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Cytotoxic effects of cadmium and zinc co-exposure in PC12 cells and the underlying mechanism

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Abbreviations;

amyotrophic lateral sclerosis, ALS; copper-zinc superoxide dismutase, Cu/Zn-SOD; reactive oxygen species, ROS; glutathione, GSH; bovine aorta endothelial cells, BAECs; metallothionein, MT; lactate dehydrogenase, LDH; mitochondrial membrane potential, MMP; mitochondrial permeability transition, MPT; voltage-dependent anion channel, VDAC

Abstract

Cadmium (Cd^{2+}) is a well studied inducer of cellular necrosis and apoptosis. Zinc (Zn^{2+}) is known to inhibit apoptosis induced by toxicants including Cd^{2+} both *in vitro* and *in vivo*. The mechanism of Zn^{2+} -mediated protection from Cd^{2+} -induced cytotoxicity is not established. In this study, we aimed to understand the effects of Zn^{2+} on Cd^{2+} -induced cytotoxicity and apoptosis using PC12 cells. Cell viability and DNA fragmentation assays in PC12 cells exposed to Cd^{2+} and/or Zn^{2+} revealed that Cd^{2+} (5 and 10 $\mu\text{mol/L}$) alone induced significant cell death, and co-exposure to Zn^{2+} (5, 10, and 100 $\mu\text{mol/L}$) for 48 h had a protective effect. Assessment of intracellular free sulfhydryl levels and lactate dehydrogenase activity suggested that Cd^{2+} (10 $\mu\text{mol/L}$) induced oxidative stress and disrupted cell membrane integrity. Addition of Zn^{2+} (10 and 100 $\mu\text{mol/L}$) reduced Cd^{2+} -mediated cytotoxicity. Changes in expression of the apoptotic factors Bax, Bcl-2, Bcl-x, and cytochrome c were measured *via* western blot and expression of caspase 9 was detected *via* reverse transcriptase polymerase chain reaction. Western blots showed that Zn^{2+} (10 and 100 $\mu\text{mol/L}$) suppressed Cd^{2+} -induced apoptosis (10 $\mu\text{mol/L}$) by reducing cytochrome c release into the cytosol, and downregulating the proapoptotic protein, Bax. In addition, expression of caspase 9 was lower in Cd^{2+} (5 $\mu\text{mol/L}$)-treated PC12 cells when co-treated with Zn^{2+} (2 and 5 $\mu\text{mol/L}$). These findings suggest that the effective inhibition of Cd^{2+} -induced apoptosis in PC12 cells by Zn^{2+} might be due to suppression of mitochondrial apoptosis pathway and inhibition of Cd^{2+} -induced production of reactive oxygen species.

Keywords: apoptosis, heavy metals, cytochrome c, DNA, caspase 9, glutathione

1. Introduction

Cadmium (Cd^{2+}) is one of the most toxic heavy metals due to its prolonged biological half life, low rate of excretion and high accumulation capacity in soft tissues. It is a widespread toxicant of occupational and environmental concern because environmental levels have risen steadily with increased (about 13,000 tons/year) production of Cd^{2+} for nickel-cadmium batteries, pigments, chemical stabilizers, metal coatings, and alloys [1, 2]. Major sources of human exposure to Cd^{2+} include i) occupational: primary metal industries, battery production industries, chemical stabilizer industries, among others and ii) non-occupational: cigarette smoking and consumption of contaminated foods and water [1-3]. The toxic effects of Cd^{2+} have been extensively studied in *in vivo* and *in vitro* systems. Cd^{2+} affects metabolic processes including energy metabolism, membrane transport, and protein synthesis. It may also act on DNA directly or indirectly by interfering with gene control and repair mechanisms [4, 5]. Numerous studies have shown that Cd^{2+} damages mammalian organs including the lungs, kidneys, testes and the cardiovascular, hematopoietic, and nervous systems [6, 7]. Cd^{2+} exposure has been reported as a causative factor in the progressive neurodegenerative disorder amyotrophic lateral sclerosis (ALS). The link between Cd^{2+} exposure and ALS may include reduced neuronal expression of copper-zinc superoxide dismutase (Cu/Zn-SOD) [8]. Occupational Cd^{2+} exposure was found to negatively affect psychomotor function and memory of workers and cause olfactory dysfunction and neurobehavioral defects [9, 10]. Increasing evidence indicates that Cd^{2+} disrupts integrity of the mitochondrial membrane [11]. Recently, it has been shown that Cd^{2+} can disrupt the blood-brain barrier and enter the brain [12, 13]. Neuronal exposure to Cd^{2+} eventually triggers release of mitochondrial proteins such as cytochrome c into the cytosol. These mitochondrial proteins activate catabolic hydrolases, which cleave important cellular targets resulting in cell death. Cd^{2+} toxicity may be associated with production of reactive oxygen species (ROS), making mitochondria key targets for Cd^{2+} toxicity. High levels of ROS have been suggested to increase blood-brain barrier permeability, induce tubulin alterations, and perturb synaptic transmission [9]. It has been proven that Cd^{2+} can induce apoptosis *via* the mitochondrial pathway in cell lines [14-16]. At low and moderate concentrations in cell culture systems (e.g., 0.1-10 $\mu\text{mol/L}$), Cd^{2+} causes apoptosis. At higher concentrations ($>50 \mu\text{mol/L}$), necrosis becomes evident [17].

Zinc (Zn^{2+}) is a well-known essential trace metal belonging to group IIB in the periodic table (as does Cd^{2+}), and is a required cofactor for various essential enzymes. By contrast, Cd^{2+} has no

known physiological or biochemical functions in organisms [17]. Multiple biological macromolecules contain Zn^{2+} as a structural component, and Zn^{2+} is a major regulator of normal human development [18]. According to the Protein Data bank, more than 2700 enzymes, including hydrolases, transferases, oxidoreductases, ligases, isomerases and lyases contain Zn^{2+} [19]. Additionally, Zn^{2+} exhibits antioxidant properties and can activate metal-binding proteins/chaperones. It is also capable of binding to and inhibiting oxidation of reduced glutathione (GSH) under oxidative stress conditions [20]. Several studies have reported a therapeutic role of Zn^{2+} in treating Cd^{2+} toxicity *in vivo* and *in vitro*. For example, Zn^{2+} inhibits Cd^{2+} -induced apoptosis and ROS production in HeLa cells and bovine aorta endothelial cells (BAECs) [16, 21]; Zn^{2+} supplementation improves biochemical characteristics of distal femur and femoral diaphysis in male rats chronically exposed to Cd^{2+} [22]. Enhanced Zn^{2+} consumption prevents alterations in lipid metabolism induced by Cd^{2+} in male rats [23]; and Zn^{2+} protects rats against Cd^{2+} -induced hepatotoxicity [24]. Zn^{2+} affects some enzymes involved in DNA metabolism and inhibits apoptosis *via* its effect on transcriptional factors activated during apoptosis [25]. Studies have shown that Zn^{2+} plays a crucial role in maintenance of the cellular redox balance *via* several molecular mechanisms, including modulation of oxidant production and oxidative damage [26], regulation of GSH metabolism, induction of metallothionein (MT), and scavenging of oxidants [27].

The effects of Zn^{2+} discussed above led us to test the hypothesis that Zn^{2+} might have critical regulatory effects on the pathways through which Cd^{2+} induces toxicity in PC12 cells. The presence of multiple metals in the environment and biological systems, and the possibility of simultaneous exposure to multiple metals have led to an increased interest in these studies. Currently, cytological and *in vivo* studies on the effects of simultaneous exposure to similar compounds, such as Cd^{2+} and Zn^{2+} , are limited. PC12 cell line is a rat pheochromocytoma clonal cell line, which has been used as a neuron model in molecular biology. Cd^{2+} is considered a potential etiological factor in neurodegenerative diseases [15], and the PC12 cell line has been selected as a neuron model to study the underlying mechanisms. The objective of this study was to understand the effects of Zn^{2+} on Cd^{2+} -induced toxicity, and to investigate its regulatory role in mechanisms underlying Cd^{2+} -induced apoptosis in PC12 cells. Finally, we aimed to unveil the underlying molecular mechanism by which Zn^{2+} inhibits Cd^{2+} -induced apoptosis after co-exposure by measuring cell viability, DNA integrity, leakage of lactate dehydrogenase,

intracellular levels of GSH, and changes in expression of apoptotic factors at the mRNA and protein level using PC12 cells.

2. Materials and methods

2.1. Materials

PC12 cells were purchased from the American Type Culture Collection (USA and Canada). Dulbecco's modified Eagle's medium (DMEM), ribonuclease A (RNase), ethidium bromide, and peroxidase-conjugated avidin were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was bought from HyClone (Rockville, MD, USA). SV total RNA isolation system and RT-PCR kit were purchased from Promega (Madison, WI, USA). High pure PCR product purification kit and proteinase K were purchased from Roche Diagnostics (Mannheim, Germany). Biotinylated goat anti-mouse IgG whole antibody and ECL western blotting detection reagent were purchased from Amersham Pharmacia Biotech (Buckinghamshire, England). Polyclonal antibodies against Bcl-2 (Cat#PC68, Oncogene), Bax (BIS, bs-0127R, BIOS), beta-actin (GTX 109639, GeneTEX) were purchased. Anti-cytochrome c monoclonal antibody was purchased from BD Biosciences Pharmingen (San Jose, CA, USA). Trypan blue solution (0.4%) was purchased from Bio-Rad (Hercules, CA, USA). The DNA 7500 assay and RNA 6000 nano assay kits were purchased from Agilent Technologies (Waldbronn, Germany). All other chemicals were of analytical grade.

2.2. Cell culture

PC12 cells were cultured in DMEM supplemented with 10% FBS in a humidified incubator at 37 °C with 5% CO₂. The cells were pre-incubated in 25-cm² flasks for 24 h; then, the medium was replaced with serum/serum-free DMEM with or without various concentrations of CdCl₂ and ZnCl₂, or with a mixture of both chemicals, and the cells were incubated for 48 h. When the medium was replaced with serum-free medium, cells were washed twice with serum-free DMEM. The desired concentration for treatment was selected by exposing PC12 cells to Cd²⁺ (0, 2, 5, 10, 20 μmol) and Zn²⁺ (0, 2, 5, 10, 20, 100, and 500 μmol) separately, and then the final combination was decided. The selected concentration for Cd²⁺ was 10 μmol, whereas Zn²⁺ was used at 10, 100, and 500 μmol.

