



# Natural silibinin modulates amyloid precursor protein processing and amyloid- $\beta$ protein clearance in APP/PS1 mice

Dafeng Bai<sup>1</sup> · Ge Jin<sup>2</sup> · Dajun Zhang<sup>2</sup> · Lini Zhao<sup>2</sup> · Mingyue Wang<sup>2</sup> · Qiwen Zhu<sup>2</sup> · Lin Zhu<sup>2</sup> · Yan Sun<sup>2</sup> · Xuan Liu<sup>2</sup> · Xueying Chen<sup>2</sup> · Liqian Zhang<sup>2</sup> · Wenbo Li<sup>2</sup> · Yan Cui<sup>2</sup>

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## Abstract

Silibinin has been shown to attenuate cognitive dysfunction and inhibit amyloid-beta ( $A\beta$ ) aggregation in Alzheimer's disease (AD) models. However, the underlying mechanism by which silibinin improves cognition remains poorly understood. In this study, we investigated the effect of silibinin on  $\beta$ -secretase levels,  $A\beta$  enzymatic degradation, and oxidative stress in the brains of APP/PS1 mice with cognitive impairments. Oral administration of silibinin for 2 months significantly attenuated the cognitive deficits of APP/PS1 mice in the Y-maze test, novel object recognition test, and Morris water maze test. Biochemical analyses revealed that silibinin decreased  $A\beta$  deposition and the levels of soluble  $A\beta$ 1-40/1-42 in the hippocampus by downregulating APP and BACE1 and upregulating NEP in APP/PS1 mice. In addition, silibinin decreased the MDA content and increased the activities of the antioxidant enzymes CAT, SOD, and NO. Based on our findings, silibinin is a potentially promising agent for preventing AD-associated  $A\beta$  pathology.

**Keywords** Silibinin · Alzheimer's disease ·  $A\beta$  generation ·  $A\beta$  degradation · Antioxidant

## Abbreviations

$A\beta$  Amyloid-beta  
AD Alzheimer's disease

APP Amyloid precursor protein  
PS1 Presenilin-1  
BACE Beta-site APP cleaving enzyme  
NEP Neprilysin  
MDA Malondialdehyde  
CAT Catalase

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✉ Ge Jin  
jinge1026@163.com; 1009602770@qq.com

Dafeng Bai  
dafengbai@163.com

Dajun Zhang  
zhangdajun2008@126.com

Lini Zhao  
clnsyzln@163.com

Mingyue Wang  
wangmingyue01@126.com

Qiwen Zhu  
qwzhu2017@163.com

Lin Zhu  
zhulindedream@163.com

Yan Sun  
inksun@163.com

Xuan Liu  
646818415@qq.com

Xueying Chen  
1181826628@qq.com

Liqian Zhang  
xiaoqian9604com@qq.com

Wenbo Li  
1976257702@qq.com

Yan Cui  
1652575681@qq.com

<sup>1</sup> Department of Pharmacology, The Eleventh People's Hospital of Shenyang, 103 Hai Tang Street, Sujiatun District, Shenyang, Liaoning 110016, People's Republic of China

<sup>2</sup> Department of Pharmacology, Key Laboratory of Behavioral and Cognitive Neuroscience of Liaoning Province, Shenyang Medical Colleges, 146 Huanghe North Street, Yuhong District, Shenyang, Liaoning 110034, People's Republic of China

SOD	Superoxide dismutase
NO	Nitric oxide synthase
NFTs	Neurofibrillary tangles
IDE	Insulin-degrading enzyme
ICV	Intracerebroventricular
NOR	Novel object recognition
DI	Discrimination index
PI	Preferential index
MWM	Morris water maze

## Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative disease characterized by learning and memory deficits, A $\beta$  plaque deposits, neurofibrillary tangles (NFTs), and the loss of neurons [1–3]. A $\beta$  is generated upon the cleavage of amyloid precursor protein (APP) by  $\beta$ -secretase (BACE1) and  $\gamma$ -secretase [4]. A $\beta$  clearance includes enzymatic degradation and receptor-mediated pathways. Neprilysin (NEP) and insulin-degrading enzyme (IDE) are two major endopeptidases that participate in the enzymatic degradation of A $\beta$  [5].

Increasing numbers of A $\beta$  plaque deposits in the brains of patients with AD induce some pathological changes, including the formation of NFTs, glia overactivation, neuroinflammation, synapse injury, oxidative damage, mitochondrial dysfunction, neurotransmitter loss, increased intracellular calcium signaling, and excitotoxicity [6]. Therefore, a major strategy of researchers developing anti-AD drugs is to target the imbalance between A $\beta$  production and clearance. BACE1 (which is involved in the cleavage of APP), NEP and IDE (which are involved in A $\beta$  clearance) are important molecular targets in this strategy.

Oxidative stress is observed in the brains of patients with AD and AD animal models [7]. A $\beta$  induces lipid peroxidation, protein and DNA oxidation, and subsequently causes neuronal cell death [8]. Because the pathological changes occurring in AD are very complex, agents with multiple targets (antioxidants and anti-A $\beta$  drugs) may exert better pharmacological effects on AD progression.

Silibinin is a natural flavonoid derived from *Silybum marianum* (milk thistle). Silibinin exerts protective effects on cognitive deficits and oxidative damage in D-galactose-injected mice and ICV-A $\beta$  mice [9, 10]. Silibinin also decreases the surface area of A $\beta$  plaques in the brains of APP/PS1 mice [11]. However, researchers have not clearly determined whether silibinin affects A $\beta$  production and clearance. The aim of the present study was to examine the effect of silibinin on APP processing and the expression of enzymes involved in A $\beta$  clearance (APP, BACE1, NEP, and IDE).

