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ORIGINAL ARTICLE

Comparative effects of *meso*-2,3-dimercaptosuccinic acid, monensin, and salinomycin on cadmium-induced brain dysfunction in cadmium-intoxicated mice

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ABSTRACT

Cadmium (Cd) is a risk factor for neurodegenerative diseases. The purpose of this study was to compare the effects of *meso*-2,3-dimercaptosuccinic acid (DMSA) and the polyether ionophorous antibiotics monensin and salinomycin on Cd-induced neurodegenerative alterations in mice. The results show that subacute intoxication of mice with Cd (II) acetate (20 mg/kg body weight (BW) for 14 days) caused a significant accumulation of cadmium (Cd) in the brain. Treatment of Cd-exposed mice with DMSA (20 mg/kg BW for 14 days) significantly increased the Cd concentration in the brains compared to those of the Cd-treated group. However, administration of monensin (20 mg/kg BW for 14 days) or salinomycin (20 mg/kg BW for 14 days) significantly reduced the Cd concentration in the brains of Cd-treated mice compared to the toxic control group. Histopathological analysis of brain tissues from the Cd-treated mice revealed that Cd induced neuronal necrosis, characterized by many shrunken, darkly stained pyknotic neurons with prominent perineuronal spaces. Whereas monensin and salinomycin significantly reduced the adverse effects of Cd on brain morphology of Cd-treated mice, DMSA did not. Monensin slightly increased the copper and iron endogenous levels in the brains of Cd-exposed mice compared to those of the untreated mice. Salinomycin did not affect the concentrations of biometal ions in the brain of Cd-exposed mice compared to untreated controls. The results demonstrated salinomycin to be a better potential chelating agent for treatment of Cd-induced brain injury compared to DMSA and monensin.

KEY WORDS: DMSA; monensin; salinomycin; cadmium; neurodegenerative diseases

Introduction

Cadmium (Cd), a non-essential metal, is one of the world's most toxic environmental pollutants. It is released by various natural and anthropogenic sources to the atmosphere, water, and soil. Thus, human non-occupational exposure to Cd primarily occurs through inhalation of cigarette smoke and ingestion of contaminated food or water (Satarug *et al.*, 2010).

Cadmium possesses a low rate of excretion and induces toxicity at very low dosages, resulting in renal, skeletal, pulmonary, hepatic, reproductive, and cardiovascular dysfunctions (Bernhoft, 2013; Vukićević, 2012). It also elicits neurotoxic effects that result in histopathological and ultrastructural sequelae, neurological dysfunction, neurochemical and behavioral changes (Haider *et al.*, 2013). Wang *et al.* (2017) demonstrated a direct relationship between Cd exposure and cognitive as well as olfactory impairments in an animal model. Clinical studies suggest that blood Cd is positively related with depression and neurodegenerative diseases, including Parkinson's disease, Huntington's disease and death from Alzheimer's disease (Johnson, 2001; Okuda *et al.*, 1997; Panayi, 2002, Min & Min, 2016).

A possible mechanism by which Cd induces these disorders may involve alteration of copper (Cu), zinc (Zn), iron (Fe), and calcium (Ca) homeostasis (Wang & Du, 2013).

Chelation therapy is the most commonly used therapeutic strategy for the treatment of toxic metal poisonings (Flora & Pachauri, 2010). *Meso*-2,3-dimercaptosuccinic

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acid (DMSA) is a Food and Drug Administration (FDA) approved agent for the treatment of lead intoxication. DMSA is suitable for oral administration, which is one of the advantages of this chelating agent over other antidotes to lead poisoning. However, the potential application of DMSA as antidote to cadmium poisoning is largely unclear (Sarić *et al.*, 2004). Presently, no effective chelation therapy for Cd intoxication in humans exists (Rinaldi *et al.*, 2017). Our previous studies on mice demonstrated that the polyether ionophorous antibiotic monensin (administered as tetraethylammonium salt) attenuates Cd-induced toxicity in the liver, kidneys, spleen, lungs, and testes, and significantly lowers the Cd content in the organs of mice subjected to subacute Cd exposure (Gegova *et al.*, 2012; Gluhcheva *et al.*, 2013; Ivanova *et al.*, 2014a; Ivanova *et al.*, 2014b; Ivanova 2012a, Pavlova *et al.*, 2012). These results motivated us to undertake a detailed investigation on the potential application of polyether ionophorous antibiotics as antidotes to Cd-poisoning.

