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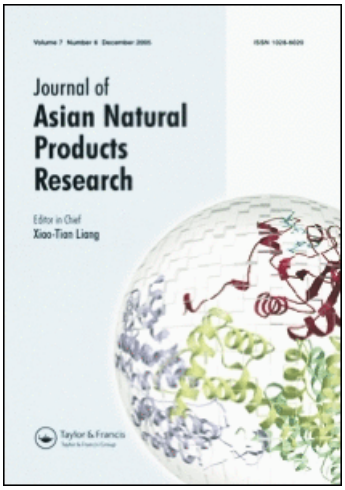

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Protective effects of xanthoceraside on learning and memory impairment induced by $A\beta_{25-35}$ in mice

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This study examined the effects of xanthoceraside (**1**) on learning and memory impairment induced in mice by intracerebroventricular injection of aggregated peptide β-amyloid 25–35 ($A\beta_{25-35}$). Learning and memory functions in mice were examined using step-through, Y-maze and water maze tests. Administration of **1** reduced the number of errors and prolonged latency in the step-through test in mice impaired by $A\beta_{25-35}$. Likewise, latency to find the terminal platform was decreased and the number of right reflects was increased in the water maze test, and the percentage of alternation behaviors in the Y-maze test was increased. Biochemical studies showed that decreased activities of superoxide dismutase, glutathione peroxidase, and acetylcholinesterase, and increased content of malondialdehyde in mice impaired by $A\beta_{25-35}$ were significantly ameliorated by administration of **1**. The present results suggest that **1** may provide a potential treatment strategy for Alzheimer's disease.

Keywords: xanthoceraside; $A\beta_{25-35}$; Alzheimer's disease; learning and memory

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1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder associated with global mental dysfunction and impairment in cognitive faculties [1]. Characteristic pathological features include intracellular neurofibrillary tangles, extracellular senile plaques, and neuronal loss in selective brain regions [2]. Epidemiological surveys have shown that AD is estimated to affect approximately 24 million people worldwide today and that this amount will double every 20 years to 42 million by 2020 [3]. AD is thus becoming a greater medical and social problem worldwide with the aging of the population.

The amyloid cascade hypothesis postulates that amyloid β (Aβ) peptide, the major component of senile plaques, plays a causative role in the development and progression of AD [4]. Aβ deposition may be the common path that leads all the factors to AD. Aβ triggers many kinds of immune/inflammatory responses and neurotoxicity cascade reactions, which cause widespread neuron degeneration and even neuron death, leading to memory and cognitive impairment [5,6].

Xanthoceras sorbifolia Bunge (EXB) is a solo-type *Xanthoceras* belonging to the Sapindaceae family, specifically growing in China and containing numerous

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resources [7]. Previous studies have shown that extract of the husk of EXB could significantly improve learning and memory dysfunction induced in rats by bilateral occlusion of the common carotid arteries and in mice by intracerebroventricular (i.c.v.) injection of the aggregated peptide β-amyloid 25–35 ($A\beta_{25-35}$) and impaired by D-galactose and $A\beta_{25-35}$ in rats [8,9]. The present study examined xanthoceraside (**1**), a type of triterpene saponin monomer compound extracted from EXB (Figure 1). Both EXB and **1** extracted from the equivalent EXB have been demonstrated to significantly improve memory acquisition impairment induced by scopolamine and memory consolidation impairment induced by sodium nitrite, indicating that **1** is one of the most important elements in EXB for improving learning and memory deficits [10]. The present study therefore sought to confirm the usefulness of **1** against Aβ-induced neurotoxicity, and investigated whether **1** can improve Aβ-induced learning and memory deficits in mice.

2. Results and discussion

2.1 Learning and memory tests

2.1.1 Step-through test

We evaluated the effects of **1** on long-term memory 11 days after $A\beta_{25-35}$ injection in the step-through test. $A\beta_{25-35}$-model mice showed significantly shorter latency and higher number of errors than sham-operated mice (Table 1). Treatment with **1** (0.16 or 0.32 mg/kg) or huperzine significantly extended the latency and reduced the number of errors compared with the $A\beta_{25-35}$-model mice (Table 1).

