

Citicoline protects against lead-induced oxidative injury in neuronal PC12 cells

Azadeh Aminzadeh and Ayda Salarinejad

Abstract: Lead is a major environmental pollutant that causes serious adverse effects on biological systems and cells. In this study, we examined the effect of citicoline on lead-induced apoptosis in PC12 cells. The PC12 cells were pre-treated with citicoline and then exposed to lead for 48 h. The effect of citicoline on cell survival was examined by MTT assay. In addition, levels of lipid peroxidation (LPO), total thiol groups, total antioxidant power (TAP), catalase (CAT), superoxide dismutase (SOD), and reduced glutathione (GSH) were evaluated. The levels of Bax, Bcl-2, and caspase-3 were also measured, by Western blot analysis. Citicoline significantly increased the cell viability of PC12 cells exposed to lead. Treatment of PC12 cells with lead increased LPO levels, and citicoline effectively decreased LPO. Levels of total thiol groups and TAP, CAT, SOD, and GSH were significantly increased in citicoline-treated PC12 cells compared with the lead-treated group. Citicoline pretreatment significantly reduced Bax expression, and increased the level of Bcl-2 expression. Citicoline also reduced caspase-3 activation in PC12 cells compared with the lead-treated group. Our findings indicate that citicoline exerts a neuroprotective effect against lead-induced injury in PC12 cells through mitigation of oxidative stress and, at least in part, through suppression of the mitochondria-mediated apoptotic pathway.

Key words: lead toxicity, oxidative stress, apoptosis, PC12 cells, citicoline.

Résumé : Le plomb est un polluant environnemental important qui provoque plusieurs effets indésirables graves sur les systèmes biologiques et les cellules. Dans cette étude, les auteurs ont examiné l'effet de la citicoline sur l'apoptose induite par le plomb chez les cellules PC12. Les cellules PC12 ont été prétraitées à la citicoline puis exposées au plomb pendant 48 h. L'effet de la citicoline sur la survie cellulaire a été examiné par un dosage au MTT. De plus, la peroxydation lipidique (LPO), les thiols totaux, le potentiel antioxydant total (PAT), la catalase (CAT), la superoxyde dismutase (SOD) et le glutathion réduit (GSH) ont été mesurés. Les niveaux de Bax, de Bcl-2 et de la caspase-3 ont aussi été mesurés par bavardage de western. La citicoline pouvait accroître significativement la viabilité des cellules PC12 exposées au plomb. Le traitement des cellules PC12 au plomb augmentait la LPO alors que la citicoline diminuait efficacement la LPO. Les thiols totaux ainsi que le PAT, la CAT, la SOD et le GSH étaient significativement accrûs chez les cellules PC12 traitées à la citicoline comparativement au groupe traité au plomb. Le prétraitement à la citicoline réduisait significativement l'expression de Bax et augmentait le niveau d'expression de Bcl-2. La citicoline réduisait aussi l'activation de la caspase-3 dans les cellules PC12 comparativement au groupe traité au plomb. Les données des auteurs ont révélé que la citicoline exerce des effets neuroprotecteurs envers les dommages induits par le plomb chez les cellules PC12 par l'atténuation du stress oxydant et du moins en partie, par la suppression de la voie mitochondriale de l'apoptose. [Traduit par la Rédaction]

Mots-clés : toxicité du plomb, stress oxydant, apoptose, cellules PC12, citicoline.

Introduction

Lead toxicity is a major health concern for public and health care worldwide. Exposure to lead can cause serious adverse effects on various body systems such as the hematopoietic and reproductive systems, the kidneys, and the peripheral and central nervous systems (Flora et al. 2012; Du et al. 2015). The nervous system is the main target for lead-induced toxicity. It has been shown that lead induces neuronal cell damage and dysfunction via oxidative stress (Sanders et al. 2009; Ge et al. 2015). Oxidative stress is the result of an imbalance between the generation of reactive oxygen species (ROS) and the ability of the biological systems to detoxify the reactive intermediates (Flora et al. 2012; Zuo et al. 2015). ROS can react with cell structures such as proteins, lipid membranes, and nucleic acids, thereby activating a complex chain of reactions that cause neuronal cell damage and apoptosis (Chen et al. 2012). Bcl-2 family members such as Bax and Bcl-2 have an important role in

the regulation of apoptotic signal transduction. Bax protein is known to promote cell death, whereas Bcl-2 is an anti-apoptotic protein that promotes cell survival (Birkishaw and Czabotar 2017).

It is now recognized that an effective approach for inhibiting the formation of free radicals under oxidative stress as well as the damage they cause can prevent both the initiation and progression of neuropathy (Uttara et al. 2009).

Citicoline is the generic name of cytidine-5'-diphosphate choline (CDP-choline), an essential precursor in the synthesis of neuronal membrane phospholipids, especially phosphatidylcholine. Citicoline has been shown to be beneficial for treating several diseases, including acute ischemic stroke, traumatic brain injury, cerebrovascular disease, amblyopia, and Parkinson's disease (PD) (Yücel et al. 2006; Martynov and Gusev 2015; Iulia et al. 2017). Citicoline has demonstrated neuroprotective properties in various slowly-advancing neurodegenerative disorders such as mild

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A. Aminzadeh and A. Salarinejad. Department of Pharmacology and Toxicology, School of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran; Pharmaceutics Research Center, Institute of Neuropharmacology, Kerman University of Medical Sciences, Kerman, Iran.

Corresponding author: Azadeh Aminzadeh (emails: Azadehaminzadeh@yahoo.com, a.aminzadeh@kmu.ac.ir).

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vascular cognitive impairment and glaucoma (Parisi et al. 2008; Álvarez-Sabín and Román 2011; Takasaki et al. 2011; Ottobelli et al. 2013). One important effect of citicoline is the stimulation of phospholipid biosynthesis that is necessary for membrane repair (Grieb 2014). Interestingly, the other mechanisms that have been implicated in the protective effects of citicoline in several models, both *in vivo* and *in vitro*, include preventing the activation of phospholipase A2, reducing the levels of brain platelet-activating factor (PAF), attenuating mitogen-activated protein kinases (MAPKs) and caspase activation, and preventing loss of cardiolipin, which is a unique inner mitochondrial membrane phospholipid that is essential for mitochondrial electron transport (Krupinski et al. 2012, 2005; Hernández-Esquível et al. 2014; Zazueta et al. 2018). Recent studies have demonstrated that citicoline has an antioxidant effect and scavenges harmful free radicals, especially hydroxyl radicals (OH^{\cdot}) (Adibhatla and Hatcher 2003). Citicoline is known to increase the glutathione redox ratio and prevent staurosporine-induced oxidative stress in human SH-SY5Y neuroblastoma cells (Barrachina et al. 2002). It is also known to prevent oxidative stress in several cell types, such as endothelial cells, primary cultured retinal cells, and neuronal cultures (Ma et al. 2013; Matteucci et al. 2014; Davinelli et al. 2017). However, it has not been verified that citicoline has neuroprotective effects against lead-induced toxicity in neuronal PC12 cells.

PC12 cells, derived from a pheochromocytoma tumor of the rat adrenal medulla, have been extensively used as a cell model to study lead neurotoxicity (Xu et al. 2006; Sanders et al. 2015). Thus, our present study was designed to evaluate the neuroprotective effects of citicoline against lead induced neurotoxicity in PC12 cells.