In 2009, Gupta *et al.* found that the polyether antibiotic salinomycin is one of the most effective agents against cancer stem cells. In 2013, this antibiotic was approved for clinical trials in patients diagnosed with triple negative invasive breast carcinoma (Antoszczak & Huczyński, 2015; Naujokat & Steinhart, 2012). To the best of our knowledge, there is a lack of information regarding the potential application of this antibiotic for treatment of Cd-intoxication.

In this study, we present experimental data about the effects of DMSA, monensin, and salinomycin on Cd-induced neurodegenerative disorders in mice, thus significantly expanding the existing knowledge regarding the biological activity of these compounds.

Materials and methods

Chemicals

The sodium salts of monensin and salinomycin were obtained from Biovet Ltd. (Peshtera, Bulgaria). Tetraethylammonium hydroxide (Et_4NOH), nitric acid (HNO_3), Cd(II) acetate ($Cd(CH_3COO)_2{\times}2H_2O$) and diethyl ether ($Et_2O$) were acquired from Merck (Darmstadt, Germany).

Preparation of monensic and salinomycinic acids

The preparation of monensic acid A monohydrate and salinomycinic acid was conducted according to the procedure described in detail by Gertenbach and Popov, 1975; Ivanova *et al.,* 2010, Ivanova *et al.*, 2012b. Data for the purity and spectral characteristics of both compounds are presented by Ivanova *et al.*, 2012b, Ivanova *et al.,* 2010.

Experimental design

Mature 60-day-old adult male imprinting control region (ICR) mice were fed a standard diet and had access to food *ad libitum*. The mice were housed in individual standard hard bottom polypropylene cages at the Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences at 23 °C under a controlled 12 h light/dark cycle. The animals were left to acclimatize for a week prior to dosing, then 45 mice were randomized into 5 groups (9 mice in each group) as follows.

The first group (untreated mice) served as the control group and received distilled water for 28 days.

The mice from the second group (Cd-exposed, toxic control group) were subjected to Cd(II) acetate treatment with an average daily dose of 20 mg/kg body weight (BW) for two weeks. During the next 14 days of the experiment, the animals from this group received distilled water.

The third group (Cd+DMSA) was exposed to Cd by administration of an average daily dose of Cd(II) acetate (20 mg/kg BW) for two weeks. After 14 days, the Cd exposure was stopped and the mice from this group were treated with DMSA at an average daily dose of 20 mg/kg BW for 14 days.

The fourth group (Cd+Mon) received an average daily dose of 20 mg/kg BW Cd(II) acetate for two weeks. From the 15th to 28th days of the experimental protocol the mice from this group were treated with tetraethylammonium salt of monensic acid (average daily dose 20 mg/kg BW).

The mice from the fifth group (Cd+Sal) were treated with Cd(II) acetate for 14 days, similarly to the toxic control (Cd-exposed) group. After 14 days, the Cd treatment was stopped and the animals received tetraethylammonium salt of salinomycinic acid at an average daily dose of 20 mg/kg BW for 14 days.

All compounds were dissolved in drinking (distilled) water given orally. The dose of Cd(II) acetate was selected based on our previous study (Ivanova *et al.*, 2012). We found that mice exposed to an average daily dose of 10 mg/kg BW Cd(II) acetate for 14 days did not have any significant accumulation of the toxic metal ion in the brain (Ivanova *et al.*, 2012). Therefore, to achieve the aim of this study, we doubled the dose of the Cd(II) acetate. Higher doses of the compound were not tested because of an increased risk of mortality for the experimental animals. The dose of 20 mg/kg BW of the chelating agent DMSA corresponds to the average dose used in clinical protocols for chelation therapy (Blaurock-Busch and Busch, 2014). For comparative purposes, the antibiotics salinomycin and monensin were also administered at 20 mg/kg BW. We found the dose of 20 mg/kg BW of the selected chelating agents to be well tolerated by the mice, thus lower doses were not studied.

