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Anti-amnesic effect of ESP-102 on $A\beta_{1-42}$-induced memory impairment in mice

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ABSTRACT

The aim of this study was to characterize the effects of ESP-102 on the memory impairments and pathological changes induced by amyloid-β $(A\beta)_{1-42}$ peptide in mice. The ameliorating effect of ESP-102 on memory impairment was investigated using the passive avoidance and the Morris water maze tasks, and the pathological changes were identified by immunohistochemistry and western blotting. $A\beta_{1-42}$ peptide (3 μg/3 μl) was administered by intracerebroventricular injection. By the single administration of ESP-102 (100 mg/kg, p.o), the memory impairment induced by $A\beta_{1-42}$ peptide was significantly attenuated ($P<0.05$). Moreover, ESP-102 (100 mg/kg, p.o) significantly inhibited acetylcholinesterase (AChE) activity in the hippocampus compared to the $A\beta_{1-42}$ peptide-injected control group. In the subchronic treatment study, ESP-102 (50 or 100 mg/kg/day, p.o) administration for seven days ameliorated the memory impairments induced by $A\beta_{1-42}$ peptide. Moreover, ESP-102 inhibited lipid peroxidation induced by $A\beta_{1-42}$ peptide in the hippocampus. $A\beta_{1-42}$-induced increases in the expression of GFAP (an astrocyte marker) and inducible nitric oxide synthase (iNOS) in the hippocampal region were also attenuated by ESP-102 treatment. These results suggest that the ameliorating effect of ESP-102 on $A\beta_{1-42}$ peptide-induced memory impairment is mediated via its AChE inhibitory, antioxidative, and/or anti-inflammatory activities.

1. Introduction

Alzheimer's disease (AD) is the most common age-related neurodegenerative disorder (Koo et al., 1999), characterized by cognitive decline at its onset followed by deficits in multiple cortical functions in later stages. The disease's specific hallmarks are neurofibrillary tangles (Terry, 1963) and the formation of senile plaques (Braak and Braak, 1997). Senile plaques are composed of a core of amyloid-β (Aβ) peptide aggregates and present the more complex extracellular lesions (Cotman et al., 1996; Itagaki et al., 1989). Aβ peptide has the potential to induce oxidative stress and inflammation in the brain. These factors have been postulated to play important roles in the pathogenesis of AD (Behl, 1999; McGeer and McGeer, 1999). Numerous studies suggest that Aβ promotes oxidative stress and lipid peroxidation in neuronal cultures and rodents (Butterfield et al., 1994; Mark et al., 1997; Varadarajan et al., 2001), suggesting that antioxidants and free radical scavengers protect neurons against Aβ-induced toxicity (Butterfield and Boyd-Kimball, 2005; Morris et al., 1998).

ESP-102 is a standardized combined extract of *Angelica gigas*, *Saururus chinensis* and *Schizandra chinensis*. Previous reports indicated that ESP-102 showed a significant neuroprotective activity against neurotoxicity induced by glutamate in primary cultures of rat cortical cells (Ma et al., 2009). Moreover, acute and prolonged daily oral treatments with ESP-102 significantly reduced scopolamine-induced memory deficits in both the passive avoidance and the Morris water maze tasks in mice (Kang et al., 2005). We also observed that the extracts of *A. gigas* or *S. chinensis* ameliorate cognitive dysfunction induced by scopolamine, a nonspecific muscarinic receptor antagonist, in mice (unpublished data). Therefore, it is valuable to know whether ESP-102 containing these herbal materials attenuates cognitive dysfunction caused by $A\beta_{1-42}$ peptide. In the present study, we measured the ameliorating effects of ESP-102 on the $A\beta_{1-42}$ peptide-induced memory dysfunction with behavioral studies. In addition, we also investigated whether ESP-102 attenuates $A\beta_{1-42}$ peptide-induced inflammatory responses, such as astrocyte activation and enhancement of inducible nitric oxide synthase (iNOS) expression.

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2. Materials and methods

2.1. Animals

Animal maintenance and treatment were carried out in accordance with the Principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1985) and the Animal Care and Use Guidelines of Kyung Hee University, Korea. Male ICR mice (25–30 g) were purchased from the Orient Co., Ltd. (a branch of Charles River Laboratories, Seoul). Animals were housed 5 per cage, allowed access to water and food ad libitum, and maintained under a constant temperature (23 ± 1 °C) and relative humidity ($50 \pm 10\%$) under a 12-h light/dark cycle (light on 07.30–19.30 h).

2.2. Materials

ESP-102 and donepezil were donated by Daewoong Pharmaceuticals Co. (Seoul, Korea). ESP-102 is a combined preparation of the standardized extract of *A. gigas* roots, *S. chinensis* herb and *S. chinensis* fruits. The ratio of the three different components adjusted for ESP-102 was 8:1:1 (*A. gigas*: *S. chinensis*: *Shizandra sinensis*). This ratio had been shown to be the highest effective in the previous study and its effects had been higher than that of *A. gigas* alone (Kang et al., 2005). Decursin in *A. gigas*, sauchinone in *S. chinensis*, and schizandrin in *S. sinensis* were used to ensure preparation consistencies. The mean levels of decursin, sauchinone, and schizandrin in ESP-102 were $3.02 \pm 1.49\%$, $0.05 \pm 0.03\%$, and $0.04 \pm 0.03\%$, respectively. ESP-102 was suspended in a 0.5% carboxymethylcellulose (CMC)-saline solution according to the desired concentration (0.1, 1 and 10 mg/ml for doses of 1, 10 and 100 mg/kg body weight, respectively). Anti-glial fibrillary acidic protein (GFAP), anti-iNOS, and anti-β-actin antibodies were purchased from Chemicon (Temecula, CA). $A\beta_{1-42}$ peptide was purchased from Sigma Chemical Co. (St. Louis, MO). All other materials used were of the highest grades available from normal commercial sources.

2.3. $A\beta_{1-42}$ peptide injection and drug administration

$A\beta_{1-42}$ peptide was dissolved in sterile saline (1 μg/μl) in tubes, which were then sealed and incubated for 24 h at 37 °C to cause the peptide to aggregate. Mice were injected with aggregated $A\beta_{1-42}$ peptide or vehicle (3 μl/3 min, i.c.v.) into the right lateral ventricle at stereotaxic coordinates (AP, −0.2 mm; ML, ±1.0 mm; DV, −2.5 mm) taken from the atlas of mouse brain (Paxinos and Franklin, 2001) under anesthesia (a mixture of N_2O and O_2 (70:30) containing 2% isoflurane). After 5 min, the needle was removed using three intermediate steps with 1 min inter-step delay to minimize backflow, and mice were kept on a warm pad until awakened. Sham animals were injected in an identical manner with the same amount of sterile saline (3 μl). In an acute treatment study, mice were administered $A\beta_{1-42}$ and ESP-102 (12.5, 25, 50, or 100 mg/kg, p.o) 1 h before behavioral testing at 7 days after the administration of $A\beta_{1-42}$. To examine the effects of ESP-102 in terms of its memory-ameliorating and neuroprotective properties, mice were administered $A\beta_{1-42}$ and then 1 h later ESP-102 (12.5, 25, 50, or 100 mg/kg, p.o) treatment was started, ESP-102 was administered once a day for 7 days. Mice in the control group were orally given 0.5% CMC-saline. Behavioral tests were conducted 24 h after the last administration of ESP-102 (Fig. 1). To investigate the effect of ESP-102 alone on memory in unimpaired animals, the treatments and experiments were conducted under the same condition of $A\beta_{1-42}$ treatment study without $A\beta_{1-42}$ injection.

2.4. The passive avoidance task

The passive avoidance task was conducted seven or eight days after $A\beta_{1-42}$ peptide injection in acute and subchronic administration studies, respectively. Testing was carried out in identical illuminated and non-illuminated boxes ($20 \times 20 \times 20$ cm), separated by a guillotine door (5×5 cm). The illuminated compartment contained a 50 W bulb, and the floor of the non-illuminated compartment ($20 \times 20 \times 20$ cm) was composed of 2 mm stainless steel rods spaced 1 cm apart as described elsewhere (Kim et al., 2008). For an acquisition trial, mice were initially placed in the illuminated compartment. The door between the two compartments was opened 10 s later. When mice entered the non-illuminated compartment, the door automatically closed and an electrical foot shock (0.5 mA) of 3 s duration was delivered through the stainless steel rods. Twenty-four hours after the acquisition trial, a retention trial was conducted by returning individual mice to the illuminated compartment. The time taken for a mouse to enter the dark compartment after door opening was defined as latency for both trials. Latencies were recorded for up to 300 s. The drug administration schedule for each study is shown in Fig. 1. The administration of ESP-102 or donepezil (1 mg/kg, p.o), a positive control, was conducted 1 h before the acquisition trial in the acute administration study. In the subchronic administration study, ESP-102 was administered for 7 days, and the acquisition trial was conducted 24 h after the last treatment of ESP-102. To investigate the effect of ESP-102 alone on memory in unimpaired animals, the treatment and experiment were conducted under the same condition of $A\beta_{1-42}$ treatment study without $A\beta_{1-42}$ injection.

