

Dual effects of zinc on cadmium toxicity in *Meriones shawi* adult neurons: A successful model of environmental pollutants assessment

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ABSTRACT

Cadmium is a well-known environmental pollutant that leads to several neurodegenerative diseases during adulthood. The role of zinc in cadmium toxicity has been controversial and there are reports suggesting its synergistic as well as antagonistic effects when treated with cadmium. The current study was conducted to determinate the effect of zinc on cadmium-induced toxicity in the brain of a semi desert rodent *Meriones shawi*. Therefore, to imitate human environmental exposure modeling to these pathologies, a neuronal hippocampal culture model was targeted from adult rodent that supports the long-term survival and physiological regeneration of mature cells. The viability of neurons was assessed by TUNEL assay. The results showed that cadmium induces neuronal death as Cd concentration increases with IC₅₀ of 2.5 µM, following 5 days of treatment. Neuron viability was significantly attenuated by low concentrations of zinc treatment. However, high zinc concentration reveals to be very toxic to selected culture. The present innovative data support that low concentrations of zinc protect against cadmium cytotoxicity. Thus, zinc provides therapeutic potential for brain lesions.

KEYWORDS: Cadmium, zinc, adult neuronal cells, *in vitro*, *Meriones shawi*, immunocytochemistry

INTRODUCTION

Cadmium (Cd), a nonessential heavy metal, is widely distributed in the environment due to its use in primary metal industries and phosphate fertilizers used in agriculture (Novelli et al., 1998; Bernard, 2008). Cd has important ecological repercussions and causes a significant health damages to humans and animals (Waalkes, 2000; Fernández-Pérez et al., 2010). In humans, Cd exposure can induce several neurodegenerative diseases such as learning disabilities, olfactory dysfunction (Mascagni et al., 2003) and retinal diseases (Kalariya et al., 2009). It can lead also to many neurological disorders (Gulisano et al., 2009; Satarug et al., 2010), neurobehavioral defects, and reduces learning-memory functions in rats and in contaminated areas of workers (Viaene et al., 2000; Ishitobi et al., 2007). Even low concentrations of Cd (10 nM Cd) induced suppressive effects on dendritic and synaptic development in cortical neurons (Ohtani-kaneko et al., 2008). Recently, it was reported that

the treatment of human neuroblasts with different concentrations of Cd significantly affects cell growth. Usai et al. (1999) demonstrated that cadmium permeation and accumulation in mammalian neuron are favored by cell membrane depolarization and through the same pathways of calcium (Ca²⁺) influx.

Several lines of evidence suggests that injury to adult brain in Neurodegenerative diseases is due to the inability of mature neurons to regenerate and results finally in cumulative cell loss. (Bambrick et al., 1995; Butler et al., 2003; Hernández-Ortega et al., 2011; Pizza et al., 2011). It have been reported that Cd enters the central nervous system (CNS) through the olfactory pathway or by altering the permeability of the blood-brain barrier. It is suggested that elevation of intracellular reactive oxygen species (ROS) may increase permeability of the blood-brain barrier and perturbation in synaptic transmission (Matés et al., 2009).

Neurotoxic effects of Cd have been also observed in various brain cell cultures including, rat cortical neurons (Lopez et al., 2003), isolated rat optic nerve preparation (Fern et al., 1996), anterior pituitary cells (Poliandri et al., 2004), glioma and neuron blastoma cells (Huang et al., 1993) and primary mid-brain neuroglia cultures (Varghese et al., 2009). Several studies showed that mutation in mitochondrial DNA (mtDNA) and the oxidative stress both contribute to ageing and cell death-related neurodegenerative (Lopez et al., 2003; Lin and Beal, 2006). Chen et al. (2008a) have demonstrated that Cd induces the generation of reactive oxygen species (ROS) by up regulating the expression of the nicotinamide adenine dinucleotide phosphate-oxidase 2 (NADPH oxidase 2) and its regulatory proteins in rat and human neuronal cells. Recently, it was proposed that Cd induces neuronal apoptosis through activation of the mammalian target of rapamycin (mTOR) pathway (Chen et al., 2011). Cd also was shown to cause apoptosis in various cells through activation of mitogen-activated protein kinases (MAPK) cascade (Chen et al., 2008b). Cd increases with smoking and it can also induce cell death in retinal pigment epithelial cells in a dose- and time-dependent manner (Kalariya et al., 2009).

