

# *Houttuynia cordata* Improves Cognitive Deficits in Cholinergic Dysfunction Alzheimer's Disease-Like Models

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## Abstract

Cognitive impairment is a result of dementia of diverse causes, such as cholinergic dysfunction and Alzheimer's disease (AD). *Houttuynia cordata* Thunb. (Saururaceae) has long been used as a traditional herbal medicine. It has biological activities including protective effects against amyloid beta ( $A\beta$ ) toxicity, via regulation of calcium homeostasis, in rat hippocampal cells. To extend previous reports, we investigated the effects of water extracts of *H. cordata* herb (HCW) on tauopathies, also involving calcium influx. We then confirmed the effects of HCW in improving memory impairment and neuronal damage in mice with  $A\beta$ -induced neurotoxicity. We also investigated the effects of HCW against scopolamine-induced cholinergic dysfunction in mice. In primary neuronal cells, HCW inhibited the phosphorylation of tau by regulating p25/p35 expression in  $A\beta$ -induced neurotoxicity. In mice with  $A\beta$ -induced neurotoxicity, HCW improved cognitive impairment, as assessed with behavioral tasks, such as novel object recognition, Y-maze, and passive avoidance tasks. HCW also inhibited the degeneration of neurons in the CA3 region of the hippocampus in  $A\beta$ -induced neurotoxicity. Moreover, HCW, which had an  $IC_{50}$  value of 79.7  $\mu$ g/ml for acetylcholinesterase inhibition, ameliorated scopolamine-induced cognitive impairment significantly in Y-maze and passive avoidance tasks. These results indicate that HCW improved cognitive impairment, due to cholinergic dysfunction, with inhibitory effects against tauopathies and cholinergic antagonists, suggesting that HCW may be an interesting candidate to investigate for the treatment of AD.

**Key Words:** *Houttuynia cordata*, Cognitive impairment, Amyloid beta, Cholinergic dysfunction, Neuroprotection

## INTRODUCTION

Cholinergic neurons containing acetylcholine (ACh) as a direct indicator of cholinergic neurotransmitter are involved in memory and cognition by enhancing afferent input, synapses, and maintenance of novel information in where memory is encoded in the brain (Drachman and Leavitt, 1974; Schliebs and Arendt, 2011). Much attention has been focused on neuronal dysfunction, especially cholinergic dysfunction in the brain, which can eventually lead to cognitive impairment and the decline into dementia (Schliebs and Arendt, 2011). In the basal forebrain cholinergic system, it has been found that a decrease in the ACh concentration occurs in cholinergic synaptic clefts in the brain of a patient suffering from cognitive im-

pairment (Farlow and Cummings, 2008). Thus, re-increasing the ACh concentration in the cholinergic synaptic cleft could be useful to treat the cognitive impairment in patients with dementia including Alzheimer's disease (AD) which is the most common neurodegenerative disease (Farlow and Cummings, 2008).

Additionally, although there are various causes of cholinergic dysfunction, amyloid beta ( $A\beta$ ) deposition and neurofibrillary tangles bring about the cholinergic dysfunction usually found in the hippocampus and basal forebrain cholinergic system in AD, leading to cholinergic deficiency and synapse loss (Zheng *et al.*, 2002; Schliebs and Arendt, 2011). Accumulation of  $A\beta$ , a peptide of 40-43 amino acids, acts as a neurotoxin because it forms a  $\beta$ -sheet structure and then induces neuronal

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death and synaptic loss (Grace *et al.*, 2002). A $\beta$  also interacts directly with neurons, leading to apoptosis via increasing intracellular calcium levels, generation of reactive oxygen species, cholinergic dysfunction, and the abnormal overphosphorylation of tau (Busciglio *et al.*, 1995). Thus, in helping to improve cholinergic dysfunction due to A $\beta$  toxicity, agents against tau phosphorylation and cholinergic antagonists could be effective for the development of new drugs for treating cognitive impairment (Terry *et al.*, 1991; Van der Zee and Luiten, 1999).

The aerial part of *Houttuynia cordata* Thunb. (Hottuyniae Herba, Saururaceae) is a traditional herbal medicine for furunculosis, disorders of urines and fever in East Asia and, recently, it has been shown to have effective anti-inflammatory, anti-oxidant, anti-virus, and anti-leukemic effects (Chen *et al.*, 2003; Toda, 2005; Nuengchamnonng *et al.*, 2009; Shin *et al.*, 2010; Tian *et al.*, 2011). Additionally, there is a report that *H. cordata* enhances memory and learning in a mouse model via an anti-oxidant effect (Shi *et al.*, 2004). In a previous study, we reported that *H. cordata* had a protective effect against A $\beta$ -toxicity in regulating intracellular calcium levels, preventing reactive oxygen species overproduction, and inhibiting mitochondria-mediated apoptosis in rat primary neuronal cells (Park and Oh, 2012). Although there are a few reports of *H. cordata* and effects in memory impairment, there is no reported research about improving cholinergic dysfunction with *H. cordata* by inhibiting tauopathies, confirmed with *in vivo* experiments.

