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

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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 μmol/L) alone induced significant cell death, and co-exposure to $Zn^{2+}$ (5, 10, and 100 μmol/L) for 48 h had a protective effect. Assessment of intracellular free sulfhydryl levels and lactate dehydrogenase activity suggested that $Cd^{2+}$ (10 μmol/L) induced oxidative stress and disrupted cell membrane integrity. Addition of $Zn^{2+}$ (10 and 100 μmol/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 μmol/L) suppressed $Cd^{2+}$-induced apoptosis (10 μmol/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 μmol/L)-treated PC12 cells when co-treated with $Zn^{2+}$ (2 and 5 μmol/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.



## 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

*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.

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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 μmol/L), $Cd^{2+}$ causes apoptosis. At higher concentrations (>50 μmol/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 Cd2+-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, BIOSS), 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% $CO_2$. The cells were pre-incubated in 25-$cm^2$ flasks for 24 h; then, the medium was replaced with serum/serum-free DMEM with or without various concentrations of $CdCl_2$ and $ZnCl_2$, 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 $Cd^{2+}$ (0, 2, 5, 10, 20 μmol) and $Zn^{2+}$ (0, 2, 5, 10, 20, 100, and 500 μmol) separately, and then the final combination was decided. The selected concentration for $Cd^{2+}$ was 10 μmol, whereas $Zn^{2+}$ 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 $1 \times 10^5$ cells/flask and pre-incubated for 24 h. Then, the cells were treated with $Cd^{2+}$ (0, 2, 5, 10, and 20 μmol/L and 0, 5, 10, 100, and 500 μmol/L) and $Zn^{2+}$ (0, 2, 5, 10, and 20 μmol/L and 10, 100, and 500 μmol/L) separately; in addition, they were co-exposed to $Cd^{2+}$ (10 μmol/L) and $Zn^{2+}$ (10, 100, and 500 μmol/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