2.3. Cell viability

Cell viability was determined using trypan blue exclusion assay. PC12 cells were seeded at a density of 1×10^5 cells/flask and pre-incubated for 24 h. Then, the cells were treated with Cd^{2+} (0, 2, 5, 10, and 20 $\mu\text{mol/L}$ and 0, 5, 10, 100, and 500 $\mu\text{mol/L}$) and Zn^{2+} (0, 2, 5, 10, and 20 $\mu\text{mol/L}$ and 10, 100, and 500 $\mu\text{mol/L}$) separately; in addition, they were co-exposed to Cd^{2+} (10 $\mu\text{mol/L}$) and Zn^{2+} (10, 100, and 500 $\mu\text{mol/L}$). The cells were then incubated for 48 h. Total cells and trypan blue-stained cells were counted using a Bio-Rad automated cell counter (Hercules, CA, USA). Cell viability was expressed as percentage of the counted trypan blue-stained cells. Each experiment was carried out at least in triplicate to ensure biological reproducibility and statistical validity.

2.4. Cytotoxicity assay

2.4.1. Lactate dehydrogenase (LDH) activity assay

Cytotoxicity was assessed by measuring the activity of LDH in the treatment medium using a nonradioactive cytotoxicity assay kit (Promega) as described by Kihara *et al.* [28]. PC12 cells (1×10^5 cells/flask) were cultured in the medium with/without Cd^{2+} (0, 10 $\mu\text{mol/L}$) or Cd^{2+} (10 $\mu\text{mol/L}$) + Zn^{2+} (10, 100, and 500 $\mu\text{mol/L}$) for 48 h. After 48-h incubation, 50 μL of the medium was transferred to a 1.5-mL tube, and then 50 μL of a substrate mixture containing tetrazolium salts was added to the tube. After 30-min incubation at room temperature (25 °C), 50 μL of the stop solution was added, and the amounts of formazan dye formed were determined by measuring the absorbance at 490 nm using a DU-65 spectrophotometer (Beckman, CA, USA). LDH activity was expressed as LDH activity/ 1×10^6 cells. This experiment was carried out in triplicate for ensuring reproducibility.

2.4.2. Measurement of intracellular free sulfhydryl (SH) levels

Intracellular free SH levels were investigated as previously described by Kihara *et al.* [28]. Cells (1×10^5) were pre-incubated for 24 h. Then, they were exposed to Cd^{2+} (10 $\mu\text{mol/L}$) or Cd^{2+} (10 $\mu\text{mol/L}$) + Zn^{2+} (10–500 $\mu\text{mol/L}$) for 48 h. The cells were harvested, washed with $1 \times$ phosphate-buffered saline (PBS), added to 150 μL of a lysis buffer, and then incubated at room temperature (25 °C) for 10 min. Two freeze-thaw sonication cycles were performed in order to rupture the cell membranes, and the resultant solution was centrifuged at 1,500 rpm for 10 min to collect the supernatant. The total protein contents were measured spectrophotometrically by

using protein assay dye reagent (Bio-Rad, Hercules, CA, USA). Intracellular free SH levels were determined using 2.5 $\mu\text{mol/L}$ 5,5'-dithiobis-2-nitrobenzoic acid (DTNB, pH 7). DTNB (final concentration; 200 $\mu\text{mol/L}$) was added to the cell lysate, and then the absorbance was measured at 412 nm by using a DU-65 spectrophotometer (Beckman, CA, USA). The concentration of free SH in PC12 cells was determined using a molecular coefficient factor of 13,600 per cell number (1×10^5). The experiment was carried out in triplicate to ensure mechanical reproducibility.

2.5. Isolation of genomic DNA from PC12 cells

After treatment of PC12 cells with various concentrations of CdCl_2 and ZnCl_2 , the genomic DNA was isolated using high pure PCR template preparation kit according to the manufacturer's instruction as described by Kawakami *et al.* (2008) [29]. After 48-h incubation, the cells were harvested using a scraper. Then, the obtained cells were centrifuged at 1,500 rpm for 5 min to remove the supernatant. After addition of 3 mL of PBS, the mixture was centrifuged again at 1,500 rpm for 5 min to wash the cells. The obtained solution containing DNA was mixed with 2 μL of 500 $\mu\text{g/mL}$ RNase and incubated for 15 min at 37 $^\circ\text{C}$. After incubation, 500 μL of ethanol and 20 μL of 3 M NaOAc buffer (pH 4.5) were added for ethanol precipitation, and the solution was allowed to stand overnight in a freezer to precipitate the DNA. On the next day, DNA was separated using microcentrifugation at 15,000 rpm for 8 min, and then washing with 70% ethanol at the same speed for 3 min was carried out. Then, the DNA sample was dried for approximately 10 min, and DNA concentration was measured after reaction with 1 $\times$ Tris/Borate/EDTA (TBE) using a UV-visible spectrophotometer.

2.6. Agarose gel electrophoresis of genomic DNA

The ladder pattern of DNA was analyzed *via* agarose gel electrophoresis. The DNA was collected from PC12 cells after treatment with Cd^{2+} (0, 2, 5, 10, and 20 $\mu\text{mol/L}$) and Zn^{2+} (0, 2, 5, 10, and 20 $\mu\text{mol/L}$) for 48 h. Approximately 3–5 μg of DNA with the loading dye was subjected to electrophoresis on a 1.5% agarose gel. Electrophoresis was carried out for 40 min at 100 V in 1.5% of agarose gel by using a submarine-type electrophoresis system (Mupid-ex, Advance, Tokyo, Japan). To visualize the DNA strand breaks, the gel was soaked in ethidium bromide solution for 5–10 min. Images of the agarose gel were taken under UV illumination using a ChemiDoc XRS (Bio-Rad, Hercules, CA, USA). To evaluate cell apoptosis, the fluorescence intensity of DNA in the gel was analyzed by a software named Quantity one. Apoptosis was

expressed as the ratio of DNA to the DNA ladder. This experiment was conducted at least in triplicate.

2.7. Determination of gene expression *via* RT-PCR

Gene expression of caspase 9 was detected using the access RT-PCR kit and access RT-PCR introductory kit according to the manufacturers' instructions. Total RNA in PC12 cells treated with 5 $\mu\text{mol/L}$ Cd^{2+} and 0, 2, 5 $\mu\text{mol/L}$ Zn^{2+} for 48 h was extracted using SV total RNA isolation kit. The PCR primers for caspase 9 used were similar to those described by [29] Kawakami *et al.* (2008). The detailed sequences of the primers, expected sizes of PCR products, annealing temperatures, and cycles are summarized in Table 1. The PCR conditions were as follows: 48 °C for 45 min and 94 °C for 2 min. The cycles were as follows: 94 °C for 30 s, 60 °C for 1 min, and 68 °C for 2 min. Final extension was carried out for 7 min at 68 °C. Forty cycles were performed, and an annealing temperature of 60 °C was used. β -Actin was selected as an internal control. PCR products were verified using a DNA 7500 assay with an Agilent 2100 Bio analyzer according to the manufacturer's instructions (Santa Clare, CA, USA). This experiment was carried out at least in triplicate.

2.8. Western blot analysis for determination of protein expression

Cytochrome c release in the cytosol was quantified using cytochrome c release apoptosis assay kit (Merk-Millipore, Darmstadt, Germany). PC12 cells were cultured in 5 mL of DMEM containing 10% FBS, 0, 10 $\mu\text{mol/L}$ Cd^{2+} , and 0, 10, 100, and 500 $\mu\text{mol/L}$ Zn^{2+} . After 48-h treatment, the cells were harvested and centrifuged at 1,500 rpm for 5 min to remove the supernatant. After addition of 10 mL of ice-cold PBS, the mixture was centrifuged again at 1,500 rpm for 5 min. After removal of the supernatants, the cells were resuspended in 150 μL of cytosol extraction buffer mix (1 $\times$ cytosol extraction buffer containing 1 mL of protease inhibitor cocktail and 2 mmol/L DTT). The mixture was allowed to stand on ice for 10 min, and subsequently the cells were disrupted by sonication for 30 s using a Sonicator 250 (Branson). To remove the unbroken cells, the lysed cells were centrifuged at 3,000 rpm for 5 min. The supernatant was transferred into a 1.5-mL tube and centrifuged at 11,000 rpm for 30 min. The obtained final supernatant was collected as the cytosolic fraction for cytochrome c analysis. For determination of Bcl-2, Bax, and Bcl-x, β -actin lysis buffer (100 mmol/L HEPES, 1 mol/L NaCl,

300 mmol/L EGTA, 0.1 M PMSF, 100 mmol/L Na₃VO₄, 10 mmol/L Na₂MgO₄, 100 mmol/L 2-glycerophosphoric acid, 1 mmol/L MgCl₂, 100 mmol/L DTT, 100 mmol/L NaF, and triton X-100) was used to collect the protein fraction after two cycles of sonication followed by centrifugation at 1500 rpm for 10 min. After that, the protein concentration was determined spectrophotometrically by using protein assay dye reagent (Bio-Rad, Hercules, CA, USA). Then, the equal amount (25 µg) of protein was separated via polyacrylamide gel electrophoresis (12.5–15%), and the electrophoresed proteins were transferred to nitrocellulose membranes with a semidry blotting system, type-AE6678 (ATTO, Tokyo, Japan). The membranes were incubated overnight at 4 °C in 5% skimmed milk as a blocking agent. The membranes were incubated for 60 min at 37 °C with the primary antibodies, washed three times, and then incubated with the secondary antibody for 60 min. After washing for five times (each 3 min), the protein bands were visualized using enhanced chemiluminescence. The images of the detected bands were analyzed using a ChemiDoc XRS (Bio-Rad, USA). Each experiment was conducted at least in triplicate to ensure reproducibility.

2.9. Statistical analysis

All data are expressed as the mean ± standard error of mean (SEM). Statistical analyses were performed using single-factor analysis of variance (ANOVA) followed by unpaired Student's *t*-test.

Table 1

Primers used in RT-PCR analyses of caspases 9 and β -actin.