In this study, we investigated the effects of *H. cordata* on improving memory impairment by inhibiting tauopathies caused by increasing intracellular calcium levels as an extension of our previous study. Factors involved in tauopathies, the p25/CDK5 complex and p-tau205 were analyzed and the cognitive effects of *H. cordata* were confirmed by *in vivo* studies. Also, the development of cholinergic dysfunction and the effects of *H. cordata* were examined using a scopolamine-injected *in vivo* model.

## MATERIALS AND METHODS

### Materials

Neurobasal medium and B27 supplement were purchased from Gibco (Carlsbad, CA, USA). Penicillin and Streptomycin were purchased from Hyclone Lab Inc. (Logan, UT, USA). Chlorogenic acid, caffeic acid, poly-L-lysine (PLL), A $\beta_{25-35}$ , A $\beta_{1-42}$ , dimethyl sulfoxide (DMSO), glutamine, sodium chloride, phosphate-buffered saline (PBS), glycine, trizma base, 9-amino-1,2,3,4-tetrahydroacridine hydrochloride (tacrine), scopolamine hydrobromide, acetylthiocholine iodide, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), and sodium bicarbonate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tetramethylethylenediamine, Tween-20, ammonium persulfate, acrylamide, enhanced chemiluminescence (ECL) reagent, and skimmed milk were purchased from Bio-Rad Lab (Hercules, CA, USA). Donepezil hydrochloride was supplied by Eisai Korea Co., Ltd. (Aricept, Seoul, Korea). Mouse anti- $\beta$ -actin, rabbit anti-p35 (C-19), and rabbit anti-phospho-tau (p-tau, Thr205) antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Mouse anti-neuronal nuclei (NeuN) antibody was obtained from Millipore Corp. (Billerica, MA, USA). Biotinylated anti-rabbit antibody, normal goat serum, and an avidin biotin peroxidase complex (ABC) standard kit were purchased from Vector Lab (Burlingame, CA, USA). Anti-rabbit

and anti-mouse horseradish peroxidase secondary antibodies were purchased from Assay Designs Inc. (Ann Arbor, MI, USA). Zoletil 50 $^{\circ}$  and Rompun $^{\circ}$  were purchased from Virbac (Carros, France) and Bayer Korea (Seoul, Korea), respectively. A $\beta_{25-35}$  and A $\beta_{1-42}$  were reconstituted in sterile water at a concentration of 500  $\mu$ M and 1 mg/ml for *in vitro* and *in vivo* assays, respectively. Aliquots were incubated at 37 $^{\circ}$ C for 72 h or 120 h to form aggregated amyloid. A dried Houttuyniae Herba was purchased from Jung Do herbal Drug Co. (Seoul, Korea) and the voucher specimen (KHUOPS-MH022) was deposited in the herbarium of the College of Pharmacy, Kyung Hee University (Seoul, Korea). The *Houttuynia cordata* water extract (HCW) was prepared according to methods published previously (Park and Oh, 2012).

### Primary cultures of neuronal cells

Primary cultures of cortical neurons were prepared according to methods published previously (Park and Oh, 2012).

### Western blot

The primary culture cells were lysed with a triple-detergent lysis buffer to detect p35, p25 and tau according to the manufacturer's instructions. The lysates were separated by 10% SDS-PAGE and then transferred to a membrane. Membranes were incubated with 5% skimmed milk in Tris-buffered saline and Tween 20 for 1 h and then with the primary antibodies (1:500 dilution of p35, p25, p-tau, and  $\beta$ -actin) overnight at 4 $^{\circ}$ C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h. Immunoreactive bands were detected using an enhanced chemiluminescence detection kit, and a LAS-4000 mini system (Fujifilm Corporation) was used for visualization. The intensities of the bands were normalized to the non-phospho-form band or  $\beta$ -actin band using Multi Gauge software (Fujifilm Corporation).

### Animals

Male ICR mice (8 weeks, 27-30 g) were purchased from the Daehan Biolink Co. Ltd. (Eumseong, Korea). Animals were housed 5 or 6 per cage, had free access to water and food, and maintained under a constant temperature (23  $\pm$  1 $^{\circ}$ C), humidity (60  $\pm$  10%), and a 12 h light/dark cycle. Animal treatment and maintenance were carried out in accordance with the Principle of Laboratory Animal Care (NIH publication No. 85-23, revised 1985) and the Animal Care and Use Guidelines of Kyung Hee University, Seoul, Korea.

