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Effects of cadmium and zinc ions on mice brain lipid peroxidation and amounts of thiol-rich compounds

Rasa Bernotiene[1,2], Laima Ivanoviene[2], Ilona Sadauskiene[1,2], Arunas Liekis[1], and Leonid Ivanov[2]

[1]Neuroscience Institute and [2]Department of Biochemistry, Medical Academy, Lithuanian University of Health Sciences, Kaunas, Lithuania

Key words
cadmium – zinc – oxidative stress – brain – antioxidants

Abstract. Objective: Mechanisms underlying neurotoxicity of Cd ions are not completely understood. The present study aimed at investigating the effects of intraperitoneally injected Cd and Zn ions alone and in combination on lipid peroxidation in mice brain as well as on the contents of metallothioneins (MT) and reduced glutathione (GSH). Methods: Experiments were done on outbred white laboratory mice using intraperitoneal (IP) injections of $CdCl_2$ and/or $ZnSO_4$ solutions. The exposure-time was 24 hours or 14 days. Content of MT in mice brain was evaluated using the colorimetric method with Ellman's reagent. GSH was measured by reaction with Ellman's reagent to give a compound that absorbs 412 nm light wavelength. Lipid peroxides were estimated by measuring thiobarbituric-acid-reactive substances and were expressed as malondialdehyde (MDA). Results: The obtained results indicated that Cd increased MT content in mice brain after 24 hours and decreased after 14 days of exposure. Contrary to Cd, Zn decreased MT content in mice brain after 24 hours, but increased after 14 days. Meanwhile, Zn reduced the influence of Cd on the MT content in mice brain after 24 hours and 14 days of exposure. In our experiments we also determined that Cd decreased GSH content in mice brain and Zn partly reduced this affect after 14 days of exposure. In another series of experiments our results showed that Cd and Zn increased MDA content in mice brain after 24 hours and 14 days of exposure. Zn also protected lipids from peroxidation induced by Cd after only 14 days. Conclusions: Our studies showed that manifestation of oxidative stress estimated by MDA level under acute exposure of brain to Cd^{2+} and Zn^{2+} is little dependent on MT and GSH amounts. Following 14 days, Zn-induced increasing and Cd^{2+}+Zn^{2+}-induced decreasing in MDA level coincided with pronounced increasing in quantities of MT and mild changes in quantities of GSH.

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Correspondence to
Dr. Rasa Bernotiene
Laboratory of Molecular Neurobiology, Neuroscience Institute,
Lithuanian University of Health Sciences,
Eiveniu 4, 50009,
Kaunas, Lithuania
Rasa.Bernotiene@lsmuni.lt

Introduction

Heavy metal cadmium (Cd) and its compounds have a wide variety of industrial applications such as electroplating, pigments, plastics, and Cd-Ni batteries; therefore, it is a very common natural and anthropogenic pollutant. Because of the unusually slow elimination rate from human organism (Cd biological half-life varies between 10 and 30 years), Cd is known to induce a variety of health-threatening disturbances [1]. In the animal model it has been shown that Cd can accumulate in soft tissues, including brain causing neurological alterations [2]. Different mechanisms are proposed to be responsible for Cd-induced toxicity. One of them is oxidative stress, characterized as an imbalance between pro-oxidant and antioxidant homeostasis [3]. Evidences about the link between Cd and oxidative stress come from three types of experimental investigations: 1) evaluation of adverse effects of Cd on cellular antioxidant defense systems and status of thiol-compounds; 2) evaluation of effects of Cd on enzymes with antioxidant activity; 3) evaluation of lipid peroxidation by Cd. Cd-induced depletion of cellular antioxidant system components, particularly thiol-containing antioxidants, such as glutathione (GSH) and metallothionein (MT), and enzymes is evidenced by experimental studies [4]. Effects of Cd on lipid peroxidation in mice liver have been recently studied and reported by authors of the present investigation [5]. The brain is especially sensitive to oxidative damage as a result of its high oxygen consumption rate, abundant lipid content, and relative paucity of antioxidant

Table 1. Mice assignment into experimental groups.