All animals were sacrificed on the 29th day of the experiment. Craniotomy was performed and the intact brains of 4 animals from each group were excised, weighed and processed for histological studies. The brains of 5 animals from each group were frozen at –20 °C prior to atomic absorption analysis.

The animal studies were approved by Ethics Committee of the Institute of Experimental Morphology, Pathology and Anthropology with Museum, BAS and were conducted according to the ARRIVE guidelines (Kilkenny *et al.*, 2010).

Atomic absorption analysis

A wet digestion procedure was used for the preparation of brain tissue for atomic absorption analysis. Each organ was washed with deionized water and blotted dry with filter paper, then weighed and poured using 5 mL of concentrated nitric acid. After 24 h, the samples were heated on a sand bath to a sample volume of 1 mL. The samples were then transferred to centrifuge tubes and diluted with deionized water to a volume of 10 mL. Blanks were prepared in duplicate. An atomic absorption spectrometer (Perkin Elmer Analyst 400, air-acetylene flame) was used to assess Cu, Fe, Zn, and Ca concentrations and an electrothermal atomic absorption spectrometer (Zeeman Perkin Elmer 3030, HGA 600) to determine the Cd concentration in the brains of the experimental animals. Analytical accuracy was checked by analysis of Certified Reference Materials from the International Atomic Energy Agency (IAEA-H-8 (kidney) and IAEA-H-4 (animal muscle). Good agreement between the experimental and certified values was achieved. The limits of detections for Cd, Cu, Zn, Fe, and Ca were 0.01 ng/mL, 0.2 mg/L, 0.1 mg/L and 0.5 mg/L, respectively.

Statistical calculations

The results for all groups studied are expressed as the mean value ± SD, n=5. Student's *t*-test was used to assess the significance of the differences between experimental results of two groups. The results were considered significantly different at $p<0.05$.

Histological analysis

Brains from control and treated mice were fixed in Bouin fixative for 24 h, then paraffin-embedded. Briefly, after fixation, the samples were dehydrated in a graded series of ethanol, cleared with xylene, impregnated in molten paraffin, embedded in fresh molten paraffin, then sectioned into 5-µm thick sections using a microtome. Subsequently, the sections were stained with hematoxylin and eosin and observed under a light microscope (Leica DM 5000B; Leica Microsystems, USA).

Results

Atomic absorption analysis

Exposure of mice to 20 mg/kg BW Cd(II) acetate daily for two weeks resulted in significantly higher accumulation of Cd in the brain compared to that of the untreated control animals (Figure 1).

Treatment of the Cd-intoxicated mice with DMSA significantly raised the brain concentrations of Cd to approximately 2-fold of the toxic control group value, demonstrating that DMSA might not be an appropriate antidote to Cd-poisoning. In contrast, the administration of the tetraethylammonium salt of monensic acid to Cd-intoxicated animals significantly reduced the brain Cd concentration by 87% compared to that of the toxic control group. A similar result was observed for the group subjected to treatment with tetraethylammonium salt of salinomycinic acid. Thus, the results presented in Figure 1 clearly demonstrate that monensin and salinomycin can mobilize Cd from the brain.

Table 1 lists Cu, Zn, Fe, and Ca concentrations in the brains of experimental mice. The administration of 20 mg/kg BW Cd(II) acetate to mice for 2 weeks did not significantly alter the homeostasis of biometal ions in the brains of Cd-intoxicated mice compared to those of untreated control mice. The treatment of the Cd-intoxicated mice with DMSA did not change the brain levels of Cu, Zn, Fe, and Ca. These results imply that accumulation of Cd in the brain (~16.7 ng/g) does not affect the homeostasis of these essential metal ions.