Materials and methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), horse serum (HS), and penicillin-streptomycin were purchased from the Gibco-Invitrogen Corporation (Carlsbad, California, USA). MTT and Pb-acetate were obtained from Sigma (Saint Louis, Missouri, USA). Polyclonal antibodies against Bax and Bcl-2 were obtained from Abcam (Cambridge, England). Caspase-3 antibody was acquired from Cell Signaling (Danvers, Massachusetts, USA). Horseradish peroxidase linked anti-rabbit secondary antibody and anti- β -actin were purchased from Cell Signaling. 5,5'-Dithiobiis (2-nitrobenzoic acid) (DTNB), 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), and Tris(hydroxymethyl)aminomethane were acquired from Sigma. An enhanced chemiluminescence (ECL) detection kit was purchased from Amersham Biosciences (Amersham, Buckinghamshire, UK). All of the other reagents were purchased from Sigma-Aldrich.

Cell culture

The undifferentiated PC12 cell line was purchased from the Pasteur Institute (Tehran, Iran). The cells were grown in DMEM supplemented with 5% (v/v) FBS, 10% (v/v) HS, and 1% (v/v) penicillin-streptomycin, and the cells were incubated at 37 °C with 5% CO_2 .

Cell viability

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is used in cytotoxicity and proliferation studies to determine the number of viable cells. This is a colorimetric assay based on the reduction of yellow soluble tetrazolium compound to purple insoluble formazan crystals in living cells. Briefly, PC12 cells were seeded in 96-microplates at a density of 7000 cells per well. The next day, the cells were pretreated with different concentrations of citicoline (30, 50, and 100 $\mu\text{mol/L}$) for 72 h and treated with lead for 48 h. The MTT solution (0.5 mg/ml) was added and the cells were incubated at 37 °C for 4 h. The culture medium was removed and 100 μL of DMSO was added to dissolve the formazan crystals. The absorbance was measured using an ELISA reader at 570 nm.

Measurement of lipid peroxidation

A thiobarbituric acid reactive substance (TBARS) assay was used to measure lipid peroxidation (LPO). Malondialdehyde (MDA) is one of the end products of lipid peroxidation, and it is widely used as a marker of cell membrane injury. In this method, thiobarbituric acid (TBA) reacts with MDA to produce a pink color. First, to precipitate the proteins of the sample, 500 μL of supernatant was added to 2.5 mL of trichloroacetic acid (TCA) (20% w/v) and the sample was centrifuged for 10 min at 3000g. Then, 2.5 mL of sulfuric acid (0.05 mol/L), and 2 mL TBA (0.2% in 2 mol/L sodium sulfate) were added, and the mixture was incubated in a boiling water bath for 30 min. Finally, after adding 4 mL n-butanol, the absorbance was measured at 532 nm.

Measurement of total thiol levels

DTNB is used as a reagent for measuring the total sulphydryl levels in plasma. This method is based on the reaction of thiol molecules with DTNB to form a yellow complex. In this assay, 0.2 mL of supernatant was added to Tris(hydroxymethyl)aminomethane, ethylene diamine tetra acetic acid (EDTA) buffer (20 mmol/L, pH = 8.2), and DTNB (10 mmol/L). The mixture was then centrifuged for 10 min at 4200g at room temperature. The absorbance was measured at 412 nm.

Measurement of total antioxidant power (TAP)

The ferric reducing antioxidant power (FRAP) test was used to assess TAP. This test is based on the ability of cells to reduce ferric tripyridyltriazine to a ferrous blue complex. For the preparation of the stock solution of this assay, acetate buffer (pH = 3.6) was mixed with acetic acid, ferric chloride (FeCl_3), and 2,4,6-tripyridyl-s-triazine (TPTZ) in hydrochloric acid. Then, the supernatant was added to FRAP solution. The absorbance was measured at 593 nm.

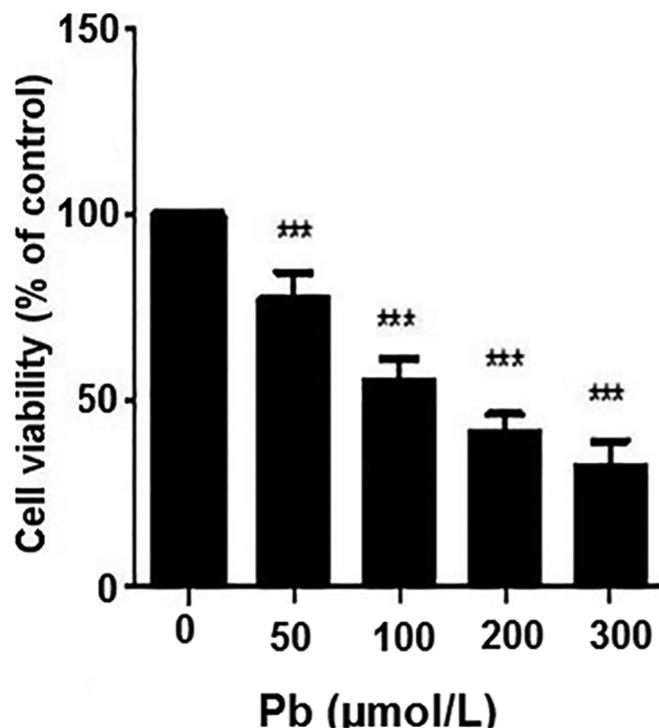
Measurement of CAT, SOD, and GSH

The CAT activity was measured using hydrogen peroxide (H_2O_2) as the substrate. Briefly, 50 μL of supernatant and 1.0 mL of hydrogen peroxide were mixed with 1.95 mL of phosphate buffer. A decrease in absorbance as a result of H_2O_2 degradation was determined at 240 nm for 1 min. SOD activity was assayed by evaluating its ability to inhibit the photoreduction of nitroblue tetrazolium (NBT) as a superoxide generator via the xanthine-xanthine oxidase system. Briefly, 20 μL of the supernatant was mixed with 200 μL of tetrazolium and 20 μL of enzyme solution. The absorbance was measured at 412 nm after incubation at 37 °C for 20 min. The intracellular GSH content was determined using a colorimetric method based on the reaction of GSH with DTNB to produce yellow colored TNB. A 100 μL aliquot of supernatant was mixed with 200 μL of 5% TCA and centrifuged. The supernatant was collected and combined with the DTNB solution. Absorbance was measured at 412 nm.