Despite compelling evidence that mature neurons should be used in neurodegenerative model systems, most of the available *in vitro* studies have focused on the use of embryonic or early postnatal brain of laboratory animals

(Ohtani-Kaneko et al., 2008; Varghese et al., 2009) due to the difficulties to maintain culturing adult cells. As a result, most of the current *in vitro* models fall short of adequately representing the *in vivo* conditions and parameters that lead to these neurodegenerative diseases during adulthood and the virtual absence of neurogenesis in the adult mammalian brain underscores the necessity of ensuring the survival of neurons over many years.

A growing interest has been focused on factors that can protect against Cd toxicity. It has been mainly noted that zinc (Zn), which is essential transition metal for cellular growth and differentiation (Smart et al., 2004), can protect several tissues from the harmful effects of Cd on the antioxidant system in several organs (kidney, (Brzóška and Moniuszko-Jakoniuk, 2001), liver, brain (Kumar et al., 1996; Rogalska et al., 2009), and bone (Brzóška et al., 2008). However, the available studies are contradictory about the effect of Zn during Cd exposure which, appears to be dual. Zn was also found to be neurotoxic for the mammalian CNS (Araki et al., 1987; Bao and Knoell, 2006; Bao et al., 2006) and can induce both necrotic and apoptosis in primary cultures of rat cerebellar granule neurons. Zn has been reported to disturb neurofilament assembly *in vitro* (Banerjee et al., 1982) and neurofilament aggregates *in vitro* (Kress et al., 1981) and is increase in affected areas in parkinsonian brains (Dexter et al., 1989). Duncan et al. (1992) have suggested that long term consumption of 1 g of Zn per day could be responsible for the initiation of neurodegenerative processes in humans.

Also it is evident that the pollutant environmental exposure situation for humans and animals is usually quite different from those laboratory animals. The availability and toxicity of heavy metals are strongly influenced by the developed physiological mechanisms of the organism. Laboratory animals are usually given excessive doses of Cd during a short time while humans and animals on the environmental have a low daily Cd intake (Elinder and Piscator, 1978) and usually for long time. To fill this gap, the present study was carried out first, to explore the direct effect of acute Cd exposure on CNS neurons using primary hippocampal neurons of adult rodent *Meriones shawi*, recently considered as a good marker model for heavy metals land pollution (Bouyatas and Gamrani, 2007; Mbarek et al., 2011). Secondly to explore whether zinc can reverse or inhibit the Cd toxicity. Hippocampal neurons are among the most sensitive to stress-induced neuron death (Stein-Behrens et al. 1994).

To the knowledge of researchers, this is the first study to elucidate the damages of cadmium toxicity on mature hippocampal neurons and whether zinc can play a major role in the detoxification of heavy metal.

MATERIALS AND METHODS

Cell culture

Primary neuron cultures were prepared from hippocampus of adult brains of 3-4 month-old *Meriones shawi* using a modification of a previously described method (Bremer et al., 1993). All experiment protocols were carried out in accordance with French standard ethical guidelines for laboratory animals (agreement 75-178, 5_16_2000), and all efforts were made to minimize the number of animals used

