Therapeutic efficacy of chlorogenic acid on cadmium-induced oxidative neuropathy in a murine model

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Abstract. The aim of the present study was to determine whether chlorogenic acid (CA) is able to modulate cadmium (Cd)-induced oxidative brain damage. Cd-treated rats displayed numerous pathological effects, including the inhibition of acetylcholinesterase, elevated lipid peroxidation, the depletion of enzymatic and non-enzymatic antioxidants, the reduction of membrane-bound ATPase activity, mitochondrial dysfunction and DNA fragmentation. Pretreatment of the rats with CA significantly attenuated these effects. These results lead to the hypothesis that the mechanisms by which CA attenuates the effects of Cd-induced oxidative brain damage include the maintenance of antioxidant homeostasis, inhibition of the membrane effects and the perpetuation of mitochondrial dysfunction. These data support the potential of CA as a beneficial intervention in the prevention of heavy metal poisoning due to Cd exposure.

Introduction

Heavy metal pollution due to cadmium (Cd), arsenic and lead is under vital scrutiny as a result of potential neurotoxicological hazards to the human population (1,2). Cd is a universal ecological toxicant frequently encountered through metal industries, in aerosolized form in cigarette smoke and in certain industrial workplaces, including those where battery manufacture and smelting take place (3). In addition to the noxious effect of Cd on vital organs such as the kidneys, liver and testes, Cd also elicits damaging effects on the central nervous system (CNS) due to a variety of factors, such as its toxicity at low concentration, extended half-life (15-30 years in humans) and low clearance rate (4). Although the blood-brain barrier (BBB) is impermeable to chronic low levels of Cd exposure, Cd may affect the permeability of the barrier and reach the brain (5).

Preclinical studies have indicated that Cd induces behavioral disorders, as well as morphological and biochemical changes in the CNS (6,7). The brain region displays a high potential for oxidation and a low antioxidant defense capacity relative to the hepatic system. The neurotoxic potential of Cd has been attributed to the changes it induces in the brain enzyme network involved in counteracting oxidative stress and the disturbance of brain metabolism. Further, Cd increases the generation of free radicals, in particular superoxide ions, in the brain and hinders the antioxidant defence system, thus increasing lipid peroxidation. Moreover, free radicals generated by Cd interact with mitochondrial sites, leading to the breakdown of mitochondrial potentials, a consequence of reductions in intracellular glutathione levels (8). The mechanism by which Cd induces oxidative stress appears to be based mainly on the disruption of the prooxidant/antioxidant balance, which may lead to brain injury via oxidative damage to critical biomolecules, such as thiols, lipids, proteins and DNA (9).

Dietary polyphenols are proposed to be major antioxidants that provide health benefits due to their free radical-scavenging and metal-chelating properties, and the modulation of enzymatic activity and signal transduction pathways (10). Chlorogenic acid (CA) is a potent polyphenolic antioxidant abundantly found in apples, coffee beans, tomatoes, potatoes and apricots (11,12). CA is an ester formed between caffeic acid and quinic acid that is hydrolyzed by intestinal bacteria to various aromatic acid metabolites (13). The antioxidant potential of CA is due to the presence of vicinal hydroxyl groups on an aromatic residue, which enable it to scavenge free radicals in the aqueous phase (14) and prevent oxidative DNA damage (15). Previous studies have reported the antioxidant potential (16,17) and widespread bioactive effects of CA, such as antimutagenic, antiviral (18), anticarcinogenic (19) and tissue protection (20) effects. The neuroprotective potential of CA against oxidative insult to the brain has been well documented (21-23). The present study was carried out to investigate the neuroprotective efficacy of CA against the changes to the brain provoked by cadmium.

Materials and methods

Chemicals. CA, cadmium chloride ($CdCl_2$) and other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of the highest available commercial grade.

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Animals. Male Wistar rats weighing 170-200 g were obtained from the animal facility at Shandong University (Jinan, China). The animals were maintained under standard laboratory conditions of relative humidity (55±5%), temperature (25±2°C) and light (12 h light/12 h dark). They were fed standard diet pellets and water was provided *ad libitum*.

