Influence of Cadmium on Nerve Cells of the Brain and the Neuroprotective Effect of Ca^{2+} Chelator and N-Acetyl-L-Cysteine

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ABSTRACT

This paper discussed the mechanism of damage and apoptosis caused by cadmium (Cd) to the nerve cells of the brain and the influence of Cd on the mRNA expression of Bcl-2 and NO synthase (NOS) genes. In addition, the protective influence of BAPTA-AM and N-Acetyl-L-cysteine (NAC) to the nerve cells was analyzed. Experiments were conducted to measure cell survival and concentrations of Ca^{2+} and ROS under different concentration of cadmium acetate. The results showed that with the increase in the concentration of cadmium acetate, the survival of nerve cells declined dramatically, while the intercellular Ca^{2+} and ROS concentrations increased significantly. This indicated considerable damage caused by Cd to the nerve cells. The mRNA expression of Bcl-2 decreased significantly with the increase in Cd concentration, while the mRNA expression of Bax increased to varying degrees. At a higher concentration of Bcl-2, Bcl-2/Bax heterodimer was detected in nerve cells, which slowed down the apoptosis of the cells; at a low concentration of Bcl-2, Bcl-2/Bax homodimer was formed, thus accelerating the apoptosis of the nerve cells. Fluorescence staining showed that the nerve cells in the control group were intact, uniformly stained and had elliptical nuclei. For the experimental group, the nuclei in most cells shrank in size or even became fragmented due to the presence of Cd. BAPTA-AM reversed the sudden increase of Ca^{2+} concentration in the nerve cells treated by Cd, while NAC reduced cell apoptosis by inhibiting the breaking and mutation of the DNA strand in cells. Compared with BAPTA-AM, NAC exhibited less significant inhibitory effect on Cd-induced cell apoptosis and offered limited neuroprotective effect on nerve cells.

Key Words: Nerve Cell of the Brain, Cadmium, Cell Apoptosis, mRNA Expression, NAC, Calcium Chelator

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Introduction

Cadmium (Cd) is a heavy metal element widely present in the living environment of human beings. Cd is the metal toxin that is most likely to accumulate in the human body, with a half-life of over 10 years and causing irreversible damage to multiple organs, especially the nerve cells of the brain. Some reports have shown that Cd can cause extensive apoptosis of the nerve cells of the brain (Chargui *et al.,* 2011; Maiuri *et al.,* 2007; Torres, 2003; Yuan *et al.,* 2015; Green, 2005. Kroemer *et al.,* 2009).

Balance of intracellular calcium ions, mitochondrial membrane potential (MMP) and reactive oxygen species (ROS) plays an important role in maintaining the normal shape, functioning and signal transduction of nerve cells. Cd, however, can cause imbalance of intracellular calcium ions, MMP and ROS (Shintani and Klionsky, 2004; Dong *et al.,* 2009; Yang *et al.,* 2009; Antoniio *et al.,* 1999; Gui *et al.,* 2005;

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Cantarella *et al.,* 2007). The mRNA expression of Bcl-2 gene is closely associated with the regulation of nerve cell apoptosis. In particular, the expressions of Bcl-2, Bax and Bcl-2/Bax are considered as the most important indicators of the health status of nerve cells. NOS is a neurotransmitter closely related to memory and learning ability and regulation of blood supply to the brain. Therefore, investigating the influence of Cd content on the mRNA expressions of nNOS and Bcl-2 in nerve cells can inform the understanding of the mechanism of Cd-induced apoptosis (Livak and Schmittgen, 2012; Son *et al.,* 2010; Lin *et al.,* 2009; Ramirez and Gimenez, 2003; Wang *et al.,* 2008; Wang *et al.,* 2015; Wang *et al.,* 2009). Study has shown that calcium chelator BAPTA-AM can inhibit the damage caused by Cd to the nerve cells. N-acetylcysteine can also protect the cells against DNA damage, promoting cell viability and suppressing cell apoptosis.

This study focused on the mechanism of damage and apoptosis induced by Cd to the nerve cells of the brain, as well as the influence of Cd on mRNA expression of Bcl-2 and gene encoding for NOS in the nerve cells. We also analyzed the neuroprotective effect of BAPTA-AM and NAC on the nerve cells. The changes in cell survival rate, Ca^{2+} and ROS concentrations of cells under different concentrations of cadmium acetate were measured by experiments.

