Original Article

Oxidative Stress and Apoptotic Changes of Rat Cerebral Cortical Neurons Exposed to Cadmium *in Vitro**

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Abstract

Objective To investigate the cytotoxic mechanism of cadmium (Cd) on cerebral cortical neurons.

Methods The primary cultures of rat cerebral cortical neurons were treated with different concentrations of cadmium acetate (0, 5, 10, and 20 μmol/L), and then the cell viability, apoptosis, ultrastructure, intracellular $[Ca^{2+}]_i$ and reactive oxygen species (ROS) levels, mitochondrial membrane potential (ΔΨ) , activities of catalase (CAT) and superoxide dismutase (SOD) were measured.

Results A progressive loss in cell viability and an increased number of apoptotic cells were observed. In addition, Cd-induced apoptotic morphological changes in cerebral cortical neurons were also demonstrated by Hoechst 33258 staining. Meanwhile, ultrastructural changes were distortion of mitochondrial cristae and an unusual arrangement. Simultaneously, elevation of intracellular $[Ca^{2+}]_i$ and ROS levels, depletion of ΔΨ were revealed in a dose-dependent manner during the exposure. Moreover, CAT and SOD activities in the living cells increased significantly.

Conclusion Exposure of cortical neurons to different doses of Cd led to cellular death, mediated by an apoptotic mechanism, and the apoptotic death induced by oxidative stress may be a potential reason. And the disorder of intracellular homeostasis caused by oxidative stress and mitochondrial dysfunction may be a trigger for apoptosis in cortical neurons.

Key words: Cadmium; Apoptosis; Oxidative stress; Cerebral cortical neurons

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INTRODUCTION

Cadmium (Cd) is an extremely toxic metal commonly found in industrial workplaces, a food contaminant and a major component of cigarette smoke. It is toxic even at low doses since the metal accumulates and has a long biological half-life in humans (10-30 years)[1] . In addition to peripheral organs, the central nervous system is also subjected to Cd toxicity[2]. Cd can enter into the brain parenchyma and neurons causing neurological alterations in humans and animal models, leading to lower attention, hypernociception, olfactory dysfunction and memory deficits[3-4]. Moreover, there are studies showing the neurotoxicity of Cd at μM range on cell culture models like neurons and glial cells[5-6]. In the past years, Cd has been shown to induce apoptosis

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in vivo[7-8] and *in vitro*[9-11] at various concentrations ranging from 1 to 300 μmol/L. Therefore, Cd-mediated toxicity is thought to involve, at least in part, the induction of apoptosis. However, the mechanisms of Cd-induced apoptosis have not been made clear. Furthermore, there is little information available on the Cd-induced apoptosis in cerebral cortical neurons.

Apoptosis is characterized by cell shrinkage, cytoplasmic, nuclear and chromatin condensation, membrane blebbing, protein fragmentation, and DNA degradation, and finally breakdown of a cell into apoptotic bodies[12]. Although extensive research has been undertaken to elucidate signal pathways in apoptosis, at present, oxidative stress has been considered an important possible mechanism of Cd toxicity[13]. Accumulated evidence has also shown that Cd increased cellular reactive oxygen species (ROS) levels, lipid peroxidation and alteration in glutathione (GSH) levels in many cell types[14-16], suggesting that Cd-induced apoptosis may be connected with oxidative stress. ROS are known to be able to affect mitochondrial membrane potential and trigger a series of mitochondria-associated events including apoptosis[17]. A high level of ROS in the mitochondria can result in free radical attack of membrane phospholipids, preceding mitochondrial membrane depolarization, which is considered as an irreversible step in the apoptosis process, can trigger a cascade of caspases[18]. In addition, it is known that cellular toxicity of Cd is, in part, related to alteration of intracellular calcium (Ca) homeostasis, which can competitively reduce extracellular Ca influx or increase intracellular Ca levels by inhibiting Ca-dependent ATPase[10,19].

Since Cd is a toxic agent for several cells, the aim of this work is to study the mechanism through which Cd induces cell death on cerebral cortical neurons, and the relationship between oxidative stress and apoptosis in the Cd-induced neurotoxicity *in vitro*.

MATERIALS AND METHODS

Materials

Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit was obtained from Pharmingen (Becton Dickinson Company, USA). Fetal calf serum (FCS) was purchased from Sijiqing Biological Engineering Material (Hangzhou, China). Neurobasal A medium and B27 were purchased from Gibco (Grand Island, NY, USA). SOD and CAT analysis kits were obtained from Jiancheng Bioengineering Institute (Nanjing, China). Dulbecco's modified Eagle's medium (DMEM)-F_{12}(1:1), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2′, 7′ -dichlorofluorescein diacetate (DCFH-DA), Fluo-3/AM, Rhodamine 123 (Rh 123), cadmium acetate ($CdAc_2$), Hoechst 33258 staining, trypsin, Penicillin/streptomycin and all other chemicals were purchased from Sigma-Aldrich (USA).