Primer's name	Sequence (5'→3')	annealing site (bp)	Length (bp)	T _m (°C)	Cycle (number)
Bact F	ATGGATGACGATATCGCTG		19	61.1	40
Bact R	ATGAGGTAGTCTGTCAGGT		19	53.1	40
Casp 9F	CAAAGGAGCAGAAAGTAGTGAAG	411-433	23	60.95	40
Casp 9R	GAGGAAGGGCAGAAGTTCAC	687-668	20	62.73	40

3. Results

3.1. Cell viability

To examine whether zinc (Zn^{2+}) affected cadmium (Cd^{2+}) toxicity, the viability of PC12 cells exposed to 0, 10 $\mu\text{mol/L}$ Cd^{2+} and to 10, 100, and 500 $\mu\text{mol/L}$ Zn^{2+} for 48 h separately, as well as to a combination of both was measured *via* trypan blue staining (Fig. 1). The cell viability of PC12 cells was not reduced after treatment with Zn^{2+} 10 $\mu\text{mol/L}$ and 100 $\mu\text{mol/L}$ for 48 h, but significantly decreased upon exposure to Zn^{2+} 500 $\mu\text{mol/L}$ (Fig. S1). Cell viability significantly reduced after exposure to Cd^{2+} (10 $\mu\text{mol/L}$), whereas it significantly increased after co-exposure to Cd^{2+} (10 $\mu\text{mol/L}$) and Zn^{2+} (10, 100 $\mu\text{mol/L}$). However, it decreased upon exposure to Cd^{2+} (10 $\mu\text{mol/L}$) and Zn^{2+} (500 $\mu\text{mol/L}$) (Fig. 1). For comparison between the control, Cd^{2+} -, Zn^{2+} -, and Cd^{2+} + Zn^{2+} (1:1)-treatment groups, cell viability was also studied after 48-h exposure (data not shown). It was found that Zn^{2+} had no effect on cell viability (<100 $\mu\text{mol/L}$), whereas Cd^{2+} caused a significant decrease in cell viability (2, 5, 10, and 20 $\mu\text{mol/L}$), and the Cd^{2+} + Zn^{2+} -treatment group showed significantly higher cell viability than the Cd^{2+} -treatment group. The cell viability study confirmed that the combined exposure to Cd^{2+} and Zn^{2+} resulted in significantly higher cell viability than Cd^{2+} treatment only. On the basis of these results (Fig. 1), the treatment groups were decided as follows: control group (no treatment), Cd^{2+} -treatment group (10 $\mu\text{mol/L}$), Cd^{2+} (10 $\mu\text{mol/L}$) + Zn^{2+} (10 $\mu\text{mol/L}$)-treatment group, Cd^{2+} (10 $\mu\text{mol/L}$) + Zn^{2+} (100 $\mu\text{mol/L}$)-treatment group, Cd^{2+} (10 $\mu\text{mol/L}$) + Zn^{2+} (500 $\mu\text{mol/L}$)-treatment group.

3.2. LDH activity after combined exposure to Cd^{2+} and Zn^{2+}

To investigate the combined effects of Cd^{2+} and Zn^{2+} on cytotoxicity, LDH activity assay was carried out in the culture medium of PC12 cells after treatment with/without Cd^{2+} (10 $\mu\text{mol/L}$) and Zn^{2+} (10, 100, and 500 $\mu\text{mol/L}$). The results showed that there was a significant difference between the treatment groups ($p < 0.05$). LDH activity significantly increased after treatment with 10 $\mu\text{mol/L}$ Cd^{2+} only (Fig. 2). However, after combined exposure to 10 $\mu\text{mol/L}$ Cd^{2+} along with 10 and 100 $\mu\text{mol/L}$ Zn^{2+} , a reduction in LDH activity was observed, and increase in Zn^{2+} concentration to 500 $\mu\text{mol/L}$ resulted in further increase in LDH activity. These results suggested that Zn^{2+} had protective effects against the cytotoxicity induced by Cd^{2+} (10 $\mu\text{mol/L}$) in PC12 cells up to a certain concentration. Our findings are in agreement with the results of the cell viability study (Fig. 1). In addition, to confirm whether effects of Zn^{2+} on Cd^{2+} toxicity are observed in other cell line, we have done same experiments using HepG2 cell, a well-

differentiated hepatocellular carcinoma cell. As shown in Fig. S2, Zn^{2+} has recovered Cd^{2+} toxicity in HepG2 cells as well as PC12 cells.

3.3. Effects of simultaneous exposure of Cd^{2+} and Zn^{2+} on intracellular free SH levels

Glutathione (GSH), a major thiol component of the cellular antioxidant system, has been shown to play an important role in protecting the cells against ROS, such as free radicals and peroxides [30]. GSH is usually oxidized to GSSG after stress stimuli, particularly oxidative stress. To examine the stress status in PC12 cells after combined exposure of Cd^{2+} and Zn^{2+} , intracellular free SH levels were determined in cell lysates after treatment with/without Cd^{2+} and Zn^{2+} separately and in combinations. A significant reduction in free SH levels was observed after treatment with Cd^{2+} (10 $\mu\text{mol/L}$) compared to the control group (Fig. 3). After combined exposure to Cd^{2+} (10 $\mu\text{mol/L}$) and Zn^{2+} (10 or 100 $\mu\text{mol/L}$), the levels of intracellular free SH increased significantly ($p < 0.05$) compared to the Cd^{2+} (10 $\mu\text{mol/L}$)-treatment group alone (Fig. 3). However, free SH level in the Cd^{2+} (10 $\mu\text{mol/L}$) + Zn^{2+} (500 $\mu\text{mol/L}$)-treatment group decreased to the same level as in the Cd^{2+} (10 $\mu\text{mol/L}$) treatment group. These results indicated that Zn^{2+} (up to 100 $\mu\text{mol/L}$) could reduce oxidative stress induced by Cd^{2+} in PC12 cells. This finding is also in accordance with the cell viability results (Fig. 1).

3.4. DNA fragmentation analysis using agarose gel electrophoresis

To clarify whether apoptosis was induced in PC12 cells treated with Cd^{2+} , and to examine the effect of Zn^{2+} on Cd^{2+} toxicity, the genomic DNA extracted from PC12 cells treated with various concentrations of Cd^{2+} (0, 2, 5, 10, and 20 $\mu\text{mol/L}$) and/or Zn^{2+} (0, 2, 5, 10, and 20 $\mu\text{mol/L}$) for 48 h was electrophoresed using 1.5% agarose gel. The morphological characteristics of apoptosis are frequently accompanied by multiple cleavages of DNA resulting in fragments of 180–200 base pairs. The oligonucleosomal fragments can be visualized as a characteristic DNA ladder following agarose gel electrophoresis [31]. This DNA ladder pattern was observed for the cells treated with more than 5 $\mu\text{mol/L}$ Cd^{2+} (Fig. 4A). These results indicated that apoptosis was induced by Cd^{2+} . In addition, DNA ladder pattern was significantly reduced upon addition of Zn^{2+} (1:1) in the medium containing Cd^{2+} (Fig. 4B), which confirmed the suppressive effects of Zn^{2+} on Cd^{2+} -induced cytotoxicity and/or apoptosis. To evaluate the degree of apoptosis in the

cell, the fluorescence intensity of DNA in the gels was analyzed. The relative intensity of DNA ladder after treatment with $\text{Cd}^{2+} + \text{Zn}^{2+}$ was higher than that after treatment with Cd^{2+} (0, 2, 5, 10, and 20 $\mu\text{mol/L}$) only (Fig. 4C). These results indicated that apoptosis induced by Cd^{2+} (5, 10, and 20 $\mu\text{mol/L}$) was suppressed by Zn^{2+} (2, 5, 10, and 20 $\mu\text{mol/L}$).

3.5. Western blot analysis for determination of protein expression

To examine whether Zn^{2+} could affect the proapoptotic environment induced by Cd^{2+} treatment, western blot analysis of the lysate of PC12 cells treated with Cd^{2+} (10 $\mu\text{mol/L}$) or with Cd^{2+} (10 $\mu\text{mol/L}$) + Zn^{2+} (10, 100, and 500 $\mu\text{mol/L}$) was performed to assess the protein expression of Bax, Bcl-2, Bcl-x, β -actin, and cytochrome c. The western blot results are shown in Fig. 5(A-D). As shown in Fig. 5A, the proapoptotic Bax significantly increased after treatment with 10 $\mu\text{mol/L}$ Cd^{2+} compared to the control group. However, after combined exposure to Cd^{2+} (10 $\mu\text{mol/L}$) + Zn^{2+} (10, 100 $\mu\text{mol/L}$), Bax protein expression was reduced, whereas it increased again after co-treatment with Cd^{2+} (10 $\mu\text{mol/L}$) + Zn^{2+} (500 $\mu\text{mol/L}$). In addition, the levels of the antiapoptotic protein, Bcl-2 were relatively unchanged after both treatments (Fig. 5C); however, the levels of Bcl-x were found to be lower in the Cd^{2+} (10 $\mu\text{mol/L}$) + Zn^{2+} (10, 100 $\mu\text{mol/L}$)-treatment group compared to the Cd^{2+} (10 $\mu\text{mol/L}$)-treatment group and Cd^{2+} (10 $\mu\text{mol/L}$) + Zn^{2+} (500 $\mu\text{mol/L}$)-treatment group. From these results, it was supposed that there was a net imbalance in Bax/Bcl-2 ratio, which indicated the occurrence of apoptosis in PC12 cells after treatment with Cd^{2+} , whereas co-exposure to Cd^{2+} and Zn^{2+} resulted in reduction in proapoptotic Bax protein level. Consequently, the release of cytochrome c into the cytosol was also investigated (Fig. 5D). Results showed that the cells treated with Cd^{2+} (10 $\mu\text{mol/L}$) only exhibited high content of cytochrome c, which was reduced after co-treatment with Cd^{2+} (10 $\mu\text{mol/L}$) + Zn^{2+} (10, 100 $\mu\text{mol/L}$); however, co-exposure to Cd^{2+} (10 $\mu\text{mol/L}$) + Zn^{2+} (500 $\mu\text{mol/L}$) further increased cytochrome c levels. From these results, it was suggested that Cd^{2+} mainly induced the mitochondrial pathway of apoptosis, and Zn^{2+} suppressed Cd^{2+} -induced cytochrome c release upon simultaneous exposure. To investigate the effects of the low exposure level, we examined the effects of co-exposure to Cd^{2+} (5 $\mu\text{mol/L}$) + Zn^{2+} (2, 5, 10, and 20 $\mu\text{mol/L}$). We observed that Zn^{2+} (2, 5, 10, and 20 $\mu\text{mol/L}$) suppressed cytochrome c release induced by Cd^{2+} (5 $\mu\text{mol/L}$) (data not shown). Thus, it was confirmed that Cd^{2+} induced

apoptosis in PC12 cells *via* activation of the mitochondrial pathway, whereas Zn^{2+} (10, 100 $\mu\text{mol/L}$) had a suppressive effect upon co-exposure with Cd^{2+} (5, 10 $\mu\text{mol/L}$).