Treatment of Cd-intoxicated mice with the tetraethylammonium salt of monensin increased brain concentrations of Cu and Fe by 20% and 26%, respectively, compared to those of untreated control animals. In

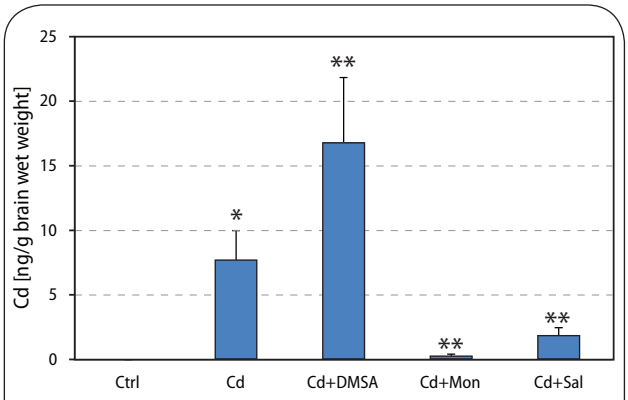

Figure 1. Effects of Cd, DMSA, tetraethylammonium salts of monensic acid and salinomycinic acid on the concentration of Cd in brains of experimental mice subjected to subacute Cd intoxication. Legend – Ctrl: No detectable Cd concentration in the brains of untreated control mice; Cd: Cd concentration in brains of Cd-treated mice; Cd+DMSA – Cd concentration in brains of Cd-intoxicated mice subjected to treatment with DMSA; Cd+Mon: Cd concentration in brains of Cd-intoxicated mice subjected to treatment with tetraethylammonium salt of monensic acid. Cd+Sal: Cd concentration in brains of Cd-intoxicated mice, subjected to treatment with tetraethylammonium salt of salynomicinic acid. Each column represents mean ± SD; n=5; asterisk (*) indicates the significant difference between the toxic control group (Cd) and the untreated control animals (Ctrl); double asterisk (**) indicates the significant difference between the DMSA, monensin or salinomycin treated group and the Cd-treated controls ($p<0.05$).

Table 1. Concentration of some essential metal ions in the brain of experimental animals.

	Cu, mg/kg, n=5	Zn, mg/kg, n=5	Fe, mg/kg, n=5	Ca, mg/kg, n=5
Ctrl	2.70±0.33	13.1±1.1	22.7±1.5	108±15
Cd	2.72±0.37	13.2±0.6	24.7±2.1	138±21
Cd+DMSA	3.01±0.43	11.2±2.1	18.1±4.1	105±11
Cd+Mon	3.36±0.41*	10.9±1.7	26.3±1.9*	110±7
Cd+Sal	3.03±0.41	13.2±0.6	22.6±2.3	157±44

Asterisk (*) denotes the significant difference between the untreated control group and the group treated with tetraethylammonium salt of monensic acid.

