Role of Nitric Oxide and Metallothionein in Cytotoxic Activity of Cadmium in Caco-2 Cells

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Abstract- Cadmium (Cd) induces carcinogenicity and cytotoxicity through a variety of mechanisms. Metallothioneins (MTs) play critical roles in metal detoxification and radical scavenging. Here we evaluated the possible involvement of NO and MT in Cd-induced toxicity and resistance development. By utilizing Cd-resistant Caco-2 cells as a model of chronic exposure to Cadmium, we observed that Cd decreased Caco-2 cell proliferation, whereas Cd-resistant cells showed a lower sensitivity to Cd cytotoxicity. L-NAME as an iNOS inhibitor and cPTIO as an NO scavenger induced a significant reduction in Cd-mediated toxicity of parent Caco-2 in spite of resistant cells. In resistant cells, iNOS mRNA expression was declined; however, MT protein synthesis was increased following acute and chronic Cd exposure. It seems that NO synthesis involves in Cd-induced cytotoxicity, while elevated MT expression is associated with Cd detoxification and resistance.

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Introduction

Cadmium (Cd) is a toxic heavy metal which accumulates in the environment due to industrial and agricultural activities (1). Cd has been categorized as a group I cumulative carcinogen because of its long half-life (15-20 years) (2) and induces carcinogenicity in various cell lines following chronic exposure (3). Although not a Fenton metal, Cd indirectly enhances the production of various radicals including superoxide, hydroxyl and nitric oxide (NO) (4).

Recent studies have indicated that NO is a key signaling molecule in regulation of tumorigenesis (5). In addition, increased expression of inducible nitric oxide synthase (iNOS) is observed in tumors of the colon, lung, bladder, reproductive organs, breast, and central nervous system (6). NO produced by tissue injury can be considered as an important factor in cadmium toxicity (7). On the other hand, the oxidative stress induced by NO is prevented by cytoprotective mechanisms such as metallothioneins (8).

Metallothioneins (MTs), cysteine-rich low-molecular weight proteins, play critical roles in detoxification of heavy metals as well as radical scavenging (9,10). It is described that NO can compete with various metals for cellular binding sites including Cd-binding sites to MT (11).

In this study, we aimed to investigate the possible involvement of NO pathway in Cd cytotoxicity. The expression of iNOS and MT was also evaluated in short and long-term exposure of Caco-2 cells to Cd.

Materials and Methods

Reagents

L-NAME (Nω-Nitro-L-arginine methyl ester hydrochloride) and MTT (3-(4,5-dimethyl thiazole-2-yl)-2,5-diphenyl tetrazolium bromide) were purchased

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from Sigma, St. Louis, MO. cPTIO (2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) was obtained from Tocris, UK.

Cell culture and cytotoxicity test

Human epithelial colorectal carcinoma, Caco-2, cells (Pasteur Institute, Iran) were cultured in RPMI-1640 (Biosera, UK) supplemented with 35% DMEM/Ham`s F-12 (Biosera), 15% fetal bovine serum (FBS; Gibco, UK), 100 IU/ml penicillin and 100 µg/ml streptomycin (Biosera) in 5% CO_2 at 37 ºC. To develop Cd-resistant Caco-2, cells were exposed to incrementing doses of $CdCl_2$ (Merck, Germany) up to 10 µM for at least 2 months.

Caco-2 cells were cultured in 24-well plates (SPL, South Korea) and a dose–response analysis (10-200 µM) for Cd cytotoxicity was accomplished by MTT assay. To study the possible contribution of NO pathway in Cd cytotoxicity, cells were co-treated with $CdCl_2$ and 400 µM L-NAME (an iNOS inhibitor) or 50 µM of cPTIO (an NO scavenger) for 48 h. At the end of treatments, cells were washed with PBS and subsequently incubated with MTT (5 mg/ml in PBS) for 4 h at 37 ºC. Then, formazan crystals were dissolved in DMSO. The optical density was finally measured on a microplate reader (Biotek, USA) at 570 nm with 690 nm as reference. The data are presented as mean±SD of at least two independent experiments (n=3).

