomarker for detection of heavy metal contamination.

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Authors' Contribution
CFZ and XGL designed the study, analysed thr data and wrote the manuscript. YXL, LFH and FJS collected background information. WTY performed manuscript review.

Key words
***Antheraea pernyi*, Uranyl acetate, Antioxidant enzyme, mRNA expression, Activity of enzymes**

INTRODUCTION

Exposure to heavy metals produces many physiological changes in organisms, including the production of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide (H_2O_2) and hydroxyl free radical. The production and removal of reactive oxygen species in organisms are in a dynamic equilibrium state. When the concentration of ROS exceeds the physiological limit, breaking this balance, the biological macromolecules will be damaged including lipid peroxidation, DNA oxidative damage, protein oxidation and monosaccharide oxidation (Yamamoto *et al*., 2005). Lipid peroxidation is a process of ROS oxidation of biofilms after enhanced oxygen stress, such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE), which changes the fluidity and permeability of cell membranes and ultimately leads to changes in cell structure and function. The antioxidant enzyme system include superoxide dismutase (SOD) which converts $\cdot O_2$ to H_2O_2 and oxygen (O_2), catalase (CAT) which transforms H_2O_2 into H_2O and O_2, and glutathione peroxidase (GSH-Px) with its function to break down H_2O_2 while simultaneously oxidizing reduced glutathione (GSH) to oxidized glutathione (GSSG) (Yuan *et al*., 2016). In adult zebrafish, with copper (Cu as copper chloride dihydrate), cadmium (Cd as cadmium acetate), lead (Pb as lead nitrate) and chromium (Cr) exposure, the MDA level and SOD activity are up-regulated by the four heavy metals (Yin *et al*., 2018). Mercury increased levels of reactive oxygen species and the expression of GPx 1 and GPx4 in rats (Martinez *et al*., 2017).

Uranium (U) which is one of the heaviest elements that can be found in nature, is a radioactive metal within the earth. Uranium is mainly used as nuclear fuel for nuclear weapons and nuclear reactors. It is also used to make alloy steel, catalysts for organic chemistry, preservatives and hardeners for rubber industry, and colorants for glass, ceramics and enamel. Uranium concentrations in natural environments can increase as a consequence of anthropogenic activities such as mining and the utilization of nuclear power (Muscatello and Liber, 2010). Although numerous data have been demonstrated the heavy metal toxicity, most of them concentrated on the developmental delay, malformations, mortality of different

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living organisms (Labrot, 1999; Kuhne, 2002; Lagisz and Laskowski, 2008). Little is known about the effects of U on wild macro-invertebrates. Therefore, there is a particular need for more data to further understand mechanism of its toxicity to such organisms.

Antheraea pernyi has been used as a well-known wild silk moth in Chinese sericultural industry and medicine industry for hundreds of years. In recent years, due to the development of biotechnology, the comprehensive utilization of *A. pernyi* has attracted more and more attention. High-tech products such as hormone, interleukin, antimicrobial peptide, interferon, etc. extracted from silkworm, pupae and moth, as well as medical and health products, have provided tremendous market potential in China. On the other hand, it is usually used as an invertebrate insect model to study pesticide technique, metabolism regulation, and immune response by its convenience, low cost and no ethical issues (Li *et al*., 2010; Zhang *et al*., 2015; Panthee *et al*., 2017; Xin *et al*., 2017; Liu *et al*., 2018). Here, change in MDA content and reduced glutathione (GSH) and the activities as well as gene expression of oxido-reductases including SOD, GST, CAT and GSH-Px were studied with injection of uranyl acetate in the Chinese silk worm *A. pernyi* pupae. The results suggested that oxido-reductase plays a significant role in heavy metal stress and the activities and expression of ROS enzymes can be used as biomarker for detection of heavy metal contamination.