## Materials and methods

### Animals and drug administration

Male mice carrying the HuAPP Swedish mutations (K595N/M596L) and Hu PS1 deltaE9 mutation (APP/PS1 mice,  $30 \pm 2$  g, 6 months) were procured from Beijing HFK Bioscience Co. Ltd (Beijing, China). Mice were housed on a 12-h light–dark cycle in a temperature- ( $22 \pm 2$  °C) and humidity-controlled ( $50 \pm 5\%$ ) environment, with free access to food and water. Age-matched C57BL/6 mice were used as the controls. APP/PS1 mice were randomly divided into four groups: a placebo group, 2.6 mg/kg memantine group, and 100 mg/kg and 200 mg/kg silibinin groups. Each group comprised ten animals. Mice in the treatment groups were orally administered silibinin (dissolved in 5% carboxymethyl cellulose sodium, silibinin powder purity > 96%, Green Biological Development Co., Ltd., Panjin, China) at a dose of 100 or 200 mg/kg once per day, while mice in the placebo group and C57BL/6 mice were consecutively administered an equal volume of 5% carboxymethyl cellulose sodium by gavage for 2 months from 6 to 8 months of age. All animal studies were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals (the P.R. China legislation on the use and care of laboratory animals: Permit Number: SYPU-IACUC-C2015-0831-203). Animal experiments were performed at Shenyang Pharmaceutical University.

### Y-maze test

The Y-maze was performed after 2 months of silibinin treatment as described in our previous report [12]. The maze was composed of wood with three arms. Each arm was 40 cm long, 12 cm high, and 10 cm wide. Each mouse was placed at the end of one arm and allowed to move freely through the maze for 5 min. The total number of arm entries was recorded. Alternation was defined as the successful consecutive entry of the mouse into each of the three arms. The alternation behavior (%) was calculated using the equation [number of successful alternations/(total number of arms entries – 2)  $\times$  100].

### Novel object recognition test

The novel object recognition (NOR) test was performed after the Y-maze test as described in our previous report [12]. Briefly, the test consisted of three phases: habituation, training, and retention. The apparatus was a square wooden box (50  $\times$  50  $\times$  15 cm, length  $\times$  width  $\times$  height). In the habituation phase, each mouse was placed in the empty box for

5 min per day for 2 days to become familiar with the arena. In training phase, two identical objects, A1 and A2, were placed in a symmetric position from the center of the arena. Each mouse was placed in the box and allowed to explore the objects for 5 min. Exploratory behavior was defined as directing the nose toward the object at a distance of less than 2 cm and/or touching the object with the nose. In the retention phase, the familiar object (A2) was replaced with a novel object (B). After a 1-h intertrial interval, the mice were returned to the box and allowed to explore the objects for 5 min. The objects and box were cleaned with alcohol (70%) to eliminate olfactory cues. The time the mice spent exploring each object (s) in the trials was recorded. The discrimination index (DI) was calculated with the following equation:  $DI = (tB - tA1)/(tA1 + tB)$ . The preferential index (PI) was calculated as [time spent exploring novel object/total exploration time].

### Morris water maze test

The Morris water maze (MWM) test was performed after the novel object recognition test as described in our previous report [12]. The apparatus was a black stainless-steel circular pool (100 cm in diameter and 50 cm deep) filled with water ( $25 \pm 1$  °C and 30 cm deep). A hidden circular platform (10 cm in diameter placed 1 cm below the water surface) was located in one of the four equal quadrants. Animal adapted to the water maze for the first two days and then carried out the experiments for 6 consecutive days. Mice were trained twice a day for five consecutive days with an inter-trial interval of 2 h. Mice were individually placed into the water facing the wall and were allowed 60 s to find the hidden platform. If the mouse failed to find the platform, the experimenter placed the mouse on the platform for 10 s. The platform was removed on the sixth day; mice were individually administered a probe test. The mouse was placed in the water at a random site. A computer system (equipped with a video camera) automatically captured the escape latency (time spent in the target quadrant), swimming distance, and swimming speed for 60 s. After the MWM test, mice were decapitated under chloral hydrate anesthesia, and the cortex and hippocampus were dissected and stored at  $-80$  °C.

### ELISA

Hippocampal tissues were homogenized in ice-cold TBS (containing 2 mM EDTA) supplemented with protease inhibitors, followed by centrifugation at  $300,000 \times g$  for 20 min. Supernatants were collected and soluble A $\beta$ 1-40 and A $\beta$ 1-42 levels were analyzed using ELISA kits (Bangyi, Biotechnology Co., Ltd., Shanghai, China). The absorbance was measured at 450 nm using an ELISA reader (Thermo Fisher Scientific, USA).

### Estimation of the oxidative stress level induced by silibinin in APP/PS1 mice

Cortical tissues were homogenized in 0.1 M PBS and centrifuged separately at  $3000 \times g$  for 10 min at 4 °C. The contents of MDA, NO, SOD, and CAT were measured in cortical extracts in ice-cold cell lysis buffer using assay kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's protocol.

### Immunohistochemistry

Anesthetized mice were transcranially perfused with ice-cold PBS, followed by 4% paraformaldehyde in PBS. Brains were removed and fixed with 4% paraformaldehyde at 4 °C overnight and embedded in paraffin. Coronal sections (4  $\mu$ m thickness) were cut with a microtome. Sections were incubated with rabbit anti-A $\beta$  primary antibodies (1:200) overnight at 4 °C. After washes, sections were incubated with a biotinylated secondary antibody for 1 h at room temperature. The staining process was performed in accordance with a modified ABC immunohistochemistry procedure. Images were acquired with an inverted microscope.