Western blot analysis

To analyze protein expression levels, PC12 cells were trypsinized, washed twice with ice-cold phosphate-buffered saline. Then, the cells were lysed with RIPA buffer, sodium orthovanadate solution (10 $\mu\text{M}/\text{mL}$), phenylmethylsulphonyl fluoride (PMSF) solution (10 $\mu\text{L}/\text{mL}$), and protease inhibitor cocktail solution (10–20 $\mu\text{L}/\text{mL}$). The supernatant fractions were collected following centrifugation at 13 000 g for 20 min at 4 °C. The total protein concentration was determined using Bradford assay (Bradford 1976). Lysate protein was loaded onto an SDS-PAGE gel and then transferred to polyvinylidene difluoride (PVDF) membranes. Subsequently, the protein blots were blocked with non-fat milk for 1 h. The blots were immunoblotted with the primary antibodies overnight at 4 °C. After that, the blots were incubated with horseradish-peroxidase-conjugated secondary antibodies for 2 h at room temperature. Protein bands were developed for visualization using an enhanced chemiluminescence (ECL) detection kit. The protein bands were quantified

Fig. 1. Effect of various concentrations of lead (50, 100, 200, or 300 $\mu\text{mol/L}$) on cell viability in PC12 cells. Cell viability was measured by MTT assay. Results reported are the mean \pm SEM ($n = 5$). ***, $P < 0.001$ compared with the control cells.



using measurement of densitometry, using Total Lab Quant 12 software (Wales, UK).

Statistical analysis

The data presented are the mean \pm SEM. Comparisons of multiple groups were performed using one-way analysis of variance (ANOVA), and for detection of significant differences among groups, Tukey's post-hoc tests were performed. Values for $P < 0.05$ were considered statistically significant.

Results

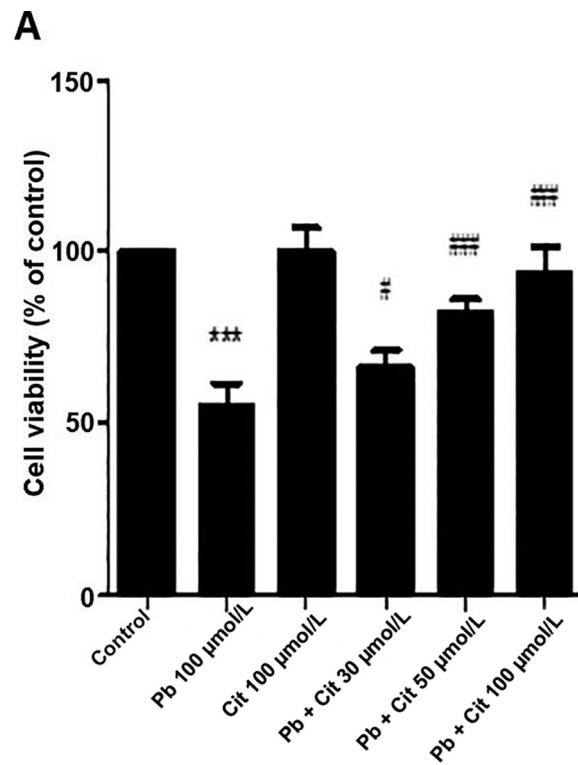
Effect of lead on cell viability

The effect of various concentrations of lead (50, 100, 200, and 300 $\mu\text{mol/L}$) on the viability of PC12 cells was investigated using the MTT assay. As shown in Fig. 1, lead reduced the percentage of viable cells in a concentration-dependent manner, by comparison with the control group. Based on our results, the lead concentration of 100 $\mu\text{mol/L}$ was selected for further study ($P < 0.001$).

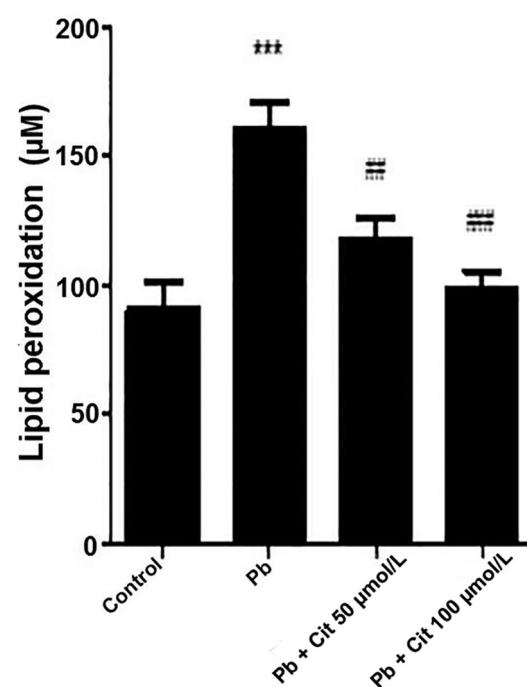
Effect of citicoline on lead-induced neurotoxicity

To investigate the neuroprotective effect of citicoline, the cells were treated with different concentrations of citicoline at various time-points, and cell viability was examined by MTT assay. We have observed that a 72 h treatment of PC12 cells with citicoline produces neuroprotective effects in this neurotoxicity model (data not shown). As shown in Fig. 2A, pretreatment with citicoline alleviated lead cytotoxicity in a concentration-dependent manner. Cell viability after pre-treatment with citicoline (50 or 100 $\mu\text{mol/L}$) increased significantly compared with the lead-treated group ($P < 0.001$ for both concentrations), so these concentrations of citicoline were used for performing next round of experiments.

Fig. 2. Effect of citicoline on cell viability (A) and lipid peroxidation (B) in lead-treated PC12 cells. The cells were pretreated with different concentrations of citicoline (30, 50, or 100 $\mu\text{mol/L}$) for 72 h and then incubated with lead (100 $\mu\text{mol/L}$) for 48 h. The results reported are the mean \pm SEM ($n = 5$). ***, $P < 0.001$ compared with the control cells; #, $P < 0.05$; ##, $P < 0.01$; and ###, $P < 0.001$ compared with the lead-treated cells.



B



Effect of citicoline on lipid peroxidation

Our results showed that lead significantly increased the MDA levels compared with the control group. In addition, we found that administration of citicoline (50 and 100 $\mu\text{mol/L}$) to the lead-treated cells significantly inhibited the increases in MDA levels by comparison with the lead-treated that were not administered citicoline ($P < 0.01$ and $P < 0.001$, respectively) (Fig. 2B).

Effect of citicoline on total thiol levels

As shown in Fig. 3A, lead treatment markedly decreased the total thiol levels compared with the control group. However, when PC12 cells were exposed to lead in the presence of citicoline (100 $\mu\text{mol/L}$) the levels of total thiols increased ($P < 0.05$).

Effect of citicoline on total antioxidant properties

The results, as illustrated in Fig. 3B, indicated that treatment of PC12 cells with lead significantly decreased the cell's total antioxidant capacity, whereas treatment with citicoline (50 or 100 $\mu\text{mol/L}$) effectively increased the total antioxidant capacity ($P < 0.05$ and $P < 0.01$, respectively).

Effects of citicoline on CAT, SOD, and GSH

Lead markedly decreased the levels of CAT, SOD, and GSH compared with the control cells ($P < 0.001$ for all). However, incubating the cells with citicoline (100 $\mu\text{mol/L}$) significantly increased the levels of CAT, SOD, and GSH when compared with the lead-treated groups ($P < 0.001$ for all) (Figs. 4A–C).

Effect of citicoline on the Bax:Bcl-2 ratio

To explore the neuroprotective mechanism of citicoline on lead toxicity, we evaluated the expression of Bax and Bcl-2 proteins in PC12 cells by Western blot analysis. The results presented in Figure 5 show that the Bax:Bcl-2 ratio was significantly increased by treatment with lead ($P < 0.001$), and that this increase was inhibited by pretreatment with citicoline.

