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Original Article

Aluminium-Maltolate-induced Impairment of Learning, Memory and Hippocampal Long-term Potentiation in Rats

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Abstract: Recently, aluminium (Al) has been proposed to be one of the environmental factors responsible for cause Alzheimer's disease (AD). However, the relationship between Al and AD is controversial. To investigate the effects of subchronic Aluminium-maltolate (Al (mal)$_3$) exposure on the behavioral, electrophysiological functions. Forty Sprague-Dawley (SD) rats were randomly distributed into five groups. Over two months, rats in the saline group received daily intraperitoneal (i.p.) injections 0.9% saline, rats in the maltolate group received 7.56 mg/kg maltolate, and rats in the 0.27, 0.54, 1.08 mg/kg Al (mal)$_3$ groups received i.p. administrations of these three doses, respectively. Neural behavior was assessed in Morris water maze. Long-term potentiation (LTP) in hippocampus was recorded. Al content in the neocortex was determined using a graphite furnace atomic absorption spectrophotometer. Our studies indicate that subchronic Al (mal)$_3$ exposure significantly impaired spatial learning and memory abilities, suppressed the LTP in the CA1 hippocampal area, and elevated Al levels in cerebral cortex in a dose-dependent fashion. In conclusion, low doses of Al (mal)$_3$ can still lead to dramatic Al accumulation in the brain, severely impair learning and memory capacities, and hippocampal LTP.

Key words: Aluminium-maltolate, Learning and memory, Hippocampus, Long-term potentiation, Rat

Introduction

Aluminium (Al) is the most abundant metal and the third most common chemical element in the earth's crust[1–5]. This metal is widely used in our everyday life, and in the course of industrial production, it has easy access to our body through cooking utensils, foods, antacids, drinking water, Al-containing dust and fumes[6]. Some studies have reported the potential toxicity of Al in experimental animal models and in humans[3, 7]. Regardless of the host, the route of administration, and the chemical speciation, Al has been found to be a potent neurotoxicant[8]. Al has also been proposed to be one of the critical environmental factors related to several serious neurodegenerative diseases, such as Alzheimer's disease (AD)[3, 9–12].

AD is the most common form of dementia, and with increasing life expectancy across the world, AD is a rapidly

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growing socioeconomic and medical problem[13]. AD is clinically characterised by progressive learning impairment and memory loss followed by cognitive decline. This disease is pathologically characterised by severe neuronal loss, the formation of neurofibrillary tangles (NFTs) and senile plaques (SPs)[3, 14]. SPs are an early toxic event that are formed by a gradual accumulation of β-amyloid protein (Aβ). Crapper *et al*. first showed the increased level of Al in the brains of AD patients[15]. Sakae Yumoto *et al.* demonstrated that Al was colocalized with Aβ in amyloid fibers in the cores of SPs[16]. Other studies have also shown that Al increased the level of Aβ, promoted Aβ aggregation, and increased Aβ neurotoxicity[10, 17]. Walton verified that Al played an important role in the formation of SPs and NFTs in patients with AD[18]. Some epidemiologic and experimental studies have revealed that high Al consumption from drinking water may be a risk factor for AD[19–21]. Nevertheless, the role of Al in AD remains controversial, and there is little direct evidence correlating Al with AD[14, 17, 22]. Furthermore, understanding the molecular mechanism of Al neurotoxicity is hampered due to intrinsic difficulties in discerning the role of chemical speciation in biological systems[3, 22, 23]. *In vitro* and *in vivo* studies have demonstrated that the physiological activity and the biological availability of Al depend strongly on the chemical form of the metal ion[24, 25]. Differences in the chemical form of Al are important factors affecting Al transfer into the brain[26]. The specific ligands for Al can differentially modulate its uptake and toxicity, and can also impact the apparent rate of Al accumulation in neurons *in vivo*[1]. Hence, studying the appropriate Al compound is very important to understanding Al toxicity[22].