2.1.2 Y-maze test

We evaluated the effects of **1** on short-term memory 13 days after $A\beta_{25-35}$ injection in the Y-maze test. No significant difference in the total number of arm entries was seen between groups, indicating that $A\beta_{25-35}$, **1**, and huperzine did not affect spontaneous locomotor activity in mice. $A\beta_{25-35}$-model mice showed significantly reduced spontaneous alternation behavior compared with sham-operated mice

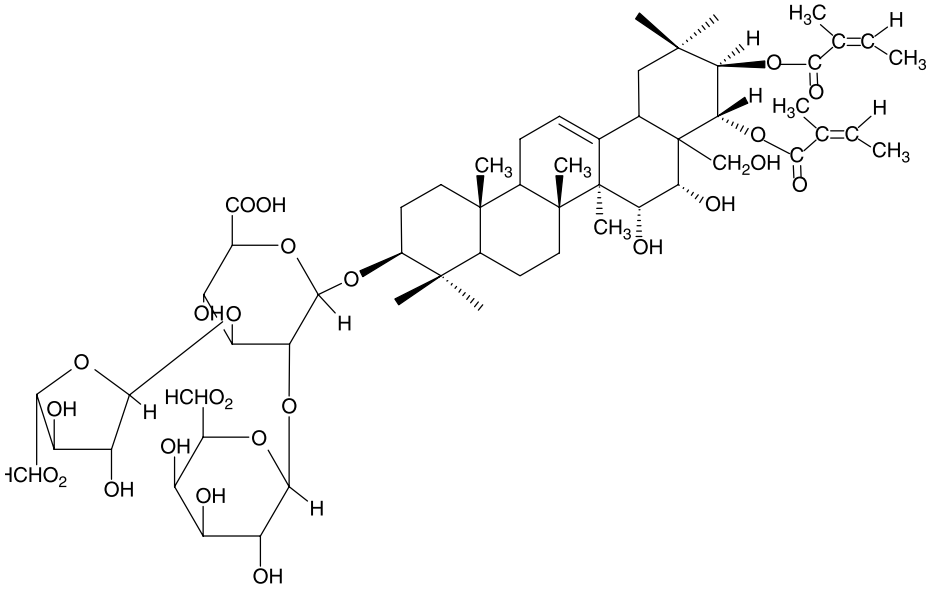

Figure 1. Chemical structure of **1**.

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Table 1. Effect of **1** in mice with i.c.v. $A\beta_{25-35}$ in the step-through test.

Group	Dose (mg/kg)	Latency (s)	Errors
Sham-operated	–	267.2 ± 12.5	1.6 ± 0.2
Model	–	195.1 ± 21.1$^{\#\#}$	2.6 ± 0.2$^{\#}$
1	0.08	218.6 ± 25.0	2.1 ± 0.2
	0.16	263.5 ± 12.2*	1.7 ± 0.3*
	0.32	274.9 ± 11.4**	1.5 ± 0.2*
Huperzine	0.039	274.5 ± 16.9**	1.7 ± 0.2*

Notes: $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with the sham-operated group; $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the model group. $n = 16–20$.

($P < 0.05$; Table 2). Treatment with **1** (0.16 or 0.32 mg/kg) or huperzine attenuated the impairment of spontaneous alternation behavior in $A\beta_{25-35}$-model mice in a dose-dependent manner ($P < 0.05$; Table 2).

2.1.3 *Water maze test*

We evaluated the effects of **1** on spatial recognition memory 14 days after $A\beta_{25-35}$ injection in the water maze test. Starting from day 2 of the water maze test, swimming time to the safe platform was significantly prolonged in Aβ-model mice (Figure 2(A)). From day 3 of the water maze test, the number of right reflects of Aβ-model mice was significantly reduced compared with the sham-operated group (Figure 2(B)). Treatment with **1** caused a dose-dependent reversal of impairments in spatial recognition memory for $A\beta_{25-35}$-model mice. The huperzine group exhibited a pattern similar to the **1** groups in terms of the swimming time and the number of right reflects (Figure 2(A),(B)).

