Impact of Sub-chronic Aluminium-maltololate Exposure on Catabolism of Amyloid Precursor Protein in Rats

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Abstract

Objective To investigate the impact of sub-chronic Aluminium-maltololate [Al(mal)]₃ exposure on the catabolism of amyloid precursor protein (APP) in rats.

Methods Forty adult male Sprague-Dawley (SD) rats were randomly divided into five groups: the control group, the maltololate group (7.56 mg/kg BW), and the Al(mal)₃ groups (0.27, 0.54, and 1.08 mg/kg BW, respectively). Control rats were administered with 0.9% normal saline through intraperitoneal (i.p.) injection. Maltololate and Al(mal)₃ were administered to the rats also through i.p. injections. Administration was conducted daily for two months. Rat neural behavior was examined using open field tests (OFT). And the protein expressions and their mRNAs transcription related with APP catabolism were studied using enzyme-linked immunosorbent assay (ELISA) and real-time polymerase chain reaction (RT-PCR).

Results The expressions of APP, β-site APP cleaving enzyme 1 (BACE1) and presenilin-1 (PS1) proteins and their mRNAs transcription increased gradually with the increase of Al(mal)₃ doses (P<0.05). The enzyme activity of BACE1 in the 0.54 and 1.08 mg/kg Al(mal)₃ groups increased significantly (P<0.05). The expression of β-amyloid protein (Aβ) 1-40 gradually decreased while the protein expression of Aβ1-42 increased gradually with the increase of Al(mal)₃ doses (P<0.05).

Conclusion Result from our study suggested that one of the possible mechanisms that Al(mal)₃ can cause neurotoxicity is that Al(mal)₃ can increase the generation of Aβ1-42 by facilitating the expressions of APP, β-, and γ-secretase.

Key words: Aluminium-maltololate; Amyloid precursor protein; β-amyloid protein; Rat

INTRODUCTION

Aluminum (Al) is the third most abundant element in the crust of earth and has been suspected to be an important environmental risk factor for Alzheimer’s disease (AD)¹⁵. However, the role of Al in the pathogenesis of AD remains currently controversial¹⁵. AD, the most common form of dementia, is characterized clinically by the progressive loss of...
increased the formation of Aβ by directly promoting Aβ synthesis. However, the exact mechanism of this remains elusive. Further research on the effect of Al on the metabolism of APP and the formation of Aβ is therefore needed and it is critical.

The physiological activity and the bioavailability of Al largely depended on its chemical form and the equilibrium state. Aluminum-maltolate [Al(mal)₃] is stable between pH 3.0 and 10.0 and it does not form aluminum hydroxide precipitates at physiological pHs. Al(mal)₃ may be formed in vivo in the gastrointestinal tract and it has strong neurotoxic effect. This compound is therefore needed to be studied in terms of its toxicity and neuropathology of AD. And the aim of this study is to investigate the impact of sub-chronic Al(mal)₃ exposure on the catabolism of APP in rats and, at present, no relevant researches have been reported both home and abroad.

**MATERIALS AND METHODS**

**Material**

The AlCl₃·6H₂O (purity 99%), maltolate (purity 99%), and Sensizyme BACE1 activity assay kit were from Sigma-Aldrich Chemical Co. The BCA protein quantitative kit was from Wuhan Boster Bio-engineering Limited Company. The rat APP, BACE1, PS1, Aβ1-40, and Aβ1-42 ELISA kits were from Wuhan Life Science, Inc. Primers, the reverse transcriptase reagent, and the fluorescence quantitative kit were from Takara Biotechnology (Dalian) Co., LTD. All chemicals were analytical grade unless otherwise indicated.

**Al(mal)₃** Preparation

Al(mal)₃ was prepared according to a previous publication. AlCl₃·6H₂O was dissolved in distilled water to final concentration of 80 mmol/L. And maltolate was dissolved in phosphate-buffered saline (PBS) to final concentration of 240 mmol/L. The solutions were then mixed in equal volumes, and the pH was adjusted to 7.4 with 1 mol/L NaOH. The resulting Al(mal)₃ was freshly prepared for each experiment and all solutions were filter sterilized using 0.22 μm syringe filters immediately following preparation.