and their suffering. Hippocampi were dissected under a binocular loupe and devoid of meninges and blood and then transferred to a 35-mm plate containing PBS plus 0.5% bovine serum albumin. Sterile scalpel-minced tissue was then removed to a fresh 15-ml tube where the tissue pieces were allowed to settle to the bottom. After discarding the supernatant, 5 ml of a solution of 0.05% trypsin/EDTA was added. The tissue was triturated with a fire-polished pipette followed by incubation of the cell suspension at 37°C for 10 min or until the clumps of tissue were digested. The cells were removed from the incubator, and DNase (1:10 dilution) was added, followed by trituration with a fire-polished pipette. An equal volume of Dulbecco's modified Eagle's medium (DMEM)/F12 plus 10% fetal bovine serum (FBS) was added to stop the activity of trypsin, followed by centrifugation of the cell suspension for 5 min at 1200 rpm. The pellet was re-suspended in 5 ml of DMEM/F12 + 10% FBS. Additional DNase (1:10 dilution) was added and followed by another round of trituration. The washes and DNase treatment were repeated (1-2 more times) until a homogeneous single-cell suspension without cell clumps was obtained. The final pellet was re-suspended in DMEM/F12, the final volume was determined, and viable cells count was performed by the trypan blue dye exclusion method (Kim et al., 2005; Jackson et al., 2010). The isolated cells were seeded at 5×10^5 /well to 24-well culture plates pre-coated with poly-D-lysine (20 µg/ml) slides, and maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air in minimum essential medium containing 10% fetal bovine serum (FBS), 1 g/l glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 µM nonessential amino acids, 50 U/ml penicillin, and 50 µg/ml streptomycin. Fresh medium was added to each well 3 days later.

In situ cell death detection

Neurons viability was assessed by terminal deoxy-uridine triphosphate nick-end labeling (TUNEL, Roche Diagnostics Meylan, France) according to the manufacturer's instructions.

Briefly, fixed cells were permeabilized with 0.1 % Triton X-100 for 5 minutes and then saturated with the saturation buffer for 30 minutes. The primary antibody was diluted in the saturation buffer and let overnight at 4°C. Cells were washed three times for 10 min with 0.01 M PBS. Freshly prepared TUNEL reaction mixture was applied on each section and left in a humid dark chamber for 1 h at 37°C. Cells were then washed thoroughly and secondary antibody incubation was performed in a dark humid chamber at room temperature for 2 h with DAPI and corresponding secondary antibody. Cells were again washed three times with PBS 0.01 M and finally mounted in glycerol/PBS solution (V/V). TUNEL positive neurons were counted in within area equivalent to 16mm² and compared to the total number of neurons.

Immunocytochemistry detection

Nuclear Morphology of DAPI-stained Neurons

To distinguish between programmed or non-apoptotic cell death, neuronal cultures were examined after staining with 4,6-diamidino-2-phenylindole (DAPI), which selectively stains DNA allowing the evaluation of chromatin distribution

and nuclear morphology. Hippocampal neurons were prepared from adult *Meriones*, cells were treated with 2 μM of cadmium chloride (CdCl_2 from Sigma-Aldrich) during 5 days. For zinc treatment, hippocampal neurons were pre-incubated first during 24h with 10 and 20 μM zinc sulfate (ZnSO_4 from Sigma- Aldrich) in culture medium and then treated with 2 μM of CdCl_2 for 4 days more. Cells incubated in culture medium were considering as controls, for the whole incubation time (positive control of apoptosis); after they were washed twice with phosphate buffered saline (PBS) 1X and then fixed with 4% paraformaldehyde (PFA) for 30 min at room temperature. Fixed cells were washed with PBS and stained with 2 $\mu\text{g}/\text{ml}$ DAPI. The cells were determined as neuron by using microtubule-associated protein-2 (MAP-2), a chicken polyclonal anti-MAP-2 antibody IgY (Neuronal marker (Abcam)) as primary antibody. The secondary antibody was Alexafluor 488 goat polyclonal to chicken IgY, diluted (1/400).

Nuclei are considered to have the normal phenotype when glowing bright and homogenously. Apoptotic nuclei can be identified by the condensed chromatin gathering at the periphery of the nuclear membrane or a total fragmented morphology of nuclear bodies.

The cultures were rinsed in PBS, mounted, and cover slipped. Lamellae were mounted with PBS/glycerol (V/V) and examined under a fluorescence microscope (Leica DM 4000B, Germany), with appropriate filters. Images were taken with a Nikon Coolpix camera. set and images were computed for quantitative analysis with imageJ. All images within an individual experiment were captured at the same exposure time. Cell numbers were calculated in each captured images within area equivalent to 16mm^2 .