Cell Isolation and Culture

The cerebral cortical neurons were obtained from brains of newborn Sprague-Dawley rats within 24 h according to the procedure reported with minor modification[20]. Briefly, neonatal brain tissues were removed from rats and placed on 35 mm glass petri dishes containing 2 mL ice-cold D-Hanks. The cortex was cut into tiny particles and digested with trypsin for 20 min at 37 °C. The cortex was easily dissociated by triturating in DMEM/F_{12} containing 10% fetal calf serum, and using a sterile, long-neck Pasteur pipette. The cell concentration was adjusted to 1×10^6/mL and the suspension was planted in 96-, 24- or six-well plates coated with 100 mg/L poly-L-lysine. Cells were incubated at 37 °C in a humidified incubator containing 5% CO_2 for 4 h. Then, the medium was changed and cells were incubated in serum-free neurobasal A medium supplemented with 2% B27, 5 μmol/L glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin. On Day 3, the cultures were incubated with 2.5 mg/L cytosine arabinoside for 24 h to suppress the growth of glial cells. The cells were used on Day 6. Cell viability was assessed by the trypan blue exclusion and it was routinely higher than 95%. Cell purity was checked by staining cells with tolridine blue to identify neurons.

Cd Treatment

Regarding the toxic doses chosen for this experiment, cells were incubated in the presence of 0, 5, 10, and 20 μmol/L $CdAc_2$, in a serum-free medium at different time intervals. Primarily, events of Cd exposure over a 12-h period were chosen to investigate the Cd neurotoxicity.

Measurement of Cell Viability

Cells were seeded in 96-well plates. After the preprocessing, the cells were treated with various Cd doses (0, 5, 10, and 20 μmol/L) for a time range of 3, 6, 12, and 24 h. At the designated time points, cell viability was measured by the MTT assay, which is based on the conversion of the tetrazolium salt to the colored product, formazan. In brief, 20 μL

MTT solution (5.0 g/L in PBS) was added into each well of the 96-well plate (containing 100 μL medium and cells) 4 h before the end of incubation. The supernatant was then discarded, and 150 μL DMSO was added to dissolve the formazan. The absorbance was measured at 570/630 nm by the microplate reader (Sunrise, Austria).

Cell Morphological Analysis

Cells were seeded in a six-well plate. On Day 6, various Cd doses (0, 5, 10, and 20 μmol/L) were added. After incubation for 12 h, the culture plates were examined and photographed by a T-DH inverted phase microscopy (Nikon, Japan) equipped with the Quick Imaging system.

Ultrastructural Analysis

For transmission electron microscopy observation, after being treated with various Cd doses (0, 5, 10, and 20 μmol/L) for 12 h, the cells were trypsinised, collected and centrifuged. The pellets were immediately fixed in ice-cold glutaraldehyde (2.5% in 0.1 mol/L cacodylate buffer, pH7.4) for 24 h and cells were postfixed in osmium tetroxide. After dehydration with a graded series of alcohol concentrations, the samples were rinsed in propylene oxide and impregnated with epoxy resins. The ultrathin sections were contrasted with uranyl acetate and lead citrate for electron microscopy. Electron micrographs were taken with a PHILIPS CM-120 transmission electron microscope.

Hoechst 33258 Staining

Apoptotic morphological changes in the nuclear chromatin were detected by staining with the DNA binding fluorochrome Hoechst 33258 (bisbenzimide). Cerebral cortical neurons were seeded on sterile cover glasses placed in the 24-well plates. After incubation with various concentrations of Cd (0, 5, 10, and 20 μmol/L) for 12 h, cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 10 min, and then incubated with 50 μmol/L Hoechst 33258 staining solution for 10 min. After three washes with PBS, the cells were observed under an inverted fluorescence microscope (Olympus, IX-70, Japan) at an excitation wavelength of 352 nm through FITC filter (blue fluorescence).

Flow Cytometric Analysis

All the following assays were carried out on BD-FACS Aria flow cytometer. Cells were cultured in six-well plates and treated with various Cd concentrations (0, 5, 10, and 20 μmol/L) for 12 h. After the treatment, the adherent cells were collected with 1.25 g/L trypsin solution. The detached and adherent cells were pooled and centrifuged at 2 000 r/min for 10 min. The harvested cells were washed twice with PBS and incubated with various fluorescent dyes for the flow cytometric analysis. Cell debris, characterized by a low forward scatter/side scatter, was excluded from the analysis. The data were analyzed by the Cell Quest program, and mean fluorescence intensity was obtained by histogram statistics.

