

The effect of cadmium on A β levels in APP/PS1 transgenic mice

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Abstract. Cadmium (Cd), which is a poisonous trace element, has been reported extensively to lead to morphological and biochemical abnormalities of the central nervous system, memory loss and mental retardation. We studied the Alzheimer's disease-related toxicity of Cd in a mouse model [amyloid precursor protein (APP)/presenilin 1 (PS1) transgenic mice, dual transfection of APP695swe and mutated PS1 genes]. Behavioral changes were detected using the Morris water maze test. The β -amyloid protein (A β) levels were determined using immunohistochemistry and ELISA. The free zinc ion concentration in mouse brain was determined using autometallography. The protein expression of α -secretase, soluble APP α (sAPP α) and neutral endopeptidase (NEP) in the mouse cerebral cortex and hippocampus was detected using western blotting. We found that Cd treatment increased the latency and distance of the platform search and reduced the number of platform crossings. The number and size of senile plaques in the brains of Cd-treated mice were significantly increased. The levels of A β ₁₋₄₂ and free zinc ions were increased. The expression of ADAM10, sAPP α and NEP protein was reduced. We speculated that Cd reduced the expression of ADAM10, sAPP α and NEP protein, which caused an increase in the levels of A β ₁₋₄₂ and free zinc ions and led to the accelerated A β deposition found in the experimental animals and their abnormal behavior.

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease that is characterized by dementia as the main clinical feature. AD mainly affects the elderly and has become one of the major fatal diseases. Typical pathological features of AD include β -amyloid protein (A β) deposition in the brain that forms senile

plaques (SPs), neurofibrillary tangles (NFTs) and neuronal apoptosis.

A variety of hypotheses exist for AD risk factors, and the trace element neurotoxicity theory has drawn an increasing amount of attention. Zinc (Zn) ions are capable of causing A β aggregation through the linking of histidines (the 13th amino acid) of adjacent A β molecules, and there is evidence that Zn ions play a key role in the pathogenesis and pathological process of AD (1-6). Cadmium (Cd) is a harmful trace element that causes morphological and biochemical abnormalities of the central nervous system (CNS), memory loss and mental retardation (7). Concentrations of Cd and Cd/Zn are significantly higher in the blood and hair of AD patients than in healthy people (8,9). However, the correlation between Cd and A β has seldom been studied.

A β is central to the development of AD and has become the focus of current research. A β levels are determined by biosynthesis and enzymatic degradation. For biosynthesis, A β is generated from its precursor, amyloid precursor protein (APP). APP has two metabolic pathways, namely the α -secretase pathway and the β -secretase pathway. Under physiological conditions, the majority of APP is cleaved by α -secretase into soluble APP α (sAPP α) and a transmembrane fragment (C83); sAPP α is further cleaved by γ -secretase into P3 and AICD. The cleavage site of α -secretase is located at the A β segment of APP, which prevents the generation of A β with a complete molecular sequence. A small part of APP is cleaved by β -secretase at the N-terminal of A β , which generates a secretive soluble APP derivative (sAPP β) and a transmembrane component [the C-terminal fragment containing 99 amino acids (C99)]. C99 is further cleaved by γ -secretase into A β and AICD (10,11). For enzymatic degradation, neutral endopeptidase (NEP) is a major A β -degrading enzyme, and a large number of studies have shown that the levels of NEP and A β deposition are negatively correlated. In healthy people, the synthesis and degradation of A β is balanced, and a steady low level of A β is maintained.

In the present study, we examined the toxic effects of Cd on A β levels in APP/PS1 transgenic mice. Special learning capacity was detected using the Morris water maze test. The change in free Zn ion concentration in the mouse brain was detected using autometallography (AMG) to study the effect of Cd on Zn. The number and size of SPs in brains was detected using immunohistochemistry. The change of A β ₁₋₄₂ level was detected using ELISA. Changes in the protein expression of APP, α -secretase (ADAM10), sAPP α and NEP were detected

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using western blotting. We aimed to explore the metal ion metabolism in the AD brain and its correlation with the pathophysiology of AD.

