Mangiferin Ameliorates Scopolamine-Induced Learning Deficits in Mice

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The aim of this study was to evaluate the effects of Anemarrhena asphodeloides BUNGE (AA) on cholinergic memory deficits in mice. This agent has previously been used as an antipyretic, anti-inflammatory, anti-diabetic, and antidepressant in traditional Chinese medicine. Mangiferin was isolated from AA and showed a dose-dependent inhibition of acetylcholinesterase (AChE) activity (IC_{50} value, 62.8 μg). Cholinergic dysfunction was induced in mice by administering scopolamine, and the animals were then tested using the passive avoidance test as well as the Morris water maze test. Mangiferin (20 mg/kg, p.o.) significantly reversed scopolamine-induced deficits in the passive avoidance test, and also improved escape latencies in training trials and increased swimming times in the Morris water maze test (p<0.05). Mangiferin also reduced acetylcholine and tumor necrosis factor (TNF)-α levels induced by scopolamine in mice brain (p<0.05) and inhibited nuclear factor (NF)-κB activation in scopolamine or TNF-α-stimulated BV-2 microglial cells. These results suggest that mangiferin can improve long-term cholinergic memory deficits by AChE inhibition or cholinergic receptor stimulation and inhibition of NF-κB activation.

Key words mangiferin; memory; acetylcholinesterase; tumor necrosis factor-κ; BV-2 cell

Cholinergic neurons in the central nervous system (CNS) degenerate in patients with Alzheimer's disease and senile dementia in a manner that correlates with functional loss.1 sexual intercourse. Many attempts have been made to reverse cognitive deficits by increasing brain cholinergic activity via acetylcholinesterase (AChE) inhibitors or cholinergic agonists, with a selective AChE inhibitor, donepezil, approved to treat mild Alzheimer’s disease. With the current few approved medicines to treat patients with memory impairment, medicinal plants may provide valuable alternatives to conventional therapies.

Anemarrhena asphodeloides BUNGE (AA), which primarily contains mangiferin and steroidal saponins such as sarsasapogenin and timosaponin AII and BII, has been used as an antipyretic, anti-inflammatory, anti-diabetic, anti-platelet aggregator, and antidepressant in traditional Chinese medicine. Mangiferin was isolated from AA and showed a dose-dependent inhibition of acetylcholinesterase (AChE) activities (IC_{50} value, 62.8 μg). Cholinergic dysfunction was induced in mice by administering scopolamine, and the animals were then tested using the passive avoidance test as well as the Morris water maze test. Mangiferin (20 mg/kg, p.o.) significantly reversed scopolamine-induced deficits in the passive avoidance test, and also improved escape latencies in training trials and increased swimming times in the Morris water maze test (p<0.05). Mangiferin also reduced acetylcholine and tumor necrosis factor (TNF)-α levels induced by scopolamine in mice brain (p<0.05) and inhibited nuclear factor (NF)-κB activation in scopolamine or TNF-α-stimulated BV-2 microglial cells. These results suggest that mangiferin can improve long-term cholinergic memory deficits by AChE inhibition or cholinergic receptor stimulation and inhibition of NF-κB activation.

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MATERIALS AND METHODS

Materials Tacrine (9-amino-1,2,3,4-tetrahydroacridine hydrochloride), (→) scopolamine hydrobromide acetylthiocholine (ATCh), 5,5'-dithiobis-[2-nitrobenzoic acid] (DTNB), AChE (electric eel type VI-S), radio-immunoprecipitation assay (RIPA) lysis buffer, and dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). TNF-α and the antibody against NF-κB (phospho-p65 and p65) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The protease inhibitor cocktail was purchased from Roche Applied Science (Mannheim, Germany). Enzyme-linked immunosorbent assay (ELISA) kits were from Pierce Biotechnology, Inc., (Rockford, IL, U.S.A.). The enhanced chemiluminescence (ECL) immunoblot system was from Pierce Co. (Rockford, IL, U.S.A.). All other materials were of the highest grade available.