Effect of citicoline on activation of caspase 3

Western blot analysis revealed that exposure to lead causes significant upregulation of cleaved caspase-3 levels ($P < 0.001$), and that pretreatment with citicoline (50 and 100 $\mu\text{mol/L}$) effectively prevented lead-induced changes in cleaved caspase-3 levels in PC12 cells ($P < 0.05$ for both concentrations of citicoline) (Fig. 6).

Discussion

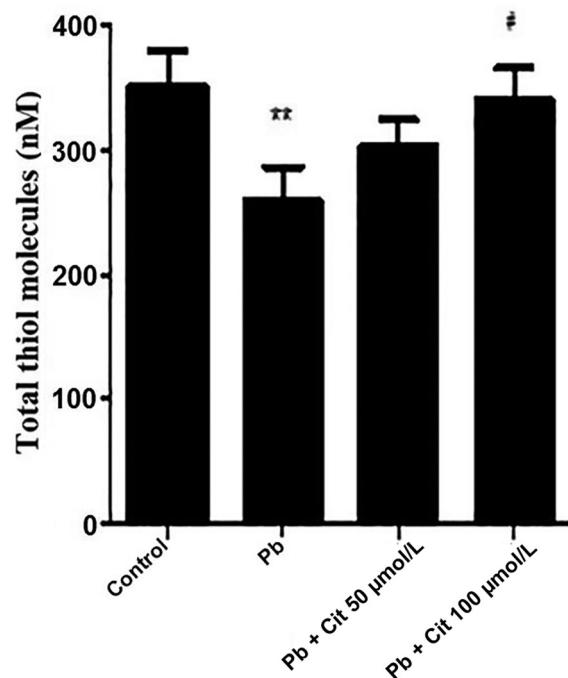
In this study, we investigated the protective effects of citicoline against lead-induced injury in PC12 cells. We demonstrated, for the first time, that citicoline can exert neuroprotective effects against lead-induced oxidative stress and cell damage in neuronal PC12 cells.

Our results revealed that citicoline increased the viability of PC12 cells, which was decreased by lead toxicity. This is in agreement with previous studies showing that citicoline improved viability and also exerted a neuroprotective effect against glutamate-induced excitotoxicity in cerebellar granule cells (Mir et al. 2003).

Lead toxicity is a global health problem, and is highly destructive to most organ systems. Studies have shown that lead causes cell damage through the induction of oxidative stress (Xu et al. 2008). Oxidative stress can damage cellular components and alter their functions. Products of lipid peroxidation have been identified as biomarkers of oxidative damage. MDA, end product of lipid peroxidation, is involved in stress signaling pathways in the progression of various degenerative disorders (Siddique et al. 2012; Aminzadeh and Mehrzadi 2018a). Our results indicate that lead increased the MDA levels in PC12 cells, whereas citicoline diminished the MDA levels compared with the lead-treated group. This finding is in agreement with the evidence indicating that citicoline prevents MDA formation and hydroxyl radical (OH^{\cdot}) generation

Fig. 3. Effect of citicoline on total thiol levels (A) and total antioxidant capacity (B) in lead-treated PC12 cells. The cells were pretreated with citicoline for 72 h and then incubated with lead (100 $\mu\text{mol/L}$) for 48 h. The results reported are the mean \pm SEM ($n = 3$). **, $P < 0.01$ compared with the control cells; #, $P < 0.05$, and ##, $P < 0.01$ compared with the lead-treated cells.

A



B

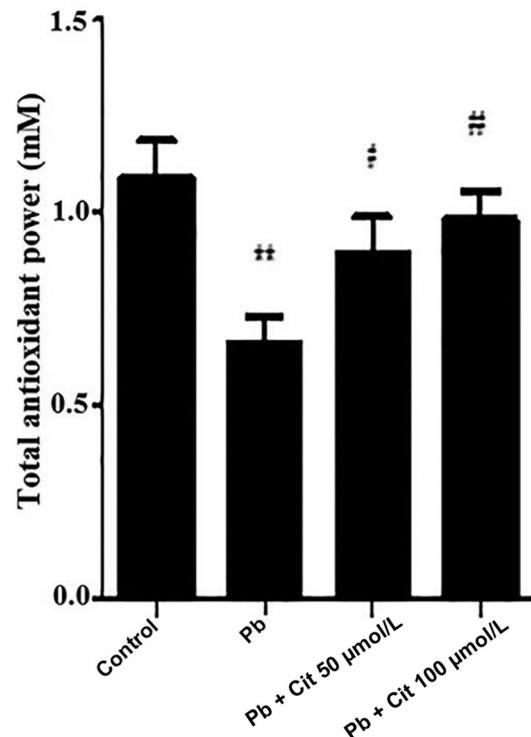


Fig. 4. Effect of citicoline on (A) catalase (CAT); (B) superoxide dismutase (SOD); and (C) levels of reduced glutathione (GSH) in lead-treated PC12 cells. The cells were pretreated with citicoline for 72 h and then incubated with lead (100 $\mu\text{mol/L}$) for 48 h. The results reported are the mean \pm SEM ($n = 3$). ***, $P < 0.001$ compared with the control cells; #, $P < 0.05$; ##, $P < 0.01$; and ###, $P < 0.001$ compared with the lead-treated cells.

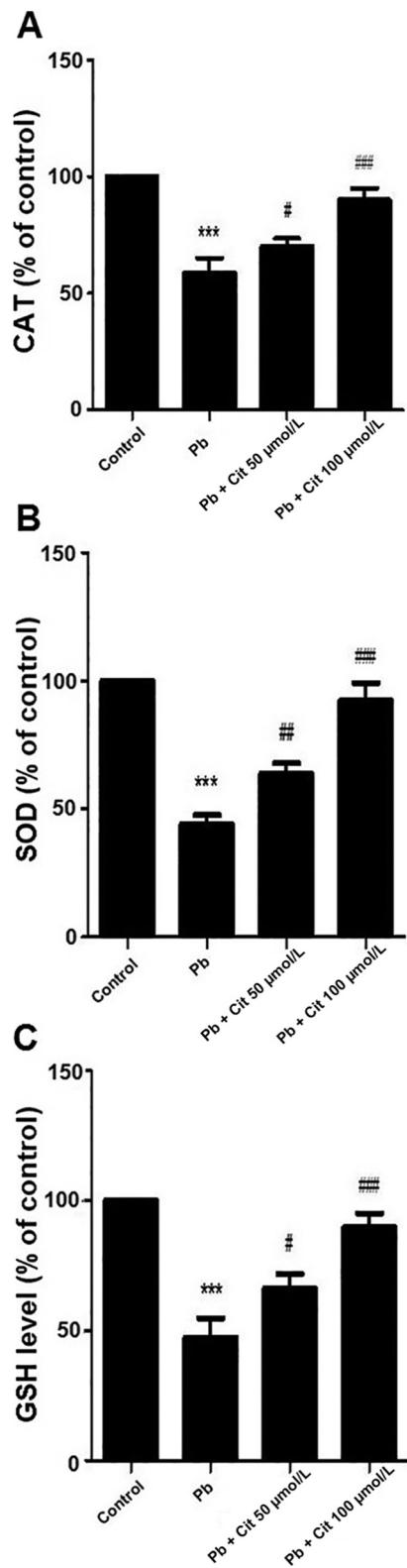
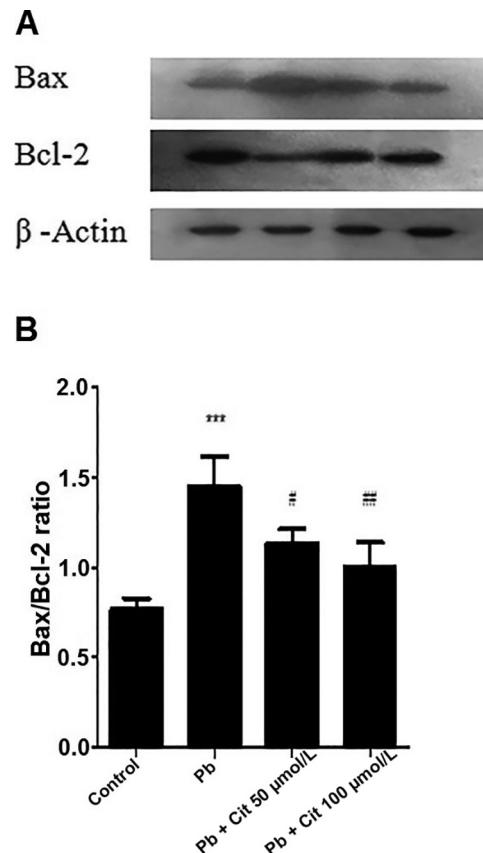


