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Research Article

Botanical drug composed of *C. chinensis, T. kirilowii*, and *P. vulgaris* ameliorates cognitive dysfunction induced by scopolamine or $A\beta_{1-42}$ in mice

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Abstract: Cognitive impairment brought by the agglomeration of aggregated β-amyloid (Aβ) and hyperphosphorylation of neurofibrillary tangles in the brain are the characters of Alzheimer's disease (AD). It has been treated with several prescribed drugs, however, their low efficacy and adverse effects remain to be overcome. We focused on the anti-inflammatory effect of three herbal complex (*Clematis chinensis* Osbeck, *Trichosanthes kirilowii* Maximowicz and *Prunella vulgaris* Linne) extract (HCE), and explored its effects on cognitive function. We conducted several behavioral tests including the Y-maze, the passive avoidance test, the novel object recognition test to investigate the effects of acute or sub-chronic administration of HCE on cognitive function in hypocholinergic state or $A\beta_{1-42}$ protein accumulated mice. The Western blot was tried to unveil the underlying mechanism of HCE on its cognitive function. In the results, HCE improved short-term, long-term, and object recognition memory dysfunction. After the administration of HCE, ERK/CaMKII/CREB signaling pathway was activated, BDNF expression levels increased, and GFAP, Iba-1, and caspase 3 levels decreased. These findings revealed that HCE could increase synaptic plasticity in scopolamine- or $A\beta_{1-42}$-induced memory damaged mice, and reduce neuronal inflammation in $A\beta_{1-42}$-induced cognitive function impaired mice. To summarize, HCE could ameliorate cognitive impairment brought by scopolamine or $A\beta_{1-42}$ via increase of ERK/CaMKII/CREB signaling pathway activation, the increase BDNF expression level, and the decrease of the neuroinflammatory factors. HCE could therefore be used as a herbal medicine to treat conditions like AD that cause cognitive dysfunction.

Keywords: *herbal drug; learning & memory; ERK-CaMKII-CREB pathway; BDNF; neuroinflammation*

1. Introduction

The most widespread neurodegenerative disorder, and presented by aphasia, personality and behavior abnormalities, performance issues, and cognitive deficits, is Alzheimer's disease (AD) (Soria Lopez, González, & Léger, 2019). Tremendous studies have presented that the extracellular aggregation of amyloid β (Aβ) protein and intracellular neurofibrillary tangles (NFT) composed of hyperphosphorylated tau protein are the major pathogenic factors for progressing damage of synapses and neurons in AD patients (Bondi, Edmonds, & Salmon, 2017; Graff-Radford et al., 2021). Aducanumab, an Aβ directed antibody therapy, has thus recently received FDA approval (Rabinovici, 2021). However, its efficacy against AD still remains unclear (Schneider, 2020). Moreover, acetylcholinesterase inhibitors (donepezil, galantamine, rivastigmine) or N-methyl-D-aspartate (NMDA) receptor antagonist (memantine) are prescribed to AD patients in clinic, but these drugs have difficulties in restraining the progression of neuronal degradation and have been associated with severe adverse effects such as vomiting, anorexia, diarrhea, nausea, or insomnia (Dunn, Pearce, & Shakir, 2000; Thomas & Grossberg, 2009). Therefore, the efforts have been shifted towards prevent in the early stages of AD (Crous-Bou, Minguillón, Gramunt, & Molinuevo, 2017; Ossenkoppele, van der Kant, & Hansson, 2022; Porsteinsson, Isaacson, Knox, Sabbagh, & Rubino, 2021).

An herbal extract medicine titled JOINS® (SKI306X, SK Chemicals, Seongnam, Korea) is an analgesic used to treat rheumatoid arthritis (RA) composed of a combination of three kinds of herbs [Clematis chinensis Osbeck (Ranunculaceae), Trichosanthes kirilowii Maximowicz (Cucurbitaceae) and Prunella vulgaris Linne (Lamiaceae)] (C. H. Choi et al., 2014). Furthermore, many studies indicated the anti-inflammatory effects of JOINS® and its component

herbal extracts (H. R. Kim et al., 2019; Meng et al., 2017). It is noteworthy to note that recent studies have revealed a decline prevalence of AD in people who have used non-steroidal anti-inflammatory medicines (NSAIDs) for a prolonged period of time (Jenkinson, Bliss, Brain, & Scott, 1989; Kinney et al., 2018; Zhu, Wang, Sun, Schultzberg, & Hjorth, 2018). Moreover, several epidemiological studies have shown that some NSAIDs, such as aspirin (Zmudka et al., 2017) and celecoxib (Jordan et al., 2020), have a protective effect against AD. Therefore, we hypothesized that JOINS® might be a potential candidate for treating AD if JOINS® could reduce inflammatory activity and ameliorate cognitive dysfunction against Aβ-mediated neuroinflammation. In our previous study, a botanical drug containing active constituents of JOINS® ameliorated MK-801-induced cognitive dysfunction in mice (Koo et al., 2020), and P. vulgaris which is one of the constituents of JOINS® has an effect on enhancing memory function in cognitive dysfunction mice induced by scopolamine (Park et al., 2010). However, no studies have been conducted on the roles of anti-inflammatory effects of JOINS® against cognitive dysfunction induced by hypocholinergic or neuroinflammatory states.