RNA extraction and RT-PCR

Total RNA was extracted using Trizol (Roche, Germany) and cDNA was synthesized by MuLV H-reverse transcriptase (Fermentas, Latvia). iNOS expression was then evaluated using a semi-quantitative PCR (95 °C/30 s, 64 °C/1 min, 72 °C/40 s) with specific primers (sense: 5′-TGGAAGCGGTACCAAAGGAGATAG-3′ and antisense: 5′-CACGTGTCTGCAGATGTGTTCAA-3′). The intensity of iNOS band at the exponential phase of amplification was finally normalized to the corresponding β-actin.

Western blot analysis

Cells were exposed to different concentrations of Cd for 48 h and lysed with RIPA buffer (10 mM Tris pH 8, 1.5 mM $MgCl_2$, 5 mM KCl, 1% NP-40, 0.1% SDS, 0.5% Na-deoxycholate, 0.5 mM dithiothreitol, 1 µM phenylmethylsulfonyl fluoride and 10 µM leupeptin). Protein samples (50 µg) were subjected to SDS-PAGE and then transferred to polyvinylidenedifluoride (PVDF) membranes (Roche) using a semi-dry apparatus (Peqlab, Germany). After blocking in 1% casein, membranes were incubated overnight with either iNOS (1:1000; Abcam, UK) or MT (1:1000; Stressgen, NY, USA) antibodies. Thereafter, the blots were stripped and re-probed for β-actin (1:2000; Santa Cruz, CA, USA). Protein bands were finally revealed using horseradish peroxidase-conjugated secondary antibodies and chemiluminescence substrates (Roche) on X-ray films (Fujifilm, Japan). Bands were digitalized with ImageJ software (National Institutes of Health, Bethesda, MD, USA) and normalized to the corresponding β-actin band intensity.

Immunocytochemistry

Parent and Cd-resistant Caco-2 cells were seeded at the density of 8×10^3 cells in chamber slides (Nunc, Denmark) and treated with $CdCl_2$, L-NAME or 10 µg/ml of lipopolysaccharide (LPS, Roche). Subsequently, cells were fixed with methanol-acetone (9:1) and incubated overnight with primary antibodies against iNOS (1:50) or MT (1:100) at 4 ºC. Following incubation with biotinylated secondary antibodies (1:200) for 30 min at room temperature, DAB chromogen (Santa Cruz) was used as HRP substrate. Cells were then washed, slightly counterstained with Mayer's hematoxylin (Sigma) and slides were mounted with Faramount (Dako, Denmark). Microscopic evaluation of three randomly selected fields in each experiment was accomplished using OLYSIA software on Olympus microscope (Olympus, Center valley, PA).

Statistical analysis

The reported data are shown as the mean ± standard deviation (SD). Non-linear regression with least square roots was used to determine the IC_{50} values. Two-way analysis of variance (ANOVA) was applied to study the differences in Cd cytotoxicity in parent and resistant cells as well as the possible contribution of NO pathway. One-way ANOVA was used to compare iNOS and MT expression in different groups. Prism 5 (Graph Pad, San Diego, CA) was applied for statistical analysis and $P<0.05$ was considered statistically significant.

Results

The growth pattern of parent and Cd-resistant Caco-2 cells

Growth curves of parent and Cd-resistant Caco-2 cells were determined by trypan blue dye exclusion assay. Both parent and resistant cells were proliferating exponentially; however Cd-resistant cells showed a

slower rate of growth (data not shown). Cd-resistant cells were used as a model of long-term exposure to Cd in this study.

The effects of NO modulators on Cd cytotoxicity

Cd elicited a dose-dependent cytotoxicity in Caco-2 cells, and this effect was greater in parent (IC_{50}: 51.1±4.2 µM; r^2: 0.91) compared to resistant cells (IC_{50}: 72.9±2.4 µM; r^2: 0.96; P<0.05; Figure 1A). As depicted in Figure 1B, L-NAME (400 µM) and cPTIO did not induce cytotoxicity by themselves; however, L-NAME relatively decreased Cd cytotoxicity in parent cells (IC_{50} (Cd+L-NAME): 81.27±3.02 µM; r^2: 0.93; P<0.01; Figure 2A). Moreover, concurrent treatment of parent cells with cPTIO (50 µM) and different concentrations of Cd, resulted in a rightward shift in Cd dose-response curve (IC_{50} (Cd+cPTIO): 70.5±2.9 µM; r^2: 0.95; P<0.05; Figure 2A). On the other hand, there was no significant difference between Cd and Cd+L-NAME (IC_{50}: 75.9±3.7 µM; r^2: 0.92) or Cd+cPTIO (IC_{50}: 74.2±3.4 µM; r^2: 0.9) in resistant cells (Figure 2B).

