BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 21(4), 299-306 (2013)

Ethanolic Extract of the Seed of *Zizyphus jujuba* var. *spinosa* Ameliorates Cognitive Impairment Induced by Cholinergic Blockade in Mice

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Abstract

In the present study, we investigated the effect of ethanolic extract of the seed of *Zizyphus jujuba* var. *spinosa* (EEZS) on cholinergic blockade-induced memory impairment in mice. Male ICR mice were treated with EEZS. The behavioral tests were conducted using the passive avoidance, the Y-maze, and the Morris water maze tasks. EEZS (100 or 200 mg/kg, p.o.) significantly ameliorated the scopolamine-induced cognitive impairment in our present behavioral tasks without changes of locomotor activity. The ameliorating effect of EEZS on scopolamine-induced memory impairment was significantly reversed by a sub-effective dose of MK-801 (0.0125 mg/kg, s.c.). In addition, single administration of EEZS in normal naïve mouse enhanced latency time in the passive avoidance task. Western blot analysis was employed to confirm the mechanism of memory-ameliorating effect of EEZS. Administration of EEZS (200 mg/kg) increased the level of memory-related signaling molecules, including phosphorylation of extracellular signal-regulated kinase or cAMP response element-binding protein in the hippocampal region. Also, the time-dependent expression level of brain-derived neurotrophic factor by the administration of EEZS was markedly increased from 3 to 9 h. These results suggest that EEZS has memory-ameliorating effect on scopolamine-induced cognitive impairment, which is mediated by the enhancement of the cholinergic neurotransmitter system, in part, via NMDA receptor signaling, and that EEZS would be useful agent against cognitive dysfunction such as Alzheimer's disease.

Key Words: *Zizyphus jujuba* var. *spinosa*, Scopolamine, Memory impairment

INTRODUCTION

Dementia is a series of symptoms that is represented by lack of cognitive capabilities or memory impairment, and mainly caused by Alzheimer's disease (AD) or vascular dementia. Especially, the pathological hallmark of AD is the loss of cholinergic neurons in the basal forebrain that innervate the neocortex and hippocampus involved in learning and memory (Daulatzai, 2010; Schliebs and Arendt, 2011). Therefore, the most effective agent for dementia would be expected to have neuroprotective and enhancing effects on the cholinergic neurotransmitter system. Donepezil, developed by those hypotheses, is a prescribed drug for AD (Birks *et al.*, 2000). However, there are several issues to be overcome such as nausea, diarrhea, anorexia, or vomiting (Shintani and Uchida, 1997; Farlow *et al.*, 2011). We are working on the development for anti-amnesic or anti-dementic agent using *in vivo* screening from herbal materials which are traditionally known to be active to the central nervous system (CNS).

The seed of *Zizyphus jujuba* var. *spinosa* (Rhamnoceae) has been used for traditional medicine in many Asian countries as a hypnotic agent. Most of the previous studies about the seed of *Z. jujuba* var. *spinosa* have been focused on its traditional use as sedative or hypnotic agent. Jujubosides, some of the main components in *Z. jujuba* var. *spinosa*, have been suggested to play roles in hypnotic effects, and serotonergic neurotransmitter systems may be involved in their hypnotic effects (Cao *et al.*, 2010). Jujubogenin, which easily pen-

Open Access **http://dx.doi.org/10.4062/biomolther.2013.043**

Received May 15, 2013 **Revised** Jul 8, 2013 **Accepted** Jul 22, 2013

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etrates into blood brain barrier, is one of active constituents in *Z. jujuba* var. *spinosa* against insomnia by the activation of the $GABA_A$ receptor (Chen *et al.*, 2008). In addition, methanolic extract of the seed of *Z. jujuba* var. *spinosa* protects NMDA-induced neuronal cell damage in cultured rat cerebellar granular cells *in vitro* (Park *et al.*, 2004). However, there is no report regarding with the cognitive function of the seed of *Z. jujuba* var. *spinosa*.

In the present study, we wanted to investigate whether the ethanolic extract of the seed of *Z. jujuba* var. *spinosa* (EEZS) has memory-ameliorating effect on scopolamine-induced cognitive impairment or memory enhancing effect in normal naïve mice. We employed several behavioral tests including the passive avoidance task, the Y-maze task and the Morris water maze task. Moreover, Western blot analysis was also employed to confirm the effect of EEZS on the levels of memory-related biochemical parameters, including phosphorylation levels of extracellular signal-regulated kinase (ERK) or cAMP response element-binding protein (CREB), or the expression levels of brain-derived neurotrophic factor (BDNF).

