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The ameliorating effect of the extract of the flower of *Prunella vulgaris* var. *lilacina* on drug-induced memory impairments in mice

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ABSTRACT

Prunella vulgaris var. *lilacina* is widely distributed in Korea, Japan, China, and Europe, and its flowers are used to treat inflammation in traditional Chinese medicine. In the present study, we studied the effects of the ethanolic extract of the flower of *P. vulgaris* var. *lilacina* (EEPV) on drug-induced learning and memory impairment using the passive avoidance, the Y-maze, and the Morris water maze tasks in mice. EEPV (25 or 50 mg/kg, p.o.) significantly ameliorated scopolamine-induced cognitive impairments in the passive avoidance and Y-maze tasks ($P < 0.05$). In the Morris water maze task, EEPV (25 mg/kg, p.o.) significantly shortened escape latencies in training-trials. Furthermore, swimming times within the target zone during the probe-trial were significantly increased as compared with scopolamine-treated mice ($P < 0.05$). In addition, the reduced latency induced by MK-801 treatment in the passive avoidance task was ameliorated by EEPV (25 mg/kg, p.o.) ($P < 0.05$). Additionally, the ameliorating effect of EEPV on scopolamine-induced memory dysfunction was antagonized by a sub-effective dose of MK-801. These results suggest that EEPV would be useful for treating cognitive impairments induced by cholinergic dysfunction, and that it exerts its effects via NMDA receptor signaling.

1. Introduction

It is well known that learning and memory are closely related to the cholinergic and glutamatergic neurotransmitter systems in brain (Bartus et al., 1982; Durand et al., 1996). Blockade of muscarinic receptors by scopolamine, a muscarinic receptor antagonist, impairs learning and memory in mice (Bartus et al., 1982; Renner et al., 2005). Furthermore, scopolamine-induced amnesic animal models are used to screen for potential treatments for cognitive dysfunction. It has been established that cognitive impairment can be caused by normal aging and stress as well as by specific neurodegenerative and psychiatric disorder such as Alzheimer's disease (AD), vascular dementia, and schizophrenia (Hsiao et al., 1996; McEwen, 1999; Gooding and Tallent, 2004; Haenschel et al., 2009). Muscarinic and nicotinic acetylcholine receptor ligands and acetylcholinesterase (AChE) inhibitors, such as tacrine, donepezil, rivastigmine, and galantamine, are being successfully used for the therapeutic approaches for cognitive loss. However, AChE inhibitors have side effects such as nausea, diarrhea, and vomiting (Terry and Buccafusco, 2003; Cummings et al., 2008). Therefore, the development of new side effect-free drugs to treat cognitive impairments would be highly desirable. Furthermore, because treatment with herbal agents is generally cheap and relatively free of side effects, several research groups have focused on the identification of anti-amnesic agents in herbal materials used as traditional medications (Howes et al., 2003).

Prunella vulgaris var. *lilacina* Nakai (Labiatae) is widely distributed in Korea, Japan, China, and Europe, and it continues to be used

Abbreviations: AChE, acetylcholinesterase; AD, Alzheimer's disease; CREB, cAMP response element-binding protein; EEPV, the ethanolic extract of the flower of *Prunella vulgaris* var. *lilacina*; ERK, extracellular signal-regulated kinase; NMDA, *N*-methyl-D-aspartate; LTP, long-term potentiation.

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to treat inflammation, eye pain, headache, and dizziness in traditional Chinese medicine (Zhu, 1998). Previous studies have shown that *P. vulgaris* var. *lilacina* contains several active compounds including oleanolic acid, betulinic acid, ursolic acid, flavonoids, and rosmarinic acid (Lamaison et al., 1991; Xu et al., 1999; Ryu et al., 2000). Furthermore, this herb has been shown to have anti-allergic, anti-inflammatory, anti-oxidative, anti-microbial, and anti-viral effects (Ryu et al., 2000; Psotová et al., 2003). However, to the best of our knowledge, there have not been any reports on the anti-amnesic effects of the flower of *P. vulgaris* var. *lilacina* or of its constituents. In the present study, we examined the effects of an ethanolic extract of the flower of *P. vulgaris* var. *lilacina* on drug-induced cognitive impairment in mice using the step-through passive avoidance, the Y-maze, and the Morris water maze tasks.

2. Material and methods

2.1. Animals

Male ICR mice (6 weeks old, 25–30 g) were purchased from the Orient Co. Ltd., a branch of Charles River Laboratories (Seoul, Korea). Mice were housed in groups of five. Animals were provided with food and water ad libitum and kept under a 12 h light/ dark cycle (light on 07:00–19:00) at room temperature. After delivery, animal maintenance and treatment were carried out in accordance with the Principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1985) and with the Animal Care and Use Guidelines issued by Kyung Hee University, Republic of Korea.

2.2. Materials

(−)-Scopolamine hydrobromide, dizocilpine (MK-801), and 9-amino-1, 2, 3, 4-tetrahydroacridine hydrochloride hydrate (Tacrine) were purchased from Sigma Chemical Co. (St. Louis, MO). Dried *P. vulgaris* var. *lilacina* flowers were obtained from an herbal supplier in Seoul, Korea and voucher specimens (KHUOPS-08-33) were deposited at the herbarium of the College of Pharmacy, Kyung Hee University. The material was authenticated by Emeritus Professor Chang Soo Yook (Department of Oriental Pharmaceutical Science, College of Pharmacy, Kyung Hee University). All other materials were of the highest grades available and were obtained from normal commercial sources. Tacrine, MK-801, and scopolamine were dissolved in 0.9% saline solution. To obtain an ethanolic extract of the flower of *P. vulgaris* var. *lilacina* (EEPV), flowers were extracted with 70% ethanol twice for two hours in an ultrasonic bath. The obtained extract was then filtered, concentrated in a water bath under vacuum, frozen, lyophilized (model FD-5N; Eyela, Tokyo), and then stored at −20 °C until required (yield: 16.85 ± 1.72%). EEPV concentrations were standardized based on the amount of rosmarinic acid, a major constituent (Lamaison et al., 1991). The mean level of rosmarinic acid in EEPV was 11.86 ± 2.05% (n = 3).