Materials and methods

Experimental animals. A total of 24 male APP/PS1 transgenic mice (3 months old, weighing 25–27 g) were purchased from the Experimental Animal Center of China Medical University. Mice were randomly divided into two groups, a control group and a Cd treatment group, with 12 mice each. CdCl₂ (Cd 2.5 mg/kg.d⁻¹) was added to the drinking water of the Cd treatment group, and drinking water was normal for the control group. The protocols in this study were approved by the Institutional Review Board and the Animal Care and Use Committee of China Medical University (Shenyang, China).

Morris water maze

Place navigation test. For 2 days prior to the experiments, mice were familiarized with the water maze environment in the morning and afternoon. For each training session, the mice were placed into the water 4 times, from the southeast, northeast, southwest and northwest. Formal experiments began on day 3 and lasted 4 days. A quadrant was selected randomly, and the mice were placed into the water along the wall with their back against the platform. Escape latency was the time that the mice took to reach the platform. If mice could not find the platform within 1 min, they were led to it by the experimenter, and the latency was recorded as 60 sec. The swimming trajectory and movement distance were also recorded to judge the learning capacity of mice.

Spatial probe test. On the 5th day of the experiment, the platform was withdrawn. A quadrant was selected randomly, and the mice were placed into the water with their face toward the wall. The swimming trajectory and the number of crossings of the original platform within 1 min were recorded to judge the memory capacity of mice.

Immunohistochemical staining. After behavioral testing, APP/PS1 transgenic mice were decapitated. Their brains were quickly removed, and one-half was rapidly placed into 4% paraformaldehyde for fixation and the preparation of conventional paraffin sectioning. The other half was used to separate the cortex and hippocampus, which was stored at -80°C. Brain tissue paraffin sections of mice in each group were dewaxed in xylene, washed with PBS, boiled using a microwave for antigen retrieval, naturally cooled, washed with PBS, and incubated with 5% BSA at room temperature for 1 h, followed by incubation with a mouse A β antibody (Sigma, 1:5000) at 4°C overnight. The sections were washed with 0.01 M PBS thoroughly, incubated with biotin-labeled goat anti-mouse IgG at room temperature for 2 h, washed with 0.05 M Tris-HCl thoroughly, and incubated with SABC at room temperature for 1 h. DAB coloration was monitored under a microscope. The sections were washed with distilled water to stop the reaction and counterstained with hematoxylin. The sections were dehydrated using conventional techniques, made transparent and mounted. Each group included 6 mice. Five sections of the same part of the brain

per mouse were selected, and images were captured under an optical microscope. The optical density value of positive SPs in the hippocampus and cortex was analyzed using IPP 6.0 software and compared statistically.

ELISA. The A β ₁₋₄₂ level was detected using an ELISA kit (Biosource International) according to the manufacturer's instructions. Brain tissues were lysed with 5 M guanidine hydrochloride diluted with standard dilution buffer (1:50) and centrifuged (12,000 \times g, 4°C, 25 min). The supernatant was collected. A total of 50 μ l of standard or sample was added to each well followed by 50 μ l of antibody. The solution was incubated on a shaker at room temperature for 3 h. The supernatant was removed, and the plate was washed 4 times. Enzyme-conjugated secondary antibody (100 μ l) was added and incubated at room temperature for 30 min. The supernatant was removed, and the plate was washed 4 times. Substrate (100 μ l) was added and incubated at room temperature for 30 min. The stop solution (100 μ l) was added, and OD₄₅₀ values were detected on a microplate reader. The A β ₁₋₄₂ level was calculated according to the standard curve.