MATERIALS AND METHODS

Animals

Male ICR mice (25-30 g, 6 weeks old) were purchased from the Orient Co. Ltd, a branch of the Charles River Laboratories (GyeongGi-do, Korea). Mice were housed 5 per cage, provided with food and water *ad libitum*, and kept under a 12 h light/dark cycle (light on 07:30-19:30 h) at a constant temperature (23 ± 1°C) and relative humidity (60 ± 10%). Animal treatment and maintenance were carried out in accordance with the Animal Care and Use Guidelines issued by Kyung Hee University. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Kyung Hee University (approved number; KHP-2011-01-03).

Materials

Donepezil hydrochloride monohydrate, scopolamine hydrobromide, and dizocilpine (MK-801) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Rabbit polyclonal anti-phosphorylated ERK (pERK) antibody was purchased from Cell Signaling Technology (Cell Signaling, MA, USA). Rabbit polyclonal anti-ERK, rabbit polyclonal anti-CREB, rabbit polyclonal anti-BDNF and mouse polyclonal β-tubulin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). An anti-phosphorylated CREB (pCREB) antibody was purchased from Chemicon (Temecula, CA, USA). All other materials were obtained from normal commercial sources and were of the highest grade available.

Preparation of herbal extracts and drug administration

The seed of *Z. jujuba* var. *spinosa* were cultivated at Jinan, Shandong province, China, harvested in October 2009, and authenticated by Dr. J. H. Lee, Dongguk University. A voucher specimen was deposited in the Herbarium of the Traditional Herb Research Center, Korea Food and Drug Administration (No.11E-1001). The seed of *Z. jujuba* var. *spinosa* (1,240 g) were crushed and extracted two times with 70% ethanol (3 L) under reflux (60°C) for 2 h. The extract was evaporated to dryness under reduced pressure to give the 70% ethanolic extract (94.5 g; yield, 7.6%). To ensure the quality, EEZS was standardized based on the amount of jujuboside A, A2, B or naringin using a Shisheido C18 CAPCELL PAK column (5 μm, 4.6×250 mm; Shisheido, Tokyo) (Fig.1). The mean level of jujuboside A in EEZS was 0.54%.

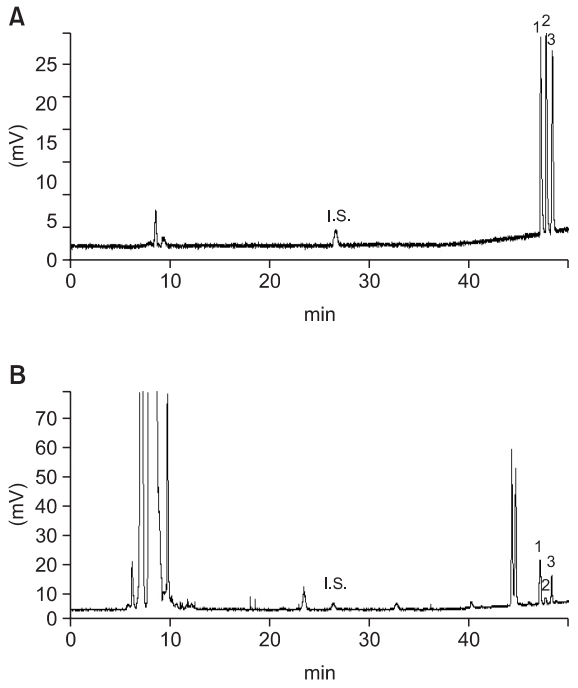

Fig. 1. Representative HPLC chromatogram of standard mixture (15 μg/ml) (A) and the 50% aq-methanolic extract of *Z. jujuba* var. *spinosa* (EEZS) (B). Jujuboside A was detected at around 47.09 min in this system. In the presented case, the concentration of jujuboside A in EEZS was 11.0 μg/ml. 1: jujuboside A, 2: jujuboside A2, 3: jujuboside B, I.S: naringin.

Passive avoidance task

Assessment of acquisition and retention trials of the passive avoidance task was carried out for 2 days. Testing was performed in a box consisting of two identical chambers (20×20 ×20 cm) with one illuminated with 50 W bulb and another non-illuminated chamber that were separated by a guillotine door (5×5 cm). The floor of the non-illuminated compartment was composed of 2 mm stainless steel rods spaced 1 cm apart as described previously (Park *et al.*, 2012). Mice were administered of EEZS (25, 50, 100 or 200 mg/kg, p.o.) or donepezil (5 mg/kg, p.o.) 1 h before an acquisition trial. The control group received 10% Tween 80 solution rather than EEZS. Mice were treated with scopolamine (1 mg/kg, i.p.) or 0.9% saline 30 min before the acquisition trial. Mice were initially placed in the illuminated compartment during the acquisition trial. The door between the two compartments was opened 10 s later. The door automatically closed when the mice entered the non-illuminated compartment, and a 3 s electrical foot shock (0.5 mA) was delivered through the stainless steel rods. Mice that did not enter non-illuminated compartment within 60 s after the opening of the door were excluded from retention trial. The retention trial was conducted 24 h after the acquisition trial by returning individual mice to the illuminated compartment. The time for the mouse to enter the dark compartment after the door opening was defined as latency in both trials. Latencies