Group No	Duration of mice brain exposure to IP injected salt solutions		
	24 hours (acute intoxication)	Group No	14 days (sub-acute intoxication)
1	A single IP injection of $CdCl_2$ (14 mmol Cd/kg body weight)	4	A single daily IP injection of $CdCl_2$ (14 mmol Cd/kg body weight)
2	A single IP injection of $ZnSO_4$ (24 mmol Zn/kg body weight)	5	A single daily IP injection of $ZnSO_4$ (24 mmol Zn/kg body weight)
3*	Co-exposure to $ZnSO_4$ and $CdCl_2$ in aforementioned doses	6**	A single daily injection of $ZnSO_4$ and $CdCl_2$ in aforementioned doses

*Mice of 3rd experimental group received IP injections of $ZnSO_4$ solution and after 20 min – IP injection of $CdCl_2$ solution. Termination after 24 hours following $CdCl_2$ injection. **For 14 days, mice of 6th experimental group were pre-treated daily by IP injections of $ZnSO_4$ solution, and after 20 min they received an IP injection of $CdCl_2$ solution. Mice were terminated after 24 hours following the last $CdCl_2$ injection.

enzymes compared to other tissues [6]. Several functions are ascribed to GSH and MT in brain: they act as antioxidants and as buffers, particularly MT, implicated in maintenance of homeostasis of many physiological metal ions, such as Cu^{2+} and Zn^{2+} as well as in detoxification of Cd^{2+} [7].

Zn^{2+} being a physiological metal ion is involved in numerous reactions of cellular metabolism. It is known that Zn^{2+} may be substituted by toxic Cd^{2+} in enzymatic reactions. Low Zn/Cd ratio can be used to predict Cd toxicity, which is increased during Zn deficiency. In animal model study, Zn^{2+} were shown to protect mice liver against Cd-induced peroxidation of lipids [5].

The present study was conducted to determine how Cd and Zn ions alone and in combination could affect lipid peroxidation and MT and GSH amounts in the brain of experimental mice.

Materials and methods

The subject of research

Experiments were done on 4 – 6 week-old out-bred white laboratory mice weighing 20 – 25 g. All experiments were performed according to the Republic of Lithuania Law on the Care, Keeping, and Use of Animals (License of State Veterinary Service for working with laboratory animals No. 0221). We have chosen the model of acute single-dose intoxication (24 h) and the model of sub-acute prolonged 14 days of intoxication with the metal salts as indicated in Table 1.

Mice were randomly assigned into eight groups: six experimental and two control groups. First control group animals received single intraperitoneal (IP) injection of the same volume of physiological solution. Second control group animals received daily IP injections of the same volume of physiological solution for 14 days. The number of mice in each group ranged from 8 to 15.

MT content assay in mice brain

MT were determined in mice brain according to our method described earlier [5]. To the aliquots of 1 mL of supernatant were added 1.05 mL of cold (–20 ºC) absolute ethanol and 80 µL of chloroform; then the samples were centrifuged at 6,000 × g for 10 min. The collected supernatant was combined with 3 volumes of cold ethanol (–20 ºC), kept at –20 ºC for 1 hour and centrifuged at 6,000 × g for 10 min. The MT-containing pellets were then rinsed with 87% ethanol and 1% chloroform and centrifuged at 6,000 × g for 10 min. The MT content in the pellet was evaluated using the colorimetric method with Ellman's reagent. The pellet was re-suspendent in 150 µL 0.25 M NaCl and subsequently 150 µL 1 N HCl containing 4 mM EDTA (ethylenediaminetetraacetic acid) was added to the sample. A volume of 4.2 mL 2 M NaCl containing 0.43 mM DTNB (5,5′-dithiobis(2-nitrobenzoic acid)) buffered with 0.2 M Na-phosphate, pH 8.0 was then added to the sample at room temperature. The sample was finally centrifuged at 3,000 × g for 5 min; the supernatant absorbance was evaluated at wave 412 nm, and MT content was expressed as mg/g of wet weight of brain.