**Animals and Treatment**

The male Sprague-Dawley (SD) rats (200-220 g BW) were purchased from the Experimental Animal
Centre of Shanxi Medical University. The rats were group-housed in standard laboratory cages for one week of habituation in an environment at 22-24 °C and 65% humidity on a 12:12 h light-dark cycle with access to food and water ad libitum prior to experiment. The rat behavioral screenings were conducted using the Morris water maze test. Forty SD rats were selected and were divided randomly into five groups: the control group, the maltolote group (7.56 mg/kg BW) and the Al(mal)$_3$ groups (0.27, 0.54, and 1.08 mg/kg BW respectively). Control rats were administered with 0.9% normal saline through intraperitoneal (i.p.) injection. Maltolote and Al(mal)$_3$ were administered to the rats also through i.p. injections. Administration was conducted daily for two months and all injections were performed at the same time.

**Open Field Test (OFT)**

General motor activities and behavioral responses of tested rats to a novel environment were measured via an open field apparatus. The measure device consisted of a wooden 100 cm × 100 cm square surrounded by a 30-cm-high wall and an overhead video camera. The area within the walls was divided into 25 segments with approximately equal area (20 cm × 20 cm) and the 16 squares along the walls were defined as the periphery. The remaining nine squares were defined as the central lattice. The tested rats were placed in the center of the apparatus at the beginning of each test, and they were allowed to move freely around the open field for 5 min. The retention time in the central lattice and the number of rearings were scored. Fecal pellets were removed, and the floor was wiped with clean damp tissues after each trial.

**Sample Preparation**

Four rats randomly selected from each group were anesthetized and killed and their cerebral cortex was dissected, weighed and frozen shortly after death. All subsequent operations were performed on ice and the samples were frozen in cryogenic refrigerators at -80 °C for further analysis.

**ELISA for APP, BACE1, PS1, Aβ1-40, and Aβ1-42 Proteins**

Approximately 200 mg cerebral cortex of rats was homogenized in 3 mL organization cracking liquid (PMSF was diluted prior to use, and its final concentration was 100 ng/ml) by ultrasonic cracking (ultrasonic time for 5 s, takt time for 6 s, total for 5 times). The homogenates were then centrifuged at 12 000 rpm for 15 min at 4 °C and the supernatants were collected afterwards. All experimental procedures were operated according to manufacturer's instructions.

**RT-PCR of APP, BACE1, and PS1 Gene Expression**

Total cellular RNA from cerebral cortex of tested rats was isolated using Trizol reagent. First-strand cDNA was synthesized from 3 μg total RNA with SuperScript II. All primers were synthesized by Takara Biotechnology (Dalian) Co., LTD. The primers were designed using Oligo 6.0 primer analysis software for each analyzed gene: APP forward primer 5'-AACATGTGCATGTTGGA-3' and reverse primer 5'-CACGGCAGGGACTGTGAGA-3'; BACE1 forward primer 5'-AGCTGGATTATGGTGCGG-3' and reverse primer 5'-CCTGAGTCGGTCTCTCCTC-3'; PS1 forward primer 5'-ATGGACCCGATGGCTC-3' and reverse primer 5'-TGCAACCAGTGCAGAATGGA-3'; β-actin forward primer 5'-GGAGATTACTGCTGTCCTCACA-3' and reverse primer 5'-GACTCAGTGTACTCCTGCGC-3'. Each PCR reaction contained SYBR Premix Ex Taq II (10 μL), forward/reverse primers (0.8 μL), ROX reference dye II (0.4 μL), dH$_2$O (6 μL) and DNA template (2 μL). And PCR was performed using the following two-cycle programs: (1) denaturation of cDNA (one cycle: 95 °C for 30 s); (2) amplification (40 cycles: 95 °C for 5 s, 60 °C for 30 s); and (3) drawing melting curve.

