

CADMIUM EXPOSURE INDUCES OXIDATIVE STRESS BY DECREASING EXPRESSION OF ANTIOXIDANT ENZYMES IN MICE LIVER

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ABSTRACT:

Purpose: Heavy metals are known to generate oxidative stress which is the root cause for all health hazards. However the mechanism by which heavy metals generate oxidative stress is not yet fully understood. Cadmium is a toxic heavy metal and its deleterious effect is due to oxidative damage to macromolecules. This study intended to elucidate the mechanism of its toxicity.

Methodology: Swiss albino mice were administered cadmium for three weeks intraperitoneally. The total antioxidant status and MDA levels were measured. The enzyme activity of Glutathione S-transferase (GST) and catalase was measured spectrophotometrically using kinetic methods. Their mRNA expression was analysed by real time PCR.

Results: There was a significant decrease in total antioxidant levels and increase in lipid peroxidation in mice treated with cadmium in comparison to controls. Cadmium decreased both enzyme activity and gene expression of GST and Catalase.

Conclusion: This study shows that cadmium generates oxidative stress by affecting the transcription of antioxidant genes, GST and Catalase.

Keywords: Cadmium; GST; Catalase; Gene expression

INTRODUCTION

Environmental pollution and increased exposure to heavy metals is one of the major health concerns in the modern world. Heavy metals are toxic because they may have cumulative deleterious effects that can cause chronic degenerative changes (1), especially to the nervous system, liver, and kidneys, and, in some cases, they also have teratogenic and carcinogenic effects (2). The importance of these metals as environmental health hazards is readily evident from the fact that they ranked in the top 10 on the current Agency for Toxic Substances and Disease Registry Priority List of Hazardous Substances (3).

Cadmium (Cd) is one of the most toxic heavy metals known to mankind. It affects numerous organs and systems, specially the liver, kidneys and nervous system. The high toxicity of cadmium can be attributed to its long biological half life (10-30 years) in human beings (4). Humans are exposed to cadmium due to cigarette smoking, agricultural practices and industrial sources like electroplating cadmium-nickel battery, and paint pigment manufacturing units (5, 6, 7). Liver is the major target organ for both acute and chronic cadmium exposure. After acute exposure it causes hepatocyte swelling and massive necrosis resulting in marked elevation of enzymatic biomarkers (8).

Although many studies about Cd-induced toxicity have been performed, the molecular mechanisms underlying Cd toxicity have not been explicitly defined. The generation of reactive oxygen species (ROS) and as a consequence oxidative

damage of macromolecules plays a major role in the pathogenesis of cadmium toxicity (9). However, it is not yet known whether Cd directly generates ROS by reacting with molecular oxygen or it inhibits the antioxidant defense system and thereby enabling the normally generated ROS to increase unabated.

The total antioxidant capacity of a cell reflects the sum total of all non enzymatic antioxidant molecules present in the cell. This include macromolecules like albumin, ceruloplasmin and ferritin and small molecules like reduced glutathione, vitamin E, ascorbic acid, β -carotene uric acid and Bilirubin. It does not include the contribution of antioxidant enzymes (10). As the cell is subjected to oxidative stress the antioxidant reserve is used up resulting in decrease in total antioxidant capacity. Since it is the cumulative effect of all the antioxidants present, it gives a more comprehensive idea about the oxidative stress faced by the cells or tissues in which it is measured.

Glutathione S-transferase (GST) and Catalase are two important antioxidant enzymes which deals with oxidative stress in the biological system. GST gene is located in Chromosome 6 (NC_000072.6)* and Catalase is located in chromosome 2 (NC_000068.7)* as described in NCBI gene database for Mus musculus. Glutathione S-transferase catalyse the conjugation of the tripeptide glutathione with reactive unsaturated carbonyls, reactive DNA bases, epoxides and organic hydroperoxides produced during oxidative stress. Thus GST play a vital role in protecting tissues

against oxidative damage (11). Catalase enzyme acts on peroxides generated during oxidative stress and forms water and molecular oxygen and protects macromolecules against oxidative damage (12).