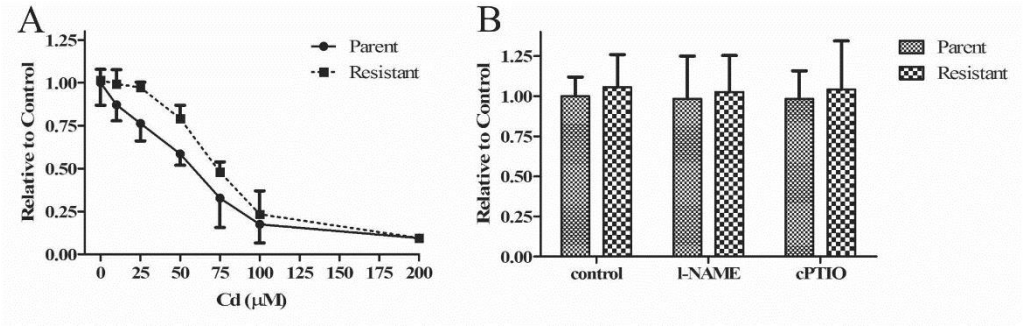

Figure 1. Effects of Cd, L-NAME, and cPTIO on Caco-2 cells viability

Caco-2 cells were incubated with increasing doses of $CdCl_2$ up to 10 µM for at least 2 months and then treated with indicated doses of Cd for 48 h (A). Parent and Cd-resistant Caco-2 cells were treated with L-NAME (400 µM) and cPTIO (50 µM) for 48 h (B), and a viability test was conducted using MTT assay

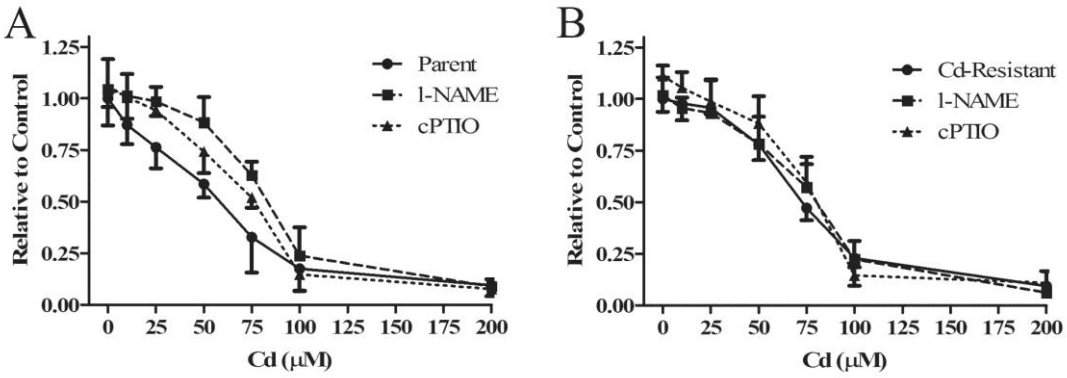

Figure 2. Effects of L-NAME and cPTIO on Cd cytotoxicity

Parent (A) and Cd-resistant (B) Caco-2 cells were treated with indicated doses of $CdCl_2$ with/without L-NAME (400 µM) or cPTIO (50 µM) for 48 h, and a viability test was performed using MTT assay

Effect of Cd on iNOS and MT expression

We observed by RT-PCR that iNOS expression was reduced (by 67±9 %) in Cd-resistant cells (P<0.05) in comparison to parent Caco-2 cells. But, acute treatment of neither parent nor resistant cells with different doses of Cd (10, 25, 50, 75 µM) did not alter iNOS mRNA expression (Figure 3). The iNOS protein level was also assessed by western blotting, and we observed a 55±8 % reduction of iNOS protein expression in chronic Cd treatment. However, acute exposure to Cd changed iNOS band intensity neither in parent nor in resistant cells (Figure 4A).

