Effect of magnesium pretreatment on reduced glutathione levels in tissues of mice exposed to acute and subacute cadmium intoxication: a time course study

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Abstract. The study was designed to investigate the role of magnesium (Mg) pretreatment on reduced glutathione (GSH) levels in kidney, liver and testis of mice intoxicated with cadmium (Cd). Animals were divided into four groups: I – controls, II – Cd group: mice intoxicated with Cd, III – Mg+Cd group: mice given Mg 1 h before Cd, and IV – Mg group: mice given only Mg. In acute time – response studies, the single oral dose of Cd was 20 mg Cd/kg b.w. and 40 mg Mg/kg b.w. GSH levels were determined after 4, 6, 12, 24, and 48 h. In subacute experiments, mice were given 10 mg Cd/kg b.w. orally every day and 20 mg Mg/kg b.w., and GSH content was determined in investigated organs after 1 and 2 weeks. Acute cadmium intoxication significantly decreased the GSH content in liver 4, 6 and 12 h after Cd administration and increased GSH in kidney after 12, 24 and 48 h, but did not cause significant GSH alterations in testis. Mg pretreatment reduced the observed changes of GSH content in kidney and liver. Subacute Cd intoxication induced diminished renal GSH levels compared with the controls while the increased GSH levels were observed in liver and testes after 2 weeks Cd treatment. Mg pretreatment was efficient in restoring renal and testis GSH levels towards the control group, but had no effect on hepatic GSH.

Key words: Cd intoxication, Mg pretreatment, Cd–Mg interactions, reduced glutathione, mice

Cadmium (Cd) is an abundant, nonessential element that is present as a contaminant in food, water, air and soil. This ubiquitous environmental and professional pollutant, classified as a group I carcinogen by the International Agency for Research in Cancer (IARC), has a long biological half-life of 17-30 years in humans and can result in damage to a variety of tissues such as the lung, liver, kidney and testis [1]. The exact mechanism by which Cd produces adverse biological effects is not well understood.

Several studies have shown that among numerous mechanisms proposed for Cd toxicity are oxidative stress and disturbances in the metabolism and function of bioelements [2-5]. Although cadmium has no redox activity, it could indirectly induce the production of hydroxyl radicals...
[6], superoxide anions, nitric oxide and hydrogen peroxide [7]. Increased lipid peroxidation, as a result of cadmium exposure was observed in various in vitro and in vivo studies [2, 8-10]. Up-to-date investigations indicate that cadmium can decrease the intracellular glutathione content and activities of cellular antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT) and glutathione-S-transferase (GST) which lead to the accumulation of reactive oxygen species (ROS) and an increase in intracellular oxidative stress [11-13]. Short-term exposure to cadmium has been shown to decrease the activities of almost all of these enzymes, whereas with more elevated doses and extended exposure, enhancement of activities was found, probably because of an adaptive induction of genes [13]. Casalino et al. [12] demonstrated that a single intraperitoneal dose of CdCl₂ (2.5 mg/kg b.w.) induces decreased activity of CuZn-SOD and CAT in rat liver and kidney and that the inhibition is imputable to a cadmium/enzyme interaction. One month of oral Cd exposure (15 mg Cd/day/kg) decreased the total SOD, CAT and GSH-Px but increased the GST activity in the liver of rats, while a 2-week injection of the same doses produced the opposite effect. Furthermore, Eybl et al. [22] demonstrated that Cd acute administration reduced hepatic GST in mice but increased its concentration in rat liver.

In rats chronically exposed to cadmium, increased GSH tissue levels were observed [23, 24]. In a year-long Cd treatment, Kamiyama et al. [25] reported increased GSH levels in both liver and kidney. Moreover, in vitro studies of Gaubin et al. [26] showed that low Cd concentrations induced a significant increase, while higher Cd concentrations resulted in a 30% decrease of cell glutathione level.