2.2 *Biochemical assays*

The levels of malondialdehyde (MDA) were significantly higher in the Aβ group than in the sham-operated group, and the activity of superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) was significantly decreased after i.c.v. Aβ compared to the sham-operated group. Treatment with **1** reversed these effects (Table 3). Compared to the sham-operated group, acetylcholinesterase (AChE) activity in mouse brains was significantly reduced after i.c.v. injection with aggregated $A\beta_{25-35}$. However, treatment with **1** (0.32 mg/kg) prevented the decrease in AChE activity in the brain of $A\beta_{25-35}$-model mice. Huperzine did not significantly change the levels of AChE (Table 3).

2.3 *Histology*

The Nissl-stained slices of mouse brain showed intense, rich Nissl bodies in the sham-operated group, with clearly depicted axons and no obvious abnormalities in

Table 2. Effect of **1** on mice with i.c.v. $A\beta_{25-35}$ in the Y-maze test.

Group	Dose (mg/kg)	Entries	Alternation behavior (%)
Sham-operated	–	33.0 ± 3.0	72.8 ± 3.5
Model	–	30.1 ± 1.5	59.5 ± 3.9$^{\#}$
1	0.08	35.3 ± 3.2	64.7 ± 1.9
	0.16	36.0 ± 2.9	71.1 ± 2.2*
	0.32	37.7 ± 2.6	72.2 ± 1.8*
Huperzine	0.039	33.0 ± 2.9	75.5 ± 2.6*

Notes: $^{\#}P < 0.05$ compared with the sham-operated group; $^{*}P < 0.05$ compared with the model group. $n = 10$.

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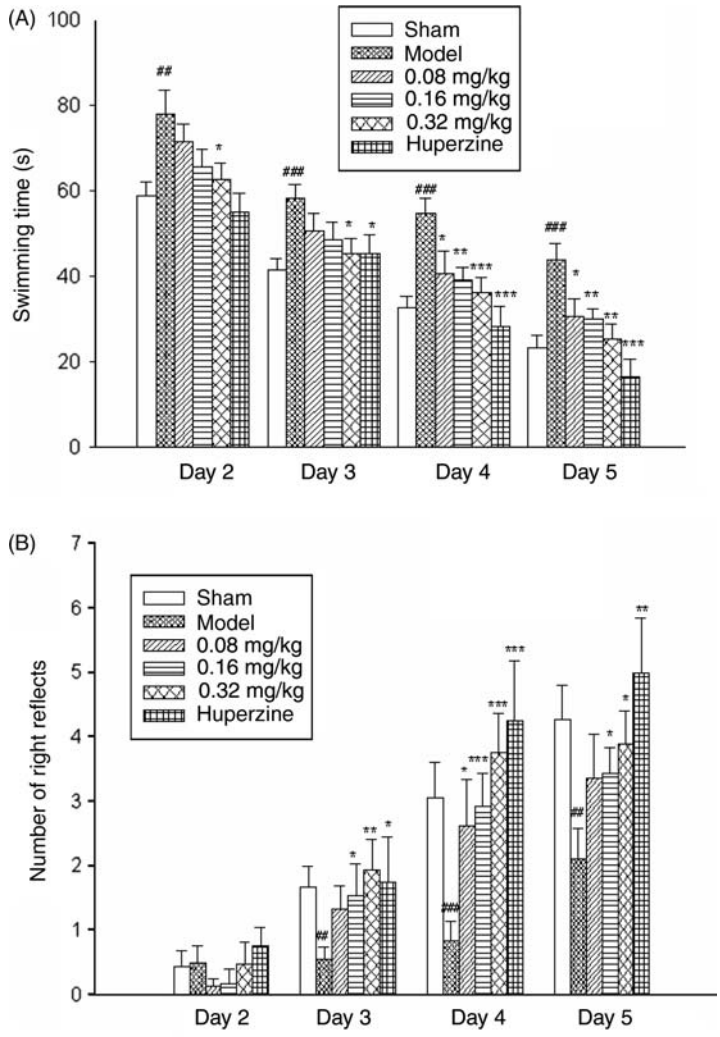

Figure 2. Effect of **1** on mice with i.c.v. $A\beta_{25-35}$ in the water maze test. (A) Swimming time. (B) Number of right reflects. Results are expressed as mean ± SEM ($n = 15–20$). $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared with the sham-operated group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the model group.