were recorded for up to 300 s. In a separate antagonism study, EEZS (100 or 200 mg/kg) was administered 1 h before the acquisition trial, and sub-effective dose of MK-801 (0.0125 mg/kg), an N-methyl-D-aspartate (NMDA) receptor antagonist, was treated after the administration of EEZS. Scopolamine (1 mg/kg) was administered 5 min after the treatment with MK-801. The acquisition trial was conducted at 25 min after the administration of scopolamine. The dose of MK-801 in the present study was used not to impair the passive avoidance task performance when administered alone (Kim *et al.*, 2010; Park *et al.*, 2010). In the memory enhancing study, EEZS (100 or 200 mg/kg) alone without scopolamine was administered 1 h before the acquisition trial. When the mice entered the non-illuminated compartment, a 3 s electrical foot shock (0.25 mA) was delivered through the stainless steel rods to avoid ceiling effects. Other procedures were the same as described above.

Y-maze task

The Y-maze is a three-arm horizontal maze (40 cm-long and 3 cm-wide with 12 cm-high walls) in which the arms are symmetrically disposed at 120° angles from each other. The maze floor and walls were constructed of dark opaque polyvinyl plastic as described elsewhere (Kim *et al.*, 2006). Mice were initially placed within one arm, and the sequence (i.e., ABCCAB, etc.) and number of arm entries were recorded manually for each mouse over an 8 min period. An actual alternation was defined as entries into all three arms on consecutive choices (i.e., ABC, CAB, or BCA but not BAB). One hour before the test, mice were administered EEZS (100 or 200 mg/kg, p.o.) or donepezil (5 mg/kg, p.o.). Control group animals received 10% Tween 80 solution rather than EEZS or donepezil. Scopolamine (1 mg/kg, i.p.) was introduced to induce memory impairment 30 min before the test. Maze arms were thoroughly cleaned with water spray between each test to remove residual odors and residues. The alternation score (%) for each mouse was defined as the ratio of the actual number of alternations to the possible number (defined as the total number of arm entries minus two) multiplied by 100 as shown by the following equation: % Alternation=[(Number of alternations)/(Total arm entries–2)]×100. The number of arm entries was used as an indicator of locomotor activity.

Morris water maze task

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 to a depth of 30 cm with water containing 500 ml of black pigment (24 ± 1°C). The tank was placed in a dimly lit, soundproof test room with four visual cues. A black platform (6 cm in diameter and 29 cm high) was then placed in one of the pool quadrants. The first experimental day was dedicated to swimming training for 60 s in the absence of the platform. During the five subsequent days the mice were given two trials per session per day with the platform in place. 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 time interval between each trial per session was 30 min (Kim *et al.*, 2006). During each trial session, the time taken and distance moved to find the hidden platform (latency time) was recorded using a video camera-based Ethovision System (Nodulus, The Netherlands). One day after the last training trial session, mice were subjected to a probe trial session in which the platform was removed from the pool, allowing the mice to swim for 60 s to search for it. A record was kept of the swimming time in the pool quadrant where the platform had previously been placed. EEZS (100 or 200 mg/kg, p.o), or donepezil (5 mg/kg, p.o.) was given 1 h before the first trial at each session at every consecutive day. Memory impairment in mice was induced by scopolamine administration (1 mg/kg, i.p.) at 30 min before the first trial in each session. Control group received 10% Tween 80 solution only.

Open field test

The open field test was conducted in clear black Plexiglas box (40×40×40 cm) equipped with a video-based Ethovision System (Noldus, The Netherlands) as described previously (Jung *et al.*, 2006). Mice were administered EEZS (100 or 200 mg/kg, p.o.) or 10% Tween 80 solution 1 h before the test. Mice were initially placed in the center of the apparatus, and total distance moved was recorded for 1 h. The horizontal locomotor activity is expressed in terms of the total ambulatory distance.