On the other hand, experimental studies have shown that cadmium can markedly alter the metabolism and function of bioelements [4, 27-29]. One of them is Mg, the deficiency of which causes several complications in the organism, including increased susceptibility to oxidative stress [30, 31]. A possible mechanism by which Mg deprivation increases cellular vulnerability to oxidation is by depleting reduced glutathione [32, 33].

In vitro studies of Guiet-Bara et al. [34, 35] and in vivo data of Soldatović et al. [36, 37] represent a useful contribution to cadmium toxicology and the understanding of the Mg antagonism mechanism. Moreover, our experimental studies and some other findings indicated that an excessive intake of magnesium may antagonize the toxic effects of metals such as cadmium [36-42].

Based on literature data on cadmium and magnesium interaction and their effects on free radical generation, the objective of the present study was to determine the effect of increased oral magnesium pretreatment on GSH levels in kidney, liver and testes of mice exposed to acute and subacute Cd intoxication.

Materials and methods

Animals

Swiss albino male mice (184 animals) with a body weight ranging from 25-28 g were used for the present study. The experimental treatment protocol was based on the Guidelines for Animal Study no. 282-12/2002 (Ethics Committee of the Military Medical Academy, Belgrade, Serbia) and has been reviewed on behalf animal protection ethics.

All animals were kept under the standard laboratory conditions (temperature 22 ± 2 °C; relative humidity of 50 ± 10%, 12 h/12 h light–dark cycle) and housed in cages (4 mice/cage). They were freely fed standard pellet LM₄ diet with metabolizable energy of at least 11.5 MJ/kg. The diet was composed of min. 19% protein, max. 7% cellulose, min. 1.0% calcium, min. 60 mg/kg, copper min. 6 mg/kg and iron min. 40 mg/kg. The animals had access to tap water (Mg-15 mg/L, Cd-under detection limit 0.02 μg/mL) and the diet (Mg-1.8 g/kg and Cd-0.546 μg/kg) ad libitum.
Chemicals

Cadmium chloride (CdCl$_2$H$_2$O) and Magnesium acetate [Mg(CH$_3$COO)$_2$4H$_2$O] were purchased from Merck, Darmstadt, Germany. All reagents and chemicals used were of analytical grade quality or higher purity.

Experimental design

The experiment was divided into acute and subacute cadmium treatment. After the adaptation to our conditions mice were randomly divided into groups.

Acute intoxication: 128 male mice were divided into four groups: I - control group (n = 8): untreated animals; II - Cd group (n = 40): animals given a single oral dose of 20 mg Cd/kg b.w. as an aqueous solution of CdCl$_2$; III - Mg+Cd group (n = 40) i.e. mice given 40 mg Mg/kg b.w. orally as an aqueous solution of Mg(CH$_3$COO)$_2$ 1 h before Cd intoxication; IV - Mg group (n = 40): animals given 40 mg Mg/kg b.w. orally as an aqueous solution of Mg(CH$_3$COO)$_2$. I group-control was sacrificed as a time 0 control. The animals from the other three groups were sacrificed by decapitation at 4, 6, 12, 24 and 48 h (8 animals for each five time interval). Tissues (kidney, liver and testis) were excised and stored frozen (-70 °C) until analysis.

Subacute intoxication: 56 males were divided into four groups: I - control group (n = 8): untreated animals; II - Cd group (n = 16) was intoxicated orally, every day with 10 mg Cd/kg b.w. as aqueous solution of CdCl$_2$; III - Mg+Cd group (n = 16) was pretreated with 20 mg Mg/kg b.w. as aqueous solution of Mg(CH$_3$COO)$_2$ 1 hour before subacute intoxication with same dose of Cd as in group II; IV - Mg group (n = 16) was given orally every day 20 mg Mg/kg b.w. as aqueous solution of Mg(CH$_3$COO)$_2$. I group-control was sacrificed at zero time. The animals from the other three groups were sacrificed by decapitation after 1 and 2 weeks (8 animals for each group after 1 week and after 2 weeks). Kidneys, liver and testis were removed and stored as described above.