MATERIALS AND METHODS

Experimental animals and challenge

The pupae were provided by the Sericultural Research Institute of Henan. The larvae were reared on fresh oak leaves at 25 ± 1 °C in 14 h light:10 h dark (a long day length) with 70% humidity. The pupae were collected and kept at room temperature. Three individuals were used for different uranyl acetate concentration challenge. Fat bodies was used for experiment materials. Fat bodies were collected at different time through dissection. In order to study the influencing mechanism of U on *A. pernyi*, the pupae of diapause were chosen to be injected with 6.25 mM, 12.5 mM and 25 mM in10 µl uranyl acetate dissolved in PBS (Han *et al*., 2009)($C_4H_6O_6U$, Sangon Biotech Co., Ltd., Shanghai). The control pupae were injected with 10 µl PBS solution at different time.

Biochemical determinations

The fat bodies of the pupae were prepared for each treatment. The fat bodies were dissected on ice, immediately placed into liquid nitrogen, and stored at −70 °C until biochemical assay (within 1 week). MDA, SOD, CAT, and GST were determined individually. Approximately 0.1 g of preserved sample was homogenized in 0.9 mL ice-cold physiologic saline (50 mmol L^{-1} at pH 8.6). The homogenates were centrifuged at 10,000×g for 10 min and 4 °C, and the resulting supernatants were used for MDA, SOD, CAT, and GST contents and enzyme activities measurements. The enzymes contents and activities of the antioxidative enzymes were determined using substrates obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to protocols provided by the manufacturer. The protein concentrations in the fat body samples were determined by the method of Coomassie Blue Staining using bovine serum albumin as a standard. All protein measurements were performed in triplicate.

Effect of U on antioxidant enzymes

Malondialdehyde (MDA)

MDA is a common index of membrane lipid peroxidation. Under acidic and high temperature conditions, it can react with thiobarbituric acid (TBA) and form red-brown trimethylchuan (3,5,5-trimethyloxazole-2,4-dione), which maximum absorption wavelength is 532 nm.

Superoxide dismutase (SOD)

The SOD activity in the resulting supernatant was measured at 550 nm with the xanthine and xanthineoxidase system. The one unit of SOD activity was defined as the amount of SOD required for 50 % inhibition of the enzymatic reaction in 1 mL enzyme extraction of per milligram of protein.

Catalase (CAT)

The samples were treated with excess hydrogen peroxide and incubated at 37°C for 1 min, and the remaining hydrogen peroxide (not decomposed by catalase) reacted with ammonium molybdate and produced a yellowish complex, which had a maximum absorption at 240 nm. One unit of CAT activity was defined as 1 mmol H_2O_2 decomposed in 1 mg of protein per second.

Reduced glutathione (GSH)

GSH was measured using 5, 5′-dithio-bis (2-nitrobenzoic acid) (DTNB) as described by Griffith (1980). The absorbance was read at 405 nm. GSH was measured as milligram GSH per gram protein.

Analysis of enzyme gene expression by quantitative RT-PCR

Total RNAs from fat bodies of three pupae treatment with 6.25 mM uranyl acetate for 4 h, were reverse transcribed into cDNAs. Primer Premier 5.0 software was used to design fluorescent quantitative PCR primers. The

housekeeper gene 18S rRNA (GenBank: DQ347469) was used as the internal reference gene. The primer sequences of *SOD* (GenBank: AFC35302.1), *CAT* (GenBank: NM_001043447.1), *GST* (GenBank: EU541490.1) and GSH-Px (GenBank: DQ31144) were shown in Table I. Before quantitative PCR, routine PCR amplification was carried out to find out the best reaction conditions and optimize the reaction system. After obtaining a single and clear band, quantitative PCR test was carried out. According to the instructions of SYBR Premix ^{Ex}TaqTM (TaKaRa) kit, the fluorescence quantitative PCR reaction was carried out. Three replicates were set for each reaction, and the reaction system was prepared according to the following proportion: 2 x SYBR Premix ^{Ex}TaqTM 10 μ L, upstream primers (10 mμ M) 0.5 mμL, downstream primers (10 mμM) 0.5 mμ L, template 1 μL, ddH_2O 8 L. The final volume is 20 m L.