Experimental design. Twenty four rats were divided into four groups (n=6 per group) and subjected to treatment as follows: Group 1: Rats received distilled water 1 ml/100 g body weight once daily by oral gavage for 30 days and served as a control group. Group 2: Rats orally received Cd as $CdCl_2$ in saline (5 mg/kg body weight) each day for 30 days. Group 3: Rats received CA alone (60 mg/kg body weight) dissolved in distilled water, intragastric administration once daily for 30 days. Group 4: Rats orally received Cd (5 mg/kg body weight) followed by intragastric administration of CA (60 mg/kg body weight) in distilled water once daily for 30 days.

At the end of the experimental period, rats were fasted overnight and sacrificed by decapitation. Blood was collected in a heparinized BD vacutainer (BD Biosciences, Franklin Lakes, NJ, USA) and plasma samples were collected by centrifugation at 2,000 x g for 20 min. The whole brain was immediately excised and rinsed in ice-cold saline to remove the blood. The brain tissues were homogenized (25%, w/v) in 25 mmol/l phosphate buffer (pH 7.0) containing 0.25 mol/l sucrose (w/v) and centrifuged at 1,000 x g for 10 min. The clear supernatant was used for various biochemical assays to assess oxidative stress.

Preparation of mitochondrial fraction. This was conducted as previously described (24). Brain homogenate (25%, w/v) was prepared and the supernatant was subject to centrifugation at 12,000 g for 10 min at 4°C (Remi cooling centrifuge, C-24 BL; Remi Laboratory Instruments, Mumbai, India). The mitochondrial pellets thus obtained were washed twice with phosphate buffer (pH 7.4) to remove sucrose and homogenized in phosphate buffer containing 0.5% Tween-80 (v/v). The supernatant was collected and used for enzyme analysis, then washed and resuspended in the same buffer.

Biochemical assays

Estimation of acetylcholinesterase (AChE) activity. The level of AChE in the brain homogenate was measured according to a previously described method (25) using acetylthiocholine iodide (ATCI) as a substrate. In this method, AChE in the sample hydrolyzed ATCI into thiocholine and butyric acid. The thiocholine reacted with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to form 5-thio-2-nitrobenzoic acid. The yellow color that developed was measured spectrophotometrically at 412 nm. The values are expressed as μmol of ATCI hydrolyzed/min/mg protein.

Assessment of enzymatic antioxidants. The brain tissue level of superoxide dismutase (SOD) was estimated by the method of Kakkar *et al* (26). The reaction was based on the inhibition of the reduction of the nitro blue tetrazolium chromogen by NADH and phenazine methosulfate. One unit of SOD activity corresponds to the amount of enzyme that causes a 50% suppression of the reduction of nitro blue tetrazolium/min/mg of protein.

The catalase (CAT) activity in the brain homogenate was analyzed by the method of Sinha *et al* (27) wherein the breakdown of H_2O_2 was evaluated by measuring the UV absorption of H_2O_2 at 240 nm. The CAT activity is expressed as μmol H_2O_2 consumed/min/mg of protein.

The brain levels of glutathione peroxidase (GPx) were assayed by the method of Rotruck *et al* (28). The assay system was based on the reaction between a measured amount of enzyme preparation H_2O_2 and reduced glutathione (GSH) for a specified time period. The unreacted GSH was then measured by reaction with DTNB. The GPx activity is expressed as nmol GSH oxidized/min/mg of protein.

Glutathione S-transferase (GST) was estimated by the method of Habig *et al* (29). The increase in absorbance was measured at 340 nm using 1-chloro-2,4-dinitrochlorobenzene (CDNB) as substrate. GST activity is expressed in units of nmol of GSH-CDNB conjugate formed/min/mg of protein.

Estimation of protein content. The protein content of the brain homogenate was estimated by the method of Lowry *et al* (30) using bovine serum albumin as a standard.

Estimation of non-enzymatic antioxidants. The GSH levels in the brain were determined by the method of Moron *et al* (31). The nonprotein sulfhydryl content of cells is in the form of GSH. DTNB is a disulfide compound that is readily reduced by sulfhydryl compounds to form a highly colored yellow anion. The optical density of this yellow substance was measured at 412 nm. Results are expressed as μg/mg of protein.

The concentration of ascorbic acid (vitamin C) in the brain was determined by the method of Omaye *et al* (32). In this method dehydroascorbic acid, the oxidized product of ascorbic acid, reacts with 2,4-dinitrophenylhydrazine to form bis-2,4-dinitrophenylhydrazone. This undergoes further rearrangement to form a product with an absorption maximum at 520 nm. Thiourea provides the reducing medium to prevent interference from non-ascorbic acid chromogens. Results are expressed in μg/mg of protein.