### Western-blot analysis

The hippocampus was homogenized in 400  $\mu$ l of ice-cold extraction buffer containing phosphatase and protease inhibitors. A BCA Protein Assay Kit (BOSTER Biological Technology, China) was used to determine the protein concentration. The following antibodies were used in this study: anti-APP (1:800), anti-BACE1 (1:800), anti-NEP (1:800), anti-IDE (1:800) and anti- $\beta$ -actin (1:1000), anti-mouse IgG and anti-rabbit IgG (1:6000, Santa Cruz, USA). Equal amounts of protein (30  $\mu$ g) were separated on 8–15% SDS-PAGE gels, transferred to PVDF membranes (Millipore, USA), blocked with 5% skim milk, and incubated with primary antibodies overnight at 4 °C. The proteins were detected with secondary antibodies. Protein bands were visualized with an ECL Western-blot kit (Kangwei Biotechnology, China). The intensity was quantified by densitometry using Quantity One 4.6.2 software (Bio-Rad, USA).

### Statistical analysis

Data are presented as means  $\pm$  SEM. Statistical significance was determined using one-way or two-way ANOVA, followed by Fisher's least significant difference (LSD) multiple comparison test;  $p < 0.05$  was considered statistically significant. The data were analyzed with SPSS 17.0 software.

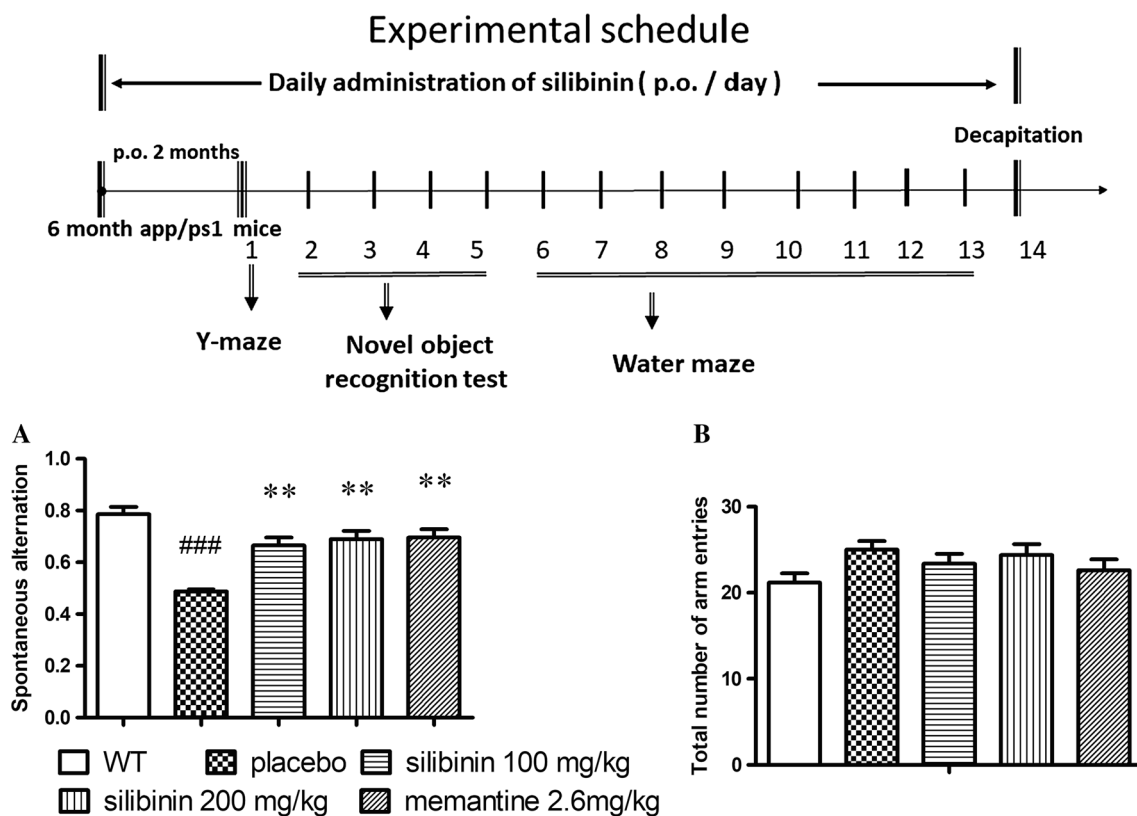
## Results

### Effects of silibinin on the impairment of spontaneous alternation behavior in the Y-maze in APP/PS1 mice

The effect of silibinin on spontaneous alternation behaviors was evaluated using the Y-maze test. As shown in Fig. 1, APP/PS1 mice showed a significant impairment in spontaneous alternation behavior compared with the WT mice ( $p < 0.05$ , Fig. 1a). Treatment with silibinin (100 and 200 mg/kg) for 2 months significantly increased spontaneous alternation behaviors ( $p < 0.05$ , Fig. 1a). The memantine (2.6 mg/kg) group was used as a positive control, and this treatment significantly alleviated the impairments. No significant differences in the total number of arm entries were observed among the groups (Fig. 1b).