In the current investigation, we explored whether the botanical drug composed of three kinds of herbs which are the working constituents of JOINS®, could improve memory dysfunction caused by scopolamine and Aβ in mice through several behavioral tests. Also, we conducted Western blotting to explore the underlying mechanism(s) of a botanical medication that has cognitive-improving effects in mice with scopolamine- and Aβ-induced cognitive function deficit.

2. Materials and Methods

2.1. Animals

CD-1 mice (Eaton, Johnson, Custer, & Crane, 1980) weighing 26-28 g were bought from Orient Co. Ltd, a branch of Charles River Laboratories (Seongnam-si, Gyeonggi-do, Korea) and kept in the Kyung Hee University Animal Care Unit in groups of five per cage with unlimited approach to feed and drinking water. The mice were cared under a persistent humidity (60 ± 10%) and temperature (23 ± 1 °C) with 12 h light and dark cycles (illumination time 7:30-19:30). Animal care and experimental procedures were conducted in accordance with the Animal Care and Use Guidelines issued by Kyung Hee University, Republic of Korea. The institutional animal care and use committee of Kyung Hee University approved the all experimental orders using animals (approval No, KHSASP-21-406). All behavioral tests were executed after the adaptation for 6 days in the Animal Care Unit of Kyung Hee University.

2.2. Materials

Scopolamine hydrobromide, donepezil hydrochloride, and $A\beta_{1-42}$ peptide were bought from Sigma Aldrich (St Louis, MO, USA). Anti-phosphorylated extracellular signal-regulated kinase (pERK), anti-cAMP response element-binding protein (pCREB), anti-CREB, and anti-phosphorylated calcium/calmodulin-dependent protein kinase II (pCaMKII) antibodies were acquired from Cell Signaling Technology (Danvers, MA, USA). Anti-ERK, anti-CaMKII, and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Anti-glial fibrillary acidic protein (GFAP), antibody was bought from GeneTex Co. (Irvine, CA, USA). Anti-brain-derived neurotrophic factor (BDNF), anti-allograft inflammatory factor 1 (Iba-1), and anti-caspase 3 antibodies were purchased from Abcam (Cambridge, UK). All other materials were bought from general market source, and they were of the highest quality available.

2.3. Herbal preparation and extraction

The extraction and preparation of herbal drug composition were described in the previous study (Koo et al., 2020). In brief, three herbs were bought from Edam Pharm. Co. (Seoul, Korea), and all examples were stored at the herbarium of the College of Pharmacy, Kyung Hee University [*C. chinensis* Osbeck (Ranunculaceae), KHUOPS-2017-03; *T. kirilowii* Maximowicz (Cucurbitaceae), KHUOPS-2017-04; and *P. vulgaris* Linne (Lamiaceae), KHUOPS-2017-05]. The herbal ingredients (*C. chinensis*: *T. kirilowii*: *P. vulgaris* = 1 : 2 : 1) were minced and twice extracted with 30% ethanol at reflux (60 °C) for 3 h. The extract was filtered, concentrated, and partitioned with n-butanol. The n-butanol partitions

were freeze-dried into a powder, and then yielded 2.39 ± 0.05 % of a herbal complex extract (HCE). HCE was standardized with rosmarinic acid for quality assurance. The average concentration of rosmarinic acid in HCE was 14.35 ± 0.04 mg/g.

2.4. Drug administration

Mice were parted into 6 groups (n = 10) for the acute study at random. The groups were vehicle, vehicle with scopolamine, scopolamine with HCE (30, 100 or 300 mg/kg), and scopolamine with donepezil (5 mg/kg). HCE was liquefied in 10% Tween 80 solution. Scopolamine and donepezil were liquefied in 0.9% saline solution. Mice were injected with scopolamine (1 mg/kg, i.p.) 30 min before the behavioral tests and sacrifice for Western blotting. HCE (30, 100 or 300 mg/kg, p.o.), donepezil (5 mg/kg, p.o.) or the same volume of vehicle was administered 1 h before the behavioral tests and sacrifice for Western blotting.

Mice were parted into 4 groups (n = 10) for the sub-chronic research. The groups were vehicle, vehicle with $A\beta_{1-42}$ protein, $A\beta_{1-42}$ protein with HCE (300 mg/kg), and $A\beta_{1-42}$ protein with donepezil (5 mg/kg). For aggregating $A\beta_{1-42}$ peptide, the $A\beta_{1-42}$ peptide was liquefied in sterile saline (1 μg/μl) and incubated at 37 °C for 4 days with sealing. After anesthesia with Avertin (5 mg/kg, i.p.), the aggregated $A\beta_{1-42}$ peptide or same volume of vehicle were injected at 1 μl/min for 3 min into the right lateral ventricle of mice at stereotaxic coordinates (AP, -0.2 mm; ML, +1.0 mm; DV, -2.5 mm) taken from the atlas of the mouse brain (Xiong et al., 2017). After the completion of injection, the needle was held for extra 2 min to reduce backflow. After removing the needle, mice were placed to a warm incubator (32-33 °C) until they wake up. The day after injection, HCE, donepezil, or vehicle were administered for 7 days. Each behavioral test was started on the 7th day after the treatment.

2.5. Y-maze task

The Y-maze task as a behavioral test for measuring the working memory abilities by the mice wiling to search for new environment was conducted as described elsewhere (Bae, Kim, Jeon, et al., 2020). The opaque flat arms (40 x 4 x 12 cm) are positioned at 120 degree to one another in the experiment maze. Each mouse was first positioned in the center of the maze, and throughout the course of 6 min, the course of arm entries (i.e. ACBABC) was written down. An entry into all three arms successively was considered to be an actual alternation (i.e. ABC, BCA or CAB). The ratio of the number of actual alternations to the number of possible alternation [(the total number of arm entries - 2)] was used to calculate the spontaneous alternation (%), as presented in the following equation: % spontaneous alternation = [(number of alternations) / (total arm entry numbers - 2)] x 100.