Western blot analysis

Mice were decapitated immediately after the retention trial of the passive avoidance task or designated time points after the administration of EEZS in normal naïve mice, and the brains were isolated for Western blotting. Isolated hippocampal tissues from both hemispheres were homogenized in ice-cold 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 subjected to SDS-PAGE (8% gel for pERK, ERK, pCREB and CREB, 12% gel for BDNF and β-tubulin) under reducing conditions. Proteins were transferred to PVDF membranes in 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 incubated for 2 h with a blocking solution (5% skim milk) at room temperature followed by incubation in a 1:5,000 dilution of anti-pERK, anti-ERK, anti-pCREB and anti-CREB antibodies, or a 1:1,000 dilution of anti-BDNF and anti-β-tubulin antibodies for 24 h at 4°C. The blots were washed ten times with a solution of Tris-buffered saline/Tween 20 (TBST) and incubated in a 1:10,000 (for pERK, ERK, pCREB, CREB) or a 1:3,000 (for BDNF and β-tubulin) dilution of horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The blots were washed ten times with TBST, and developed using enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL). Immunoreactivity was analyzed using the Multi-gauge, bio-imaging program on an LAS-4000 mini (Fujifilm Lifescan USA, Stamford, CT, USA).

Statistics

Values are expressed as means ± S.E.M. The latencies in the passive avoidance task, the spontaneous alternation in the Y-maze task, and the swimming time in the probe trial of the Morris water maze test were analyzed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test for multiple comparisons. Western blot analysis was analyzed by one-way ANOVA followed by Tukey's *post-hoc* analysis for multiple comparisons. The interactions between the agonist and the antagonist in the passive avoidance task

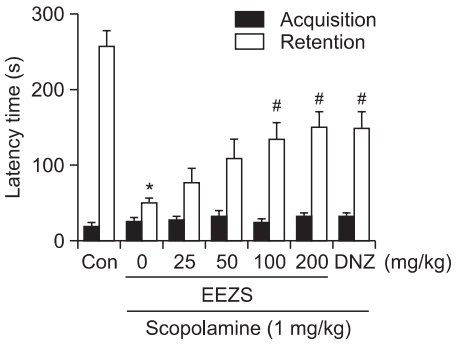

Fig. 2. The effects of the ethanolic extract of the seed of *Z. jujuba* var. *spinosa* (EEZS) on scopolamineinduced memory impairment in the passive avoidance task. EEZS (25, 50, 100 or 200 mg/kg, p.o.), donepezil (DNZ, 5 mg/kg, p.o.), or the same volume of vehicle (10% Tween 80 solution) was administered to mice 1 h before the acquisition trial. Memory impairment was induced by scopolamine (1 mg/kg, i.p.) 30 min before the acquisition trial. Twenty four hours after the acquisition trial, a retention trial was conducted for 300 s. Data represent means ± S.E.M (n=8-10/group) (*p<0.05, versus the vehicle-treated controls, #p<0.05, versus the scopolamine-treated group).

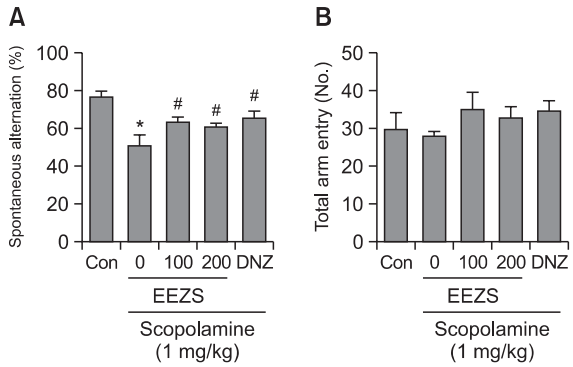

Fig. 3. The effects of the ethanolic extract of the seed of *Z. jujuba* var. *spinosa* (EEZS) on scopolamineinduced memory impairment in the Y-maze task. Mice were administered EEZS (100 or 200 mg/kg, p.o.), donepezil (DNZ, 5 mg/kg, p.o.), or the same volume of vehicle (10% Tween 80 solution) 60 min before the Ymaze test. Memory impairment was induced by scopolamine (1 mg/kg, i.p.) 30 min before the Y-maze tests. Spontaneous alternation behavior (A) and the numbers of arm entries (B) during an 8 min session were recorded. Data represent means ± S.E.M (n=8-10/group) (*p<0.05, versus the vehicle-treated controls, #p<0.05, versus the scopolamine-treated group).

were analyzed by two-way ANOVA, and Tukey's *post-hoc* test was used to perform pair-wise comparisons to determine antagonist or agonist effects. Training-trial latencies in the Morris water maze test were analyzed by two-way ANOVA followed by Tukey's *post-hoc* analysis using the day as one variable and treatment as a second. Statistical significance was set at p<0.05.