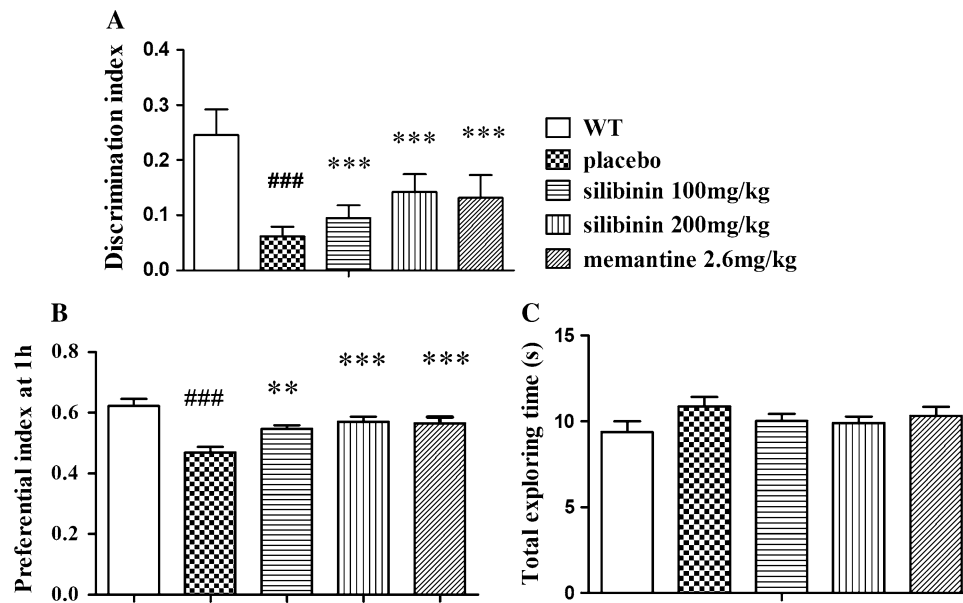
### Effects of silibinin on visual recognition impairment in the novel object recognition test in APP/PS1 mice

The NOR test was employed to evaluate visual recognition impairments. In the training phase, the mice spent an equal amount of time exploring the two identical objects (A1 and A2), indicating the absence of a biased exploratory preference. In the 1-h retention phase, when object A2 was replaced with the novel object B, the DI and PI of APP/PS1 mice were significantly decreased compared with the WT mice ( $p < 0.01$ , Fig. 2a, b). Treatment with silibinin at the doses of 100 or 200 mg/kg for 2 months significantly improved the discrimination index and exploratory preference index ( $p < 0.05$ , Fig. 2). The memantine group showed significant improvements compared with the placebo group. No significant differences in the total exploratory time were observed among the groups (Fig. 2c). All data were analyzed using one-way ANOVA.



**Fig. 1** Silibinin increases spontaneous alternation behavior in the Y-maze effects of silibinin on learning and memory deficits of APP/PS1 mice in the Y-maze test. The 8-week silibinin treatment significantly increased spontaneous alternation behaviors in APP/PS1 mice. **a** Alternations (%) in the Y-maze test. **b** The total number of arm

entries in the Y-maze test. The data are presented as means  $\pm$  SEM and were analyzed with a one-way ANOVA,  $N=10$  animals per group. ### $p < 0.001$  compared with the WT mice; \*\* $p < 0.01$  compared with the placebo group



**Fig. 2** Silibinin improves visual recognition in the novel object recognition test. Effects of silibinin on learning and memory deficits of APP/PS1 mice in the novel object recognition test. Silibinin groups showed significant improvements in the DI and PI. **a** The discrimination index recorded in the 1-h novel object recognition test. **b** Preferential index recorded in the retention session of the 1-h novel

object recognition test. **c** Total time spent exploring objects A1 and A2. The data are presented as means  $\pm$  SEM and were analyzed with a one-way ANOVA,  $N=10$  animals per group, ### $p < 0.001$  compared with the WT mice; \* $p < 0.01$  and \*\*\* $p < 0.001$  compared with the placebo group

### Effects of silibinin on spatial memory impairment in the Morris water maze test in APP/PS1 mice

In the training period, APP/PS1 mice consistently required more time to find the platform than the WT mice (Fig. 3b). Silibinin groups showed significant decreases in escape latency. In the probe test, APP/PS1 mice spent less time in the target quadrant than the WT mice ( $p < 0.05$ , Fig. 3c). Silibinin groups spent less time searching for the target quadrant than mice in the placebo group ( $p < 0.05$ , Fig. 3c). Silibinin-treated mice improved in their search accuracy, as indicated by the increased number of platform crossings compared to the untreated APP/PS1 mice ( $p < 0.05$ , Fig. 3d). Mice treated with memantine, a positive control, showed decreased escape latencies ( $p < 0.05$ ) in the acquisition phase and spent significantly more time in the target quadrant ( $p < 0.01$ ) and exhibited an increased number of platform crossings. No significant differences in the swimming speed were observed among all groups of mice, indicating that the swimming ability and motivation were not altered in probe tests ( $p = 0.314$ , Fig. 3a). Based on these data, silibinin attenuates the learning and memory deficits of APP/PS1 mice.

