Original Article

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

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Abstract

Objective To investigate the impact of sub-chronic Aluminium-maltolate [$Al(mal)_3$] 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 maltolate 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. Maltolate 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-maltolate; *Amyloid precursor protein*; *β-amyloid protein*; Rat

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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)[1-5]. However, the role of Al in the pathogenesis of AD remains currently controversial[1,5].

AD, the most common form of dementia, is characterized clinically by the progressive loss of

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memory and other cognitive functions, and pathologically by senile plaques (SPs) which is composed of *β-amyloid protein* (*Aβ*), neurofibrillary tangles (NFTs) that is composed of hyperphosphorylated tau, and neurons loss[5-7]. The excessive production and accumulation of *Aβ* plays a critical role in the pathogenesis of AD at early stage and may be a common pathway to AD induced by various factors[8-10]. Al accelerated *Aβ* generation and aggregation, induced structural changes of *Aβ* from an α-helix to a β-sheet, and increased the formation and stability of *Aβ* oligomers[11-12]. Abnormally high concentration of Al^{3+} was present along with *Aβ* in the SPs in AD patients[13]. *Aβ* are derived from the sequential proteolysis of the amyloid precursor protein (*APP*) by *β-* and *γ-secretase*[14-16]. Up-regulated expression of the *APP* gene occurred early in the cascade of events that led to the formation of SPs in the brain of AD patients[17]. And the potential relationship of Al with the expression of *APP* has been investigated extensively[18]. Chronic exposure to $AlCl_3$ through drinking water or food led to the overexpression of *APP* in the brain of rats[17,19]. Yet, some other studies showed that Al had no effect on the expression or processing of *APP*[20]. Moreover, The β-site *APP* cleaving enzyme 1 (*BACE1*) is the most important *β-secretase*[21]. *BACE1* cleavage of APP is a prerequisite for *Aβ* formation and is putatively the rate-limiting step in *Aβ* generation from *APP*[22-23]. It was shown that elevated *BACE1* expression and activity were associated with the accumulation of *APP* products in AD[24]. And *BACE1* suppression or knockout could prevent *Aβ* generation and completely abolish *Aβ* pathology in *APP* transgenic mice[24-25]. The mice treated with $AlCl_3$ combined with D-galactose showed memory impairment and the high expression of *Aβ* and *BACE1* in cortex and hippocampus[26]. Some previous reports demonstrated that exposure to Al increased the expression of *APP* and *Aβ* in the brains of experimental animals[27-28]. However, Castorina A thought that the effects of $AlCl_3$ on the mRNA transcription of *β-secretase* were subtle[8]. Lin R found that PC12 cells treated with Al^{3+} (50-100 mmol/L) did not increase the expression of either *APP* or *BACE1*[21]. *γ-Secretase* is a highly hydrophobic complex and presenilin-1 (*PS1*) is its crucial catalytic components. The final cleavage of *APP* by *γ-secretase* determines the length of *Aβ* peptides. *Aβ1-42* may be more hydrophobic and amyloidogenic than other *Aβ* peptides and increase *Aβ1-42* levels likely provide the core for oligomerization, fibrillation, and SPs generation[29]. All these studies suggested that Al increased the formation of *Aβ* by directly promoting *Aβ* synthesis. However, the exact mechanism of this remains elusive[10,13]. 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[30]. 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[31-32]. This compound is therefore needed to be studied in terms of its toxicology and neuropathology of AD[31]. 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 $AlCl_3 \cdot 6H_2O$ (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[33]. $AlCl_3 \cdot 6H_2O$ 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 maltolate 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. Maltolate 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 (Dailian) Co., LTD. The primers were designed using Oligo 6.0 primer analysis software for each analyzed gene: *APP* forward primer 5'-AACATGTGCGCATGGTGGA-3' and reverse primer 5'-CACGGCAGGGACGTTGTAGA-3'; *BACE1* forward primer 5'-AGCTGGATTATGGTGGCCTGAG-3' and reverse primer 5'-CCTGCAGCTTTCAGGGTCTTC-3'; *PS1* forward primer 5'-ATGGACCGCATGGCTCATC-3' and reverse primer 5'-TCGACCAGCATACGAAGTGGA-3'; *β-actin* forward primer 5'-GGAGATTACTGCCCT GGCTCCTA-3' and reverse primer 5'-GACTCATCGT ACTCCTGCTTGCTG-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_2O (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 placed 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 shaked 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±481.77 pg/mL, n=4) were signifi- cantly 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) ($P<0.05$).