Aluminium-maltolate (Al $(mal)_3$) is a suitable compound for toxicology and neuropathology with relevance to AD. It is highly soluble and stable between pHs of 3.0 and 10.0, possesses hydrolytic stability at pH 7.0[22, 25, 27, 28]. However, most other Al salts (e.g., Aluminium chloride ($AlCl_3$)) produce insoluble complexes at a neutral pH[29]. Al $(mal)_3$ was reported to have strong toxicity[1, 29], and maltolate is a common component of the human diet. Maltolate is found in coffee, soybeans, baked cereals, caramelised, and browned foods[5, 30], and was reported to be a strong enhancer of Al accumulation in the brain[23]. Given the extremely high affinity of maltolate for Al, there is a potential for Al $(mal)_3$ to form in the gastrointestinal tract[23, 28]. We therefore selected Al $(mal)_3$ as a source of Al in this study.

The hippocampus is a crucial element of the neurobiology basis of higher cognitive function[31]. Many experiments have demonstrated that behavioral performance was followed by and correlated with an increase or depression in synaptic efficiency in the hippocampus[32, 33]. Long-term potentiation (LTP) is an activity-dependent and persistent functional increase in synaptic efficacy. Hippocampal LTP has been accepted as a well-known electrophysiological model for the cellular basis of learning and memory[33]. Chronic, low Al exposure may alter fundamental mechanisms involved in learning and memory[33]. Recently, studies have reported that Al impairs hippocampal LTP[33–35]. However, the relationship between LTP and learning is far from conclusive[33].

In the present study, the effect of subchronic intraperitoneal (i.p.) injections Al $(mal)_3$ was assessed at behavioral and electrophysiological levels to investigate the possible pathophysiology associated with Al toxicity and to suggest a link between high level Al and AD progression. AD is characterised by impairment in the understanding of time and place. If Al accumulation in the brain induces spatial memory impairment, it is conceivable that Al is one of the important factors impairing brain function and a potential contributing factor in the etiology and/or pathology of AD[23]. However, the exact mechanism of Al neurotoxicity in AD has not yet been reported[36]. Measurements of LTP may provide a sensitive index of the integrity of cognitive processes affected by Al exposure. However, there has been very little study on the effects of Al $(mal)_3$ exposure on LTP. The aim of this study was to investigate the behavioral effects following Al $(mal)_3$ exposure and to clarify the *in vivo* effects of Al on hippocampal LTP that are induced by Al $(mal)_3$.

Materials and Methods

Material

Aluminium chloride hexahydrate ($AlCl_3 \cdot 6H_2O$), maltolate, and urethane were purchased from Sigma-Aldrich Chemical Co. A standard Al solution (1,000 mg/l) was obtained from Tianjin Aoran Fine Chemical Institute. All chemicals used were of analytical grade unless otherwise indicated. Ultrapure water (18 MΩ) was obtained by ultrafiltration of distilled water using a Heal Force ultrapure water system. The Morris water maze was purchased from the Institute of Materia Medica of the Chinese Academy of sciences. The stereotaxic apparatus was purchased from Narishige, Japan. A pair of parallel stimulating/recording electrodes was obtained from Sequim, WA, USA. An electronic stimulator (SEN-3301) and a coupled isolator (ss-102 J) were created in Kohden, Japan. The

multichannel biological signal acquisition/processing system was the product of Chengdu Instruments Ltd, China. The graphite atomic absorption spectrometer (AA240Z) was ordered from Varian, USA.

Al (mal)$_3$ preparation

Al (mal)$_3$ was prepared according to the procedure described in a previous publication[37]. $AlCl_3 \cdot 6H_2O$ was dissolved in distilled water until the final concentration was 80 mM. Maltolate was dissolved in phosphate buffered saline (PBS) to a final concentration of 240 mM. The solutions were mixed in equal volumes, and the pH was adjusted to 7.4 with NaOH 1 M. The resulting Al (mal)$_3$ was freshly prepared for each experiment. All solutions were filter sterilised using 0.22 μm syringe filters immediately following preparation.

Animal and treatment

Male Sprague-Dawley (SD) rats were purchased from the Experimental Animal Centre. Prior to experimentation, rats were group-housed in standard laboratory cages for one week of habituation in an environment of 22–24°C, 65% humidity, a 12:12 h light-dark cycle and access to food and water *ad libitum*. Behavioral screenings were conducted using the Morris water maze test. Forty rats (200–220 g body weight) were selected for the present study. These rats were randomly assigned to five groups according to body weight. Groups receiving 0.27, 0.54, or 1.08 mg/kg Al (mal)$_3$ were given intraperitoneal administrations of these concentrations for two months. The maltolate group received daily i.p. injections of maltolate at 7.56 mg/kg, and the saline group received daily injections of an equal volume of normal saline solution. All injections were performed at the same time.