### Effects of silibinin on soluble A $\beta$ 1-42 and A $\beta$ 1-40 levels and A $\beta$ plaques in APP/PS1 mice

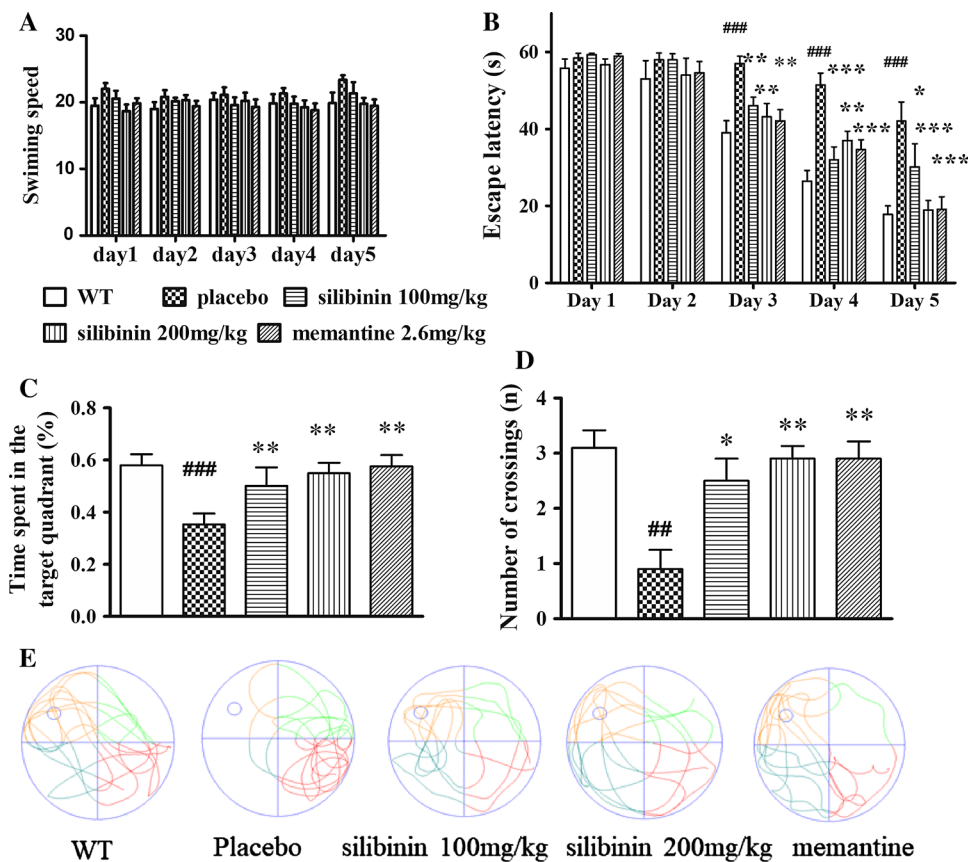
A $\beta$  plaques were assessed using anti-A $\beta$  antibodies (CST,  $\beta$ - amyloid, D12B2). The number of A $\beta$  plaques was significantly increased in the cortex and hippocampus of WT mice. Treatment with silibinin significantly decreased the density of A $\beta$  deposits in the cortex and hippocampus ( $p < 0.001$ , Fig. 4). The levels of soluble A $\beta$ 1-40 and A $\beta$ 1-42 in the brain were evaluated using an ELISA. The A $\beta$ 1-40 and A $\beta$ 1-42 levels were significantly increased in the brains of 8-month-old APP/PS1 mice compared with the WT mice ( $p < 0.01$ , Fig. 4). Treatment with silibinin at a dose of 200 mg/kg significantly decreased the levels of soluble A $\beta$ 1-40 and A $\beta$ 1-42 in the hippocampus ( $p < 0.01$ , Fig. 4).

### Effects of silibinin on cortical oxidative damage (lipid peroxidation, nitrite, superoxide dismutase, and catalase levels)

Compared with WT mice, APP/PS1 mice exhibited significantly increased MDA levels and decreased levels of the



**Fig. 3** Silibinin improvement of spatial memory in Morris water maze test. Effects of silibinin on learning and memory deficits in the Morris water maze. Silibinin groups exhibited significant increases in time spent in the target quadrant and number of platform crossings. **a** Swimming speed in the training period. **b** Escape latency in the training period. **c** Mean time spent in the target quadrant in probe trial. **d** Mean number of platform crossings during the probe trial. **e** Representative traces of each group. The data are presented as means  $\pm$  SEM and were analyzed with a one-way ANOVA, from ten mice per group,  $##p < 0.01$  and  $###p < 0.001$  compared with the WT mice;  $*p < 0.05$ ,  $**p < 0.01$ , and  $***p < 0.001$  compared with the placebo group