### A $\beta$ injection, scopolamine injection and drug administration

Mice were immediately anesthetized by mixture of Zoletil 50 $^{\circ}$  and Rompun $^{\circ}$  solution (3:1 ratio, 1 mL/kg, *i.p.*) and mounted in a stereotaxic apparatus (myNeuroLab, St. Louis, MO, USA). Each mouse was unilaterally injected (at rate 0.5  $\mu$ L/min) with 3  $\mu$ L of A $\beta_{1-42}$  (1 mg/mL) into the hilus of dentate gyrus (DG) of hippocampus (coordinates with respect to bregma in mm: AP -2.0, ML 1.5, DV 2.0), according to the stereotaxic atlas of mouse brain (Paxinos and Franklin, 2001). The sham-operated mice were injected with the same volume of saline alone. The accuracy of stereotaxic injection to the targeted region was monitored in all animals by examination of the needle tract within brain sections. The mice were randomly divided into 5 groups (n=6 in each group); (1) Control group (sham-operated plus intraorally saline-treated group), (2) A $\beta$  toxicity group (A $\beta_{1-42}$ -lesioned plus intraorally saline-treated

group), (3) A $\beta$  toxicity+HCW 100 mg/kg/day group (A $\beta$ <sub>1-42</sub>-lesioned plus intraorally HCW 100 mg/kg/day treated group), (4) A $\beta$  toxicity+HCW 200 mg/kg/day group (A $\beta$ <sub>1-42</sub>-lesioned plus intraorally HCW 200 mg/kg/day treated group). HCW dissolved in saline was administered once per day for 3 consecutive days before stereotaxic injection and 6 consecutive days after stereotaxic injection.

In the scopolamine-induced memory impairment study, mice were randomly divided into 6 groups (n=10 in each group). HCW (100, 200, and 400 mg/kg, *p.o.*) were given as a single administration 1 h before the acquisition trial in the animal behavior test. Memory impairment was induced by scopolamine injection (1.1 mg/kg, *i.p.*) 30 min before the acquisition trial in the animal behavior test.

### Novel object recognition task

The novel object recognition test (NORT) was performed according to the method described previously (Karasawa *et al.*, 2008). The experiments were carried out in a gray open field box (45×45×50 cm). Prior to the test, mice were habituated to the test box for 5 min without objects. After a habituation period, mice were placed into the test box with two identical objects and allowed to explore for 3 min. The objects used in this study were wooden blocks of the same size but different shape. The time spent by the animal exploring each object was measured (defined as the training session). Twenty-four hours after training session, mice were allowed to explore the objects for 3 min, in which familiar object used in the previous training session was placed with a novel object. The time that the animals spent exploring the novel and the familiar objects were recorded (defined as the test session). The animals were regarded to be exploring when they were facing, sniffing or biting the object. The test box and objects were cleaned with 70% ethanol between sessions. Results were expressed as percentage of novel object recognition time (time percentage=novel/[novel+familiar]×100).

### Y-maze task

The Y-maze is the three arms horizontal maze which the arms are disposed at 120° angles from each other (40 cm long and 3 cm wide with 12 cm high walls). The constituent of the maze floor and walls has been described previously (Kim *et al.*, 2014). Each arm has the sequence like A, B and C and mice were placed within any arm. Numbers 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 like ABC, CAB, or BCA. Maze arms were thoroughly cleaned between tasks to remove residual odors. The result was expressed as percentage of alternation to the following equation: % Alternation=[(Number of alternations)/(Total arm entries-2)]×100.

### Passive avoidance task

Learning and memory test was performed using a two-compartment step-through passive avoidance apparatus. The box was divided into bright (21×21×21 cm) and dark (21×21×21 cm) compartments by a guillotine door. The bright compartment contained an 50 W electric lamp, and the floor of the dark compartment was composed of 2 mm stainless steel rods spaced 1 cm apart. Mice were treated with HCW or vehicle 1 h before the acquisition trial and initially placed in the bright compartment for the acquisition trial. The door between the

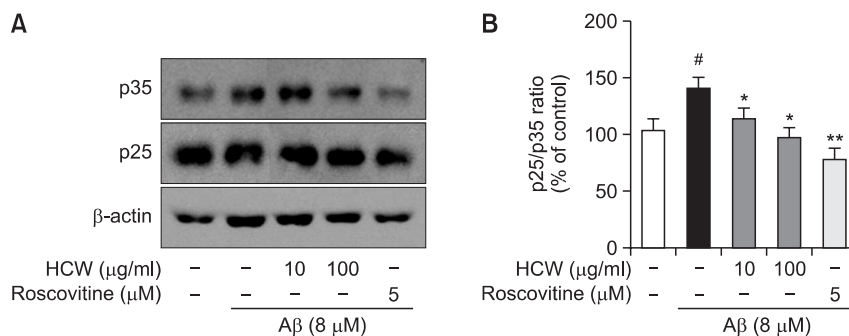
two compartments was opened 10 s later. Then, when the hind legs of the mice entered into the dark chamber, the guillotine door was closed and electrical foot shock (0.6 mA) was delivered through the grid floor for 3 s. The mice were again placed in the bright chamber for the retention trial 24 h after the acquisition trial. The time taken for a mouse to enter the dark chamber after the door opening was defined as the latency time. The latency time was recorded for up to 300 s.