2.5. Morris water maze task

The Morris water maze task was started seven days after $A\beta_{1-42}$ peptide injection. The Morris water maze is a circular pool (90 cm in diameter and 45 cm in height) with a featureless inner surface. The pool was filled with water containing 500 ml of milk (20 ± 1 °C). The tank was placed in a dimly lit, soundproof test room with various visual cues. The pool was conceptually divided into quadrants. A white platform (6 cm in diameter and 29 cm high) was then placed in one of the pool quadrants and submerged 0.5 cm below the water surface so that it was invisible at water level. The first experimental day was dedicated to swimming training for 60 s in the absence of the platform. During the four subsequent days the mice were subject to four trials per session per day with the platform in place as previously described elsewhere (Kim et al., 2007). When a mouse located the platform, it was permitted to remain on it for 10 s. If the mouse did not locate the platform within 60 s, it was placed on the platform for 10 s. The animal was taken to its home cage and was allowed to dry under an infrared lamp after each trial. The time interval between each trial was 30 s. During each trial, the time taken to find the hidden platform (latency) was recorded using a video camera-based Ethovision System (Nodulus, Wageningen, The Netherlands). For each training trial, mice were placed in the water facing the pool wall at one of the pool quadrants within a day. In an acute treatment study, ESP-102 or donepezil (1 mg/kg, p.o.), a positive control, was administered 1 h before the first trial on each training day (Fig. 1). In a subchronic administration study, the Morris water maze task was conducted from 7 days after the initiation of daily ESP-102 administration; drugs were not administered during training. One day after the last training trial, mice were subjected to a probe trial in which the platform was removed from the pool, allowing the mice to swim for 60 s in search of it. We recorded swimming time in the pool quadrant and the number of times mice crossed the zone where the platform had previously been placed, as well as swimming speed. The dosage employed in the Morris water maze task was obtained from the results of the passive avoidance task. To investigate the effect of ESP-102 alone on memory in unimpaired animals, the treatments and experiments were conducted under the same condition of $A\beta_{1-42}$ treatment study without $A\beta_{1-42}$ injection.

2.6. Acetylcholinesterase activity assay

Seven days after $A\beta_{1-42}$ peptide injection, mice were decapitated 1 h after drug administration, and hippocampi were separated. Samples

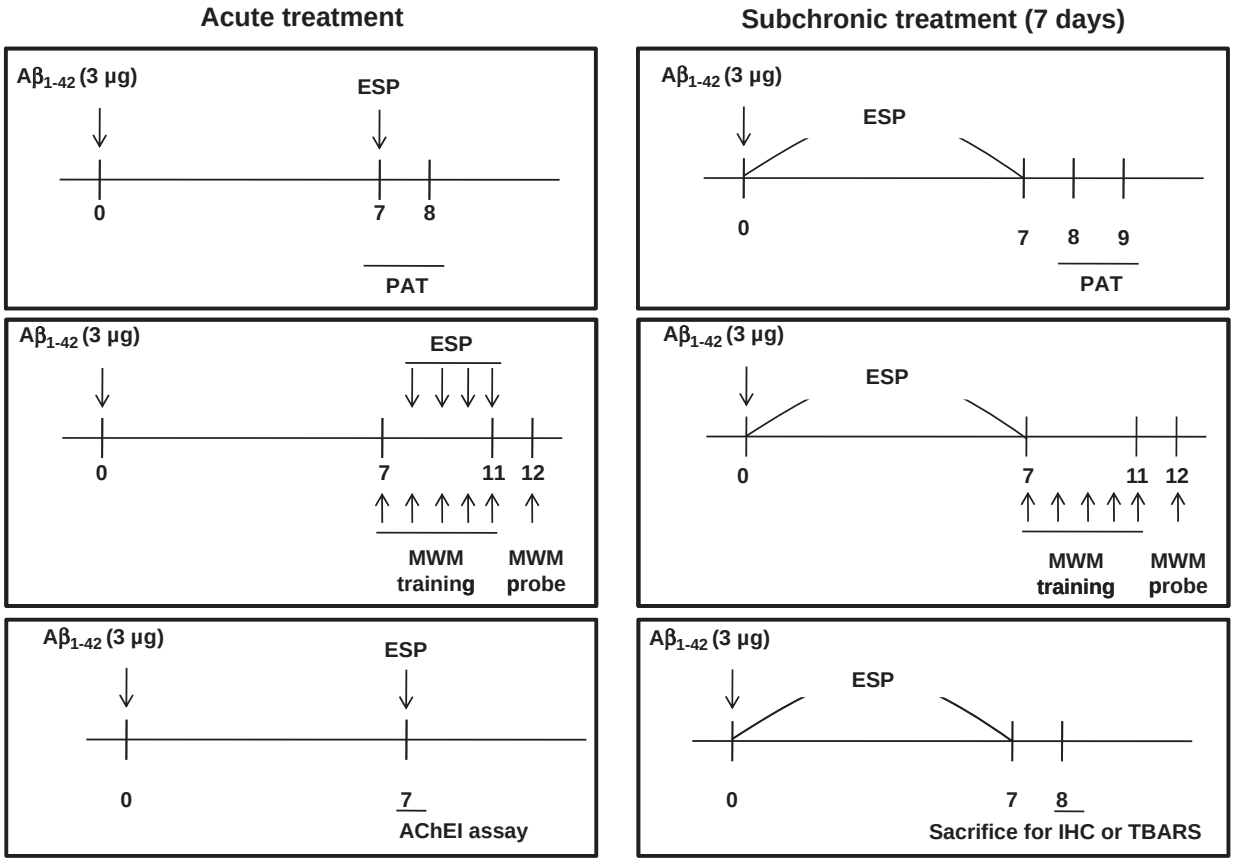

Fig. 1. Experimental schedules. Animals were intracerebroventrically injected with $A\beta_{1-42}$ peptide (3 μg/3 μl). For the investigation of the effect of single administration of ESP-102 (ESP), ESP-102 was administered 7 days after $A\beta_{1-42}$ peptide injection. Mice were then introduced to the behavioral tasks [passive avoidance (PAT) or Morris water maze task (MWM)]. The acetylcholinesterase inhibition (AChEI) assay was also conducted 1 h after the administration of ESP-102 at 7 days after $A\beta_{1-42}$ peptide injection. The neuroprotective effect of ESP-102 was examined after the subchronic administration of ESP-102 for 7 days (once a day) from 1 h after $A\beta_{1-42}$ injection using immunohistochemistry (IHC) and measurements of thiobarbituric acid reactive substance (TBARS). The final administration was completed 24 h before behavioral testing.

were immediately homogenized in a glass Teflon homogenizer (Eyela, Japan) containing 4 vol of homogenization buffer (12.5 mM sodium phosphate buffer pH 7.0, 400 mM NaCl), and then centrifuged at 1000×*g* for 10 min at 4 °C. The supernatant obtained was used as source of enzyme for the assay. Then 500 μl of sodium phosphate buffer (100 mM, pH 8.0), 100 μl of buffered Ellman's reagent [10 mM 5, 5′-dithio-bis (2-nitrobenzoic acid) and 15 mM sodium bicarbonate], and enzyme (400 μl) were mixed and incubated for 30 min. The reaction was initiated by adding 20 μl of acetylthiocholine iodide solution (75 mM) and stopped by adding 10 μl of neostigmine (10 μM). Absorbance was measured at 410 nm immediately after adding 20 μl of acetylthiocholine iodide solution (75 mM) to the reaction mixtures (OPTIZEN 2120UV, Mecasys Co. Ltd., Korea). Readings were taken 3 min after the reaction. To investigate the effect of ESP-102 alone on basal acetylcholinesterase activity, the treatment and experiment were conducted under the same condition of $A\beta_{1-42}$ treatment study without $A\beta_{1-42}$ injection.