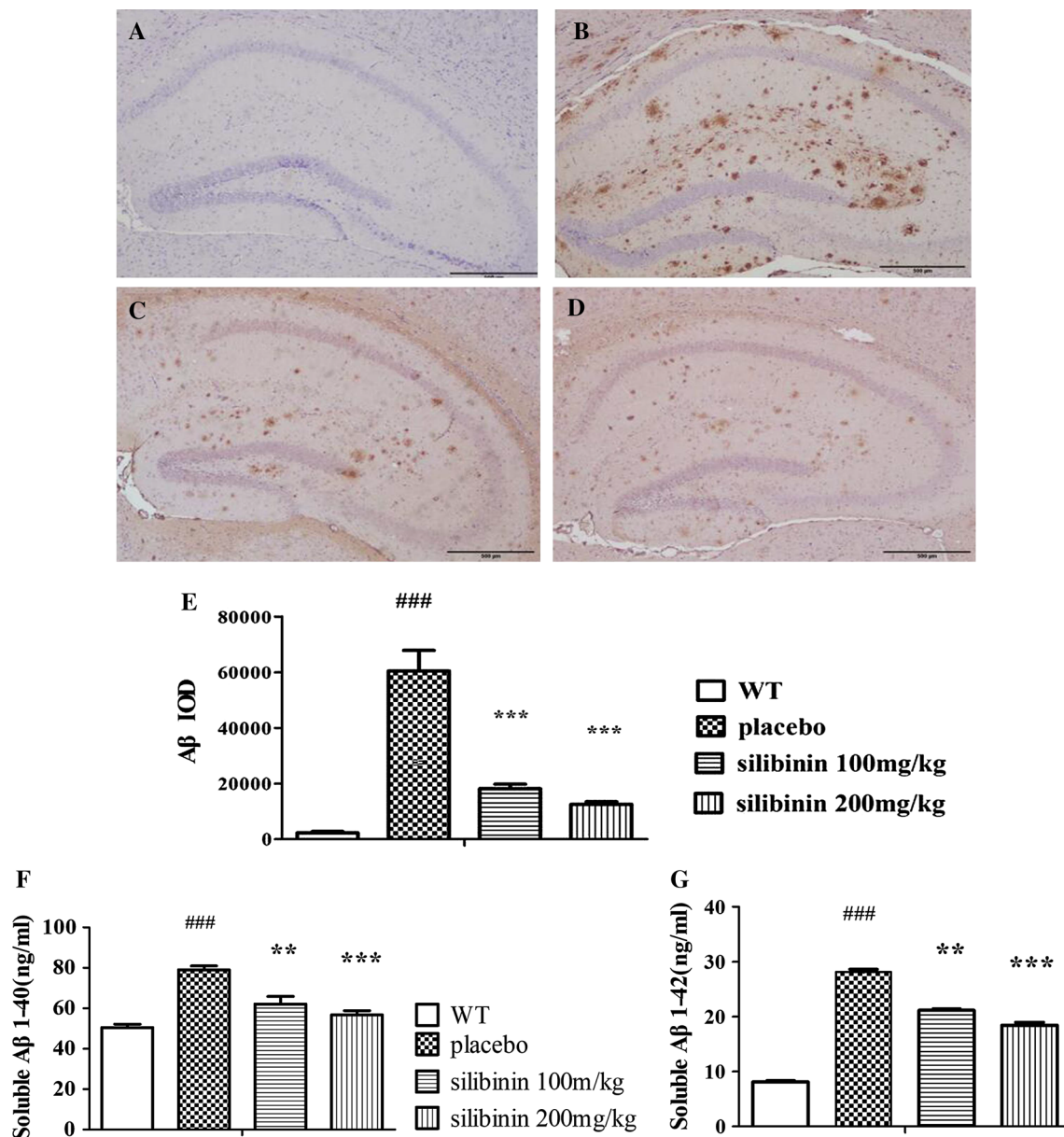
endogenous antioxidant enzymes (NO, CAT, and SOD) in the cerebral cortex ( $p < 0.01$ , Fig. 5). Silibinin-treated groups showed a significant attenuation of the cortical oxidative stress (decreased MDA contents and increased NO, CAT and SOD levels).

### Effects of silibinin on the levels of enzymes that produce and degrade A $\beta$ in the brains of APP/PS1 mice

BACE1 is the rate-limiting enzyme that cleaves APP and further promotes A $\beta$  formation. NEP and IDE, two pivotal endopeptidases, regulate A $\beta$  enzymatic degradation. We examined the levels of APP, BACE1, NEP, and IDE in the brains using Western-blot analysis to examine the effect of silibinin on A $\beta$  processing. Treatment with silibinin at the dose of 200 mg significantly decreased the levels of APP and BACE1 and increased the levels of NEP in the hippocampus ( $p < 0.05$ , Fig. 6). However, neither dose of silibinin altered the IDE level.

### Discussion

Silibinin has been reported to be effective in attenuating cognitive dysfunction in many animal models. Silibinin inhibits the inflammatory response induced by ICV-A $\beta$ 25-35 or chronic AIC3 administration (decreasing iNOS, IL-1 $\beta$  and TNF- $\alpha$  levels) [13], attenuates the decreases in dopamine and serotonin levels in the prefrontal cortex and hippocampus of methamphetamine-injected mice [14], and protects against D-galactose-induced ROS production [9]. Silibinin increases mitochondrial complex activity (I, IV, and V), restores ATP levels, the expression of the AChE mRNA and activity of the enzyme, and increases  $\alpha$ -7-nAChR mRNA expression in the brains of LPS-injected or ICV-STZ mice [15]. Silibinin also prevents A $\beta$ -induced neurotoxicity in SH-SY5Y cells and inhibits A $\beta$  aggregation and plaque deposits [16]. Based on these studies, silibinin potentially acts on multiple targets to inhibit AD pathologies. However, the effects of



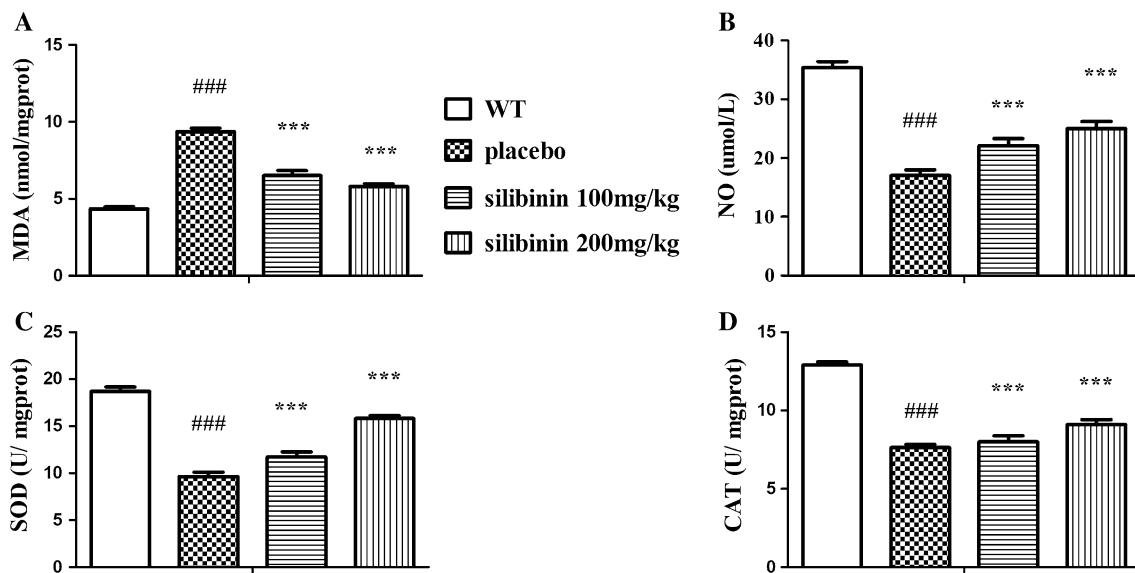
**Fig. 4** Silibinin reduces A $\beta$  plaque deposits and soluble A $\beta$ 1-42, A $\beta$ 1-40 in APP/PS1 mice. Effects of silibinin on attenuating A $\beta$  deposition in the hippocampus of APP/PS1 transgenic mice. The number of A $\beta$  plaques and levels of soluble A $\beta$ 1-42 and A $\beta$ 1-40 were significantly reduced in the silibinin-treated groups. Images of the hippocampus from the **a** WT mice, **b** placebo group, **c** 100 mg/kg silibinin group, and **d** 200 mg/kg silibinin groups (images were captured