Quantitation of Apoptosis The apoptosis induced by Cd was analyzed by Annexin V binding and propidium iodide (PI) uptake. After treatment with Cd for 12 h, harvested cells were suspended in a binding buffer (1×). An aliquot of 100 μL was incubated with 10 μL Annexin V-FITC and 5 μL PI for 15 min in dark, and 400 μL binding buffer (1×) was added to each sample. The stained cells were analyzed directly by flow cytometry with the Cell Quest program. The percentage of apoptosis was calculated from early apoptosis (annexin V^+/PI^-) and late apoptosis (annexin V^+/PI^+).

Reactive Oxygen Species (ROS) Measurement Generation of ROS was monitored by measurement of hydrogen peroxide (H_2O_2) generation with the fluorescent probe dichlorofluorescin diacetate (DCFH-DA). This dye is cleaved to form non-fluorescent dichlorofluorescin (DCFH) in the cells, which gets oxidized to fluorescent dichlorofluorescein (DCF) by ROS. Thus, the DCF fluorescence intensity is proportional to the amount of peroxides produced by the cells. Briefly, the harvested cells were incubated with DCFH-DA (10 μmol/L final concentration) for 30 min in the dark at 37 °C. After treatment, cells were immediately washed twice and re-suspended in PBS. ROS generation was measured by the fluorescence intensity (FL-1, 530 nm) of 10 000 cells on flow cytometer.

Mitochondrial Membrane Potential (ΔΨ) Assay For the detection of mitochondrial ΔΨ, the harvested cells were incubated with Rh 123 (10 mg/L final concentration) for 30 min in the dark at 37 °C, harvested and re-suspended in PBS. The mitochondrial ΔΨ was measured by the fluorescence intensity (FL-1, 530 nm) of 10 000 cells.

Analysis of Intracellular Free Ca^{2+} Concentration ($[Ca^{2+}]_i$) Fluo-3/AM was chosen to be used as

an intracellular free Ca^{2+} fluorescent probe for the analysis of $[Ca^{2+}]_i$ in Cd-exposed cerebral cortical neurons. In brief, the harvested cells were incubated with Fluo-3/AM (1 μmol/L final concentration) for 30 min in the dark at 37 °C and then washed with D-Hank's solution. Intracellular $[Ca^{2+}]_i$ levels were represented with the fluorescent intensity (FL-1, 530 nm) of 10 000 cells on the flow cytometer.

Antioxidant Enzyme Detection

Cells were seeded in six-well plates and treated with different concentrations of $CdAc_2$ (0, 5, 10, and 20 μmol/L) for 12 h. After the treatment, the adherent cells (live cells) were collected to detect the antioxidant enzymes.

Measurement of Catalase (CAT) Activity CAT activity was assayed with a spectrophotometer by measuring the decrease in absorbance of H_2O_2 at 240 nm, as previously described[21]. The activity was expressed as units per milligram protein (U/mg prot).

Determination of Superoxide Dismutase (SOD) Activity The total (Cu-Zn and Mn) superoxide dismutase (t-SOD) enzyme activity was determined based on the inhibition of nitroblue tetrazolium (NBT) reduction by O^{2-}, generated by the xanthine/XO system[22]. One unit of SOD was defined as the amount of enzyme causing 50% inhibition of the NBT reduction rate. SOD activity was expressed as U/mg prot.

Statistical Analysis

The results were expressed as mean±SD from at least three independent experiments with different batches of cells, each one performed in duplicate or triplicate. Statistical comparisons were made by using one-way analysis of variance (ANOVA; Student's *t*-test) after ascertaining the homogeneity of variance between the treatments. Differences between groups were considered significant if $P<0.05$.

RESULTS

Action of Cd on Cellular Viability

The MTT assay showed that the toxic effect of Cd on cell viability in cerebral cortical neurons was time- and concentration- dependent (Figure 1). When 20 μmol/L Cd effectively affected cell viability after a 3 h incubation period ($P<0.01$), the lower concentration with 5 and 10 μmol/L Cd, significantly decreased the cell viability after a period of 6-h exposure ($P<0.05$ or $P<0.01$). The cellular death caused by 20 μmol/L Cd was always higher than that by 5 and 10 μmol/L Cd with the same exposure time. At the longest exposure time (24 h), almost 62% cells remained viable at 5 μmol/L Cd compared to ~47% at 10 μmol/L Cd and ~37% at 20 μmol/L Cd.