RESULTS

EEZS ameliorates cognitive dysfunction induced by scopolamine on the passive avoidance task

The passive avoidance task was employed to investigate whether EEZS ameliorates memory impairment induced by scopolamine (1 mg/kg, i.p) (Fig. 2). There were significant group effects in terms of step-through latency [F(6, 54)= 27.96, p<0.05]. The reduction of step-through latency was observed in the scopolamine-injected group, but the administration of EEZS significantly reversed the latency reduction (100 or 200 mg/kg, p<0.05; Fig. 1), as observed in the donepezil-treated group. There were no significant differences in the latency time during the acquisition trial across all groups. Thereafter, we adopted 100 or 200 mg/kg of EEZS for further study.

EEZS ameliorates cognitive dysfunction induced by scopolamine on the Y-maze task

The Y-maze task was performed to examine the effect of EEZS on the spontaneous alternation behavior. A significant group effect was observed on the spontaneous alternation behavior by the administration of EEZS [F(4, 39)=7.561, p<0.05, Fig. 3A]. The percentage of spontaneous alternation in the scopolamine-treated group was significantly lower than that in the vehicle-treated control group (p<0.05), and the reduction of the spontaneous alternation was significantly ameliorated by EEZS (100 or 200 mg/kg, p.o.) or donepezil (5 mg/kg, p.o.) (p<0.05, Fig. 3A). However, the mean numbers of the arm entries were similar across all experimental groups (Fig. 3B), which demonstrated that general locomotor activity was not affected by EEZS (Sarter *et al*., 1988).

EEZS ameliorates cognitive dysfunction induced by scopolamine on the Morris water maze task

The effect of EEZS (100 or 200 mg/kg, p.o.) on spatial learning and memory was evaluated using the Morris water maze task. As shown in Fig. 4A, the scopolamine-treated group exhibited longer escape latencies throughout the training days than the vehicle-treated control group. However, escape latencies of the EEZS (200 mg/kg, p.o.)- or donepezil (5 mg/kg, p.o.)-treated group were significantly shortened compared with those of the scopolamine-treated group during the training trial sessions 4 and 5 [trial session 4, F (4, 45)=5.439, p<0.05; trial session 5, F(4, 45)=5.520, p<0.05]. On the day following the final day of training sessions, the shortened swimming time within the target zone induced by scopolamine was significantly reversed by EEZS (100 or 200 mg/kg) or donepezil (p<0.05, Fig. 4B). However, there were no significant differences in swimming speed within the target zone across all groups (data not shown).

EEZS did not affected the locomotor activity in the open field test

To determine whether EEZS modifies stimulatory effect on the exploratory behavior, the open field test was performed and spontaneous locomotor activity was observed. EEZS-treated groups (100 or 200 mg/kg) had no significant changes in total ambulatory distances compared to the control group (p>0.05, Fig. 5).

The effect of EEZS is antagonized by MK-801 in the passive avoidance task

To determine whether the memory-ameliorating effect of EEZS is mediated via NMDA receptor signaling, sub-effective dose of MK-801 (0.0125 mg/kg) was co-treated with scopolamine to the EEZS-treated mice (100 or 200 mg/kg), and the