MT protein expression was also assessed and we found that exposure to $CdCl_2$ (10 µM) in parent cells led to 4.3±0.9 fold increase in MT protein synthesis compared to control (P<0.01), but this effect was not dose dependent and incrementing Cd doses up to 75 µM did not further increase MT band intensity compared to

10 µM Cd. Comparing parent and resistant cells, there was higher MT expression in resistant cells (P<0.01), but this amount of protein expression was not affected by acute treatment of resistant cells with Cd (Figure 4B). In addition, an inverse correlation was found between iNOS and MT expression in Cd-resistant Caco-2 cells (Pearson's r= -0.67, P<0.05).

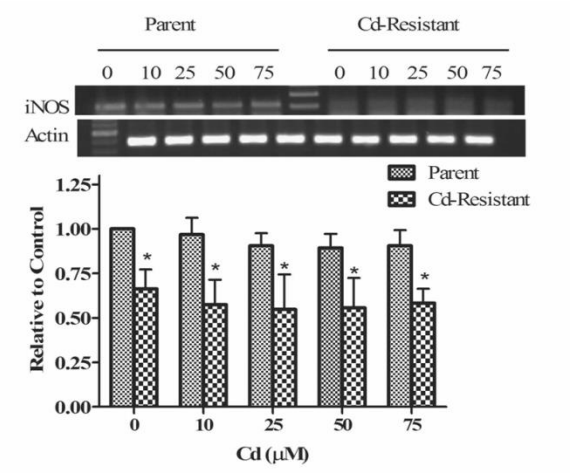

Figure 3. Effects of $CdCl_2$ on iNOS mRNA expression

Parent and resistant Caco-2 cells were incubated with indicated doses of $CdCl_2$ for 48 h, and a semi-quantitative PCR was performed for the analysis of iNOS mRNA expression. Densitometric analysis of iNOS mRNA was accomplished, and data were normalized to the corresponding β-actin band intensity (mean ± SD, n=3); *P<0.05 compared to parent cells

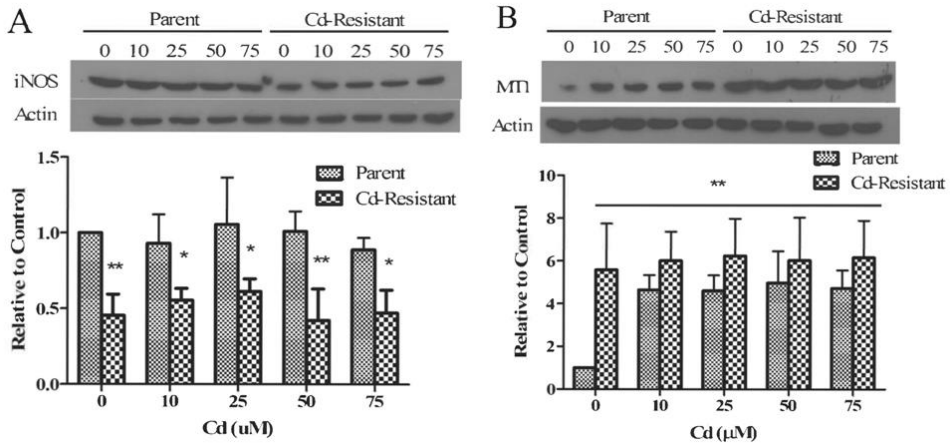

Figure 4. Effects of $CdCl_2$ on iNOS and MT protein expression

Parent and resistant Caco-2 cells were incubated with indicated doses of $CdCl_2$ for 48 h, and a western blot was performed for analysis of iNOS (A) and MT (B) protein expression. Band intensity of each protein was normalized to the corresponding β-actin as the internal control (mean ± SD, n=3); *P<0.05, **P<0.01 compared to control

Immunocytochemical analysis of iNOS and MT expression

Immunocytochemical (ICC) analysis of iNOS expression in parent Caco-2 cells showed a reduction of iNOS expression due to L-NAME (400 µM) or an induction due to LPS (10 µM) in both parent and resistant cells compared to control (data not shown). As shown in Figure 5, acute administration of Cd (50 µM) caused no change in iNOS expression of parent and resistant cells relative to control. However, we observed that prolong treatment of cells with Cd (10 µM) diminished iNOS expression. In addition, Cd caused MT synthesis induction in both parent and resistant cells (Figure 6).

