

# Ursolic acid attenuates beta-amyloid-induced memory impairment in mice

Ácido ursólico atenua a perda de memória induzida por beta-amilóide em ratos

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## ABSTRACT

**Objective:** Increasing evidence demonstrates that oxidative stress and inflammatory are involved in amyloid  $\beta$  ( $A\beta$ )-induced memory impairments. Ursolic acid (UA), a triterpenoid compound, has potent anti-inflammatory and antioxidant activities. However, it remains unclear whether UA attenuates  $A\beta$ -induced neurotoxicity. **Method:** The aggregated  $A\beta_{25-35}$  was intracerebroventricularly administered to mice. **Results:** We found that UA significantly reversed the  $A\beta_{25-35}$ -induced learning and memory deficits. Our results indicated that one of the potential mechanisms of the neuroprotective effect was attenuating the  $A\beta_{25-35}$ -induced accumulation of malondialdehyde (MDA) and depletion of glutathione (GSH) in the hippocampus. Furthermore, UA significantly suppressed the upregulation of IL-1 $\beta$ , IL-6, and tumor necrosis- $\alpha$  factor levels in the hippocampus of  $A\beta_{25-35}$ -treated mice. **Conclusion:** These findings suggest that UA prevents memory impairment through amelioration of oxidative stress, inflammatory response and may offer a novel therapeutic strategy for the treatment of Alzheimer's disease.

**Keywords:** amyloid; memory; oxidative stress; ursolic acid.

## RESUMO

**Objetivo:** Há evidências crescentes de que o estresse oxidativo e a inflamação estão envolvidos na perda de memória induzida pelo peptídeo beta-amilóide ( $\beta A$ ). O ácido ursólico (AU), um composto triterpenóide, apresenta atividades anti-inflamatórias e antioxidantes potentes. Entretanto, não se sabe ainda se o AU atenua a neurotoxicidade induzida pelo  $\beta A$ . **Método:** O agregado  $\beta A_{25-35}$  foi administrado aos ratos por via intracerebroventricular. **Resultados:** Observou-se que o AU reverteu significativamente os déficits de aprendizado e de memória induzidos pelo  $\beta A_{25-35}$ . Portanto, um dos potenciais mecanismos do efeito neuroprotetor seria a atenuação do acúmulo de malondialdeído e a depleção de glutatona no hipocampo induzidos pelo  $\beta A_{25-35}$ . Além disso, o AU suprimiu significativamente a supra regulação dos níveis de IL-1 $\beta$ , IL-6 e do fator de necrose tumoral  $\alpha$  no hipocampo dos ratos tratados com  $\beta A_{25-35}$ . **Conclusão:** Esses achados sugerem que o AU previne a perda de memória através da melhora do estresse oxidativo e da resposta inflamatória, podendo proporcionar uma nova estratégia terapêutica para o tratamento da doença de Alzheimer.

**Palavras-chave:** amiloide; memória, estresse oxidativo; ácido ursólico.

Alzheimer's disease (AD) is a progressive neurodegenerative disease that is associated with global mental dysfunction and cognitive deterioration. Common pathological features of AD are the accumulation of intraneuronal tau and extracellular amyloid  $\beta$  ( $A\beta$ ) peptide<sup>1</sup>. Accumulation of  $A\beta$  leads to the deposition of insoluble neuritic or senile plaques, thereby initiating a pathological cascade, which results in synaptic dysfunction, synaptic loss, neuronal death, and cognitive impairments<sup>2</sup>. Moreover, oxidative stress and inflammation are involved in  $A\beta$ -induced neuronal death and neurotransmitter deficits and the progression of AD<sup>3</sup>.

$A\beta_{25-35}$  peptide is the core fragment of the full-length  $A\beta$  and possesses the biological activity and toxicity of the full-length  $A\beta$  monomer<sup>4</sup>. Histological and biochemical alterations, oxidative damage, inflammatory responses, and cognitive dysfunction have been induced by intrahippocampal or intracerebroventricular (i.c.v.) injections of  $A\beta_{25-35}$ <sup>5,6</sup>. Therefore, this animal model is used to investigate the pathogenesis and progression of AD and to screen new candidates for AD therapy<sup>5</sup>.