Behavioral assessment

The Morris water maze test was performed to assess the spatial memory and learning abilities of the rats. The test was performed following the method described by Morris[38], with the minor modifications described below. The water maze consisted of a black, painted circular water pool (180 cm in diameter, 80 cm in high), which was divided into four quadrants. The tank was filled with water, which was maintained at 25°C ± 1.0°C. A circular escape platform (10 cm in diameter) was submerged approximately 1.0 cm below the surface of the water and placed at the midpoint of the target quadrant such that the rat could escape swimming. White curtains containing various prominent visual cues surrounded the pool. The water maze had 4 starting positions: north, south, east, and west. The Morris water maze training was recorded by a video camera mounted overhead, and data were analysed by a behavior software system. The Morris water maze test was conducted in two phases, the hidden platform test and the probe trial. For the hidden platform test, a trial was initiated each day by placing each rat in the water in one of the four quadrants such that the rat was facing the pool wall. For each trial, rats were required to search for the platform for a maximum of 120 s. If a rat failed to find the platform within 120 s, it was guided to reach the platform by the experimenter, allowed to remain on the platform for 30 s, and the time recorded for the trial was 120 s. Four trials per rat per day were performed, and there was an inter-trial interval of 2 min. Each rat was exposed to the task between 8:00 AM to 12:00 AM for five consecutive days. The daily escape latency for each rat was calculated as the average of the four trials. The probe trial was performed on the 6th day. The hidden platform was removed from the pool, and the animals were given 60 s to swim in the pool to measure the spatial bias of the rats. The swimming time in the quadrant of platform and the number of crossings over the previous platform location were recorded. All behavioral tests were conducted in a quiet environment with subdued lighting.

Electrophysiological recordings

Following the behavioral investigations, electrophysiological tests were performed. The rats were anaesthetised with urethane (1.5 g/kg, i.p.), and the heads were fixed in a stereotaxic device for surgeries and recordings. Body temperature was monitored throughout the experiment, and a heating pad was used to maintain the temperature of the animals at 37°C ± 0.5°C. The skull was exposed, and a pair of parallel stimulating/recording electrodes were inserted into the hippocampus of rats, with the bipolar stimulating electrode at the Schaffer collateral/commissural pathway (3.4 mm posterior to bregma, 2.5 mm lateral to the midline) to induce LTP. The mono-polar recording electrode was positioned at the stratum radiatum of area CA1 of the hippocampus (4.2 mm posterior to bregma, 3.8 mm lateral to the midline) to record the field excitatory postsynaptic potential (fEPSP). The correct placement of electrodes in the CA1 region was confirmed primarily by electrophysiological criteria. The amplitude of baseline fEPSP was chosen as 50% of the maximum fEPSP amplitude by adjusting the stimulation pulse intensity. LTP was induced by a high-frequency stimulus (HFS) protocol consisting of 20 pulses at 200 Hz. Baseline fEPSP recording

(response to test stimulation prior to HFS) was monitored for a minimum of 30 min to ensure the steady state of synaptic responses. Test stimuli were applied again after following for l h to monitor the changes in fEPSP amplitude. The average value of the fEPSP amplitude over the 30 min of baseline recording was defined as 100%, and all recoded fEPSPs were normalised to this baseline value.

The general natural situation

Because of rat's body weight was correlated with its Al intake, data of body weight could be used to control the confounding factors in the present study. Under subchronic exposure of Al $(mal)_3$, the body weight of rats were measured weekly. The growth and development situation, the health appearance, and behavior of rats were observed every day. After the electrophysiological test, rats were weighted and were sacrificed immediately to get brain weights. The ratio of brain to body weight of each rat was calculated and compared.