### Immunohistochemistry and quantification

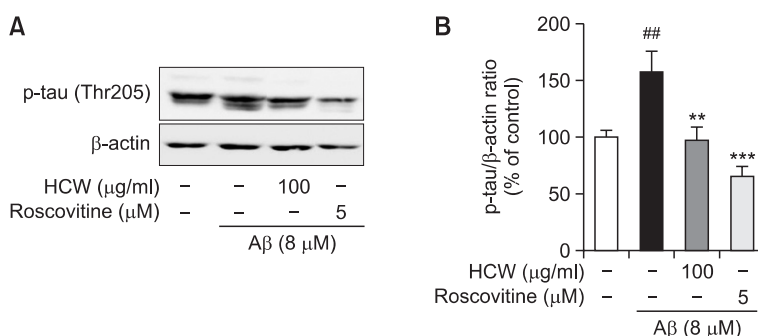
At behavioral tests, mice were perfused transcardially with 0.05 M PBS, and then fixed with cold 4% PFA in 0.1 M phosphate buffer. Brains were removed and post-fixed in 0.1 M phosphate buffer containing 4% PFA overnight at 4°C and then immersed in a solution containing 30% sucrose in 0.05 M PBS for cryoprotection. Serial 30  $\mu$ m-thick coronal sections were cut on a freezing microtome (Leica, Nussloch, Germany) and stored in cryoprotectant (25% ethylene glycol, 25% glycerol, and 0.05 M phosphate buffer) at 4°C until use. For immunohistochemical study, brain sections were briefly rinsed in PBS and treated with 1% hydrogen peroxide for 15 min. The sections were incubated with a mouse anti-NeuN antibody (1:1000 dilution) overnight at 4°C in the presence of 0.3% triton X-100 and NGS. After rinsing in PBS buffer, the sections were then incubated with biotinylated anti-mouse IgG (1:200 dilution) for 90 min and with ABC (1:100 dilution) for 1 h at room temperature. Peroxidase activity was visualized by incubating sections with DAB in 0.05 M tris-buffered saline (pH 7.6). After several rinses with PBS, sections were mounted on gelatin-coated slides, dehydrated, and cover-slipped using histomount medium. The optical density of NeuN-immunoreactivities in the CA3 of hippocampus region was analyzed with ImageJ software (Bethesda, MD, USA). For measurement of the optical density of NeuN-immunoreactivity, the total region of interest was manually outlined and averaged optical densities were acquired in images with converted eight-bit indexed color. The images were photographed at 200×magnification using an optical light microscope (Olympus Microscope System BX51; Olympus, Tokyo, Japan) equipped with a 20× objective lens. Data are presented as percentages of sham group values.

### Acetylcholinesterase inhibition assay

The acetylcholinesterase inhibition assay we used is to examine in a colorimetric method, as described Ellman's method (Ellman *et al.*, 1961). We used acetylthiocholine iodide as a substrate, eel enzyme in purification as the source of enzyme. Each drug was initially dissolved in distilled water and diluted to several concentrations (HCW, 62.5-1000  $\mu$ g/ml) immediately before use. The positive control was Tacrine (0.1 M), which was used to treatment AD like donepezil. An aliquot of diluted drug solution was the mixed with 715  $\mu$ l of phosphate buffer (0.1 M, pH 8.0), 25  $\mu$ l of buffered Ellman's reagent [10 mM5, 50-dithio-bis (2-nitrobenzoic acid), 15mM sodium bicarbonate], and the enzyme source (25  $\mu$ l), and the mixture was pre-incubated at room temperature for 5 min. Absorbance was measured at 410 nm 5 min after For blank, each mixture divided in half in each column and the one of them, the addition of 2.5  $\mu$ l of acetylthiocholine iodide solution (75 mM) was added for reaction and the other had no addition to exclude the interference pigment in each drug. After additional 5 min incubation time in room temperature, Absorbance was measured at 410



**Fig. 1.** Effects of HCW on inhibition of the cleavage p35 to p25 induced by Aβ toxicity in rat primary neuronal cells. Neuronal cells were treated with HCW 30 min before the treating with 8 μM Aβ for a total of 24 h and Western blot was performed (A) to measure the cleavage p35 to p25 level (B). Values are expressed as the mean ± SEM (n=4). #*p*<0.05 compared with the control group, \**p*<0.05 and \*\**p*<0.01 as compared to the Aβ-treated group.



**Fig. 2.** Effect of HCW on inhibition of the phosphorylated tau induced by Aβ toxicity in rat primary neuronal cells. Cortical neurons were treated with HCW 30 min before the treating with 8 μM Aβ for a total of 24 h and Western blot was performed (A) to measure the p-tau 205 level (B). Values are indicated as the mean ± SEM (n=4). ##*p*<0.01 compared with the control group, \*\**p*<0.01 and \*\*\**p*<0.001 as compared to the Aβ-treated group.

nm. The concentration of drug required to inhibit acetylcholinesterase (AChE) activity by 50% (IC<sub>50</sub>) was calculated using an enzyme inhibition dose-response curve.

### Statistical analysis

All statistical parameters were calculated using Graphpad Prism 4.0 software. Values were expressed as the mean ± Standard Error of the Mean (S.E.M.). The results of NORT was analyzed by Student's *t*-test followed by the Mann-Whitney test. Other results were analyzed by one-way ANOVA analysis followed by the Tukey's post hoc test. Differences with a *p* value less than 0.05 were considered statistically significant.