Sample preparation

The tissues were dissected and homogenized with 0.5 mL cold (10%) sulphasalicylic acid followed by centrifugation at 9000 x g for 20 min. GSH content was determined in the supernatants; precipitant was dissolved in 1 mL 1 mol/L NaOH and used for protein content estimation.

Reduced glutathione determination

The reduced glutathione (GSH) content in kidney, liver and testis was determined by the method of Ellman [43]. Standards of glutathione were prepared from a stock solution (0.1 mM) within a concentration range from 0 to 50 nmol/mL. Tissue supernatant (0.1 mL) was allowed to react with 0.1 mmol/L 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB). The solution was then kept at room temperature for 20 min. and the extinction was read spectrophotometrically at 412 nm. The results were expressed as μmol of GSH per mg of protein (μmol GSH/mg protein).

Protein determination

Protein estimation was done by the method of Lowry et al. [44] using bovine serum albumin as standard.

Statistical analysis

The statistical significance of differences between the groups was determined by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test, considering the significance at a level of P < 0.05.

Results

Reduced glutathione levels in acute Cd intoxication

The results in table 1 demonstrate changes of GSH concentration in the kidney, liver and testes of mice 4, 6, 12, 24 and 48 h after Cd intoxication (Cd group), mice pretreated with Mg (Mg+Cd group) and mice given only Mg.

The mean control GSH levels were 7.89 ± 1.07, 96.23 ± 14.17 and 30.91 ± 5.01 μmol/mg protein in the mice kidney, liver and testes, respectively. Cadmium acute treatment (20 mg Cd/kg b.w.) induced a strong, significant increase of GSH content in kidney 12, 24 and 48 h after the exposure (highest increase observed after 12 h – about 70% if compared with controls). On the contrary, a significant decrease of liver GSH content was observed after 4, 6 and 12 h. Surprisingly, no significant effect was found in testis during the intervals investigated.

In the group of mice intoxicated with Cd but pretreated with Mg, a significant increase of hepatic GSH content was observed after 6 and 12 h if compared with the group of animals intoxicated with Cd. Mg pretreatment also produced enhancement of GSH content in testes but only after 12 h if compared...
with the Cd group. On the contrary, renal GSH content was statistically reduced after 12 h if compared with the Cd group.

In the group of animals given only Mg, the increased GSH levels were observed after 4, 6 and 24 h in kidney and decreased hepatic GSH levels at 24 and 48 h (compared with control) with no significant changes in testis during the intervals investigated (table 1).

Reduced glutathione levels in subacute Cd intoxication

Figure 1 presents the levels of renal GSH in the control group and in mice given Cd, in a group pretreated with Mg and mice given Mg only, every day for two weeks. After one week, a statistically significant decrease of renal GSH was observed in animals intoxicated with Cd (compared with the control). However, Mg pretreatment was efficient in restoring renal GSH levels towards the control group. A significant decrease of renal GSH was detected in animals given only Mg.

No alterations of hepatic GSH were observed between the groups after one week of investigation. At the end of the experiment, the values obtained for hepatic GSH were nearly the same in Cd intoxicated mice and in ones pretreated with Mg, and were significantly increased if compared with the control.

Table 1. Effect of single treatment with Cd, Mg+Cd, and Mg alone on GSH content in kidneys, liver and testes of mice.