This study was done to evaluate the effect of Cd on catalase and glutathione-s-transferase, in mice liver. The study intended to see whether cadmium generates oxidative stress by affecting the activity or genetic expression of these enzymes.

Foot Note: * NC_000072.6 and NC_000068.7 are NCBI gene database reference number of GST and Catalase, respectively.

MATERIALS AND METHODS

Animals: Adult swiss albino mice (weighing 18-20 g) were used for the study. The animals were obtained from the Central Animal House, University College of Medical Sciences, Delhi, India and housed in standard laboratory conditions (natural light and dark cycle, 23±1°C temp. and 50±2% humidity) with pellet diet and water available ad libitum. The animals were allowed a period of one week for acclimatisation to laboratory conditions before the experiments. Permission was taken from Institutional Animal Ethics Committee and the care of animals was done as per “CPCSEA Guidelines for laboratory animal facilities.”

Drugs: Cadmium chloride was procured from Sigma Aldrich. The drug was dissolved in distilled water and administered daily in dosages of 1.2mg/kg/ i.p for 3 weeks. Control group was injected with distilled water. The dosing was done every day at 11 AM and was not related to meal as the mice were fed ad libitum.

Experimental Groups: Study design was case control study. The animals were divided into two groups; Control (n=8) and Cadmium treated group (n=8).

After completion of dosing for 3 weeks, the animals were sacrificed by ether anesthesia after 24 hours of the last dose. The liver was quickly dissected out, washed with ice-cold sodium phosphate buffer, weighed and stored over ice. The

liver tissues were further processed within half an hour of dissection, and the estimation of oxidative stress was done in the same working day. Liver tissue was homogenized with 10 times (w/v) phosphate buffer (pH 7.4) with the help of sonicator. The homogenate was then centrifuged at 3000 rpm for 15 min. The supernatant was used to measure oxidative stress parameters and catalase and GST activity

Estimation of oxidative stress: The parameters of oxidative stress used were Total Antioxidant status and malondialdehyde (MDA).

Estimation of Total anti-oxidant status: Total Antioxidant Assay kit (Cayman's, USA) was used for the estimation of the total antioxidant capacity of tissue lysate. The instructions provided by the manufacturer were followed for the estimation of 2, 2'-Azino-di-[3-ethylbenzthiazoline sulphonate], which was oxidized in the reaction. Antioxidants in the sample cause suppression in the oxidation, which results in decrease in the absorbance (at 750/405 nm) proportional to the concentration of the anti-oxidants. A water based tocopherol analogue, Trolox, was used as the control for comparison and the result was quantified as millimolar Trolox equivalents.

Malondialdehyde estimation (MDA): Estimation of liver lipid peroxidation was done by measuring malondialdehyde (MDA) levels, as described by Ohkawa et al. (13).

Estimation of Glutathione-S-transferase activity: GST estimation was done by a kinetic method as described by Mannervik and Danielson, 1988 with slight modification (14). Briefly 10 µL of samples were transferred to wells of a 96 well plate in duplicates. 190 µL of substrate solution, consisting of 300 mM phosphate buffered saline at pH 6.5, 200 mM of reduced glutathione solution and 100 mM of CDNB in the ratio of 98:1:1, was added to each well. The plate was read in a multimode reader at 340 nm for 5 mins. $\Delta A_{340}/\text{min}$ was calculated by finding the difference between the initial read and final read and dividing it by 5 mins. GST activity was calculated by the following formula:

$$\text{GST Activity} = \frac{\frac{\Delta A_{340}}{\text{min}} \times \text{reaction volume}(200\mu\text{L}) \times 1000}{\text{extinction Coefficient (9.6)} \times \text{sample volume}(10\mu\text{L}) \times \text{protein (mg)}}$$

Protein concentration was measured using nanodrop 2000c spectrophotometer (Thermo Scientific, USA) by taking absorbance of 2 µL sample at 260nm.