## RESULTS

### Effects of HCW on inhibiting the cleavage of p35 to p25/CDK5 complex in rat primary neuronal cells

To investigate the effects of HCW on Aβ toxicity-induced cleavage of p35 to p25 in primary cortical cells, we performed Western blots to recognize the p35 and p25 forms. This cleavage induces p25/CDK5 complex formation, an activated form that causes tau protein phosphorylation. Exposure to 8 μM Aβ increased the ratio of p25/p35 versus the control group (Fig. 1, 141.29 ± 8.61% of the control). Treatment with roscovitine, a specific inhibitor of CDK5 as a positive control, alleviated the

effect of Aβ toxicity (106.77 ± 30.62%). Treatment with HCW at 10 and 100 μg/mL decreased the ratio of p25/p35 significantly (113.83 ± 9.41% and 97.06 ± 8.70%, respectively). Thus, treatment with HCW had the effect of inhibiting the conversion of p35 to p25, induced by Aβ toxicity in primary cortical cells at low and high doses. Also, the effect of treatment with HCW at 100 μg/mL was comparable to the positive control.

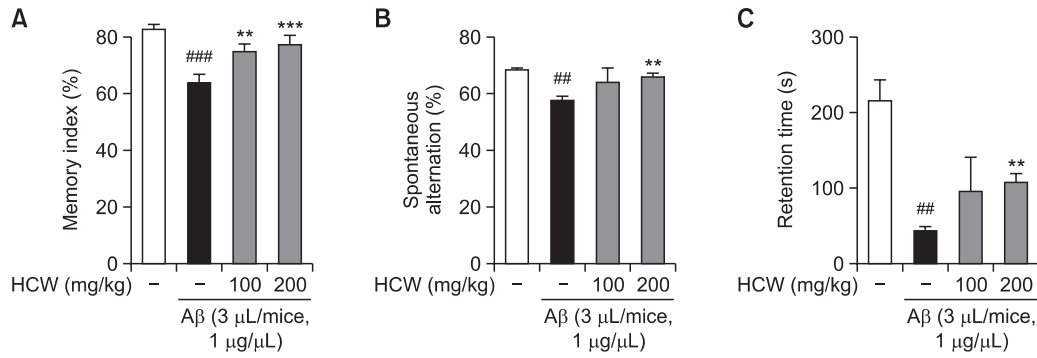
### Effects of HCW in inhibiting tau hyperphosphorylation in rat primary neuronal cells

Tau in neurofibrillary tangles in AD brain is hyperphosphorylated. To examine the degree of phosphorylation of tau protein in Aβ toxicity, we performed Western blots with anti p-tau (Thr 205). The level of p-tau was increased by exposure to 8 μM Aβ versus the control group (Fig. 2, 157 ± 18.76% of the control). Treatment with roscovitine, as a positive control, had the effect of reducing the phosphorylation of tau induced in Aβ toxicity (65.21 ± 8.81%). Treatment with HCW at 100 μg/mL also attenuated tau phosphorylation significantly (96.72 ± 12.01%). Thus, HCW has the effect of inhibiting the phosphorylation of tau protein. Indeed, the effect of treatment with HCW at 100 μg/mL was comparable to the positive control.

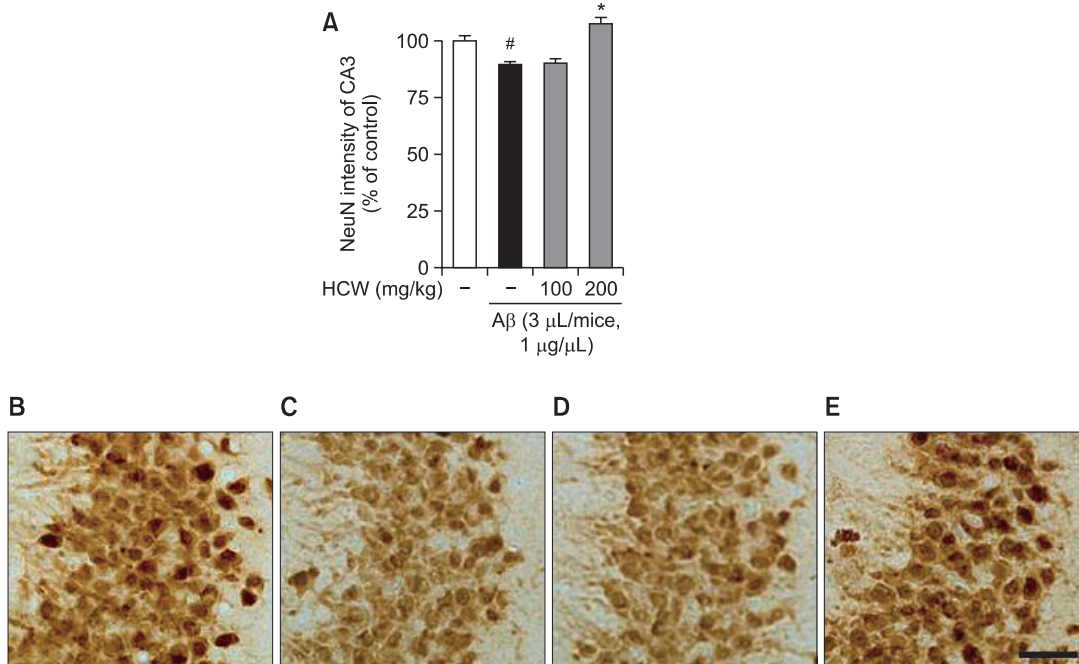
### Effects of HCW on memory development in an Aβ model with NORT, Y-Maze, and PAT