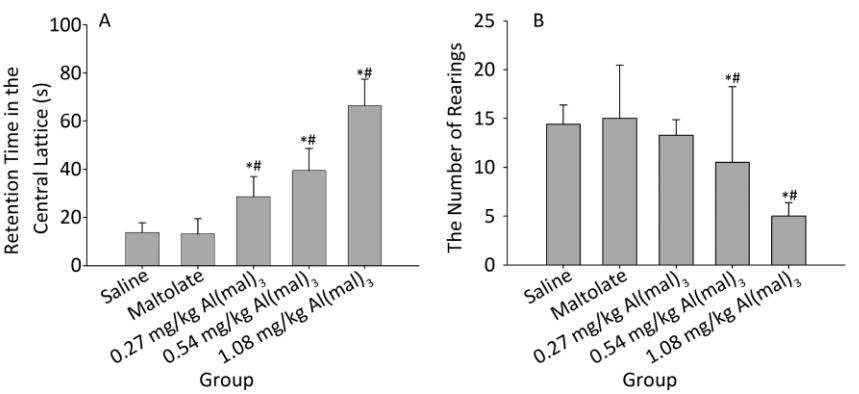

Figure 1. Al(mal)$_3$ treatment impaired anxiety state and exploratory behavior. A: The retention time in the central lattice of the OFT. B: The number of rearings of the OFT. Bars represent means±SD. *Note*. $^{*}P<0.05$ *vs*. control group, $^{\#}P<0.05$ *vs*. maltolate group.

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).

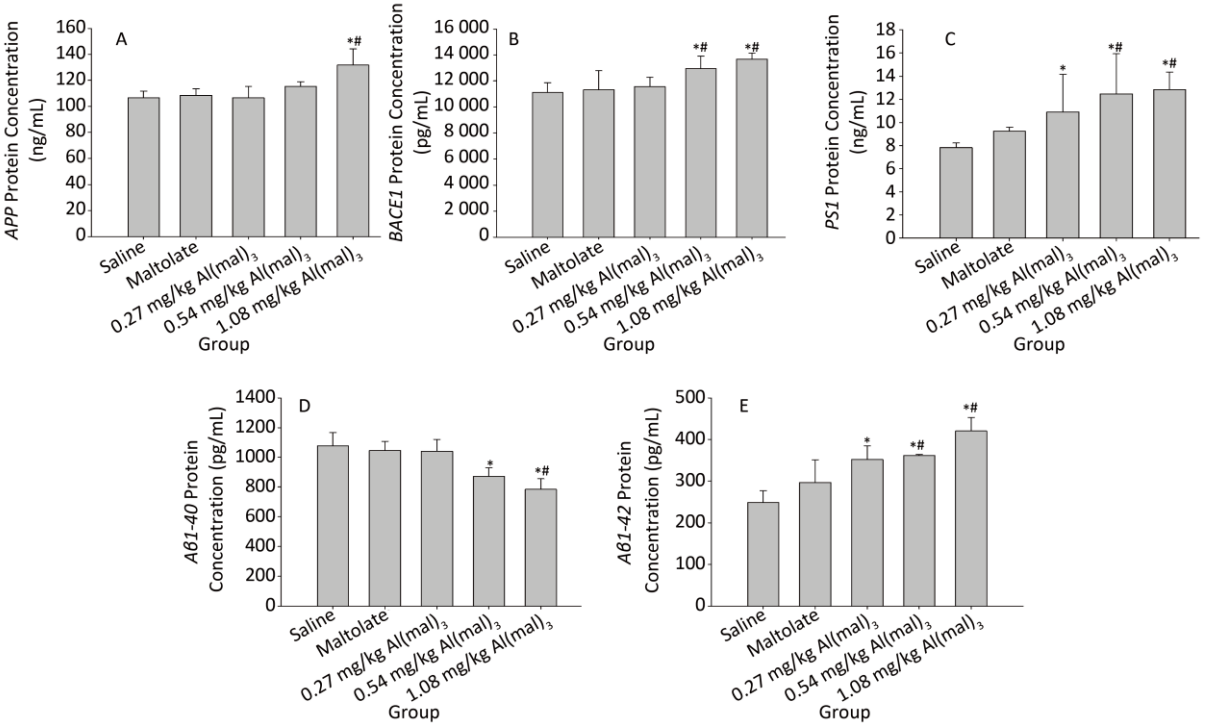

Figure 2. Expressions of *APP*, *BACE1*, *PS1*, *Aβ1-40*, and *Aβ1-42* proteins in cerebral cortex of tested rats. A, B, C, D, and E represent protein expressions of *APP*, *BACE1*, *PS1*, *Aβ1-40,* and *Aβ1-42* in cerebral cortex of tested rats of the control group, maltolate group, and Al(mal)$_3$ groups, respectively. Bars represent the means±SD. *Note*. $^{*}P$<0.05 *vs*. saline group, $^{\#}P$<0.05 *vs*. maltolate group.