2.7. Lipid peroxidation

After $A\beta_{1-42}$ peptide injection, mice were treated with ESP-102 or vehicle for 7 days. One day after the last administration of drugs or vehicle, mice were decapitated, and brains were isolated. Isolated hippocampal tissue was homogenized in an ice-chilled KCl buffer (1.15% KCl–10 mM phosphate buffer with 5 mM EDTA, pH 7.4, 200 μl and protease inhibitor). Samples (100 μg for protein) were mixed with trichloroacetic acid (1 ml of a 10% w/v solution) and 1 ml of 0.67% (w/v) thiobarbituric acid solution was added, and then the reaction mixture was heated at 100 °C for 30 min. After cooling and centrifugation at 3000×*g* for 10 min, thiobarbituric acid reactive substance (TBARS) levels were determined by measuring chromogen absorbance at 535 nm (OPTIZEN 2120UV, Mecasys Co. Ltd., Korea). Tetramethoxypropane was used as standard. To investigate the effect of ESP-102 alone on basal lipid peroxidation, the treatments and experiment were conducted under the same condition of $A\beta_{1-42}$ treatment study without $A\beta_{1-42}$ injection.

2.8. Tissue preparation

After $A\beta_{1-42}$ peptide injection, mice were treated with drugs or vehicle for 7 days. One day after the last administration of drugs or vehicle, mice were anesthetized with Zoletil 50® (10 mg/kg, i.m.) and transcardially perfused with 0.1 M phosphate buffer (pH 7.4) followed by an ice-cold 4% paraformaldehyde. Brains were removed and postfixed in the phosphate buffer (0.05 M, pH 7.4) containing 4% paraformaldehyde overnight and then immersed in a solution containing 30% sucrose in 0.05 M phosphate-buffered saline (PBS) and stored at 4 °C until sectioned. Frozen brains were coronally sectioned on a cryostat at 30 μm and then stored in a storage solution at 4 °C.

2.9. Immunohistochemistry

Free floating sections were incubated for 24 h in PBS (4 °C) containing monoclonal anti-GFAP antibody (1:1000 dilution), anti-iNOS antibody (1:500 dilution), 0.3% Triton X-100, 0.5 mg/ml bovine serum albumin, and 1.5% normal horse serum. The sections were then incubated for 90 min with biotinylated secondary antibody (1:200 dilution), treated with avidin–biotin–peroxidase complex (1:100 dilution) for 1 h at room

temperature, and reacted with 0.02% 3, 3′-diaminobenzidine and 0.01% H_2O_2 for approximately 3 min. After each incubation step, sections were washed three times with PBS. Finally, they were mounted on gelatin-coated slides, dehydrated in an ascending alcohol series, and cleared in xylene. Cells in the hippocampal CA1 region were counted in 5 sections (using one of every six sections from −1.5 mm of bregma) per mouse in 3 mice per group by one person unaware of treatment history.

2.10. Western blot analysis

After $A\beta_{1-42}$ peptide injection, mice were treated with ESP-102 or vehicle for 7 days. One day after the last administration of drugs or vehicle, mice were decapitated, and brains were isolated. Isolated hippocampal tissues were homogenized in an ice-chilled Tris–HCl buffer (20 mM, pH 7.4) containing 0.32 M sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM sodium orthovanadate and one tablet of protease inhibitor (Roche, Seoul, Korea) per 50 ml of buffer. Samples of homogenates (15 μg of protein) were then subjected to SDS-PAGE (8% gel) under reducing conditions. Proteins were transferred to PVDF membranes in the transfer buffer [25 mM Tris–HCl buffer (pH 7.4) containing 192 mM glycine and 20% v/v methanol] at 400 mA for 2 h at 4 °C. Western blots were then incubated for 3 h with a blocking solution (5% skim milk) at room temperature, then with a 1:2000 dilution of anti-iNOS antibody for 24 h at 4 °C, washed ten times with Tris-buffered saline/Tween 20 (TBST), incubated with a 1:5000 dilution of horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature, washed ten times with TBST, and finally developed by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL). Blots were then stripped and incubated with anti-β-actin antibody (1:2000). Film densitometry was performed using the Quantity One Image Analysis System (version 4.6.3, Bio-Rad Laboratories, CA). To investigate the effect of ESP-102 alone on basal iNOS expression, the treatment and experiment were conducted under the same condition of $A\beta_{1-42}$ treatment study without $A\beta_{1-42}$ injection.

2.11. Statistics

Values are expressed as the means ± S.E.M. Passive avoidance task results were analyzed using a Kruskal–Wallis non-parametric ANOVA test, and when results were found significant, treatment groups were compared using Dunn's post-hoc test. In a particular, group differences in the Morris water maze escape latencies were analyzed using two-way ANOVA with repeated measures and data from probe trials were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons. TBARS, immunohistochemical, and western blot results were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey's post-hoc test for multiple comparisons. Statistical significance was accepted at the $P<0.05$ level.

3. Results

3.1. Effect of acute administration of ESP-102 on $A\beta_{1-42}$-induced memory impairment

A significant group effect was observed on step-through latency during the retention trial in the passive avoidance task [$F(6, 63) = 10.270$, $P<0.001$] (Fig. 2A). The step-through latency of the $A\beta_{1-42}$-treated group was significantly shorter than that of the sham group

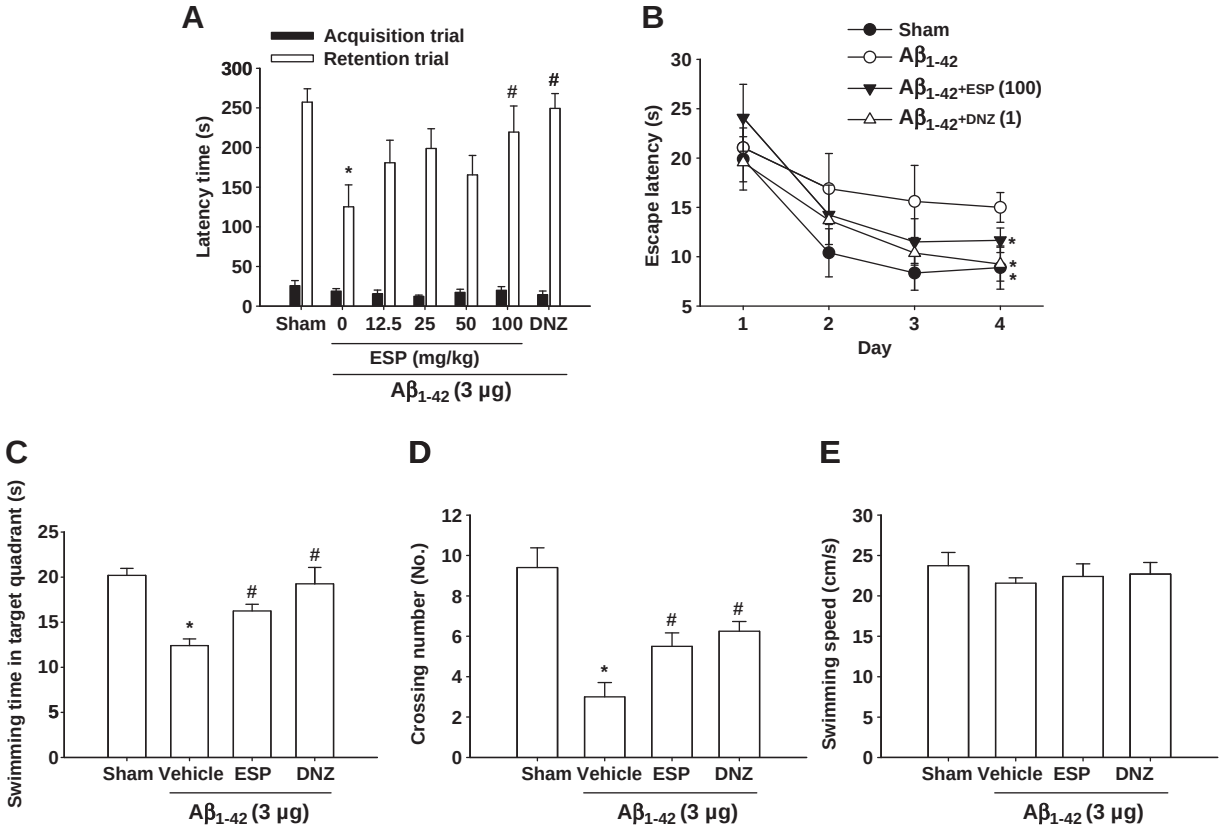

Fig. 2. Anti-amnesic effect of acute administration of ESP-102 (ESP) on $A\beta_{1-42}$-induced memory deficits. A. The results of the passive avoidance task. ESP-102 (12.5, 25, 50, or 100 mg/kg, p.o) or the same volume of 5% carboxymethyl cellulose (CMC) solution was administered 1 h before an acquisition trial. The retention trial was performed 24 h after the acquisition trial. Donepezil (DNZ, 1 mg/kg, p.o) was used as a positive control. B–E. The results of the Morris water maze task. ESP-102 (100 mg/kg, p.o) or the same volume of 5% CMC solution was administered 1 h before the first training trial of each consecutive day. Escape latency during each training trial session (B), swimming time in the pool quadrant where the platform had previously been placed (C), number of times crossed the zone where the platform had previously been placed (D), and swimming speed (E) during the probe trial session were presented. Data represent means ± S.E.M. *$P<0.05$, compared with the sham group. #$P<0.05$, compared with the $A\beta_{1-42}$ control group.

