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RESEARCH PAPER

Silibinin prevents amyloid β peptide-induced memory impairment and oxidative stress in mice

P Lu[1,2], T Mamiya[1], LL Lu[1,3], A Mouri[1], LB Zou[3], T Nagai[4], M Hiramatsu[5], T Ikejima[2] and T Nabeshima[1,6]

[1]*Department of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, Meijo University, Nagoya, Japan,* [2]*China-Japan Research Institute of Medical and Pharmaceutical Sciences, Shenyang Pharmaceutical University, Liaoning, China,* [3]*Department of Pharmacology, Shenyang Pharmaceutical University, Liaoning, China,* [4]*Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University, Nagoya, Japan,* [5]*Laboratory of Neuropsychopharmacology, Meijo University, Nagoya, Japan, and* [6]*Japanese Drug Organization of Appropriate Use and Research, Nagoya, Japan*

Background and purpose: Accumulated evidence suggests that oxidative stress is involved in amyloid β (Aβ)-induced cognitive dysfunction. Silibinin (silybin), a flavonoid derived from the herb milk thistle (*Silybum marianum*), has been shown to have antioxidative properties; however, it remains unclear whether silibinin improves Aβ-induced neurotoxicity. In the present study, we examined the effect of silibinin on the memory impairment and accumulation of oxidative stress induced by $A\beta_{25-35}$ in mice.
Experimental approach: Aggregated $A\beta_{25-35}$ (3 nmol) was intracerebroventricularly administered to mice. Treatment with silibinin (2, 20 and 200 mg·kg^{-1}, once a day, p.o.) was started immediately after the injection of $A\beta_{25-35}$. Locomotor activity was evaluated 6 days after the $A\beta_{25-35}$ treatment, and cognitive function was evaluated in a Y-maze and novel object recognition tests 6–11 days after the $A\beta_{25-35}$ treatment. The levels of lipid peroxidation (malondialdehyde) and antioxidant (glutathione) in the hippocampus were measured 7 days after the $A\beta_{25-35}$ injection.
Key results: Silibinin prevented the memory impairment induced by $A\beta_{25-35}$ in the Y-maze and novel object recognition tests. Repeated treatment with silibinin attenuated the $A\beta_{25-35}$-induced accumulation of malondialdehyde and depletion of glutathione in the hippocampus.
Conclusions and implications: Silibinin prevents memory impairment and oxidative damage induced by $A\beta_{25-35}$ and may be a potential therapeutic agent for Alzheimer's disease.
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Keywords: amyloid β; silibinin; memory deficits; oxidative stress

Abbreviations: Aβ, amyloid β; AD, Alzheimer's disease; CMC, carboxymethylcellulose; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); GSH, glutathione; MDA, malondialdehyde; MES buffer, 0.2 M 2-(N-morpholino)ethanesulphonic acid buffer; TBARS, thiobarbituric acid-reactive substance

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder associated with global mental dysfunction and impairment of cognitive function (Palmer, 2002). Common pathological features of AD are senile plaques, neurofibrillary tangles and neuronal loss in the medial temporal lobe structures and cortical areas of the brain (Blennow *et al.*, 2006). The deposition of amyloid β (Aβ) peptide in neuritic plaques is the defining feature for diagnosis of AD, and the level of Aβ peptide correlates well with the extent of cognitive impairment (Naslund *et al.*, 2000). Aβ, a spontaneously aggregating peptide of 39–43 amino acids, is the primary protein component of senile plaques, a pathological hallmark of AD in the brain (Shen *et al.*, 2002). Several studies have demonstrated that oxidative stress is involved in Aβ-induced neurotoxicity and the progression of AD (Schubert *et al.*, 1995; Yankner, 1996). In particular, Aβ fragment 25–35 ($A\beta_{25-35}$) seems to be responsible for toxic and oxidative events leading to brain damage, such as oxidative stress-mediated changes in hippocampal long-term potentiation (Trubetskaya *et al.*, 2003), protein nitration, induction of inducible nitric oxide synthase (Tran *et al.*, 2001; Alkam *et al.*, 2008) and protein oxidation in fibroblasts derived from AD patients (Choi *et al.*, 2003).