Vitamin E was analyzed by the method of Desai (33). Ferric ions were reduced to ferrous ions in the presence of vitamin E, and a pink-colored complex was formed. The chelating agent orthophosphoric acid was added to minimize carotene interference in the assay. Results are expressed as μg/mg of protein.

Measurement of tissue lipid peroxides (LPOs). The LPO levels in the brain homogenate were measured according to the method of Ohkawa *et al* (34). The final colored end-product was assayed spectrophotometrically at 532 nm. The LPO level was expressed as nmol malondialdehyde (MDA)/mg protein.

Determination of membrane-bound ATPase activities. Na^+/K^+-ATPase activity in the brain homogenate was assayed by the method of Bonting (35). In this method, 0.2 ml brain homogenate was added to a reaction mixture containing Tris HCl buffer (pH 7.5), 50 mM $MgSO_4$, 50 mM KCl, 600 mM NaCl, 1 mm EDTA, 40 mM ATP and 2.5% ammonium molybdate and incubated for 15 min at 37°C. The reaction was then arrested by the addition of 1 ml ice cold 10% trichloroacetic acid. Then the amount of inorganic phosphorus (Pi) liberated in the protein-free supernatant was estimated. The activity of Mg^{2+}-ATPase in the tissue homogenate was assayed by the method of Ohinishi (36). Brain levels of Ca^{2+}-ATPase level were estimated as described by the method of Hjertan and Pan (37). The activities of these ATPase enzymes in tissue

Table I. Effects of Cd and CA on the activities of enzymatic antioxidants in the brain.

Groups	SOD (U/mg protein)	CAT (U/mg protein)	GPx (nmol/min/mg protein)	GST (nmol/min/mg protein)
Control	7.22±0.19	4.35±0.1	2.87±0.13	5.59±0.45
Cd (5 mg/kg)	4.11±0.13[a]	1.56±0.07[a]	1.38±0.06[a]	3.18±0.21[a]
CA (60 mg/kg)	7.91±0.17[a,b]	3.70±0.08[a,b]	3.95±0.18[a,b]	4.62±0.17[b]
Cd (5 mg/kg) + CA (60 mg/kg)	7.10±0.09[b]	4.20±0.14[b]	4.10±0.15[a,b]	5.43±0.10[b]

Values are expressed as mean ± standard deviation for 6 rats in each group. [a]P<0.05 compared with the control group; [b]P<0.05 compared with the Cd group. Units of measurement are: for SOD, U/mg protein where 1 unit is equal to the amount of enzyme that causes a 50% suppression of the reduction of nitro blue tetrazolium/min/mg of protein; for CAT, U/mg protein where 1 unit corresponds to 1 nmol H_2O_2 consumed/min/mg protein; for GPx, nmol GSH oxidized/min/mg protein; and for GST, nmol GSH-CDNB conjugate formed/min/mg protein. Cd, cadmium; CA, chlorogenic acid; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GST, glutathione S-transferase; GSH, reduced glutathione; CDNB, 1-chloro-2,4-dinitrochlorobenzene.

homogenate were expressed as μmol of Pi liberated/min/mg protein.

Determination of mitochondrial citric acid cycle enzymes. The activity of α-ketoglutarate dehydrogenase (KDH) was estimated from the rate of reduction of NAD$^+$ in the presence of α-KDH at 340 nm (38). The activity of isocitrate dehydrogenase (ICDH) was assayed by the method of King (39). Succinate dehydrogenase (SDH) was assayed by the method of Nulton-Persson and Szweda (40) and the activity was estimated from the rate of reduction of dichloroindophenol (DCIP) in the presence of sodium succinate at 600 nm. The levels of malate dehydrogenase (MDH) were estimated by the method of Mehler *et al* (41). In this reaction the rate of oxidation of NADH was measured in the presence of oxaloacetate at 340 nm.