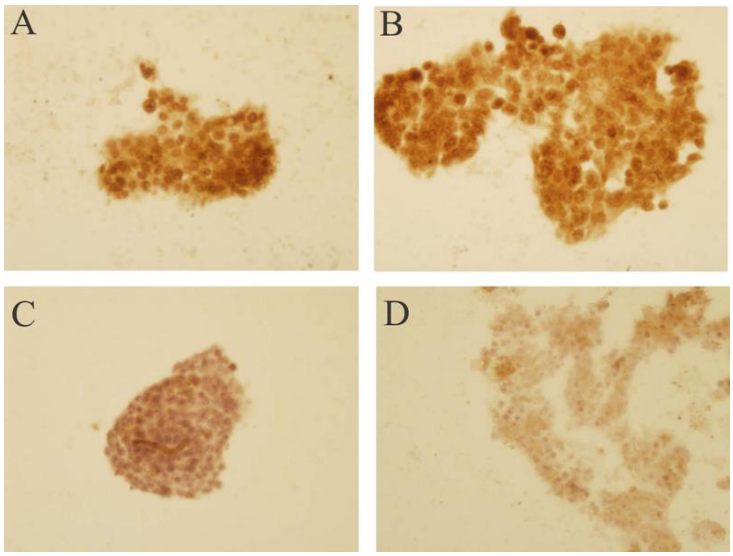

Figure 5. Immunocytochemical analysis of iNOS localization in Caco-2 cells
The expression of iNOS was investigated in Caco-2 parent (A) parent cells treated with Cd (50 µM) for 48 h (B), Cd-resistant (C) and Cd-resistant cells treated with Cd (50 µM) for 48 h (D)

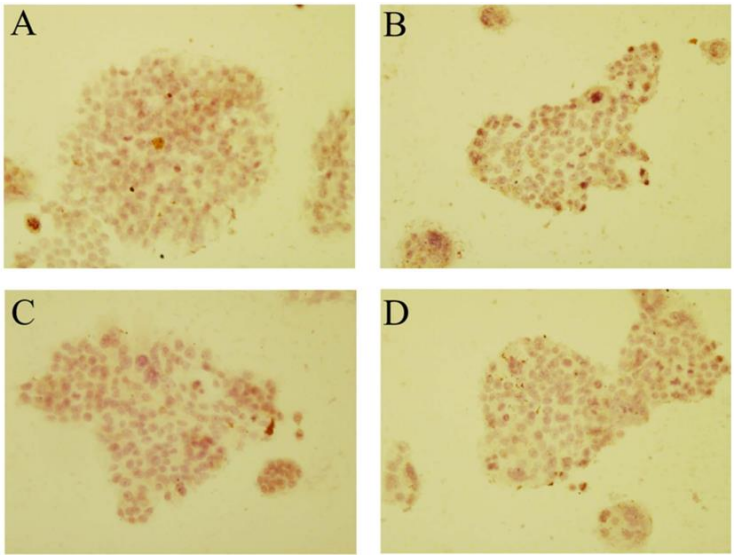

Figure 6. Immunocytochemical analysis of MT localization in Caco-2 cells
The expression of MT was investigated in Caco-2 parent (A) parent cells treated with Cd (50 µM) for 48 h (B), Cd-resistant (C) and Cd-resistant cells treated with Cd (50 µM) for 48 h (D)

Discussion

Cadmium (Cd) is considered as an important environmental and industrial contaminant (12). Previous studies showed the principal role of intestinal epithelium in Cd absorption in mammals (13,14). Although Cd^{2+} does not contribute to any biological function (15), it is associated with carcinogenesis (16) and cytotoxicity through the production of free radicals (17). Results of the present study showed that Cd dose-dependently reduced the number of viable Caco-2 cells. A variety of mechanisms has been suggested for Cd-mediated toxicity. Galan *et al*., showed that incubation of U-937 human promonocytic cells with $CdCl_2$ (2 h at 200 µM) caused intracellular oxidation as assessed by the peroxide and/or anion superoxide accumulation (18).