(Fig. 2A, $P<0.05$). In donepezil (a positive control) plus $A\beta_{1-42}$-treated group, step-through latency was significantly greater than that of the $A\beta_{1-42}$-treated group. Moreover, the reduction in step-through latency induced by $A\beta_{1-42}$ was significantly reversed by ESP-102 (100 mg/kg, $P<0.05$). In the ESP-102 alone-treated study on the passive avoidance task, the latency time was significantly increased in the ESP-102 (100 mg/kg)-treated group compared to vehicle-treated control group (Fig. 3A, $P<0.05$).

In the Morris water maze task, the $A\beta_{1-42}$-treated group exhibited longer escape latencies (the time taken to find the platform) throughout training sessions in comparison to those of the vehicle-treated controls [$F(1, 72)=22.392$, $P<0.001$, Fig. 2B]. ESP-102 (100 mg/kg) significantly attenuated the effects of $A\beta_{1-42}$ on escape latency [$F(1, 72)=10.053$, $P=0.002$, Fig. 2B], as did donepezil [$F(1, 72)=20.905$, $P<0.001$, Fig. 2B]. On the day of probe testing, a significant group effect on swimming times [$F(3, 36)=10.198$, $P<0.001$, Fig. 2C] and crossing number [$F(3, 36)=27.604$, $P<0.001$, Fig. 2D] in the probe test was observed. Swimming times and crossing number within the platform quadrant in the $A\beta_{1-42}$-treated group were significantly lower than those in the vehicle-treated control group (Fig. 2C and D, $P<0.05$). Moreover, the shorter swimming times and crossing number within the platform quadrant induced by $A\beta_{1-42}$ peptide were significantly reversed by ESP-102 or donepezil (Fig. 2C and D, $P<0.05$). Swimming speed during the probe trial was not affected by any drug treatment (Fig. 2E). However, in the ESP-102 alone-treated study, there was no significant change in all of behavioral parameters in both training and probe trials (Fig. 3B–E, $P>0.05$).

In the AChE assay, ESP-102 significantly inhibited AChE activity in the hippocampus compared to $A\beta_{1-42}$ peptide-treated mice [$F(6, 21)=4.461$, $P=0.005$, Fig. 4A]. Moreover, AChE activity was significantly inhibited in the ESP alone-treated group compared to the vehicle-treated control group (Fig. 4B, $P<0.05$).

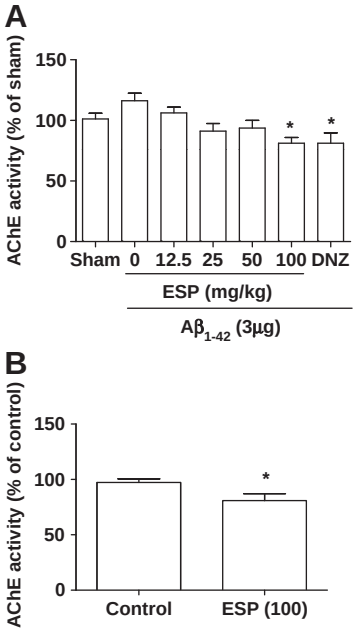

Fig. 4. Inhibitory effect of ESP-102 (ESP) on acetylcholinesterase (AChE) activity in $A\beta_{1-42}$-injected (A) and normal (B) mouse hippocampal tissue. A. Animals ($n=4$ per group) were treated with vehicle, ESP-102 (100 mg/kg, p.o.), or donepezil (DNZ, 1 mg/kg, p.o.) 7 days after $A\beta_{1-42}$-injection and sacrificed at 1 h after each treatment. AChE activities were determined as described in Materials and methods. Data represent means ± S.E.M. ($n=4$/group). *$P<0.05$ versus the $A\beta_{1-42}$ control group. B. Animals ($n=4$ per group) were treated with vehicle or ESP-102 (100 mg/kg, p.o.) and sacrificed at 1 h after each treatment. AChE activities were determined as described in Materials and methods in the manuscript. Data represent means ± S.E.M. ($n=4$/group). *$P<0.05$ versus the control group.

3.2. Effect of subchronic administration of ESP-102 on $A\beta_{1-42}$-induced memory impairment

In the ESP-102 alone-treated study, administration of ESP-102 for 7 days (behavioral studies were conducted 24 h after the last drug treatment) had no effect on the passive avoidance (Fig. 5A, $P>0.05$) and the Morris water maze tasks (Fig. 5B–E, $P>0.05$). However, a significant group effect was observed for step-through latency during the retention trial in the passive avoidance task [$F(6, 63)=12.721$, $P<0.001$, Fig. 6A]. The step-through latency in the $A\beta_{1-42}$-treated group was significantly

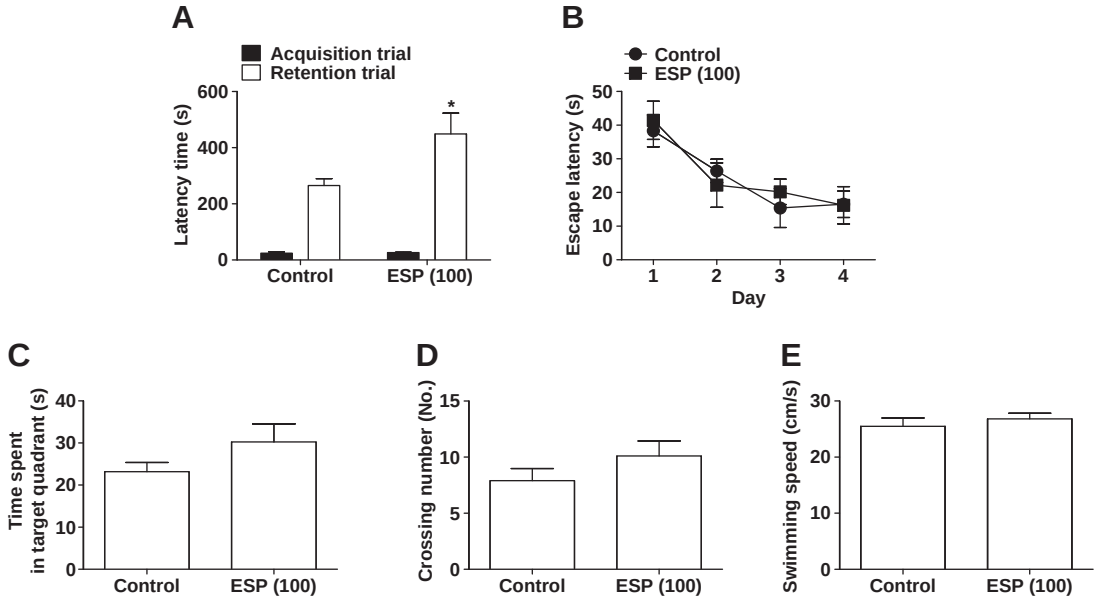

Fig. 3. The effect of acute administration of ESP-102 (ESP) alone on learning and memory in normal naive mouse. A. The results of the passive avoidance task. Normal naïve mouse was administered ESP-102 (100 mg/kg, p.o) or the same volume of 5% carboxymethyl cellulose (CMC) solution 1 h before an acquisition trial. The retention trial was performed 24 h after the acquisition trial. B–E. The results of the Morris water maze task. ESP-102 (100 mg/kg, p.o) or the same volume of 5% CMC solution was administered 1 h before the first training trial of each consecutive day. One session comprised 2 training trials. Escape latency during each training trial session (B), time spent in the pool quadrant where the platform had previously been placed (C), number of times crossed the zone where the platform had previously been placed (D), and swimming speed (E) during the probe trial session were presented. Behavioral studies were determined as described in Materials and methods in the manuscript. Data represent means ± S.E.M. ($n=10$/group). *$P<0.05$ versus the control group.