Influence of Cd on apoptosis and mRNA expression of nerve cells of the brain

Bcl-2 gene and the gene encoding for NOS play a decisive role in the growth and development of nerve cells of the brain, and also in the regulation of blood supply to the brain and anti-apoptotic effect. Therefore, it is of high importance to study the influence of Cd concentration on mRNA expression of nNOS and Bcl-2 genes.

Materials and methods

Reagents used in this experiment included DMEM, B-27, Gibco, cytarabine, Hoechst, BBI, Amresco, cadmium acetate, RNA extraction reagent, active enzymes and serum.

Adult rats that were born and reared in a large laboratory were used. Before formal experiment, the rats were acclimatized for 2 weeks. The female and male rats were kept in the same cage at a proportion of 3:1. Pregnancy of female rats was detected and the pregnant rats were labeled. Nerve cells of the brain were harvested from fetal rats aged 20 days and placed in the culture medium. Different amount of cadmium acetate was added into the culture media. Control group was set up.

The influence of Cd on the apoptosis rate and morphological changes of apoptosis was analyzed by experiment. The cells were washed and centrifuged, and the filtrate was collected, washed and photographed. To discuss the influence of Cd on mRNA expression of nNOS and Bcl-2 genes, Cd-contaminated RNA was extracted and its concentration and purity were analyzed, using uncontaminated RNA as control.

Influence of Cd on mRNA expression of Bcl-2 gene

Fig. 1 shows the survival of nerve cells when the concentration of cadmium acetate was 0μmol/L, 5μmol/L, 10μmol/L and 20μmol/L in the culture medium, respectively. It can be seen that as the concentration of cadmium acetate increased, the survival rate of nerve cells decreased considerably. To be specific, the survival rates at the concentration of 5$\mu\mu$mol/L, 10$\mu\mu$mol/L and 20μmol/L were 88.7%, 79.6% and 61.3%, respectively, indicating the severe damage caused by Cd.

Fig. 2 shows the mRNA expressions of Bcl-2 under different cadmium acetate concentrations. It can be seen that the mRNA expressions of Bcl-2 decreased considerably in all treatments. The reduction was about 23.1% under the concentration of 5μmol/L. As the concentration of cadmium acetate increased, the mRNA expression of Bcl-2 decreased, by 15.5% and 8.2% under the concentration of 10μmol/L and 20μmol/L, respectively.

Fig. 3 shows the mRNA expression of Bax under different concentrations of cadmium acetate. Fig. 4 shows the expression ratio of Bcl-2 to Bax. Compared with Bcl-2, mRNA expression of Bax increased to varying extent. The increase was largest under the concentration of 10μmol/L, with the value of 57.3%; however, the mRNA expression decreased by 15.5% and 8.2% under the concentration of 5μmol/L and 20μmol/L, respectively. It can be seen from Fig. 4 that the Bcl/Bax ratio was 0.585, 0.559 and 0.868 under the three cadmium acetate concentrations, respectively. Bcl/Bax ratio was a measure of the survival of nerve cells. When Bcl-2 content was higher, Bcl-2/Bax heterodimer was formed in the cells, thus slowing down the apoptosis of nerve cells; otherwise, Bcl-2/Bax homodimer was formed, which accelerated cell apoptosis.

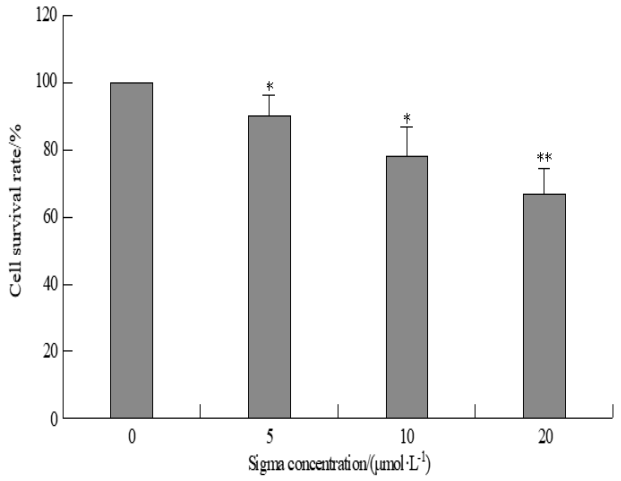


Figure 1. Viability of brain nerve cells under different cadmium acetate concentrations