DNA fragmentation analysis. The DNA fragmentation pattern obtained using agarose gel electrophoresis was analyzed by the method of Watabe *et al* (42). Briefly, the brain samples were washed twice with phosphate-buffered saline and were lysed in a solution of 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% (w/v) sodium dodecyl sulfate and 0.1% (w/v) RNase A, with incubation for 60 min at 50°C. The lysate was then further incubated for 60 min at 50°C with 1 mg/ml proteinase K and subjected to electrophoresis on 1% agarose gel for 60 min at 50 V using 40 mM Tris acetate (pH 7.5) containing 1 mM EDTA. Subsequent to electrophoresis, DNA was visualized by staining with ethidium bromide.

Statistical analysis. The results are expressed as mean ± standard deviation for 6 rats per group. Statistical analysis was performed by one-way analysis of variance using the SPSS software package for Windows (version 10.0; SPSS, Inc., Chicago, IL, USA. Post hoc testing was performed for inter-group comparisons using the least significance difference (LSD) test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of CA and Cd on brain AChE status. Fig. 1 displays the activity of AChE in the brains of control and experimental
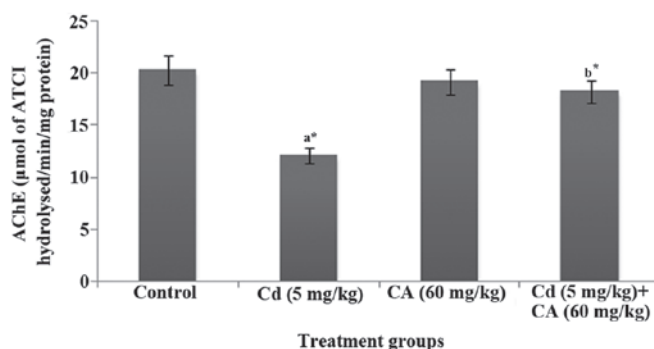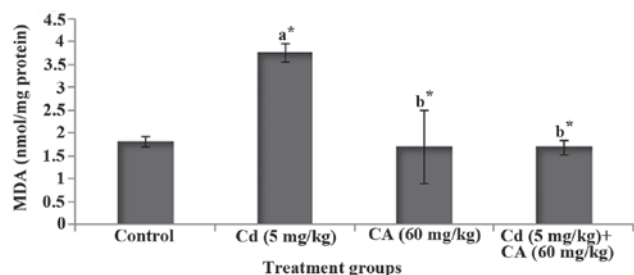

Figure 1. Effect of Cd and CA on the level of AChE in rat brain homogenate. Values are mean ± standard deviation (n=6 per group). [a*]P<0.05 vs. the control group; [b*]P<0.05 vs. the Cd group. Cd, cadmium; CA, chlorogenic acid; AChE, acetylcholinesterase; ATCI, acetylthiocholine iodide.

rats. In the present study, the levels of AChE in the brain were significantly (P<0.05) decreased in the Cd-treated rats whereas the administration of CA (60 mg/kg body weight) significantly (P<0.05) attenuated the Cd-induced reduction in AChE activity, resulting in normal AChE levels.

Effects of CA and Cd on brain enzymatic antioxidant levels. The activities of the enzymatic antioxidants SOD, CAT, GPx and GST in the brains of control and experimental rats are displayed in Table I. Cd-treated rats displayed significant (P<0.05) reductions in the activities of SOD, CAT, GPx and GST when compared with the control rats. The intragastric administration of CA reversed the noxious effect of Cd and elicited a significant (P<0.05) restoration of the levels of antioxidant enzymes to normal values.

Effects of CA and Cd on brain non-enzymatic antioxidant levels. Table II displays the levels of the non-enzymatic antioxidants GSH, vitamin C and vitamin E in the brains of control and experimental rats. The levels of GSH and of vitamins C and E were significantly (P<0.05) diminished in the brain tissues of Cd-treated rats when compared with control rats. Treatment with CA significantly (P<0.05) restored the depleted levels of GSH, vitamins C and E to near normal levels.

Table IV. Effect of Cd and CA on the activities of brain mitochondrial citric acid cycle enzymes.