Table I. The primers used in this study.

Primer name	Primer sequence (5'-3')
18SRNA	GTCCGGGCCTGGTGAGATT
	CGATCCGCCGACGTTACTAC
GST	AAATCGACGGCAAGCAGTAC
	TCCAGTTCCGCCTTCTTCTT
SOD	CTTCGTGGTGATGTTAGCGG
CAT	TGCCTCAATGTTACCGAGGT
	AAAACGGCGATACAAACGGT
	CGGTGTCCTTTTGCCTATGG
GSH-Px	AATGTTGCTTCTCAGTGCGG
	TGACTTTACGCTCAGAGGCA

The PCR reaction was carried out on Bio-rad CFX96 fluorescent quantitative polymerase chain reaction (FQPCR) instrument. The reaction conditions were as follows: pre-denaturation at 95°C for 5 seconds and denaturation at 95°C for 5 seconds. According to the pre-experimental results, the optimum annealing temperature (58-62 °C) was selected for 30 seconds and extension at 72 °C. Using 18S rRNA as internal reference, the relative expression levels of some immune genes in tissues of *A. pernyi* silkworm larvae injected with uranyl acetate and control at different times were analyzed by $2^{-\Delta\Delta Ct}$ relative quantitative method (Livak and Schmittgen, 2001). Additionally, total RNA that was extracted from fat bodies of pupae, treated with uranyl acetate (6.25 mM, 12.5 mM and 25 mM) and PBS was detected using the same method. Each independent experiment was conducted in triplicate.

Statistical analysis

The descriptive data were analyzed statistically using SPSS software (version 20.0 for Windows) to calculate the mean, SD, maximum and minimum. One-way analysis of variance (ANOVA) was applied to determine significant differences among various groups. Differences were considered to be statistically significant when $P < 0.05$ and such differences were indicated by an asterisk.

RESULTS

MDA content in the fat bodies

In vivo, free radicals act on lipid peroxidation, and the end product of oxidation is MDA, which can cause cross-linking polymerization of proteins, nucleic acids and other living macromolecules. As an end product of lipid oxidation, MDA affects mitochondrial respiratory chain and key enzyme activities in mitochondria. MDA is one of the most important products of membrane lipid peroxidation. Its production can also aggravate membrane damage. MDA can be used to understand the extent of membrane lipid peroxidation, and indirectly measure the degree of membrane system damage. The MDA content was elevated with increasing uranyl acetate concentrations at 4 h, compared to the control (Fig. 1). The maximal increase in MDA after treatment with 25 mM uranyl acetate was 147.7%, relative to the control (P<0.01). Similar results have been reported in the testis of *Bombyx mori*, the maximal increase was 231.4 % after treatment with 50 mg kg^{-1} Cd (Yuan *et al*., 2016).

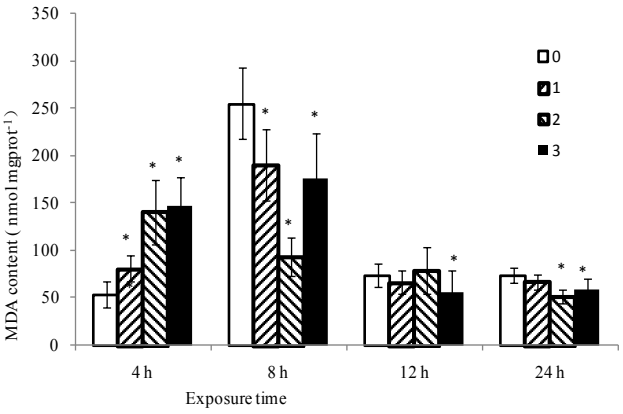

Fig. 1. MDA content in the fat bodies of *A. pernyi* exposed to different concentration of uranyl acetate. The values represent mean±SE (n=3). For each experiment, samples were analyzed in triplicate. (0, Control; 1, 6.25 mM; 2, 12.5 mM; 3, 25 mM.).