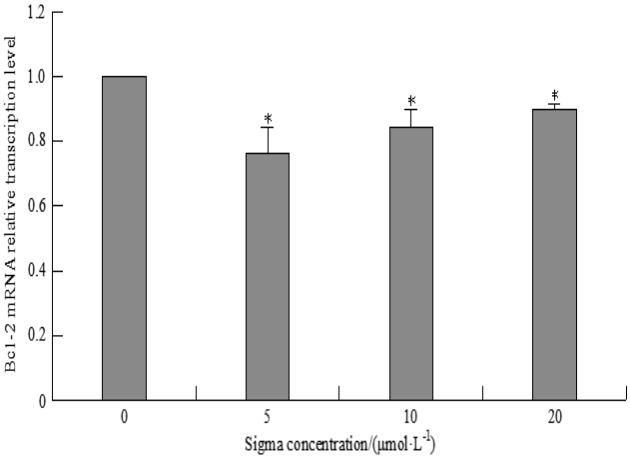


Figure 2. mRNA expressions of Bcl-2 in primary cultured cerebral cortical neurons in different treatments

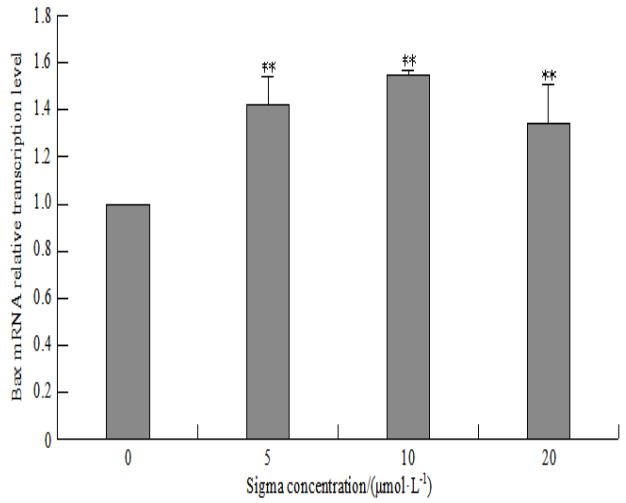


Figure 3. mRNA expressions of Bax in primary cultured cerebral cortical neurons in different treatments

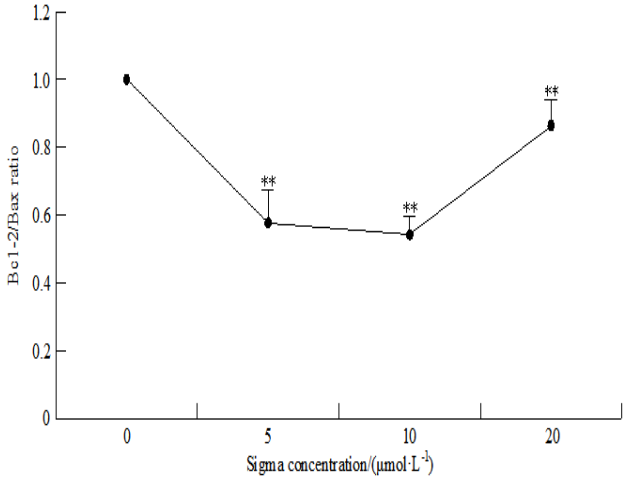


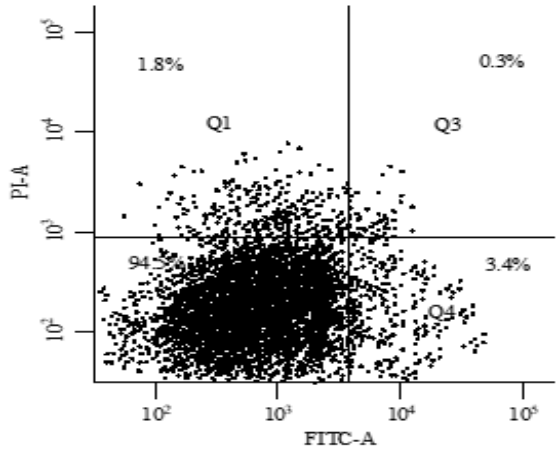
Figure 4. Changes in Bc1-2/Bax ratio in primary cultured cerebral cortical neurons in different treatments

It can be known from the above analysis that Cd caused severe damage to the normal structure of nerve cells, inducing considerable apoptosis. Bcl-2 is an anti-apoptotic gene, while Bax is a pro-apoptotic gene. As the Cd concentration in the cell increased, the mRNA expression of Bcl-2 decreased and that of Bax increased.