2.6. Passive avoidance task

A fear-stimulated experiment intended to test capacity of mice for learning and memory, was carried out the passive avoidance test as previously reported (J. Kim et al., 2020). The box for using the task was divided into two indistinguishable compartments (20 x 20 x 20 cm) that one of the boxes had 50 W bulb to illuminate and another had none, with a guillotine door (5 x 5 cm) between the two. The boxes had stainless steel rods (diameter: 2 mm) on the floor of the box. The experiment was required for 2 days, acquisition and retention trial days. Each mouse was positioned gently in the corner of the light box for the acquisition trial and permitted to move freely. The guillotine door was uplifted silently after 30 s, and if mouse went inside the dark box, mouse was given an electric foot shock (0.5 mA, 3 s) through the steel rod. The retention trial was executed the day after the acquisition trial. It was executed in the equal way as the acquisition trial, but without electric foot shock. In each trial, the latency time for going mice into the dark box was recorded. The maximum latency for the acquisition trial and the retention trial was set at 60 s and 300 s, respectively.

2.7. Novel object recognition task

The novel object recognition task was employed to measure recognition memory in mice. For habituation session, each mouse was permitted to move freely in the polyvinyl test box (25 x 25 x 25 cm) 10 min for 2 days without any objects. 24 h after habituation session, the training session was conducted. Each mouse was let to move without restraint in the test box for 5 min, and two indistinguishable objects placed diagonally in the test box. Each mouse was given 5 min to investigate and identify the objects. The day after training session, the probe session was executed. The probe session proceeded in the same way as the training session, but one object was swapped out for another that had a similar material but a different shape. The behaviors of mice were recorded with a video camera, and the object preference ratio and discrimination ratio were estimated by measuring the time used inspecting the familiar object (Tfamiliar) and the novel object (Tnovel) as follows: object preference ratio = Tobject / (Tnovel + Tfamiliar) x 100 (%); discrimination ratio = (Tnovel - Tfamiliar) / (Tnovel + Tfamiliar) x 100 (%)

2.8. Western blot

The day after the retention trial of passive avoidance test, mice were dislocated cervically to sacrifice, and the cortex and the hippocampus were removed. These tissues were homogenized in the 20 mM Tris-HCl buffer (pH 7.4) with protease and phosphatase inhibitor cocktail [1 mM phenylmethylsulfonyl fluoride, 1 mM orthovanadate, 1 mM sodium fluoride, 1 mM ethylene-diamine-tetra-acetic acid, 0.32 M sucrose, and protease inhibitor (1 tablet for 50 mL)]. The homogenized tissues were centrifuged for 20 min (4 °C 14,000 rpm, 2 times), and the supernatants were gathered. These supernatants were quantified by BCA assay (15 μg of protein). The samples containing proteins were subjected to sodium dodecyl sulfate - polyacrylamide gel electrophoresis (10 - 12 %) under reduced conditions. The proteins transferred to polyvinylidene fluoride membrane (300 mA, 2 h) in transfer buffer [25 mM Tris-HCl buffer (pH 7.4) with 192 mM glycine and 20 % v/v MeOH]. After transfer, membranes were blocked with blocking buffer (5 % skim milk) for 2 h. After blocking, membranes were incubated with primary antibodies with following dilution overnight in 4 ℃: ERK, pCREB, CREB, Iba-1, caspase 3, 1:1000; BDNF, 1:1500; pERK, pCaMKII, CaMKII, GFAP, GAPDH, 1:3000. The next day, membranes were washed with Tris-buffered saline with 0.1 % Tween 20 (TBST) for 10 min 6 times, and incubated with appropriate secondary antibodies for 2 h in room temperature. After incubating, membranes were washed TBST for 10 min 6 times, and Multi Gauge bio-imaging program on the LAS-4000 mini (Fujifilm Life Science USA, Stamford, CT) was used to analyze membranes. The immunoreactivity ratios were normalized to the control or sham group.

2.9. Statistics

The data were shown as means ± standard error of the means (S.E.M.), The results from the Y-maze test, the passive avoidance test, discrimination ratio in the novel object recognition test, and immunoreactivity in the Western blot analysis were analyzed by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test for multiple comparisons. The results from object preference ratio in the novel object recognition test were analyzed by two-way ANOVA followed by Bonferroni's post hoc test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Effect of HCE on cholinergic blockade-caused cognitive impairment in the Y-maze task

The working or short-term memory capability of mice with scopolamine-caused memory disorder was inspected using the Y-maze task. Spontaneous alternation activity was significantly different between each group [F (5, 52) = 3.760, p = 0.0056, Fig. 1A]. The administration of HCE (100 and 300 mg/kg) and donepezil significantly improved the cholinergic blockade-caused decrease in the percentage of spontaneous alternations ($p < 0.05$). Across the experimental groups, there was no significant difference in the total arm entries [F (5, 52) = 2.013, p = 0.0921, Fig. 1B], indicating that the improving effect of HCE on working memory performance was not affected by general locomotor activity.