3.6. Expression of caspase 9

To investigate the mechanism of apoptosis induced by Cd^{2+} and the effects of Zn^{2+} on the apoptotic pathways, changes in apoptotic factors of PC12 cells after treatment with Cd^{2+} and Zn^{2+} were determined using RT-PCR. The mRNA expression levels of caspase 9 in PC12 cells treated with Cd^{2+} (0, 5 $\mu\text{mol/L}$) and Zn^{2+} (2, 5 $\mu\text{mol/L}$) for 48 h are shown in Fig. 6. Caspase 9 mRNA levels were significantly ($p < 0.05$) higher in PC12 cells treated with Cd^{2+} (5 $\mu\text{mol/L}$) compared to those in the control. Co-treatment with Zn^{2+} (2, 5 $\mu\text{mol/L}$) and Cd^{2+} (5 $\mu\text{mol/L}$) resulted in the significant ($p < 0.05$) reduction in caspase 9 mRNA levels to the same level as that in the control cells.

4. Discussion

This study was conducted to test the hypothesis that co-exposure to the essential trace element, Zn^{2+} , will antagonize the cytotoxicity and apoptosis induced by the toxic heavy metal Cd^{2+} in PC12 cells, a widely studied neuron model. The two metals were selected because of their similar chemical characteristics and the high possibility of co-exposure. The present study showed that Cd^{2+} (5 $\mu\text{mol/L}$) induced apoptosis in PC12 cells. Similar results have been observed in previous studies using HeLa cells and BAECs [16]. Cd^{2+} -induced DNA fragmentation and formation of apoptotic bodies have been observed by several investigators under different experimental conditions, including Cd^{2+} -induced apoptosis in the testis of freshwater crab [32] and in pancreatic β -cells [33]. Our results also showed DNA fragmentation in PC12 cells exposed to Cd^{2+} (Fig. 4). Various mechanisms could be involved in Cd^{2+} -induced DNA damage, including activation of Ca^{2+} -dependent endonucleases resulting in DNA fragmentation and interference with the DNA repair process [13, 15, 34, 35]. In addition, Cd^{2+} toxicity could be partly due to oxidative stress induced by ROS produced in the cells in response to metal ions [36, 37]. Cd^{2+} induces the expression of hemeoxygenase enzyme, which generates ROS, in various cell types [38, 39]. Some evidence suggests that Cd^{2+} -MT complexes can induce ROS, which in turn cause DNA damage in cells [40]. Intracellular levels of free SH groups determined in this study confirmed that Cd^{2+} (10 $\mu\text{mol/L}$) exposure induces oxidative stress, and that co-exposure to Zn^{2+} (10 or 100 $\mu\text{mol/L}$) prevented this effect (Fig. 3). In our study, co-

exposure to Zn^{2+} (10 or 100 $\mu\text{mol/L}$) significantly increased the level of GSH relative to that found in cells treated with Cd^{2+} only. A further increase in Zn^{2+} concentration (to 500 $\mu\text{mol/L}$) reduced the level of GSH. These results indicate that Cd^{2+} -induced oxidative stress decreases in the presence of Zn^{2+} in PC12 cells, which confirms the antioxidant effects of Zn^{2+} in cells exposed to Cd^{2+} . The reduction of Cd^{2+} -induced oxidative stress by Zn^{2+} may also be due to the combined influence of both metal ions as described in a previous study [22]. Supplementation with Zn^{2+} may also diminish the inhibitory potency of Cd^{2+} on the antioxidant enzymes SOD, peroxidase, and catalase [20, 40]. Furthermore, LDH activity in cell culture medium significantly increased after treatment with Cd^{2+} (10 $\mu\text{mol/L}$). Simultaneous exposure to Zn^{2+} (10 or 100 $\mu\text{mol/L}$) and Cd^{2+} (10 $\mu\text{mol/L}$) markedly inhibited leakage of LDH into the culture medium of PC12 cells (Fig. 2). Again, increasing the concentration of Zn^{2+} to 500 $\mu\text{mol/L}$ increased the leakage of LDH into the culture medium. These findings suggest that Zn^{2+} might act as a scavenger of ROS produced by Cd^{2+} ; alternatively, Zn^{2+} could compete with Cd^{2+} for accumulation sites in PC12 cells. The viability of PC12 cells exposed to Cd^{2+} decreases in a concentration-dependent manner (data not shown). However, when cells are co-exposed to Zn^{2+} (10 or 100 $\mu\text{mol/L}$), their viability significantly increases (Fig. 1). A further increase in Zn^{2+} concentration (to 500 $\mu\text{mol/L}$) synergistically promoted Cd^{2+} (10 $\mu\text{mol/L}$)-induced apoptosis. Similarly, DNA fragmentation caused by Cd^{2+} was suppressed by addition of Zn^{2+} (Fig. 4). These results suggest that apoptosis induced by Cd^{2+} in PC12 cells is inhibited by the addition of Zn^{2+} up to 100 $\mu\text{mol/L}$. This finding agrees with the results of previous studies, which showed that addition of Zn^{2+} significantly inhibits Cd^{2+} -induced apoptosis in HeLa cells and reduces ROS production in BAECs [16]. Previous studies have also shown that Zn^{2+} ions can inhibit DNA fragmentation and apoptosis induced by other stimuli in multiple *in vivo* and *in vitro* biological systems [16, 22, 24].

To clarify the mechanism by which Zn^{2+} suppresses Cd^{2+} -induced apoptosis, western blot (Fig. 5A-D) and RT-PCR (Fig. 6) analyses were carried out. The results indicated that Cd^{2+} (10 $\mu\text{mol/L}$)-mediated increased expression of caspase 9 and mitochondrial release of cytochrome c were suppressed by addition of Zn^{2+} (2, 5, 10 or 100 $\mu\text{mol/L}$). Apoptosis can be triggered by the activation of death receptors (extrinsic pathway) or induction of mitochondria-mediated signaling pathways (intrinsic pathway). The intrinsic apoptotic pathway is regulated by members of the Bcl-2 protein family, which also play a central role in regulating mitochondrial membrane

permeability [41, 42]. Cytochrome c is a key regulator of the intrinsic pathway, activating caspase-dependent apoptosis. Release of cytochrome c from the mitochondria to the cytosol depends on certain factors, including mitochondrial membrane potential (MMP) and membrane integrity. The proapoptotic Bcl-2 family member Bax can interact with the mitochondrial permeability transition (MPT) pore components, particularly the voltage-dependent anion channel (VDAC), to release cytochrome c into the cytosol. In addition, it can alter the MMP and initiate swelling of mitochondrial organelles. Under conditions of cellular homeostasis, cytochrome c binds to the inner mitochondrial membrane in association with the anionic phospholipid, cardiolipin. Studies have suggested that dissociation of cardiolipin from cytochrome c could be the critical step in its release into the cytosol [43, 44]. Several studies have shown that changes in levels or chemical structure of cardiolipin can create a soluble pool of cytochrome c within the mitochondrial intermembrane space, which can promote release of cytochrome c into the cytosol upon permeabilization of the outer mitochondrial membrane [45, 46]. In our study, the increased level of cytochrome c in the cytosol upon Cd^{2+} (10 $\mu\text{mol/L}$) exposure suggests that Cd^{2+} influences MMP in PC12 cells. The release of cytochrome c from mitochondria into the cytosol accelerates activation of caspase 9, initiating a downstream caspase cascade, ultimately leading to cell death [15, 26]. In this study cytosolic levels of the proapoptotic proteins Bax and cytochrome c increased upon Cd^{2+} (10 $\mu\text{mol/L}$) exposure, and co-treatment with Zn^{2+} (10 or 100 $\mu\text{mol/L}$) significantly suppressed these increases. A further increase in Zn^{2+} concentration to 500 $\mu\text{mol/L}$ has an opposite effect in Cd^{2+} (10 $\mu\text{mol/L}$)-treated cells. One explanation is that co-treatment with Zn^{2+} might ameliorate Cd^{2+} -induced production of excess ROS in PC12 cells. Previous studies reported similar effects in Zn^{2+} on Cd^{2+} exposure in different cell lines [16, 23, 24].

In the present study, we showed the suppressive effects of Zn^{2+} on Cd^{2+} -induced apoptosis in PC12 cells for the first time. Zn^{2+} is a redox-inactive metal, which is considered to be an important part of the cellular antioxidant system. The ability of Zn^{2+} to maintain cellular redox balance can be attributed to several possible molecular mechanisms, including regulation of oxidant production, induction of oxidative damage [23], induction of the Zn^{2+} -binding protein, MT [24], involvement in regulation of GSH metabolism [47], and direct or indirect regulation of redox signaling [20]. The normal cellular amount of Zn^{2+} is very small, and most of it strongly binds to proteins and other molecules, including GSH. One study showed that 5–10 pmol of Zn^{2+}

is present in the cytosol and nucleus of resting PC12 cells [15]. Zn^{2+} can modulate cellular oxidant production, and bind to SH groups to protect them from oxidation. In our present study, Cd^{2+} (10 $\mu\text{mol/L}$) induced oxidative stress, leading to the cell death and this may be due to displacement of Zn^{2+} bound to thiol groups (Figs. 1 and 2). Co-treatment with Zn^{2+} (10 or 100 $\mu\text{mol/L}$) suppressed cell death and LDH activity relative to that in cells treated with Cd^{2+} only, possibly due to the increased supply of Zn^{2+} . Zn^{2+} may also modulate oxidant levels by binding to SH groups of thiols. In addition, it may enhance/initiate synthesis of metal binding proteins such as MT. In neural cells, Cd^{2+} exposure is reported to significantly decreases Zn^{2+} content in proteins, hinder the enzymatic activity of Cu/Zn-SOD, and induce significant conformational changes in proteins that ultimately lead to neural cell apoptosis [48]. In this study, we observed a significant increase in levels of GSH in co-treated cells (Cd^{2+} 10 $\mu\text{mol/L}$ + Zn^{2+} 10 or 100 $\mu\text{mol/L}$) compared to that in Cd^{2+} (10 $\mu\text{mol/L}$)-treated cells. Therefore, Zn^{2+} (10 or 100 μmol) supplementation effectively protects PC12 cells against Cd^{2+} (10 $\mu\text{mol/L}$)-induced oxidative stress, possibly through modulation of intracellular GSH levels. Moreover, Cd^{2+} can induce MT synthesis, resulting in the formation of intracellular Cd^{2+} -MT complexes, which can cause apoptosis. Similar to Cd^{2+} , Zn^{2+} exposure can also initiate synthesis of MT. Although we did not investigate the involvement of metal-binding proteins/chaperones, this possibility cannot be neglected. It can be speculated from our study that co-treatment of PC12 cells with Cd^{2+} and Zn^{2+} can affect the formation and chemistry of MT. Additional studies are needed to evaluate this hypothesis. Suzuki *et al.* [49] showed that co-treatment with Cd^{2+} and Zn^{2+} salts might increase synthesis of MT in different organs, including the liver, kidneys, and pancreas, relative to that reported for treatment with Cd^{2+} or Zn^{2+} alone. When cells are exposed to Cd^{2+} and Zn^{2+} simultaneously, both metals compete for MT binding sites [7]. We measured caspase mRNA levels after exposure to Cd^{2+} , or Cd^{2+} and Zn^{2+} . Caspase 9 mRNA levels significantly ($p < 0.05$) increased after treatment with Cd^{2+} (5 $\mu\text{mol/L}$); however, the levels decreased significantly ($p < 0.05$) upon co-treatment of Cd^{2+} -treated cells with Zn^{2+} (2 or 5 $\mu\text{mol/L}$). Thus, Zn^{2+} effectively inhibited Cd^{2+} -induced expression of caspase 9, possibly explaining its mechanism of inhibiting Cd^{2+} -induced apoptosis in PC12 cells. Previous studies have shown similar results, suggesting that Zn^{2+} can inhibit maturation of caspase 3, an apoptotic protease, which acts upstream of the endonuclease in apoptotic cell death [22, 50]. In addition, Kumari *et al.* (2011) [51] showed that prepubertal dietary Zn^{2+} deficiency induced apoptotic changes in the testes of