<table>
<thead>
<tr>
<th>Time after CdCl₂ administration (hours)</th>
<th>Kidneys</th>
<th>Liver</th>
<th>Testes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GSH (μmol/mg protein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cd groupᵃ</td>
<td>Mg+Cd groupᵇ</td>
<td>Mg groupᶜ</td>
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<tr>
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<td>Cd groupᵇ</td>
<td>Mg+Cd groupᵇ</td>
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<td>Cd groupᶜ</td>
<td>Mg+Cd groupᵇ</td>
<td>Mg groupᶜ</td>
</tr>
<tr>
<td></td>
<td>Kidneys</td>
<td>Liver</td>
<td>Testes</td>
</tr>
<tr>
<td>Controlᵈ</td>
<td>7.89 ± 1.07ᵃ</td>
<td>96.23 ± 14.17ᵃ</td>
<td>30.91 ± 5.01ᵇ</td>
</tr>
<tr>
<td>4</td>
<td>9.32 ± 2.43ᵇ</td>
<td>8.18 ± 0.74ᵇ</td>
<td>11.77 ± 3.18ᵇ</td>
</tr>
<tr>
<td>6</td>
<td>9.39 ± 1.72ᵇ</td>
<td>10.11 ± 1.15ᵇ</td>
<td>53.01 ± 8.42ᵇ</td>
</tr>
<tr>
<td>12</td>
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<td>9.37 ± 2.78ᵇ</td>
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<tr>
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<td>10.73 ± 2.2ᵇ</td>
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<tr>
<td>48</td>
<td>10.08 ± 2.6ᵇ</td>
<td>8.62 ± 1.4ᵇ</td>
<td>85.06 ± 25.7ᵇ</td>
</tr>
</tbody>
</table>

Data represent the means of eight experimental values ± SD; Means not sharing the same letter are significantly different (P < 0.05, for the same hour of the acute study; including the respective control group values for each studied organ). (P-values were obtained by one-way ANOVA followed by Tukey-Kramer’s test).

ᵃ Cd group was intoxicated orally with 20 mg Cd/kg b.w.
ᵇ Mg+Cd group was given 40 mg Mg/kg b.w. before Cd.
ᶜ Mg group was given 40 mg Mg/kg b.w.
ᵈ Control group – animals without treatment.
Figure 1. Levels of renal GSH in mice after subacute treatment with Cd, Mg+Cd, or Mg. Control: control mice; Cd-group intoxicated orally every day with 10 mg Cd/kg b.w.; Mg+Cd-group given 20 mg Mg/kg b.w. before Cd during treatment; Mg-group given every day only 20 mg Mg/kg b.w. Values are means ± SD (n = 8). Means not sharing the same letter are significantly different (P < 0.05, for the same week of the study). (P-values were obtained by one-way ANOVA followed by Tukey-Kramer’s test).

Figure 2. Levels of hepatic GSH in mice after subacute treatment with Cd, Mg+Cd, or Mg. Control: control mice; Cd-group intoxicated orally every day with 10 mg Cd/kg b.w.; Mg+Cd-group given 20 mg Mg/kg b.w. before Cd during treatment; Mg-group given every day only 20 mg Mg/kg b.w. Values are means ± SD (n = 8). Means not sharing the same letter are significantly different (P < 0.05, for the same week of the study). (P-values were obtained by one-way ANOVA followed by Tukey-Kramer’s test).
Cadmium, although with no redox properties, can indirectly increase the production of reactive oxygen and nitrogen species [6, 7] or alter activities of cellular antioxidant enzymes and intracellular glutathione content [11, 12]. It is well known that GSH has a high affinity for cadmium and that plays a major role in cellular defense against Cd-induced oxidative damage.

Magnesium is the second most abundant and ubiquitous intracellular cation and it activates many steps in the cellular metabolic pathways. Overall Mg is important for more than 300 different enzyme systems, among them enzyme(s) for glutathione synthesis (γ-glutamylcysteine synthetase and glutathione synthetase). Various investigations show that magnesium deficiency induces a decrease in cellular GSH which renders the tissue susceptible to oxidative damage [31, 33]. On the other hand, investigations on interactions between Cd and bioelements indicate that an excessive intake of bioelements, particularly magnesium, may antagonize cadmium effects [37-42, 45]. For example, the results of Poirier et al. [45] demonstrated clearly that the development of tumors at the site of s.c. cadmium chloride (CdCl₂) injection can be prevented by a simultaneous injection of magnesium acetate (Mg(CH₃COO)₂). Wershmana [40] found that magnesium administration prevented embryo-, haemato-, hepat- and nephro-defects that were induced by Cd. Our recent investigations on Cd-Mg interaction pointed to the beneficial effect of Mg supplementation on mice kidney in acute and subacute Cd intoxication: Mg pretreatment reduced the Cd kidney content after 4 and 6 h in acute Cd intoxication, and after 2 weeks under the conditions of subacute Cd intoxication [41].