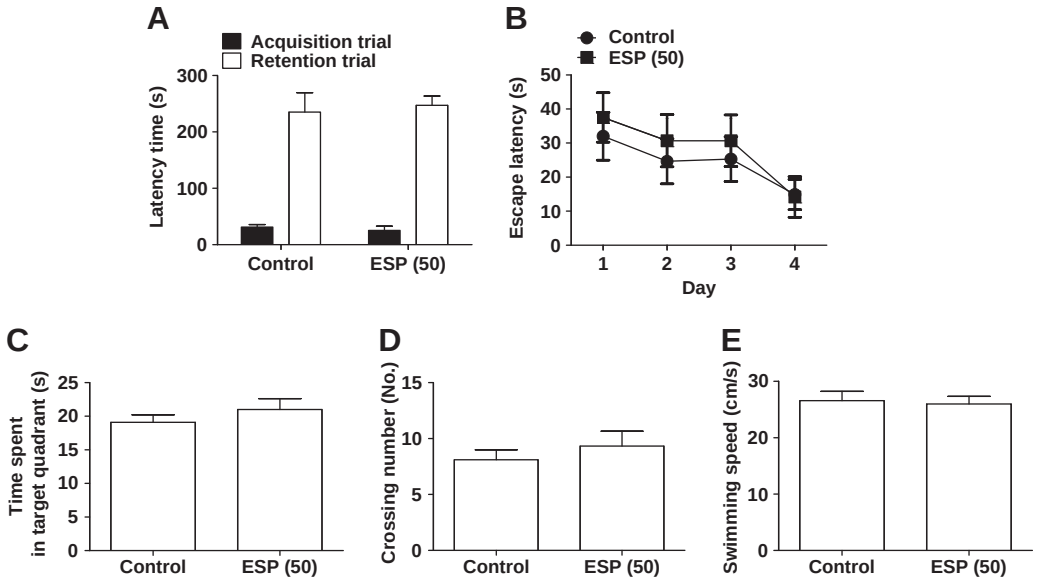

Fig. 5. The effect of subchronic administration of ESP-102 (50 mg/kg, for 7 days) alone on learning and memory in normal naïve mice. A. The results of the passive avoidance task. Normal naïve mouse was administered ESP-102 (50 mg/kg, p.o) or the same volume of 5% carboxymethyl cellulose (CMC) solution for 7 days. The acquisition trial was conducted 24 h after the last administration of drugs and the retention trial was performed 24 h after the acquisition trial. B–E. The results of the Morris water maze task. ESP-102 (50 mg/kg, p.o) or the same volume of 5% CMC solution was administered for 7 days. The first training trial of the first training session was conducted 24 h after the last administration of drugs. One session comprised 2 training trials. Escape latency during the training trial session (B), time spent in the pool quadrant where the platform had previously been placed (C), crossings of the zone where the platform had previously been placed (D), and swimming speed (E) during the probe trial session were presented. Data represent means ± S.E.M. (n = 10 per group).

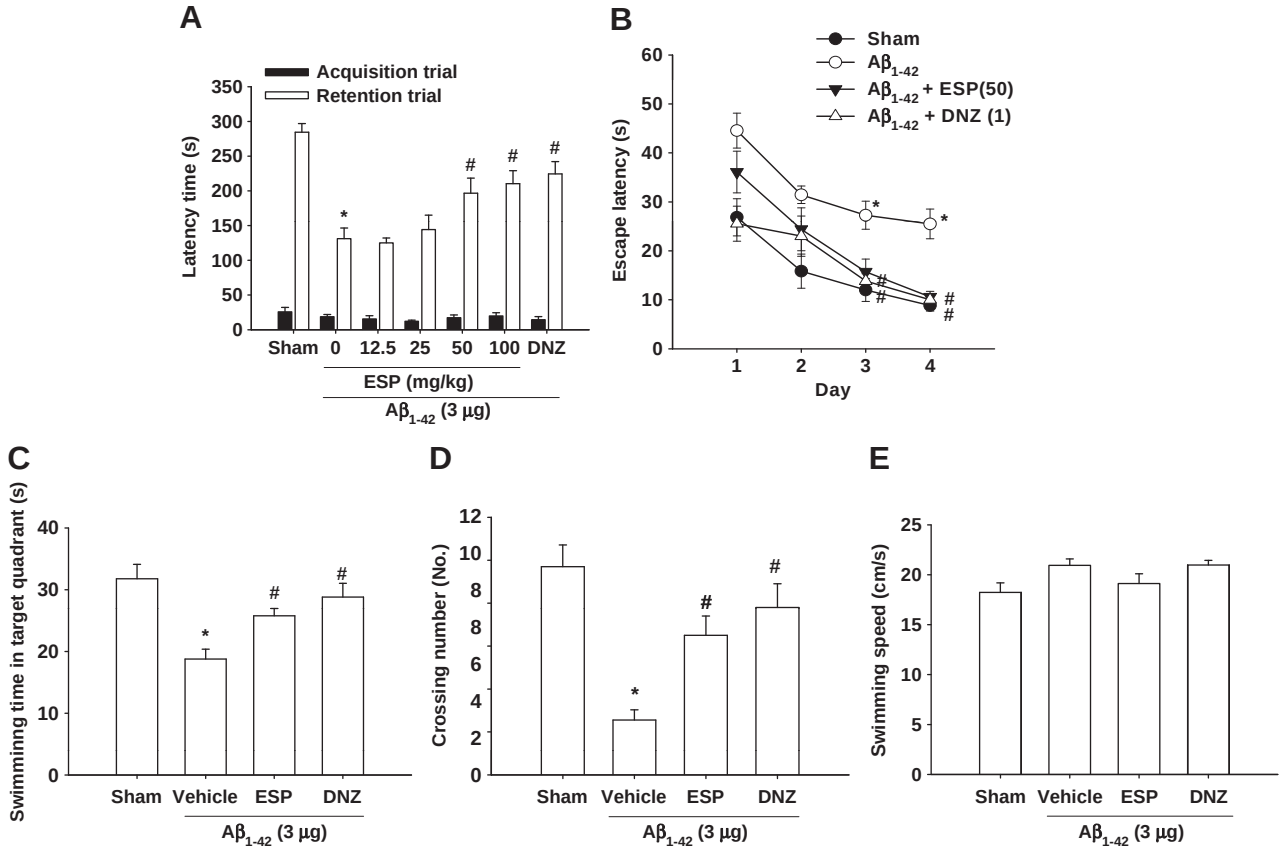

Fig. 6. Anti-amnesic effect of subchronic administration of ESP-102 (ESP) on $A\beta_{1-42}$-induced memory deficits. A. The results of the passive avoidance task. ESP-102 (12.5, 25, 50, or 100 mg/kg, p.o), donepezil (DNZ, 1 mg/kg, p.o), or the same volume of 5% carboxymethyl cellulose (CMC) solution was administered for 7 days from 1 h after $A\beta_{1-42}$ injection. The acquisition trial was conducted 24 h after the last administration of drugs and retention trial 24 h after the acquisition trial. B–E. The results of the Morris water maze task. ESP-102 (50 mg/kg, p.o), donepezil (1 mg/kg, p.o), or the same volume of 5% CMC solution was administered for 7 days from 1 h after $A\beta_{1-42}$ injection. The first training trial of the first training session was conducted 24 h after the last administration of drugs. Escape latency during the training trial session (B), swimming time in the pool quadrant where the platform had previously been placed (C), crossings of the zone where the platform had previously been placed (D), and swimming speed (E) during the probe trial session were presented. Data represent means ± S.E.M. (n = 10 per group). *P<0.05, compared with the sham group. #P<0.05, compared with the $A\beta_{1-42}$ control group.

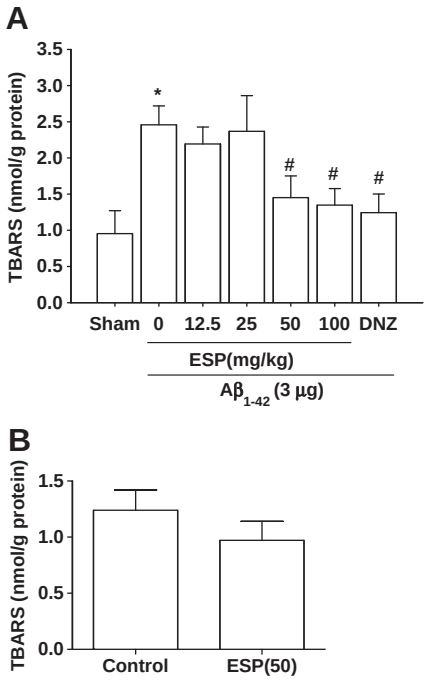

Fig. 7. Effects of ESP-102 (ESP) on lipid peroxidation in $A\beta_{1-42}$-induced (A) and in normal naïve mice (B) as determined by thiobarbituric acid reactive substance (TBARS) assay. A. ESP-102 (12.5, 25, 50, or 100 mg/kg/day, p.o), donepezil (DNZ, 1 mg/kg/day, p.o) or the same volume of 5% carboxymethyl cellulose (CMC) solution was administered for 7 days from 1 h after $A\beta_{1-42}$-injection. Mice were sacrificed 24 h after the last administration of ESP-102 for TBARS assay. TBARS were determined as described in Materials and methods. Data represent means ± S.E.M. (n = 4/group). *P<0.05, compared with the sham group. #P<0.05, compared with the $A\beta_{1-42}$-treated control group. B. ESP-102 (50 mg/kg/day, p.o) or the same volume of 5% carboxymethyl cellulose (CMC) solution was administered for 7 days in normal naïve mouse. Mice were sacrificed 24 h after the last administration of ESP-102 for those assays. Data represent means ± S.E.M. (n = 3/group).

shorter than that of the sham-treated group (Fig. 6A, P<0.05). In the donepezil (a positive control) plus $A\beta_{1-42}$-treated group, step-through latency was significantly greater than that of the $A\beta_{1-42}$-treated group. Moreover, the reduction in step-through latency induced by $A\beta_{1-42}$ was significantly reversed by ESP-102 (50 or 100 mg/kg/day, P<0.05).