SOD activity

The effect of U on SOD activity in the fat bodies of silkworms is presented in Figure 2. With low concentrations of uranyl acetate injection (6.25 mM), the activity of total superoxide dismutase increased and reached the highest level 215.42 U/mg at 8 h, and then decreased to near normal level at 24 h. After the injection of higher concentrations of uranium (12.5 mM and 25 mM) at 4 h, the enzyme activity reached 203.5 U/mg and 211.09 U/mg respectively. Then the level gradually decreased and approached the control group at 24 h. Similar trends were up-regulated in zebrafish by four heavy metals exposure including copper (Cu), cadmium (Cd), lead (Pb) and chromium (Cr) (Yin *et al*., 2018).

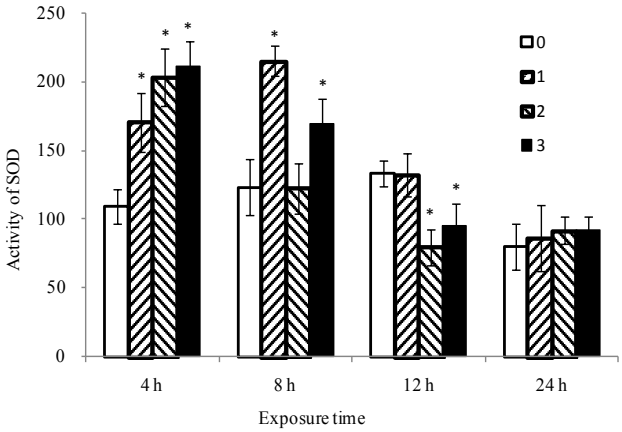

Fig. 2. Activity of super oxide dismutase in the fat bodies of *A. pernyi* exposed to different concentration of uranyl acetate. The values represent mean±SE (n=3). For each experiment, samples were analyzed in triplicate. (0: Control; 1: 6.25 mM; 2: 12.5 mM; 3: 25 mM.).

GSH content of glutathione S-transferase(GST) in the fatbodies

Glutathione is a polypeptide consisting of glutamic acid, glycine and cysteine. It is the main non-protein sulfhydryl compound in tissues. It is also the substrate of GSH-Px and GSH-ST enzymes. It is necessary for these two enzymes to decompose hydrogen peroxide. Reduced glutathione is the most important non-enzymatic antioxidant in the body. It has many important functions, such as eliminating free radicals, detoxifying, promoting iron absorption, maintaining the integrity of erythrocyte membrane, maintaining DNA biosynthesis and playing a part in normal cell growth and development, and cell immunity. GSH is a low molecular scavenger, which can scavenge. O_2, H_2O_2, LOOH. Therefore, the amount of GSH is an important factor to measure the body's antioxidant capacity. Oxidative stress leads to altered changes in both enzymatic and non-enzymatic components of its management system. As shown in Figure 3, in the fat bodies of *A. pernyi*, a significant decrease in GSH content was found at levels of about 33.18, 50.63, 70.73 and 63.45 compared with the control of 79.74, 73.68, 85.88 and 84.96 respectively. And the degree of inhibition were reduced in a time-dependent manner. Similarly, a concentration-dependent significant decrease ($p < 0.001$) in GSH content (~26%) was observed in the *Drosophila melanogaster* brain ganglia of 20.0 mg/ml Cr(VI) exposed for 48 h (Singh and Chowdhuri, 2018). And in liver and kidney of the fish, *Channa punctatus* exposure to 5 mg/L Zn^{2+}, a decrement in percentage values of GSH was observed as 44.09%(T1) and 49.63%(T2) for liver and 49.85%(T1) and 45.82% (T2) for kidney in comparison to unexposed fishes after 28 day (Ratn *et al*., 2018). And, in hepatic homogenates of rats contaminated by uranyl acetate, the level of GSH in the treatment group (9.08± 1.45) mg/gprot was significantly lower than that in the control group (13.32±0.56) mg/gprot (Yuan *et al*., 2017).