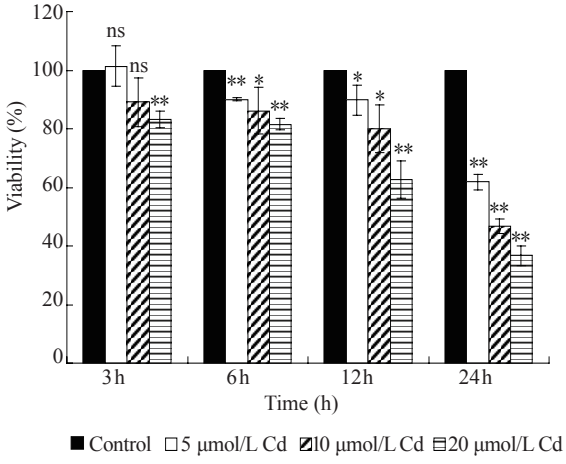

Figure 1. Effect of Cd on cell viability measured by MTT assay. The cells were incubated with increasing concentrations of $CdAc_2$ for different periods of time. Cell viabilities of the control group at the designated time points were assumed as 100%, and the variations were represented as percentage. Absorbance was measured at 570/630 nm. Each point represents mean±SD (n=6). ns Not significant, $^{*}P<0.05$, $^{**}P<0.01$ as compared to respective control, using one-way ANOVA.

Effect of Cd Treatment on Neuronal Morphology

Results from Figure 2 were obtained after the treatment of the cells with different Cd concentrations by phase-contrast microscopic observation. Cd changed the neuronal morphology after a 12-h treatment at different Cd concentrations. The decreased cell density and neural network were observed from these figures (Figure 2B-D); furthermore, the cells showed a great loss of neuronal integrity mainly evidenced by the disappearance of the axons and dendrites caused by Cd at 20 μmol/L (Figure 2D).

Ultrastructural Changes of Cerebral Cortical Neurons after Treatment with $CdAc_2$

Transmission electron microscopy showed that the normal cerebral cortical neurons had a well-defined outline and that spherical or oval mitochondria possessed well-defined transversal cristae (Figure 3A). After treatment with Cd for 12 h, we found that changes in mitochondria

ultrastructure included disruption and loss of mitochondrion cristae (Figure 3B-D), mitochondria swelling, vacuoles formed in cytoplasm(Figure 3C), Cell plasma membrane disruption (Figure 3D).

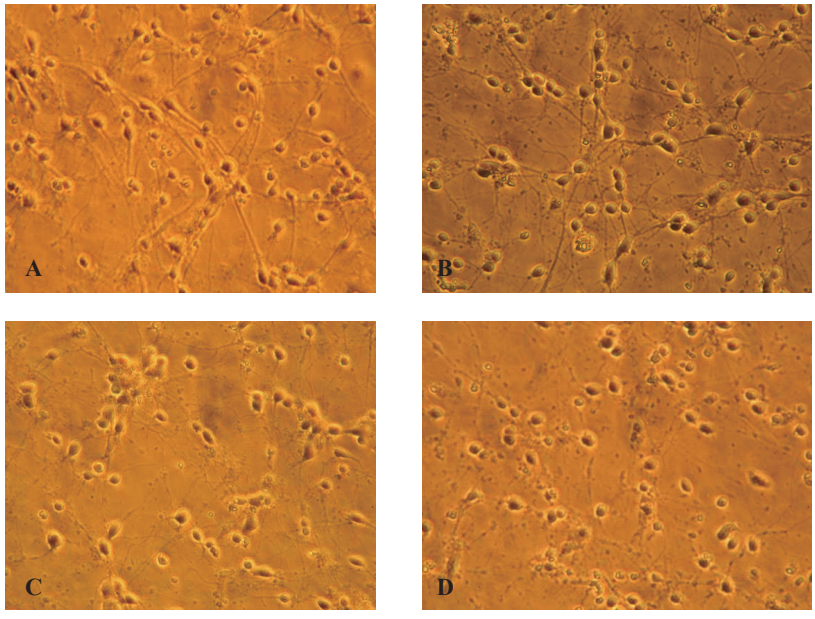

Figure 2. Cd effect on morphology of cerebral cortical neurons. The morphology of cerebral cortical neurons after exposure to the different doses of $CdAc_2$ for 12 h was examined and photographed by the phase microscope (200×). A, Control; B, 5 μmol/L $CdAc_2$; C, 10 μmol/L $CdAc_2$; D 20 μmol/L $CdAc_2$.