Correspondence: Toshitaka Nabeshima, Department of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, Meijo University, 150 Yagotoyama, Tempaku-ku, Nagoya 468-8503, Japan. E-mail: tnabeshi@ccmfs.meijo-u.ac.jp
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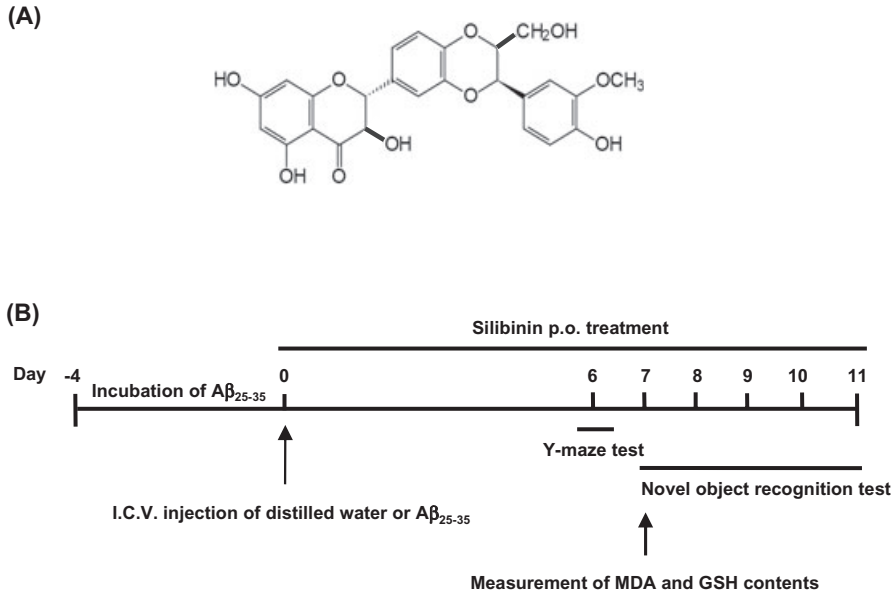

Figure 1 (A) Chemical structure of silibinin and (B) protocol used in this study.

In animal experiments, intrahippocampal or i.c.v. injections of $A\beta_{25–35}$ induced histological and biochemical changes, learning deficits (Maurice *et al.*, 1996; Meunier *et al.*, 2006; Alkam *et al.*, 2008) and dysfunction of the cholinergic system, which plays an important role in the cognitive deficits associated with aging and neurodegenerative diseases (Tran *et al.*, 2001). Thus, $A\beta_{25–35}$-injected animals are useful models for understanding the pathogenesis and progression of AD, and for evaluating new therapeutic agents for AD (Maurice *et al.*, 1996).

Several synthetic antioxidants are available, but there is a growing trend towards the use of natural products (polyphenols, flavonoids, vitamins, carotenes and lycopenes) as antioxidants. Of these, bioflavonoids are a ubiquitous group of polyphenolic substances present in most plants (Nijveldt *et al.*, 2001). Silibinin (silybin) is a flavonoid derived from the herb milk thistle (*Silybum marianu*m) (Kren and Walterová, 2005; Figure 1A) and used as a hepatoprotectant in the clinical treatment of liver disease (Kren and Walterová, 2005). Silibinin seems to protect against oxidative stress as it has been reported to decrease lipid peroxidation, a sensitive marker of oxidative lipids, in liver microsomes and isolated hepatocytes (Bosisio *et al.*, 1992). Furthermore, it has been demonstrated that the antioxidative activity of silibinin is related to the scavenging of free radicals (De Groot and Rauen, 1998; Trouillas *et al.*, 2008) and activation of antioxidative defenses: increases in cellular glutathione (GSH) content (Valenzuela *et al.*, 1989) and superoxide dismutase levels (Müzes *et al.*, 1991). These findings suggest that silibinin could attenuate oxidative stress-induced brain dysfunction. In fact, there are a few reports that silymarin, a mixture of flavonoids present in milk thistle and whose main component is silibinin, has protective effects on the central nervous system against ethanol-induced brain injury (La Grange *et al.*, 1999) and lipopolysaccharide-induced neurotoxicity (Wang *et al.*, 2002). However, it remains unclear whether silibinin protects against Aβ-induced neurotoxicity.

In the present study, to confirm the usefulness of silibinin against Aβ-induced neurotoxicity, we investigated whether silibinin prevents memory impairment in an $A\beta_{25–35}$-injected animal model of AD. Moreover, we examined the protective effect of silibinin on oxidative events indicated by an increase in malondialdehyde (MDA), the end product of lipid peroxidation, and decrease in GSH, an endogenous antioxidant, in the hippocampus of $A\beta_{25–35}$-injected mice.

Methods

Animals

Male ICR mice, 5 weeks old at the beginning of experiments, were obtained from Japan SLC Inc. (Shizuoka, Japan). They were housed in plastic cages and kept in a regulated environment (23 ± 0.5°C, 50 ± 5% humidity) with a 12/12 h light/dark cycle (lights on from 08.00 h to 20.00 h). The mice received food (CE2; Clea Japan Inc., Tokyo, Japan) and water *ad libitum*. Behavioural experiments were carried out in a sound-proof and air-regulated experimental room, to which mice were habituated for at least 1 h. All experiments were performed in accordance with the Guidelines for Animal Experiments of the Faculty of Pharmaceutical Sciences Meijo University and the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society (2006). The procedures involving animals and their care conformed to the international guidelines set out in the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