Al content in rat cerebral cortex

Approximately 200 mg (wet weight) of fresh neocortical tissue from the right hemisphere of the brain was immediately dissolved in a solution consisting of 1.6 ml of concentrated nitric acid and 0.4 ml of perchlorate acid. This solution was heated at 120–150°C for four hours, after which the digested tissue was diluted to 2 ml with Al-free water. The concentration of Al in the brain was determined by graphite furnace atomic absorption spectrometry using a wavelength of 396.2 nm, slit width of 0.5 nm, lamp current of 10.0 mA, and an injection volume of 10 µl.

All experimental protocols used in the present work were approved by the Ethics Committee for Animal Studies of Shanxi Medical University. All efforts were made to minimise the number of animals used and their suffering.

Statistical analysis

All of the data are displayed as the mean ± SD. All statistic analyses were performed with the software Statistical Package for the Social Sciences 13.0. Repeated measures analysis of variance (ANOVA) and one-way ANOVA were used to analyse behavioral, electrophysiological and Al content data. The statistical significance level was defined as $p<0.05$.

Results

The general natural situation

Rats in control groups had fine growth and development, keep their fur lustrous and glowing, and were lively and active. Rats in Al $(mal)_3$ groups remain health. Although subchronic exposure to Al $(mal)_3$ resulted in slight loss of total body weight and of brain weight, such effect was not statistically significant. However, the ratios of brain to body weight were significantly reduced in 0.54 mg/kg, 1.08 mg/kg Al $(mal)_3$ groups compared to the control groups ($p<0.05$)(data not shown). Furthermore, rats of Al $(mal)_3$ groups appeared fur lustrous poorly, fur loss, unresponsive of different degrees along with the increase of Al $(mal)_3$ dose. None of rats died unexpectedly during the experimental period.

Al $(mal)_3$ treatment impaired learning and memory behavior

Figure 1, the impairment of Al on spatial learning and memory was investigated by the Morris water maze test in rats. The ability to acquire spatial information was assessed by a hidden platform test. (A) depicts the mean escape latencies in the learning acquisition test in different groups. As expected, the average latencies in searching for the hidden underwater platform decreased progressively with the increase in training days ($p<0.05$). However, when performances on the same training day were analysed, escape latencies of the Al $(mal)_3$ groups were prolonged but no significant differences were found when compared to those of the saline and maltolate groups ($p>0.05$). (B, C) In the probe trail, the swimming time in the quadrant of platform was significantly decreased in the Al $(mal)_3$ groups compared to those of saline and maltolate groups ($p<0.05$), at the same time, the Al $(mal)_3$ effect was detected on the average number of crossings of the platform site within 60 s, which was significantly reduced in the Al $(mal)_3$ groups compared to those of saline and maltolate groups ($p<0.05$).

Al $(mal)_3$ treatment significantly suppressed LTP in the CA1 hippocampal area

To investigate the effects of Al $(mal)_3$ on hippocampal LTP in vivo, we successfully induced LTP in the CA1 hippocampal area of SD rats by applying HFS to the synaptic Schaffer collateral/commissural pathway (Fig. 2). (A) When compared with the saline and maltolate groups, a significant suppression of LTP by Al $(mal)_3$ treatment was observed. The strength of the LTP gradually decayed in

