大韓本草學會誌 제35권 제5호(2020년 9월)
Kor. J. Herbol. 2020 ; 35(5) : 33-39

ISSN 1229-1765(Print), ISSN 2288-7199(Online)
http://dx.doi.org/10.6116/kjh.2020.35.5.33.

사이프러스 에센셜 오일의 흡입이 전임상 실험동물의 손상된 학습능력과 기억력에 미치는 영향

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Cypress Essential Oil Improves Scopolamine-induced Learning and Memory Deficit in C57BL/6 mice

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ABSTRACT

Objectives : Increasing evidence supports the biological and pharmacological activities of essential oils on the central nervous system such as pain, anxiety, attention, arousal, relaxation, sedation and learning and memory. The purpose of present work is to investigate the protective effect and molecular mechanism of cypress essential oil (CEO) against scopolamine (SCO)-induced cognitive impairments in C57BL/6 mice.

Methods : A series of behavior tests such as Morris water maze, passive avoidance, and fear conditioning tests were conducted to monitor learning and memory functions. Immunoblotting and RT-PCR were also performed in the hippocampal tissue to determine the underlying mechanism of CEO.

Results : SCO induced cognitive impairments as assessed by decreased step-through latency in passive avoidance test, relatively low freezing time in fear conditioning test, and increased time spent to find the hidden platform in Morris water maze test. Conversely, CEO inhalation significantly reversed the SCO-induced cognitive impairments in C57BL/6 mice comparable to control levels. To elucidate the molecular mechanisms of memory enhancing effect of CEO we have examined the expression of brain-derived neurotrophic factor (BDNF) in the hippocampus. CEO effectively elevated the protein as well as mRNA expression of BDNF via activation of cAMP response element binding protein (CREB).

Conclusions : Our findings suggest that CEO inhalation effectively restored the SCO-impaired cognitive functions in C56BL/6 mice. This learning and memory enhancing effect of CEO was partly mediated by up-regulation of BDNF via activation of CREB.

Key words : brain-derived neurotrophic factor, cAMP response element binding protein, cypress essential oil, learning and memory impairment, scopolamine

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· Received : 26 March 2020 · Revised : 08 September 2020 · Accepted : 25 September 2020

Ⅰ. INTRODUCTION

Alzheimer's disease (AD) is a representative progressive neurodegenerative which is characterized by deposition of amyloid plaques, formation of neurofibrillary tangles, and the deficits in the cholinergic system[1]. Cholinergic system has been reported to play a critical role in learning and memory functions and scopolamine (SCO), a muscarinic acetylcholine receptor (mAChR) antagonist has been widely used as a standard and/or reference drug for inducing cognitive deficits in diverse experimental models[2]. Based on the cholinergic hypothesis in AD, inhibition of acetylcholine metabolizing enzymes such as acetylcholinesterase (AChE) and butrylcholinesterase (BuChE) have been regarded as a pivotal target for the management of AD[1]. Therefore, cholinesterase inhibitors including tacrine, donepezil, galantamine, and rivastigmine were developed and used for the treatment of AD.

However, because of the limitation in current therapeutic approved for AD such as relatively low efficacy with adverse effects and ineffectiveness in the late stage of AD, there are great needs for development of new drug candidates from natural resources as well as synthetic derivatives[3]. In this regard, natural essential oils with high efficacy and low side effects have been screened for the prevention and/or treatment of learning and memory loss and one candidate in this study is cypress essential oil (CEO). Plant-derived essential oils have been widely applied to numerous mental disorders and reported to exhibit diverse biological functions such as analgesic, anxiolytic, anti-stress, attention and arousal promoting, relaxing, sedative, hypnotic, anticonvulsive, and learning and memory improving effects[4].

Cypress is the common name of many plants in the cypress family Cupressus (Cupressaceae) comprising more than ten species distributed at high altitudes in North America, the Mediterranean region and subtropical Asia[5]. Especially in South Korea, they are known to mainly inhabit in southern part and Jeju-island. Phytopreparations obtained from *C. sempervirens* were reported to have antiseptic, antirheumatic, antihemorrhoidal, antidiarrheic, vasoconstrictive[6], antiviral[7], anticoagulant[8], and lipid-lowering[9] activities. The methanol extract from leaves of this plant was shown to exert hepatoprotective and free radical scavenging effects[10]. CEO was also known to exhibit antimicrobial and antifungal properties[11]. However, the biological roles of CEO in the central nervous system (CNS) have not been identified and the underlying molecular mechanisms remain unclarified.