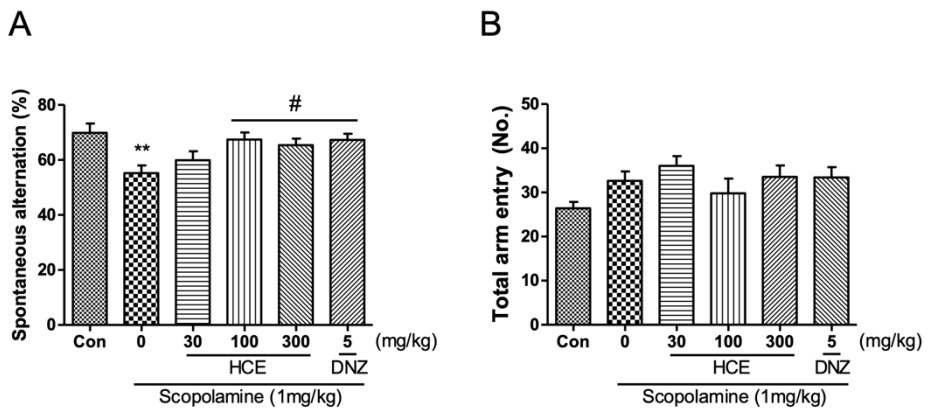

Figure 1. The ameliorating effect of HCE on scopolamine-induced cognitive impairment in the Y-maze test. The mice were administered of HCE (30, 100, and 300 mg/kg), or the same volume of vehicle solution (10% Tween 80 solution) 1 h before the test. Donepezil (5 mg/kg, p.o.) was used as a positive control. Memory impairment was induced by scopolamine (1 mg/kg, i.p.) 30 min before the test. The results from the spontaneous alternation (A) and total arm entry (B) were presented. Data represent the means ± S.E.M. (n = 8 - 10/group) (**p < 0.01, versus the vehicle-treated control; #p < 0.05, versus the scopolamine-treated group). Con, control; DNZ, donepezil.

3.2. Effect of HCE on cholinergic blockade-caused cognitive dysfunction in the step-through passive avoidance task

To examine the effects of HCE on hippocampal dependent learning and memory in cholinergic blockade-caused cognitive disorder in mice, the passive avoidance test was conducted. The significant group effects were discovered in the step-through latencies in the retention trial [F (5, 54) = 43.21, p < 0.001, Fig. 2]. HCE administration (100 and 300 mg/kg) and donepezil significantly attenuated the reduction of step-through latency of scopolamine treatment in retention trial (p < 0.05). Additionally, there were no significant differences in the step-through latency of the acquisition trial across the groups [F (5, 53) = 3.346, p > 0.05, Fig. 2].

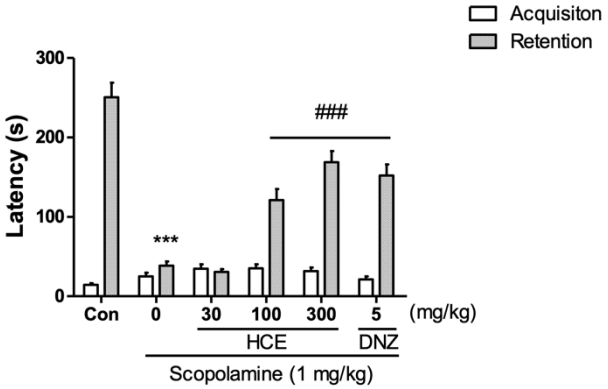

Figure 2. The effect of HCE on scopolamine-induced memory impairment in the step-through passive avoidance test. The mice were administered of HCE (30, 100, and 300 mg/kg), or the same volume of vehicle solution (10% Tween 80 solution) 1 h before the acquisition trial. Donepezil (5 mg/kg, p.o.) was used as a positive control. Memory impairment was induced by scopolamine (1 mg/kg, i.p.) 30 min before the acquisition trial. A retention trial was conducted 24 h after the acquisition trial without drug administration for 300 s. The results from the acquisition trial and retention trial were presented. Data represent the means ± S.E.M. (n = 9 - 10/group) (***p < 0.001, versus the vehicle-treated control; ###p < 0.001, versus the scopolamine-treated group). Con, control; DNZ, donepezil.

3.3. Effect of HCE on cholinergic blockade-caused memorial dysfunction in the novel object recognition task

We executed the novel object recognition task in mice with memory disorder provoked by scopolamine in order to clarify whether HCE improves recognition memory. The object preference ratio showed significant group effects [object, F (1, 104) = 16.64, p < 0.001; treatment, F (5, 104) = 80.93, p < 0.001; interaction object x treatment, F (5, 104) = 0, p = 1; Fig. 3A] and the discrimination ratio also showed significant group effects [F (5, 54) = 5.636, p = 0.0003, Fig. 3B]. HCE and donepezil significantly reversed the scopolamine-caused decrease in cognitive deficits, as indicated by the lowered preference for the novel object and a lower discrimination ratio between the novel and familiar objects (p < 0.05). The total exploration times, however, were not changed significantly between treatment groups [F (5, 54) = 0.3091, p = 0.9054, Fig. 3C]. These results indicated that HCE improve the recognition memory against the cholinergic blockade state.