AMG. Mice were decapitated, and their brains were quickly removed. Fresh tissue slices approximately 2 mm thick were cut from the middle part of the hippocampus. According to the modified AMG protocol described by Danscher *et al.* (18), the slices were immediately immersed in phosphate buffer (pH 7.4) containing 0.1% sodium sulfide and 3% glutaraldehyde, incubated on a shaker at 4°C for 3 days, and washed with 0.1 M PBS for 10 min. The slices were immersed in a 30% sucrose solution at 4°C until they sank to the bottom of the glass. Frozen sections (30- μ m thick) were prepared. The slices were placed in a staining cylinder that contained metal developing incubation buffer (60 ml gum arabic solution, 10 ml citrate buffer, 15 ml hydroquinone solution and 15 ml silver emulsion solution), incubated in a 26°C water bath for 60 min, and immersed in a 5% sodium thiosulfate solution for 10 min to stop the reaction. The sections were washed with deionized water, dehydrated gradually with ethanol, made transparent with xylene, and mounted with neutral gum. Each group included 6 mice. Five sections of the same part of the brain per mouse were selected, and the images were acquired under an optical microscope. The optical density value of positive Zn ion plaques in the cortex was analyzed using IPP 6.0 software and compared statistically.

Western blotting. The cerebral cortex and hippocampus tissues of APP/PS1 transgenic mice were weighed and cut into pieces using small scissors on ice. A 5X volume of protein lysis buffer was added, and the tissues were sonicated and lysed at 4°C overnight. The samples were centrifuged at 4°C 12,000 rpm for 30 min, and the supernatant was collected. The protein level was determined using the Coomassie Brilliant Blue assay. Protein (60 μ g/10 μ l) was loaded, and the electrophoresis was stopped when the bromophenol blue reached the bottom of the gel. The protein was transferred to film at 4°C at 45 V overnight. The membranes were incubated with primary antibodies against ADAM10 (1:1000), sAPP α (1:500), NEP (1:500) and GAPDH (1:12000) at room temperature for 2 h, washed with TTBS 3 times for 10 min, incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000) at room temperature

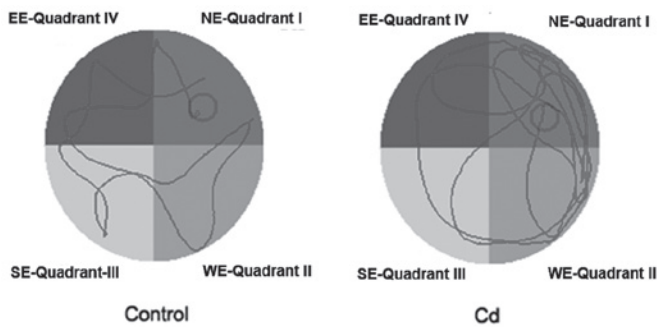


Figure 1. The movement trajectory of the two groups of mice across quadrants. Cd, cadmium.

for 2 h, and washed with TTBS 3 times for 10 min. ECL luminescence was performed, and the resulting images were captured and analyzed using a Bio-Rad gel image analyzer.

Statistical analysis. A T-test analysis of the data was performed using SPSS 15.0 software, and the results were presented as the means \pm standard deviation (SD). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Morris water maze test. APP/PS1 transgenic mice displayed significant behavioral symptoms of AD. To examine whether

Cd affected the behavioral change, we used the Morris water maze test to detect the memory ability of these two groups of mice (9 months old). During the place navigation test that was conducted over 4 days, the search latency of these two groups of mice decreased. Compared to the control group, the movement trajectory of the Cd treatment group was mainly along the wall and away from the platform (Fig. 1), and the search latency and distance were longer. The number of crossings of the platform was significantly reduced (Fig. 2, $p < 0.01$).

$A\beta$ immunohistochemistry. The number and size of SPs in the cerebral cortex and hippocampus increased significantly in the Cd treatment group (Fig. 3), and the results of the optical density analysis showed that the difference was statistically significant ($p < 0.01$).

$A\beta_{1-42}$ levels in brains. We used an ELISA kit to further detect changes in $A\beta_{1-42}$ levels. The $A\beta_{1-42}$ levels in the Cd treatment group (94.32 ± 2.83 pg/mg) increased significantly compared to those in the control group (67.25 ± 3.45 pg/mg) ($p < 0.01$, Fig. 4).