Fig. 5. Effect of citicoline on the protein expression levels of Bax and Bcl-2 in lead-treated PC12 cells. The cells were pretreated with citicoline for 72 h and then incubated with lead (100 $\mu\text{mol/L}$) for 48 h (A). The densities of Bax and Bcl-2 were analyzed and the Bax:Bcl-2 ratio was determined (B). The results reported are the mean \pm SEM ($n = 3$). ***, $P < 0.001$ compared with the control cells; #, $P < 0.05$, and ##, $P < 0.01$ compared with the lead-treated cells.



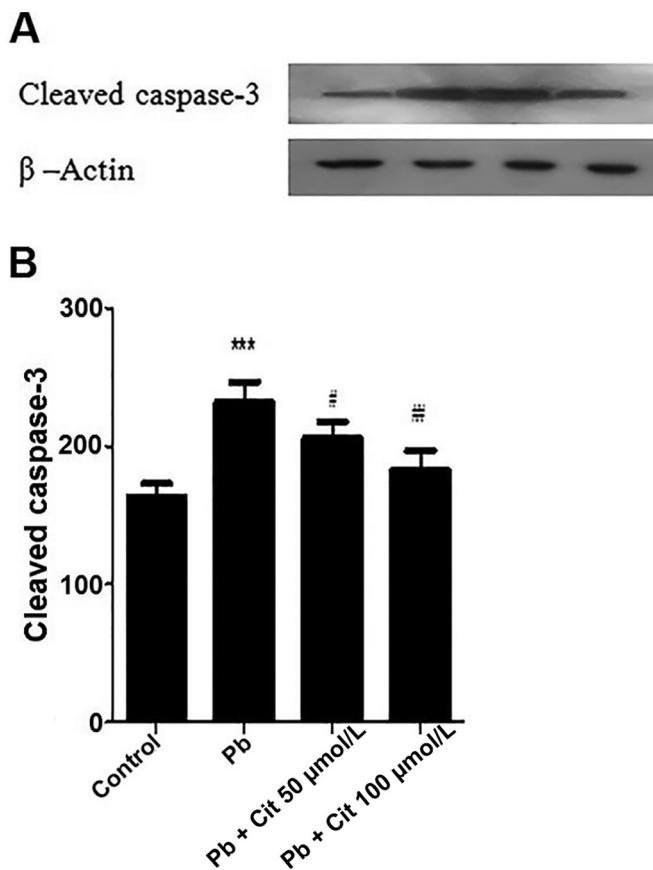
after cerebral ischemia (Adibhatla and Hatcher 2003; Adibhatla et al. 2003).

The antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) are important in the first-line antioxidant defence system, which plays a critical role in the total defence strategies in biological systems (Birben et al. 2012). In this study we found that, under conditions of lead toxicity, citicoline increased the total antioxidant capacity, and the concentrations of CAT, SOD, and GSH in PC12 cells. Consistent with our finding, there is evidence indicating that citicoline increases total glutathione and glutathione reductase activity after transient cerebral ischemia and reperfusion (Adibhatla et al. 2001). Another study has shown that citicoline increases the levels of GSH and reversed 6-hydroxydopamine (6-OHDA)-induced cell death in human SH-SY5Y neuroblastoma cells (Barrachina et al. 2003).

In addition, our results reported that the decrease in total thiol levels caused by lead toxicity was reversed by treatment with citicoline. These results are in agreement with recent studies indicating that citicoline exerts neuroprotective effects via reducing hypoglycemia-induced oxidative injury by preserving neuronal GSH levels in a rat model (Kim et al. 2018).

Previous studies have indicated that citicoline is safe for long-term treatments because it has no side effects. Therefore, citicoline may be used in the treatment and management of Parkinson's disease, cognitive disorders, cerebrovascular disease, amblyopia, glaucoma, and Alzheimer's disease (Putignano et al. 2012; Gareri

Fig. 6. Effect of citicoline on the activation of caspase-3 in lead-treated PC12 cells. The cells were pretreated with citicoline for 72 h and then incubated with lead (100 $\mu\text{mol/L}$) for 48 h (A). The density of cleaved caspase-3 was determined (B). The results reported are the mean \pm SEM ($n = 3$). ***, $P < 0.001$ compared with the control cells; #, $P < 0.05$, and ##, $P < 0.01$ compared with the lead-treated cells.



et al. 2015; Iulia et al. 2017). Citicoline is an important precursor for the synthesis of structural phospholipids of cellular membranes, particularly phosphatidylcholine (Grieb 2014). It has been reported that citicoline slows phospholipid breakdown, thereby stabilizing the cell membrane, and accelerates the resynthesis of structural phospholipids necessary for membrane repair (D'Orlando and Sandage 1995; Grieb 2014).

Citicoline has been shown to have neuroprotective effects against various noxious stimuli in in-vitro and in-vivo models, including cerebral ischemia and stroke (Adibhatla and Hatcher 2005), spinal cord injury caused by trauma in rats (Yücel et al. 2006), in-vitro glutamate-induced apoptotic pathway (Matyja et al. 2008; Mir et al. 2003), and in-vivo beta-amyloid induced neurodegeneration (Álvarez et al. 1999).