Ursolic acid (UA, 3 $\beta$ -hydroxy-urs-12-en-28-oic acid) is a lipophilic, pentacyclic triterpenoid compound that is widely present in fruits and many types of herbs, such as *Perilla*

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*frutescens*, *Glechoma hederaceae*, *Rosemarinus officinalis*, and *Eriobotrya japonica*<sup>7</sup>. UA has many pleiotropic biological activities, such as anti-oxidant, -inflammatory, -hyperlipidemic, -ulcer, -microbial, and -tumoral activities<sup>7,8,9,10</sup>. Moreover, *in vitro* studies indicate that it suppresses A $\beta$ -induced injury in PC12 cells<sup>11</sup>. In particular, UA attenuates oxidative and inflammatory injury in the brain and cognitive impairments induced by D-galactose<sup>7,12</sup>. However, to the best of our knowledge, no study has been conducted to determine whether UA has an effect against A $\beta$ <sub>25-35</sub>-induced cognitive deficits and neurotoxicity in a mouse model. Here, we addressed this issue and investigated the potential mechanisms underlying the neuroprotective effect of UA.

## METHOD

### Animals

In total, 80 male Kunming mice (10 weeks old) were obtained from the Branch of National Breeder Center of Rodents (Shanghai). They were housed in plastic cages and kept in a regulated environment (22 ± 2°C, 50 ± 5% humidity) with a 12:12-h light/dark cycle (lights on from 8:00 h to 20:30 h). The mice were allowed to acclimate for 1 week, with food and water *ad libitum*. All experiments were performed as per the Chinese legislation on the use and care of laboratory animals.

### Drugs and treatment

UA (Sigma-Aldrich, MO, USA) was suspended in distilled water containing 0.1% Tween-80 (dH<sub>2</sub>O/0.1% Tween-80). A $\beta$ <sub>25-35</sub> (Sigma-Aldrich, MO, USA) was dissolved in double-distilled water at a concentration of 1 mg/ml and stored at -20°C. A $\beta$ <sub>25-35</sub> was aggregated, by incubating it in distilled water at 37°C for 4 days before the injection as described previously<sup>5</sup>. Mice were injected with aggregated A $\beta$ <sub>25-35</sub> or vehicle (3  $\mu$ l/3 min, i.c.v.) into lateral ventricles on day 0<sup>6</sup> under mild anesthesia with ether and then returned to their home cages. In addition, mice received UA (10, 20, or 40 mg/kg) or the distilled water containing 0.1% Tween-80 was by oral gavage for 11 days after the treatment with A $\beta$ <sub>25-35</sub> and the time of treatment was based on our pretest study. The dose of UA in the experiments was

based on previous reports<sup>7,12</sup>. The schedule of our study is demonstrated in Figure 1.

### Open-field test

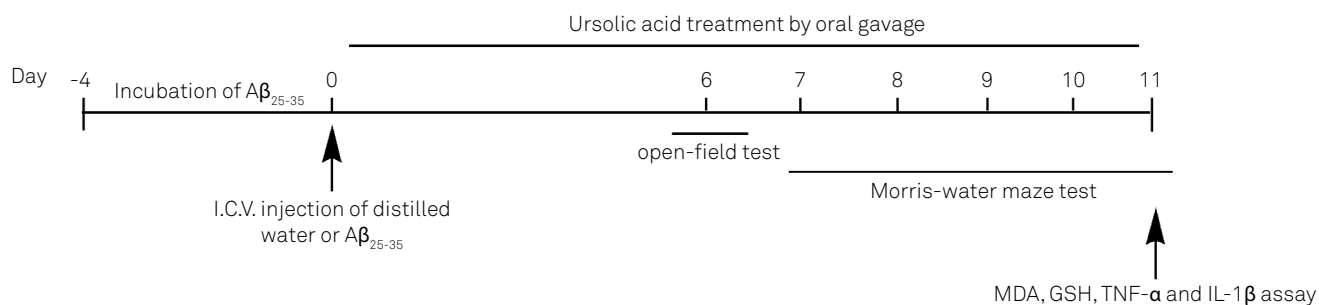
An open-field test was conducted on day 6 after the injection of A $\beta$ <sub>25-35</sub> as described previously<sup>7</sup>. Each mouse was placed in the center of an open-field apparatus with internal dimensions of 30 cm × 30 cm and walls of height 30 cm. The open field was divided into 6 × 6 cm squares marked with white lines. In the test, mice were individually placed in the center of the arena and permitted for free explorations. After 1 min of adaptation, the behavior of each mouse was recorded for 5 min by two observers 1 m away from the open field area. Between trials, the mice were returned to their home cages in the same room, and the open field was wiped clean with a slightly damp cloth and dried prior to occupancy by another mouse. The behavioral parameters were assessed as follows:

- ambulation: the number of grids crossed in adrena during the observation period;
- rearing/leaning: the number of times the mouse stands on its hind legs;
- grooming: the number of times the mouse “washes” itself by licking, wiping, combing, or scratching any part of the body.

### Morris water maze test

Following the open-field test, the Morris water maze (MWM) task was performed as described previously<sup>7</sup> with minor modifications. The experimental apparatus consisted of a circular pool (120 cm in diameter, 60 cm in height) located in a test room with various prominent visual cues on white walls. With its inner surface painted black, the pool was filled with water to a depth of 40 cm (maintained at 25 ± 1°C). The pool was virtually divided into 4 quadrants and the platform was placed at a fixed position in the center of a quadrant. An escape platform (black), 10 cm in diameter, was submerged approximately 1.5 cm below the surface of the water and placed at the midpoint of one quadrant. Four different start points (NE, SE, SW, and NW) were equally spaced around the circumference of the pool.

Each mouse received 4 training periods per day for 5 consecutive days. For each trail, the mice were placed in the



**Figure 1.** The experimental design of the study demonstrating subchronic treatment of ursolic acid.

water facing the wall at one of the 4 possible starting points, and their latency to the platform was recorded. If a mouse did not find the escape platform within 90 s, it was given a latency score of 90 s. At the end of each session, all animals were dried and returned to their home cages. On day 6, the probe test was conducted by removing the platform and allowing each mouse to swim freely for 90 s. The time spent in the target quadrant (where the platform was located during hidden platform training) and the swimming path length by the mouse were measured.

### Brain homogenate

For biochemical studies, animals were deeply anesthetized and sacrificed. The hippocampus was promptly isolated after the animals were sacrificed. The hippocampus was weighed, homogenized in 9 volumes (1:9 w/v) of cold saline to prepare a 10% tissue homogenate in an ice bath, and centrifuged at 1699  $\times g$  for 15 min (Eppendorf Centrifuge 5804R, Hamburg, Germany). Then, the supernatant was collected for the subsequent determination of oxidative alterations in brain.

### Determination of oxidative alterations in hippocampus

The contents of malondialdehyde (MDA) and glutathione (GSH) in the hippocampus were measured as per their corresponding kits (Jiancheng Bio-tech, Nanjing, China) as per the manufacturer's instructions. Measurement of all assays described above was conducted in triplicate. Protein contents were determined by the bicinchoninic assay.

### Determination of TNF- $\alpha$ and IL-1 $\beta$ levels in hippocampus

The hippocampus was homogenized in ice-cold normal saline and then centrifuged 12,000 rpm for 5 min at 4°C. TNF- $\alpha$  and IL-1 $\beta$  levels were respectively measured using an enzyme-linked immunosorbent assay with mouse TNF- $\alpha$ /IL-1 $\beta$  kits (R&D Systems) as per the manufacturer's instructions.

### Statistical analysis

All statistical analyses were performed using SPSS software, version 13.0 (SPSS Inc., Chicago, IL, USA). Values are expressed as mean  $\pm$  SEM. For the MWM task, data were analyzed by Kruskal-Wallis non-parametric analysis of variance (ANOVA). If the results were significant, the intergroup variance was assessed by Tukey's *post hoc* test. For the biochemical and neuroendocrine assays, differences between groups were analyzed by one-way ANOVA followed by the Dunnett's *post hoc* test. The results were considered statistically significant if  $p < 0.05$ .

## RESULTS

### Effect of UA on the behavioral alterations induced by A $\beta_{25-35}$ in the open-field test

Figure 2 demonstrated that A $\beta_{25-35}$  injection produced behavioral effects in mice. These behavioral alterations included a reduction in crossings ( $p < 0.001$ ), rearings ( $p < 0.01$ , and groomings ( $p < 0.05$ ). UA treatment markedly suppressed the decrease in open-field activity in A $\beta_{25-35}$ -treated mice ( $p < 0.05$  or  $p < 0.01$ ). There was no significant difference in open-field activity between the distilled water-injected mice and distilled water/UA group ( $p > 0.05$ ).