Free Zn ion levels in brains. Incubation with an AMG developer achieved the silver amplification of Zn sulfide microcrystals that formed on slices, which is highly specific and sensitive for the detection of free Zn ions. The AMG-positive reaction product was brown, which indicates the presence of Zn ions. Free Zn ion levels in the Cd treatment group increased

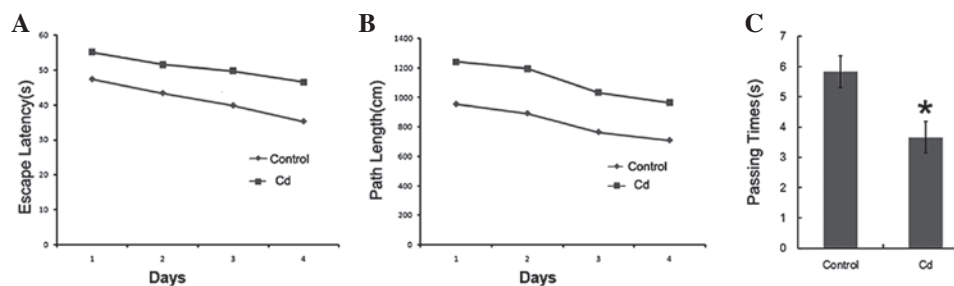


Figure 2. Comparison of the (A) search latency and (B) distance and (C) the number of crossings of the platform of the two groups of mice, * $p < 0.01$. Cd, cadmium.

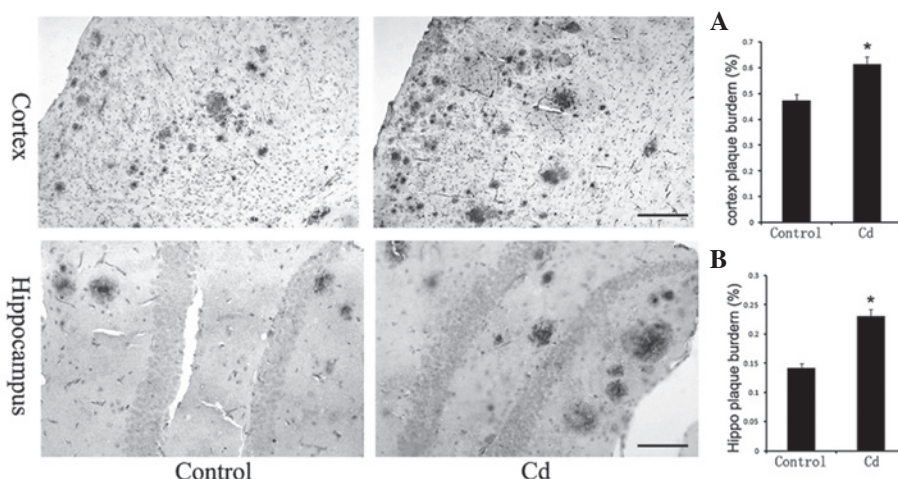


Figure 3. Representative $A\beta$ immunohistochemical results of the two groups of mice. Scale bar for cortex, 200 μ m; for hippocampus, 50 μ m. (A and B) Results of the optical density analysis of $A\beta$ immunohistochemistry, * $p < 0.01$. Cd, cadmium; $A\beta$, β -amyloid protein.

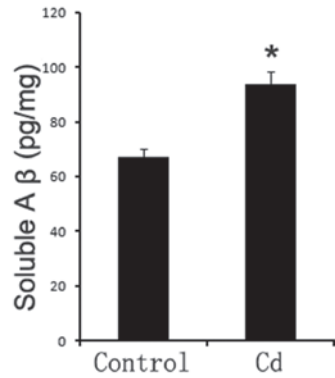


Figure 4. A β_{1-42} levels in the two groups of mice detected using ELISA, * $p<0.01$. Cd, cadmium; A β , β -amyloid protein.

significantly compared to those in the control group ($p<0.01$, Fig. 5).