Wistar rats. Our results indicate that Cd^{2+} induces apoptosis *via* the mitochondrial pathway, and Zn^{2+} supplementation inhibits this induced cell death process. It has also been reported that Zn^{2+} can inhibit activation of caspase 9 in different cells [22, 23, 52]. Additionally, it is known that Zn^{2+} reduces Cd^{2+} accumulation in biological systems both *in vivo* and *in vitro* [15–24]. Low concentrations of Zn^{2+} may inhibit activation of caspase 9, while high concentrations inhibit accumulation of Cd^{2+} in cells. Therefore, our findings confirm the antioxidative effects of Zn^{2+} against Cd^{2+} -induced oxidative stress and its inhibitory effects against Cd^{2+} -induced apoptosis in PC12 cells.

5. Conclusion

Our present findings indicate that Zn co-treatment protected PC12 cells against Cd induced oxidative stress, loss of cell membrane integrity, DNA fragmentation, and ultimately, apoptotic cell death via the mitochondrial pathway. In addition, Zn^{2+} and Cd^{2+} may cooperatively induce synthesis of metal binding proteins/chaperones to neutralize the possible adverse effects of Cd^{2+} in neuron-like PC12 cells.

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Figure legends

Fig. 1. Viability of PC12 cells with and without $\text{Cd}^{2+}/\text{Cd}^{2+} + \text{Zn}^{2+}$ treatment at different concentrations after 48-h incubation measured *via* trypan blue staining method. Each experiment was conducted five times independently to ensure biological reproducibility. Cells co-exposed to 10 $\mu\text{mol/L}$ Cd^{2+} and 10–500 $\mu\text{mol/L}$ Zn^{2+} . Error bars indicate the mean $\pm$ SEM ($n = 5$), * denotes significance at $p < 0.05$ compared to control. There is also a significant difference ($p < 0.05$) between a and b.

Fig. 2. LDH activity in the culture medium of PC12 cells with and without $\text{Cd}^{2+}/\text{Cd}^{2+} + \text{Zn}^{2+}$ treatment at different concentrations after 48 h measured by non-radiative cytotoxicity assay kit. Each experiment was conducted three times for reproducibility. Error bars indicate mean $\pm$ SEM ($n=3$). Error bars indicate the mean $\pm$ SEM ($n = 3$), * denotes significance at $p < 0.05$ compared to control. There is also a significant difference ($p < 0.05$) between a and b.

Fig. 3. Intracellular free-SH levels in PC12 cells with and without $\text{Cd}^{2+}/\text{Cd}^{2+} + \text{Zn}^{2+}$ treatment at different concentrations after 48 h determined *via* DTNB assay method. Each experiment was carried out three times independently for ensuring reproducibility. Error bars indicate the mean $\pm$ SEM ($n = 3$), * denotes significance at $p < 0.05$ compared to control. There is also a significant difference ($p < 0.05$) between a and b.

Fig. 4. Agarose gel electrophoresis of genomic DNA extracted from PC12 cells cultured in the medium with and without $\text{Cd}^{2+}/\text{Cd}^{2+} + \text{Zn}^{2+}$ for 48 h. It was conducted four times to confirm biological reproducibility. A) DNA from the cells treated with 0–20 $\mu\text{mol/L}$ Cd^{2+} , B) DNA from the cells treated with 0–20 $\mu\text{mol/L}$ Cd^{2+} and 0–20 $\mu\text{mol/L}$ Zn^{2+} , C) the fluorescent intensity of genomic DNA extracted from PC12 cells cultured in the medium for 48 h after treatment with 0–20 $\mu\text{mol/L}$ Cd^{2+} and 0–20 $\mu\text{mol/L}$ Zn^{2+} . Lane M in the Figs. A and B means λ DNA digested with *Hind*III for the marker. The values are calculated following formula; (DNA/ DNA and DNA ladder) $\times 100$. Error bars indicate mean $\pm$ S.E.M. ($n=4$). Significant difference between a and b is observed ($P < 0.05$).

Fig. 5. Western blot analyses for the relative contents of A; Bax, B; Bcl2, C; Bclx and D; cytochrome c 48 h after treatment with and without $\text{Cd}^{2+}/\text{Cd}^{2+} + \text{Zn}^{2+}$ treatment. Each experiment was conducted three times separately to ensure reproducibility. Immunostaining bands are present above the bar diagram for respective protein. M means Error bars indicate the mean $\pm$ SEM ($n = 3$), * denotes significance at $p < 0.05$ compared to control. There is also a significant difference ($p < 0.05$) between a and b.

Fig. 6. Expression levels of caspase 9 mRNA in PC12 cells cultured in the medium containing 5 $\mu\text{mol/L}$ Cd^{2+} and 0–5 $\mu\text{mol/L}$ Zn^{2+} for 48 h using of RT-PCR method. Expression level of caspase 9 mRNA is expressed as nmol / nmol of β -actin which used as an internal control. Error bars indicate the mean $\pm$ SEM ($n = 4$), * denotes significance at $p < 0.05$ compared to control. There is also a significant difference ($p < 0.05$) between a and b.

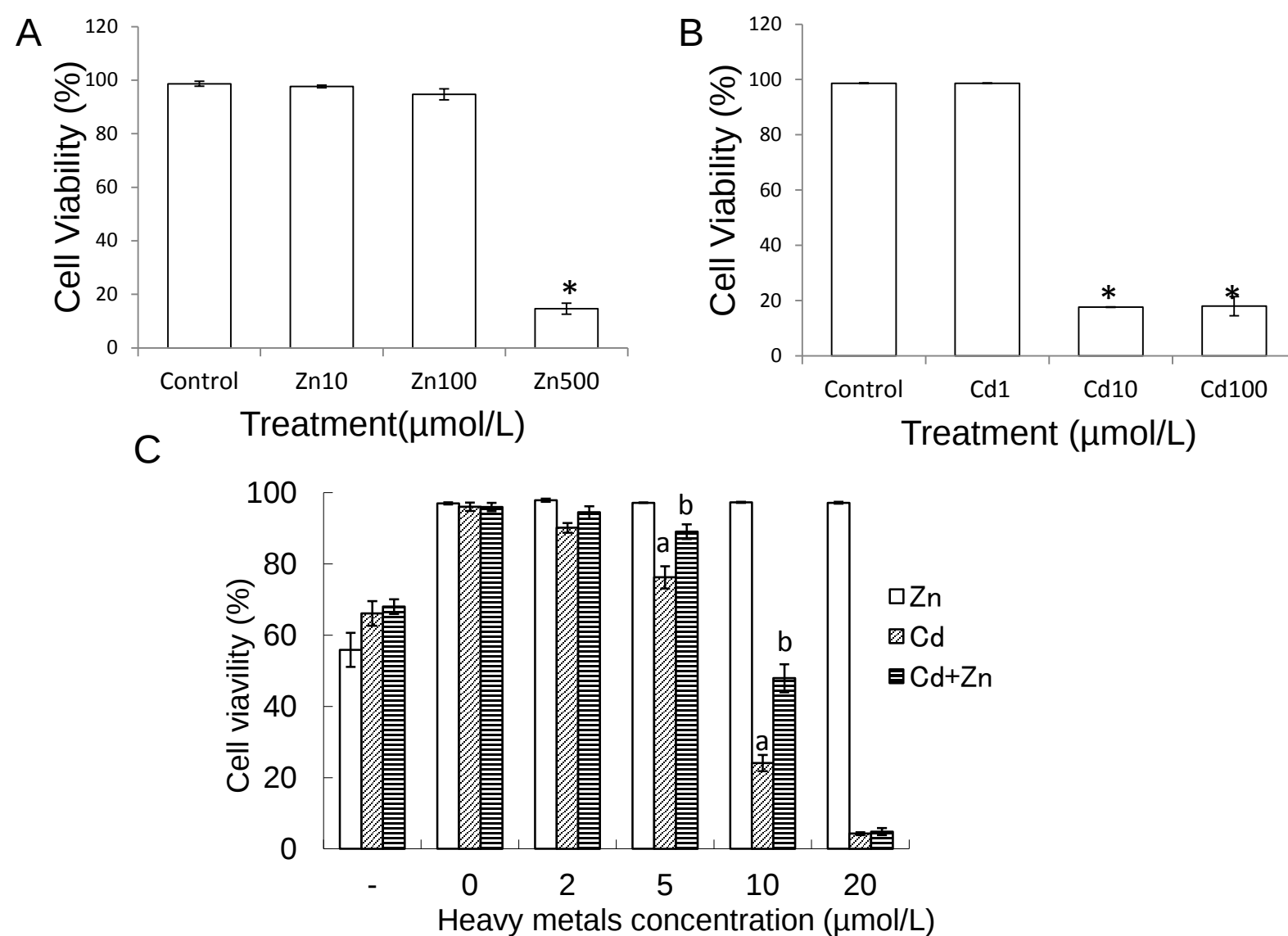


Fig. S1. Cell viability of PC12 cells exposed to different concentration of Cd^{2+} (A) , Zn^{2+} (B) and Cd^{2+} , Zn^{2+} and both (C) for 48 h. Each experiment was conducted at least three times for reproducibility. Error bars indicate mean $\pm$ SEM (n=4). * denotes significance at $p < 0.05$ compared to control. There is also a significant difference ($p < 0.05$) between a and b.