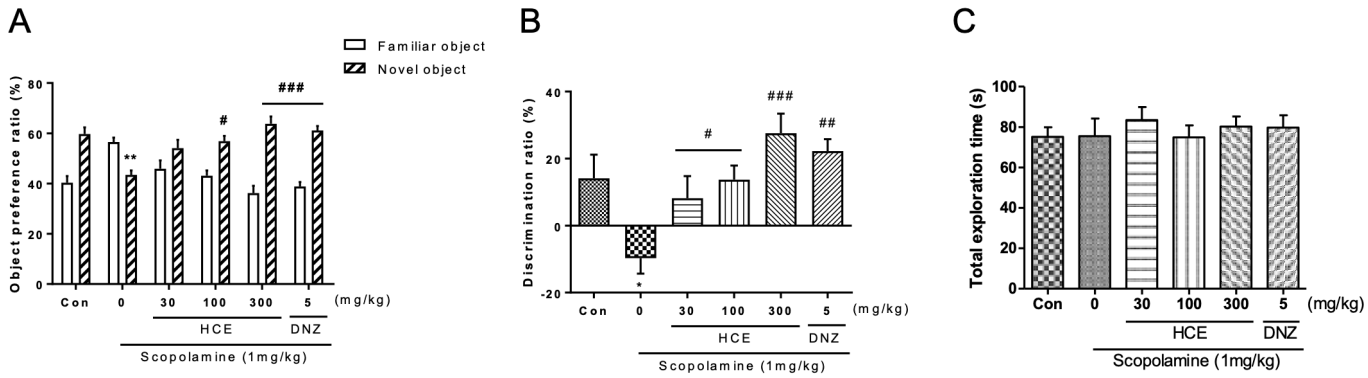

Figure 3. The effects of HCE on scopolamine-induced cognitive dysfunction on the novel object recognition test in mice. The mice were administered of HCE (30, 100, and 300 mg/kg), or the same volume of vehicle solution (10% Tween 80 solution) 1 h before the training session. Donepezil (5 mg/kg, p.o.) was used as a positive control. Memory impairment was induced by scopolamine (1 mg/kg, i.p.) 30 min before the training session. A probe session was performed 24 h after the training session without drug administration. The results from the percentage of preference on the novel or the familiar objects (A), discrimination ratio (B), and the total exploration time (C). Data represent the means ± S.E.M. (n = 8 - 10/group) (*p < 0.05, versus the vehicle-treated control; **p < 0.01, versus the vehicle-treated control; #p < 0.05, versus the scopolamine-treated group; ##p < 0.01, versus the scopolamine-treated group; ###p < 0.001, versus the scopolamine-treated group). Con, control; DNZ, donepezil.

3.4. Effects of HCE on ERK-CREB signaling pathway under hypocholinergic state

To investigate which signaling molecules are associated with memory ameliorating activity of HCE, the Western blot analysis was conducted. There were significant differences in the phosphorylation level of ERK across each group [F (4, 15) = 4.036, p =0.0204, Fig. 4B]. Phosphorylation level of ERK, which was reduced by scopolamine, was significantly increased by the administration of HCE (300 mg/kg) or donepezil (p < 0.05). Also, there were significant differences in the phosphorylation level of CREB between administration groups [F (4, 20) = 3.231, p = 0.0337, Fig. 4B]. Scopolamine-induced decreased phosphorylation level of CREB was significantly increased by the administration of HCE (100 or 300 mg/kg) or donepezil (p < 0.05).

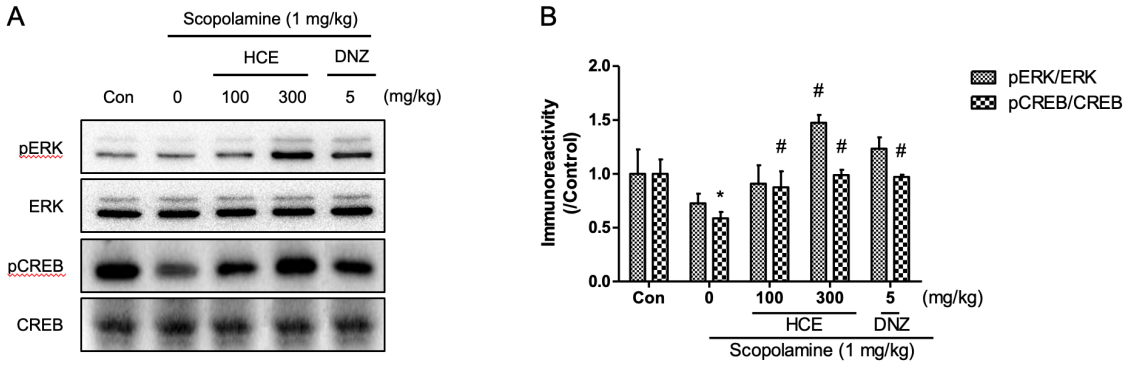

Figure 4. The effects of HCE on ERK-CREB signaling pathway in the hippocampal tissue. HCE (30, 100, and 300 mg/kg) or the same volume of vehicle solution (10% Tween 80 solution) was administered 1 h before sacrifice. Donepezil (5 mg/kg, p.o.) was used as a positive control. Memory impairment was induced by scopolamine (1 mg/kg, i.p.) 30 min before sacrifice Immunoreactivity (A), and quantitative analysis were represented. The immunoreactivity of pERK/ERK and pCREB/CREB were normalized to the control group (taken as 1.0). Data represent the means ± S.E.M. (n = 4 - 5/group) (*p < 0.05, versus the vehicle-treated control; #p < 0.05, versus the scopolamine-treated group). Con, control; DNZ, donepezil.