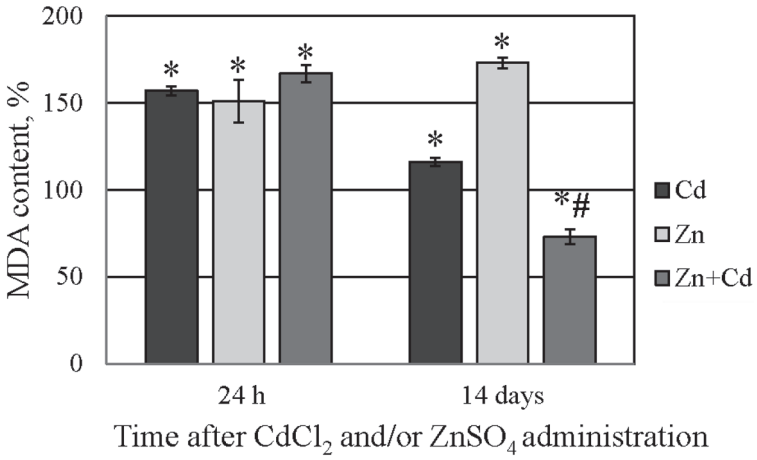

Figure 1. Dependence of malondialdehyde (MDA) content in mice brain on the time of exposure to Cd^{2+} and/or Zn^{2+}. The content of MDA in the brain of control mice group after 24 hours and 14 days of exposure (80 nmol/g and 96 nmol/g wet weight of brain, respectively) was set at 100%. *$p < 0.05$ as compared to the control mice group; #$p < 0.05$ as compared to the group of Cd-treated mice. Data represents results of 6 – 10 separate experiments.

Measurement of GSH in mice brain

GSH were determined by the method described in [5]. GSH was measured by reaction with DTNB to give a compound that absorbs at wave 412 nm. Each sample cuvette contained 2 mL 0.6 mM DTNB in 0.2 M sodium phosphate, pH 8.0, 0.2 mL supernatant fraction and 0.8 mL 0.2 M phosphate buffer to the final volume of 3 mL. GSH content was expressed as mmol/g of wet weight of the brain.

Determination of MDA in mice brain

Lipid peroxidation in brain tissue was estimated by the TBA (thiobarbituric acid) reaction method for MDA [5]. The MDA results were expressed as nmol/g wet tissue. The brain was removed and homogenized with 9 volumes (as compared with brain weight) with cold 1.15% KCl to make 10% homogenate. To 0.5 mL of this homogenate were added 3 mL 1% H_3PO_4 and 1 mL 0.6% TBA aqueous solution. The mixture was heated for 45 min in a boiling water bath. After cooling, 4 mL of n-butanol was added and mixed vigorously. The butanol phase was separated by centrifugation and supernatant absorbance was determined at 535 and 520 nm.

Statistical analysis

The data was expressed as the mean ± SEM. Differences among means were analyzed by one-way ANOVA. $p < 0.05$ value was considered statistically significant (SPSS version 19.0, SPSS).

Results

The present study aimed at investigating the effects of Cd^{2+} and Zn^{2+} on lipid peroxidation and contents of thiol-rich compounds MT and GSH in mice brain homogenates. Although Cd^{2+} is a redox inactive ion, it can indirectly contribute to production of reactive oxygen species (ROS). ROS can randomly react with lipids and initiate the lipid peroxidation process, a chain reaction that produces multiple breakdown molecules such as MDA. Therefore, in our experiments we determined the content of MDA in brain tissue following 24 hours and 14 days of mice treatment with solely $CdCl_2$ solution and in combination with $ZnSO_4$. The data obtained is presented in 1. Significant increasing of MDA content was detected under short-time (24 h) exposure of mice brain to either Cd^{2+} or Zn^{2+} and to their combination. Following 14 days of mice treatment with those metal salt solutions, MDA contents were increased by 16% in Cd-exposed brain, by 75% in Zn^{2+}-exposed brain, and decreased by 27% in Cd^{2+}+Zn^{2+}-exposed brain in regard to the controls. The results about mild effects of Cd^{2+} but potent effects of Zn^{2+} on MDA content in mice brain tissue under sub-acute exposure to these ions were rather unexpected.

It was proposed that Cd^{2+} could enter into brain cells via divalent metal transporter-1 (DMT1) in competition with other ions [8]. In our previous studies we demonstrated that intracellular Cd^{2+} are powerful inducers of MT in mice liver [5].