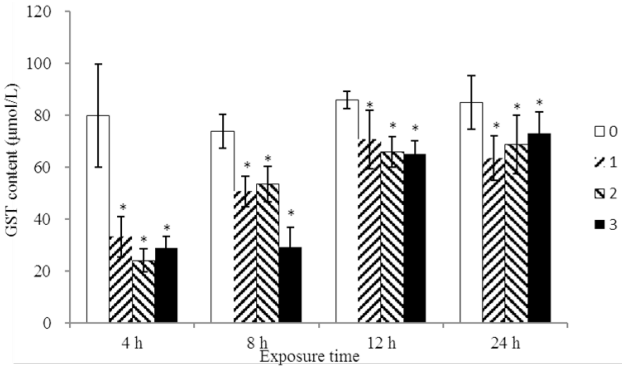

Fig. 3. Content of reduced glutathione (GSH) in the fat bodies of *A. pernyi* exposed to different concentration of uranyl acetate. The values represent mean±SE (n=3). For each experiment, samples were analyzed in triplicate. (0: Control;1: 6.25 mM; 2: 12.5 mM; 3: 25 mM.)

CAT activity in the fat bodies

Catalase can decompose hydrogen peroxide into water and harmless oxides, reducing the danger of hydrogen peroxide accumulation in the body. CAT activity reflects this ability. It can be seen from the Figure 4 that the activity of *A.pernyi* was inhibited by the injection of uranyl acetate. The trend was similar to the change of GST activity as shown in Figure 4, which the degree of inhibition were reduced with a time-cource relationship. As an important enzyme involved in the antioxidant defense system, catalase is generally inhibited by metals. In the crayfish, *Procambarus clarkii*, a significant decrease in CAT activity was observed at T4 in gills of crayfish exposed to both

types of U including depleted uranium (DU) and ^{233}U (Al Kaddissi *et al*., 2012a). Additionally, in zebrafish (*Danio rerio*) after a depleted uranium (500mg/L) waterborne exposure, the CAT activity showed a significantly reduced (~-57%) (Barillet *et al*., 2011).

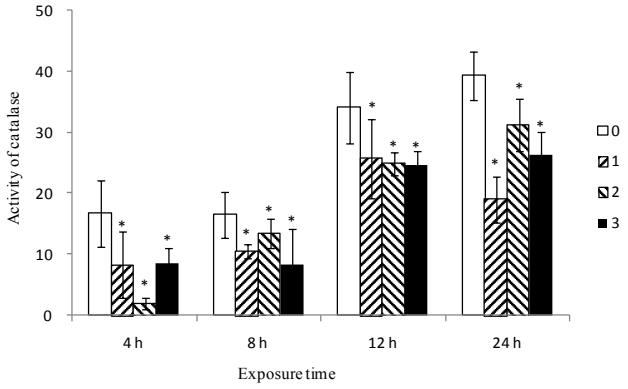

Fig. 4. Activity of catalase in the fat bodies of *A. pernyi* exposed to different concentration of uranyl acetate. The values represent mean±SE (n=3). For each experiment, samples were analyzed in triplicate. (0: Control; 1: 6.25 mM; 2: 12.5 mM; 3: 25 mM.).