Based on these ideas, the purpose of the present work is to investigate the memory enhancing effect of CEO against the SCO-induced amnesia in C57BL/6 mice by conducting diverse behavior tests. Furthermore, to elucidate an underlying molecular mechanism, we have examined the activation of cAMP response element binding (CREB) protein and subsequent expression of brain-derived neurotrophic factor (BDNF) in the hippocampus.

Ⅱ. MATERIALS AND METHODS

1. Chemicals and reagents

Scopolamine (SCO) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Cypress essential oil (CEO) was supplied by CPL Aromas (Bishop's Stortfod, UK). Anti-BDNF antibody was the product of Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti- phospho-CREB (p-CREB) and anti-CREB antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA).

2. GC/MS analysis for cypress essential oil

To analyze the composition of CEO, GC/MS was performed in a 7890A-5975C GC/MSD System (Agilent Technologies, California, USA) using a split ratio of 1:50. An HP-5 capillary column (30 m × 0.25 ㎜ ID, film thickness of 0.25 ㎛) was directly coupled to the mass spectrometry. The carrier gas was helium with a flow rate of 1 ㎖/min. Oven temperature was programmed initially kept at 70℃ for 1 min, then gradually increased to 200℃ at a rate of 5℃/min, and subsequently held isothermal for 14 min. The temperature of injector port was 280℃ and volume of injection was 0.1 ㎕. Components of CEO identified by GC/MS analysis were indicated in Table 1.

3. Experimental animals

Male C57BL/6 mice (8-week old, weighing 20-25 g) were supplied from the Dae Han Bio-Link. Co., Ltd. (Eumseong-gun, Chungcheongbuk-do, South Korea). The experimental animals were maintained with a regular 12 h light-12 h dark cycle under controlled temperature (21 ± 2℃) and humidity (45-60%). They were allowed to freely access to standard chow and tap water. All the animal experiments were conducted in compliance with the institutional guidelines of NIH and Daegu Haany University for the Care and Use of Laboratory Animals.

Table 1. Chemical Composition of Cypress Essential Oil (CEO)

Peak Number	Compounds	Retention Time (min)	Relative %
1	α-Pinene	5.504	24.03
2	Bicyclo	5.806	1.15
3	Camphene	5.860	0.91
4	Sabinene	6.627	1.53
5	2-β-Pinene	6.774	1.44
6	β-Myrcene	7.317	2.19
7	δ-3-Carene	8.580	22.25
8	di-Limonene	9.471	5.88
9	γ-Terpinen	11.168	0.51
10	α-Terpinolene	13.671	3.65
11	α-Terpinolene	14.771	1.08
12	Cyclopentanone	19.079	1.06
13	Borneol	19.792	1.78
14	4-Terpineol	20.691	2.43
15	Cuminol	21.047	0.91
16	Fenchyl alcohol	21.598	0.91
17	endo-Bornyl acetate	27.308	2.43
18	Camphene	27.975	4.40
19	4-Carene	30.152	0.71
20	Camphenone	30.338	0.16
21	β-Caryophyllene	33.747	0.93
22	α-Humulene	35.258	0.32
23	γ-Muurolene	36.351	0.47
24	δ-Cadinene	38.342	0.85
25	Caryophyllene oxide	40.659	0.30
26	α-Cedrol	41.419	1.58
	Total		83.86

4. Experimental groups and drug administration

The experimental mice were divided into six groups (6-7 animals per group) as follows, Group 1 : sham control group, Group 2 : SCO-induced amnesia group, Croup 3 and 4 : SCO + CEO treatment groups, Group 5 and 6 : CEO alone-exposed groups (1 and 3 hours per day, respectively). The inhalation apparatus (Hyochang Science, Daegu, South Korea) was made of acrylic fiber capped with an air filter paper, which allowed minimum respiration air to pass. After pre-saturation of the inhalation cage for 30 min with CEO (an undiluted original solution obtained from CPL Aromas), the mice were exposed to CEO by inhalation for 1 h/day or 3 h/day, 1 h before starting the behavior tests. Sham control mice were exposed to fresh air instead. SCO (1 ㎎/㎏) was intraperitoneally injected 30 min prior to conducting behavior tests.