Our present study showed that content of MT in mice brain were dependent on the duration of mice IP treatment with $CdCl_2$: in regard to controls, 19% increasing and 14%

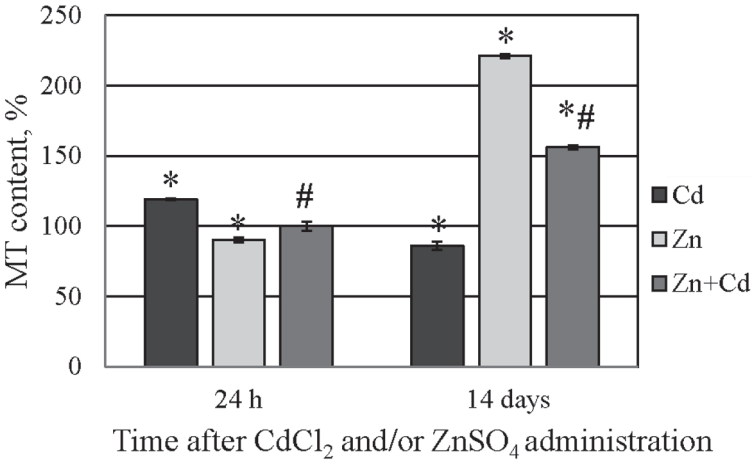

Figure 2. Dependence of metallothionein (MT) content in mice brain on the time of exposure to Cd^{2+} and/or Zn^{2+}. The content of MT in the brain of control mice group after 24 hours and 14 days of exposure (31 mg/g and 33 mg/g wet weight of brain, respectively) was set at 100%. *$p < 0.05$ as compared to the control mice group; $^{\#}p < 0.05$ as compared to the group of Cd-treated mice. Data represents results of 6 –10 separate experiments.

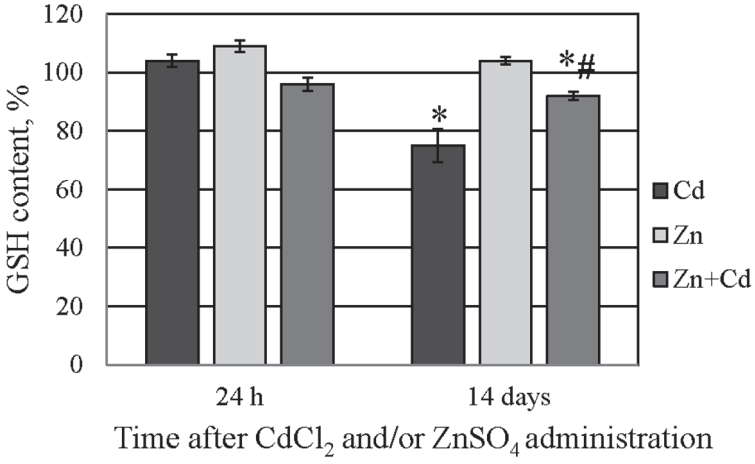

Figure 3. Dependence of reduced glutathione (GSH) content in mice brain on the time of exposure to Cd^{2+} and/or Zn^{2+}. The content of GSH in the brain of control mice group after 24 hours and 14 days of exposure (2.3 mmol/g and 2.4 mmol/g wet weight of brain, respectively) was set at 100%. *$p < 0.05$ as compared to the control mice group; $^{\#}p < 0.05$ as compared to the group of Cd-treated mice. Data represents results of 6 – 10 separate experiments.

decreasing of MT content was observed respectively after 24 hours and 14 days of the treatment (Figure 2). Although Zn^{2+} are also shown as inducers of MT expression [9], in our study, effects of Zn^{2+} were time-delayed: in compare to controls, MT content was decreased by 10% following 24 hours of a single IP injection of $ZnSO_4$ solution. After a 14 day-period of mice treatment with daily injections of $ZnSO_4$ solution, more than twice increasing of MT content (by 121% as compared to control mice group) was detected in mice brain tissue. Under combined action of $CdCl_2$ and $ZnSO_4$, mice brain MT content was increased after only 14 days of IP injections of those salts solutions. Notably, Cd^{2+}+Zn^{2+}-caused increase of MT content which was half of MT present in solely Zn-exposed mice brain (56% vs. 121%).

GSH has been shown to act as a metal-binding ligand, which can decrease toxic effects of heavy metal ions [10]. In the next series of experiments, we studied effects of Cd^{2+} and Zn^{2+} on the content of GSH in mice brain after 24 hours and 14 days of injections of solely a $CdCl_2$ solution and under its combination with $ZnSO_4$ solution. The data presented in Figure 3 showed that none of the considered ions (alone or in combinations) influenced levels of mice brain GSH following 24 hours of exposure. Daily IP injections of either solely the $CdCl_2$ solution or in combination with $ZnSO_4$ for 14 day-period caused a small but significant decreasing of GSH content in mice brain – 25% and 8%, respectively. At this time-period, Zn^{2+} did not influence content of GSH. Taken together our data shows that in short term of mice brain exposure to Cd^{2+}+Zn^{2+}, manifestation of oxidative stress estimated by MDA level is only a little dependent on MT and GSH amounts. However, a 27% decreasing of MDA content coincided with 56% increasing in MT and with 8% decreasing in GSH contents following a 14-day brain exposure to combination aforementioned ions.