3.5. Effects of sub-chronic administration of HCE on $A\beta_{1-42}$ protein-caused memory damaged mouse model in the step-through passive avoidance test

The passive avoidance test was executed to measure the effect of sub-chronic administration of HCE on long-term memory in $A\beta_{1-42}$ protein-caused cognitive deficit model. There were significant differences in step-through latency of the retention trial between each group [F (3, 34) = 53.20, $p < 0.001$, Fig. 5]. When compared to the group that induced cognitive impairment by $A\beta_{1-42}$ protein, step-through latency was significantly increased in HCE and donepezil treatment group ($p < 0.05$). In addition, there was no significant differences in step-through latency of the acquisition trial across the groups [F (3, 35) = 0.5149, p = 0.6747, Fig. 5].

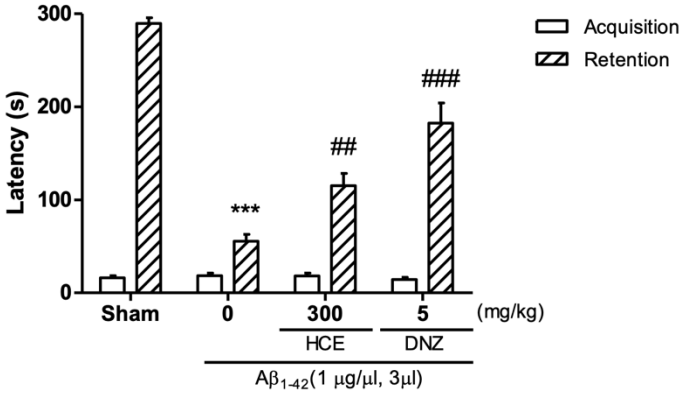

Figure 5. The effects of HCE on $A\beta_{1-42}$-induced memory impairment in the step-through passive avoidance test. Animals were intracerebroventricularly injected with $A\beta_{1-42}$ protein (3 μg/3 μL) or sterile saline (3 μL). Immediately following $A\beta_{1-42}$ protein injection, HCE (300 mg/kg), donepezil (5 mg/kg), as a positive control, or the same volume of vehicle solution (10% Tween 80 solution) were administered for 6 days (once a day, p.o.). The last administration of HCE, donepezil or vehicle solution was conducted at 1 h prior to acquisition trial. A retention trial was performed 24 h after the acquisition trial without drug administration. The results from acquisition trial and retention trial are presented. Data represent the means ± S.E.M. (n = 9 - 10/group) (***$p < 0.001$, versus the sham control group; ##$p < 0.01$, versus the $A\beta_{1-42}$-injected group; ###$p < 0.001$, versus the Aβ1-42-injected group). DNZ, donepezil.

3.6. Effects of sub-chronic administration of HCE on $A\beta_{1-42}$ protein-caused recognition memory impaired mouse model in the novel object recognition test

To explore the effect of sub-chronic administration of HCE in object recognition function, we executed the novel object recognition test. There were significant group differences in object preference ratio between groups [object, F (1, 70) = 14.87, $p < 0.001$; treatment, F (3, 70) = 94.85, $p < 0.001$; interaction object x treatment, F (3, 70) = 0, p = 1; Fig. 6A], and discrimination ratio [F (3, 35) = 7.091, p = 0.0008, Fig. 6B]. The novel object preference ratio and discrimination index were significantly increased in HCE or donepezil administration group compared to $A\beta_{1-42}$ peptide-induced cognitive dysfunction group ($p < 0.05$). Also, there was no significant difference between the groups in total exploration time [F (3, 35) = 0.7446, p = 0.5328, Fig. 6C].

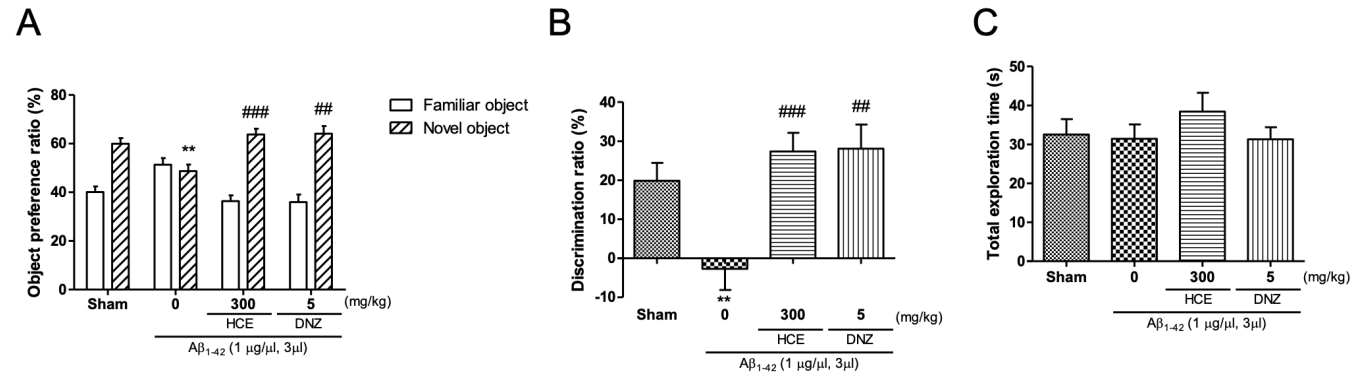

Figure 6. The effects of HCE on $A\beta_{1-42}$-induced cognitive dysfunction on the novel object recognition test. Animals were intracerebroventricularly injected with $A\beta_{1-42}$ protein (3 μg/3 μL) or sterile saline (3 μL). Immediately following $A\beta_{1-42}$ protein injection, HCE (300 mg/kg), donepezil (5 mg/kg), as a positive control, or the same volume of vehicle solution (10% Tween 80 solution) were

administered for 6 days (once a day, p.o.). The last administration of HCE, donepezil or vehicle solution was conducted at 1 h prior to training session. A probe session was performed 24 h after the training session without drug administration. The results from the percentage of preference on the novel or the familiar objects (A), discrimination ratio (B), and the total exploration time (C). Data represent the means ± S.E.M. (n = 9 - 10/group) (**p < 0.01, versus the sham control group; ##p < 0.01, versus the $A\beta_{1\text{-}42}$-injected group; ###p < 0.001, versus the Aβ1-42-injected group). DNZ, donepezil.