I.c.v. injection

$A\beta_{25–35}$ was dissolved in distilled water at a concentration of 1 mM as a stock solution and stored at –20°C. In the present study, the concentration and dose were selected according to a previous study (Maurice *et al.*, 1996). It has been shown that

i.c.v. administration of $A\beta_{25-35}$ at the dose of 9 nmol leads to memory impairment with severe neurotoxicity in mice, and at the dose of 3 nmol produces mild brain dysfunction, whereas it fails to cause cognitive deficits at the dose of 1 nmol (Maurice *et al.*, 1996). The $A\beta_{25-35}$ (3 nmol = 3 μg) is greatly diluted by cerebrospinal fluid and interstitial fluid to a level of about 6000 $ng \cdot g^{-1}$ tissue, which is in the range of tissue levels (21.0–89.1 $ng \cdot g^{-1}$ tissue: water-soluble Aβ; 377.3–3000.0 $ng \cdot g^{-1}$ tissue: water-insoluble Aβ) found in brains of AD patients (Kuo *et al.*, 1996), when it diffuses throughout the whole brain (average weight is about 0.5 g). Moreover, a certain amount of $A\beta_{25-35}$ is cleared or moved out from brain, which further decrease its concentration. Therefore, $A\beta_{25-35}$ at the dose of 3 nmol was used in this study. The $A\beta_{25-35}$ was aggregated, or 'aged', by incubating it in distilled water at 37°C for 4 days. Aggregated $A\beta_{25-35}$ [3 $nmol \cdot (3\ \mu L)^{-1}$] or distilled water (3 μL) was injected i.c.v., as described previously (Maurice *et al.*, 1996). Briefly, a microsyringe with a specially made 28-gauge stainless steel needle, 3 mm in length, was used for microinjections. Mice were anaesthetized lightly with ether, and the needle was inserted unilaterally 1 mm to the right of the midline point equidistant from each eye, at an equal distance between the eyes and the ears and perpendicular to the plane of the skull (A, –0.22 mm; L, 1 mm from the bregma; V, 2.5 mm from the skull). $A\beta_{25-35}$ [3 $nmol \cdot (3\ \mu L)^{-1}$] or distilled water (3 μL) was delivered gradually within ~3 s. Mice exhibited normal behaviour within 1 min after injection. In preliminary experiments the injection site was confirmed by injecting Indian ink. Neither insertion of the needle nor injection of the distilled water had a significant influence on survival, behavioural responses or cognitive functions.

Drug treatment

Silibinin was suspended in a 0.3% carboxymethylcellulose solution. Mice were administered silibinin (2, 20 and 200 $mg \cdot kg^{-1}$, p.o.) or the 0.3% carboxymethylcellulose solution by oral gavage 60 min before the Y-maze test and the training session of the novel object test. All compounds were administered systemically in a volume of 0.01 $mL \cdot g^{-1}$ body weight (Figure 1B).

Behavioural procedures

Previously it has been shown that acute exposure to aged $A\beta_{25-35}$ induces apoptosis-mediated neuronal toxicity during 6 days of incubation in hippocampal cultures, and cognitive dysfunction in several learning and memory tests in mice (Maurice *et al.*, 1996). The behavioural tests were started 6 days after the $A\beta_{25-35}$ injection and were carried out sequentially according to the experimental schedule shown in Figure 1B. The present study was conducted in a blind manner.

Measurement of locomotor activity

The measurement of locomotor activity in a novel environment was carried out on day 6 after the start of silibinin administration. Spontaneous locomotor activity was measured as previously reported with a minor modification (Miyamoto *et al.*, 2001). Mice were placed individually in a transparent acrylic cage with a black frosted Plexiglas floor (45 × 26 × 40 cm) for 90 min, and locomotor activity was measured in 5 min intervals by using digital counters with infrared sensors. The system was equipped with photosensor frames in the side walls. Locomotor activity was defined as the total number of beam cuts due to horizontal movement measured by the photosensors. The acrylic cage was wiped with a paper towel between uses and kept clean.

Spontaneous alternation in a Y-maze test

This behavioural test was performed 6 days after the $A\beta_{25-35}$ injection, according to a previous study (Mouri *et al.*, 2007), with minor modifications. The maze was made of black painted wood; each arm was 40 cm long, 12 cm high, 3 cm wide at the bottom and 10 cm wide at the top. The arms converged at an equilateral triangular central area that was 4 cm at its longest axis. Each mouse was placed at the centre of the apparatus and allowed to move freely through the maze for 8 min. The series of arm entries was recorded visually. Alternation was defined as successive entry into the three arms on overlapping triplet sets. Alternation behaviour (%) was calculated as the ratio of actual alternations to possible alternations (defined as the number of arm entries minus two) multiplied by 100.

Novel object recognition test

The novel object recognition test was performed 7–11 days after the $A\beta_{25-35}$ injection, according to a previous study (Mouri *et al.*, 2007), with minor modifications. The task consisted of three sessions: habituation, training and retention. Each mouse was individually habituated to the box (30 × 30 × 30 high cm), with 10 min of exploration in the absence of objects for 3 days (habituation session). During the training session, two objects were placed in the middle of the box. A mouse was then placed midway at the front of the box, and total time spent exploring the two objects was recorded for 10 min. During the retention session, animals were placed back into the same box 24 h after the training session, in which one of the familiar objects used during training was replaced with a novel object. The animals were then allowed to explore freely for 5 min, and the time spent exploring each object was recorded. Throughout the experiments, the objects were used in a counterbalanced manner in terms of their physical complexity and emotional neutrality. A preference index, a ratio of the amount of time spent exploring any one of the two objects (training session) or the novel object (retention session) over the total time spent exploring both objects, was used to measure cognitive function.