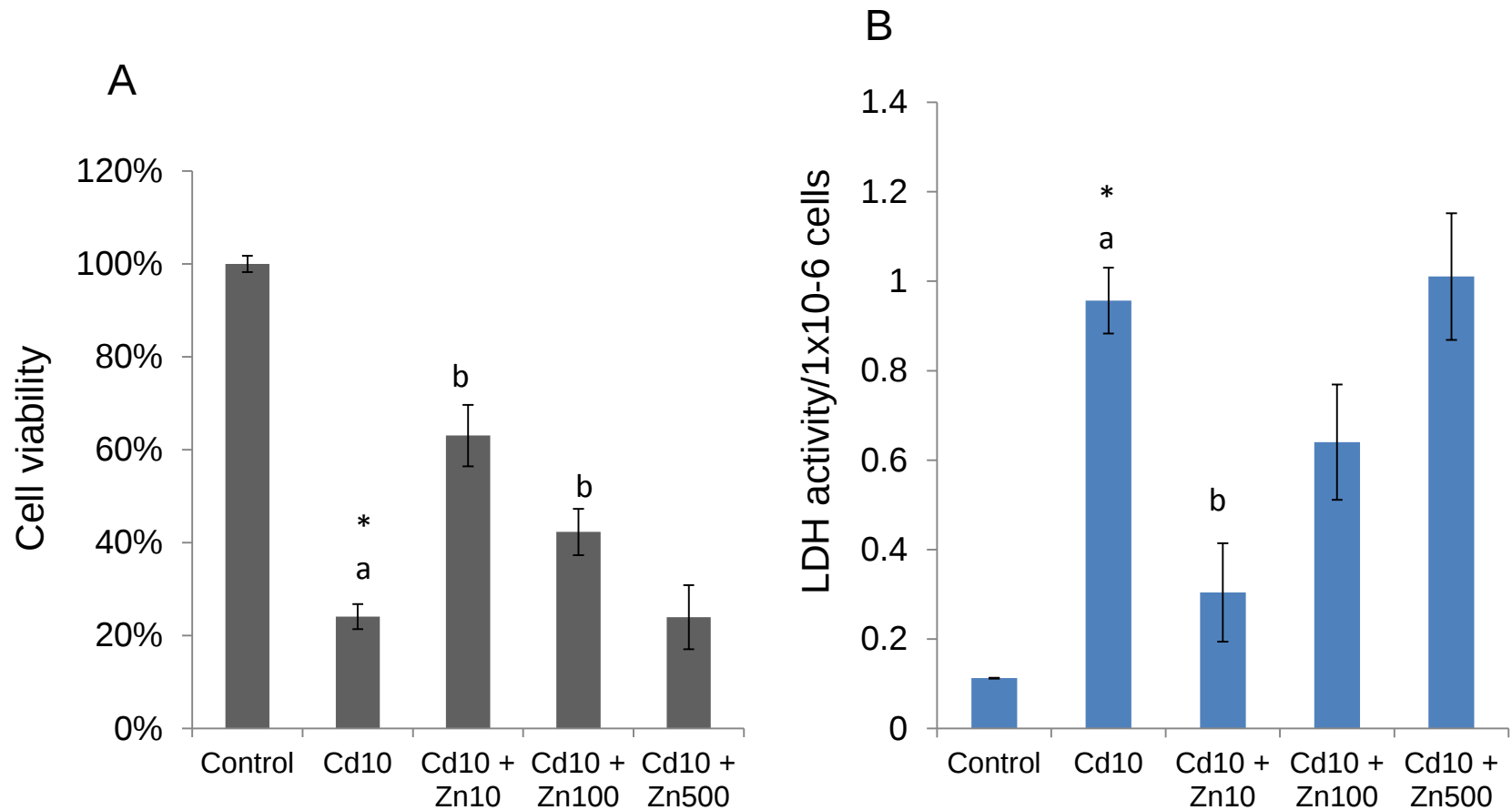


Fig. S2 Viability (A) and LDH activity (B) of HepG2 cells with and without Cd²⁺/Cd²⁺ + Zn²⁺ treatment at different concentrations after 48-h. Each experiment was conducted three times independently to ensure biological reproducibility. Cells co-exposed to 10 μ mol/L Cd²⁺ and 10–500 μ mol/L Zn²⁺. Error bars indicate the mean $\pm$ SEM, * denotes significance at $p < 0.05$ compared to control. There is also a significant difference ($p < 0.05$) between a and b.