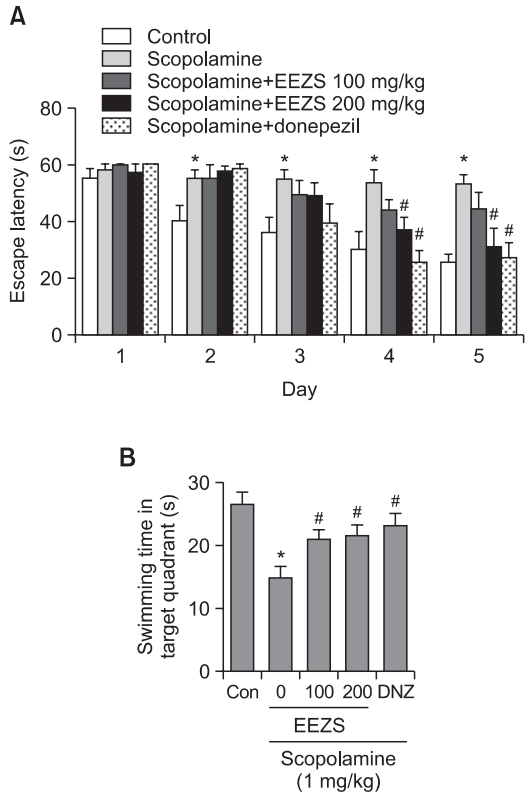

Fig. 4. The effects of the ethanolic extract of the seed of *Z. jujuba* var. *spinosa* (EEZS) on the scopolamineinduced memory dysfunction in the Morris water maze task. The training sessions were conducted for 5 days. Mice were administered EEZS (100 or 200 mg/kg, p.o.), donepezil (DNZ, 5 mg/kg, p.o.), or the same volume of vehicle (10% Tween 80 solution) 60 min before the first training trial of each session. Memory impairment was induced by the administration of scopolamine (1 mg/kg, i.p.) 30 min before the first training trial. Training trial and probe trial sessions were performed over 60 s as described in materials and methods section. Escape latencies during the training sessions (A), the swimming time in the target quadrant (B) during the probe-trial session in the Morris water maze task were measured. Data represent means ± S.E.M (n=10/group) (**p*<0.05, versus the vehicle-treated controls, #*p*<0.05, versus the scopolamine-treated group).

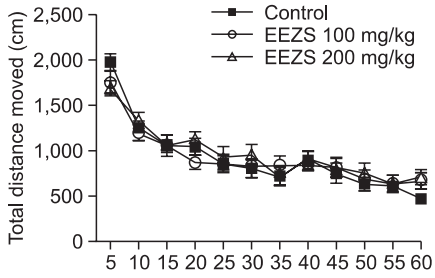

Fig. 5. The effects of the ethanolic extract of the seed of *Z. jujuba* var. *spinosa* (EEZS) on the locomotor activity in the open field test. The exploratory behaviors of mice in the open field test were observed for 60 min. Mice were administered EEZS (100 or 200 mg/kg, p.o.) or the same volume of vehicle (10% Tween 80 solution) 1 h before the test. Data are expressed as means ± S.E.M (n=10/group).

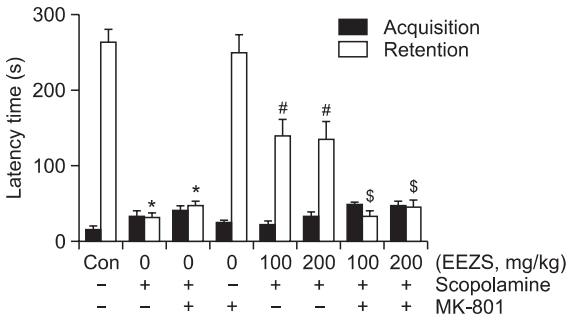

Fig. 6. The role of NMDA receptor signaling in the effects of the ethanolic extract of the seed of *Z. jujuba* var. *spinosa* (EEZS) on scopolamine-induced memory impairments in mice. EEZS (100 or 200 mg/kg, p.o.) or the same volume of vehicle (10% Tween 80 solution) was administered 60 min before the acquisition trial, and subeffective dose of MK-801 (0.0125 mg/kg, s.c.) was injected to the mice 30 min after the oral injection. Scopolamine (1 mg/kg, i.p.) was treated 5 min after the injection of MK-801. The retention trial was conducted 24 h after the acquisition trial. Data represent means ± S.E.M (n=8-10/group) (**p*<0.05, versus the vehicle-treated controls, #*p*<0.05, versus scopolamine-treated groups, $*p*<0.05, versus EEZS plus scopolamine-treated group).

passive avoidance task was conducted. The reduced step-through latency induced by scopolamine was significantly reversed by EEZS (100 or 200 mg/kg) [F(7, 71)=33.150, *p*<0.05] (Fig. 6). The ameliorating effect of EEZS on scopolamine-induced memory impairment was disappeared by the administration of MK-801, and the step-through latency was reached to that of scopolamine alone-treated group. There were significant group effects between EEZS-treated group and MK-801-treated groups determined by two-way ANOVA [F(1, 52)= 23.740, *p*<0.05]. In the acquisition trial, significant differences in step-through latency were not observed across all groups.

EEZS enhances cognitive performance in naive mice measured by the passive avoidance task

The memory-enhancing effect of EEZS in the nave animal was evaluated using the passive avoidance task (Fig. 7). During the acquisition trial, there were no significant differences in the step-through latency between groups. The retention trial was performed 24 h after the acquisition trial. As a result, dose-dependent increase of the step-through latency was observed in the EEZS-treated group [F(2, 28)=3.946, *p*<0.05, Fig. 7]. EEZS (200 mg/kg) significantly increased step-through latency compared with the vehicle-treated control (*p*<0.05).