Table 3. Influence of **1** on biochemical parameters in the brains of mice with i.c.v. $A\beta_{25-35}$.

Group	Dose (mg/kg)	MDA (nmol/mg protein)	SOD (U/mg protein)	GSH-PX (U/mg protein)	AChE (U/mg protein)
Sham-operated	–	21.0 ± 1.6	108.2 ± 9.5	11.2 ± 1.0	4.1 ± 0.3
Model	–	35.6 ± 5.5$^{\#}$	83.3 ± 3.5$^{\#}$	7.9 ± 0.4$^{\#\#}$	3.2 ± 0.2$^{\#}$
1	0.08	23.3 ± 3.8	85.7 ± 7.4	6.8 ± 0.7	3.5 ± 0.7
	0.16	20.7 ± 2.1*	83.4 ± 3.2	7.2 ± 0.7	4.0 ± 0.1
	0.32	19.7 ± 2.3*	97.7 ± 4.0*	10.2 ± 1.1*	4.3 ± 0.3*
Huperzine	0.039	18.8 ± 2.0*	97.2 ± 2.9*	9.1 ± 0.9	3.6 ± 0.4

Notes: $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with the sham-operated group; $^{*}P < 0.05$ compared with the model group. $n = 10–12$.

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neurons. In the Aβ-model group, Nissl bodies were significantly reduced, and neurons were swollen or missing in the region of the hippocampus CA1 formation and in the cerebral cortical layer. Treatment with **1** or huperzine protected neurons from Aβ-induced damage (Figure 3).

2.4 Discussion

According to the Aβ cascade hypothesis, Aβ represents a potential cause leading to cognitive impairment in AD patients [11]. Injection of $A\beta_{25-35}$ into areas related to learning/memory in the brain (e.g. cerebral ventricles and hippocampus) has been confirmed to lead to significant declines in learning and memory ability [12–14]. Maurice *et al.* reported that mice show missing cerebral neurons and amyloid deposition after i.c.v. injection of aggregated $A\beta_{25-35}$. Such mice also show significant cognitive impairment in the behavioral tests [15]. From recent research, reducing the formation and deposition of Aβ appears to offer a promising direction for future research in AD [16].

Therefore, in the present study, we examined the effect of **1** on memory impairment induced by $A\beta_{25-35}$ in mice. Compound **1** prevented $A\beta_{25-35}$-induced long-term, short-term, and spatial recognition memory impairment in the step-through, Y-maze, and water maze tests, respectively. The mechanisms to reverse learning and memory disorders may be related to the reversal of nerve cell toxicity caused by $A\beta_{25-35}$.

According to the oxidative stress hypothesis in AD, the inclusion of Aβ into the bilayer lipid membrane of nerve cells leads to increases in reactive oxygen species, and decreases in a series of anti-oxidant enzymes and anti-oxidants. This destroys the balance of elimination and the formation of free radicals, resulting in lipid peroxidation and protein oxidation [17]. Increased free radicals may also promote the amyloid accumulation and enhance the neuron damage caused by Aβ. At the same time, the loss of membrane integrity results in cell dysfunction, such as calcium overload and disorder of signal transduction, and activation of nuclear transcription factors and apoptotic pathways, ultimately leading to nerve cell death [18].