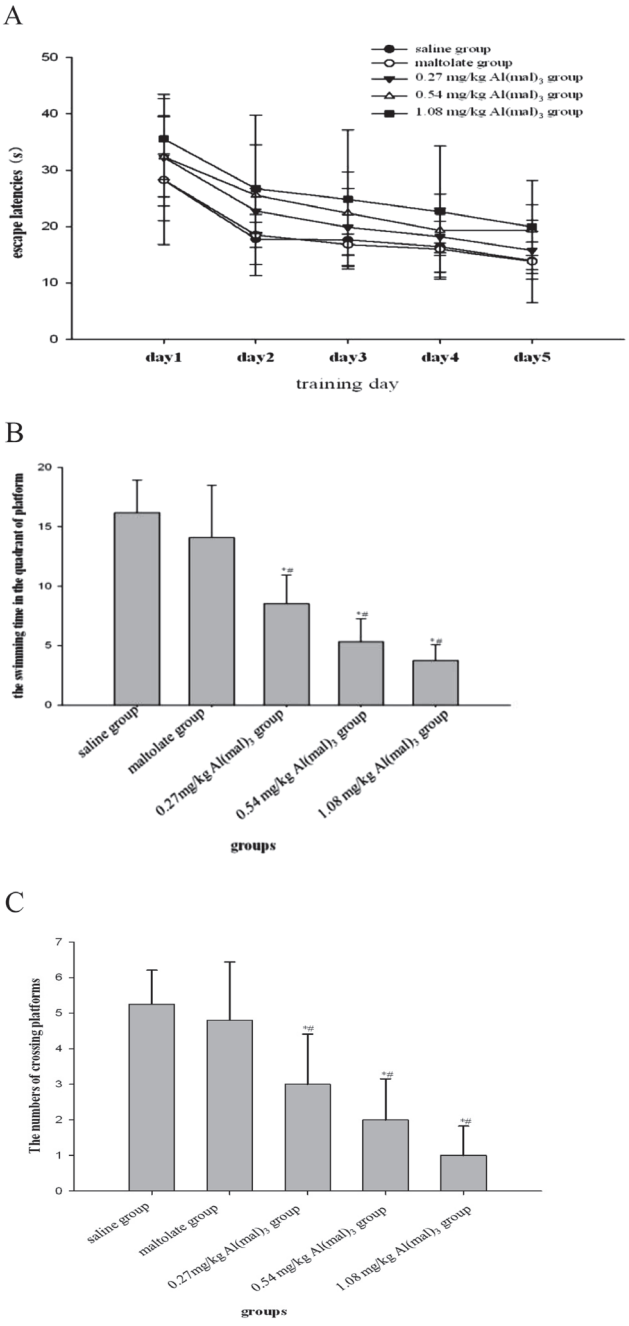

Fig. 1. Al (mal)$_3$ impaired learning and memory behavior as assessed by the Morris water maze. (A) The hidden platform test shows the average escape latencies required for searching for the hidden underwater platform. Each point represents the mean ± SD. (B, C) The probe trials indicate the swimming time in the quadrant of platform and the average number of crossings of the platform site within 60 s. Each column represents the mean ± SD. For this and all other figures, different superscripts (* and #) indicate significant differences ($p < 0.05$) as compared to saline or maltolate groups.

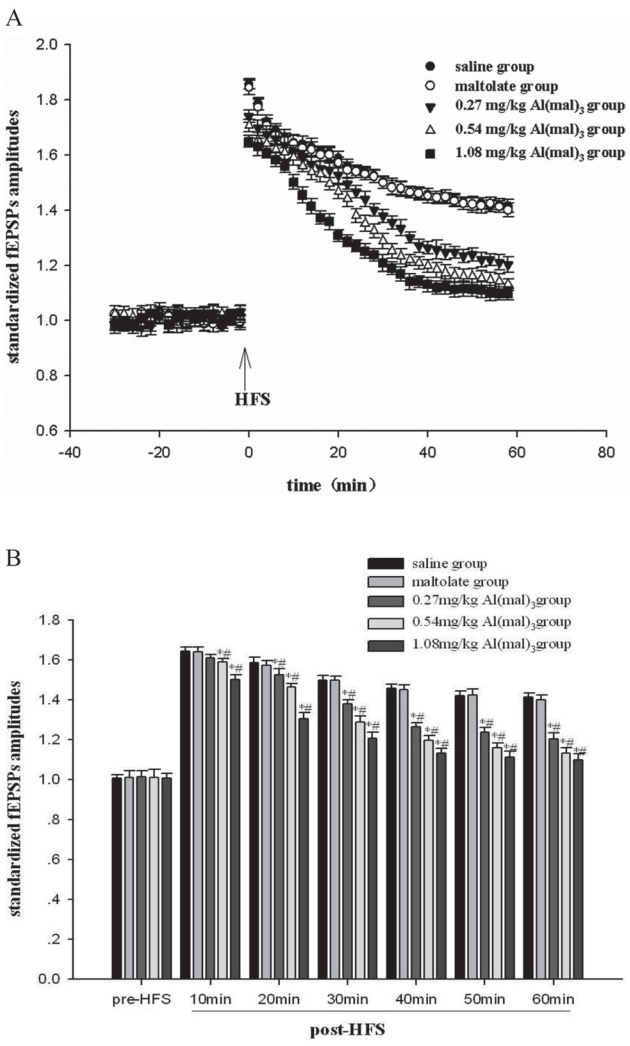

Fig. 2. Al (mal)$_3$ suppressed hippocampal LTP in the CA1 region. (A) The suppressive effect of Al (mal)$_3$ on LTP in the hippocampal CA1 region. Each point represents the mean ± SD. (B) Histograms showing the average standardised fEPSP amplitudes in five subgroups at different time points prior to and following HFS. Each column represents the mean ± SD of the fEPSP amplitude.