Statistical analysis

Data are shown as the standard error of the mean (SEM). All results were compared with control cells culture as well as to the Cd-exposed cells (2 μM Cd). For all experiments, a one-way ANOVA was used to analyze the differences between groups, followed by a Dunnett's test with a threshold of significance of $p < 0.05$ and $p < 0.01$ to detect specific differences, using a statistical software package (Statistica, version 9).

RESULTS

1. Effect of Cd on hippocampal mature neurons viability of *Merionesshawi*

Cytotoxic effect of Cd, was assessed using TUNEL staining. *Meriones hippocampal* mature neurons were treated with various doses of CdCl_2 (0.5; 1; 1.5; 2; 2.5; 3; 5 or 10 μM) and their response was examined after 5 days of exposure. As illustrated in figure 1, Cd caused a pronounced decrease in cells viability as concentration increases, with a $\text{IC}_{50} = 2.5 \mu\text{M}$. The decrease in cells viability started statistically significant at 1.5 μM concentration ($p < 0.05$). The cytotoxic effect was more notable in cultures treated with the highest concentration (10 μM) and Cd statistically significantly reduced viability by up to 97% ($p < 0.01$). This suggests that the concentration of Cd appeared to play a major role in the induction of programmed cell death.

2. Effect of Zn on hippocampal mature neurons viability of *Meriones shawi*

To identify the neuron-mediated toxicity of Zn to hippocampal neurons, *Meriones* primary cultures were continuously exposed to different amount of Zn (0; 2; 5; 10; 15; 20 and 50 μM) during 5 days as expected in figure 2.

There was no damaging effect of Zn when hippocampal neurons were exposed at low concentrations of Zn (0-20 μM) in comparison to control neurons, while Zn induced neuronal damaging statistically significant ($p < 0.01$) (about 40% of mortality) under high concentration. These results indicate that Zn is safe under low concentration, but a high dose of Zn is very toxic to cultured neurons.

3. Effect of zinc on the Cd-induced neuronal viability

To explore whether Zn can reverse or inhibit the Cd-induced neuronal damage in primary cultures of adult *Meriones* hippocampal neurons, cultures were pre-incubated with different amounts of ZnSO_4 (0; 2; 5; 10; 15 and 20 μM). After 24 hours of Zn exposure, cells were incubated for 4 days more in medium containing CdCl_2 at a final concentration of 2 μM . This concentration was chosen because it is slightly lower than the IC_{50} (2.5 μM).

Treatment with 2 μM Cd reduced 53% the viability of the primary cultured cells of the hippocampal neurons. As shown in figure 3, the lowest concentration of Zn used (2 μM) did not have any significant effect on Cd-induced damaging on cells viability when incubated for 2 μM Cd. However, Cd cytotoxicity was dramatically inhibited by the addition of ZnSO_4 to the culture medium in a dose-dependent manner. Concentrations of 5, 10, 15 and 20 μM ZnSO_4 statistically significant increased the cell viability ($p < 0.05$) as compared to the cells under Cd exposure condition ($54 \pm 2.16\%$, $59 \pm 5.53\%$, 65 ± 0.3 and $72.0 \pm 2.68\%$ respectively). It is shown that final concentration amounting 20 μM in the culture medium is statistically significant more effective against the Cd-induced cell death ($p < 0.01$) than 10 μM of ZnSO_4 .

DISCUSSION

The main and original of this work is first to explore the direct effect of acute Cd exposure on CNS using hippocampal mature neurons of adulte *Meriones shawi*, following 5 days of continuous exposure. Second, the effects of various concentrations of ZnSO_4 were tested in order to verify whether Zn can reverse or inhibit Cd-induced neuronal death. Viability assays showed clearly that Cd is toxic to *Meriones* primary hippocampal neuron cultures with IC_{50} of 2.5 μM . The cytotoxicity is remarkably increased as the concentration of Cd increases. A concentration of 10 μM of CdCl_2 seems to be very toxic to primary neuron culture. In agreement with research finding, several studies demonstrated neurotoxicity effects of Cd in both *in vivo* and *in vitro* (Fern et al., 1996; Lopez et al., 2003).