After mice receive i.c.v. injection of $A\beta_{25-35}$, more peroxide is reportedly generated in the brain, weakening anti-oxidant abilities [19]. The present results were consistent with the existing literature. Compared to the sham-operated group, the brains of AD model mice with i.c.v. injection of $A\beta_{25-35}$ showed the accumulation of lipid peroxide (MDA), and decrease in anti-oxidant enzymes (SOD and GSH-PX). It suggests that $A\beta_{25-35}$ induces the free radical damages in neurons. Compared with AD model mice, **1** led to increased SOD, GSH-PX activity, and reduced MDA content. These findings indicate that **1** protects neurons, thereby reversing learning and memory disorders, by reducing free radicals and enhancing radical scavenging against the neurotoxicity of $A\beta_{25-35}$.

Neurons of the prosencephalon and basal ganglia are damaged earlier than other brain neurons in AD patients. After further development of the disease, about 60–90% of cholinergic neurons will be lost, resulting in lower acetylcholine (ACh) levels in the nerve fiber projection areas (cerebral cortex and hippocampus). Some studies have shown that, after injection with amyloid, mice develop learning and memory impairments due to cholinergic nervous system dysfunction in the hippocampus, including reduced release of ACh and loss of cholinergic neurons [20,21].

Nitta and Nabeshima [22] found that choline acetyltransferase (ChAT) was reduced in the forebrain cortex and hippocampus of the decapitated brain 16 days after sustained injection with $A\beta_{1-40}$, but identified no significant changes in AChE for the forebrain cortex, parietal cortex, striatum, or hippocampus.

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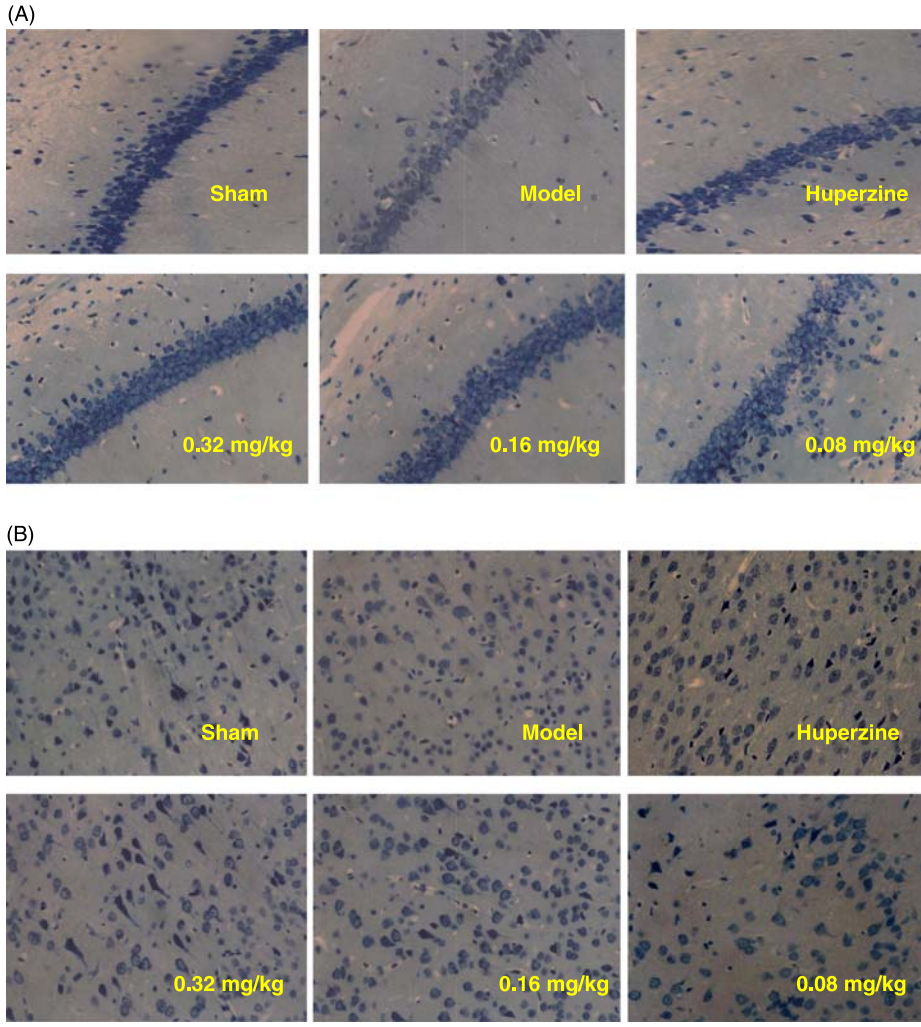

Figure 3. Influence of **1** treatment on pathological changes to (A) hippocampal CA1 formation and (B) cerebral cortical layer in mice with i.c.v. $A\beta_{25-35}$ (Nissl staining).