Previous studies have demonstrated that the Bcl-2 family and caspase-3 are involved in lead-induced apoptosis in PC12 cells (Xu et al. 2006). Bax and Bcl-2 are proteins that belong to the Bcl-2 family. Bax is a pro-apoptotic protein that induces the opening of mitochondrial membrane pores and facilitates the discharge of cytochrome c and other mediators from the mitochondria into the cytosol, which is an important step in the regulation of cell apoptosis. By contrast, Bcl-2 is an anti-apoptotic protein that inhibits the opening of the mitochondrial membrane pores, which in turn reduces the occurrence of apoptosis (Birkinshaw and Czabotar 2017). In the current study, we investigated the effect of citicoline on the expression of Bcl-2 and Bax proteins in PC12 cells. Our data indicate that the Bax:Bcl-2 ratio was increased in the lead-treated cells, whereas pretreatment with citicoline signifi-

cantly decreased the Bax:Bcl-2 ratio compared with the lead-treated group. In agreement with these results, previous reports have demonstrated that citicoline can protect heart function against reperfusion-induced damage in myocardium via preventing mitochondrial transition (Hernández-Esquível et al. 2014). It has also been shown that citicoline ameliorates the liver damage caused by ischemia and reperfusion through maintaining mitochondrial function and reducing oxidative stress (Zazueta et al. 2018).

Proteases of the caspase family play a crucial role in regulating apoptosis after activation by a series of signal transductions, including oxidative stress. Caspase-3 is known as a key executive enzyme that mediates apoptosis. Activated caspase-3 can cleave substrates implicated in the apoptotic process, such as PARP and Bcl-2 proteins, resulting in cell apoptosis (Rupinder et al. 2007; Aminzadeh and Mehrzadi 2018b). In the current study, citicoline significantly inhibited caspase-3 activity in PC12 cells undergoing lead-induced apoptosis. These results are consistent with evidence showing that citicoline decreases active caspase-3 expression and alleviates hyperoxia-induced lung injury in a neonatal rat model (Cetinkaya et al. 2013). Moreover, a study reported that citicoline reduced caspase-3 activation and prevented staurosporine-induced neurotoxicity in human SH-SY5Y neuroblastoma cells (Barrachina et al. 2002).

In summary, the results of this study indicate that citicoline is neuroprotective against lead-induced cell damage in PC12 cells, which may be related to inhibition of oxidative stress and its antioxidant effects and, at least in part, through blockade of mitochondria-mediated apoptotic pathway.

Conflict of interest statement

The authors declare that there is no conflict of interest associated with this work.

References

- Adibhatla, R.M., and Hatcher, J.F. 2003. Citicoline decreases phospholipase A2 stimulation and hydroxyl radical generation in transient cerebral ischemia. *J. Neurosci. Res.* **73**(3): 308–315. doi:[10.1002/jnr.10672](https://doi.org/10.1002/jnr.10672). PMID:[12868064](https://pubmed.ncbi.nlm.nih.gov/12868064/).
- Adibhatla, R.M., and Hatcher, J. 2005. Cytidine 5'-diphosphocholine (CDP-choline) in stroke and other CNS disorders. *Neurochem. Res.* **30**(1): 15–23. doi:[10.1007/s11064-004-9681-8](https://doi.org/10.1007/s11064-004-9681-8). PMID:[15756928](https://pubmed.ncbi.nlm.nih.gov/15756928/).
- Adibhatla, R.M., Hatcher, J., and Dempsey, R. 2001. Effects of citicoline on phospholipid and glutathione levels in transient cerebral ischemia. *Stroke*, **32**(10): 2376–2381. doi:[10.1161/hs1001.096010](https://doi.org/10.1161/hs1001.096010). PMID:[11588329](https://pubmed.ncbi.nlm.nih.gov/11588329/).
- Adibhatla, R.M., Hatcher, J.F., and Dempsey, R.J. 2003. Phospholipase A2, hydroxyl radicals, and lipid peroxidation in transient cerebral ischemia. *Antioxid. Redox Signal.* **5**(5): 647–654. doi:[10.1089/152308603770310329](https://doi.org/10.1089/152308603770310329). PMID:[14580322](https://pubmed.ncbi.nlm.nih.gov/14580322/).
- Álvarez, X., Sampedro, C., Lozano, R., and Cacabelos, R. 1999. Citicoline protects hippocampal neurons against apoptosis induced by brain beta-amyloid deposits plus cerebral hypoperfusion in rats. *Methods Find. Exp. Clin. Pharmacol.* **21**(8): 535–540. doi:[10.1358/mf.1999.21.8.794835](https://doi.org/10.1358/mf.1999.21.8.794835). PMID:[10599052](https://pubmed.ncbi.nlm.nih.gov/10599052/).
- Álvarez-Sabín, J., and Román, G.C. 2011. Citicoline in vascular cognitive impairment and vascular dementia after stroke. *Stroke*, **42**(1): S40–S43. doi:[10.1161/STROKEAHA.110.606509](https://doi.org/10.1161/STROKEAHA.110.606509). PMID:[21164117](https://pubmed.ncbi.nlm.nih.gov/21164117/).
- Aminzadeh, A., and Mehrzadi, S. 2018a. Melatonin attenuates homocysteine-induced injury in human umbilical vein endothelial cells. *Fundam. Clin. Pharmacol.* **32**(3): 261–269. doi:[10.1111/fcp.12355](https://doi.org/10.1111/fcp.12355). PMID:[29436019](https://pubmed.ncbi.nlm.nih.gov/29436019/).
- Aminzadeh, A., and Mehrzadi, S. 2018b. Cardioprotective effect of levosimendan against homocysteine-induced mitochondrial stress and apoptotic cell death in H9C2. *Biochem. Biophys. Res. Commun.* **507**(1–4): 395–399. doi:[10.1016/j.bbrc.2018.11.049](https://doi.org/10.1016/j.bbrc.2018.11.049). PMID:[30446219](https://pubmed.ncbi.nlm.nih.gov/30446219/).
- Barrachina, M., Secades, J., Lozano, R., Gómez-Santos, C., Ambrosio, S., and Ferrer, I. 2002. Citicoline increases glutathione redox ratio and reduces caspase-3 activation and cell death in staurosporine-treated SH-SY5Y human neuroblastoma cells. *Brain Res.* **957**(1): 84–90. doi:[10.1016/S0006-8993\(02\)03605-3](https://doi.org/10.1016/S0006-8993(02)03605-3). PMID:[12443983](https://pubmed.ncbi.nlm.nih.gov/12443983/).
- Barrachina, M., Domínguez, I., Ambrosio, S., Secades, J., Lozano, R., and Ferrer, I. 2003. Neuroprotective effect of citicoline in 6-hydroxydopamine-lesioned rats and in 6-hydroxydopamine-treated SH-SY5Y human neuroblastoma cells. *J. Neurol. Sci.* **215**(1–2): 105–110. doi:[10.1016/S0022-510X\(03\)00204-1](https://doi.org/10.1016/S0022-510X(03)00204-1). PMID:[14568136](https://pubmed.ncbi.nlm.nih.gov/14568136/).
- Birben, E., Sahiner, U.M., Sackesen, C., Erzurum, S., and Kalayci, O. 2012. Oxidative stress and antioxidant defense. *World Allergy Organ. J.* **5**(1): 9–19. doi:[10.1097/WOX.0b013e3182439613](https://doi.org/10.1097/WOX.0b013e3182439613). PMID:[23268465](https://pubmed.ncbi.nlm.nih.gov/23268465/).