Groups	SDH	MDH	α-KDH	ICDH
Control	43.57±2.35	875.85±32.65	54.27±3.97	155.57±3.88
Cd (5 mg/kg)	12.65±4.55[a]	553.43±26.38[a]	23.43±1.83[a]	99.41±6.25[a]
CA (60 mg/kg)	43.94±1.47	870.05±31.41	52.84±2.73	160.64±5.58
Cd (5 mg/kg) + CA (60 mg/kg)	26.25±1.35[b]	711.59±26.26[b]	38.46±1.52[b]	134.02±3.57[b]

Units of measurement are: for SDH, nmol succinate oxidized/min/mg protein; for MDH, nmol NADH oxidized/min/mg protein; for KDH, nmol ferricyanide formed/h/mg protein; and for ICDH, nmol α-ketoglutarate formed/h/mg protein. Values are expressed as mean ± standard deviation for 6 rats in each group. [a]$P<0.05$ compared with the control group; [b]$P<0.05$ compared with the Cd group. CD, cadmium; CA, chlorogenic acid; SDH, succinate dehydrogenase; MDH, malate dehydrogenase; α-KDH, α-ketoglutarate dehydrogenase; ICDH, isocitrate dehydrogenase.
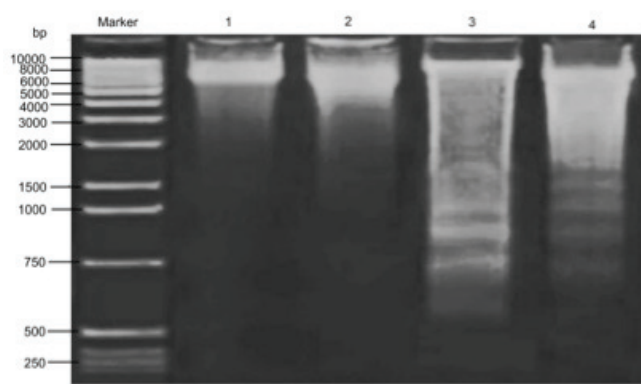

Figure 3. Effect of Cd and CA on brain DNA fragmentation. Marker, DNA molecular weight marker; lane 1, control; lane 2, Cd (5 mg/kg); lane 3, CA (60 mg/kg); lane 4: Cd (5 mg/kg) + CA (60 mg/kg). Cd, cadmium; CA, chlorogenic acid.

neurotoxicity of heavy metals. There is evidence to suggest that free radicals provoked by heavy metals are responsible for diminished AChE activity in the brain (48). In the brain, low levels of AChE could lead to noxious events such as the accumulation of acetylcholine, which causes the rapid firing of neurons leading to convulsions and status epilepticus (49). The modulation of AChE in the brain displayed by Cd is concentration dependent; at a low concentration (0.01 mM) activation of AChE occurs whilst at higher concentrations (>0.1 mM) AChE activity is inhibited (7). Further, Cd induces a conformational change in AChE that leads to the enzyme becoming unreactive (50). In the present study toxic doses (5 mg/kg) of Cd were reflected by diminished levels of AChE and treatment with CA restored the activity of AChE in the brain. The neuroprotective effects that CA elicited in the present study may be due to antioxidant and free radical-scavenging actions. Furthermore, regulation of the ionic homeostasis imbalance in neurons and the highly lipophilic nature of CA may be responsible to its neuroprotective efficacy. Studies suggest that CA has the ability to cross the BBB so that it may effectively block the deleterious effects of free radicals within the brains vascular and cellular compartments (51,52).

In the oxidative stress cascade, lipid peroxidation is the chief culprit and plays an imperative role in the toxicity of many xenobiotics (53,54). In the present study, the oxidative insult of Cd increased the MDA level, an oxidative product of LPO in brain tissue. Previous studies suggest that Cd-induced lipid peroxidation is due to the generation of hydroxyl radicals, superoxide anions, nitric oxide and H_2O_2 (55-57). Indeed, in the present study, treatment with CA effectively reduced the MDA level and this may be due to the ability of CA to transfer electrons, scavenge free radicals, chelate metals and activate antioxidant enzymes. GSH, the first line of defense against reactive oxygen species (ROS), is a readily available source of endogenous sulfhydryl (-SH) groups. It has been demonstrated that Cd exposure causes a marked decline in GSH levels, which may be ascribed to direct conjugation with free or protein-bound -SH groups (58). Further, GSH depletion may be due to the inhibition of glutathione reductase, the enzyme responsible for the catalytic conversion of glutathione disulfide (GSSG) to GSH (59). Cd interferes with the -SH group of metallothionein, a metal chelating protein (60) and thus reduces the GSH level. Vitamin C is a vital antioxidant that also acts as an anti-stress factor. Vitamin E, a lipophilic chain-breaking antioxidant, also plays a critical role in the detoxification of Cd. The present study exemplifies the depletion of non-enzymatic antioxidant levels in the brain, which may be due to increased utilization of these biomolecules to reduce the Cd-induced oxidative stress. CA directly acts as a scavenger to inhibit the Cd-mediated lipid peroxidation and thus reduces the utilization of non-enzymatic antioxidants, consequently leading to improvements of GSH and vitamin C and E levels in the brain. The findings observed in the present study are consistent with previous findings (22).