Gene expression patterns of SOD, CAT, GST *and* GSH-Px *exposed to uranyl acetate*

Exposure to heavy metals not only produces many physiological changes including alterations in the metabolic activities in *A. pernyi*, but also influences some gene expression patterns. According to the relative gene expression of in *A. pernyi* silkworm fat bodies treated with uranium acetate at different time, we can see that the level of SOD increased significantly at 4 h, then decreased gradually (Fig. 5). In the zebrafish mRNA levels of SOD1 and SOD2 were up-regulated by non-lethal concentration Cu, Cr, Cd and Pb exposure (Yin *et al*., 2018). In addition, in *Mytilus coruscus* (Wu *et al*., 2017), the highest point of SOD mRNA appeared at 15 d after being exposed to copper (7-fold at 0.5 mg/L and 13.2-fold at 1.5mg/L), but plumbum at 1 d (2.4-fold at 1.0 mg/L and 4.4-fold at 3.0 mg/L) and at 15 d (2.1-fold at 0.2 mg/L).

After injecting low concentration of uranium acetate in *A.pernyi* pupae, the gene expression level of catalase increased (Fig. 5), reached the highest level 1.82-fold at 4 h, and slowed down at 8, 12 and 24 h. While in hepatopancreas of *Mytilus coruscus*, after Cu^{2+} and Pb^{2+} exposure, the highest point of CAT mRNA appeared at different times for exposure to heavy metals with copper at day 5 (0.1 mg/L 30-fold, 0.5 mg/L 15-fold,1.5 mg/L 6-fold) and plumbum at day 3 (3.0 mg/L 20-fold) (Bao *et al*., 2018). On the other hand, GST transcript gradually increased from 0.48- fold to 0.75-fold, while the level of GSH-Px gene expression pattern was reverse, which decreased from 1.02-fold to 0.48-fold (Fig. 5).

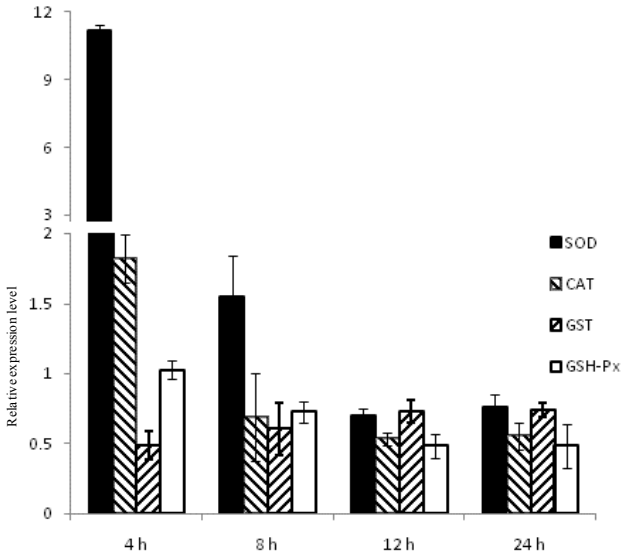

Fig. 5. Raletive expression of *super oxide dismutase* (*SOD*), *catalase* (*CAT*), *glutathione S-transferase*(*GST*) and *Glutathione peroxidase* (*GSH-Px*) gene in the fatbodies of *A. pernyi* exposed to 6.25 mM uranyl acetate for 4 h. The values represent mean±SE (n=3). For each experiment, samples were analyzed in triplicate.

DISCUSSION

Heavy metal can trigger an imbalance in the oxidant/ antioxidant system in organs by the excessive ROS/RNS production and/or antioxidant system impairment. Once this coordination and dynamic balance of the oxidant/ antioxidant system is disturbed and imbalanced, it will cause a series of metabolic disorders and immune function reduction, and forming oxygen free radical chain reaction to damage the biofilm and its function, resulting in cell transparency lesions, growth, differentiation, proliferation and apoptosis. In this paper, the effects of heavy metal uranium on the activity and genes expression of oxido-reductas in *A. pernyi* silkworm were studied. From the results, we can see that in the fat body of *A. pernyi*, with uranium acetate treatment, the contents of MDA were increased at 4 h and then decreased compared with control. And the levels of GSH were decreased with different concentration of uranium treatment at different time. However, the activities of catalase were inhibited while SOD activity increased. On the other hand, the fat body of *A. pernyi*, as an important antioxidant tissue, can increase the expression of oxido-reductase system enzymes by

promoting the expression of SOD, CAT and GST to improve the body's response to heavy metal stimulation injury.