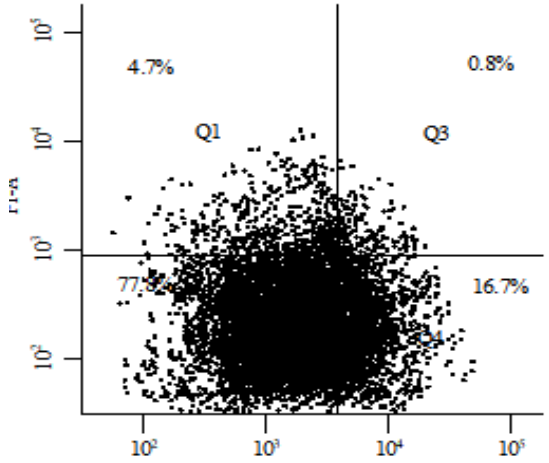
Influence of Cd on mRNA expression of NOS in nerve cells

We further discussed the influence of Cd on mRNA expression of NOS in nerve cells. Fig. 5 shows the survival of nerve cells after immersion in the Cd-containing culture medium for 24h, with black dots indicating the distribution of the nerve cells. Cells falling into quadrant Q1 of the dot plot were in good health status, and those in quadrant Q4 were apoptotic. Fig. 5(a) is the dot plot of nerve cells cultured in Cd-free medium. It can be seen that the majority of the cells fell into quadrant Q1, accounting for 94.5%; those in quadrant Q4 experienced natural death, accounting for about 3.6%. In Fig. 5(b), there was extensive apoptosis of nerve cells after adding Cd, and the cells falling in quadrant Q4 accounted for as high as 16.7%; those in quadrant Q1 accounted for 77.8%.

Fluorescence staining was performed on the nerve cells in the control group and experimental group. The nerve cells in the control group were intact, uniformly stained and had elliptical nuclei. In contrast, many cells in the experimental group had shrunk or even fragmented nuclei. The normal structure of the cells was damaged with chromatic condensation.



(a) Control group



(b) Experimental group

Figure 5. Influence of cadmium on the neuron apoptosis rate

Fig. 6 shows the mRNA expression of nNOS in the presence of Cd. It can be seen that as the concentration of cadmium acetate in the culture medium increased, the mRNA expression of nNOS first increased and then decreased. The degree of increase was 30.8% and 64.1% under the concentration of 5μmol/L and 10μmol/L, respectively, as compared with the control group. However, the mRNA expression of nNOS was similar at 20μmol/L as compared with the control group.

Fig. 7 shows the mRNA expression of iNOS under different concentrations of cadmium acetate. It can be seen that the mRNA expression of iNOS was similar to that of the control group under the concentration of 5μmol/L and 10μmol/L, respectively. When the Cd concentration increased to 20μmol/L, the mRNA expression level of iNOS was 4.7 times of that of the control group.

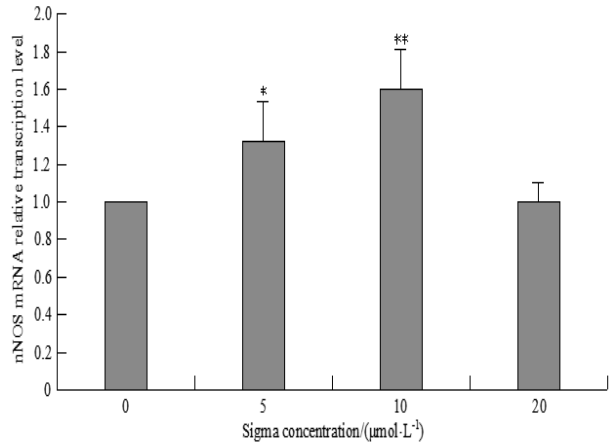


Figure 6. Relative mRNA expression of nNOS in primary cultured cerebral cortical neurons of embryonic rats