**BACE1 Activity Assay**

BACE1 activity in rat cerebral cortex was measured using a series of multistep reactions according to manufacturer’s instructions. Briefly, 100 μL of standard, blank and test sample were pipetted into the each corresponding wells of the plates respectively and incubated for 2 h at 4 °C. The solution was aspirated from the wells and wells were washed with 100 μL wash buffer for 4 times. The plate was blotted on tissue paper in order to remove any residual solution. 50 μL substrate working solution was pipetted into each well and the plates were incubated in a humidified chamber at room temperature overnight. Then, 50 μL reagent mixture was added to each well and the plates were shaken for 20 s and incubated at room temperature for 3 h. After removing the lid, the absorbance for each well was measured at 405 nm using a microplate reader.

All experimental protocols used in the present study were approved by the Ethics Committee for
Animal Studies of Shanxi Medical University and efforts were made to minimize use and suffering of animals.

**Statistical Analysis**

All data were analyzed using SPSS 16.0 software and were expressed as mean±SD. One-way ANOVA was performed, and the statistical significance level was defined as \( P<0.05 \).

**RESULTS**

**Al(mal)_3 Treatment Impaired Anxiety State and Exploratory Behavior**

The OFT was performed as a general measure in order to evaluate the potential neurotoxicity of Al. The retention times in the central lattice in 0.27, 0.54, or 1.08 mg/kg BW Al(mal)_3 groups (28.58±8.34 s, \( n=8 \); 39.40±9.28 s, \( n=8 \); 66.33±11.06 s, \( n=8 \), respectively) were significantly longer compared with those in the control group (13.75±3.96 s, \( n=8 \)) as well as maltolate group (13.10±6.37 s, \( n=8 \)) (\( P<0.05 \)) (Figure 1A). Also, the number of rearings in the 0.54 mg/kg and 1.08 mg/kg BW Al(mal)_3 groups (10.50±7.74, \( n=8 \); 5.00±1.37, \( n=8 \), respectively) were significantly lower than those in the saline group (14.40±1.95, \( n=8 \)) as well as the maltolate group (15.00±5.47, \( n=8 \)) (\( P<0.05 \)) (Figure 1B).

**Expressions of APP, BACE1, PS1, Aβ1-40, and Aβ1-42 Proteins**

The protein expressions of APP, BACE1, PS1, Aβ1-40, and Aβ1-42 in the cerebral cortex of the tested rats in each group were measured. As the result, the expression of APP protein in 1.08 mg/kg BW Al(mal)_3 group (131.76±12.33 ng/mL, \( n=4 \)) was significantly higher than those in the control group (106.62±5.07 ng/mL, \( n=4 \)) as well as the maltolate group (108.49±5.14 ng/mL, \( n=4 \)) (\( P<0.05 \)) (Figure 2A). The expressions of BACE1 protein in 0.54 and 1.08 mg/kg BW Al(mal)_3 groups (12 933.01±974.37 pg/mL, 13 676.34±81.77 pg/mL, \( n=4 \)) were significantly higher than those in the control group (11 110.08±743.75 pg/mL, \( n=4 \)) as well as the maltolate group (11 319.44±1 463.8 pg/mL, \( n=4 \)) (\( P<0.05 \)) (Figure 2B). The expressions of PS1 protein in 0.27, 0.54, and 1.08 mg/kg BW Al(mal)_3 groups (10.91±3.26 ng/mL, 12.48±3.45 ng/mL and 12.85±1.51 ng/mL, \( n=4 \)) increased significantly compared with the control group (7.83±0.40 ng/mL, \( n=4 \)) (\( P<0.05 \)) (Figure 2C). The expressions of PS1 protein in 0.54 and 1.08 mg/kg BW Al(mal)_3 groups were significantly higher than that of the maltolate group (9.24±0.36 ng/mL) (\( P<0.05 \)). The expressions of Aβ1-40 protein in 0.54 and 1.08 mg/kg BW Al(mal)_3 groups (872.55±57.86 pg/mL, 784.49±72.08 pg/mL, \( n=4 \)) were significantly lower than that in saline group (1077.02±90.22 pg/mL) (\( P<0.05 \)) (Figure 2D). The expression of Aβ1-40 protein in 1.08 mg/kg BW Al(mal)_3 group decreased significantly compared with the maltolate group (1045.79±61.75 pg/mL) (\( P<0.05 \)). The expressions of Aβ1-42 protein in all Al(mal)_3 groups (352.19±33.05 pg/mL, 361.33±2.88 pg/mL and 420.33±32.57 pg/mL, \( n=4 \)) increased significantly compared with the control group (249.00±27.81 pg/mL, \( n=4 \)) (\( P<0.05 \)) (Figure 2E). The expressions of Aβ1-42 protein in 0.54 and 1.08 mg/kg BW Al(mal)_3 groups were significantly higher than that in the maltolate group (296.72±54.43 pg/mL, \( n=4 \)) (\( P<0.05 \)).