With the development of science and technology, more and more living organisms are faced with xenobiotica stress, such as heavy metals like Cd (II) (Han *et al*., 2009; Lai, 2015; Li *et al*., 2018), Cu (II) (Zhang *et al*., 2014), U (Goulet *et al*., 2015; Goussen *et al*., 2015), Pb (II) (Labrot, 1999; Chinni *et al*., 2002), Fe(II), TiO2 (Han *et al*., 2009; Xue *et al*., 2017). To unveil the basis of individual organisms and metal ion populations interaction, many insects have been developed as environmental pollution bioindicators based on their experimental advantages, i.e., low cost, ease of maintenance, high throughput capabilities, similarity to xenobiotica stimulation. When *S. litura* larvae were exposed to lead (Pb) (25–200 mg /kg) (Han *et al*., 2009), there was a significant inhibition in Vg of female adults. On the other hand, upon cadmium iron stress, the transfer in mRNA level up-regulated in fat body, while the expression were suppressed it in the fat body, muscle, and Malpighian tubules of the silk worm *Bombyx mori* (Zhou *et al*., 2015). Although uranium can be less toxic than other metals or metalloids produced by mining activities (Bergmann *et al*., 2018) to some freshwater invertebrates (*Schizopelex festiva, Proasellus* sp., *Theodoxus fluviatili*), specimens of *Schizopelex festiva* and *Chironomus tentans* larvae appeared to decrease after exposure to aqueous uranium (Muscatello and Liber, 2010; Bergmann *et al*., 2018). The GST activity was reduced to about 46±3.76% and 48.6±14.33% in the gills and the hepatopancreas of crayfish *Procambarus clarkii,* respectively after treatment with U (Al Kaddissi *et al*., 2012b). In our study, GSH contents were reduced in a time-dependent manner after injection of uranyl acetate.

Heavy metal-induced imbalance between oxidant/antioxidant has largely been discussed in many data. However, the effects of different metals in different organisms are not always straightforward to interpret. For example, in adult SD male rats after *in vivo* effect of uranium, the activities of SOD, CAT and GST were up-regulated except for MDA (Yuan *et al*., 2017). While in *Hydra magnipapillata* incubation with cobalt, SOD and GST expressions were up-regulated and CAT gene level down-regulation (Zeeshan *et al*., 2017). In stream shredder *Calamoceras marsupus*, the catalase activity increased with increased copper concentration, and the activity of GST enzymes did not differ after copper and uranium treatments (Tagliaferro *et al*., 2017). In white shrimp *Litopenaeus vannamei* by adding copper sulfate to 5‰ seawater, significant increases in ROS production and the relative expression levels of Cu-Zn SOD and CAT were up-regulated in a dose-dependent and time-dependent manner by quantitative real-time PCR analysis (Guo *et al*., 2017). Similar results were obtained for samples from our larvae exposed to uranyl acetate for 4 h, in which *SOD, CAT, GST* and *GSH-Px* transcripts were up-regulated 11.15, 1.82, 0.48 and 1.2 folds, respectively. These results completely support the theory that antioxidant enzymes play important role in heavy metal contamination. Though the ecotoxicology and environmental toxicology of heavy metals were studied and discussed, there is needed more data to investigate the mechanisms of enzymatic activities and the expression patterns of oxidant/antioxidant enzyme in *A. pernyi* upon heavy metal stress, which may include some signal transduction or insect cellular immune factor functions.

Statement of conflict of interest

The authors declare there is no conflict of interest.

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