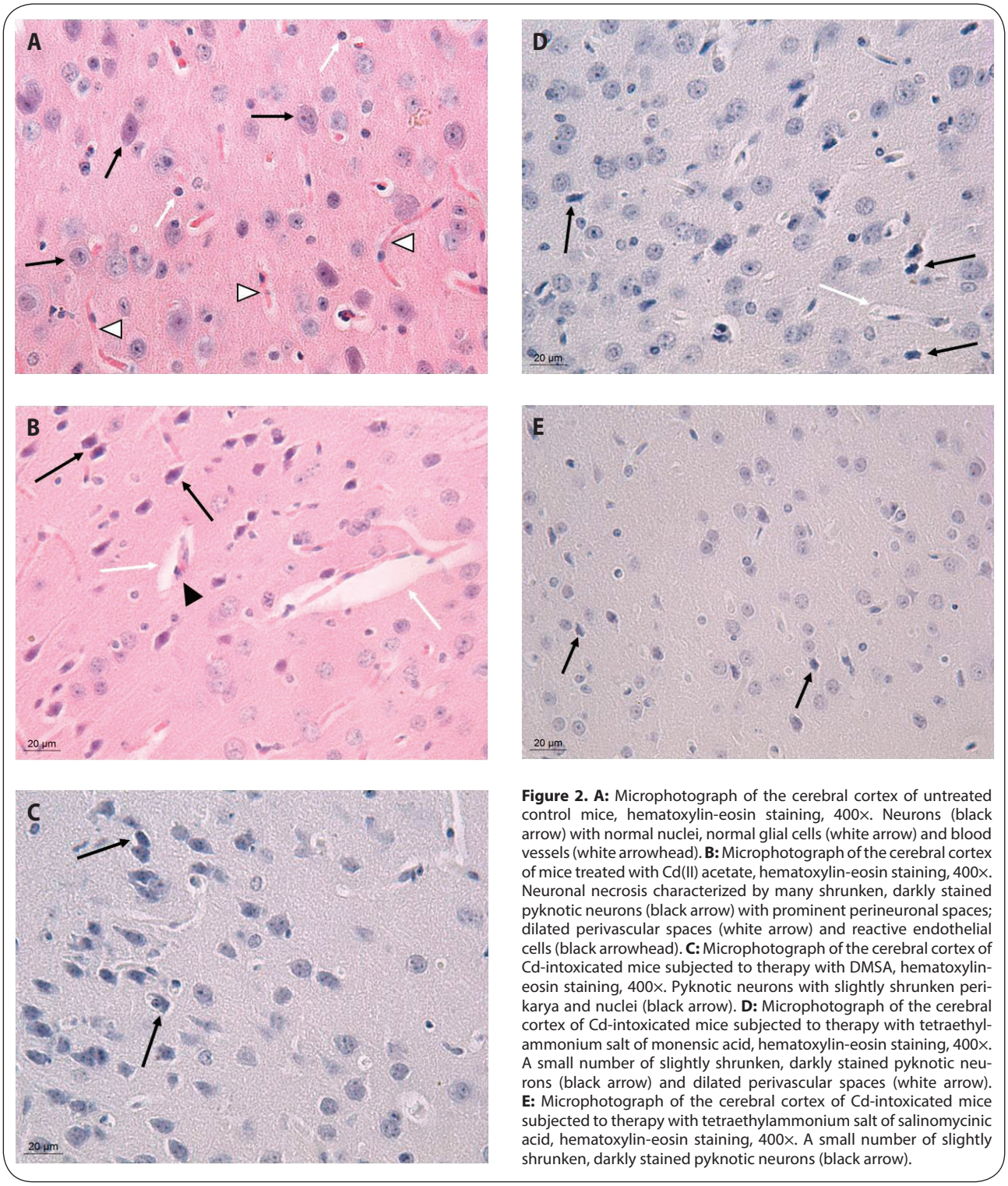

Figure 2. A: Microphotograph of the cerebral cortex of untreated control mice, hematoxylin-eosin staining, 400×. Neurons (black arrow) with normal nuclei, normal glial cells (white arrow) and blood vessels (white arrowhead). **B:** Microphotograph of the cerebral cortex of mice treated with Cd(II) acetate, hematoxylin-eosin staining, 400×. Neuronal necrosis characterized by many shrunken, darkly stained pyknotic neurons (black arrow) with prominent perineuronal spaces; dilated perivascular spaces (white arrow) and reactive endothelial cells (black arrowhead). **C:** Microphotograph of the cerebral cortex of Cd-intoxicated mice subjected to therapy with DMSA, hematoxylin-eosin staining, 400×. Pyknotic neurons with slightly shrunken perikarya and nuclei (black arrow). **D:** Microphotograph of the cerebral cortex of Cd-intoxicated mice subjected to therapy with tetraethylammonium salt of monensic acid, hematoxylin-eosin staining, 400×. A small number of slightly shrunken, darkly stained pyknotic neurons (black arrow) and dilated perivascular spaces (white arrow). **E:** Microphotograph of the cerebral cortex of Cd-intoxicated mice subjected to therapy with tetraethylammonium salt of salinomycinic acid, hematoxylin-eosin staining, 400×. A small number of slightly shrunken, darkly stained pyknotic neurons (black arrow).

contrast, treatment of Cd-intoxicated mice with tetraethylammonium salt of salinomycinic acid did not affect the brain levels of Cu, Zn, Fe, and Ca.

Histological analysis

Results from the histological study of the cerebral cortex of the experimental mice are presented in Figure 2. Figure 2A illustrates the architecture of normal cortex in the untreated control animals. The neurons had spherical or pyramidal perikaryon with round, centrally located nuclei and prominent nucleoli. Morphologically normal glial cells and brain capillaries were also observed. No pyknotic neurons were seen.