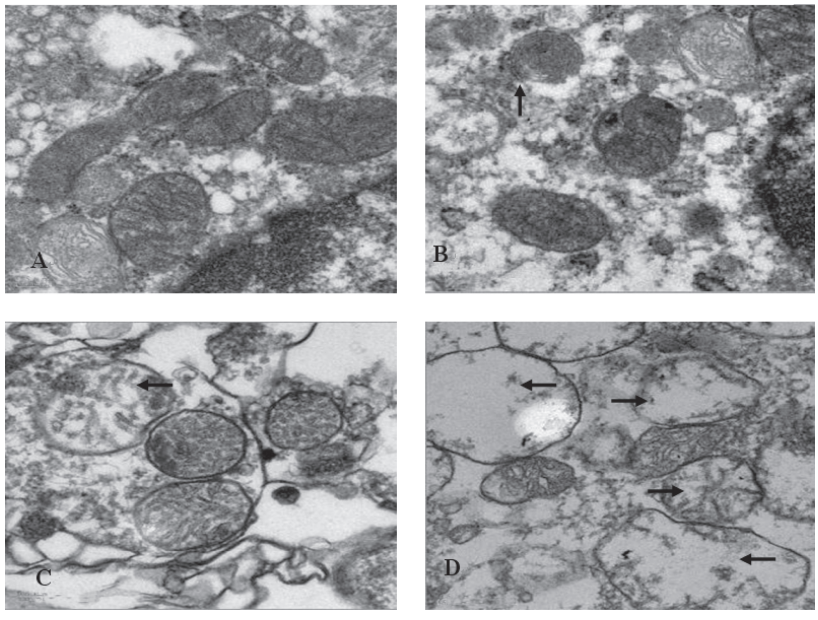

Figure 3. Effects of $CdAc_2$ treatement on the ultrastructural morphology of cerebral cortical neurons by transmission electron microscopy (26 500×). Cells were grown and treated with various doses of $CdAc_2$ for 12 h. The changes were indicated by arrows. A, Control; B, 5 μmol/L $CdAc_2$, disruption of mitochondrion cristae; C, 10 μmol/L $CdAc_2$, vacuoles formed in cytoplasm and swelled mitrochondrion; D, 20 μmol/L $CdAc_2$, mitrochondrion swelling, loss of mitochondrion cristae, cell plasma membrane disruption, cytoplasmic organelles breakdown.

Induction of Apoptosis by Cd

In the present study, flow cytometric measurement (Annexin V/FITC and PI double staining) was used to quantify the extent of apoptosis in the total cell population. After incubation with different concentrations (5, 10, and 20 μmol/L) of $CdAc_2$ for 12 h, the percentage of total (early+late) apoptotic cells increased to 6.6%, 8.1%, and 19.9%, respectively, compared to 0.5% in the control group (Figure 4), which indicated that Cd induced apoptosis in cerebral cortical neurons.

Effect of Cd on Apoptotic Morphological Changes

Apoptotic morphological changes induced by Cd were assessed by Hoechst 33258 staining (Figure 5). In the control group, the majority of cells had uniformly stained nuclei and the chromatin of normal nuclei was unaltered and spread uniformly throughout the entire nucleus (Figure 5A). After exposure to $CdAc_2$ (5, 10, and 20 μmol/L) for 12 h, nuclear morphological changes typical of apoptosis were observed, i.e., nuclear chromatin was condensed and fragmented chromatin characterized by a scattered, drop-like structure. The nuclei of apoptotic cells appeared smaller and shrunken when compared to intact cells (Figure 5 B-D).

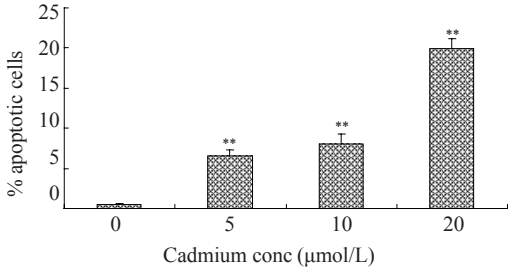

Figure 4. The apoptosis induced by Cd in cerebral cortical neurons measured by the flow cytometry. The cells were treated with different concentrations of Cd (0, 5, 10, and 20 μmol/L) for 12 h for the apoptosis assay. Results are expressed as percentages of apoptosis with regard to the total cells. Data are mean±SD of three separate experiments with each one performed in triplicate. $^{**}P<0.01$ compared to the control, using one-way ANOVA.