Western blotting. APP has two metabolic pathways, namely the α -secretase pathway and the β -secretase pathway. The cleavage site of α -secretase is located between the 16-17th amino acids of the A β fragment of APP, which produces sAPP α and a transmembrane fragment (C83) that prevents A β generation. In this study, we detected the protein expression of ADAM10 (α -secretase) in the brains of the two groups of mice using western blotting. The results showed that ADAM10 and sAPP α protein levels were significantly lower in the Cd treatment group (Fig. 6A-B, $p<0.01$). NEP is an enzyme that is related

to A β degradation. NEP knockout or specific NEP inhibitors increase A β levels. Our results indicated that the NEP protein level was decreased in the Cd treatment group (Fig. 6C, $p<0.01$).

Discussion

Cd has a number of biological toxicities, including carcinogenesis, damage to kidney and bone, fetal toxicity and teratogenic effects. In addition, Cd affects the nervous system (12). Cd directly inhibits thiol-containing enzymes; decreases the levels of norepinephrine, serotonin, and acetylcholine; and has adverse effects on brain metabolism (13). Exposure to Cd decreases children's IQ, visual development and learning ability (7). Cd is also involved in the formation of NFTs (14). Cd and Cd/Zn are significantly higher in the blood and hair of AD patients than in healthy people (8,9). Our results from the water maze test revealed that the movement trajectory of the Cd treatment group was mainly along the wall and away from the platform and that the search latency and distance was longer, which indicated that Cd treatment worsened the learning ability of the APP/PS1 transgenic mice. The number of crossings of the platform was significantly reduced in the Cd treatment group, which indicated that these mice also had worse memory ability. Our results revealed that Cd treatment aggravated the behavioral symptoms of AD.

Danscher *et al* confirmed that Zn ion levels in the cerebral cortex and hippocampus of AD patients are significantly higher than those in healthy people using atomic absorption spectroscopy and X-ray microanalysis (15). This result suggests that Zn ions play a key role in the pathogenesis and pathological

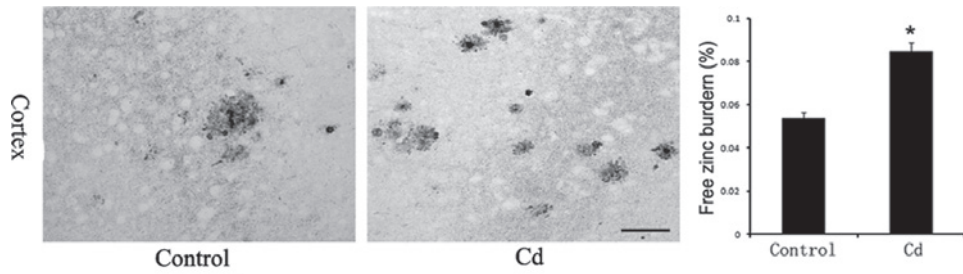


Figure 5. The detection of free zinc ion levels in the cortex of the two groups of mice using AMG and the resultant optical density analysis. Scale bar, 50 μ m, * $p<0.01$. AMG, autometallography; Cd, cadmium.

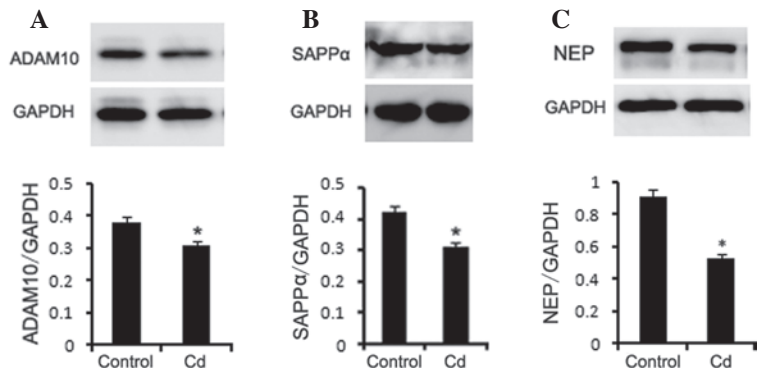


Figure 6. Detection of the protein expression of (A) ADAM10, (B) sAPP α , and (C) NEP in the brains of the two groups of mice using western blotting, * $p<0.01$. Cd, cadmium; ADAM10, α -secretase; sAPP α , soluble amyloid precursor protein α ; NEP, neutral endopeptidase.