Estimation of Catalase activity: Catalase activity was assayed following the method of Luck, 1974 (15). H₂O₂-phosphate buffer (3.0ml) was taken in an experimental cuvette, followed by the rapid addition of 40µl of lysate and mixed thoroughly. The time required for a decrease in absorbance by 0.05 units was recorded at 240nm in a nanodrop spectrophotometer (Thermo Scientific, USA). The enzyme solution containing H₂O₂-free phosphate buffer served as control. One enzyme unit was calculated as the amount of enzyme required to decrease the absorbance at 240nm by 0.05 units. It was expressed as IU/mg protein.

Real Time-PCR for Glutathione-S-transferase and Catalase: Since the gene expression is reflected by relative amount of mRNA transcribed, the expression of catalase and GST was measured by using specific primers in real time PCR reaction. Real Time-PCR for analyzing the expression of above mentioned antioxidant genes was done following the method described by Livak et al, (16). Total RNA was isolated from liver tissue using Tri-reagent (Sigma Aldrich).

Total RNA was converted to cDNA using reverse transcriptase enzyme. Briefly, cDNA synthesis was carried out using 50 U Stratascript reverse transcriptase from Stratagene, USA, 50µM random hexamer, 50mM Tris-HCl (pH8.3), 75mM KCl, 10mM DTT, 3 mM MgCl₂, 0.5mM dNTPs, 20 U RNase inhibitor (Promega Corp, Madison, USA) and 2 µg of RNA, and the final volume made to 20µl with DEPC treated water. The reaction was allowed to proceed at 42°C for 1 hour following which the reverse transcriptase was inactivated at 95°C for 5 minutes. This cDNA was used for quantification of gene expression by real time PCR.

Specific primers for GST and catalase were used to estimate their expression. Housekeeping gene G-6-PD was used as internal control. 10µl Hot start PCR mix (2x) from thermo scientific, 1 µl each of forward and reverse specific primers (10 µM) , 2 µl of nuclease free water and 1 µl of syto 9 dye was mixed with 5 µl cDNA and run in real time PCR machine (Rotor gene, Qiagen; Netherlands). The hot start enzyme was activated at 95°C for 4 minutes. The reaction cycle was; 1. Denaturation at 95°C for

10 seconds, 2. Annealing at 54°C for 15 seconds and 3. Extension at 72°C for 30 seconds. These 3 steps were repeated for 40 cycles. Fluorescence acquisition was done at 72°C after the end of the extension step. Gene expression was quantified by 2^{-ΔΔCt} method (16). The sequence of primers are given in table 1

Table 1: Primer Sequence

Primers	Sequence
GST Forward	CGCATTCCAGAGGATAACCAACA
GST Reverse	CCTTCTCGTCAGTGCGAACAAAC
Catalase Forward	GCAGATACCTGTGAACTCTC
Catalase Reverse	GTAGAATGTCCGCACCTGAG
GAPDH Forward	GGTGAAGGTCGGTGTGAACG
GAPDH Reverse	CTCGCTCCTGGAAGATGGTG

Disposal of animals: The dead animals were disposed by incineration

STATISTICAL ANALYSIS

The data was analysed using unpaired t test. P<0.05 was considered statistically significant.

RESULTS

Cadmium treatment increases oxidative stress in mice liver: The total antioxidant levels in liver of cadmium treated mice were significantly decreased as compared to controls. The antioxidant levels in controls and cadmium treated mice were 14±1.57 and 6.7±0.89 mmol Trolox equivalents respectively (Fig1). The difference was statistically significant (p<0.05)

MDA levels were raised in cadmium treated mice as compared to controls indicating increased lipid peroxidation. The MDA levels in controls and cadmium groups were 2.07±0.57 and 3.94±0.95 µmol/mg protein respectively (fig 1). The difference was statistically significant (p<0.05).