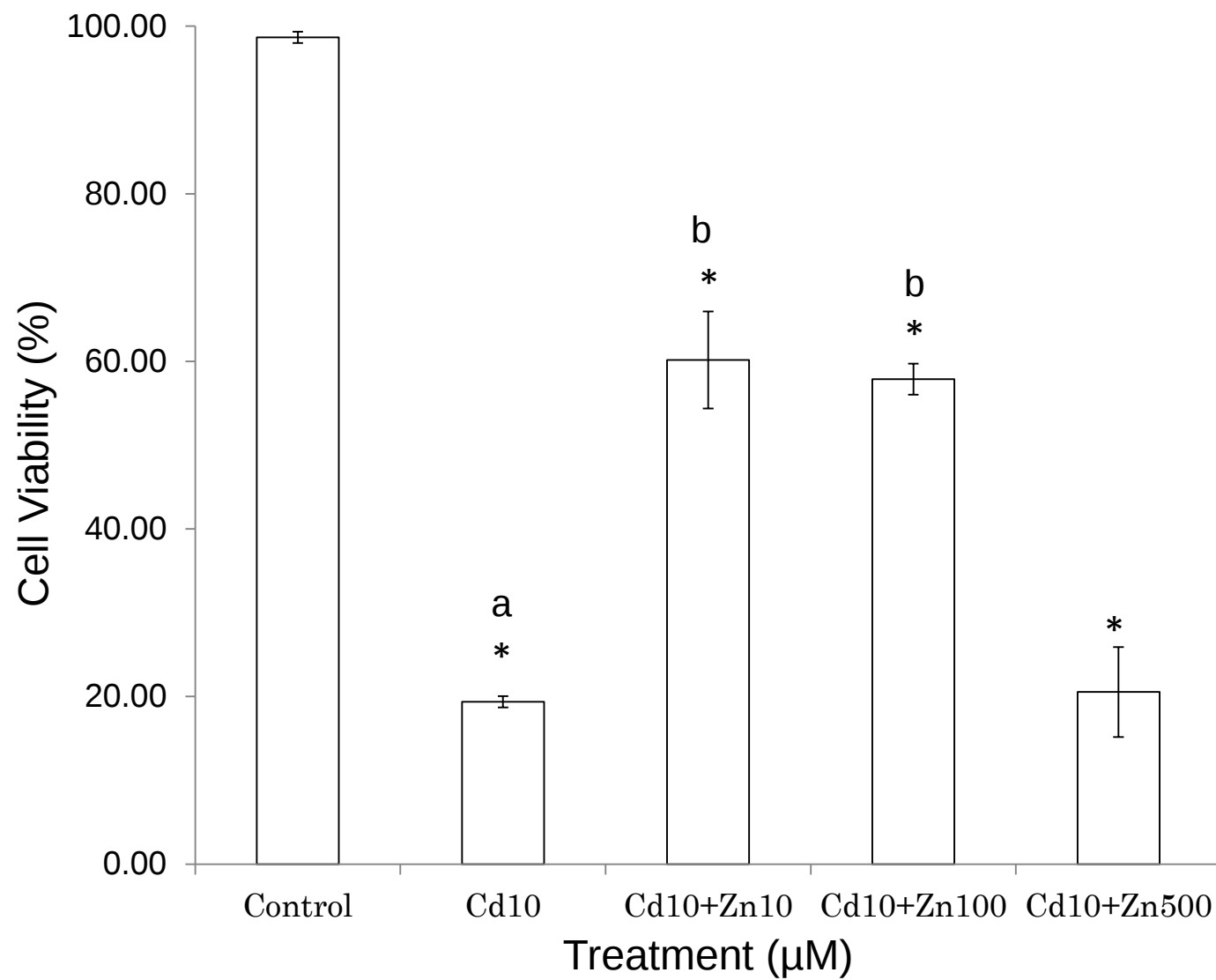


Fig. 1

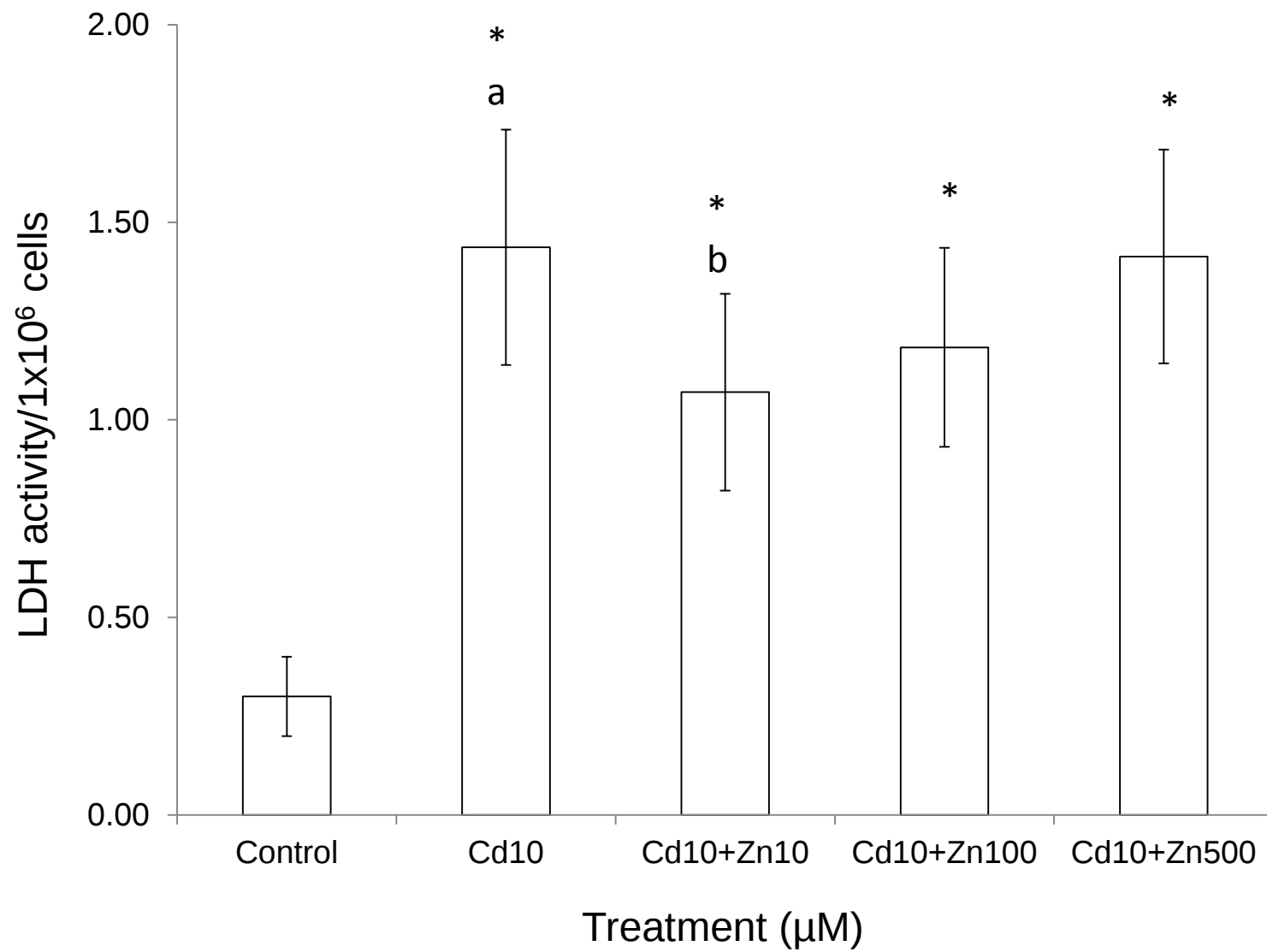


Fig. 2

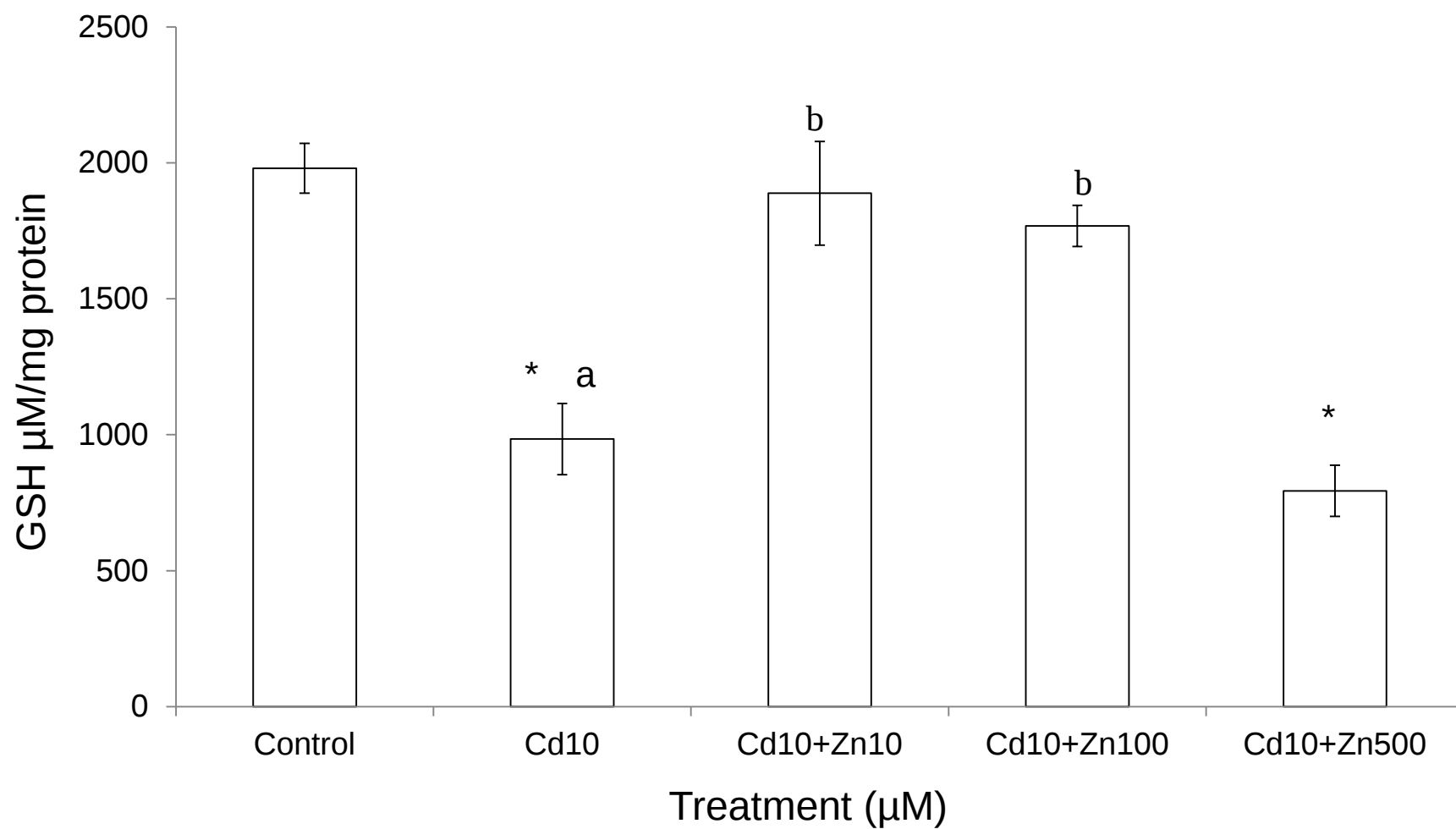


Fig. 3

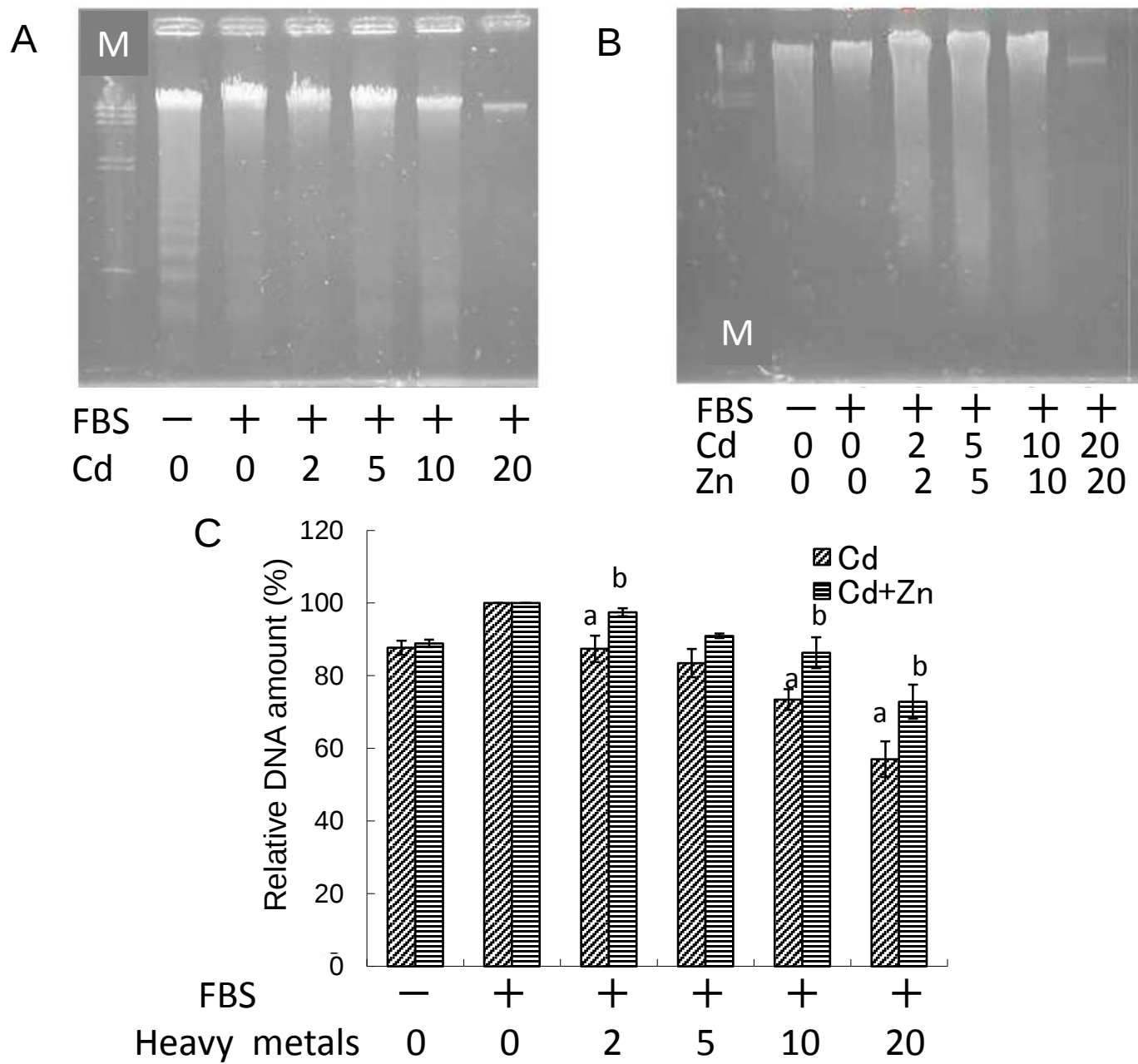


Fig. 4