using a high power 40 $\times$  objective). **e** Quantification of A $\beta$  deposits in each group. Results were calculated as the percentage of the A $\beta$  area. **f** Soluble A $\beta$ 1-40 levels in the hippocampus of APP/PS1 mice. **g** Soluble A $\beta$ 1-42 levels in the hippocampus of APP/PS1 mice. Data are presented as the means  $\pm$  SEM from five animals per group and four sections from each animal. ### $p$  < 0.001 compared with the WT mice and \*\* $p$  < 0.01, \*\*\* $p$  < 0.001 compared with the placebo group

silibinin on A $\beta$  production and removal have not been reported.

In the present study, learning and memory deficits were assessed using the Y-maze test, novel object recognition test and Morris water maze test, which are widely used methods to investigate the behaviors of mice [17–20]. The contents of soluble and insoluble A $\beta$  and A $\beta$  plaques are increased in 6-month-old APP/PS1 mice, comparable to patients with

AD [21, 22]. Therefore, APP/PS1 mice are a good model to mimic the process of AD and evaluate the potential therapeutic effects of anti-AD agents [23]. In this study, 8-month-old APP/PS1 mice exhibited impairments in spontaneous alternation behaviors, visual recognition and spatial memory. Treatment with silibinin significantly attenuated the cognitive deficits of APP/PS1 mice in the Y-maze test, novel object recognition test, and Morris water maze test.



**Fig. 5** Effects of silibinin on cortical oxidative damage (lipid peroxidation, nitrite, superoxide dismutase, and catalase levels). Silibinin exerted antioxidant effects on the cortex of APP/PS1 mice. Treatment with silibinin for 8 weeks reduced the MDA content and increased

SOD, NO, and CAT activity in APP/PS1 mice. Data are presented as the means  $\pm$  SEM from five animals per group. ### $p < 0.001$  compared with the WT mice; \*\*\* $p < 0.001$  compared with the placebo group

In the amyloid cascade hypothesis, soluble and insoluble A $\beta$ 1-40 and A $\beta$ 1-42 induce oxidative damage, which may contribute to synaptic dysfunction, neuritic injury, and cognitive impairments in patients with AD [11]. Based on this hypothesis, we used ELISA and immunohistochemistry to determine the A $\beta$  levels in the brains of APP/PS1 mice, and further evaluated the therapeutic effects of silibinin. After the 2-month silibinin treatment, the soluble A $\beta$ 1-40 and A $\beta$ 1-42 levels were decreased in cortex and hippocampus and the area of A $\beta$  plaque deposits was also reduced, indicating that the mechanism by which silibinin improved learning and memory deficit may be related to alterations in A $\beta$  levels.