![Figure 1](image) **Figure 1.** Al(mal)_3 treatment impaired anxiety state and exploratory behavior. A: The retention time in the central lattice of the OFT. B: The number of rearings of the OFT. Bars represent means±SD. Note. ¹ \( P<0.05 \) vs. control group, ² \( P<0.05 \) vs. maltolate group.
Relative Expressions of APP, BACE1, and PS1 mRNA

The relative expressions of APP, BACE1, and PS1 mRNA are presented in Figure 3. The relative expressions of APP and BACE1 mRNA in 1.08 mg/kg BW Al(mal)$_3$ group (2.60±1.47, 1.53±0.13, n=4) were significantly higher than those in the control group (1±0, 1±0, n=4) as well as the maltolate group (1.24±0.34, 1.02±0.11, n=4) ($P<0.05$) (Figure 3A and B). The relative expressions of PS1 mRNA in 1.08 mg/kg BW Al(mal)$_3$ group (1.54±0.38, n=4) was significantly higher than that in the control group (1±0, n=4) (Figure 3C).

**Figure 2.** Expressions of APP, BACE1, PS1, A61-40, and A61-42 proteins in cerebral cortex of tested rats. A, B, C, D, and E represent protein expressions of APP, BACE1, PS1, A61-40, and A61-42 in cerebral cortex of tested rats of the control group, maltolate group, and Al(mal)$_3$ groups, respectively. Bars represent the means±SD. Note. *$P<0.05$ vs. saline group, **$P<0.05$ vs. maltolate group.

**Figure 3.** Relative expressions of APP, BACE1, and PS1 mRNA in the cerebral cortex of tested rats. A, B, and C represent the relative expressions of APP, BACE1, PS1 mRNA in the control, maltolate, and Al(mal)$_3$ groups, respectively. Bars represent the means±SD. Note. *$P<0.05$ vs. saline group, **$P<0.05$ vs. maltolate group.
**BACE1 Activity**

Higher BACE1 activity levels were observed in rats in the Al(mal)3 groups compared with the rats in control as well as in maltolate groups (Figure 4) and significant differences in BACE1 activity levels were fund among the control group (0.23±0.11 ng/mL, n=4), maltolate group (0.37±0.19 ng/mL, n=4), and Al(mal)3 groups (0.53±0.07 ng/mL, 1.06±0.63 ng/mL, and 1.87±0.17 ng/mL, n=4).

![Figure 4. BACE1 activity levels in cerebral cortex of tested rats. Bars represent the means±SD. Note. *P<0.05 vs. saline group, **P<0.01 vs. maltolate group.](image)

**DISCUSSION**

Behavioral changes as indicators of neurotoxicity may be more sensitive than neurochemical alterations and these changes may be observed during early Al exposure. The OFT is a classic behavioral experiment in order to assess an anxiety state and exploratory behavior of tested animals in a novel environment. Findings from our study showed that Al(mal)3 sub-chronic exposure led to significant reductions in spontaneous locomotor and exploratory activities in OFT. And some previous investigations have interestingly found the similar altered behaviors in Al-treated animals, yet our findings contrast with those of some other studies.