Isolation of AChE-Inhibitory Constituent(s) from AA Anemarrhena asphodeloides was purchased from Kyung-Dong Market, Seoul, Korea, and authenticated by Professor Nam Jae Kim, Kyung Hee University, Korea. A voucher specimen (KHOPS-06-017) was deposited at the Herbarium of College of Pharmacy, Kyung Hee University, Seoul, Korea. The dried AA (3.5 kg) was extracted five times with MeOH in a boiling water bath. The MeOH extracts were combined and evaporated to dryness under reduced pressure to yield 846 g. This extract was partitioned with n-hexane and H₂O. Dried powder was extracted in a stepwise manner with CHCl₃ (71 g), ethyl acetate (20 g), and n-butanol (200 g). The butanol extract, which most potently inhibited AChE, was separated by column chromatography on silica gel, and eluted with a gradient of MeOH in CH₂Cl₂. Six fractions (FB1-FB6) were collected on the basis of their thin layer chromatography (TLC) profiles and tested for AChE inhibition. FB5, the most potent inhibitor, was separated by silica gel column chromatography and eluted with 7 : 2 : 0.5 (CH₂Cl₂ : MeOH : H₂O) to afford several subfractions, one of which was further subjected to semi-preparative HPLC (35% CH₃CN in 50% MeOH at a flow rate of 7 ml/min over 60 min, GS-320 column, 30×500 mm, Japan Analytical Instrument) to afford mangiferin (4.3 g).

Mangiferin (Fig. 1): Colorless amorphous solid, electron spray ionization (ESI)→MS/MS 421, 321 [M−Na]⁻.

Assay of AChE Activity AChE activity was measured using a modified method of Ellman et al. The reaction mixture consisted of 125 μl of 3 mM DTNB, 25 μl of 15 mM ATCh, 50 μl of 50 mM Tris–HCl, pH 8.0, and 25 μl of test
agents in a microplate. The mixture was pre-incubated for 10 min, after which 25 μl of AChE (0.226 U/ml) was added and the reaction was then scanned at 405 nm for 10 min using a microplate reader, Model Biotek uQuant MQX200 (Winooski, VT, U.S.A.).

**Animals** All the experiments were performed using male Institute of Cancer Research (ICR) mice weighing 28—30 g and purchased from the Orient Co., Ltd., a branch of Charles River Laboratories (Seoul, Korea), according to the guidelines of the Principle of Laboratory Animal Care (NIH publication No. 85-23, revised 1985). The mice were housed 5 or 6 per cage, allowed access to water and food ad libitum, and maintained at an ambient temperature of 22±1°C with 50±10% humidity and a 12 h diurnal light cycle (lights on 07:30—19:30 h), prior to testing. All behavioral experiments were carried out in a room adjacent to the housing room under the same ambient conditions.

**Passive Avoidance Test** The passive avoidance test was performed in identical illuminated and non-illuminated boxes (Gemini Avoidance System, San Diego, CA, U.S.A.) according to the previous method of Kim et al. The illuminated compartment (20×20×20 cm) contained a 100 W bulb, and the floor of the non-illuminated compartment (20×20×20 cm) was composed of 2 mm stainless steel rods spaced 1 cm apart. These compartments were separated by a guillotine door (5×5 cm). For the acquisition trial, mice were initially placed in the illuminated compartment and the door between the two compartments was opened 10 s later. When the mice entered the dark compartment, the door automatically closed and an electrical foot shock (0.5 mA) of 3 s durations was delivered through the stainless steel rods. One hour before the acquisition trial, mice received mangiferin (10, 20, 40 mg/kg, p.o) or tacrine (10 mg/kg, p.o) as a positive control. Memory impairment was induced by scopolamine treatment (1 mg/kg, i.p.) 30 min after the administration of test agent, tacrine, or 10% Tween 80 solution alone. The lyase was centrifuged (15000×g, 4°C) for 15 min, and the supernatant transferred to 96-well ELISA plates. TNF-α concentration was determined using commercial ELISA kits (Pierce Biotechnology, Inc., Rockford, IL, U.S.A.).