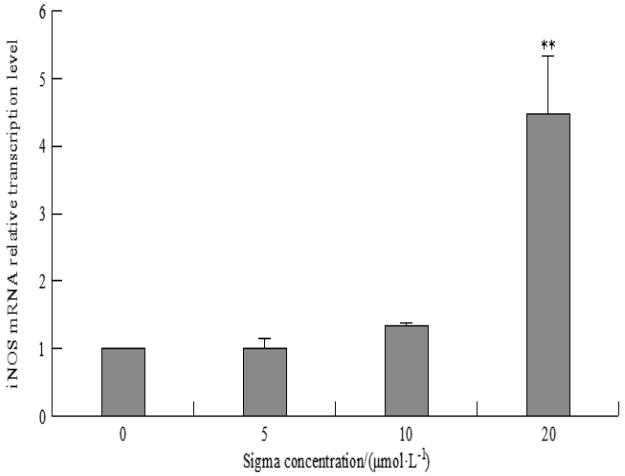


Figure 7. Relative mRNA expression of iNOS in primary cultured cerebral cortical neurons of embryonic rats

Influence of Cd on calcium homeostasis of nerve cells and the neuroprotective effect of NAC

Balance of intracellular calcium ions in nerve cells is crucial for maintaining normal shape, functioning and signal transduction of nerve cells. When treated by Cd, the nerve cells would experience an imbalance of intracellular calcium ions, which led to severe cell damage or even death. NAC proves effective in protecting against intracellular DNA damage, enhancing cell viability and inhibiting cell apoptosis. This section examined the response of nerve cells to Cd and the neuroprotective effect of NAC on nerve cells.

Influence of Cd on calcium homeostasis of nerve cells

Existing studies have shown that calcium chelator BAPTA-AM can inhibit the Cd-induced damage on nerve cells. Fig. 8 shows the average fluorescence

intensity detected in the nerve cells after adding different concentrations of cadmium acetate (0μmol/L, 5μmol/L, 10μmol/L and 20μmol/L) and mixture of cadmium acetate and calcium chelator into the culture medium. It can be seen that as the concentration of cadmium acetate increased, the intracellular calcium concentration increased significantly. As compared with the control group, the increase was 20.5%, 43.9% and 58.3% under the cadmium acetate concentration of 5μmol/L, 10μmol/L and 20μmol/L, respectively. After adding the mixture of cadmium acetate and BAPTA-AM, [Ca^{2+}] production was inhibited, and the increase of [Ca^{2+}] was only 5.2% and 4.8% under the concentration of 10μmol/L and 20μmol/L, as compared with the control group, respectively.