5. Step-through passive avoidance test

The passive avoidance system (GEMINI^{TM}, San Diego, CA, USA) was composed of illuminated and non-illuminated compartments (25 × 20 × 20 ㎝) which were connected by an automatically operated guillotine door. On the first day of trial, mice were released into the illuminated compartment and allowed to explore for 30 sec. When the light was on, the door between the two chambers was open and the mice entered the non-illuminated chamber. Then the door was automatically closed and an electrical foot shock (0.5 mA, 5 sec) was delivered through the stainless-steel rods. The time taken to enter the non-illuminated chamber was recorded as the step-through latency (acquisition trail). After 24 h, the mice were placed again in the illuminated chamber and without electric foot shock the time to enter the dark chamber was measured (retention trial). When the mice did not enter the dark chamber within 300 sec, the step-through latency was recorded as 300 sec. Both chambers were cleaned thoroughly with 70% ethanol before and after conducting each test to remove any confusing olfactory cues.

6. Morris water maze test

The Morris water maze test was conducted in a circular pool (120 ㎝ in diameter and 45 cm in height) filled with water (21 ± 2℃), which was made to be opaquely white with non-fat milk powder. The maze was divided into four quadrants containing visual external cues and an escape platform (10 ㎝ in diameter and 30 ㎝ in height) was located in one quadrant submerged 2 ㎝ below the water surface. The location and swimming parameter of each experimental mouse, from the start point to the platform, was recorded and analyzed by a video tracking system (Ethovision system, Noldus, Wageningen, Netherlands). During five successive days of training session, the mice were given three trials per day. Once the mouse found and climbed onto the platform, the escape latency was recorded, and it was allowed to remain on it for additional 10 sec. If the mouse failed to find the hidden platform within 120 sec, the trial

session was stopped, and it was gently guided to and placed on the platform for 10 sec. After the last acquisition trial, mice were subjected to a probe test. In this test the hidden platform was removed from the maze and mice were allowed to swim for 60 sec to search for it and the time staying in the disappeared platform quadrant was recorded.

7. Contextual fear conditioning test

Fear conditioning studies were performed using an automated video acquisition based on Startle and Fear Combined System (Panlab, Barcelona, Spain). Contextual fear conditioning is an important behavioral paradigm for studying the neurobiology of learning and memory and the mnemonic function of the hippocampus[12]. For training (conditioning session), mice were located in the conditioning chamber for a 1-min acclimation period, and then 85 dB auditory tone (conditioned stimulus, CS) stimuli was delivered for 15 sec, which was co-terminated with 0.5 mA electric foot shock for 1 sec (unconditioned stimuli, US). A total of two auditory tone and electric foot shock pairings were offered with 150-sec interval. Twenty-four hours later (retention session), mice were placed back into the original conditioning chamber and the freezing response was measured for 300 sec. The total freezing response during this session was monitored via video camera and automatically scored when there was no movement except respiration.

8. Immunoblotting

The hippocampal tissue was thoroughly homogenated with RIPA buffer [25 mM Tris · HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, Thermo Scientific, IL, USA] using automatic homogenizer (Precellys 24, Bertin Technologies, Montigny le Bretonneux, France) and centrifuged at 14,000 *g* for 15 min on 4℃. The protein concentrations of the tissue lysates were determined by BCA protein assay (Pierce Biotechnology, IL, USA). The isolated proteins (30 ㎍) solubilized in SDS sample buffer were loaded to 10-12% SDS-PAGE gel and separated proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Roche diagnostics, IN, USA) at 300 mA for 3 h. Membranes were blocked with 5% non-fat dried milk in PBST (phosphate-buffered saline containing Tween-20) for 1 h at room temperature (RT) and then incubated with primary antibodies at 4℃ for overnight, followed by reaction with horseradish peroxidase (HRP)-conjugated secondary antibodies at RT for 1 h. The signals were developed using the enhanced chemiluminescence (ECL) Western blotting detection reagent (Amersham Biosciences, Piscataway, NJ, USA) and the relative density of the bands were analyzed using the ImageQuant LAS 4000 Multi Gauge software (Fujifilm, Tokyo, Japan).

9. Reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA was isolated with homogenation of hippocampus with TRI reagent (Molecular Research Center, OH, USA) and converted to cDNA by using M-MLV reverse transcriptase (Promega, WI, USA) in the presence of oligo dT and dNTP according to manufacturer's instructions. cDNA was further amplified by polymerase chain reaction (PCR) using Taq polymerase (SolGent, Daejeon, Korea) and synthetic primers specific to BDNF or glyceraldehydes-3-phosphate dehydrogenase (GAPDH). The sequences of primers are as follows. BDNF : 5'-AGC CTC CTC TTC TCT TTC TGC TGG A-3' (forward) and 5'-CTT TTG TCT ATG CCC CTG CAG CCT T-3' (reverse), GAPDH : 5'-GCC AAG GTC ATC CAT GAC AAC-3' (forward) and 5'-AGT GTA GCC CAG GAT GCC CTT-3' (reverse). PCR was performed by denaturation at 95℃ for 30 sec, amplification at 58℃ for 30 sec and elongation at 72℃ for 60 sec with total 33 cycles. PCR products were resolved by 1.5% agarose gel containing ethidium bromide and visualized by UV light using a gel documentation system (TransLum SOLO, Life Sciences Pro, Richmond Hill, ON, Canada).

10. Statistical analysis

The data were calculated as mean values ± standard error of the mean (S.E.M.). For statistical analysis, one-way ANOVA followed by the Tukey's test was conducted using SPSS software (SPSS 12.0 KO for windows) as multiple comparisons. When p was less than 0.05, the values were considered to be statistically significant.

Ⅲ. RESULTS AND DISCUSSION

The effect of CEO on the long-term memory was examined by the passive avoidance test. Passive avoidance test represents not only hippocampus-dependent contextual memory but also amygdala-dependent emotional memory of the fear in the dark

compartment. Under normal non-stimulated condition (acquisition trial), mice located in the illuminated compartment of passive avoidance apparatus quickly entered the non-illuminated compartment, however after stimulation with electrical foot shock (retention trial) the mice hesitated to enter the non-illuminated compartment. In the SCO-injected model group, time taken to enter into the dark compartment (step-through latency) was significantly lower than that of the sham control group (Fig. 1). Conversely, CEO-exposed groups for 1 h/day or 3 h/day exhibited higher step-through latency as compared to scopolamine-treated model group ($p<0.05$) (Fig. 1). The most effective exposure time to CEO was found to be 3 h/day, which was used in subsequent experiments. Furthermore, CEO alone-inhaled mice for 3 h/day have a tendency to perform better in the retention trial even compared with the sham control, although this result was not statistically significant. Under the same experimental condition, during the acquisition trial, the latency time was not significantly different among groups.

In the next experiment, Morris water maze test was conducted to examine the effect of CEO on the hippocampus-dependent spatial recognition and long-term memory. Spatial memory is hippocampus-mediated function that appears to be compromised in the elderly subjects and in patients suffering from AD. The mean escape latency time of the sham control group declined progressively during the training period of 5 consecutive days, which was shorter than that of the SCO-induced amnesic group (Fig. 2A).

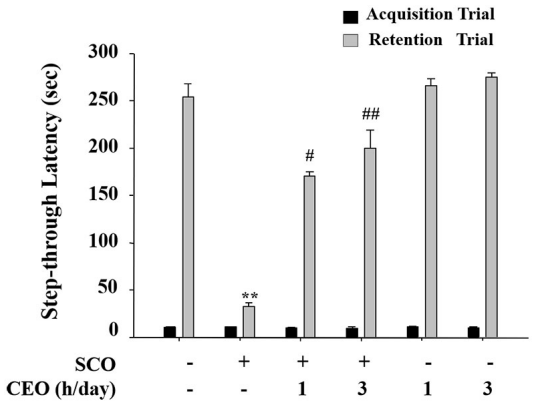

Fig. 1. Effect of CEO against SCO-induced memory impairment in the acquisition and retention trials of passive avoidance test. CEO was exposed for 1 h/day or 3 h/day and scopolamine (1 ㎎/㎏) was intraperitonically injected 30 min before acquisition trial. Retention trial was conducted 24 h after acquisition trial. Data represent mean ± S.E.M (n=7). $^{**}p < 0.01$ compared with the sham control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with the scopolamine-injected model group.