Exposure of mice to Cd(II) acetate for 2 weeks resulted in extensively damaged cerebral cortices with features of neuronal necrosis (Figure 2B). Abnormal neurons with atrophic shrunken perikarya, darkly stained pyknotic nuclei, and prominent perineuronal spaces were present,

as well as enlarged perivascular spaces and blood vessels with reactive endothelial cells. Chelation therapy with DMSA did not substantially improve the histological features, as many pyknotic neurons with slightly shrunken perikarya and nuclei were still present (Figure 2C). These results concur with the results from our atomic absorption analysis (Figure 1).

In comparison, treatment of Cd-intoxicated mice with tetraethylammonium salts of monensic acid (Figure 2D) and salinomycinic acid (Figure 2E) significantly reduced the number of pyknotic neurons. In addition, the perivascular spaces in the brains of these mice showed nearly normal appearance, confirming the ability of both antibiotics to inhibit Cd-induced neurotoxicity.

Discussion

In vivo studies have demonstrated that Cd can penetrate and accumulate in the brains of developing rats and adult rats, causing cellular dysfunction and cerebral edema (Goncëalves *et al.*, 2010; Mendez-Armenta and Rios, 2007). In humans, Cd induces severe neurotoxicity via the olfactory pathways to the brain (Mascagni *et al.*, 2003).

Our results confirmed that Cd accumulates in the brains of Cd-exposed mice. The literature data regarding the neurotoxic effect (Wang & Du, 2013) of Cd and our results demonstrate the necessity of having chelating agents that could mobilize Cd from the brain. Unfortunately, the published results regarding the effects of different chelating agents on Cd-induced brain dysfunction are sparse.

In 1983, Gale *et al.* studied the effects of different chelating agents on organ distribution and excretion of Cd in rats. They found that intraperitoneal administration of diethyldithiocarbamate (DDTC) increased the Cd burden in the lungs, testicles, and heart, while elevating the brain burden about ten-fold. In contrast, DMSA does not reduce the Cd content of any organ (Gale *et al.*, 1983). Sarić *et al.* (2004) however reported that DMSA significantly decreased the Cd concentration in the liver and kidney of Cd-exposed rats.

In this study we demonstrated for the first time that the treatment of the Cd-intoxicated mice with DMSA increased significantly the Cd concentration in the brain of Cd-exposed mice compared to that of the toxic control group. Monensin and salinomycin reduced significantly the concentration of the toxic metal ion in the brains of Cd-exposed mice compared to Cd-treated controls. These results suggest that polyether ionophorous antibiotics are better chelating agents than DMSA for treatment of Cd-induced neurodegenerative diseases.

Cadmium can disturb the homeostasis of Cu and Zn ions in the brain of Cd-intoxicated animals (Wang and Du, 2013). It might also induce apoptosis of cerebral cortical neurons by altering Ca homeostasis and the Ca-mediated signalling pathway (Xu *et al.*, 2011; Yuan *et al.*, 2013). In humans, alteration of endogenous levels of Cu and Fe in the brain causes diseases such as Alzheimer's disease, amyotrophic lateral sclerosis, autism spectrum disorders, Huntington's disease, Parkinson's disease, Wilson's disease, and prion disease (Desai & Kaler, 2008; Salvador *et al.*, 2011; Strausak *et al.*, 2001; Valensin *et al.*, 2016; Zucca *et al.*, 2017). A high concentration of Zn in the brain may accelerate the formation of fibrillar β-amyloid aggregation, leading to neurodegeneration (Cuajungco & Faget, 2003; Jomova *et al.*, 2010). Thus, essential metal ions (i.e. Cu, Zn, Fe, and Ca) should be monitored in toxicological studies of potential new Cd chelating agents.