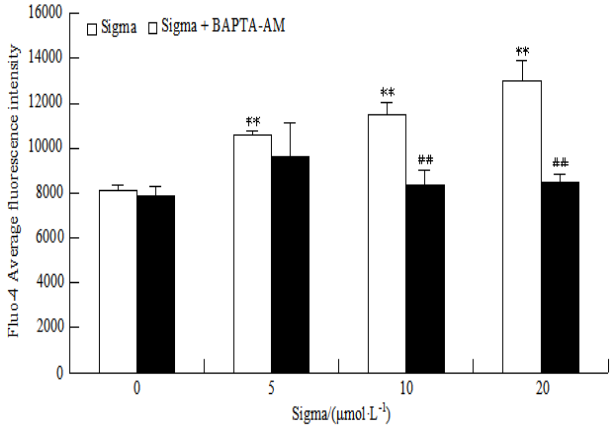


Figure 8. Influence of cadmium and BAPTA-AM on neurons [Ca^{2+}]i

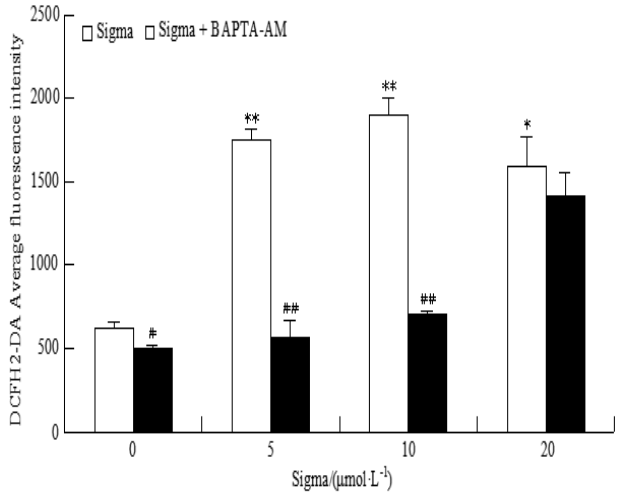


Figure 9. Influence of cadmium and BAPTA-AM on ROS levels of neurons

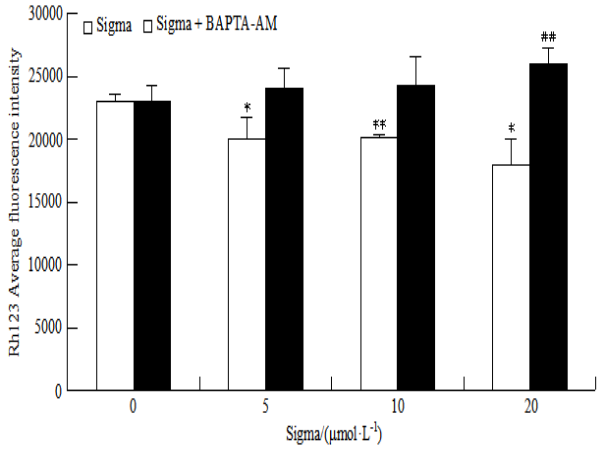


Figure 10. Influence of cadmium and BAPTA-AM on neurons ΔΨm

Fig. 9 shows the changes of ROS content after adding cadmium acetate alone or the mixture of cadmium acetate and calcium chelator. Excess ROS will cause intracellular DNA damage, inhibiting neuronal signaling, and changing gene transcription. This finally leads to the apoptosis of nerve cells. It can be seen from the figure that after the addition of cadmium acetate alone, the intracellular ROS content increased considerably. Under the cadmium acetate concentration of 10μmol/L, the increase of ROS content was 3.6 times of that of the control group. Intracellular ROS content decreased significantly after adding the mixture of cadmium acetate and calcium chelator, which was good evidence of the neuroprotective effect of BAPTA-AM on the nerve cells.

Fig. 10 shows the changes in MMP of the nerve cells after the addition of cadmium acetate alone or the mixture of cadmium acetate and calcium chelator. MMP is closely associated with normal mitochondrial functioning. When MMP is too low, the cells cannot synthesize ATP, which leads to low energy production and even death of the cells. After the addition of cadmium acetate alone, MMP decreased to varying extent. MMP at 20μmol/L was only 76.4% of that of the level in the control group. After the addition of cadmium acetate and calcium chelator, MMP increased considerably, and that at the cadmium acetate concentration of 20μmol/L was 1.37 times of that of the control group.

To conclude, BAPTA-AM is a highly effective calcium chelator, which can stabilize intracellular Ca^{2+} concentration after its sudden increase caused by Cd. This provides indirect evidence that Ca^{2+} concentration has a

considerable impact on intracellular MMP and ROS content.

Neuroprotective effect of NAC on nerve cells of the brain

Fig. 11 shows the intracellular Ca^{2+} concentration of nerve cells after Cd contamination and NAC treatment. C0 on the x-axis indicates no addition of Cd or NAC in the culture medium; C_1, C_2 and C_3 correspond to the addition of cadmium acetate at the concentration of 5μmol/L, 10μmol/L and 20μmol/L, respectively; C_1N, C_2N and C_1N correspond to the addition of NAC at the concentration of 100μmol/L, 130μmol/L and 200μmol/L on the basis of C_1, C_2 and C_3, respectively. It can be seen that when only cadmium acetate was added into the culture medium, intracellular Ca^{2+} concentration increased. With the further addition of NAC, Ca^{2+} concentration decreased, which indicated the neuroprotective effect of NAC.