The effects of EEZS on the expression levels of pERK, pCREB or BDNF in the hippocampus

The effect of EEZS on the levels of ERK or CREB phosphorylation in the hippocampus was determined using the Western blot analysis. Mice were sacrificed 1 h after the administration of EEZS (100 or 200 mg/kg). Significant group effects on the levels of memory-related signaling molecules in the hippocampus were observed in the EEZS-treated groups. The administration of EEZS (200 mg/kg) significantly increased the phosphorylation levels of ERK [F(2, 21)=3.975, *p*<0.05, Fig. 8A] or CREB [F(2, 19)=3.385, *p*<0.05, Fig. 8A] compared to the levels of vehicle-treated group. To investigate the changes of time-dependent BDNF levels in the hippocam-

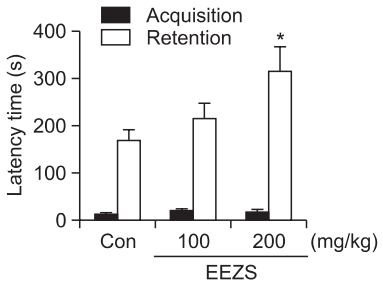

Fig. 7. The effects of the ethanolic extract of the seed of *Z. jujuba* var. *spinosa* (EEZS) on the cognitive performances in the naïve mice. EEZS (100 or 200 mg/kg, p.o.) or the same volume of vehicle (10% Tween 80 solution) was administered to mice 1 h before the acquisition trial in the passive avoidance task. In the acquisition trial, mild electrical shock (0.25 mA) was given to the mice. A 10-min retention trial was performed 24 h after the acquisition trial. Data represent the means ± S.E.M. (n=9-10/group) (**p*<0.05, versus the vehicle-treated controls).

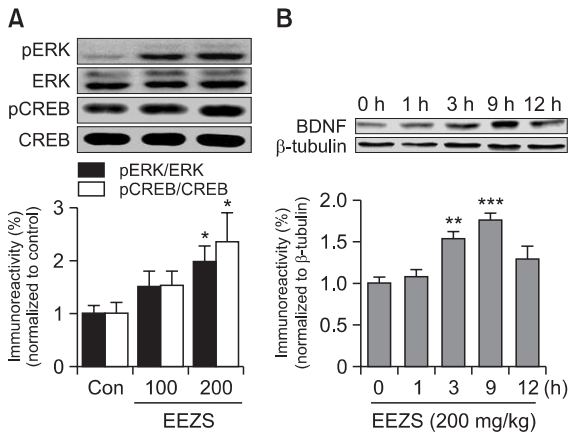

Fig. 8. The effects of the ethanolic extract of the seed of Z. jujuba var. spinosa (EEZS) on the phosphorylation levels of ERK or CREB, and the changes of time-dependent expression level of BDNF in the hippocampus. Mice were administered EEZS (100 or 200 mg/kg, p.o.) or the same volume of vehicle (10% Tween 80 solution), and sacrificed 1 h (A) or designated time points (B) after the EEZS administration. Immunoreactivity and quantitative analysis of pERK, pCREB, and BDNF were measured in the hippocampal tissue. Note that EEZS enhanced the level of phosphorylated ERK or CREB in the hippocampus (A), and the time-dependent expression level of BDNF in the hippocampus (B). Data represent the means ± S.E.M. (pERK and pCREB, n=7-8/group; BDNF, n=5/group) (**p*<0.05, versus the vehicle-treated controls; ***p*<0.01, ****p*<0.005, versus the 0 h level).

pus, mice were sacrificed 0 h, 1 h, 3 h, 9 h and 12 h after the administration of EEZS (200 mg/kg). The BDNF expression level was significantly increased from 3 to 9 h after administration of EEZS [F(4, 20)=9.292, $p<0.05$, Fig. 8B]. However, the effect of EEZS on BDNF level did not last more than 12 h.

DISCUSSION

In the present study, we investigated whether EEZS has memory-ameliorating effect on the scopolamine-induced amnesic mouse model. Scopolamine-induced amnesia model in rodents has been widely used to screen beneficial treatments for cognitive dysfunction (Polster, 1993; Potter *et al.*, 2000). To examine the effect of EEZS, several behavioral tests were employed. First, the passive avoidance task was used to examine the behavioral outcome after the administration of EEZS. Scopolamine caused memory impairments, and EEZS significantly reversed these impairments. These results suggest that EEZS rescued the cognitive dysfunctions related to learning and memory in the cholinergic dysfunction state. Second, in the Y-maze task, the administration of EEZS improved spontaneous alternation impairment induced by scopolamine, suggesting that EEZS ameliorates short-term or working memory impairment in scopolamine-induced amnesic model (Sarter *et al.*, 1988; Myhrer *et al.*, 2003). We also observed the memory-ameliorating effect of EEZS in the Morris water maze task. The Morris water maze task is dependent on hippocampal function or spatial memory that is commonly assessed using the rodents (Morris, 1984; Barnes *et al.*, 1996). EEZS significantly shortened the escape latencies during the training sessions, and also ameliorated scopolamine-induced reduction in swimming time within the quadrant where the target platform was existed during the probe trial session. According to these behavioral results, EEZS could be considered to have memory-ameliorating effect in the case of cholinergic dysfunction without affecting spontaneous locomotor activity.