3.7. Effects of sub-chronic administration of HCE on neuroplasticity and inflammation under $A\beta_{1\text{-}42}$ protein treatment

To explore the mechanism of HCE in memory improvement in AD mouse model induced by $A\beta_{1\text{-}42}$ protein, the Western blot analysis was conducted. There were significant differences in the phosphorylation level of ERK between administration groups [F (3, 20) = 3.368, p = 0.0389, Fig. 7B]. Phosphorylation level of ERK was significantly increased by the administration of HCE. Also, there were significant differences in the phosphorylation level of CaMKII across administration groups [F (3, 19) = 4.274, p = 0.0182, Fig. 7B]. Phosphorylation level of CaMKII was significantly increased by administration of HCE (p < 0.05). There were significant differences in the BDNF expression levels across administration groups [F (3, 23) = 3.375, p = 0.0387, Fig. 7B]. The expression level of BDNF was significantly increased by administration of HCE. In addition, there were significant differences in the expression level of GFAP, Iba-1, and caspase 3 [GFAP, F (3, 20) = 7.628, p = 0.0014, Fig. 7B; Iba-1, F (3, 20) = 3.724, p = 0.0282, Fig. 7B; caspase 3, F (3, 20) = 3.515, p = 0.0340, Fig. 7B]. The expression levels of GFAP, Iba-1, and caspase 3 were significantly increased in $A\beta_{1\text{-}42}$ protein injected group (p < 0.05), and the administration of HCE normalized the expression levels of GFAP, Iba-1 and caspase 3, respectively (p < 0.05).

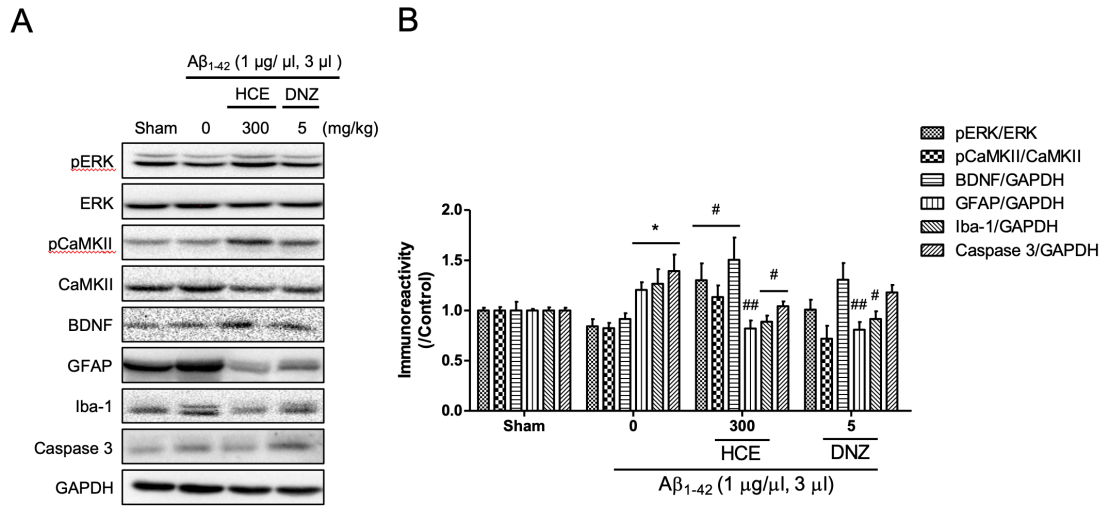

Figure 7. The effects of HCE on ERK, CaMKII, BDNF, GFAP, Iba-1 and caspase 3 expression in the cortical tissue. Animals were intracerebroventricularly injected with $A\beta_{1\text{-}42}$ protein (3 μg/3 μL) or sterile saline (3 μL). Immediately following $A\beta_{1\text{-}42}$ protein injection, HCE (300 mg/kg), donepezil (5 mg/kg), as a positive control, or the same volume of vehicle solution (10% Tween 80 solution) were administered for 6 days (once a day, p.o.). The last administration of HCE, donepezil or vehicle solution was conducted at 1 h prior to sacrifice. The phosphorylation levels of ERK and CaMKII and the expression level of BDNF, GFAP, Iba-1 and caspase 3 in cortical tissue were measured by the Western blot analysis. Immunoreactivity (A), and quantitative analysis were represented. The immunoreactivity of pERK/ERK and pCaMKII/CaMKII, BDNF/GAPDH, GFAP/GAPDH, Iba-1/GAPDH, and caspase 3/GAPDH were normalized to the sham group (taken as 1.0). Data represent the means ± S.E.M. (n = 5-6/group) (*p < 0.05, versus the sham control group; #p < 0.05, versus the $A\beta_{1\text{-}42}$-injected group; ##p < 0.01, versus the $A\beta_{1\text{-}42}$-injected group). DNZ, donepezil.