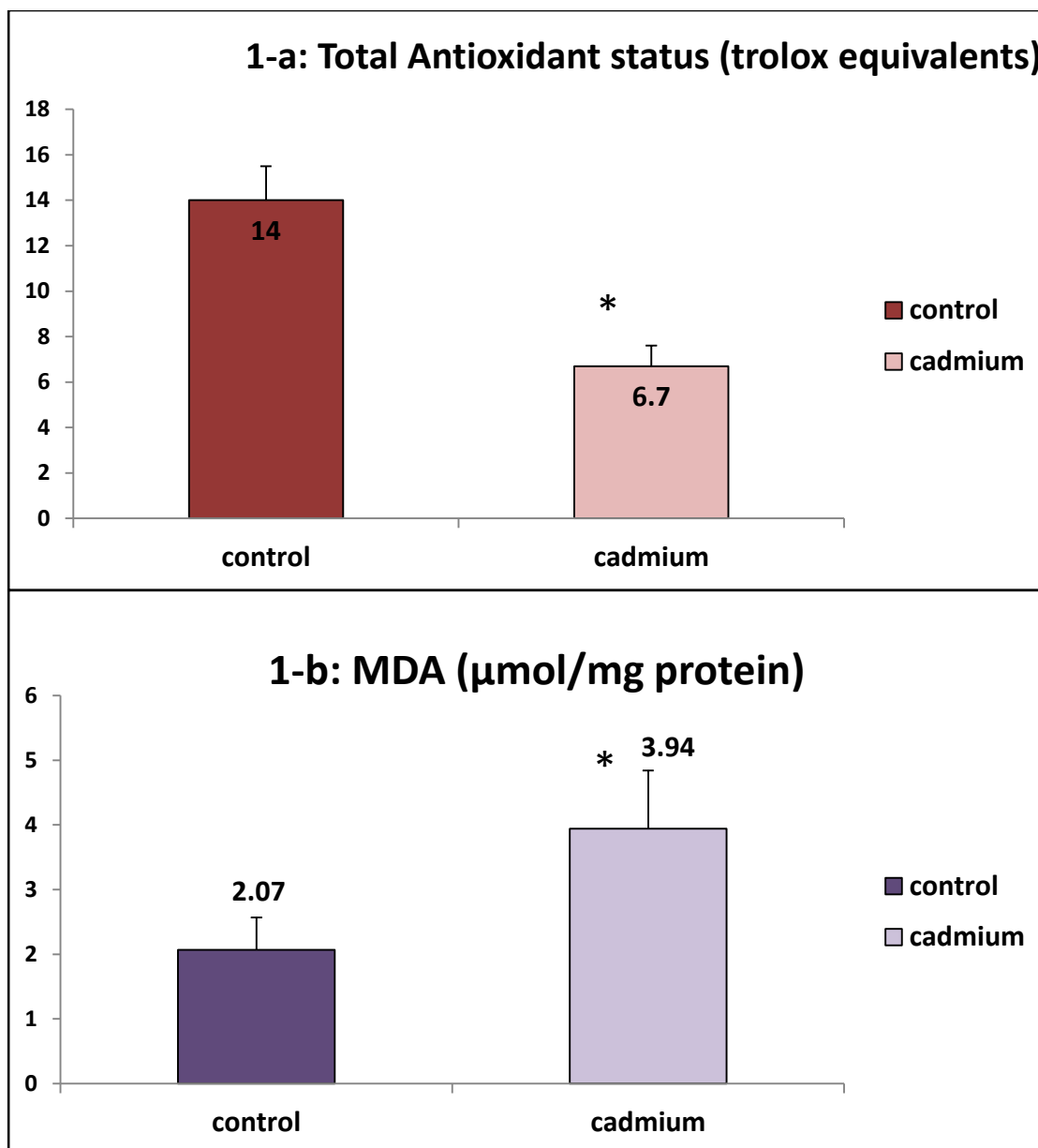


Fig. 1-a: Total antioxidant reserve of mice liver in cadmium treated and control group
Fig. 1-b: Estimation of lipid peroxidation as indicated by Malondialdehyde (MDA) levels in mice liver
cadmium treated and control group
 * $p < 0.05$

Cadmium treatment decreased activity of antioxidant enzymes GST and Catalase: The activity of GST in control group and cadmium treated mice were 0.27 ± 0.05 and 0.14 ± 0.07 IU respectively (fig 2). The activity of catalase also showed a similar trend. It was 1.47 ± 0.53 IU in controls and 0.45 ± 0.19 IU in cadmium treated group (fig 2). The decrease in activity of catalase and GST on treatment with cadmium was statistically significant $P < 0.05$.