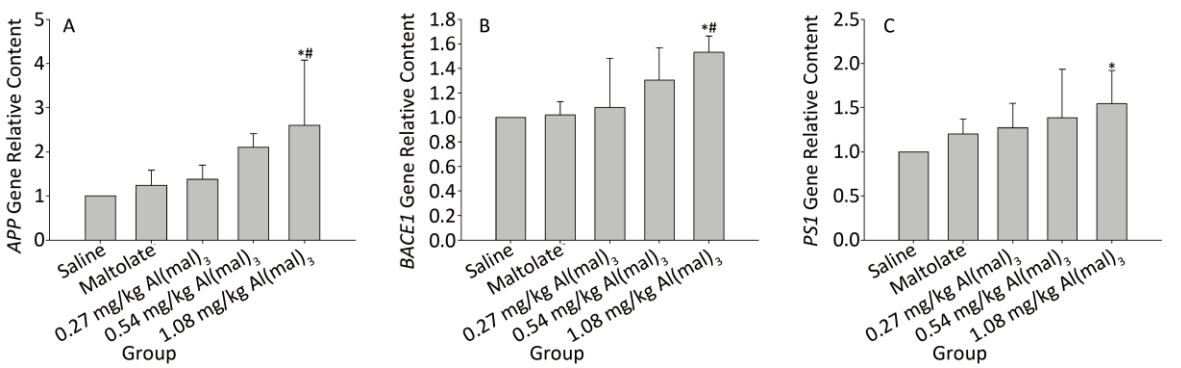

Figure 3. Relative expressions of *APP*, *BACE1*, and *PS1* mRNA in the cerebral cortex of tested rats. A, B, and C represent the relative expressions of *APP*, *BACE1*, *PS1* mRNA in the control, maltolate, and Al(mal)$_3$ groups, respectively. Bars represent the means±SD. *Note*. $^{*}P$<0.05 *vs*.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

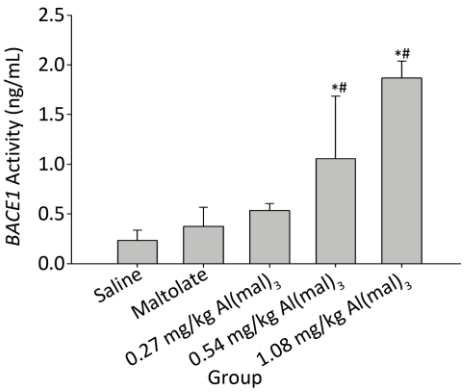

Figure 4. *BACE1* activity levels in cerebral cortex of tested rats. Bars represent the means±SD. *Note*. $^{*}P<0.05$ *vs*. saline group, $^{\#}P<0.05$ *vs*. maltolate group.

DISCUSSION

Behavioral changes as indicators of neurotoxicity may be more sensitive than neurochemical alterations and these changes may be observed during early Al exposure[34]. 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[35,37], yet our findings contrast with those of some other studies[2,36]. 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)[34,38].

APP has two alternative proteolytic pathways: the nonamyloidosis pathway and the amyloidosis pathway[15,29]. 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[15]. And abnormal amyloidosis proteolysis of *APP* and the generation of *Aβ* are key events in the pathogenesis of AD[39-40]. 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[41]. Increased *Aβ* production or reduced *Aβ* metabolism then results in the formation of aggregated *Aβ* deposits and AD[15]. Al increases the *Aβ* burden in experimental animals through a direct influence on *Aβ* anabolism or direct or indirect impact on *Aβ* catabolism[42]. The *Aβ1-42* isoform is more directly neurotoxic, and it has a greater propensity to aggregate[43-44]. 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 proteolytically cleave *APP* remains unknown[42].

Al^{3+} 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^{3+} were sufficient to influence neuronal gene expression[46-47]. Human neural cells exposed to 100 nmol/L Al up-regulated the expression of *APP* gene[42]. The inhibition of the nonamyloidosis pathway and increase of the expression of *APP* induced by Al would be in favor of *Aβ* formation[41]. 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[45]. *BACE1* triggers the amyloidogenic processing of *APP* and the deposition of *Aβ*, which is the key component of SPs in AD[21]. *BACE1* protein and its activity levels are elevated in brain and in CSF from AD patients compared with controls[46-49]. *Aβ* generation is abolished in *BACE1* knockout mice[50]. In this study, we also found that the expression of *BACE1* gene and protein and *BACE1* activity levels in cerebral cortexes 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[47,51]. 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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