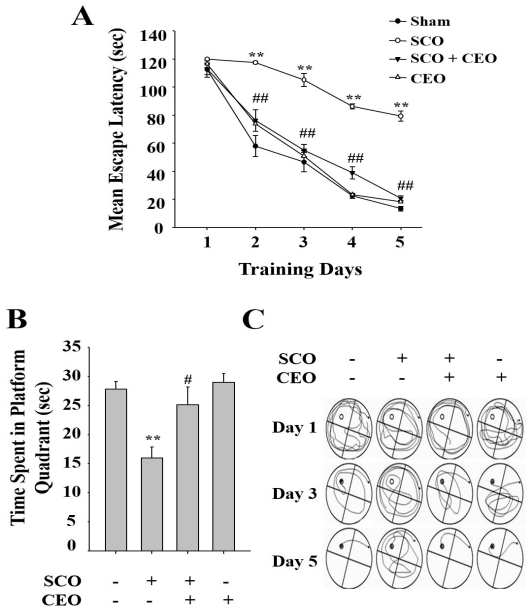

Fig. 2. Effect of CEO on SCO-induced learning and memory deficits as assessed by Morris water maze test. (A) Mean escape latency to find the hidden platform over 5 consecutive days of the training trials. (B) Swimming time spent in the target quadrant during probe trials without platform. Data are shown as ± S.E.M (n=6-7). $^{**}p < 0.01$ vs. sham control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. scopolamine-induced model group. (C) Representative pathways of Morris water maze test on days 1, 3, and 5 from each group.

The SCO-injected model group exhibited significantly delayed escape latency time particularly on days from 2 to 5 indicating long-term memory impairment (Fig. 2A). In contrast, the time spent to find the hidden platform was effectively reduced by inhalation of CEO for 3 h/day (Fig. 2A) suggesting that CEO effectively improves SCO-induced spatial cognitive deficits ($p<0.05$). On the last day of training, the platform was removed, and probe test was performed. During probe trials, the swimming time within the target quadrant without platform was greatly prolonged by CEO inhalation for 3 hr/day compared with the performance of SCO alone-treated mice (Fig. 2B).

In another experiment, hippocampus-dependent contextual fear conditioning test was conducted, which examine the ability of mice to associate the training environment with the aversive event. In this setting the mice learn to associate several features about the environment with the electric foot shock. Twenty-four hour after conditioning session, mice were returned to the conditioning chamber in order to measure contextual fear represented by freezing response. SCO-induced amnesia group showed decreased freezing time during retention session compared to sham control group (Fig. 3). However, the performance of scopolamine-injected

mice was completely restored by CEO inhalation for 3 h/day ($p<0.01$). CEO-treated alone groups did not exhibit any significant difference comparable to sham control.

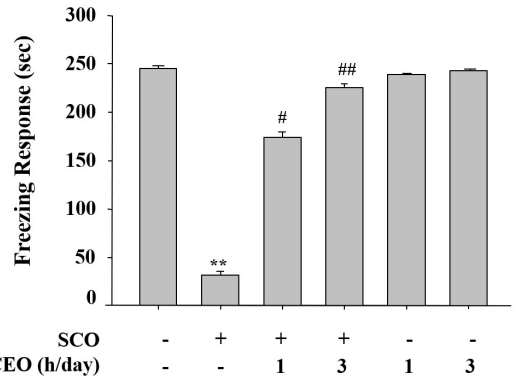

Fig. 3. Effect of CEO against SCO-induced cognitive dysfunction in the retention session of contextual fear conditioning test. Mice were exposed to CEO for 1 h/day or 3 h/day and intraperitonically injected with scopolamine (1 ㎎/㎏) 30 min before starting the retention trial. Values are expressed as means ± S.E.M. (n=7). $^{**}p < 0.01$ compared with the sham control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with the scopolamine-treated model group.

Due to some limitations of FDA-approved AChE inhibitors such as moderate efficacy, short half-lives and severe side effects, novel alternative and complementary anti-dementia therapies are urgently needed to be developed. One of these promising candidates is essential oil with diverse pharmacological and medicinal properties in CNS. Components of lemon essential oils such as limonene and perillyl alcohol was reported to improve scopolamine-impaired learning and memory in Wistar rats possibly by stimulating dopaminergic neurons and inhibiting AChE activity[13]. Essential oil extracted from *Acori graminei* rhizoma was also known to enhance cognitive function in aged animals possibly by increasing relative levels of norepinephrine, dopamine and serotonin and by decreasing the activity of AChE[14].