Neurotoxic effects of Cd have been observed in various brain cell cultures including rat cortical neurons (Lopez et al., 2003), isolated rat optic nerve preparation (Fern et al., 1996), anterior pituitary cells (Poliandri et al., 2004), glioma

and neuron blastoma cells (Huang et al., 1993) and primary mid-brain neuroglia cultures (Varghese et al., 2009). (Gulisano et al. (2009). reported that the treatment of human immature neuron cells with different concentrations of Cd significantly affects cell growth. Indeed, low concentrations of Cd stimulate cell growth, whereas higher concentrations cause a decrease in cell proliferation. It has been reported that mutation in mitochondrial DNA (mtDNA) and the oxidative stress both contribute to ageing and cell death-related neurodegenerative diseases (Lopez et al., 2003). The basis of Cd toxicity lies in the substitution of Cd with other metal ions especially Zn, Copper (Cu) and Calcium (Ca) in metalloenzymes. Several studies showed that Cd at very high concentrations (1 mM), directly crossed the membrane and induced intracellular Ca mobilization from the inositol 1,4, 5-trisphosphate-(IP₃-) sensitive calcium stores via production of inositol 1,4,5-trisphosphate (IP₃). Cd can blocking voltage-dependent Ca²⁺ channels, caused a slow increase in intracellular Ca concentration [Ca²⁺]_i. Cadmium was also demonstrated to affect metabolism, release and re-uptake of different neurotransmitters (Gerspacher et al., 2009).

Chen et al. (2008b) have demonstrated that Cd induces the generation of reactive oxygen species (ROS) by upregulating the expression of NADPH oxidase 2 and its regulatory proteins in rat pheochromocytoma (PC12) and human neuroblastoma (SH-SY5Y) cells. Recently, it was suggested that Cd induces neuronal apoptosis through activation of the mammalian target of rapamycin (mTOR) pathway (Chen et al., 2011). Cd have also been reported to cause apoptosis in various cells through activation of mitogen-activated protein kinases (MAPK) cascade (Chen et al., 2008b). Gerspacher et al. (2009) reported that a target of these damaging effects is the cytoskeleton, especially in neurons and neuronal-like PC12 cells, where the metal always induces a dramatic disassembly of neurites and the complete disappearance of microtubules, which seem to collapse onto the nuclear surface. Accumulative studies demonstrate that cadmium may disturb the natural oxidation/ reduction balance in cells through various mechanisms, which interferes with cellular signaling and gene expression systems (Buzard and Kasprzak, 2000).

Secondly, results of this study showed that low concentrations of Zn can reduce toxic effects of Cd by decreasing cell death. It appear that 20 μM of Zn are more effective than 10 μM against Cd exposure. However, high concentration of Zn (50 μM) reveals very toxic to neuron. Although the complexity of the cellular and biochemical actions of Zn on Cd-induced-toxicity have been discussed in a number of studies, the underlying mechanisms are not completely understood and the available studies are contradictory about the effect of Zn during Cd exposure which, appears to be dual. There are reports suggesting its synergistic as well as antagonistic effects when treated with Cd. Similar results were obtained by Bancila et al., (2004) when Zn at low concentration (1 μM) was applied to hippocampal cultures at mossy fibres/CA3 synapses during the ischaemic challenge.

In this study, the delayed cell death was significantly attenuated, while Zn concentrations at more than 50 μM,

exerts neurotoxic effects (Choi and Koh 1998). The authors reported that the protection afforded by zinc was mediated through the activation of pre-synaptic KATP channels.

It has been reported that zinc protects neurons from hyper-excitation, excessive transmitter release and excitotoxicity. However, one of the reasons might be cytoprotection through blockage of the membrane Ca channel pore (Bao and Knoell, 2006). Vallee and Falchuk, (1993) and Sundermann (1995) reported that Zn at low concentrations is essential for normal cellular function and has been noted mainly to protect several tissues from the harmful effects of Cd on the antioxidant system in kidney (Brzóska and Moniuszko-Jakoniuk, 2001), liver, brain (Kumar et al., 1996) and (Rogalska et al., 2009), bone (Wilson and Bhattacharyya, 1997). Gunn et al. (1965) reported that Cd induced testis tumors in experimental animals were prevented by administration of Zn. Geng et al., (2003) showed that Zn treatments protect neurons from oxidative damage *in vitro*. Contrary to these ideas, Zinc has been shown to be directly toxic to neurons (Yokoyama et al. 1986; Choi and Koh, 1998).