Determination of lipid peroxidation level

Malondialdehyde was measured with a thiobarbituric acid-reactive substance (TBARS) assay kit. Briefly, the hippocampus was isolated and homogenized in cooled RIPA buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS,1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF and protease inhibitor cocktail). Tissue homogenate from the

hippocampus was incubated with 8.1% sodium dodecylsulphate for 10 min followed by the addition of 20% acetic acid (pH 3.5). The reaction mixture was incubated with 0.6% thiobarbituric acid at 99°C for 1 h. After being cooled, the mixture was centrifuged at 1600× *g* for 10 min at 4°C. The absorbance was measured by using a plate reader (Bio-Rad 550) at 540 nm. MDA content was expressed as μmol·g^{-1} tissue.

Determination of glutathione content

A GSH assay kit was used for detecting GSH in the hippocampus. Briefly, the hippocampal tissues were isolated and homogenized in cold buffer (0.05 M phosphate, pH 6–7, containing 1 mM EDTA). The homogenates were centrifuged at 10 000× *g* for 15 min at 4°C, and the supernatant was subsequently incubated with MES buffer (0.2 M 2-(N-morpholino)ethanesulphonic acid buffer, 0.1 M phosphate and 2 mM EDTA, pH 6.0), cofactor mixture, enzyme mixture and DTNB [5,5′-dithiobis (2-nitrobenzoic acid)] in the dark on the orbital shaker at room temperature for 30 min. Absorbance at 405 nm was measured by using a plate reader (Bio-Rad 550), and GSH content was calculated as μmol·g^{-1} tissue.

Statistical analysis

All data are expressed as the means ± s.e.mean. Statistical differences among the experimental groups were tested by using a one- or two-way analysis of variance (ANOVA) for behavioural tests, and Tukey's *post hoc* test was employed for multiple comparisons. *P*-values less than 0.05 were accepted as significant.

Materials

$A\beta_{25–35}$ was purchased from Bachem (Bubendorf, Switzerland); silibinin from Panjin Green Biological Development Co., Ltd. (Panjin, China); digital counters with infrared sensors Scanet SV-10 (Melquest Ltd., Toyama, Japan); TBARS assay kit and GSH assay kit, Cayman (Ann Arbor, MI, USA).

Results

Effect of silibinin on locomotor activity

The counts of spontaneous locomotor activity of mice were measured on day 6 after $A\beta_{25–35}$ injection. There were no significant differences in the time course of locomotor activity [$F_{group}(5, 756) = 1.556$, $P = 0.170$; $F_{time}(17, 756) = 108.92$, $P < 0.01$; $F_{group \times time}(85, 756) = 0.347$, $P = 0.999$] and total locomotor activity [$F(5, 47) = 0.202$, $P = 0.96$] among the groups (Figure 2A).

Effect of silibinin on short-term memory impairment induced by $A\beta_{25–35}$ in Y-maze test

We evaluated the effects of silibinin on impairment of short-term memory 6 days after the $A\beta_{25–35}$ injection in a Y-maze test. $A\beta_{25–35}$-injected mice showed significantly reduced spontaneous alternation behaviour compared with vehicle-injected mice ($P < 0.05$, Figure 2B). Treatment with silibinin (2, 20 and 200 mg·kg^{-1}) dose-dependently attenuated the impairment of spontaneous alternation behaviour in $A\beta_{25–35}$ injected mice [$F(5, 66) = 6.325$, $P < 0.05$, Figure 3]. Silibinin doses of 20 and 200 mg·kg^{-1} significantly prevented $A\beta_{25–35}$-induced memory impairment ($P < 0.05$, Figure 3). There was no significant difference in the number of arm entries among the groups [$F(4, 66) = 0.463$, $P = 0.763$: data not shown]. Silibinin did not affect spontaneous alternation (Figure 2B) and the number of arm entries (data not shown) in mice injected with distilled water.