Cells are equipped with an array of antioxidant defence mechanisms to counteract the effects of free radicals. The present study shows that the Cd-induced increase in lipid peroxidation was accompanied by a concomitant decline in the activities of the cellular enzymatic antioxidants SOD, CAT, GPx and GST. The diminished levels of antioxidant enzymes may be due to the interaction of Cd with the -SH groups of enzymes and the decreased activity of GPx may be due to competition by Cd-metallothioneins (61). Further, a number of studies have indicated that Cd inactivates a wide array of enzymes and proteins involved in the anti-stress mechanism (62-64). In one study, the administration of CA significantly elevated the enzy-

matic antioxidant status in Cd-intoxicated rats, an effect that may be due to its anti-lipid peroxidative potential (65).

Membrane-bound ATPase enzymes are highly vulnerable to peroxidation and the actions of LPOs. They are bound to the plasma membrane and are involved in the translocation of sodium, potassium, calcium and magnesium ions. In the brain, the active transport of sodium and potassium mediated by Na$^+$/K$^+$-ATPase generates membrane potentials. In addition, Na$^+$/K$^+$-ATPase regulates the uptake and release of catecholamines (66,67) and serotonin (68). Ca^{2+}-ATPase modulates intracellular calcium levels (69), and the role of Mg^{2+}-ATPase is to maintain high brain levels of intracellular magnesium ions to control the rates of protein synthesis and cell growth (70). Thus, ATPases are vital for neuronal functions and to maintain the resting membrane potential and nerve conduction. Previous studies indicate that Cd toxicity disrupts the activities of ATPase in CNS (47,71-73), reflecting the changes to membrane and neurotransmitter functions. The reduction of ATPase activity may result from the formation of Cd-ATPase complexes through -SH groups of the enzyme and/or increased oxidative stress. Treatment with CA increased the tissue levels of ATPases, suggesting that it protects the brain by preserving its structural integrity against Cd challenge (74).

Research into cell death has focused on mitochondria due to their role as the arbiters of cell fate in response to stress. Cd is known to cause stress to the mitochondria by affecting the thiol status and depleting ATP, thereby leading to a cascade of pathophysiological events that ultimately result in necrotic cell death (75). The citric acid cycle is an essential metabolic process that is also an integral part of the oxidative defense machinery of the cells. When KDH, ICDH, SDH and MDH are inhibited, the permeability of the inner mitochondrial membrane is increased, which causes mitochondrial dysfunction, and subsequently leads to oxidative brain injury. Notably, in the present study, poisoning with Cd decreased the levels of these mitochondrial marker enzymes, whilst treatment with CA significantly attenuated these effects, possibly as a result of mitochondrial membrane stabilization (76).

Finally, in the present study Cd elevated the extent of DNA fragmentation, possibly as a result of increased ROS production during heavy metal exposure. Treatment with the CD-treated rats with CA profoundly attenuated the toxicity by diminishing the level of DNA fragmentation, possibly by inhibiting the oxidative reactive radicals induced by Cd. These results are consistent with a previous study (77).

In conclusion, the present study provides evidence for the cytoprotective potential of CA against Cd-provoked derangement of brain homeostasis. The neuroprotective effects of CA are mediated through the inhibition of lipid peroxidation and by supporting the endogenous antioxidant defense systems in brain with the subsequent restoration of AChE and membrane-bound ATPase enzyme activities, and prevention of mitochondrial dysfunction and DNA fragmentation. Recently, it was demonstrated that Cd-induced neuronal cell death involves the downregulation of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and the activation of phosphoinositide 3'-kinase/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) (78). This finding may serve as a basis for future research to explore the effect of CA on the signaling mechanisms that mediate the negative effects of Cd.

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