the Al (mal)$_3$-treated groups during 1 h of recording. (B) At 10 min, 20 min, 30 min, 40 min, 50 min and 60 min following HFS, the average standardised fEPSP amplitude in all Al (mal)$_3$ groups, except the 0.27 mg/kg Al (mal)$_3$ group at 10 min following HFS, were significantly decreased when compared to those of the saline and maltolate groups (p<0.05). These results indicate that Al (mal)$_3$ induced LTP impairment in the CA1 hippocampal area of rats.

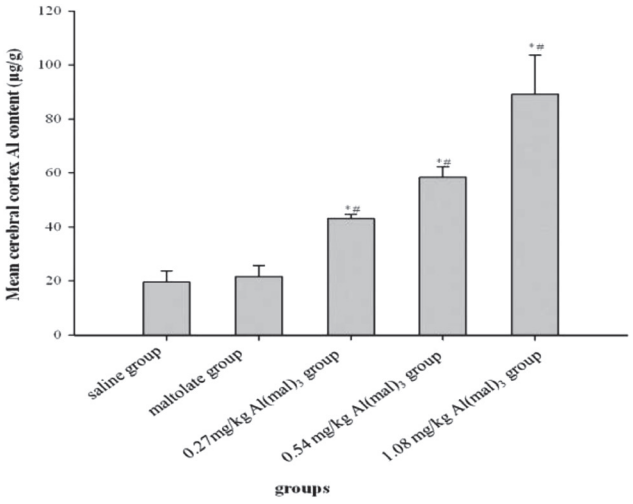

Fig. 3. Mean cerebral cortex Al content (μg/g) in each group. Each column represents the mean ± SD. Al levels (μg/g) in each Al $(mal)_3$ group were significantly increased over levels of the saline and maltolate groups ($p < 0.05$).

Differences in the Al content in rat cerebral cortex tissue in control and experimental groups

Because the observed memory impairment effects were related to Al in the central nervous system, the levels of Al in the brains of rats administered saline, maltolate or Al $(mal)_3$ were compared. Fig. 3 Al levels in the brain in the saline and maltolate groups were 19.50 ± 4.12 μg/g and 21.63 ± 4.05 μg/g, respectively, and were not significantly different from one another. However, Al content in the cerebral cortex of Al $(mal)_3$-exposed groups increased with increasing Al $(mal)_3$ dosage. The observed levels were 43.16 ± 1.54 μg/g, 58.38 ± 3.85 μg/g, and 89.20 ± 14.45 μg/g for the 0.27, 0.54, and 1.08 mg/kg Al $(mal)_3$ groups, respectively, and were all significantly higher than values from the saline and maltolate groups ($p<0.05$).

Discussion

AD is a progressive form of dementia in the elderly and is the most prevalent neurodegenerative disease in the world[13]. It is well established that exposure to Al represents a risk for the development of AD[20]. However, the relationship between Al and AD is still controversial[21, 23, 39]. The major problem in understanding Al toxicity is the complexity of Al chemistry speciation in biological systems[22]. It is thought that the biochemical and toxicological behaviors of Al depend on the chemical form of Al. Furthermore, it has been shown that Al absorption, tissue retention and deposition, and excretion depend on the properties of the Al^{3+} complexes formed with biological ligands[25]. Al $(mal)_3$ has been suggested to be able to cross the blood-brain-barrier, thereby increasing Al-induced neurotoxicity[23, 28, 40]. Moreover, when cells were treated with Al $(mal)_3$, Al was incorporated into cells[39]. It was reported that Al $(mal)_3$ had the largest toxicity effect on both neurons and glia when compared to aluminium lactate, AlCl3 and aluminium fluoride[1]. Langui D has demonstrated that the percentage of tangle-bearing neurons in culture can be increased up to approximately 20% following treatment with Al $(mal)_3$[37]. Furthermore, Tsubouchi R *et al*. reported that Al $(mal)_3$ induced apoptosis in PC12D cells by enhancing the generation of reactive oxygen species[2]. In contrast to the well-documented Al-induced cellular neurotoxicity, neurobehavioral studies of Al have generally not produced robust or consistent results[36, 41]. Therefore, the potential toxicity of Al $(mal)_3$ requires further investigation. To contribute to the available literature, the present study evaluated the effects of subchronic i.p. injections of Al $(mal)_3$ on spatial learning and memory and on LTP in the CA1 hippocampal area and on Al content in rat cerebral cortical tissue were examined for the first time.