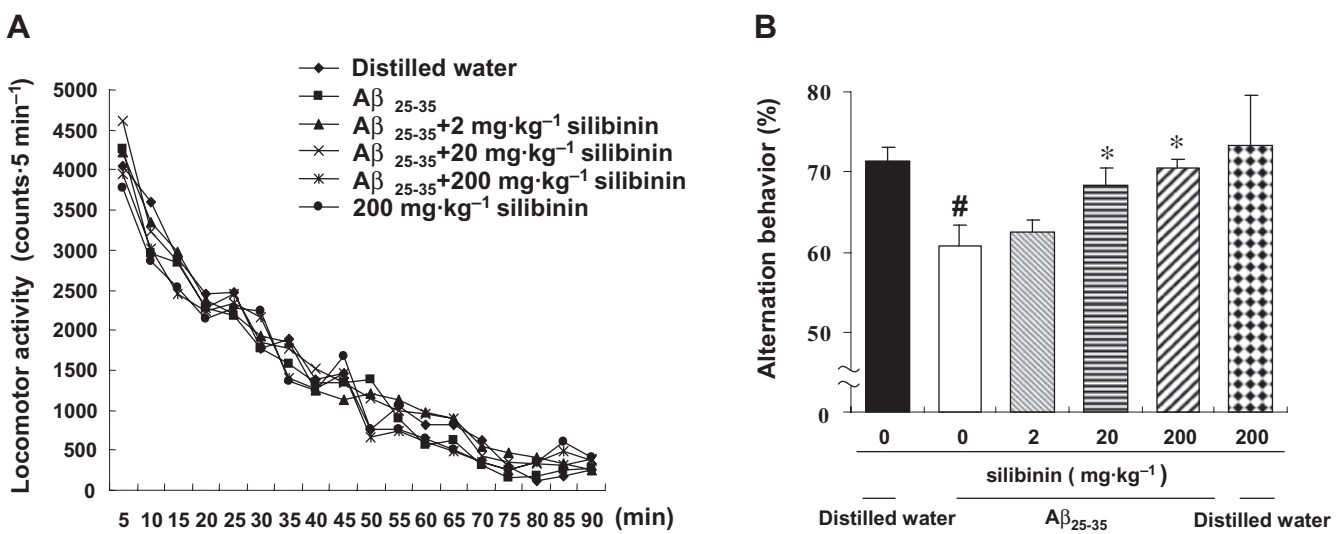

Figure 2 Effects of silibinin on locomotor activity (A) and impairment of short-term memory in Y-maze test (B) in amyloid β $(A\beta)_{25–35}$-injected mice. Results are expressed as the means ± s.e.mean (A: $n = 8$, B: $n = 13–15$) and were analysed by a one or two-way ANOVA, followed by Tukey's test for multiple comparisons. #$P < 0.05$ versus carboxymethylcellulose (CMC)-treated, distilled water-injected mice; *$P < 0.05$ versus CMC-treated, $A\beta_{25–35}$-injected mice.