It has been suggested that both cholinergic and glutamatergic neurotransmissions are related to the learning and memory processes. Furthermore, interactions between central cholinergic and glutamatergic neurotransmitter systems have been suggested to be a crucial role in memory formation (Gray *et al.*, 1996; Shimohama *et al.*, 1996; Timofeeva and Levin, 2011). Cooperations between cholinergic and glutamatergic neurotransmitter systems are also essential to induce BDNF-dependent long-lasting memory storage (Navakkode and Korte, 2012) and necessary for the induction of long-term potentiation (Jerusalinsky *et al.*, 1997). Moreover, increasing evidences indicate that NMDA receptor activity affects acetylcholine release in the basal ganglia (Ikarashi *et al.*, 1998; Knauber *et al.*, 1999; Palencia and Ragozzino, 2006). Thus, cholinergic neurotransmitter system would be associated with the NMDA receptor signaling. In the present study, the memory-ameliorating effects of EEZS on scopolamine-induced memory dysfunction were completely antagonized by a sub-effective dose of MK-801. The relation between NMDA receptor and Zizyphi semen extract *in vitro* was demonstrated by previous researchers (Park *et al.*, 2004), and was also observed in the present study *in vivo*. Moreover, the administration of EEZS enhanced memory performances in a dose-dependent manner in naive mice. Because of its weak inhibitory effect on the acetylcholinesterase inhibition activity (data not shown), these results suggest that the ameliorating effects of EEZS on the cognitive dysfunction may be attributed to the activity of cholinergic neurotransmitter system which is also regulated by glutamatergic signaling. This possibility could be supported by the changes of memory-related signaling molecules.

The administration of EEZS significantly increased the levels of pERK or pCREB in the hippocampus. Furthermore, the time-dependent expression level of BDNF was significantly increased 3 h after, and lasted at least 9 h after the administration of EEZS. A growing number of evidences show that

activation of NMDA receptors stimulates ERK signaling (Xu *et al.*, 2012), which is essential for the expression of LTP in the hippocampaus (English and Sweatt, 1996; English and Sweatt, 1997; Davis *et al.*, 2000). Others also indicate that NMDA receptor activation affects ERK-CREB signaling (Zhou *et al.*, 2009), suggesting that cognitive performance could be enhanced via NMDA receptor activation. (Bourtchuladze *et al.*, 1994; Tully *et al.*, 2003). The activation of CREB is related with transcription of many genes, including BDNF (Tao *et al.*, 1998), a member of neurotropin family of growth factors, which plays an important role in memory formation and LTP (Zheng *et al.*, 2012). Taken together, we suggest that the administration of EEZS enhances cognitive performance through the up-regulation of ERK-CREB-BDNF signaling, and these results are related with the activation of NMDA receptors.

Several biological active compounds were isolated from *Z. jujuba* var. *spinosa*. For example, dammarane-type saponins including jujubosides A and B, or fatty acids such as lauric, linoleic, arachidonic and docosanoic acid were isolated (Zhao *et al.*, 2006). Carotenes are also reported to be isolated from the extract of the seed of *Z. jujuba* var. *spinosa* (Guil-Guerrero *et al.*, 2004). We isolated several compounds mentioned above, and obtained jujuboside A and B in large amount enough to conduct behavioral tests. However, jujuboside A or B did not exert any memory ameliorating activities (data are not shown). Recently, it has been reported that jujuboside A increases the expression of $GABA_A$ receptor subunits in the hippocampal neurons (You *et al.*, 2010). If EEZS activates $GABA_A$ receptors, the cognitive functions may be impaired or no affected. Therefore, it is not likely that the cognitive functions of EEZS are associated with the $GABA_A$ receptor signaling. Further studies should be taken to investigate active constituent(s) of EEZS for cognitive function.

In conclusion, the present study demonstrated that EEZS ameliorated scopolamine-induced memory impairment in the passive avoidance, the Y-maze, and the Morris water maze tasks in mice. Moreover, the ameliorating effect of EEZS is mediated by enhancement of the cholinergic neurotransmitter system, in part, through the NMDA receptor signaling concomitant with the enhancement of ERK-CREB-BDNF signaling. Our present findings suggest that EEZS may be a potential therapeutic for treating cognitive dysfunction such as AD.

ACKNOWLEDGMENTS

This research was supported by a grant (09112KFDA890) from Korean Food & Drug Administration in 2011.

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