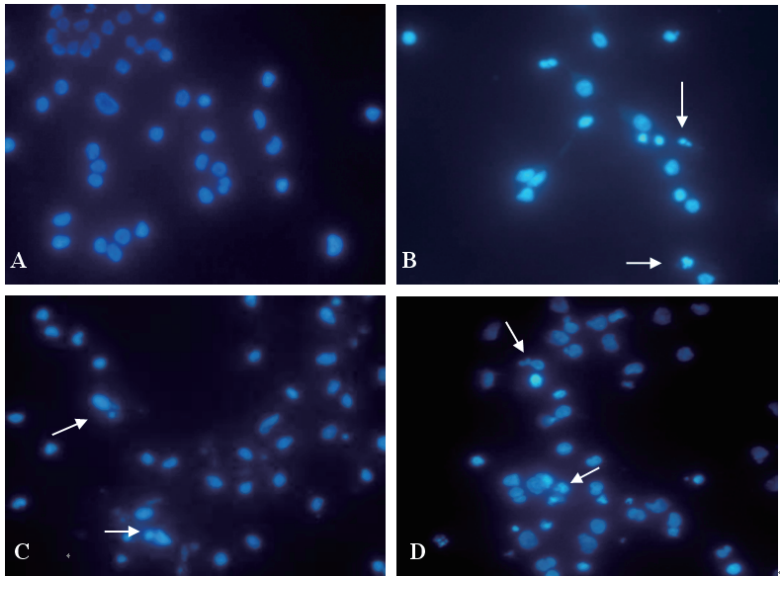

Figure 5. Effects of Cd on apoptotic morphological changes in cerebral cortical neurons. Cells were incubated with $CdAc_2$ (0, 5, 10, and 20 μmol/L) for 12 h and nuclear chromatin changes (apoptosis) were assessed by Hoechst 33258 staining. Arrows identify apoptotic nuclei. Among the groups, A, control; B, 5 μmol/L Cd; C, 10 μmol/L Cd; D, 20 μmol/L Cd.

Effect of Cd on ROS Generation and Intracellular $[Ca^{2+}]_i$

DCFH-DA, a specific dye for intracellular H_2O_2, was used to measure the level of intracellular ROS in cerebral cortical neurons treated with Cd. As shown in Figure 6A, the dose-dependent generation of ROS increased significantly at 12 h ($P<0.01$). Simultaneously,

there was a concentration-dependent enhancement in intracellular $[Ca^{2+}]_i$ (Figure 6B). Significant $[Ca^{2+}]_i$ increment was observed at 12 h (P<0.01) in groups with the three Cd concentrations (5, 10, and 20 μmol/L).

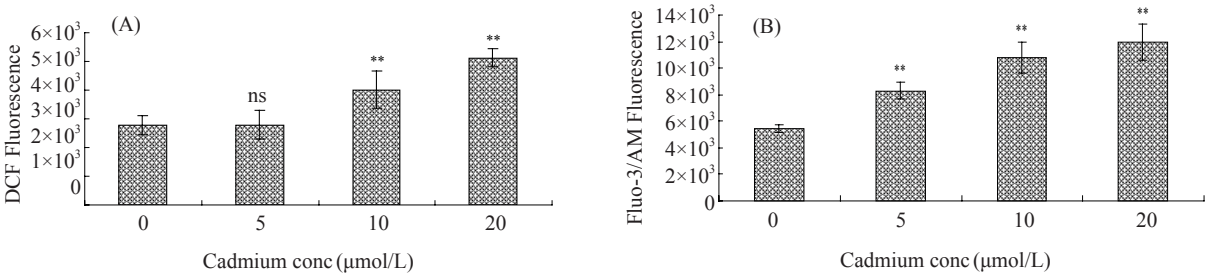

Figure 6. Effect of Cd on generation of ROS (A), intracellular $[Ca^{2+}]_i$ levels (B), measured by flow cytometry. (A), The harvested cells were incubated with 10 μmol/L DCFH-DA for 30 min at 37 °C. DCF fluorescence was measured by using the flow cytometer with FL-1 filter. (B), The harvested cells were incubated with 1 μmol/L Fluo-3/AM for 30 min at 37 °C. Fluo-3 fluorescence was measured by using the flow cytometer with FL-1 filter. Fluorescence results were expressed as mean fluorescence intensity. Each bar represents mean±SD (n=6). ns Not significant; $^{**}P$<0.01 compared to the control, using one-way ANOVA.

Cd-induced Mitochondrial Membrane Depolarization

Rh 123, a lipophilic cationic fluorescent dye, is selectively taken up by mitochondria and used to assess the mitochondrial ΔΨ of cerebral cortical neurons. As shown in Figure 7, the mitochondrial ΔΨ decreased in cerebral cortical neurons after exposure to $CdAc_2$ (5, 10, and 20 μmol/L) for 12 h. Also, its reduction was in a concentration-dependent manner. Significant mitochondrial ΔΨ reduction at 20 μmol/L was observed (P< 0.01).

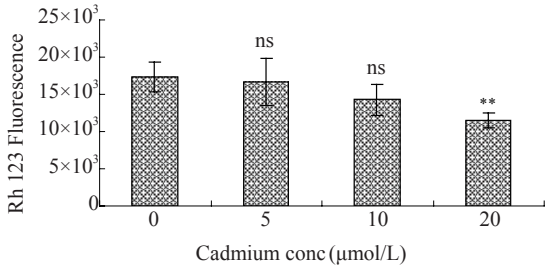

Figure 7. Effects of Cd on mitochondrial ΔΨ. Cells were treated with $CdAc_2$ (0, 5, 10, and 20 μmol/L) for 12 h. Rh 123 was added, and the harvested cells were incubated for 30 min. The fluorescence was measured by using the flow cytometer with FL-1 filter. Each bar represents mean±SD (n=6). ns Not significant; $^{**}P$<0.01 compared to the control, using one-way ANOVA.