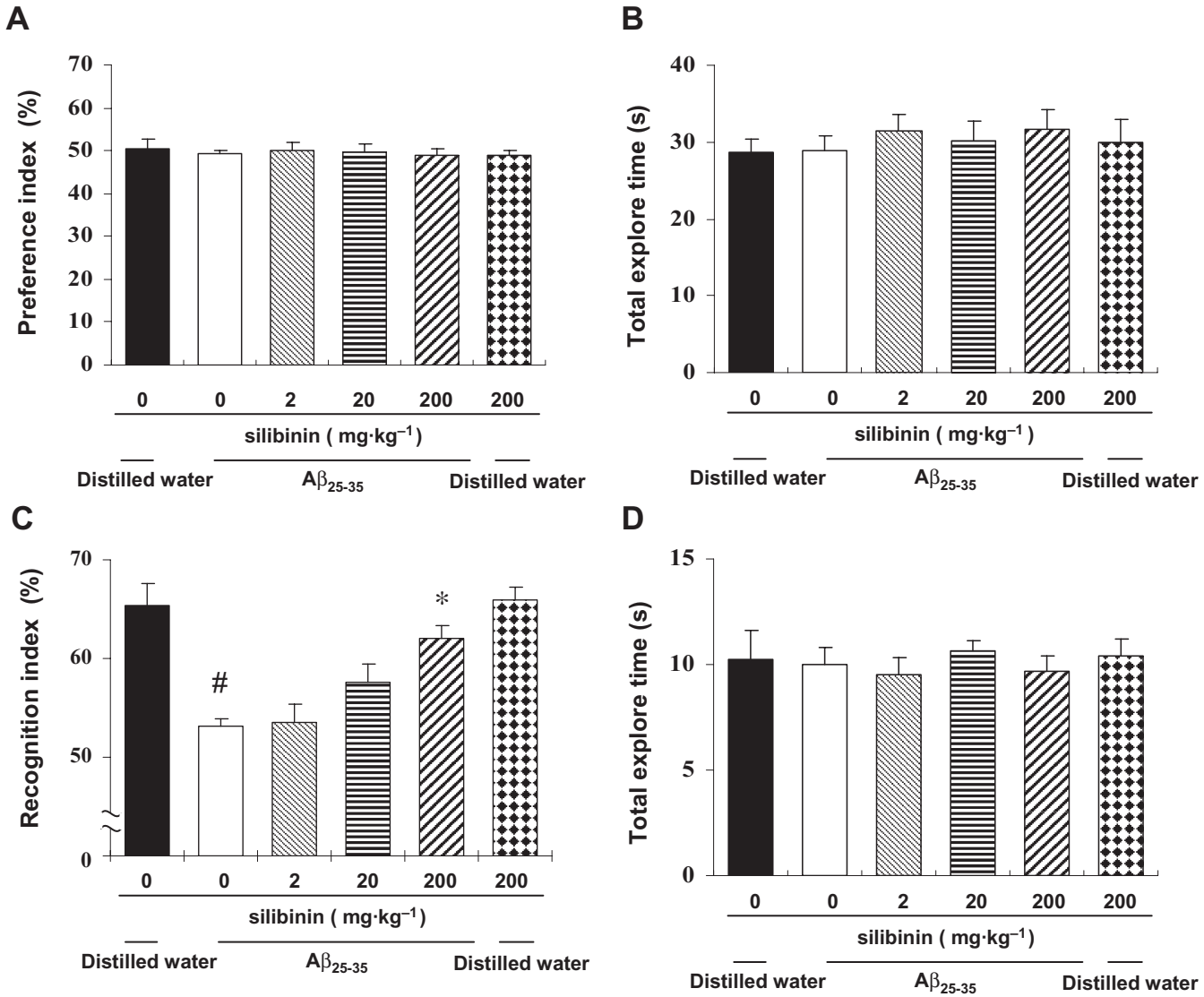

Figure 3 Effect of silibinin on recognition memory impairments induced by amyloid β $(Aβ)_{25–35}$ in the novel object recognition test. (A) Preference index in training session. (B) Total exploration time in training session. (C) Recognition index in retention session. (D) Total exploration time in retention session. Results are expressed as the means ± s.e.mean (n = 13–15) and analysed by a one-way ANOVA, followed by Tukey's test for multiple comparisons. #P < 0.05 versus carboxymethylcellulose (CMC)-treated, distilled water-injected mice; *P<0.05 versus CMC-treated, $Aβ_{25–35}$-injected mice.

Effect of silibinin on recognition memory impairment induced by $Aβ_{25–35}$ in the novel object recognition test

We evaluated the effect of silibinin on impairment of visual recognition memory 7 days after the $Aβ_{25–35}$ injection in a novel object recognition test. In the training session, mice injected with $Aβ_{25–35}$ or distilled water spent equal amounts of time exploring either of the two objects (Figure 3A), showing there was no biased exploratory preference in either group of animals. In addition, total time spent in the exploration of objects in the training and retention sessions did not differ between the groups injected with $Aβ_{25–35}$ or distilled water (Figure 3B and D).

When the retention session was performed 24 h after the training session, the level of exploratory preference for the novel objects in the $Aβ_{25–35}$-injected mice was significantly decreased compared with that in the distilled water-injected mice (P < 0.05, Figure 3C). Treatment with silibinin (2, 20 and 200 mg·kg^{-1}) dose-dependently and significantly reversed the decreased level of exploration preference in the retention session in $Aβ_{25–35}$-injected mice [$F(5, 66)$ = 16.195, P < 0.05, Figure 3C]. Tukey's *post hoc* analysis revealed that silibinin at 200 mg·kg^{-1} significantly prevented the memory impairment in $Aβ_{25–35}$-injected mice (P < 0.05, Figure 3C). Treatment with silibinin did not affect either the level of exploratory preference for the objects or the total exploration time in either the training or retention sessions for the mice injected with $Aβ_{25–35}$ or those injected with distilled water (Figure 3A–D).

Effect of silibinin on the MDA and GSH levels in the hippocampus of $Aβ_{25–35}$-injected mice

To determine whether lipid peroxidation is involved in the ameliorating effect of silibinin in $Aβ_{25–35}$-injected mice, we