4. Discussion

The cognitive function of the mixed extract of three herbs was investigated through the Y-maze test, the passive avoidance test, and the novel object recognition test in animal model induced with scopolamine or $A\beta_{1-42}$ peptide, and we observed that HCE improved cognitive function in memory impaired mice model in the present studies. In addition, HCE increased the phosphorylation level of ERK, CREB and CaMKII, and the BDNF expression level, and decreased the expression level of GFAP, Iba-1, and caspase 3 in the memory impaired mice.

HCE is prescribed for RA under the brand name of JOINS®, and its anti-inflammatory and analgesic effects have been reported (C. H. Choi et al., 2014; H. R. Kim et al., 2019; Song et al., 2007). In AD, inflammation has been suggested as a pathogenesis hypothesis along with Aβ and NFT (Cao, Hou, Ping, & Cai, 2018; Ozben & Ozben, 2019). Synaptic damage and cell death have been observed due to the activation of microglia and astrocytes in AD (Chaney, Williams, & Boutin, 2019). Microglia is an immunological cell in the central nervous system (Chaney et al., 2019) and maintains homeostasis. However, it secretes cytokines in pathological states (Heneka et al., 2015) along with astrocytes (Fakhoury, 2018). Until yet, the anti-inflammatory effect of HCE has only been reported on RA or RA-related diseases (H. R. Kim et al., 2019; J. H. Kim et al., 2005; Song et al., 2007). Therefore, we hypothesized HCE might exert anti-inflammatory effect in the brain and attenuate inflammatory associated disease, such as AD. To examine how HCE affects memory enhancement, we conducted behavioral tests through the Y-maze, the passive avoidance test, and the novel object recognition test under scopolamine- and $A\beta_{1-42}$ protein treatment states. Decreased spontaneous activity induced by cholinergic blockade was upregulated by a single-administration of HCE or donepezil in the Y-maze. The latency of entry into the dark box from the passive avoidance test was decreased in the scopolamine-treated group, and increased by both single administration of HCE and sub-chronic administration of HCE. Likewise, both the HCE single and sub-chronic administration enhanced recognition memory in the novel object recognition test. These behavioral results suggest that HCE could ameliorate cognitive dysfunction observed in hypocholinergic or Aβ-induced neuroinflammatory states.

ERK/CaMKII/CREB signaling pathway is associated long-term potentiation in the hippocampus (Lyu et al., 2020), and synaptic plasticity (Curtis & Finkbeiner, 1999), which is impaired in dementia (Jiang et al., 2021). In the memory impaired model, the activity of ERK/CaMKII/CREB signaling pathway was decreased and this leads to decrease in synaptic plasticity and in the number of neurons. BDNF is one of the neurotrophic factors which is associated with regulating neuronal survival and differentiation (Huang & Reichardt, 2001) and plays a role in memory consolidation (Bae, Kim, Kim, et al., 2020; Liao et al., 2019). In our results of the Western blot analysis, HCE administration increased phosphorylation levels of ERK, CaMKII, CREB and the expression level of BDNF, suggesting that HCE administration could contribute to recover synaptic plasticity. In pathogenic state, astrocytes are activated and produce inflammatory cytokines (Choi, Lee, Lim, Satoh, & Kim, 2014; Deng et al., 2014). Microglia is also involved in the inflammatory response in brain (Solito & Sastre, 2012). As mentioned, Aβ aggregation causes an inflammatory response, which induces to activate microglia. In addition, activated microglia also promote plaque formation in pathological state (Hansen, Hanson, & Sheng, 2018). HCE treatment normalized the expression level of GFAP, and Iba-1, indicating that the improving activity of HCE on Aβ-induced cognitive deficiency might be arisen from its anti-neuroinflammatory activity in our study. It has been reported that apoptosis is occurred in Aβ-accumulated neuronal cells (Tesco et al., 2007). Caspase 3 is a marker of cell death (D'Amelio, Cavallucci, & Cecconi, 2010) and increase in its expression level coincides with an increase in apoptosis. $A\beta_{1-42}$ protein treatment increased caspase 3 expression levels, which is compatible with previous report that neuronal cell death causes increased caspase 3 levels, resulting in the decrease of synaptic plasticity (Chan & Mattson, 1999). Thus, HCE ameliorated cognitive function through enhanced ERK/CaMKII/CREB signaling pathway under hypocholinergic state and attenuated neuroinflammation under $A\beta_{1-42}$ protein injected state, respectively.

In summary, the HCE which is used for treating rheumatoid arthritis attenuated cognitive dysfunction induced by scopolamine or $A\beta_{1-42}$ in mice. These cognitive function improvement effects were derived from activation of ERK/CaMKII/CREB signaling pathway, increased expression level of BDNF, and decreased neuroinflammation and neuronal cell death. According to these findings, HCE might be a competitor for reducing the cognitive impairment occurred in neurodegenerative disease, including AD.

Author Contributions: Jong Hoon Ryu designed the studies and wrote the paper with Keontae Park, and Young-jin Cho. Ho Jung Bae, Chang Hyeon Kong, Seo Yun Jung and Dong Hyun Kim performed and analyzed the molecular studies. Keontae Park, Woo Chang Kang, and Mijin Jeon performed and analyzed the behavioral tests. Min Seo Kim, and Won Hyung Lee prepare herbal extract and performed the gavage.

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