### Effect of UA on the behavioral alterations induced by A $\beta_{25-35}$ in the MWM task

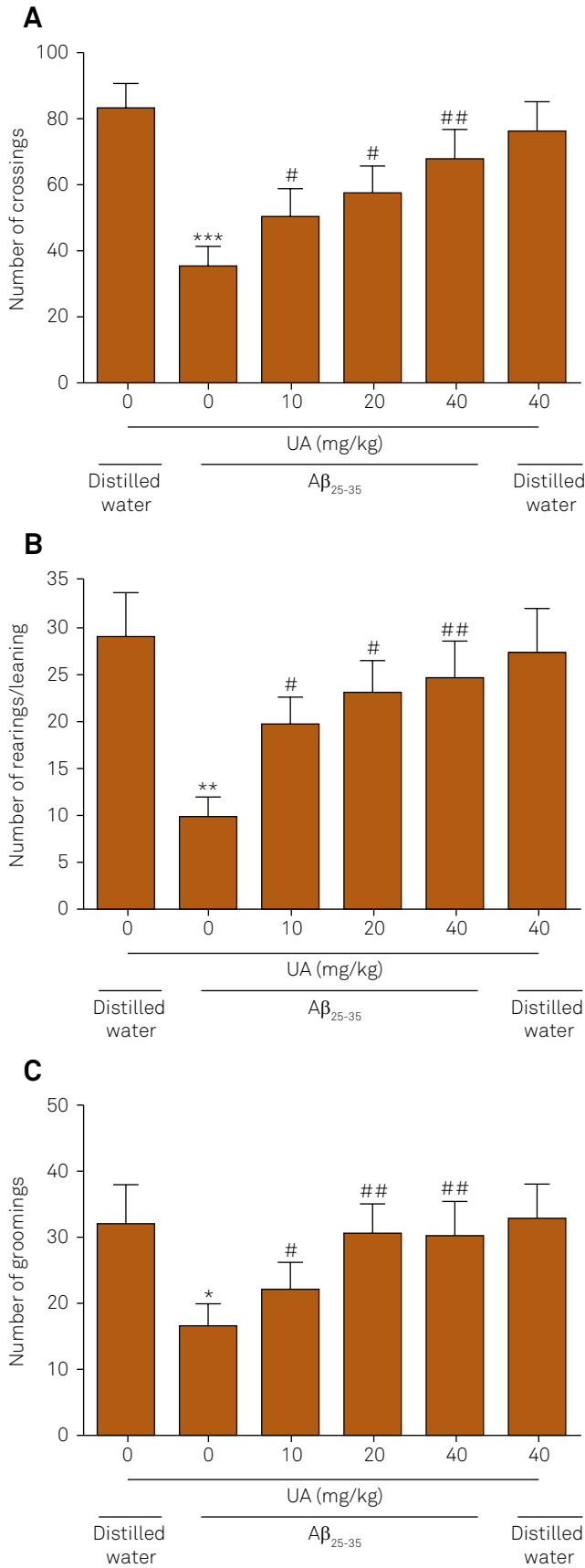
The MWM task is one of the most widely used behavioral tasks for the assessment of learning and memory in mice. The mean escape latency and swimming path length did not differ between any of the groups on the first day of testing in the MWM task ( $p < 0.05$ ; Figure 3). From the day 2 onwards, the mean latency and swimming path length in the A $\beta_{25-35}$  group were prolonged significantly compared with those of the vehicle group ( $p < 0.05$ ,  $p < 0.01$ , or  $p < 0.05$  or  $p < 0.001$ ). UA treatment significantly decreased the escape latency and swimming path length in A $\beta_{25-35}$  treatment mice ( $p < 0.05$  or  $p < 0.01$ ). However, there was no significant difference between distilled water-injected mice and distilled water/UA mice in the time taken to find the hidden platform or in swimming path length. In the probe test, a significantly shorter stay in the platform quadrant was observed in the A $\beta_{25-35}$  group, which was significantly suppressed by UA treatment ( $p < 0.05$ ). However, there was no significant difference in the time UA-treated mice spent in the platform quadrant when compared with the vehicle group ( $p > 0.05$ ).