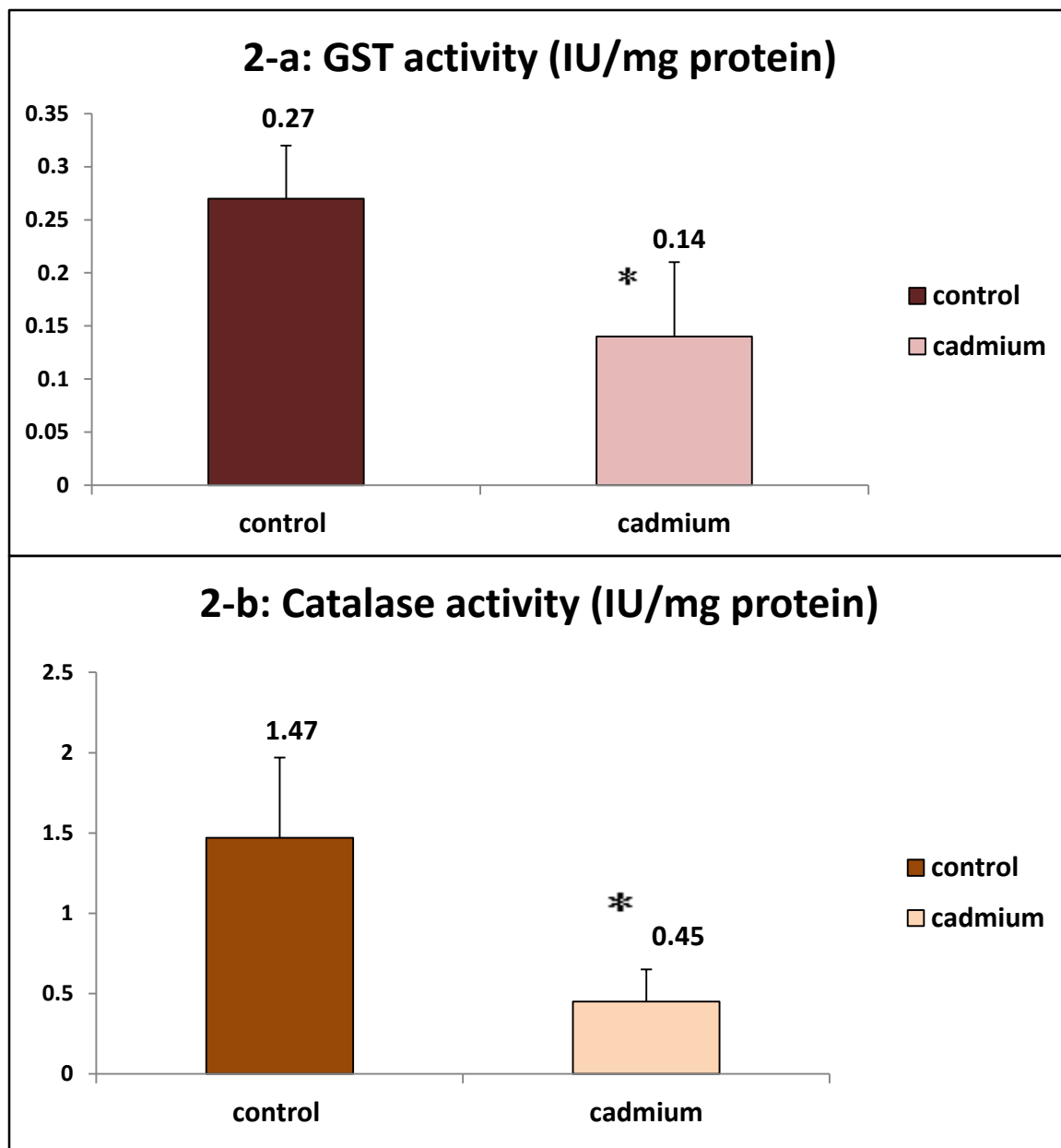


Fig. 2: Mean activity of antioxidant enzymes in mice liver in cadmium treated and control group expressed in IU/mg protein (a). Glutathione-s-transferase, (b). Catalase enzyme
* $p < 0.05$

Cadmium and chromium decreases expression of GST and catalase gene: Cadmium treatment caused 23.6 fold decrease in expression of catalase gene and 10.19 fold decrease in GST expression as compared to control. The relative expressions of the genes are shown in Fig 3a and 3b. The difference was statistically significant ($p < 0.05$).