In the Morris water maze task, the $A\beta_{1-42}$-treated group exhibited longer escape latencies (the time taken to find the platform) throughout training trial sessions than did the vehicle-treated controls [F (1, 72) = 75.613, P<0.001, Fig. 6B]. ESP-102 (50 or 100 mg/kg/day) significantly attenuated the effects of $A\beta_{1-42}$ on escape latency [F (1, 72) = 27.402, P<0.001, Fig. 6B], as did donepezil [F (1, 72) = 56.062, P<0.001, Fig. 6B]. On the day of probe testing, a significant group effect was observed on swimming times [F (3, 36) = 8.080, P<0.001, Fig. 6C] and crossing number [F (3, 36) = 11.770, P<0.001, Fig. 6D] in the probe test. Swimming times and number of crossing within the platform quadrant for the $A\beta_{1-42}$-treated group were significantly lower than those of the vehicle-treated control group (Fig. 6C and D, P<0.05). Swimming speed during the probe trial was not affected by any of the drugs examined (Fig. 6E).

3.3. Effect of ESP-102 on $A\beta_{1-42}$-induced hippocampal lipid peroxidation

The lipid peroxide levels found in the hippocampi of the $A\beta_{1-42}$-treated group are shown in Fig. 6A. $A\beta_{1-42}$ treatment increased lipid peroxide levels as determined by TBARS assay using the hippocampal tissue, and ESP-102 (50 or 100 mg/kg/day) and donepezil (1 mg/kg/day) significantly reduced these increases [F (6, 21) = 11.070, P<0.001, Fig. 7A]. However, ESP-102 alone had no effect on basal lipid peroxidation in normal naïve mouse (Fig. 7B, P>0.05).

3.4. Effect of ESP-102 on GFAP and iNOS expression

To investigate the effect of ESP-102 on $A\beta_{1-42}$-induced astrocyte activation, we conducted immunohistochemistry using GFAP antibody, a marker of astrocyte. The non-activated form of GFAP-positive cells was

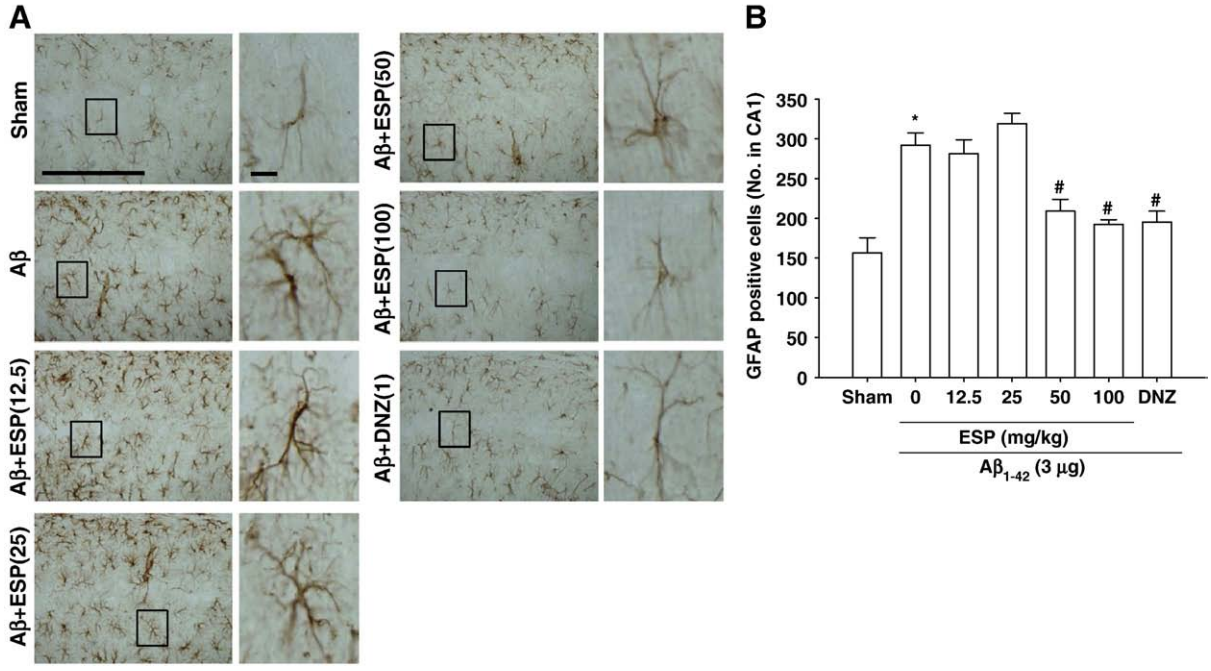

Fig. 8. Effect of ESP-102 (ESP) on astrocyte activation in the hippocampal CA1 regions. ESP-102 (12.5, 25, 50, or 100 mg/kg/day, p.o), donepezil (DNZ, 1 mg/kg/day, p.o) or the same volume of 5% carboxymethyl cellulose (CMC) solution (Sham) was administered to mice for 7 days from 1 h after $A\beta_{1-42}$ injection. Mice were perfused and fixed for immunohistochemistry 24 h after the last administration of ESP-102. A. Representative photographs of GFAP-immunopositive cells in the hippocampal CA1 region. B. Histogram representing the number of GFAP-immunopositive cells in the hippocampal CA1 region. Data represent means ± S.E.M. (n = 3/group). *P<0.05, compared with the sham group. #P<0.05, compared with the $A\beta_{1-42}$-treated control group. Low-power photographs, magnification = 400×, Bar = 250 μm; high-power photographs of rectangular region, magnification = 1000×, Bar = 25 μm.

observed in the sham group (Fig. 8A). However, in the $A\beta_{1-42}$-treated control group, the number of GFAP-positive cells in the hippocampus increased significantly and then dropped to control levels following treatment with ESP-102 (50 or 100 mg/kg/day) or donepezil (1 mg/kg/day) [$F(6, 14) = 79.032$, $P < 0.001$, Fig. 8A and B]. As shown in Fig. 9A, in the sham group, iNOS-positive cells were merely observed in the hippocampal CA1 region. However, $A\beta_{1-42}$ treatment induced hypertrophy in iNOS-positive cells, which was attenuated by the ESP-102 treatment (50 or 100 mg/kg/day, Fig. 9A). Western blotting revealed that ESP-102 also attenuated increased iNOS expression levels in hippocampal tissues [$F(6, 14) = 6.570$, $P = 0.001$, Fig. 9B and C], as did donepezil. However, ESP-102 alone had no effect on basal iNOS expression in normal naïve mouse (Fig. 9D and E, $P > 0.05$).

4. Discussion

It has been demonstrated that cerebral cholinergic dysfunction is significantly correlated with the severity of dementia and with the cognitive impairments observed in AD (Wilcock et al., 1982). Moreover, cholinesterase inhibitors and choline acetyltransferase activators may compensate for reduced acetylcholine levels in the AD brain. Experimental and clinical studies indicate that acetylcholine plays a major role in the regulation of cognitive functions (Blokland, 1995). Previously, it was reported that ESP-102 has an anti-amnesic effect on the scopolamine-induced memory impairment mouse model (Kang et al., 2005). The same group also reported that methanolic extract of the root of *A. gigas*, a major constituent of ESP-102 and its major compounds, decursin or decursinol, ameliorated scopolamine-induced memory deficits through its inhibition of AChE (Kang et al., 2003, 2001). Moreover, gomisin A or schizandrin, major compounds of the fruits of *S. chinensis*, reversed memory impairment induced by scopolamine through the enhancement of cholinergic function (Egashira et al., 2008; Kim et al., 2006). In the present study, a single administration of ESP-102 ameliorated memory impairment induced by $A\beta_{1-42}$ injection in the passive avoidance and the Morris water maze tasks. The ameliorating activities of single administration of ESP-102 on cognitive dysfunction are not likely derived from neuroprotective properties but rather from modulation of neurotransmitter systems, such as the cholinergic neurotransmitter system. In the present study, the ESP-102-treated group revealed a significant decrease of AChE activity in the hippocampal tissue compared to the $A\beta_{1-42}$-injected group. Therefore, it is likely that the effects of a single administration of ESP-102 on memory impairment induced by $A\beta_{1-42}$ injection were derived from the enhancement of cholinergic function. However, other mechanisms cannot be ruled out because various neurotransmitter systems