ChAT activity was also reported and significantly reduced in the cerebral cortex and hippocampus after injection with $A\beta_{25-35}$ compared to controls. However, AChE activity showed no significant changes [23]. Another report showed that $A\beta_{1-40}$ can impair learning and memory ability and decrease the activity of ChAT and AChE in the hippocampus and cerebral cortex in mice [17]. Dong *et al.* [24] reported significant increases in the activity of ChAT and AChE in the cerebral cortex of mice after i.c.v. injection of $A\beta_{22-35}$, and then proposed that this may represent a compensatory increase in the early stages of AD, and the increase in expression and activity of AChE may also be due to stimulation.

The present study found that, in the brains of AD model mice, AChE levels of $A\beta_{25-35}$-model mice were significantly reduced compared to the sham-operated group. This may be due to degeneration, loss of cholinergic neurons, and impairment of cholinergic function. Compared with AD model mice, **1** (0.32 mg/kg)

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significantly inhibited reduced AChE activity. This may be due to **1** that acts to reduce degeneration and prevent the loss of cholinergic neurons, and then increases AChE levels relatively.

In short, **1** can significantly reverse learning and memory impairments caused by the aggregated Aβ. In addition, **1** can not only enhance cholinergic system functions, but may also reduce the formation of free radicals, enhance radical scavenging, and act against the neurotoxicity of Aβ. Compound **1** thus offers a promising candidate for further preclinical studies aimed at the treatment of cognitive deficits in AD.

3. Materials and methods

3.1 Materials

Compound **1** was provided by the Shenyang Institute of Applied Ecology, Chinese Academy of Sciences (Shenyang, China). $A\beta_{25-35}$ was purchased from Bachem (Bubendorf, Switzerland), dissolved in distilled water at a concentration of 1 mM, and then sealed and stored at −20°C. $A\beta_{25-35}$ (1 mM) was incubated for 4 days at 37°C to transform Aβ into an 'aggregated phase'. Huperzine A (huperzine; Tailong Pharmaceutical, Zhengzhou, China) was used as the positive control drug in this study. Medicinal kits for determinations of MDA, SOD, GSH-PX, and AChE were purchased from JianCheng Bioengineering Institute (Nanjing, China). All other reagents were of chemical grade.

3.2 Animals

Healthy male Kunming mice (3–4 weeks old; body weight, 18–22 g at the start of experiments; Experimental Animal Center of Shenyang Pharmaceutical University, Shenyang, China) were used throughout the study. Animals were housed in plastic cages with a 12-h light/12-h dark cycle and controlled temperature (23 ± 1°C), and were provided with *ad libitum* access to standard laboratory food and water. Behavioral experiments were performed in a sound-attenuated and air-regulated experimental room, to which mice were habituated for ≥1 h. All animal studies were performed in strict accordance with the P.R. China legislation on the use and care of laboratory animals and with the guidelines established by the Institute for Experimental Animals at Shenyang Pharmaceutical University. Study protocols were approved by the University Committee for Animal Experiments (Certificate No. SCXK 2003-008).