In this study, we observed that L-NAME (an iNOS inhibitor) and cPTIO (an NO scavenger) could efficiently reduce Cd cytotoxicity in Caco-2 parent cells, suggesting a role for NO in Cd-induced cytotoxic effects. A previous study revealed that cPTIO prevented

hyperpermeability caused by proinflammatory cytokines in Caco-2 cells through reduced NO production (19), which might explain the protective effects of cPTIO against Cd toxicity. NO has been implicated in the modulation of carcinogenesis, mutagenicity and DNA damage (20). Moreover, increased iNOS expression has been observed in different tumors (6,21). It has been reported that prolonged exposure to sub-lethal levels of cadmium also causes human health problems (22). Cd increases carcinogenicity in various cell lines following chronic exposure (3). In this survey, continues exposure to low-dose Cd resulted in a cell line which developed resistance to Cd. In contrast to parent cells, L-NAME and cPTIO failed to produce a protective effect in resistant cells which reveals reformed NO pathway in these cells. In addition, these cells showed a lower level of iNOS mRNA and protein expression compared to parent Caco-2 cells. One may conclude that acute toxicity of Cd is mediated through NO, whereas in long-term exposure, NO is neither involved nor protective against Cd. In this regard, Poliandri *et al*., have shown that NO protects anterior pituitary cells against long term Cd^{2+} toxicity (23).

Our results illustrated that acute treatment (48 h) of parent and resistant Caco-2 cells with Cd did not change iNOS mRNA and protein expression. However, prolonged exposure resulted in a decreased iNOS mRNA and protein expression. Intriguingly, elevation of nitric oxide synthesis was realized in human skin (GM00637) and lung (IM090) fibroblast cells following Cd^{2+} exposure (24). Conversely, Cd inhibited iNOS activity in a human intestinal epithelial cell line, DLD-1 (25). Furthermore, Harstad and colleagues described that iNOS do not mediate Cd-induced acute hepatotoxicity (26). It has also been observed that chronic Cd exposure up regulated iNOS and increased NO production in macrophages (27). These differences may be due to concentration, exposure time, and the cell type (23).

Metallothionein can protect cells from Cd and oxidative stress mediated cytotoxicity (28). There is an inverse relationship between existent MT and the rate of apoptosis caused by Cd in various cell lines (29). As Kusakabe *et al*., demonstrated that Cd-induced MT protected Sertoli cells against apoptosis (30). Accordingly, our western blot, as well as ICC experiments, showed that acute exposure of Caco-2 cells to $CdCl_2$ augmented MT expression and this elevated MT was persisted in prolonged exposure. It has been described that in order to achieve cadmium resistance, most cells raise the synthesis rate of MT to sequester intracellular Cd (31). Consistent with our results, Blais *et al*., indicated that MT content was increased in Caco-2 cells exposed to long-term $CdCl_2$ (0.1, 1, 5 µM) (32). Earlier studies showed that rate of MT mRNA and protein synthesis are connected greatly with Cd at low concentrations, confirming MT expression was not increased parallel to cytotoxicity of Cd in doses higher than 10 µM (33). Increased MT expression was detected in Caco-2 cells acquired resistance to $CdCl_2$. Therefore, MT synthesis appears to be one of the mechanisms behind this resistance.

We found a reduced expression of iNOS as well as an augmented MT expression in long-term exposure to Cd which shows a negative correlation between these two proteins (Pearson's r= -0.67, $P<0.05$). Our results suggested that NO may play a role in Cd-provoked cytotoxicity and MT might be important in the development of resistance to Cd. It was previously demonstrated that MT due to nucleophilicity protects cells against nitrogen-based radicals including NO (34). MT1 and MT2 were efficient against detrimental effects of NO in the tri-nitrobenzene sulfonic acid (TNBS)-induced colitis (35). It was proposed that NO causes a conformational change in MT, releasing Zn^{2+} from this protein (36). Furthermore, NO can displace bound Zn^{2+} (and Cd^{2+}) and release them both in vitro and within the cells (37). These observations imply that MT can serve as a defensive mechanism in NO-mediated Cd cytotoxicity.

In conclusion, using Caco-2 cells as a model of Cd-resistance colon carcinoma cell, we found that induction of NO pathway gives rise to acute Cd cytotoxicity. Additionally, MT expression was increased due to Cd exposure and might be involved in Cd detoxification and resistance.

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