Our study revealed that Cd did not alter the endogenous levels of Cu, Zn, Fe, and Ca in the brains of mice exposed to 20 mg/kg bw Cd(II) acetate administered daily for 2 weeks (Table 1). Those results partially contradict a study by Syed (2016). Although as in our study, Syed 2016 did not observe significant changes in the endogenous levels of Zn and Fe in the brain of Cd-treated rats, he found that the Cu concentration in the brains of Cd-treated rats significantly declined compared to that of untreated rats. It should be noted that the experimental animals in Syed's study were treated for 120 days, and the brain concentration of Cd reached 39 ng/g. Most likely, the effect of Cd on endogenous levels of different essential metals depends on the dose, duration of exposure, and concentration of accumulated Cd in the brain. On the other hand, we found that administration of tetraethylammonium salt of monensic acid to Cd-treated mice slightly raised the concentrations of Cu and Fe in the brains compared to those of the untreated controls (Table 1). This effect should be taken into consideration when and if monensin is considered as a chelating agent for the treatment of Cd-induced neurodegenerative disorders. The treatment of the Cd-exposed mice with tetraethylammonium salt of salinomycinic acid did not change the endogeneous levels of the biometal ions in the brain, which could be one of the advantages of using this chelating agent for the treatment of Cd-induced neurodegenerative disorders.

Cerebral cortical neurons and vascular endothelium are believed to be the primary targets of Cd toxicity (Afifi & Embaby, 2016; Prozialeck *et al.*, 2006; Xu *et al.*, 2011; Yuan *et al.*, 2013). Ayannuga *et al.*,2015, observed central neuronal chromatolysis, basophilic necrotic neurons, and pyknotic glial cells in the cerebrum of both young rats and adult Cd-treated rats. Maodaa *et al.*, 2016, also reported Cd-induced neuronal degeneration, chromatolysis and pyknosis in the cerebrum, cerebellum and medulla oblongata. Other studies found dose-dependent Cd toxicity in juvenile mice brains, resulting in different degrees of hyperemia, vacuolar degeneration, and ultrastructural changes in the cerebral cortex (Yang *et al.*, 2015). Studies by Kauod and Makawy (2011), demonstrated that Cd induced neuronal necrosis in the offspring of mice. Our results (Figure 2B) correspond well with literature data, confirming that Cd can induce cerebral cortex toxicity, even in the ng/g range. In fact, our data showed that Cd can impair brain function via a mechanism that does not involve alteration of Cu, Zn, Fe, and Ca homeostasis (Table 1). Treating Cd-exposed

mice with DMSA did not reduce Cd-induced toxicity in the brain (Figure 2C). In contrast, the tetraethylammonium salts of monensic acid and salinomycinic acid significantly inhibited the adverse effect of Cd in the brains of the Cd-exposed mice (Figures 2D and 2E). These results, corroborated by the data depicted in Figure 1, confirmed that the polyether ionophorous antibiotics monensin and salinomycin are potentially suitable chelating agents for the treatment of Cd-induced neurotoxicity.

Some studies revealed that overdose or accidental ingestion of ionophorous antibiotics in non-target animal species can cause cardiotoxicity or neurotoxicity (Kart & Bilgili, 2008; Boehmerle *et al.*, 2014). Kevin *et al.* (2016), however concluded that the reported toxicity for monensin of >160.0 mg/kg (LD_{50}) in primates makes the polyether class an attractive group of molecules for optimization and development of human health applications. Further, pilot results from clinical trials demonstrated that intravenous administration of 200–250 µg/kg salinomycin every other day for 3 weeks in patients with metastatic breast, ovarian, and head and neck cancers resulted in partial regression of tumor metastases with only minor acute and long-term side effects. No severe acute and long-term side effects typical of those associated with conventional chemotherapeutic drugs were established (Naujokat & Steinhart, 2012).

In our study the tetraethylammonium salts of monensic and salinomycinic acids were administered to Cd-exposed mice orally via drinking water. The dose of 20 mg/kg BW was well tolerated by the experimental animals. No signs of neurotoxicity caused by the treatment of mice with the tetraethylammonium salts of the two antibiotics were observed.

Conclusion

The preferred chelating agent for the treatment of Cd-induced brain dysfunction should inhibit the Cd-induced neurotoxicity when orally administered. Meanwhile, it should neither raise the levels of toxic metals in the brain nor alter the homeostasis of essential metal ions. Based on these considerations, the results of our present study and the available literature data, we conclude that among the chelating agents that have been studied in animal models, salinomycin (administered as a tetraethylammonium salt) is a potential antidote for the treatment of Cd-induced brain dysfunction.

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***Conflict of interests**. The authors declare no conflict of interests.*

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