In this experiment, cadmium induced time-dependent alterations of GSH content in kidney, liver and testis. A significantly highly elevated GSH content in kidney was determined after 6, 12, 24 and 48 h (table 1). Sarkar et al. [21] observed diminished GSH kidney content in rats but 72 h after i.p. injection of 0.4 mg Cd/kg. This could be explained by the fact that they used lower Cd doses, determined GSH after a longer period of time and used rats, as experimental animals. Our previous results [41] confirmed a significant increase of renal Cd in mice given a single oral dose of 20 mg Cd/kg b.w. Could this dose induce raised levels of GSH as a defense mechanism against toxic doses of cadmium?

A single oral dose of 20 mg Cd/kg b.w. induced significantly decreased hepatic GSH contents after 4, 6 and 12 h (table 1). These results are in accordance with several previous reports [10, 22] and suggest that acute Cd-induced toxicity may be due to the exhaustion of GSH stores and the increase in oxidative stress. In addition, hepatic decrease of GSH

![Figure 3. Levels of testis GSH in mice after subacute treatment with Cd, Mg+Cd, or Mg. Control: control mice; Cd-group intoxicated orally every day with 10 mg Cd/kg b.w.; Mg+Cd-group given 20 mg Mg/kg b.w. before Cd during treatment; Mg-group given every day only 20 mg Mg/kg b.w. Values are means ± SD (n = 8). Means not sharing the same letter are significantly different (P < 0.05, for the same week of the study). (P-values were obtained by one-way ANOVA followed by Tukey-Kramer’s test).](image-url)
could be related to the fact that Cd accumulates more in liver than in kidney or testis [9, 42]. Our experimental data showed that hepatic GSH levels return to normal after 24 and 48 h, suggesting a de novo GSH synthesis and Cd elimination.

In this investigation no significant changes of GSH concentration were observed in testis after acute Cd intoxication, although a slight increase was observed after 4 h followed by a decrease after 12 h (Table 1). These data are partly in accordance with some other authors [46, 47]. Koizumi and Li [46] observed decreased testis GSH content 12 h after administration of a carcinogenic Cd dose, while Hirano et al. confirmed a decrease of testis GSH content 24 and 48 h after a single s.c. dose of 15 μmol CdCl₂/kg [47].

In general, acute exposure to metals decreases GSH levels due to the formation of metal-GSH complexes and/or consumption by the GSH-peroxidase reaction under oxidative stress induced by metals. But, comparing the results found in our experiment with the results of other studies, it is evident that the acute effect of cadmium on GSH tissue levels varies, depending on animal species, dose, route and duration of Cd exposure.