### Effect of UA on the MDA and GSH levels in the hippocampus of A $\beta_{25-35}$ -injected mice

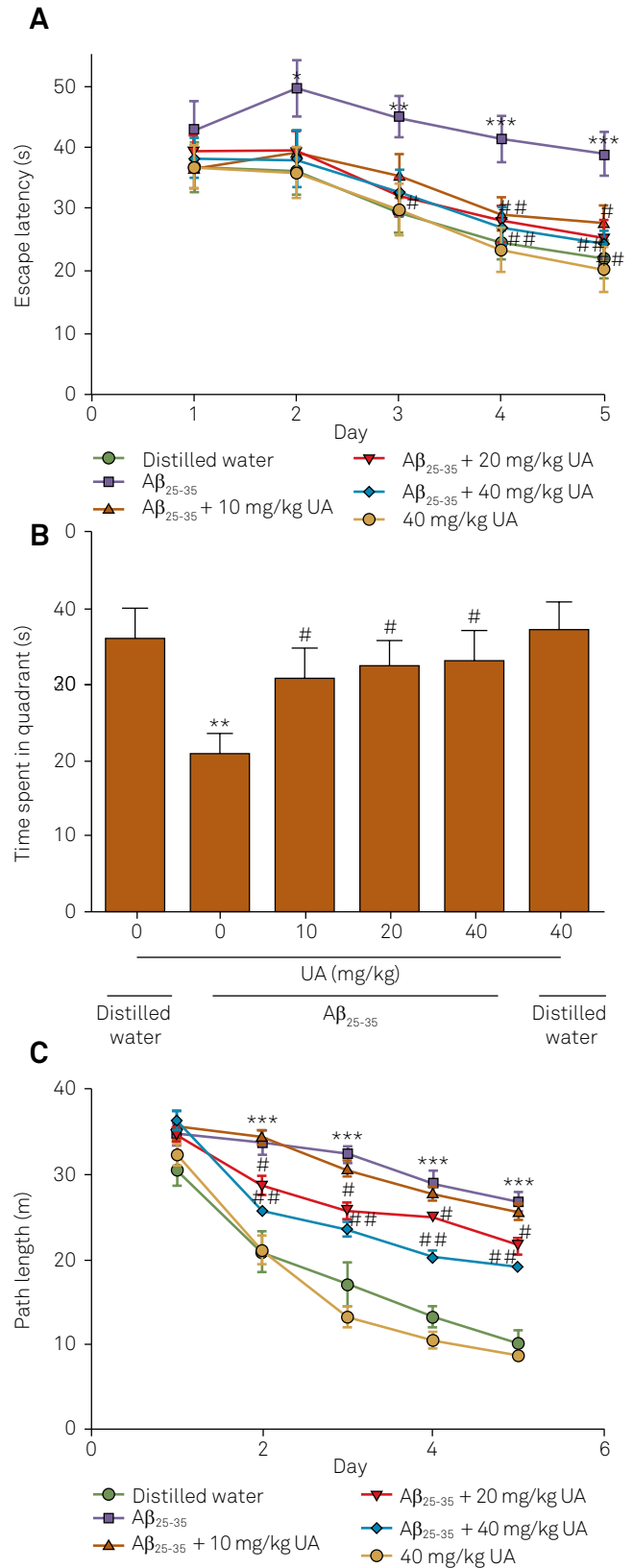
A $\beta_{25-35}$ -treated mice demonstrated a significant increase in MDA level in the hippocampus, when compared with the distilled water group ( $p < 0.05$ ; Figure 4). Similarly, the GSH levels in hippocampus of the A $\beta_{25-35}$  group were significantly increased compared with those of distilled water-injected mice, while the decrease in GSH content in the hippocampus was markedly suppressed by UA treatment ( $p < 0.05$ ). In addition, UA treatment significantly suppressed the increase in the MDA level in the hippocampus when compared with the distilled water group ( $p < 0.05$ ). However, no significant oxidative alterations were observed between the distilled water group and the distilled water/UA group ( $p > 0.05$ ).