The NORT was performed to investigate whether HCW





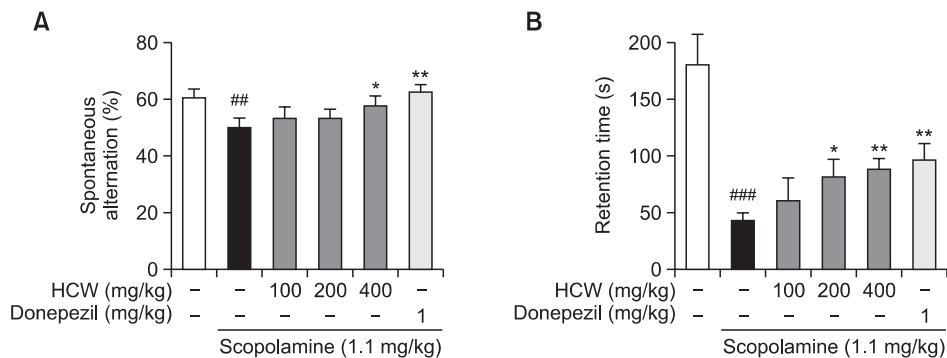
**Fig. 3.** Effects of HCW on improving memory dysfunction in intrahippocampal Aβ-injected mice. In NORT, portions of recognizing novel object were measured (A). In Y-maze test, spontaneous alternation behavior and numbers of arm entries were measured (B). In PAT, the retention time was measured (C). Values are expressed as the mean ± S.E.M. ##*p*<0.01, ###*p*<0.001 as compared to the control group, \*\**p*<0.01, \*\*\**p*<0.001 as compared to the Aβ-injected group.



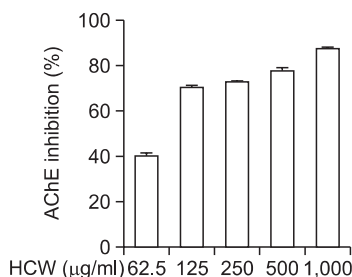
**Fig. 4.** Protective effects of HCW on neuronal cells damaged by Aβ in hippocampal CA3 region. The NeuN immunoreactivity was detected (A). Representative photomicrographs are shown for the CA3 regions of each group (B-E). Values are expressed as the mean ± S.E.M. Scale bar=30 μm. #*p*<0.05 as compared to the control group. \**p*<0.05 as compared with the Aβ-injected group.

improved memory impairment in mice with Aβ injected in the brain. The time spent exploring a novel object in the Aβ-injected group (63.89 ± 2.87%) was shorter than that in the control group (82.54 ± 1.97%; Fig. 3A). However, treatment with HCW at 100 and 200 mg/kg/day resulted in significant improvements in cognitive deficits in this task (74.91 ± 2.74% and 77.82 ± 2.89%, respectively). During the training session, no significant difference in exploratory preferences was found for any of the groups. The Y-maze and passive avoidance tasks (PAT) were also performed. The Y-maze was used to examine effects of HCW on brain impairment due to Aβ neurotoxicity. In this task, the percentage of alternating in the Aβ-injected group (57.34 ± 1.58%) was significantly lower than that of the control group (68.08 ± 1.00%). Treatment with HCW

at 100 mg/kg/day showed no significant difference with the Aβ-injected group (63.96 ± 4.82%). HCW at 200 mg/kg/day (66.03 ± 1.05%) showed a significant effect compared with the Aβ-injected group (Fig. 3B). In PAT, the retention time of the Aβ-injected group (43.59 ± 5.29 s) was significantly shorter than that of the control group (215.53 ± 26.72 s). There was no significant difference with HCW treatment at 100 mg/kg/day (95.62 ± 45.22 s) versus the Aβ-injected group, but treatment with HCW at 200 mg/kg/day (106.86 ± 12.74 s) showed a significant improvement in the cognitive deficit triggered by Aβ toxicity in this task (Fig. 3C). No significant difference was observed in passive avoidance test latencies during the acquisition trial in any group.



**Fig. 5.** Effects of HCW on memory impairment from scopolamine-induced cholinergic dysfunction in mice. Y-maze was conducted at 30-min after scopolamine injection. In Y-maze test, spontaneous alternation behavior and numbers of arm entries were measured (A). In PAT, the retention time was measured (B). Values are expressed as the mean  $\pm$  S.E.M.  $^{##}p < 0.01$  and  $^{###}p < 0.001$  compared with the control group,  $^{*}p < 0.05$  and  $^{**}p < 0.01$  as compared to the AB-treated group.