Mice subjected to subacute Cd exposure showed a decrease in the reduced glutathione levels of kidney, statistically significant after 1 week if compared to the control. The decrease in the GSH level could be explained by the increased activity of γ-glutamyl transpeptidase enzyme, as suggested by Karmakar et al. [48]. γ-glutamyl transpeptidase enzyme is an external cell-surface enzyme that can enhance the degradation of extracellular GSH to release its constituent amino acids into the cell. Another possible reason for decreased GSH levels may be due to increased levels of lipid oxidation products, which could be associated with the lesser availability of NADPH which is required for the activity of glutathione reductase to transform oxidized glutathione (GSSG) to the reduced form (GSH) [19]. Therefore, a decrease of renal GSH levels after 1 week of Cd exposure in this study could be explained by the delayed accumulation of Cd in kidney. Nordberg et al. [49] showed that Cd is first preferentially taken up by the liver where it induces metallothionein (MT), and then MT-bound Cd appears in the kidney. Similarly, lipid peroxidation, induced by Cd, was found to occur later in kidney than in liver [42]. The results of the present study show a significant elevation of GSH in liver and testes after 2 weeks Cd treatment, and are in accordance with several previous reports [23, 25]. Single i.p. injection of 0.5 and 2 mg CdCl₂/kg induced a decrease of GSH content in the liver of rats, while 2-week injection of the same doses produced the opposite effect [10], as in our experiment. Rana and Verma [23] found an increase in tissue GSH levels in rats after oral administration of CdCl₂ on alternate days over a 30-day period. In a year-long study, Kamiyama et al. [25] also reported increased GSH levels in rat tissues and suggested that orally administered Cd elevates GSH as an antioxidant. Moreover, Chin and Templeton [50] in in vitro experiments have demonstrated that cadmium produced a dose- and time-dependent increase of intracellular glutathione concentrations, while Fotakis et al. [51] in in vitro experimental studies correlated GSH synthesis and ATP levels. GSH synthesis requires γ-glutamylcysteine, glycine, ATP and magnesium and therefore the increase of Mg-ATP levels could be a response to increased GSH synthesis. In addition, measurement of the mRNA and DNA levels for γ-glutamylcysteine synthetase, the rate-limiting enzyme for glutathione biosynthesis, revealed that enhanced expression of the enzyme but not gene amplification is likely to be responsible for the elevation of cellular glutathione levels [52].

Magnesium supplementation 1 hour before acute Cd intoxication caused alterations in the GSH content of tissues, compared with GSH levels in tissues of mice intoxicated with Cd. Mg pretreatment resulted in a significant reduction of renal GSH content after 12 h and an increase of hepatic GSH after 6 and 12 h, if compared with the group of animals intoxicated with Cd. After 12 h, significance between the Mg+Cd and Cd groups was observed in all investigated organs (Table 1).

In kidney and testes of mice pretreated with Mg (in subacute experiments), the levels of reduced GSH were not altered if compared with the control group of mice, suggesting a protective role of Mg pretreatment against Cd oxidant insults (Figures 1 and 3). This is in agreement with our previous results [41] which showed that Mg supplementation reduced Cd kidney content 2 weeks after subacute Cd exposure. In addition, Boujelben et al. reported that Mg injection lowered the Cd content in kidney, liver and testes [42].

Administration of Mg 1 hour before subacute Cd intoxication induced GSH elevation in the liver of mice, statistically significant after 2 weeks Mg+Cd treatment (Figure 2). Li et al. [53] concluded that Mg may stimulate de novo GSH biosynthesis, since many enzymes require Mg as a cofactor for their activities. Thus, the detected protective action of Mg against the previously mentioned toxic effects of Cd may be
due to either the antagonistic effects of Mg and/or the stimulatory effects of Mg in producing de novo GSH.

Generally, in our acute experiment, no difference was found in the tissues level of GSH between the Mg and control groups, except at 4 h and 6 h in kidney (increase) and at 24 and 48 h in liver (decrease) (table 1). Furthermore, administration of 20 mg Mg/kg b.w induced, after 2 weeks, a significant decrease of GSH content in all investigated tissues (a significant decrease of renal GSH compared to the control and in liver and testis compared to Cd group) (figures 1, 2, 3). Li et al. [53] reported that magnesium causes a dose-dependent change of intracellular glutathione in in vitro experiments; these authors observed that the treatment of cells with Mg (1.25-5 mM) for 20 hours slightly increased cellular GSH content, while with higher Mg doses (>10 mM) it decreased.

Conclusion

The results show that exposure to cadmium induced time-dependent changes of GSH levels in tissues of mice. Under our experimental conditions, Mg supplementations imply a partly positive influence on GSH content in acute and subacute cadmium intoxication. It could be concluded that these findings contribute to investigations on the mechanism between Cd and Mg interaction and suggest the positive role of Mg in therapy of Cd poisoning.

Acknowledgements

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