We believe that the controversy of Al neurotoxicity and behavioral toxicity in experimental animals may be due to the difference of Al administration, the types of Al salt, and the animal species (rats are less susceptible to the toxic effects of Al than cats, rabbits, mice, and guinea pigs).

APP has two alternative proteolytic pathways: the nonamyloidosis pathway and the amyloidosis pathway. In the nonamyloidosis pathway, APP is cleaved by α-, γ-secretase into sAPPα, p3 and, APP intracellular domain (AICD). This pathway seems to be protective because it prevents Aβ generation. In the amyloidosis pathway, APP is cleaved by β- and γ-secretase, releasing the 39-43 aa Aβ peptide. And abnormal amyloidosis proteolysis of APP and the generation of Aβ are key events in the pathogenesis of AD. The overproduction of Aβ results in the formation of SPs and NFTs, neuronal loss in the brain. Al increases the level of Aβ, promotes Aβ aggregation, and increases Aβ neurotoxicity. Increased Aβ production or reduced Aβ metabolism then results in the formation of aggregated Aβ deposits and AD. Al increases the Aβ burden in experimental animals through a direct influence on Aβ anabolism or direct or indirect impact on Aβ catabolism. The Aβ1-42 isoform is more directly neurotoxic, and it has a greater propensity to aggregate. We found that increasing sub-chronic Al(mal)3 doses decreased gradually the expression of Aβ1-40 and gradually increased the expression of Aβ1-42 in the cerebral cortexes of rats, suggesting that Al toxicity is related to the proteolytic cleavage of APP. However, the direct influence of Al on the secretases (α, β, γ) that play the key role in the expressions of Aβ remains unknown.

Al binding to the phosphate groups of DNA and RNA affects the expression of various genes that are essential for brain functions. Nanomolar levels of Al+ were sufficient to influence neuronal gene expression. Human neural cells exposed to 100 nmol/L Al up-regulated the expression of APP. The inhibition of the nonamyloidosis pathway and increase of the expression of APP induced by Al would be in favor of Aβ formation. In our present study, the expressions of APP protein and mRNA in the 1.08 mg/kg BW Al(mal)3 group were significantly higher than those in the control group, which are in consistent with published data. BACE1 triggers the amyloidogenic processing of APP and the deposition of Aβ, which is the key component of SPs in AD. BACE1 protein and its activity levels are elevated in brain and in CSF from AD patients compared with controls. Aβ generation is abolished in BACE1 knockout mice. In this study, we also found that the expression of BACE1 gene and protein and BACE1 activity levels in cerebral cortices of rats in 0.54 and 1.08 mg/kg BW Al(mal)3 groups were significantly higher than those in the control group and the maltolate groups. The increase of BACE1 activity is predicted to be
amyloidogenic and may exacerbate AD. However, some other studies suggested that BACE1 gene variants do not influence BACE1 protein and its activity, APP levels or Aβ isoforms\(^{67,54}\). The level of BACE mRNA is not altered in AD or transgenic mouse models of AD\(^{51-53}\). PSs are the crucial catalytic components of γ-secretase. PS1 mRNA levels are significantly elevated in high plaque areas of AD brain\(^{51}\). In this study, we also found that the expression of PS1 protein and gene gradually enhanced with the increase of Al(mal)\(_3\) doses.

In conclusion, findings from our present study shows that one of the possible mechanisms that Al(mal)\(_3\) can cause neurotoxicity is that Al(mal)\(_3\) can increase the generation of Aβ1-42 by facilitating the expressions of APP, β-, and γ-secretase, and further researches on the role of Al(mal)\(_3\) in generation of Aβ are needed.

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