Zn can induced both necrotic and apoptosis in primary cultures of rat cerebellar granule neurons. Moshou et al. (2008) suggested that exposure of the sciatic nerve fibers to CdCl₂ and CdZn₂ have approximately the same inhibitory effect on the evoked compound action potential (CAP) of the nerve fibres (Ganea et al., 2007). It is seem also that Cd is toxic to neuron-glia cultures and the oxidative stress from microglia may play important roles in Cd-induced damage to dopaminergic neurons and that high concentrations of Zn attenuated serum deprivation induced apoptotic neuronal death. Taking together these observations, one can clearly confirm that Zn, at low concentrations, can protect neurons against Cd-induced toxicity. However, high concentration of Zn reveals to be toxic to hippocampal adult neurons.

Interestingly, neurotoxicity of Zn observed at high concentration may be triggered by a compromised blood-brain communication. It was also demonstrated that Zn induced cellular death in a concentration dependent manner increased intracellular ROS levels (Koh et al., 1994). Although what has been described above gives good support to the ROS theory, it does not exclude other mechanisms involved in ZnCl₂ and CdCl₂-induced neurotoxicity, such as inhibition of voltage-gated channels (Méndez-Armenta and Ríos, 2007) and voltage-gated proton channels (DeCoursey and Cherny, 2007). There is also the possibility that Cd could bind to the binding site(s) of Zn or Cd, since these cations have similar ionic radii (Suzuki et al., 1985; Lee et al., 2006) may cause neurotoxicity by direct intracellular action.

Possibilities include the zinc-binding properties of the MT protein (seven zinc ions per protein molecule) or its ability to scavenge free radicals. It is thought that the two domain structure of metallothionein permits the protein to function simultaneously in intracellular Zn²⁺ distribution and in Cd²⁺ sequestration (Smith et al 2008)); In addition, it has been proposed that MTs could have a role in protecting DNA against oxidants and playing a major in cell proliferation, as well as in inflammation and apoptosis in the CNS (Hidalgo 2001; West et al., 2008).

CONCLUSION

The present report suggests that hippocampal neurons of adult *Meriones shawi* in vitro can be used successfully for assessment of neurotoxic effects of a number of environmental pollutants. The protective effect of Zn may lead to the development of treatments for brain lesions.

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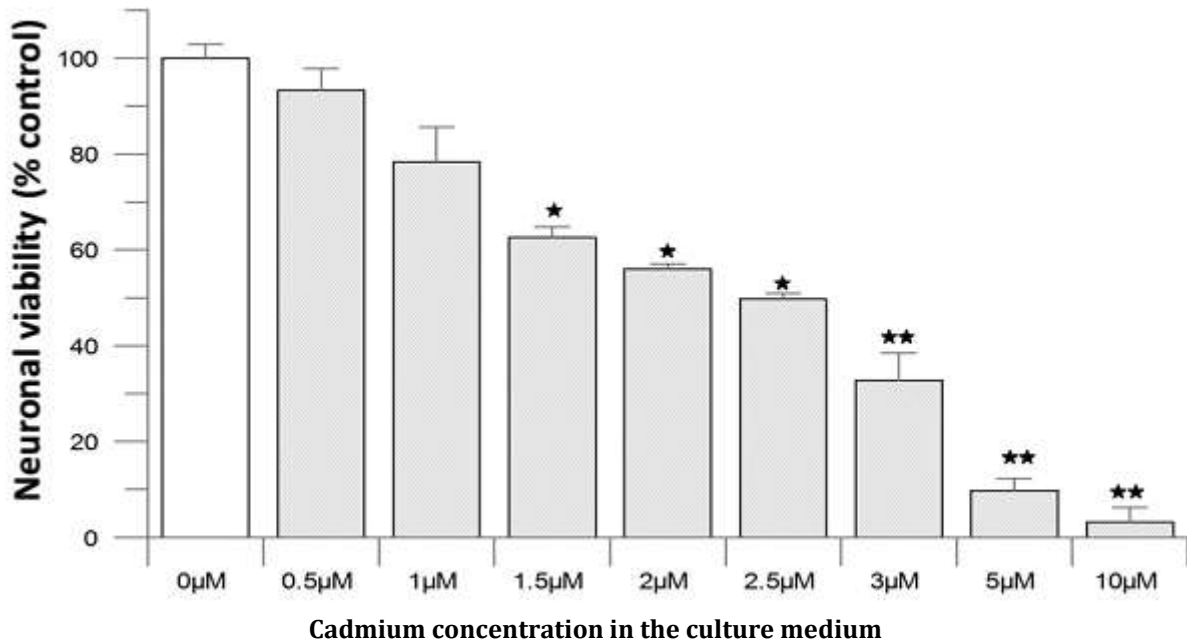