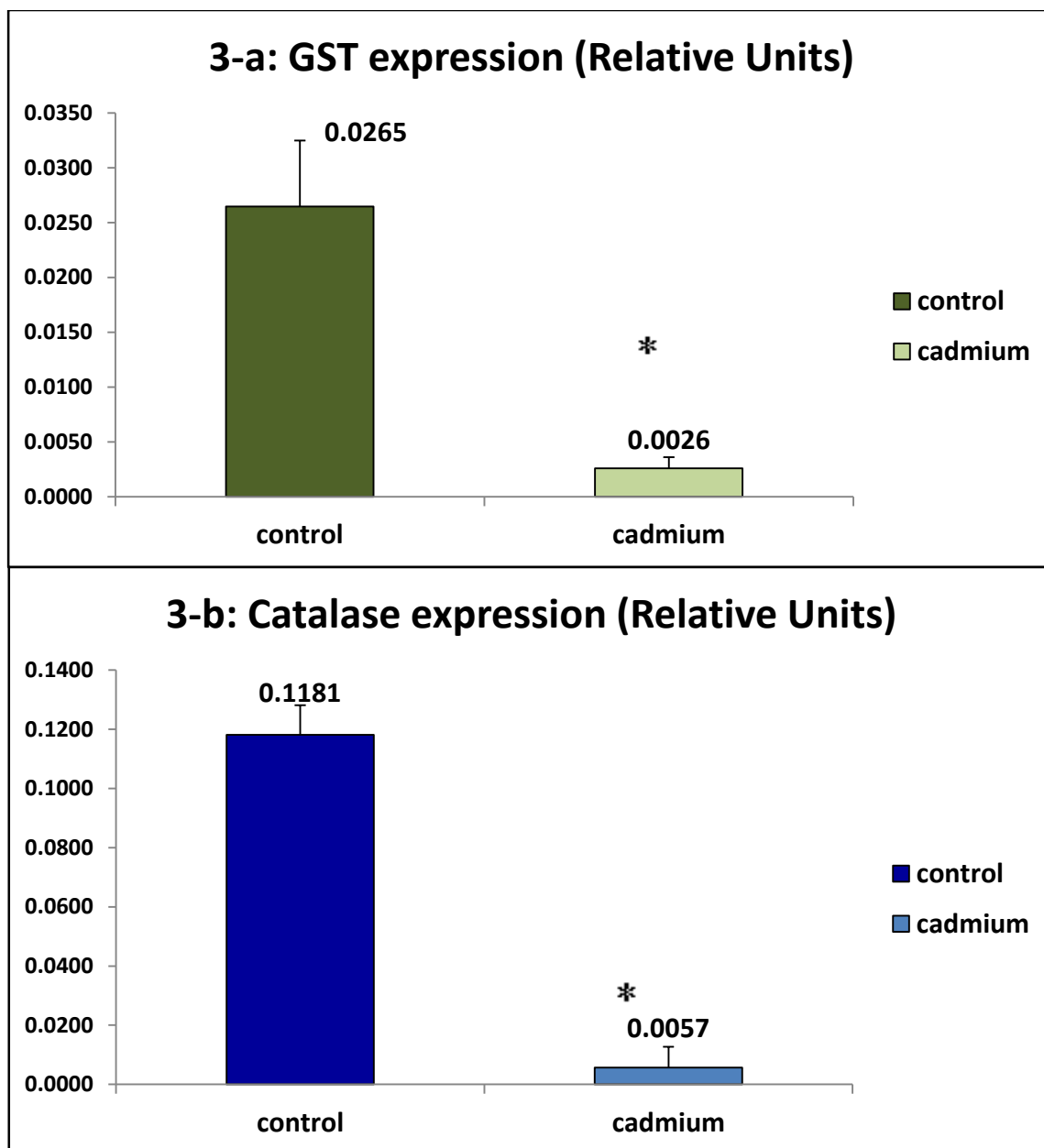


Figure 3. Gene expression of antioxidant enzymes in mice liver in cadmium treated and control group estimated by real time PCR a. Glutathione-s-transferase, b. Catalase.

* $p < 0.05$

DISCUSSION

Most of the harmful effects of heavy metals on human health are mediated through oxidative stress. Cadmium is an extremely toxic heavy metal and has been shown to generate considerable amount of reactive oxygen species (ROS) (4). Cadmium has been shown to increase lipid peroxidation and decrease antioxidant reserve of cells (17). Our study also shows increase in MDA levels and decrease in total antioxidant levels in mice liver. This increase in oxidative stress parameters could be due to increased generation of ROS. In this case the natural reaction of

the biological system would have been to increase the antioxidant enzymes to tackle the increased oxidative stress.

However, in our study cadmium was able to inhibit the activity of both GST and catalase enzymes. In the literature, there are conflicting reports on affect of Cadmium on activity of GST enzyme, some studies report that cadmium causes increase in GST activity as a compensatory mechanism to increased oxidative stress (18, 19) whereas others have shown cadmium to decrease GST activity (20). In vitro studies have also shown

that GST activity is reduced in hepatocyte cell lines treated with cadmium (21,22). On contrary to the conflicting reports on GST activity, most studies have reported that cadmium exposure resulted in decrease in catalase activity which is in accordance with our findings (23, 24).