Discussion

Although MT was isolated more than five decades ago, the cellular functions of this protein have not yet been fully defined. It is generally accepted that the principal roles of MT lie in the detoxication of potentially toxic heavy metal ions (Cd) and in the regulation of the metabolism of essential trace elements (Zn or Cu). As experimental data emerged, functions of MT were expanded to their action in free-radical scavenging [11], neurogenesis, and brain tissue restoration [12]. Isoform profiles of MT vary from tissue to

tissue: MT type I and MT type II groups are common to almost all tissues, whereas MT type III is specific for brain and MT type IV – for stratified squamous epithelia of skin and upper gastrointestinal tract [13]. Expression of MT isoforms is controlled by different ways: MT type I and type II are upregulated by many factors – metal ions, free radicals, etc. [14], whereas MT type III is almost not inducible by extracellular stimuli [15]. The present study was performed using mice brain homogenate as a source of MT containing isoforms types I, II, and III. According to our results presented in Figure 2, MT were up-regulated under short exposure (24 hours) and down-regulated under prolonged exposure (14 days) to Cd^{2+}. The responses were significant but not obvious (19% increasing and 14% decreasing, respectively). Recently, we demonstrated dramatic up-regulation of MT in mice liver following 24 hours and 14 days of exposure to the same concentrations of Cd^{2+} (increasing by 30% and 392%, respectively) [5, 16]. Such tissue-specific responses of MT to Cd^{2+} can come because of different regulations of MT genes by Cd^{2+} and limited capacity of brain to accumulate Cd^{2+} due to blood-brain barrier [17]. In mice brain, short-time exposure effects of Zn^{2+} on MT levels differed from those of Cd^{2+} – 10% decreasing of MT levels in regard to the control and 29% decreasing in regard to effects exerted by Cd^{2+}. The effects seems to be unique to the brain since our recent studies revealed almost no effect of Zn^{2+} on liver MT levels [5]. In comparison to the liver, brain MT scarcely responded to short-time exposure to metal ions such as Zn^{2+} and Cd^{2+}. The effects of Zn+Cd on lipid peroxidation quantified by MDA levels were also less pronounced in the brain (55% and 50% increasing in MDA level in response to 24 hours exposure to Cd and to Zn, respectively) than in the liver (218% and 325%, respectively) [5]. Long-time exposure effects of those ions, however, were opposite – 10% and 75% increasing in brain MDA level after 14 days of mice exposure to Cd^{2+} and Zn^{2+} compared to almost no effect in the liver. Several recent studies demonstrated that responsiveness of brain to injections of Cd^{2+} and Zn^{2+} depends on blood-brain barrier [18, 19]. In this relation, it is difficult to interpret 50% increasing of MDA and variations of MT levels in brain following 24 hours exposure of mice to either Zn^{2+}, Cd^{2+}, or in their combination. We can only speculate that under short-time exposure of mice brain to metal ions, changes in MT and MDA levels occur in coating layers of neurovascular unit, e.g. cerebral vascular endothelium, podocytes, and astrocytes. Under longer periods of an organism exposure to Cd^{2+} (up to 3 days), brain can accumulate those ions in time and dose-dependent way [20]. These findings are in tune with the results of our present study – after a 14 day-long mice treatment with respective metal ion solutions, Zn^{2+} restored amounts of MT and GSH, which were decreased by Cd^{2+}. This effect could favor towards desensitisation of brain to oxidative stress as it becomes apparent from significant decreasing in MDA levels at this time-point (Figure 1). We can propose that following 14 days of mice IP injections with $CdCl_2$ and $ZnSO_4$ solutions, the metal ions accumulate in various cells throughout the brain, therefore Zn^{2+} can mitigate Cd^{2+}-induced depletion thiol-rich compounds. It may be assumed that mechanisms of metal ion-induced acute effects on liver can't be extrapolated to brain.

Conclusions

Our studies showed that following a 14 day-long exposure of mice brain to Zn and Cd ions, Zn ions could mitigate Cd-induced peroxidation of lipids via induction of metallothionein synthesis and preservation of reduced glutathione.

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