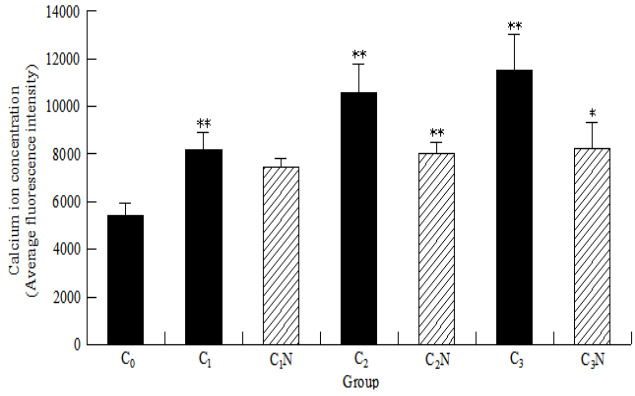


Figure 11. Influence of cadmium on primary cultured cerebral cortical neurons [Ca2+]i and protective effect of NAC

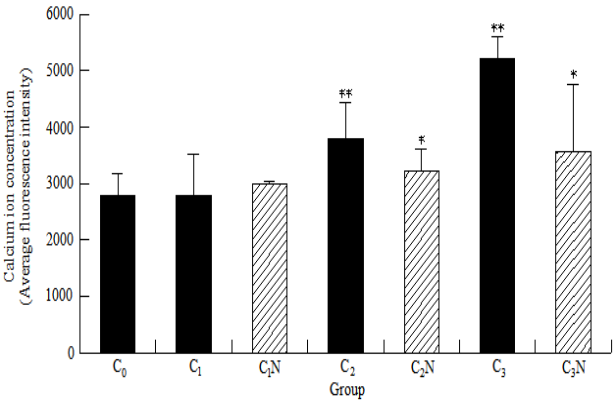


Figure 12. Influence of cadmium on ROS content in primary cultured cerebral cortical neurons

Fig. 12 shows the protective effect of NAC against ROS in nerve cells. It can be seen that after the addition of NAC, cadmium acetate at the concentration of 5μmol/L exerted very limited inhibitory effect on ROS. As the cadmium acetate concentration increased, ROS content decreased considerably, thus maintaining a favorable environment for normal mitochondrial functioning.

As shown above, Cd causes damage to nerve cells of the brain via multiple pathways. The main targets of Cd include mitochondria, intracellular Ca^{2+} and ROS content, and transcriptional levels of nNOS and Bcl. ROS proves to be an important factor that determines the accurate transcription of Bcl-2 gene. If ROS content is excessively high, the content of intracellular anti-oxidant will decrease. NAC can reduce cell apoptosis by inhibiting the breaking and mutation of intracellular DNA strands. However, the inhibitory effect of NAC on cell apoptosis was less significant than BAPTA-AM, and its neuroprotective effect was also limited.

Conclusion and outlook

We analyzed the mechanism of damage and apoptosis induced by Cd on nerve cells of the brain, as well as the effect on the transcription of intracellular Bcl-2 and NOS genes. The neuroprotective effect of BAPTA-AM and NAC was also discussed. Changes in survival rates and intracellular Ca^{2+} and ROS contents of the nerve cells were measured under different concentrations of cadmium acetate.

(1) As the concentration of cadmium acetate increased, the survival rate of nerve cells decreased significantly. The survival rate was only 88.7%. 79.6% and 61.3% under the cadmium acetate concentration of 5μmol/L, 10μmol/L and 20μmol/L, respectively. This indicated severe damage caused by Cd to the nerve cells.

(2) The mRNA expression of Bcl-2 decreased dramatically with the increase of cadmium acetate concentration, while that of Bax increased to varying extent. The Bcl/Bax ratio was 0.585, 0.559 and 0.868 under the three cadmium acetate concentrations, respectively. When Bcl-2 content was higher, Bcl-2/Bax heterodimer was formed, which slowed down cell apoptosis; otherwise, Bcl-2/Bax homodimer was formed, which accelerated cell apoptosis.

(3) Fluorescence staining indicated that the nerve cells in the control group were intact, uniformly stained and had elliptical nuclei. After Cd treatment, nuclei in most cells shrank in size or even became fragmented. As the cadmium acetate

concentration increased, the mRNA expression of nNOS first increased and then decreased. However, the mRNA expression of iNOS changed little.

(4) As the cadmium acetate concentration increased, both intracellular calcium and ROS concentrations increased significantly. BAPTA-AM stabilized the intracellular calcium concentration after a sudden increase caused by Cd. NAC reduced cell apoptosis by inhibiting the breaking and mutation of intracellular DNA strands. Compared with BAPTA-AM, NAC only exerted a limited inhibitory effect on Cd-induced cell apoptosis and its neuroprotective effect was also insignificant.

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