In order to further elucidate the underlying mechanism of CEO, we have assessed the expression of BDNF and the activation of CREB in the mice hippocampal tissues. Protein and mRNA levels of BDNF were examined by Western blot analysis and RT-PCR, respectively. BDNF, a member of the neurotrophin family, is synthesized and stored in synaptic vesicles and released into synaptic clefts in response to endogenous or exogenous stimuli[15]. BDNF is involved in various aspects of learning and memory processing-in and plays a pivotal role in the persistence and storage of memory[16]. In this study, scopolamine treatment significantly decreased the protein (Fig. 4A) as well as mRNA (Fig. 4B) levels of BDNF in the hippocampus, which were effectively restored by pre-treatment with CEO for 3 h/day. In another experiment, the activation of CREB via phosphorylation was decreased in the SCO alone-injected model group compared with the sham control, whereas hippocampal phosphorylation levels of CREB were substantially increased in the CEO-treated animals, particularly 3 h/day-exposure to CEO (Fig. 4C). Because CREB is a key transcription factor controlling the expression of BDNF, it might be a molecular trigger that leads to the increased levels of BDNF in the CEO inhalation groups.

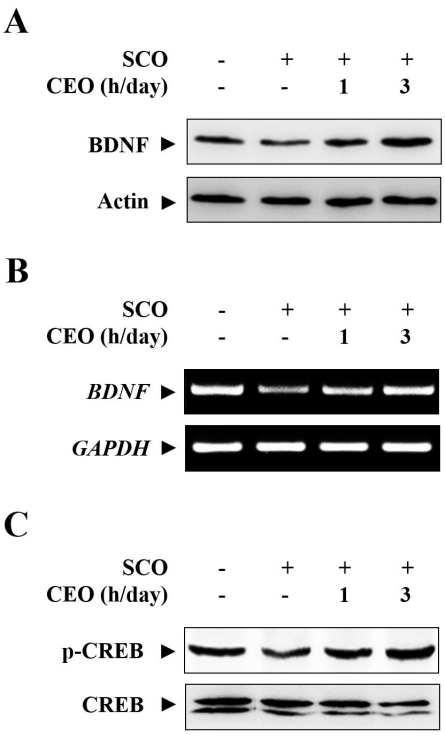

Fig. 4. Effects of CEO on the expression of BDNF and activation of CREB in the hippocampus. The protein (A) and mRNA (B) expression of BDNF was determined by immunoblotting and RT-PCR using specific antibody and primers for BDNF. Actin and *GAPDH* levels were measured for the confirmation of equal amount of protein and mRNA loading, respectively. (C) The activation of CREB was determined by Western blot analysis using anti-phospho-CREB (upper panel) and anti-CREB (lower panel) antibodies.

In this study, although we determined remarkable neuroprotective potential of CEO via *in vivo* mouse study, it is not still verified which components of CEO mainly contributed to the neuroprotective effects against SCO-induced learning and memory impairment. In our GC/MS analysis of CEO, α-pinene and δ-3-Carene were identified as the major components with contents of 24.03% and 22.25%, respectively. In other previous studies, α-pinene was demonstrated to have neuroprotective effect in cultured neuronal cells (PC12 cells) showing activation of intracellular antioxidative

system[17], and to have anxiolytic-like effect in and mouse brain with increased expression of BDNF[18]. It suggests a strong possibility that α-pinene might plays an important role in CEO-induced neuroprotective responses in mice, and further functional studies to determine key components that directly improve learning and memory will provide more reliable evidences of the neuroprotective potential of CEO.

Ⅳ. CONCLUSION

In summary, CEO inhalation effectively restored the SCO-impaired cognitive functions in C56BL/6 mice. This learning and memory enhancing effect of CEO was partly mediated by up-regulation of BDNF via activation of CREB.

ACKNOWLEDGEMENTS

This study was supported by a grant of the Traditional Korean Medicine (TKM) Project, Ministry of Health & Welfare, Republic of Korea to commercialize TKM products.

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