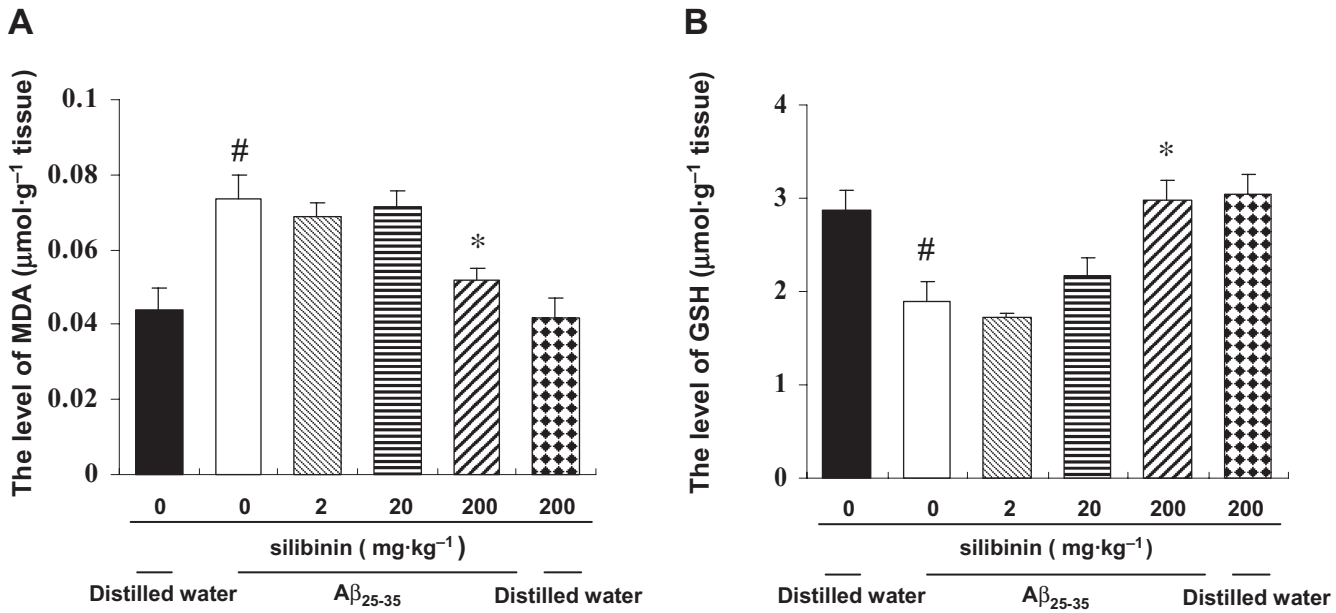

Figure 4 Effects of silibinin on amyloid β $(Aβ)_{25–35}$-induced increase in malondialdehyde (MDA) levels and decrease in glutathione (GSH) levels in the hippocampus. (A) MDA levels. (B) GSH levels. Results are expressed as the means ± s.e.mean ($n = 6–7$) and analysed by a one-way ANOVA, followed by Tukey's test for multiple comparisons. #$P < 0.05$ versus carboxymethylcellulose (CMC)-treated, distilled water-injected mice; *$P < 0.05$ versus CMC-treated, $Aβ_{25–35}$-injected mice.

examined the effect of silibinin on the levels of MDA in the hippocampus 7 days after the $Aβ_{25–35}$ injection. A significant increase in the level of MDA was observed in the hippocampus of $Aβ_{25–35}$-injected mice as compared with levels in the control group [$F(5, 34) = 8.281$, $P < 0.05$, Figure 4A]. Treatment with silibinin (200 mg·kg^{-1}) significantly prevented the increase of MDA levels in the hippocampus of $Aβ_{25–35}$-treated mice ($P < 0.05$, Figure 4A). In contrast, $Aβ_{25–35}$-injected mice showed a significant decrease of GSH levels in the hippocampus compared with the control mice. Treatment with silibinin (2, 20 and 200 mg·kg^{-1}) dose-dependently prevented the decrease in GSH levels in the hippocampus of $Aβ_{25–35}$-injected mice [$F(5, 34) = 9.351$, $P < 0.05$, Figure 4B]. Tukey's *post hoc* analysis indicated a significant effect of silibinin at the dose of 200 mg·kg^{-1} ($P < 0.05$, Figure 4B). However, silibinin did not affect levels of MDA and GSH in the hippocampus of mice injected with distilled water (Figure 4A and B).

Discussion and conclusions

In the present study, we examined the effect of silibinin on the memory impairment induced by $Aβ_{25–35}$ in mice. Silibinin prevented $Aβ_{25–35}$-induced impairment of short-term and recognition memory in the Y-maze and novel object recognition tests respectively. Furthermore, silibinin prevented the accumulation of lipid peroxide (MDA) and decrease of antioxidant (GSH) in the hippocampus after the $Aβ_{25–35}$ treatment. To our knowledge, this is the first study to show that silibinin protects against Aβ-injected neurotoxicity by regulating oxidative stress in the brain.

The accumulation of Aβ proteins is highly toxic to primary and other cell lines (Kim *et al.*, 2007; Nie *et al.*, 2008). $Aβ_{25–35}$ is most toxic Aβ fragment that has been detected in the brain of AD patients (Pike *et al.*, 1995; Kubo *et al.*, 2002; Zameer *et al.*, 2006). $Aβ_{25–35}$ is the core fragment of full-length Aβ and possesses many of the characteristics of the full-length Aβ peptide, including aggregative ability and neurotoxic properties such as learning and memory impairment, morphological alterations and cholinergic dysfunction (Pike *et al.*, 1995; Tran *et al.*, 2001; Kubo *et al.*, 2002; Alkam *et al.*, 2008). Therefore, the effect of silibinin on Aβ-induced neurotoxicity was evaluated in $Aβ_{25–35}$-injected mice. In the present study, $Aβ_{25–35}$-injected mice showed memory impairment in both the Y-maze and the novel object recognition tests. These results are consistent with our previous findings that $Aβ_{25–35}$ induces cognitive impairment in mice (Alkam *et al.*, 2007; 2008; Tsunekawa *et al.*, 2008).

Silibinin dose-dependently and significantly prevented the impairment of short-term and recognition memory induced by $Aβ_{25–35}$. It is unlikely that the protective effect of silibinin is due to changes in motivation or sensorimotor function, as various motivations are involved in these behavioural tasks, and different skills are required for better performance in each task. Actually, silibinin had no effect on locomotor activity, number of arm entries in the Y-maze test and total time spent exploring objects in the novel object test. These results suggest that silibinin attenuates cognitive impairments in the $Aβ_{25–35}$-injected mice without affecting motor function, motivation and exploratory activity.