**Fig. 6.** Effects of HCW on AChE inhibition *in vitro*. Values are expressed as the mean  $\pm$  S.E.M.

#### Effects of HCW in protecting neurons from A $\beta$ toxicity in CA3

To evaluate the effects of HCW against A $\beta$  toxicity, we performed NeuN immunohistochemistry in the mouse hippocampus. NeuN, the neuronal nuclear antigen, is commonly used as an indicator of mature postmitotic neurons. The A $\beta$ -injected group (Fig. 4C, 89.57  $\pm$  1.43%) showed decreased NeuN in the CA3 region of the hippocampus, compared with control group (Fig. 4B, 100  $\pm$  2.46%). Treatment with HCW at 100 and 200 mg/kg/day increased the level of NeuN, showing a neuroprotective effect, restoring neurons. Treatment with HCW at 100 mg/kg/day (Fig. 4D, 90.17  $\pm$  1.90%) showed no significant difference versus the A $\beta$ -injected group. In contrast, NeuN intensity in the CA3 region of the hippocampus was increased significantly by treatment with HCW at 200 mg/kg/day (Fig. 4E, 107.48  $\pm$  3.18%), like the control group.

#### Effects of HCW on memory development in a scopolamine model assessed with Ymaze and PAT

The Y-maze was used to examine the effects of HCW on cognitive deficits due to cholinergic dysfunction in scopolamine-treated mice (1.1 mg/kg, *i.p.*). In this task, the spontaneous alternations showed that the scopolamine-treated group had memory dysfunction; thus, the percentage of alternation in the scopolamine-treated group (52.44  $\pm$  1.76%) was significantly lower than that of the control group (62.71  $\pm$  2.33%). Treatment with HCW at 400 mg/kg/day and donepezil at 5 mg/kg/day groups (61.40  $\pm$  2.11% and 62.44  $\pm$  2.61%, respectively) had significant effects compared with the scopolamine-treatment group. Percentages in these groups were

similar to that of the control group. The percentages in groups treated with HCW at 100 and 200 mg/kg/day showed some effects but they were not statistically significantly different from the scopolamine-treated group (58.45  $\pm$  2.34 and 56.47  $\pm$  1.92%, respectively; Fig. 5A).

PATs were also performed. The retention time in the scopolamine-treated group (42.60  $\pm$  6.25 s) was significantly shorter than that of the control group (180.41  $\pm$  26.19 s). There was no significant difference between treatment with HCW at 100 mg/kg/day (60.36  $\pm$  19.22 s) and the scopolamine-treated group. In contrast, treatment with HCW at 200 and 400 mg/kg/day (81.36  $\pm$  15.74 and 87.73  $\pm$  9.67 s) and donepezil 5 mg/kg/day (95.63  $\pm$  15.61 s) showed significant improvement in the scopolamine-induced cognitive deficit in this task (Fig. 5B). No difference was observed in passive avoidance latencies during the acquisition trial in any group.

#### Effects of HCW on AChE inhibition

The AChE inhibition assay was carried out using an acetylthiocholine iodide substrate by a colorimetric method. HCW inhibited AChE activity in a dose-dependent manner, with IC<sub>50</sub> value was 79.67  $\mu$ g/mL where tacrine at 0.1  $\mu$ M inhibited it 80.40% (Fig. 6).

## DISCUSSION

Cholinergic dysfunction can be due to many factors, such as A $\beta$  toxicity, neurofibrillary tangles, or neurotransmitter antagonists, and induces disorders in the signaling systems in the brain. A $\beta$ , a major cause of AD, disrupts the cholinergic system via up-regulating calcium influx (León and Marco-Contelles, 2011; Arora *et al.*, 2013). When calcium influx increases, it leads to a variety of events, such as apoptosis and tau phosphorylation, leading to cognitive impairment (Härtig *et al.*, 2007; Duan *et al.*, 2013). As shown previously, we demonstrated that HCW had a neuroprotective effect via regulating calcium influx and mitochondria-mediated apoptosis, induced by A $\beta$  toxicity, in primary rat neuronal cells (Park and Oh, 2012). Based on these data, we expected that HCW might regulate a calcium-related cascade involving tau phosphorylation. Extending our previous experiments, we assessed the cleavage of p35 to p25/CDK5 complex and the phosphorylation of tau

protein, controlled by calcium influx. Hyperphosphorylated tau is found in the neurofibrillary tangles in neuronal cells, leading to cognitive impairment (Lee *et al.*, 2000; Jayapalan and Natarajan, 2013). CDK5, cyclin-dependent kinase 5, is required for a proper development of the brain and it needs the regulatory subunit, p35, which changes in form to p25, influenced by  $Ca^{2+}$  levels. The p25/CDK5 complex hyperphosphorylates tau protein, and induces apoptosis with cytoskeletal disruption (Jayapalan and Natarajan, 2013; Lee *et al.*, 2000). We assessed the levels of p25, p35, and p-tau205 protein, related to the pathway of tau hyperphosphorylation (Mondragón-Rodríguez *et al.*, 2014) due to A $\beta$  toxicity in rat primary neuronal cells. The results showed that there were significant effects of HCW in inhibiting the conversion of p35 to p25, the formation of the p25/CDK5 complex, induced by A $\beta$  toxicity in primary cortical cells. Also, HCW has the effect of inhibiting the phosphorylation of tau protein. Considering the previous study, we expected that HCW would suppress the phosphorylation of tau protein by regulating  $Ca^{2+}$  levels affected by A $\beta$  toxicity.