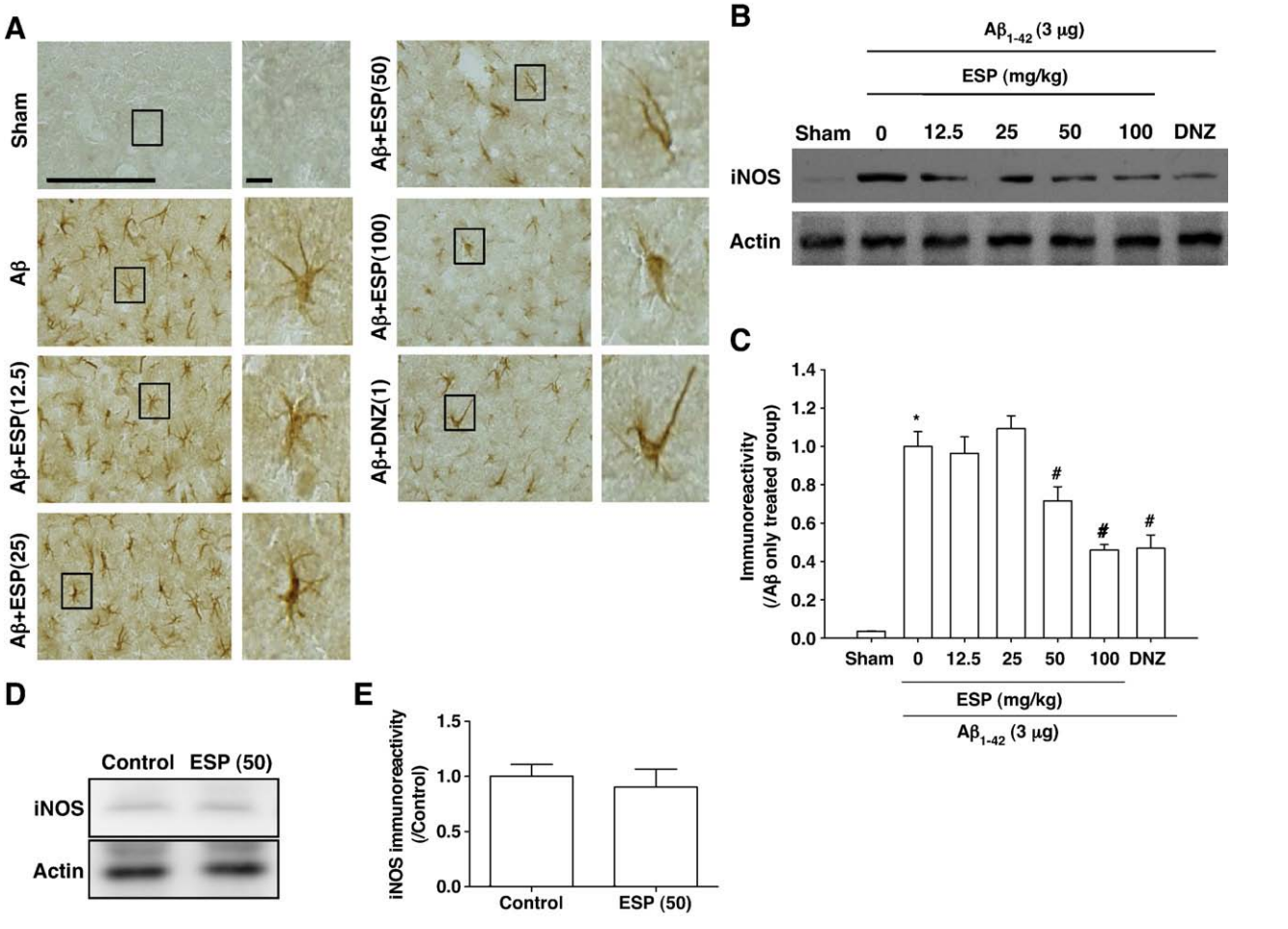

Fig. 9. Effect of ESP-102 (ESP) on iNOS expression in the hippocampus in $A\beta_{1-42}$-injected (A–C) and normal mice (D–E). A–C. ESP-102 (12.5, 25, 50, or 100 mg/kg/day, p.o), donepezil (DNZ, 1 mg/kg/day, p.o), or the same volume of 5% carboxymethyl cellulose (CMC) solution (Sham) was administered to mice for 7 days from 1 h after $A\beta_{1-42}$ injection. Mice were perfused and fixed for immunohistochemistry 24 h after the last administration of ESP-102. A. Representative photographs of iNOS-immunopositive cells in the hippocampal CA1 region. B. Photographs of iNOS western blot data. C. Densitometric analysis of iNOS western blot. Data represent means ± S.E.M. (n = 3/group). *$P < 0.05$, compared with the sham group. #$P < 0.05$, compared with the $A\beta_{1-42}$-treated control group. Low-power photographs, magnification = 400×, Bar = 250 μm; high-power photographs, magnifications of rectangular region, magnification = 1000 X, Bar = 25 μm. D–E. Normal naïve mouse was administered ESP-102 (50 mg/kg/day, p.o) or the same volume of 5% carboxymethyl cellulose (CMC) solution for 7 days. Mice were sacrificed 24 h after the last administration of ESP-102 for those assays. Photographs of iNOS western blot data (D) and densitometric analysis of iNOS western blot (E). Data represent means ± S.E.M. (n = 3/group).

including serotonergic (Geldenhuys and Van der Schyf, 2009) and glutamatergic (Francis, 2008) neurotransmitter systems also participate in $A\beta_{1-42}$-induced memory impairments. Further researches are needed to clarify these issues.

To investigate the ameliorating effects of subchronic ESP-102 administration on $A\beta_{1-42}$-induced memory dysfunction, we conducted the passive avoidance and the Morris water maze tasks one day after the last administration of ESP-102 (for 7 days from 1 h after $A\beta_{1-42}$ injection) to avoid ESP-102-induced memory enhancing effect. In this experiment, ESP-102 significantly blocked memory impairment induced by $A\beta_{1-42}$ injection in the passive avoidance and Morris water maze tasks. However, subchronic ESP-102 alone treatment exhibited no significant changes compared to vehicle treatment in all memory-related behavioral parameters. These results indicated that the memory-ameliorating activity of subchronic ESP-102 administration against $A\beta_{1-42}$ injection might be derived from its prevention of $A\beta_{1-42}$-induced neuronal damage but not from its acute memory enhancing effect.

Previously, it was reported that ESP-102 has neuroprotective effects on glutamate-induced neurotoxicity through its inhibitory effect on the increase of intracellular Ca^{2+} concentration and the decrease in the mitochondrial membrane potential induced by glutamate (Ma et al., 2009). Reports have also suggested that the antioxidant activity of ESP-102 is an important part of its mechanism of action. There is strong evidence that the neurotoxic effects of $A\beta_{1-42}$ peptide are due to the generation of reactive oxygen species (Pappolla et al., 2000, 2002). Lipid peroxidation is one of the major outcomes of free radical-mediated tissue injury that directly damages membranes and, in turn, generates a number of secondary products, such as malondialdehyde and 4-hydroxy-2-nonenal as aldehyde, and ketones (Slater, 1984). Markesbery and Lovell (1998) reported the increase of lipid peroxidation products in the hippocampus of the AD brain (Markesbery and Lovell, 1998). In the present study, our data also showed that $A\beta_{1-42}$ peptide caused significant lipid peroxidation in mouse hippocampal tissue, and ESP-102 significantly reversed the increase of lipid peroxidation caused by $A\beta_{1-42}$. This result suggests that the antioxidant effect of ESP-102 may underlie its neuroprotective effect on $A\beta_{1-42}$-induced neurotoxicity.

Astrocytes act as sensors of pathological events (including inflammatory events) in the central nervous system, and furthermore, it has been shown that astrocytes are activated in the AD brain (Schubert et al., 2001; Schubert and Rudolphi, 1998). In the present study, $A\beta_{1-42}$-treated control mice exhibited higher GFAP immunoreactivity in the hippocampal CA1 region. Moreover, a number of thick processes shown in GFAP-positive cells in the $A\beta_{1-42}$-treated control group were decreased in the ESP-102- or donepezil-treated groups following its consecutive administration for 7 days. These results indicate that ESP-102 inhibits astrocyte activation. Astrocytes activated by $A\beta_{1-42}$ peptide releases nitric oxide, one of the toxic molecules in AD, which is produced by iNOS activation (Hu et al., 1998). Therefore, iNOS inhibitors represent a possible candidate for AD treatment (Ryu and McLarnon, 2006; Tran et al., 2003; Wang et al., 2004). iNOS immunoreactivity in the hippocampal CA1 region and corresponding protein level were also increased in $A\beta_{1-42}$-treated control mice. Although we did not conduct double-immunostaining for GFAP and iNOS, it is suggested that astrocytes are a source of iNOS induction because the shape and distribution (hippocampal CA1) of iNOS were similar with those of astrocyte (Heneka et al., 2001).