- Birkinshaw, R.W., and Czabotar, P.E. 2017. The BCL-2 family of proteins and mitochondrial outer membrane permeabilisation. *Semin. Cell Dev. Biol.* **72**: 152–162. doi:[10.1016/j.semcdb.2017.04.001](https://doi.org/10.1016/j.semcdb.2017.04.001). PMID:[28396106](https://pubmed.ncbi.nlm.nih.gov/28396106/).
- Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**(1–2): 248–254. doi:[10.1016/0003-2697\(76\)90527-3](https://doi.org/10.1016/0003-2697(76)90527-3). PMID:[942051](https://pubmed.ncbi.nlm.nih.gov/942051/).
- Cetinkaya, M., Cansev, M., Kafa, I.M., Tayman, C., Cekmez, F., Canpolat, F.E., et al. 2013. Cytidine 5'-diphosphocholine ameliorates hyperoxic lung injury in a neonatal rat model. *Pediatr. Res.* **74**(1): 26–33. doi:[10.1038/pr.2013.68](https://doi.org/10.1038/pr.2013.68). PMID:[23598810](https://pubmed.ncbi.nlm.nih.gov/23598810/).
- Chen, X., Guo, C., and Kong, J. 2012. Oxidative stress in neurodegenerative diseases. *Neural. Regen. Res.* **7**(5): 376–385. PMID:[25774178](https://pubmed.ncbi.nlm.nih.gov/25774178/).
- Davinelli, S., Chiosi, F., Di Marco, R., Costagliola, C., and Scapagnini, G. 2017. Cytoprotective effects of citicoline and homotaurine against glutamate and high glucose neurotoxicity in primary cultured retinal cells. *Oxid. Med. Cell Longev.* **2017**: 2825703. PMID:[29163753](https://pubmed.ncbi.nlm.nih.gov/29163753/).
- D'Orlando, K.J., and Sandage, B.W., Jr. 1995. Citicoline (CDP-choline): mechanisms of action and effects in ischemic brain injury. *Neurol. Res.* **17**(4): 281–284. doi:[10.1080/01616412.1995.11740327](https://doi.org/10.1080/01616412.1995.11740327). PMID:[7477743](https://pubmed.ncbi.nlm.nih.gov/7477743/).
- Du, Y., Ge, M.M., Xue, W., Yang, Q.Q., Wang, S., Xu, Y., and Wang, H.L. 2015. Chronic lead exposure and mixed factors of gender × age × brain regions interactions on dendrite growth, spine maturity and NDR kinase. *PloS One*, **10**(9): e0138112. doi:[10.1371/journal.pone.0138112](https://doi.org/10.1371/journal.pone.0138112). PMID:[26368815](https://pubmed.ncbi.nlm.nih.gov/26368815/).
- Flora, G., Gupta, D., and Tiwari, A. 2012. Toxicity of lead: a review with recent updates. *Interdiscip. Toxicol.* **5**(2): 47–58. doi:[10.2478/v10102-012-0009-2](https://doi.org/10.2478/v10102-012-0009-2). PMID:[2318587](https://pubmed.ncbi.nlm.nih.gov/2318587).
- Gareri, P., Castagna, A., Cotroneo, A.M., Putignano, S., De Sarro, G., and Bruni, A.C. 2015. The role of citicoline in cognitive impairment: pharmacological characteristics, possible advantages, and doubts for an old drug with new perspectives. *Clin. Interv. Aging*, **10**: 1421–1429. PMID:[26366063](https://pubmed.ncbi.nlm.nih.gov/26366063/).
- Ge, M.M., Hu, F., Lou, Z.Y., Xue, W., Yu, H., Xu, L., et al. 2015. Role of Wnt/β-catenin signaling in the protective effect of epigallocatechin-3-gallate on lead-induced impairments of spine formation in the hippocampus of rats. *RSC Advances*, **5**(40): 31622–31628. doi:[10.1039/C5RA00315F](https://doi.org/10.1039/C5RA00315F).
- Grieb, P. 2014. Neuroprotective properties of citicoline: facts, doubts and unresolved issues. *CNS drugs*, **28**(3): 185–193. doi:[10.1007/s40263-014-0144-8](https://doi.org/10.1007/s40263-014-0144-8). PMID:[24504829](https://pubmed.ncbi.nlm.nih.gov/24504829/).
- Hernández-Esquível, L., Pavón, N., Buelna-Chontal, M., González-Pacheco, H., Belmont, J., and Chávez, E. 2014. Citicoline (CDP-choline) protects myocardium from ischemia/reperfusion injury via inhibiting mitochondrial permeability transition. *Life Sci.* **96**(1–2): 53–58. doi:[10.1016/j.lfs.2013.12.026](https://doi.org/10.1016/j.lfs.2013.12.026). PMID:[24389400](https://pubmed.ncbi.nlm.nih.gov/24389400/).
- Iulia, C., Ruxandra, T., Costin, L.B., and Liliana-Mary, V. 2017. Citicoline—a neuroprotector with proven effects on glaucomatous disease. *Rom. J. Ophthalmol.* **61**(3): 152–158. doi:[10.22336/rjo.2017.29](https://doi.org/10.22336/rjo.2017.29). PMID:[29450391](https://pubmed.ncbi.nlm.nih.gov/29450391/).
- Kim, J.H., Choi, B.Y., Kho, A.R., Lee, S.H., Jeong, J.H., Hong, D.K., et al. 2018. Acetylcholine precursor, citicoline (cytidine 5'-diphosphocholine), reduces hypoglycaemia-induced neuronal death in rats. *J. Neuroendocrinol.* **30**(1): e12567. doi:[10.1111/jne.12567](https://doi.org/10.1111/jne.12567).
- Krupinski, J., Slevin, M., and Badimon, L. 2005. Citicoline inhibits MAP kinase signalling pathways after focal cerebral ischaemia. *Neurochem. Res.* **30**(8): 1067–1073. doi:[10.1007/s11064-005-7201-0](https://doi.org/10.1007/s11064-005-7201-0). PMID:[16258856](https://pubmed.ncbi.nlm.nih.gov/16258856/).
- Krupinski, J., Abudawood, M., Matou-Nasri, S., Al-Baradie, R., Petcu, E.B., Justicia, C., et al. 2012. Citicoline induces angiogenesis improving survival of vascular/human brain microvessel endothelial cells through pathways involving ERK1/2 and insulin receptor substrate-1. *Vasc. Cell.* **4**(1): 20. doi:[10.1186/2045-824X-4-20](https://doi.org/10.1186/2045-824X-4-20). PMID:[23227823](https://pubmed.ncbi.nlm.nih.gov/23227823/).
- Ma, X., Zhang, H., Pan, Q., Zhao, Y., Chen, J., Zhao, B., and Chen, Y. 2013. Hypoxia/aglycemia-induced endothelial barrier dysfunction and tight junction protein downregulation can be ameliorated by citicoline. *Plos ONE*, **8**(12): e82604. doi:[10.1371/journal.pone.0082604](https://doi.org/10.1371/journal.pone.0082604). PMID:[24358213](https://pubmed.ncbi.nlm.nih.gov/24358213/).
- Martynov, M.Y., and Gusev, E.I. 2015. Current knowledge on the neuroprotective and neuroregenerative properties of citicoline in acute ischemic stroke. *J. Exp. Pharmacol.* **7**: 17–28. PMID:[27186142](https://pubmed.ncbi.nlm.nih.gov/27186142/).