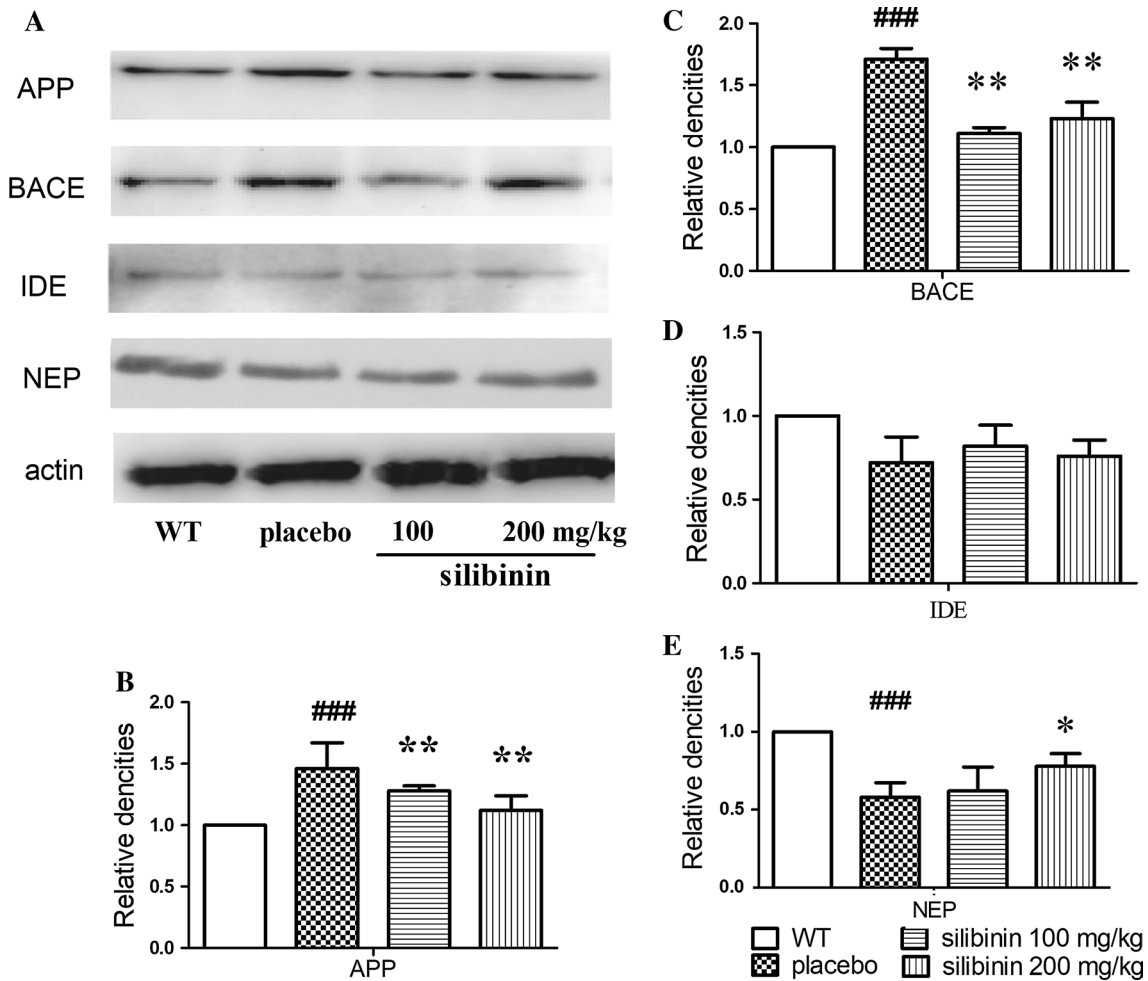
BACE1 is the rate-limiting enzyme that cleaves APP and further promotes A $\beta$  formation. BACE1 levels are increased in the brains of both AD animal models and patients with AD [24, 25], leading to A $\beta$  production and neuron death. BACE1 knockout animal models show decreased numbers of A $\beta$  plaques and a delay in the progression of A $\beta$ -related pathologies [5, 26]. NEP and IDE, two pivotal endopeptidases, regulate A $\beta$  enzymatic degradation. Downregulation of NEP and IDE significantly increases A $\beta$  levels and A $\beta$  plaques [5]. NEP levels are decreased in regions in which A $\beta$  accumulates in the brains of patients with AD and aged mice [27]. A $\beta$ -stimulated astrocytes release neuroinflammatory factors that increase the IDE level [28]. In the late stage of AD, IDE levels are increased in the brains of patients, which is closely related to the progression of neuroinflammation [29]. In the present study, we examined the effects of silibinin on APP, BACE1, NEP and IDE levels. After the

2-month silibinin treatment, APP and BACE1 levels were decreased, and NEP levels were increased. Based on these results, silibinin may prevent AD-associated A $\beta$  pathology by inhibiting A $\beta$  generation and promoting A $\beta$  degradation.

Oxidative stress has been shown to contribute to AD-induced neurodegeneration. Increasing levels of oxidative stress have been observed in neurons surrounding A $\beta$  plaques in AD transgenic mice models, and oxidative stress also causes A $\beta$  accumulation in primary neurons [30]. A $\beta$ -induced ROS production, lipid peroxidation, and antioxidant enzyme depletion (glutathione, CAT and SOD) further cause neuronal damage in the mouse brain and neuronal cell lines [31, 32]. Therefore, oxidative injury represents a potential target of AD therapy. In the present study, 8-month-old APP/PS1 mice showed significant oxidative damage, as evidenced by the increased MDA content and inhibition of the antioxidant enzymes CAT and SOD, consistent with the findings reported by Tian et al. and Wang et al. [33, 34]. However, the silibinin treatment significantly attenuated oxidative damage in APP/PS1 mice. Thus, silibinin exerted a neuroprotective effect against oxidative stress-induced injury in vivo.

In conclusion, silibinin protects against learning and memory impairments in APP/PS1 mice by altering the levels of proteins as APP, BACE, NEP involved in A $\beta$  processing and removal, subsequently decreases A $\beta$  plaques and soluble A $\beta$  levels. Silibinin also exerts antioxidant effects on APP/PS1 mice. Based on these results, silibinin acts on multiple targets (antioxidant and anti-A $\beta$  agent) and represents a potential treatment for AD.





**Fig. 6** Silibinin regulates A $\beta$  processing in APP/PS1 mice. Representative Western blots showing the levels of APP, BACE, IDE, and DEP in the hippocampus of APP/PS1 mice. After 8 weeks of treatment, silibinin significantly decreased the levels of APP and BACE in the hippocampus of APP/PS1 mice. Meanwhile, silibinin 200 mg/

kg group significantly increased levels of the NEP protein in the hippocampus. Data are presented as the means  $\pm$  SEM from four animals per group. ###*p* < 0.001 compared with the WT mice; \**p* < 0.05 and \*\**p* < 0.01 compared with the placebo group

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**Author's contribution** Dafeng Bai conceived the experiments, contributed to research data, and drafted the manuscript; Dajun Zhang, Lini Zhao, Mingyue Wang, contributed to research data; LinZhu, Yan Sun, Liqian Zhang, Xueying Chen, Liqian Zhang, Wenbo Li and Yan Cui participated in the model design and animal experiment; Qiwen Zhu revised the manuscript. Ge Jin revised the manuscript and supervised the analysis.

**Compliance with ethical standards**

**Conflict of interest** The authors have no conflicts of interest to declare.

**Ethical approval** All animal procedures performed in this study were approved by the Institute for Experimental Animals at Shen-

yang Pharmaceutical University (Permit Number: SYPU-IACUC-C2015-0831-203).

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