### Effect of UA on the TNF- $\alpha$ and IL-1 $\beta$ levels in the hippocampus of A $\beta_{25-35}$ -injected mice

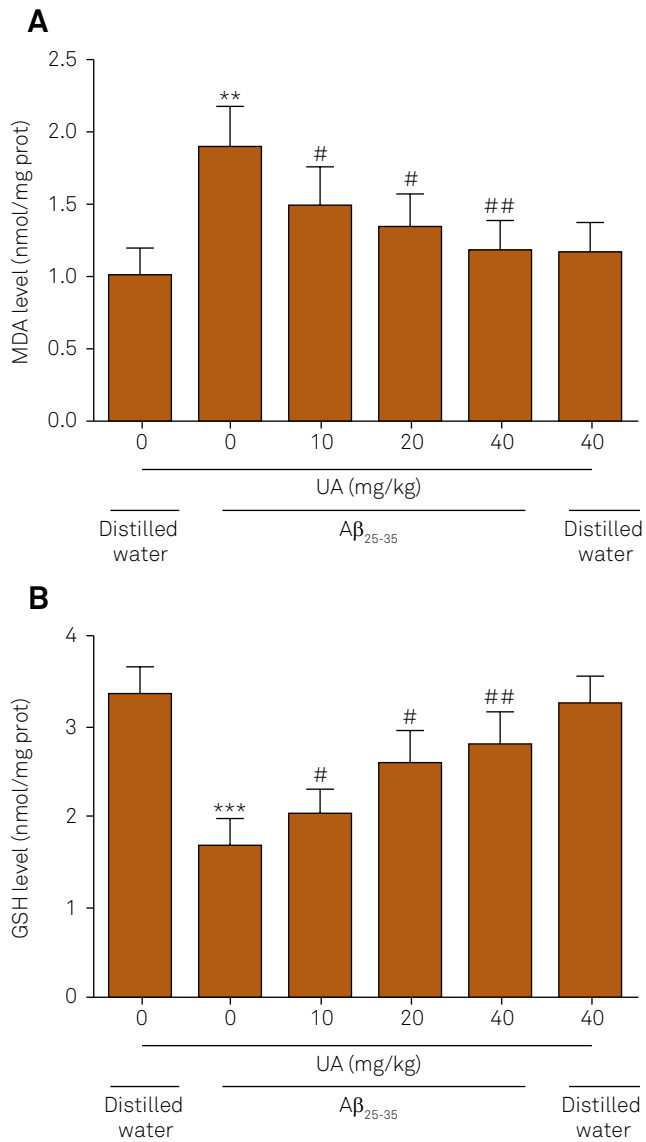
A significant increase in TNF- $\alpha$  and IL-1 $\beta$  levels was found in the hippocampus of A $\beta_{25-35}$ -treated



**Figure 2.** Number of line crossings (A), rearings (B), and groomings (C) of mice in the open-field test. Data are expressed as mean ± SEM (n = 8). \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 versus distilled water-injected mice; #p < 0.05, ##p < 0.01 versus only Aβ<sub>25-35</sub>-injected mice.



**Figure 3.** Behavioral performance of mice in the Morris water maze task. (A) Escape latency apparent during the training and probe sessions. (B) Time spent in target quadrant during the training and probe sessions. (C) Path length during the training and probe sessions. Data are expressed as mean ± SEM (n = 8). \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 versus only Aβ<sub>25-35</sub>-injected mice; #p < 0.05, ##p < 0.01 versus distilled water-injected mice.