3.3 Experimental groups

Mice were divided into six groups at random. The Aβ model group received light ether anesthesia and i.c.v. injection with 3 μl aggregated $A\beta_{25-35}$ (3 nmol/3 μl) according to the methods described by Maurice [15]. The Aβ+**1** (0.32 mg/kg body weight) group comprised Aβ model mice with per oral (p.o.) administration of **1** at 0.32 mg/kg. The Aβ+**1** (0.16 mg/kg) group used p.o. **1** at 0.16 mg/kg. The Aβ+**1** (0.08 mg/kg) group used p.o. **1** at 0.08 mg/kg. The Aβ+huperzine (0.039 mg/kg) group used p.o. huperzine at 0.039 mg/kg. Finally, the sham-operated group comprised normal mice with i.c.v. administration of 3 μl of distilled water. The Aβ model group and sham-operated group also received p.o. administration of distilled water. All compounds were administered at a volume of 0.1 ml/10 g body weight once a day from the day of i.c.v. Aβ until the end of behavioral testing. Behavioral tests started 10 days after the $A\beta_{25-35}$ injection, the step-through test was carried out firstly, and then the Y-maze test secondly, and then the water maze test finally.

3.4 Examination of learning and memory ability

3.4.1 Step-through test

The step-through test was performed 11 days after the $A\beta_{25-35}$ injection. The

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apparatus consisted of an illuminated compartment and a dark compartment. The size of each compartment was 15 × 10 × 11 cm with an interconnecting semicircular door (diameter, 3 cm). After the mouse was placed into the illuminated compartment with its back facing the door for 3 min to adapt to the environment, an electric current (30 V) was delivered through the copper grid floor. The mouse immediately received an electric foot shock when entering the dark compartment and so ran back to the safe, illuminated compartment through the door. Training was performed for 5 min as above, and the number of electric shocks that the mouse received in this learning record was recorded. The same test was then performed 24 h later, recording the latency to first entry into the dark compartment and the numbers of errors (entering the dark compartment) within 5 min as memory performance.

3.4.2 *Y-maze test*

The Y-maze apparatus has three wooden arms separated by 120° from each other. Each arm was 40 cm long, 12 cm high, 10 cm wide at the top, and 5 cm wide at the bottom. The mouse was placed at the terminus of one arm and allowed to move freely. Over a period of 8 min, the total number of arm entries (N) and the sequence of entries were recorded. One successful alternation was defined as the mouse entering each of the three arms consecutively. The number of successive alternations was recorded. Alternation behavior [number of alternations/$(N - 2) \times 100$ (%)] was used to reflect spatial working memory ability.

3.4.3 *Water maze test*

The water maze apparatus was a double-layer opaque plastic tank (63 × 36 × 20 cm), divided into five connecting parts by rectangular brown partitions. The tank included a starting point, terminal platform, and a ladder in the platform as a safe region. The maze was filled with water to a depth of 10 cm and the temperature was kept at 23 ± 1°C. The mouse was placed at the starting point facing to the wall, to start to find the safe platform and was allowed to stay on the terminal platform for 25 s to recognize the location before being placed in the water again. When the mouse reached the ladder at the terminal platform, the mouse was able to rest. Each mouse was trained seven times daily. If the mouse could reach the safe platform within 30 s, this was considered to represent a 'right reflect'. If it did not, it was manually led to the platform. The duration required for each mouse to reach the safe platform, the average swimming time, and the number of right reflects from days 2 to 5 was recorded.

3.5 *Biochemical analysis*

On the second day after the last water maze test, mice were quickly decapitated and the brains were dissected and kept at −80°C ready for analysis. To detect biochemical parameters, cold saline was added at a weight/volume ratio of 1:9 to make a 10% cerebral homogenate in an ice bath. The homogenate was then centrifuged at 3000 rpm for 15 min at 4°C. MDA content and activities of SOD, GSH-PX, and AChE in the brain were detected as described by the manufacturers of the test kits.

3.6 *Histological analysis*

As noted above, after behavioral tests, mice were killed. The brains were dissected out and placed in 4% formalin solution as fixative. Histopathological sections were made using Nissl staining to identify changes to hippocampus CA1 formation and central cortical layer. All histological assessments were made by an experienced histologist who was blinded to treatment conditions.

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3.7 Statistical analysis

All analyses were performed using SPSS for Windows version 13 software (SPSS Inc., Chicago, IL, USA). Data are expressed as the mean ± standard error of the mean (SEM). Statistical differences among the experimental groups were tested using one- or two-way analysis of variance, followed by Dunnett's *t*-test. Values of $P < 0.05$ were considered statistically significant.

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