Cd Action on Activities of CAT and SOD

After incubation with $CdAc_2$ (0, 5, 10, and 20 μmol/L) for 12 h, information on activities of CAT and SOD in living cells is shown in Figure 8A and B. At 12 h, it was found that Cd significantly increased the CAT activity (P<0.01). Simultaneously, there was a concentration-dependent significant increase of SOD activity in the groups with 10 and 20 μmol/L $CdAc_2$ (P<0.05).

DISCUSSION

Cd pollution in the environment may be accumulated in human body through direct exposure or food chain, resulting in neuro-degeneration as well as many other diseases. Cd may induce Parkinson's disease, Alzheimer's disease and other neuro-degenerative disorders primarily by triggering neuronal cell death. However, the underlying mechanism remains to be elucidated. Therefore, this study was designed to investigate the intracellular events leading to $CdAc_2$-induced neurotoxicity in primary cultures of rat cerebral cortical neurons, focusing on the relationship between apoptosis and oxidative stress. Primarily, events of Cd exposure over a 12 h period were chosen to investigate their toxic effects. The progressive loss in cell viability showed that Cd exposure induced cellular death in these cells, depending on both the Cd concentration and the exposure time (Figure 1), and the time- and dose-dependent Cd-induced toxicity has been reported also by some authors for other cell types[23-24].

With the analysis of phase microscopy and transmission electron microscopy (Figures 2 and 3),

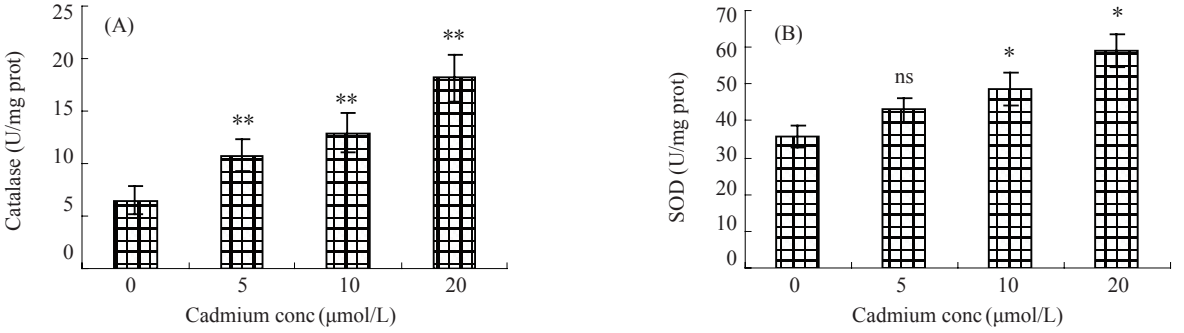

Figure 8. Effects of CdAc2 on CAT activity (A) and SOD activity (B). Cerebral cortical neurons were treated for 12 h with the indicated Cd concentrations. Data are mean±SD of three experiments with cells from different cultures, each one performed in triplicate. ns Not significant; $^{*}P<0.05$; $^{**}P<0.01$ as compared to the control, using one-way ANOVA.

the cell morphology was affected by Cd treatment for 12 h. These morphological changes were mainly located in the neural extensions (axons and dendrites), which almost disappeared after 12 h of treatment with 20 μmol/L Cd. Such morphological changes induced by Cd have also been reported by López[25]. These results indicated that Cd induced cytotoxicity on cortical neurons.

The flow cytometry analysis showed that cortical neurons exposed to 5 μmol/L, 10 μmol/L, and 20 μmol/L Cd for 12 h underwent apoptosis (Figure 4). In addition, Cd-induced apoptosis was assessed by Hoechst 33258 staining (Figure 5). Compared with the intact cells, Cd exposure resulted in obvious apoptotic morphological changes, and this change tendency was consistent with the result obtained by the flow cytometric analysis. Apoptotic morphological changes further showed that apoptosis played a critical role in Cd-induced cytotoxicity in cerebral cortical neurons.