To confirm the effects of HCW on cognition, we performed behavioral tests, such as the NORT, Y-maze, and PAT in an ADlike *in vivo* model induced by A $\beta$  toxicity. Each task has a different role in investigating effects on memory. NORT is known to evaluate the function of the hippocampal region in making new memories, such as with novel objects, and short-term memory (Grayson *et al.*, 2007). The Y-maze task evaluates the role of the hippocampal region in the consolidation of short-term to long-term memory and the memory of space (Conrad *et al.*, 1996). The PAT is related to the amygdala-hippocampus complex, which regulates memory consolidation (Roozendaal, 2002). In this study, A $\beta$  toxicity-induced memory dysfunction which is related to spatial and object recognition in NORT, spatial working memory in Y-maze task, and fear motivated in PAT was overcome by HCW treatment. Moreover, these results indicated improvement in memory regions in the brain; thus, we assessed the CA3 region of the hippocampus (Shors *et al.*, 2001) via performing neuronal cell staining using NeuN factor, which is used to measure mature neurons related to neuronal development (Collombet *et al.*, 2011). In this study, we found a statistically significant effect between the HCW-treated groups and the A $\beta$ -injected group, indicating the possible neuroprotective effect of HCW.

"Cholinergic dysfunction" involves several mechanisms, not fully understood, but blocking cholinergic receptors may be one cause related to cognitive degeneration in AD patients (Terry *et al.*, 1991; Van der Zee and Luiten, 1999). Amyloid toxicity directly affects neuronal physiology, particularly in cholinergic system, with biochemical alteration, neuroinflammation, deregulation of neuroprotective systems, resulting neurodegeneration and behavioral alterations which show symptomatic and pathophysiological similarities to AD (Zussy *et al.*, 2011). Thus, we examined the effects of HCW in A $\beta$ -injected mice model. Then we continued to work on the study using scopolamine-injected mice, a cholinergic dysfunction model of an antagonist of ACh because scopolamine induce memory deficits are similar to those found in age-related dysfunction in central nervous system and is a useful tool for investigating learning and memory studies which involved in cholinergic system (Oh *et al.*, 2013). We performed the Y-maze task and PAT to estimate memory consolidation (Conrad *et al.*, 1996; Roozendaal, 2002). Considering AD, each task is related to long-term memory and memory consolidation, so it can model

the effects of memory improvement in AD's cholinergic dysfunction. The results showed that HCW improved cholinergic dysfunction in the scopolamine model dose-dependently. In particular, the effects of treatment with HCW at 400 mg/kg/day were as effective as the positive control group in PAT. These results suggest that HCW has the potential to treat cognitive dysfunction.

We also performed an AChE inhibition assay to assess HCW's effect on the cholinergic system. In the AChE inhibition assay, the  $IC_{50}$  value of HCW was 79.67  $\mu$ g/mL, while that of ginkgo biloba is 268.33  $\mu$ g/mL (Das *et al.*, 2002). According to this result, HCW may be a competitive herbal treatment for AChE inhibition with other herbal extracts. Taken together, the results reported here indicate that HCW has neuroprotective effects against memory impairment by inhibiting tau hyperphosphorylation and cholinergic dysfunction.

In conclusion, we evaluated HCW, *in vitro* and *in vivo*, and conclude that it has effects on cognitive development in two ways, improving cholinergic dysfunction induced by tau hyperphosphorylation and blocking cholinergic receptors. In addition, HCW has a neuroprotective effect via inhibiting  $Ca^{2+}$ -induced apoptosis. Phenolic compounds are well known to have antioxidant, anti-inflammatory, and anti-apoptotic effects, then they have been reported to inhibit neurotoxin-induced damages, resulting neuroprotection (Zhao, 2009; Joseph *et al.*, 2010). Chlorogenic acid and caffeic acid have a neuroprotective effect against methylglyoxal or cryo-injury via anti-apoptotic and anti-inflammatory activities (Zhang *et al.*, 2007; Huang *et al.*, 2008). Also, chlorogenic acid has anti-amnesic effect via inhibition of AChE activity (Kwon *et al.*, 2010). In addition, quercetin and rutin were also showed protective effect from neuronal damage induced by A $\beta$  and ischemia (Ansari *et al.*, 2009; Khan *et al.*, 2009). Thus, we assumed that phenolic compounds in HCW could partially contribute the effects in the present study. From these results, HCW may be an effective treatment for improving the cholinergic system and protecting neurons from toxicity. We suggest that HCW may be an interesting candidate to investigate for the treatment of AD.

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