It has been suggested that, in some cases, behavioral changes may be more sensitive than neurochemical alterations as indicators of neurotoxicity and may be observed during early exposure to Al[41]. In recent decades, Al neurotoxicity and its possible role in cognitive function impairment have been extensively studied[3, 7]. However, results with respect to these questions are still divergent and unclear. In the present study, longer escape latencies, fewer swimming time in the quadrant of platform, and fewer crossings of the platform site in the Morris water maze task were observed in the rats exposed to Al $(mal)_3$, indicating a possible Al $(mal)_3$-induced impairment on spatial learning and long-term memory. These results are in agreement with both published data and our previous findings relating learning and memory with Al-toxicity[7, 23]. Taken together, our results indicate that Al $(mal)_3$ treatment induced spatial learning and memory deficit in rats.

Advances in neurobiology have produced significant and relevant physiological discoveries since synaptic plasticity was first proposed to explain behavioral performance. It has been widely accepted that memory formation is dependent on changes in synaptic efficiency[35]. LTP is an activity-dependent increase in synaptic efficacy and is widely used as a model to investigate the cellular mechanisms underlying some forms of vertebrate learning and memory[35, 42, 43]. LTP in the hippocampus Schaffer collateral-CA1 pathway is considered to be important for

learning and memory[44], and factors that impair learning and memory have been demonstrated to decrease the strength of LTP[45]. In the present study, LTP magnitude decreased in Al $(mal)_3$ groups, confirming results from other laboratories, which found that Al impairs hippocampal LTP in rats in vitro and in vivo[34, 35, 46, 47]. Although Al is very poorly absorbed in the gastrointestinal tract, Al-exposed rats exhibited a significant impairment of spatial memory ability and the inhibition of population spike (PS) amplitudes in the CA1 area when they were fed up with drinking water containing $AlCl_3$ in different concentrations for three months[35, 47]. Moreover, microinjection of Al into CA3 was shown to block LTP induction *in vivo*[48]. The present data demonstrate that subchronic exposure to Al $(mal)_3$ has negative effects on hippocampal functioning. Because there is general agreement that the hippocampus plays an important role in learning and memory, the observed deficiency in LTP may be responsible, at least in part, for the learning and memory deficits in Al $(mal)_3$-exposed rats.

The possibility remains that elevated levels of Al may initiate or accentuate the pathogenesis of neurological deterioration. Al accumulation in the brain was demonstrated to induce neuropathy, thereby resulting in the initial symptom of AD[23]. High Al serum levels in aged humans and Al-exposed experimental animal were found to be associated with deficient long-term memory[47, 49]. In the present study, rats treated with Al $(mal)_3$ had significantly higher Al levels in brain tissue than those in the saline or maltolate groups. These results show that subchronic Al exposure caused considerable Al accumulation in the brain, despite the fact that rats are considered to be less susceptible to Al exposure. Our previous study indicated that Al levels in the brains of rats following i.p. administration of Al $(mal)_3$ were significantly greater[7]. The results of the present study are in agreement with many other investigations in different experimental animals[26, 41, 47].

In conclusion, maltolate could strengthen Al-induced neurotoxicity. Low doses of Al $(mal)_3$ can still lead to dramatic Al accumulation in the brain and severely impair learning and memory capacities, and hippocampal LTP. The relationship between plasticity deficiencies and declines in cognitive ability clearly requires further study.

Acknowledgements

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