2.3. Passive avoidance task

Assessment of acquisition and retention of the passive avoidance task was carried out using identical illuminated and non-illuminated compartments (20 cm × 20 cm × 20 cm) containing a 50 W bulb, as previously described (Kim et al., 2006). The floor of the non-illuminated compartment was composed of 2 mm stainless steel rods spaced 1 cm apart. These two compartments were separated by a guillotine door (5 cm × 5 cm). The animals underwent two separate trials, namely, an acquisition trial, and 24 h later, a retention trial. For the acquisition trial, a mouse was initially placed in the light compartment and, 10 s later, the door between the two compartments was opened. When a mouse entered the dark compartment, the door automatically closed and an electrical foot shock (0.5 mA, 3 s) was delivered through the grid floor. One hour before the acquisition trial, mice were administered EEPV (12.5, 25, 50, or 100 mg/kg, p.o.) or tacrine (10 mg/kg, p.o.). The control group received 0.9% saline vehicle solution rather than EEPV or tacrine. Thirty minutes after the treatment with EEPV, tacrine, or saline, mice were treated with scopolamine (1 mg/kg, i.p.), MK-801 (0.1 mg/kg, s.c.), or vehicle. In our previous study, MK-801 was founded to impaired cognitive performance at 0.1 mg/kg (s.c.) in mice (Kim et al., 2009). The retention trial was conducted 24 h after the acquisition trial. Mice were again placed in the light compartment and the time required (latency) to enter the dark compartment was recorded for each mouse. If a mouse did not enter the dark compartment within 300 s, we concluded that the mouse remembered the acquisition trial. In a separate antagonism study, EEPV (25 mg/kg, p.o.)-treated mice were co-administered scopolamine (1 mg/kg, i.p.) and a sub-effective dose of MK-801 (0.0125 mg/kg, s.c.) 30 min prior to an acquisition trial. In a previous pilot study, MK-801 at this dose did not impair passive avoidance task performance. To investigate the effect of EEPV on learning and memory in unimpaired naïve animals, EEPV was administered one hour before the acquisition trial. To avoid a ceiling effect in unimpaired animals, the intensity of the electrical foot shock was set at 0.25 mA for 3 s. This lower intensity shock allowed for the study of any potential enhancing effects of EEPV.

2.4. Y-maze task

The Y-maze test was conducted in a three-arm maze with angles of 120° between the arms, which were 40 cm long and 3 cm wide with walls that were 12 cm high each. The maze floor and walls were constructed from dark opaque polyvinyl plastic as previously described (Kim et al., 2006). Mice were initially placed within one arm, and the sequence and number of arm entries were recorded manually for each mouse over an 8 min period. The percentage of triads in which all three arms were represented, i.e., ABC, CAB, or BCA but not BAB, was recorded as an 'alternation' to estimate short-term memory (Sarter et al., 1988). One hour before the test, mice were administered EEPV (12.5, 25, 50, or 100 mg/kg, p.o.) or tacrine (10 mg/kg, p.o.). Control group animals received 0.9% saline solution rather than EEPV or tacrine. Scopolamine (1 mg/kg, i.p.) or vehicle was introduced to induce memory impairment 30 min before the test. Arms were cleaned with water spray between tests to remove 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.

2.5. 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 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 1 cm below the water surface so that it was not visible. The test was conducted as previously described (Kim et al., 2006; Kim and Ryu, 2008), with slight modifications. 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 given four training-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 a mouse did not locate the platform within 60 s, then it was placed on the platform for 10 s. The animals were returned to home cages and allowed to dry under an infrared lamp after each trial. The time between training-trials was 30 s. During each training session, 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 in a randomly selected pool quadrant. The day after the last training-trial session, mice were subjected to a probe-trial session, in which the platform was removed from the pool, and mice were allowed to search for it for 60 s. A record was kept of the swimming time in the pool quadrant where the platform had been located previously. EEPV (25 mg/kg, p.o.) or tacrine (10 mg/kg, p.o.) were administered daily one hour before the first training-trial of each session. Memory impairment was induced by scopolamine (1 mg/kg, i.p.) 30 min after EEPV treatment. The control group received 0.9% saline solution only.

2.6. Statistics

The results of the behavioral studies are expressed as means ± S.E.M. Passive avoidance task latencies, Y-maze task spontaneous alternation (%), and Morris water maze test probe-trial swimming times were analyzed by one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls test for multiple comparisons. The interactions between the agonist and the antagonist that were determined by the passive avoidance task were analyzed by two-way ANOVA, and Tukey's *post hoc* test was used to perform pairwise comparisons to determine antagonist or agonist effects. The Morris water maze test training-trial latencies 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 $P < 0.05$.

3. Results

3.1. Effects of EEPV on scopolamine-induced memory impairment in the step-through passive avoidance task

We tested the effect of EEPV on scopolamine-induced memory deficit using the step-through passive avoidance task which is largely dependent on long-term memory (Myhrer, 2003). A significant group effect was observed in step-through latency in the retention trial [F (6, 109) = 18.603, $P < 0.001$] (Fig. 1A). The mean