processes of AD. Over the years, *in vitro* experiments have shown that Zn ions cause the fibrillar deposition of A β (16) and cause A β aggregation through the linking of histidines (the 13th amino acid) of adjacent A β molecules (17). In this study, we applied the latest modified AMG technology (18) to quantitatively analyze the free Zn ion distribution in the brains of two groups of mice. There were AMG-positive SPs in the brains of APP/PS1 transgenic mice, which indicated that there were numerous Zn ions in the SPs of A β deposition. Moreover, free Zn ion levels were significantly higher in the Cd treatment group. Immunohistochemistry results also revealed that the number and size of SPs in the cerebral cortex and hippocampus increased significantly in the Cd treatment group. The above results indicated that Cd increased free Zn ion levels and accelerated SP deposition in the brains of APP/PS1 mice. Since Cd is an anti-metabolite of Zn, we speculated that Cd may replace the Zn ions in Zn enzymes, which would result in an increase in extracellular Zn ions and an increase in SP deposition.

Brain A β was generated from its precursor, APP. APP has two metabolic pathways, namely the α -secretase pathway and the β -secretase pathway. Under physiological conditions, the majority of APP is cleaved by α -secretase into sAPP α and a transmembrane fragment (C83), and sAPP α is further cleaved by γ -secretase into P3 and AICD. The cleavage site of α -secretase is located in the A β segment of APP, which prevents the generation of A β with a complete molecular sequence. A very small part of APP is cleaved by β -secretase at the N-terminal of A β , which generates a secretive soluble APP derivative (sAPP β) and a transmembrane component (C99). C99 is further cleaved by γ -secretase into A β and AICD (10,11). A β_{1-42} is highly cytotoxic (19). Our ELISA results confirmed that the A β_{1-42} level was significantly higher in the Cd treatment group. To study the possible mechanism of how Cd increased the A β_{1-42} level, we used western blot analysis to detect the protein expression of α -secretase (ADAM10) and sAPP α . The results showed that the levels of these two proteins were significantly lower in the Cd treatment group. ADAM10 has a protective role in AD (20). Therefore, we speculated that Cd inhibits the activity of α -secretase, which leads to a greater metabolism of APP through the β -secretase pathway and an increase in A β . Lower α -secretase activity is associated with a reduced production of sAPP α , which has neurotrophic and neuroprotective effects on neurons (21). A decrease in sAPP α could increase the vulnerability of the surrounding neurons.

Several endopeptidases for A β degradation have been found, including NEP, endothelin-converting enzyme and insulin-degrading enzyme (22). NEP is a major A β -degrading enzyme (23), and a large number of studies have shown that the level of NEP and A β deposition is negatively correlated. In healthy people, the synthesis and degradation of A β is balanced, and a steady low level of A β is maintained.

NEP is a type II transmembrane glycoprotein in the M13 Zn metalloproteinase family and is expressed at the presynaptic membrane, axon and other neuronal parts. It is involved in the degradation of enkephalins, bradykinin, substance P, somatostatin and other neuropeptides (24). NEP has 5 cleavage sites on A β and is capable of degrading monomers, dimers and oligomers of A β_{1-40} and A β_{1-42} (24). NEP mainly degrades extracellular A β_{1-42} (25). Many researchers have suggested that NEP is an important A β -degrading enzyme in the brain; NEP

downregulation increases A β aggregation and its upregulation reduces A β levels in the brain (25,26). Our results revealed that NEP was significantly reduced in the Cd treatment group, which indicates that Cd may reduce A β degradation and increase A β aggregation through a reduction in NEP expression.

In addition, Zn plays a role in the composition of the active sites of more than 200 enzymes, including α -secretase and NEP *in vivo*. Many of the toxic effects of Cd are related to the complex interaction between Zn and Cd. Since Cd has a stronger binding capacity with thiol, carboxyl and hydroxyl than Zn, it is capable of replacing Zn in Zn enzymes. The replacement of Zn with Cd inactivates these enzymes, which causes the dysfunction of the human brain and triggers AD. The detailed mechanisms of AD require further research.

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