Figure 1: Cytotoxicity determined using the TUNEL staining in adult *Meriones* primary hippocampal cultures following 5 days of Cd exposure. Neurons were incubated with medium containing the indicated concentrations of CdCl₂ (0; 0.5; 1; 1.5; 2; 2.5; 3; 5 and 10 μM). The percentage of viability was calculated in comparison to the control, which was taken as the 100% viability value. Results are expressed as mean± SEM for four independent experiments. p<0.05, p<0.01, versus control cells (no addition of Cd).

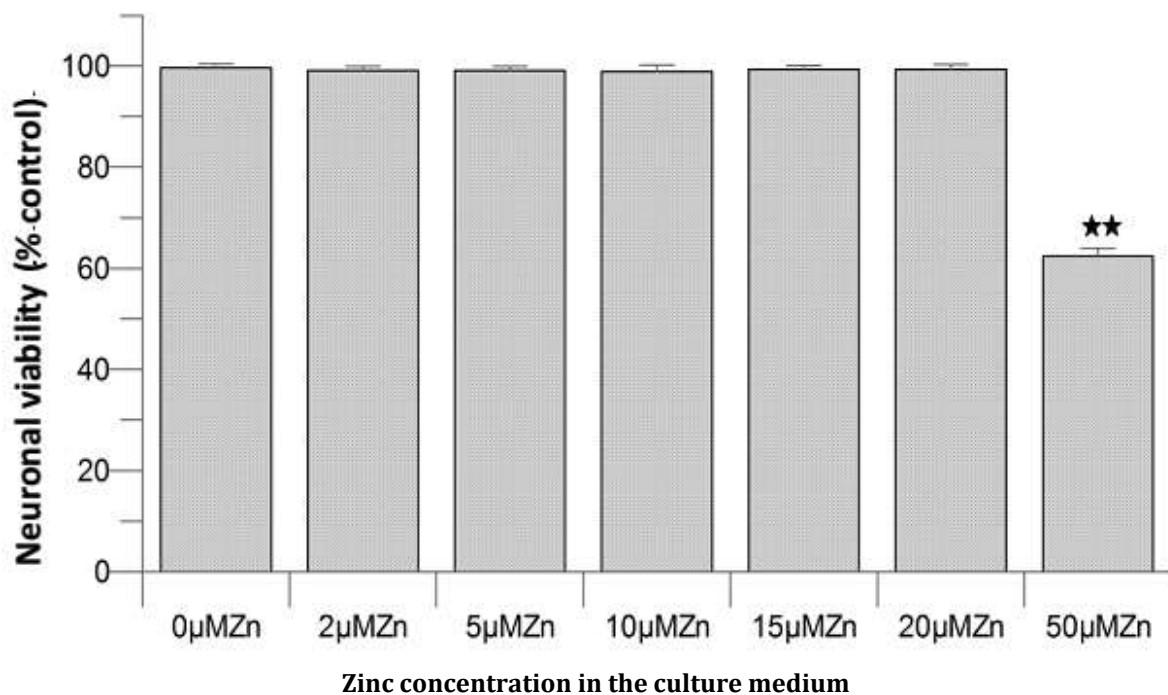


Figure 2. Viability of neurons treated by ZnSO₄ in adult *Meriones* primary hippocampal cultures following 5 days of Zn exposure. Neurons were incubated with medium containing the indicated concentrations of ZnSO₄ (0; 2; 5; 10; 15; 20, and 50 μM). After the above treatment, cell viability was determined using TUNEL staining. The percentage of viability was calculated in comparison to the no addition control, which was taken as the 100% viability value. Results are expressed as mean± SEM for four independent experiments, versus control neurons (no addition of Zn).