The decrease in activity of both the enzymes may be due to two reasons. Cd either forms transition complex with the protein enzymes, thus inhibits their activity or it may act at the transcription level and decrease the synthesis of the enzymes. In this study expression of GST and Catalase was decreased on Cd exposure. Several studies have shown that cadmium increases expression of antioxidant genes in biological systems (25, 26). However, a study in wistar rats has shown that cadmium exposure increases GST levels and decreases Catalase expression (18). Another study has shown that catalase activity is reduced in mice where metallothionein gene has been knocked out. Inverse relation between expressions of metallothionein and GST gene in prostate cell lines has been reported in a recent study (27). Therefore the variation in response of antioxidant genes reported in literature may be due to difference in expression status of upstream regulators.

This study reveals that Cd decreased the enzyme activity as well as the gene expression of GST and catalase and therefore probably affects the transcription of GST and catalase genes. Further studies on interaction of metals with promoter region of these genes and their upstream regulators like metallothionein may enable us to elucidate the mechanism of action of heavy metals on decreasing the effectiveness of antioxidant enzymes.

CONCLUSION

This study shows that cadmium generate oxidative stress by reducing activity and expression of GST and catalase. We conclude that the heavy metals in addition to generating free radicals may also have profound effects on the transcription of antioxidant genes. However, the small sample size is one of the major limitations of the study. Since we have looked into only two antioxidant enzymes the study needs to be extended to the entire array of antioxidant enzymes to get a better picture of the effect of cadmium on antioxidant system. Therefore, to delve deeper into the mechanism of oxidative stress generating potential of heavy metals, studies with other heavy metals and more number of antioxidant genes is required.

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