- Matteucci, A., Varano, M., Gaddini, L., Mallozzi, C., Villa, M., Pricci, F., and Malchioli-Albedi, F. 2014. Neuroprotective effects of citicoline in *in vitro* models of retinal neurodegeneration. *Int. J. Mol. Sci.* **15**(4): 6286–6297. doi:[10.3390/ijms15046286](https://doi.org/10.3390/ijms15046286). PMID:[24736780](https://pubmed.ncbi.nlm.nih.gov/24736780/).
- Matyja, E., Taraszewska, A., Nagańska, E., Grieb, P., and Rafałowska, J. 2008. CDP-choline protects motor neurons against apoptotic changes in a model of chronic glutamate excitotoxicity *in vitro*. *Folia Neuropathol.* **46**(2): 139–148. PMID:[18587708](https://pubmed.ncbi.nlm.nih.gov/18587708/).
- Mir, C., Clotet, J., Aledo, R., Durany, N., Argemí, J., Lozano, R., Cervós-Navarro, J., and Casals, N. 2003. CDP-choline prevents glutamate-mediated cell death in cerebellar granule neurons. *J. Mol. Neurosci.* **20**(1): 53–60. doi:[10.1385/JMN:20:1:53](https://doi.org/10.1385/JMN:20:1:53). PMID:[12663935](https://pubmed.ncbi.nlm.nih.gov/12663935/).
- Ottobelli, L., Manni, G., Centofanti, M., Iester, M., Allevena, F., and Rossetti, L. 2013. Citicoline oral solution in glaucoma: is there a role in slowing disease progression? *Ophthalmologica*, **229**(4): 219–226. doi:[10.1159/000350496](https://doi.org/10.1159/000350496). PMID:[23615390](https://pubmed.ncbi.nlm.nih.gov/23615390/).
- Parisi, V., Coppola, G., Centofanti, M., Oddone, F., Angrisani, A.M., Ziccardi, L., Ricci, B., Quaranta, L., and Manni, G. 2008. Evidence of the neuroprotective role of citicoline in glaucoma patients. *Prog. Brain Res.* **173**: 541–554. doi:[10.1016/S0079-6123\(08\)01137-0](https://doi.org/10.1016/S0079-6123(08)01137-0). PMID:[18929133](https://pubmed.ncbi.nlm.nih.gov/18929133/).
- Putignano, S., Gareri, P., Castagna, A., Cerqua, G., Cervera, P., Cotroneo, A.M., et al. 2012. Retrospective and observational study to assess the efficacy of citicoline in elderly patients suffering from stupor related to complex geriatric syndrome. *Clin. Interv. Aging*, **7**: 113–118. PMID:[22654511](https://pubmed.ncbi.nlm.nih.gov/22654511/).
- Rupinder, S.K., Gurpreet, A.K., and Manjeet, S. 2007. Cell suicide and caspases. *Vascul. Pharmacol.* **46**(6): 383–393. doi:[10.1016/j.vph.2007.01.006](https://doi.org/10.1016/j.vph.2007.01.006). PMID:[17382599](https://pubmed.ncbi.nlm.nih.gov/17382599/).
- Sanders, T., Liu, Y., Buchner, V., and Tchounwou, P.B. 2009. Neurotoxic effects and biomarkers of lead exposure: a review. *Rev. Environ. Health*, **24**(1): 15–45. PMID:[19476290](https://pubmed.ncbi.nlm.nih.gov/19476290/).
- Sanders, T., Liu, Y., and Tchounwou, P.B. 2015. Cytotoxic, genotoxic, and neurotoxic effects of Mg, Pb, and Fe on pheochromocytoma (PC-12) cells. *Environ. Toxicol.* **30**(12): 1445–1458. doi:[10.1002/tox.22014](https://doi.org/10.1002/tox.22014). PMID:[24942330](https://pubmed.ncbi.nlm.nih.gov/24942330/).
- Siddique, Y.H., Ara, G., and Afzal, M. 2012. Estimation of lipid peroxidation induced by hydrogen peroxide in cultured human lymphocytes. *Dose Response*, **10**(1): 1–10. PMID:[22423225](https://pubmed.ncbi.nlm.nih.gov/22423225/).
- Takasaki, K., Uchida, K., Fujikawa, R., Nogami, A., Nakamura, K., Kawasaki, C., et al. 2011. Neuroprotective effects of citidine-5-diphosphocholine on impaired spatial memory in a rat model of cerebrovascular dementia. *J. Pharmacol. Sci.* **116**(2): 232–237. doi:[10.1254/jphs.11013FP](https://doi.org/10.1254/jphs.11013FP). PMID:[21613753](https://pubmed.ncbi.nlm.nih.gov/21613753/).
- Uttara, B., Singh, A.V., Zamboni, P., and Mahajan, R.T. 2009. Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. *Curr. Neuropharmacol.* **7**(1): 65–74. doi:[10.2174/157015909787602823](https://doi.org/10.2174/157015909787602823). PMID:[19721819](https://pubmed.ncbi.nlm.nih.gov/19721819/).
- Xu, J., Ji, L.D., and Xu, L.H. 2006. Lead-induced apoptosis in PC 12 cells: Involvement of p53, Bcl-2 family and caspase-3. *Toxicol. Lett.* **166**(2): 160–167. doi:[10.1016/j.toxlet.2006.06.643](https://doi.org/10.1016/j.toxlet.2006.06.643). PMID:[16887300](https://pubmed.ncbi.nlm.nih.gov/16887300/).
- Xu, J., Lian, L.J., Wu, C., Wang, X.F., Fu, W.Y., and Xu, L.H. 2008. Lead induces oxidative stress, DNA damage and alteration of p53, Bax and Bcl-2 expressions in mice. *Food Chem. Toxicol.* **46**(5): 1488–1494. doi:[10.1016/j.fct.2007.12.016](https://doi.org/10.1016/j.fct.2007.12.016). PMID:[18226849](https://pubmed.ncbi.nlm.nih.gov/18226849/).
- Yücel, N., Çaylı, S.R., Ateş, Ö., Karadağ, N., Firat, S., and Türköz, Y. 2006. Evaluation of the neuroprotective effects of citicoline after experimental spinal cord injury: improved behavioral and neuroanatomical recovery. *Neurochem. Res.* **31**(6): 767–775. doi:[10.1007/s11064-006-9075-1](https://doi.org/10.1007/s11064-006-9075-1). PMID:[16794862](https://pubmed.ncbi.nlm.nih.gov/16794862/).
- Zazueta, C., Buelna-Chontal, M., Macías-López, A., Román-Anguiano, N.G., González-Pacheco, H., Pavón, N., et al. 2018. Cytidine-5'-diphosphocholine protects the liver from ischemia/reperfusion injury preserving mitochondrial function and reducing oxidative stress. *Liver Transpl.* **24**(8): 1070–1083. doi:[10.1002/lt.25179](https://doi.org/10.1002/lt.25179). PMID:[29679463](https://pubmed.ncbi.nlm.nih.gov/29679463/).
- Zuo, L., Zhou, T., Pannell, B., Ziegler, A., and Best, T.M. 2015. Biological and physiological role of reactive oxygen species — the good, the bad and the ugly. *Acta Physiol.* **214**(3): 329–348. doi:[10.1111/apha.12515](https://doi.org/10.1111/apha.12515).