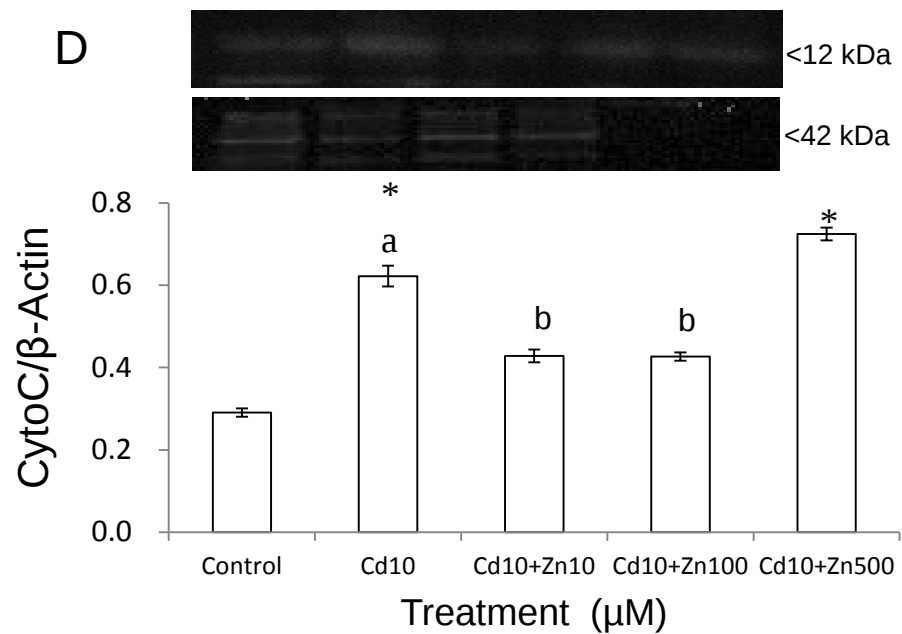
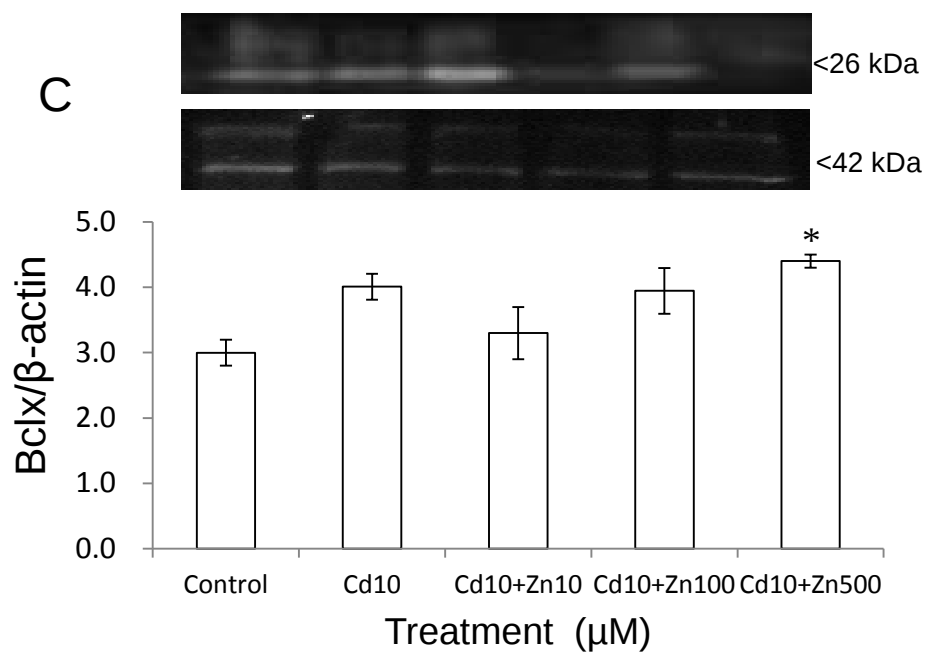
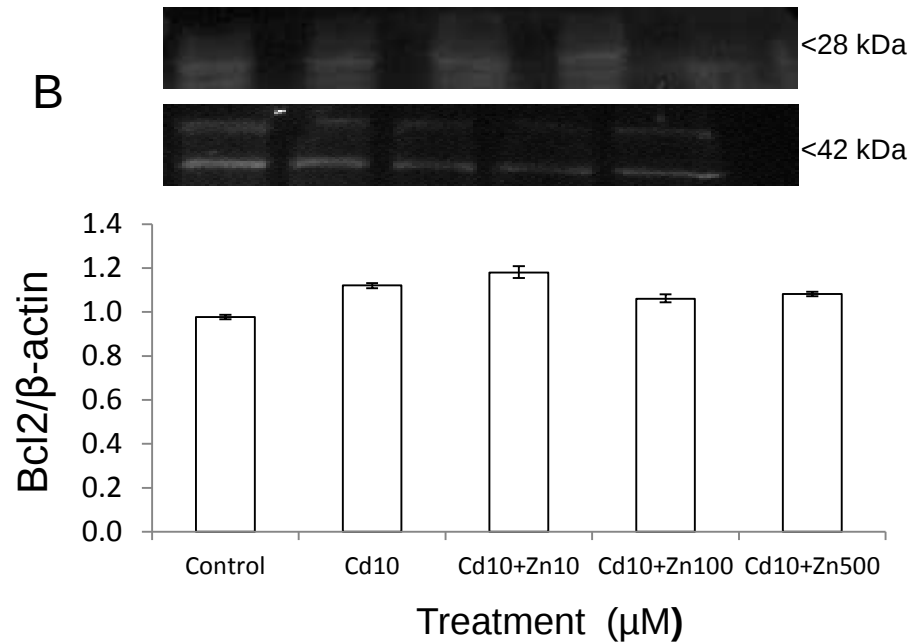
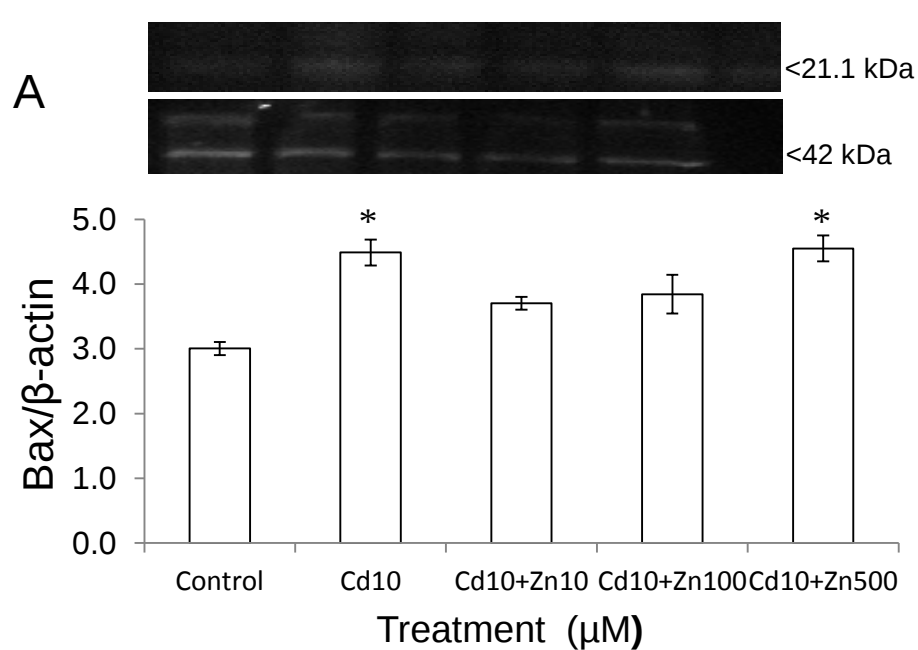


Fig. 5

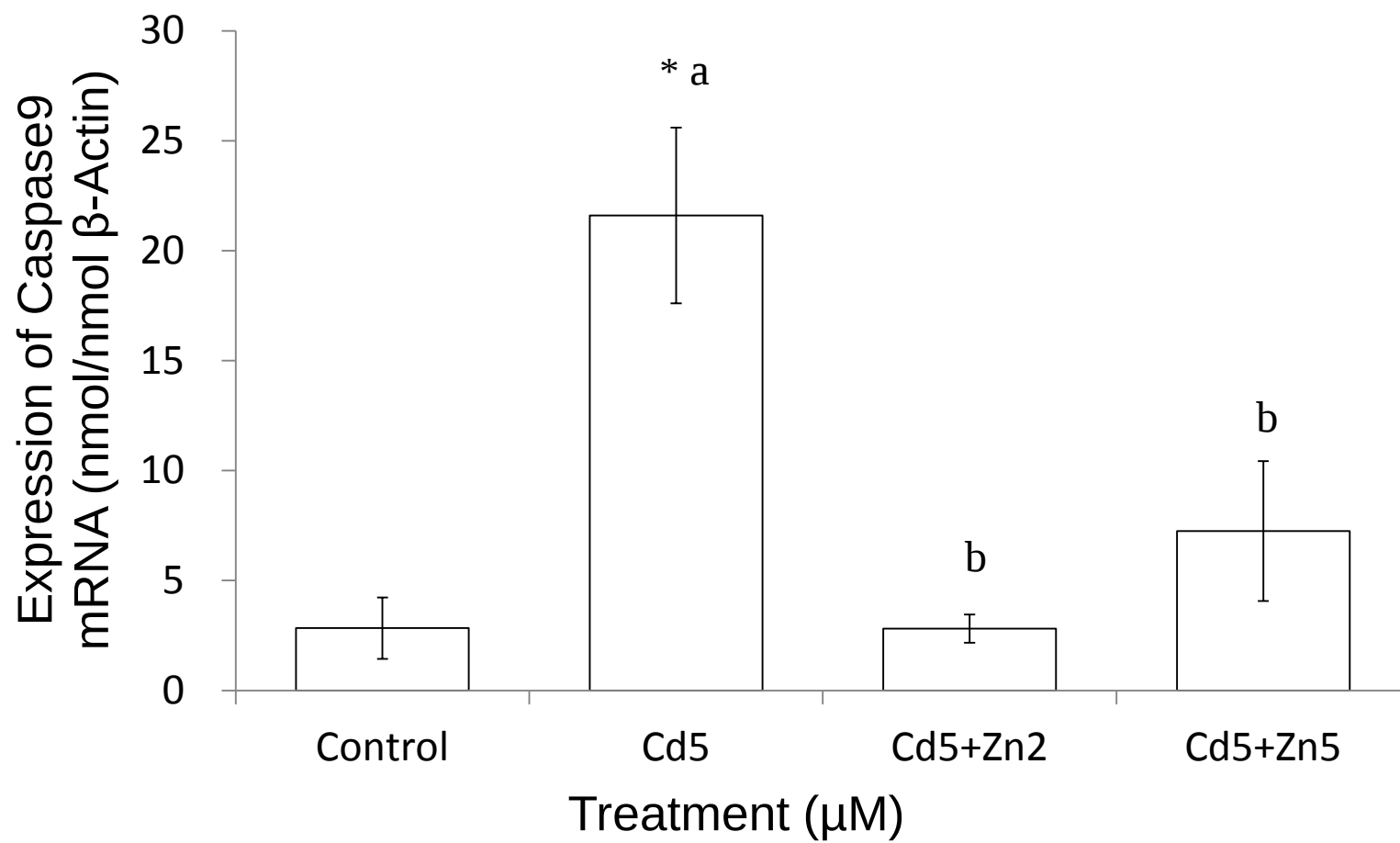


Fig. 6