Taken together, these results suggested that ESP-102 has an ameliorating effect on $A\beta_{1-42}$-induced memory impairment via its inhibitory effect on AChE activity in the hippocampus, as well as its neuroprotective effect on $A\beta_{1-42}$-induced neurotoxicity via antioxidant and anti-inflammatory effects. Although the present results do not have clinical validity, these results suggest that ESP-102 would be a valuable functional tool to enhance mental health related with learning and memory.

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References

Behl C. Alzheimer's disease and oxidative stress: implications for novel therapeutic approaches. Prog Neurobiol 1999;57:301–23.
Blokland A. Acetylcholine: a neurotransmitter for learning and memory? Brain Res Rev 1995;21:285–300.
Braak H, Braak E. Diagnostic criteria for neuropathologic assessment of Alzheimer's disease. Neurobiol Aging 1997;18:S85–8.
Butterfield DA, Boyd-Kimball D. The critical role of methionine 35 in Alzheimer's amyloid beta-peptide (1–42)-induced oxidative stress and neurotoxicity. Biochim Biophys Acta 2005;1703:149–56.
Butterfield DA, Hensley K, Harris M, Mattson M, Carney J. beta-Amyloid peptide free radical fragments initiate synaptosomal lipoperoxidation in a sequence-specific fashion: implications to Alzheimer's disease. Biochem Biophys Res Commun 1994;200:710–5.
Cotman CW, Tenner AJ, Cummings BJ. Beta-amyloid converts an acute phase injury response to chronic injury responses. Neurobiol Aging 1996;17:723–31.
Egashira N, Kurauchi K, Iwasaki K, Mishima K, Orito K, Oishi R, et al. Schizandrin reverses memory impairment in rats. Phytother Res 2008;22:49–52.
Francis PT. Glutamatergic approaches to the treatment of cognitive and behavioural symptoms of Alzheimer's disease. Neurodegener Dis 2008;5:241–3.
Geldenhuys WJ, Van der Schyf CJ. The serotonin 5-HT6 receptor: a viable drug target for treating cognitive deficits in Alzheimer's disease. Expert Rev Neurother 2009;9:1073–85.
Heneka MT, Wiesinger H, Dumitrescu-Ozimek L, Riederer P, Feinstein DL, Klockgether T. Neuronal and glial coexpression of argininosuccinate synthetase and inducible nitric oxide synthase in Alzheimer disease. J Neuropathol Exp Neurol 2001;60:906–16.
Hu J, Akama KT, Krafft GA, Chromy BA, Van Eldik LJ. Amyloid-beta peptide activates cultured astrocytes: morphological alterations, cytokine induction and nitric oxide release. Brain Res 1998;785:195–206.
Itagaki S, McGeer PL, Akiyama H, Zhu S, Selkoe D. Relationship of microglia and astrocytes to amyloid deposits of Alzheimer disease. J Neuroimmunol 1989;24:173–82.
Kang SY, Lee KY, Sung SH, Park MJ, Kim YC. Coumarins isolated from Angelica gigas inhibit acetylcholinesterase: structure–activity relationships. J Nat Prod 2001;64:683–5.
Kang SY, Lee KY, Park MJ, Kim YC, Markelonis GJ, Oh TH. Decursin from Angelica gigas mitigates amnesia induced by scopolamine in mice. Neurobiol Learn Mem 2003;79:11–8.
Kang SY, Lee KY, Koo KA, Yoon JS, Lim SW, Kim YC, et al. ESP-102, a standardized combined extract of *Angelica gigas*, *Saururus chinensis* and *Schizandra chinensis*, significantly improved scopolamine-induced memory impairment in mice. Life Sci 2005;76:1691–705.
Kim DH, Hung TM, Bae KH, Jung JW, Lee S, Yoon BH, et al. Gomisin A improves scopolamine-induced memory impairment in mice. Eur J Pharmacol 2006;542:129–35.
Kim DH, Jeon SJ, Son KH, Jung JW, Lee S, Yoon BH, et al. The ameliorating effect of oroxylin A on scopolamine-induced memory impairment in mice. Neurobiol Learn Mem 2007;87:536–46.
Kim DH, Kim S, Jeon SJ, Son KH, Lee S, Yoon BH, et al. The effects of acute and repeated oroxylin A treatments on Abeta (25–35)-induced memory impairment in mice. Neuropharmacology 2008;55:639–47.
Koo EH, Lansbury Jr PT, Kelly JW. Amyloid diseases: abnormal protein aggregation in neurodegeneration. Proc Natl Acad Sci USA 1999;96:9989–90.
Ma CJ, Kim SH, Lee KY, Oh T, Kim SY, Sung SH, Kim YC. ESP-102, a combined extract of Angelica gigas, Saururus chinensis and Schizandra chinensis, protects against glutamate-induced toxicity in primary cultures of rat cortical cells. Phytother Res 2009;23:1587–91.
Mark RJ, Lovell MA, Markesbery WR, Uchida K, Mattson MP. A role for 4-hydroxynonenal, an aldehydic product of lipid peroxidation, in disruption of ion homeostasis and neuronal death induced by amyloid beta-peptide. J Neurochem 1997;68:255–64.
Markesbery WR, Lovell MA. Four-hydroxynonenal, a product of lipid peroxidation, is increased in the brain in Alzheimer's disease. Neurobiol Aging 1998;19:33–6.
McGeer EG, McGeer PL. Brain inflammation in Alzheimer disease and the therapeutic implications. Curr Pharm Des 1999;5:821–36.
Morris MC, Beckett LA, Scherr PA, Hebert LE, Bennett DA, Field TS, et al. Vitamin E and vitamin C supplement use and risk of incident Alzheimer disease. Alzheimer Dis Assoc Disord 1998;12:121–6.
Pappolla MA, Chyan YJ, Poeggeler B, Frangione B, Wilson G, Ghiso J, et al. An assessment of the antioxidant and the antiamyloidogenic properties of melatonin: implications for Alzheimer's disease. J Neural Transm 2000;107:203–31.
Pappolla MA, Smith MA, Bryant-Thomas T, Bazan N, Petanceska S, Perry G, et al. Cholesterol, oxidative stress, and Alzheimer's disease: expanding the horizons of pathogenesis. Free Radic Biol Med 2002;33:173–81.
Paxinos G, Franklin KBJ. The mouse brain in stereotaxic coordinates, 2nd Edition. San Diego: Academic Press, 2001.
Ryu JK, McLarnon JG. Minocycline or iNOS inhibition block 3-nitrotyrosine increases and blood-brain barrier leakiness in amyloid beta-peptide-injected rat hippocampus. Exp Neurol 2006;198:552–7.
Schubert P, Rudolphi K. Interfering with the pathologic activation of microglial cells and astrocytes in dementia. Alzheimer Dis Assoc Disord 1998;12(Suppl 2):S21–8.

Schubert P, Ogata T, Marchini C, Ferroni S. Glia-related pathomechanisms in Alzheimer's disease: a therapeutic target? Mech Ageing Dev 2001;123:47–57.
Slater TF. Overview of methods used for detecting lipid peroxidation. Meth Enzymol 1984;105:283–93.
Terry RD. The fine structure of neurofibrillary tangles in Alzheimer's disease. J Neuropathol Exp Neurol 1963;22:629–42.
Tran MH, Yamada K, Nakajima A, Mizuno M, He J, Kamei H, et al. Tyrosine nitration of a synaptic protein synaptophysin contributes to amyloid beta-peptide-induced cholinergic dysfunction. Mol Psychiatry 2003;8:407–12.
Varadarajan S, Kanski J, Aksenova M, Lauderback C, Butterfield DA. Different mechanisms of oxidative stress and neurotoxicity for Alzheimer's A beta(1–42) and A beta(25–35). J Am Chem Soc 2001;123:5625–31.
Wang Q, Rowan MJ, Anwyl R. Beta-amyloid-mediated inhibition of NMDA receptor-dependent long-term potentiation induction involves activation of microglia and stimulation of inducible nitric oxide synthase and superoxide. J Neurosci 2004;24:6049–56.
Wilcock GK, Esiri MM, Bowen DM, Smith CC. Alzheimer's disease. Correlation of cortical choline acetyltransferase activity with the severity of dementia and histological abnormalities. J Neurol Sci 1982;57:407–17.