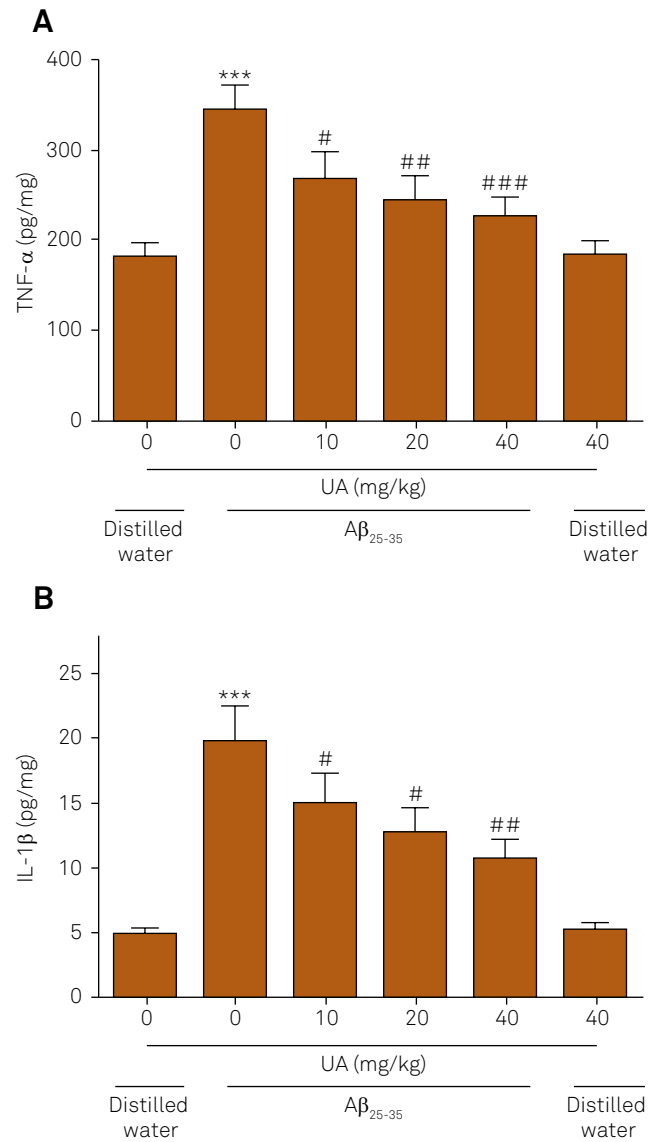


**Figure 4.** Effect of ursolic acid on MDA (A) and GSH (B) levels in the hippocampus of mice. Each value is expressed as mean  $\pm$  SEM (n = 8). \*p < 0.05, \*\*\*p < 0.001 versus distilled water-injected mice; #p < 0.05, ##p < 0.01 versus only A $\beta_{25-35}$ -injected mice.

mice compared with those of the distilled water group (p < 0.001) (Figure 5). UA treatment significantly attenuated the alterations induced by A $\beta_{25-35}$  in the hippocampus (p < 0.05, p < 0.01, or p < 0.001). However, UA did not significantly affect the TNF- $\alpha$  and IL-1 $\beta$  levels in the hippocampus of vehicle-treated mice (p > 0.05).

## DISCUSSION

Here, we demonstrated that UA produced a neuroprotective effect against A $\beta_{25-35}$ -induced neurotoxicity and memory impairment in mice. UA treatment attenuated A $\beta_{25-35}$ -induced impairment of memory in the MWM task.



**Figure 5.** Effect of ursolic acid on the TNF- $\alpha$  (A) and IL-1 $\beta$  (B) levels in the hippocampus of mice. Each value is expressed as mean  $\pm$  SEM (n = 8). \*\*\*p < 0.001 versus distilled water-injected mice; #p < 0.05, ##p < 0.01, ###p < 0.001 versus only A $\beta_{25-35}$ -injected mice.

Moreover, we demonstrated that UA suppressed the accumulation of lipid peroxide (MDA) and the reduction in GSH in the hippocampus induced by A $\beta_{25-35}$ . Furthermore, our data indicated that UA treatment is capable of suppressing the A $\beta_{25-35}$ -induced upregulation of TNF- $\alpha$  and IL-1 $\beta$  levels in the hippocampus. Collectively, these results suggest that repeated administration of UA attenuates cognitive deficits by regulating oxidative stress and inflammation in the brain.

Oxidative stress and inflammatory responses in the brain contribute to A $\beta$ -induced neuronal toxicity and cognitive deficits<sup>6,7</sup>. A $\beta_{25-35}$  is a proteolytic fragment of A $\beta$  with high neurotoxicity that is produced in the brains of AD patients<sup>13,14</sup>. Therefore, the A $\beta_{25-35}$ -treated mouse serves as a model has been used to study A $\beta$ -induced neurotoxicity and memory

impairments<sup>6,7,15</sup>. Here, the effect of UA on A $\beta$ -induced neurotoxicity was assessed in A $\beta_{25-35}$ -injected mice. Our data in the open-field test suggested that injection of A $\beta_{25-35}$  could cause motor abnormalities and deficits of novelty-induced behavioral impairment. Furthermore, A $\beta_{25-35}$ -injected mice displayed learning and memory deficits, consistent with previous reports<sup>16</sup>.