It has been proposed that oxidative stress plays a critical role in the development of AD (Smith *et al.*, 1996). Lipid peroxidation is one of the major outcomes of free radical-mediated injury that directly damages membranes and generates a number of secondary products including aldehydes, such as MDA, and 4-hydroxy-2-nonenal, ketones, etc. (Slater, 1984). Analysis of AD brains demonstrates an increase in lipid peroxidation products in the amygdala, hippocampus and parahippocampal gyrus of the AD brain compared with

age-matched controls (Markesbery and Lovell, 1998). Amyloid precursor protein transgenic mice, a genetic mouse model of AD, have shown a systemic increase in lipid peroxidation compared with wild-type littermates (Pratico *et al.*, 2001). MDA is the most abundant individual aldehyde resulting from lipid peroxidation and can be considered a marker of lipid peroxidation. To confirm the effect of silibinin on Aβ-induced oxidative stress, we measured the levels of MDA in the hippocampus. $A\beta_{25–35}$ increased the levels of MDA in the hippocampus, suggesting that it caused lipid peroxidation. Treatment with silibinin prevented the accumulation of MDA induced by $A\beta_{25–35}$. Accordingly, these results suggest that the protective effect of silibinin on $A\beta_{25–35}$-induced memory impairment is related to an accumulation of oxidative stress in the hippocampus.

It has been proposed that Aβ peptide impairs the antioxidative defenses in brain, which may contribute to the pathogenesis of AD (Mattson *et al.*, 1998). GSH is one of the most abundant intracellular non-protein thiols in the central nervous system, where it plays a major antioxidative role within both neurones and non-neuronal cells. In the present study, $A\beta_{25–35}$ decreased the level of GSH in the hippocampus, consistent with reports of the depletion of GSH in the brain of AD patients (Aksenov and Markesbery, 2001). Furthermore, the $A\beta_{25–35}$-induced decrease in the level of GSH was prevented by treatment with silibinin, indicating that the protective effect of silibinin on $A\beta_{25–35}$-induced cognitive impairment involves the activation of antioxidative defenses.

Although the mechanism by which silibinin regulates $A\beta_{25–35}$-induced oxidative stress remains to be determined, there are several possible explanations. Firstly, as a polyphenolic flavonoid, silibinin has strong free radical-scavenging activity (Trouillas *et al.*, 2008). Silibinin reacts with a damaging free radical and forms a flavonoid radical, which has greater stability, and then breaks the free radical chain reaction (Weber *et al.*, 2006). It is possible that silibinin prevents oxidative damage directly by scavenging free radicals.

Secondly, it has been demonstrated that silibinin has a metal-chelating effect (Borsari *et al.*, 2001). The neurotoxicity of Aβ is mediated by $A\beta Cu^{2+}$ or $A\beta Fe^{3+}$ forming H_2O_2, which generates lipid peroxidation adducts, protein carbonyl modifications and nucleic acid adducts in various cellular compartments. Thus, metal ions mediate the oxidative stress mechanism of Aβ toxicity. Incubation with the Fe^{3+} chelator desferrioxamine has been shown to decrease the toxicity of synthetic Aβ (Rottkamp *et al.*, 2001). It is possible that silibinin attenuates the Aβ-induced oxidative stress in the hippocampus by chelating metal ions.

Thirdly, oxidative stress occurs when the antioxidative defenses are broken down by the overproduction of reactive oxygen species. Expanding the antioxidant capacity of neurones will provide a potential strategy to protect neurones from oxidative damage (Ahlemeyer *et al.*, 2001; Alcaraz-Zubeldia *et al.*, 2001). Silibinin is known to induce the expression of antioxidative enzymes (Valenzuela *et al.*, 1989;Müzes *et al.*, 1991). Therefore, it is possible that silibinin prevents oxidative damage indirectly by activating antioxidative systems including GSH biosynthesis. It remains to be determined whether silibinin affects antioxidant enzymes in the hippocampus of Aβ-injected mice.

As another possible mechanism, silibinin may directly suppress aggregation of Aβ or stability of aggregated Aβ, so affecting Aβ conformation in the brain. Polyphenols, such as silibinin, have shown to inhibit aggregation of Aβ and exhibit significant destabilizing activity on aggregated Aβ (Ono *et al.*, 2003; Porat *et al.*, 2006; Shoval *et al.*, 2007). Because aggregated $A\beta_{25–35}$ was used in this study, it is possible that silibinin may affect the degradation of aggregated Aβ. However, further studies are needed to clarify the effect of silibinin on the conformation and levels of Aβ.

The results from the present study confirm, for the first time, that silibinin could alleviate the memory deficits induced by $A\beta_{25–35}$ in mice. The effect of silibinin may be attributed to the prevention of oxidative damage in the hippocampus, measured in terms of the amount of peroxidized lipid and the level of GSH. As a therapeutic agent, silibinin is well tolerated and largely free of adverse effects and has few negative drug interactions (Jacobs *et al.*, 2002). Therefore, silibinin is a potential candidate for further preclinical study aimed at the treatment of cognitive deficits in AD.

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Conflict of interest

The authors state no conflict of interest.

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