ROS may act as a signal molecule of the death pathway[26]. Several investigations have demonstrated that the cytotoxicity by Cd is associated with ROS[14-16]. The ability of Cd to induce oxidative stress in cortical neurons is aided by the induction of this action of ROS. Herein, cortical neurons treated with Cd at concentrations between 5 and 20 μmol/L generated ROS (Figure 6A), further demonstrating that oxidative stress played a critical role in the Cd toxicity. Mitochondria are the major source of ROS and its production is related to the level of electron transport[27] and ROS production rises when electron transport is reduced, which occurs in pathological situations[28]. Large amounts of ROS seem to be lethal by inducing the mitochondrial permeability transition, and consequently mitochondria swelling, loss of the mitochondrial ΔΨ and further ROS production[27,29]. Then, the excessive ROS contribute to cell aging because they can oxidize lipids, proteins, and DNA[30]. In this study, the induction of ROS in the cerebral cortical neurons could be mediated by mitochondria alterations because Cd caused breakdown of the mitochondrial ΔΨ (Figure 7). ROS are not only inducers of cell death due to their high toxicity but also signaling molecules in apoptosis, which can directly activate apoptosis[31]. In metal-induced apoptosis, it is thought that the mitochondria are most pertinent in mediating apoptosis, putatively via metal-induced ROS[29]. Since accumulation of ROS targeted the mitochondrial membrane to induce a collapse of mitochondrial, it is reasonable to assume that the mitochondria play a crucial role in Cd-induced apoptosis. These results indicate that the increased ROS production by Cd may lead to the reduction of ΔΨ. This in turn increases mitochondrial membrane permeability and subsequently causes a release of mitochondrial apoptogenic factors into cytosol.

Furthermore, evidence suggests that the elevation of intracellular $[Ca^{2+}]_i$ is associated with the development of apoptosis[32-33]. In this study, in order to evaluate the effect of Cd on $[Ca^{2+}]_i$ in cerebral cortical neurons, Fluo-3/AM was used as an intracellular free Ca^{2+} fluorescent probe. We observed that treatment with $CdAc_2$ (5, 10, and 20 μmol/L) resulted in abnormal manifestation in intracellular $[Ca^{2+}]_i$, showing significantly stronger $[Ca^{2+}]_i$ in the treated cells than in control cells at 12 h (Figure 6B). The fact shows that abnormal Ca^{2+} homeostasis due to Cd exposure may be another important mechanism of the development of apoptosis in these cells. Intracellular calcium overload may be related to the mitochondrial dysfunction. Since mitochondria are the major site of ATP production and mitochondrial ΔΨ is the driving force of ATP synthesis, breakdown in the mitochondrial ΔΨ could

lead to the fall of the ATP levels[34]. The resulting reduction in cellular ATP levels can give rise to a disruption of ionic homeostasis which can cause an increase in $[Ca^{2+}]_i$ and subsequent cellular apoptosis/necrosis[35]. Regarding the effect of mitochondria on the cell viability, the threatening event is the mitochondrial permeability transition, which occurs in response to calcium overload during elevated cellular ROS levels[36]. The decrease of mitochondrial $\Delta\Psi$ (Figure 7) and the increase of intracellular $[Ca^{2+}]_i$ (Figure 6B) induced by Cd in cerebral cortical neurons indicate a clear mitochondrial dysfunction together with an alteration of Ca^{2+} homeostasis.

Except cellular death mediated by Cd, there are some surviving cells which are resistant to the action of this cation. The cellular survival in the presence of the toxic effect of ROS formation is based on the equilibrium between the toxic action and the ability of living cells to protect them against ROS action. Taking this into account, the possible antioxidative defense mechanism in the living cells was investigated. SOD is one of the most effective intracellular enzymatic antioxidants, which catalyzes the dismutation of O_2^- to O_2 and to the less-reactive species H_2O_2. CAT is located in the peroxisomes, which efficiently promotes the conversion of hydrogen peroxide to water and molecular oxygen[37]. Our results indicate that in cerebral cortical neurons, exposure to Cd induced an increase in CAT (Figure 8A) and SOD (Figure 8B) activities at the three Cd concentrations. The results seem to suggest that there is a group of cells which are resistant to Cd because they are able to induce enzymes against oxidative stress producing ROS. Maybe the increase in CAT and SOD activities is an indication of increased ROS generation in living cells due to Cd treatment and this lends credence to the data in Figure 6A. Also, the increase in these enzymatic activities could be due to an increase in the enzyme expression, since it is known that oxidative damages induce a cellular response, which tries to compensate the overload of the ROS formation[38]. Another possibility is that the enzyme activity was activated by the Cd ion directly.

Regarding the Cd effect on antioxidant enzymes, there are contradictory results[6,39]. Contrary to the present findings, lower activities of SOD and CAT were strongly correlated with Cd intoxication in previous evidence. Such diversity of results may be due to the effect of Cd varies functionally with: (a) the cellular type, (b) the Cd concentration used, and (c) the exposure time. Those variations might be due to the higher concentration of Cd acetate and longer exposure time adopted in those studies compared to the lower concentration (5-20 μmol/L) and shorter exposure time (12 h) in the present investigation. Also, most of the previous experiments were based on animal studies. Whether Cd acetate influences the activities of antioxidant enzymes differently at low or high concentrations/for long or short exposure time is worthy of further investigations .

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