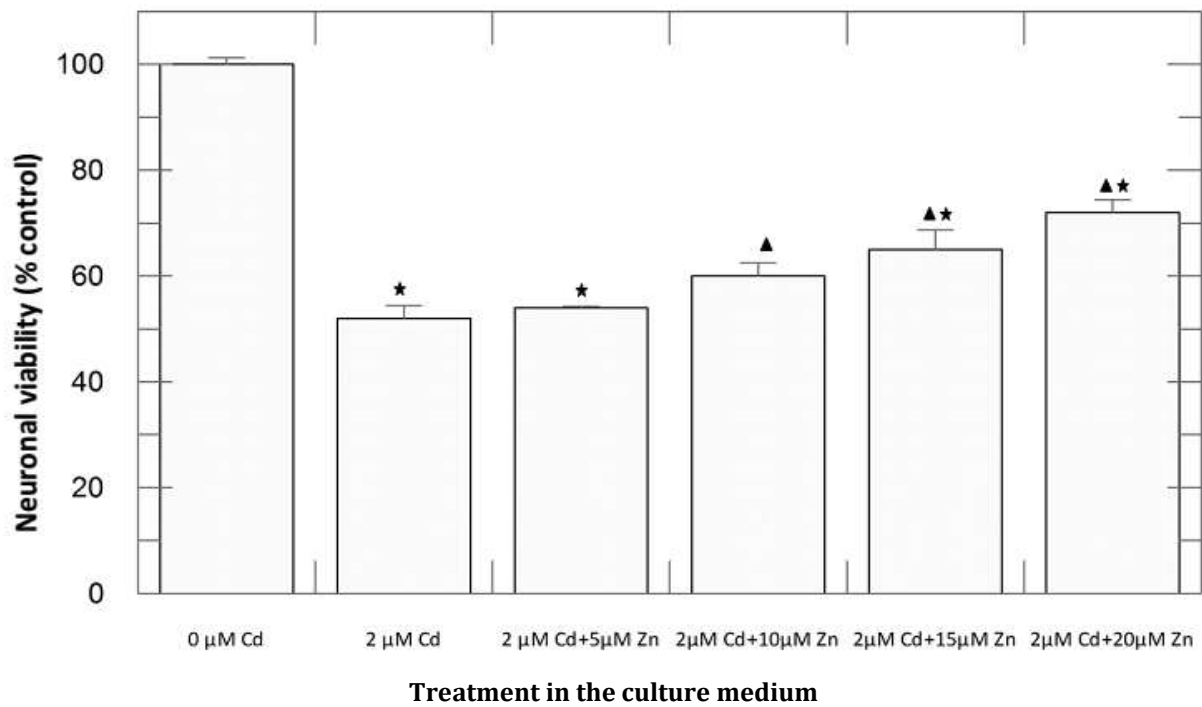


Figure 3: Effect of zinc pre-incubation against Cd-induced cytotoxicity. Hippocampal *Meriones* neurons were preincubated with medium containing the indicated concentrations of ZnSO_4 for 24 h. The medium was then replaced and either medium alone or medium plus 2 μM Cd was added for 4 days. After the above treatment, cell viability was determined using the TUNEL staining. The percentage of viability was calculated in comparison to the no addition cultures, which was taken as the 100% viability value and versus the 2 μM Cd treated cells only. Results are taken as the 100% viability value and versus the 2 μM Cd treated cells only. Results are expressed as mean \pm SEM; $p < 0.05$, versus control neurons (no addition); and $p < 0.05$, versus the no-addition Cd-treated.