Drugs isolated from traditional medicinal plants may provide a promising therapy for brain injuries caused by oxidative stress. UA (3 $\beta$ -hydroxy-urs-12-en-28-oic acid) is a lipophilic, pentacyclic triterpenoid compound. UA has a significant anti-oxidant and -inflammatory effect both *in vivo* and *in vitro*<sup>17,18</sup>. UA also exerts a neuroprotective effect against D-galactose-induced cognition deficits in mice via the reduction of its anti-oxidant properties<sup>19</sup>. Moreover, UA suppresses NF- $\kappa$ B signaling, thus attenuating the inflammatory responses of PC12 cells<sup>18</sup>. With this background, the aim of the present study was to evaluate whether UA has a protective effect against cognitive impairment induced by A $\beta_{25-35}$ -injected in mice. Here, UA significantly suppressed the behavioral alterations induced by A $\beta_{25-35}$ . However, no significant difference in behavior was found during either the open-field test or the MWM task in UA/vehicle-treated mice as compared with the vehicle group. These results suggested that UA could reverse impaired cognition induced by A $\beta_{25-35}$  in mice.

Oxidative stress plays a critical role in the pathogenicity and development of AD and mild cognitive impairment<sup>6</sup>. Lipid peroxidation has been proposed to be one of the major outcomes of free radical-mediated injury that directly damages membranes and generates numerous secondary products, including aldehydes such as MDA<sup>6</sup>. Furthermore, accumulation of lipid peroxidation products is found in multiple regions, such as amygdala, hippocampus, and parahippocampal gyrus in the AD brain<sup>20</sup>. MDA is the most abundant individual aldehyde arising from lipid peroxidation and could therefore be considered a marker of lipid peroxidation. In this study, A $\beta_{25-35}$  administration increased MDA levels in

the hippocampus, suggesting that it caused lipid peroxidation. Treatment with UA suppressed the accumulation of MDA induced by A $\beta_{25-35}$ , which suggests a beneficial effect of UA treatment in reducing lipid peroxidation caused by A $\beta_{25-35}$ . These findings suggested that protection from lipid peroxidation is involved in the improving the effects of UA on cognitive deficits.

In addition, A $\beta$  peptide impairs the anti-oxidative defenses in the brain, thereby possibly contributing to the pathogenesis of AD<sup>21</sup>. GSH is the most abundant intracellular anti-oxidant, the dysregulation of which is involved in the pathogenesis of many neurodegenerative diseases. Here, the reduction of GSH levels induced by A $\beta_{25-35}$  was suppressed by UA treatment. Thus, the protective effect of UA on A $\beta_{25-35}$ -induced cognitive deficit involves the activation of antioxidative defenses in the brain. Moreover, the antioxidant activity of UA has been studied previously<sup>7,22</sup>. Firstly, UA suppresses O<sub>2</sub><sup>-</sup> generation in xanthine-xanthine oxidase assay system<sup>23</sup>. Furthermore, UA can strengthen anti-oxidative defense against free radicals induced by D-galactose in mice<sup>7</sup>.

In addition, inflammation occurs in pathologically vulnerable regions of the AD brain<sup>24</sup>. Considerable evidence gained over the past decade has indicated that neuroinflammation is associated with AD pathology<sup>25</sup>. Moreover, treatment of microglia or astrocytes with A $\beta$  leads to the release of inflammatory factors, which may contribute to neuronal cell damage and eventual death<sup>26,27</sup>. Here, increased levels of inflammatory cytokines, such as TNF- $\alpha$  and IL-1 $\beta$  in the hippocampus were observed in A $\beta_{25-35}$ -treated mice, consistent with previous reports<sup>28,29</sup>. However, these alterations in TNF- $\alpha$  and IL-1 $\beta$  in the hippocampus were suppressed by UA treatment.

In summary, the present results demonstrate that UA can alleviate the memory impairments induced by A $\beta_{25-35}$  in mice. The effect of UA may be attributed to the prevention of oxidative stress and the inflammatory response induced by A $\beta_{25-35}$  in the hippocampus.

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# Erratum

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