**Enzyme-Linked Immunosorbent Assay (ELISA) in Brain Hippocampus** For the ELISA of TNF-α, the hippocampus was removed from the brains of scopolamine-treated mice with or without test agents and homogenized in 1 ml ice-cold lysis buffer (radio-immunoprecipitation assay, RIPA) containing 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail according to the method described by Lee et al. The lysate was centrifuged (15000×g, 4°C) for 15 min, and the supernatant transferred to 96-well ELISA plates. TNF-α concentration was determined using commercial ELISA kits (Pierce Biotechnology, Inc., Rockford, IL, U.S.A.).

**Acetylcholine Assay in Brain Hippocampus** To assay acetylcholine levels, the hippocampus was removed from the brains of mice and stored at −70°C until analysis. The brain samples were thawed unassisted at room temperature and homogenized and deproteinized using 100% methanol. After centrifuging at 20000×g for 10 min, the homogenate supernatant was injected onto an LC-MS/MS (a Waters 2795 alliance system, Waters, Milford, MA, U.S.A.) equipped with an automatic sample injector): a column, Shiseido capcell pak UG 120 CN (50×2.0 mm i.d., S-5 μm); mobile phase, 10 mM ammonium formate formate pH 3.09 (pH adjusted with formic acid) in 100% methanol; flow rate, 0.2 ml/min. The column was maintained at a temperature of 40°C during HPLC analysis and the injection volume was set to 10 μl. MS experiments were performed using a Micromass Quattro API. The mass spectrometer was operated in electrospray positive ionization mode (cone voltage: 19V). Multiple reaction monitoring (MRM) data acquisition for the transitions mass: Ach (m/z 146→87) was achieved with a collision energy of 15 eV, and a dwell time of 0.5 s. Nitrogen (set at approximately 5501/h) was used for the nebulising and desolvation gases, and the desolvation temperature was set at 350°C.

**Assay of NF-κB Activation in TNF-α-Stimulated BV-2 Cells** The immortalized murine BV-2 cells that exhibit both the phenotypic and functional properties of reactive neuronal
microglial cells were cultured in the Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum and 1% antibiotics. The cultures were maintained at 37°C in humidified 5% CO2 and split once every 2 d. In all experiments, BV-2 cells were treated with or without mangiferin (50 or 100 μM) or tacrine (5 μM) 60 min before the addition of TNF-α in serum-free DMEM. Mangiferin and tacrine were dissolved in DMSO and the final concentration of DMSO added to cells never exceeded 0.1%.

Immunoblot analyses of NF-κB (p65 and phospho-p65) and β-actin were performed according to the method described by Bae et al.[12,14] BV-2 cells (1.5 × 10⁶ cells on 60 mm dish) were incubated for a day. The cells were pretreated with test agents for 60 min. Then, the cells were stimulated with scopolamine or TNF-α for 30 min and washed in 2—3 ml of ice-cold phosphate buffered saline (PBS), centrifuged at 1000×g for 5 min. This was followed by resuspension of the pelleted cells in 1 ml of RIPA lysis buffer containing 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail. After centrifugation, the supernatant was subjected to electrophoresis on a 10% sodium dodecyl sulfate-polyacrylamide gel, and then transferred to a nitrocellulose membrane. Immunodetection for pp65 and p65 was performed using an enhanced chemiluminescence detection kit.

**Statistics** Values are expressed as means±S.E.M. For the passive avoidance and Morris water maze test, data were analyzed by a Kruskal–Wallis non-parametric ANOVA test. If the results were significant, each treatment group was compared using the Tukey’s post hoc test. Statistical significance was set at p<0.05. For the microplate assay, data were analyzed by a one-way analysis of variance (ANOVA) test. If the results were significant, each group was compared using the Dunnett’s post hoc test. Statistical significance was set at p<0.05.

**RESULTS**

**Mangiferin Inhibits AChE** We performed a bio-guided fractionation strategy using a microplate assay for AChE inhibition to identify active AA isolates. The n-BuOH fraction showed greater inhibition than the CHCl₃ and H₂O fractions. The n-BuOH fraction was further subjected to silica gel column chromatography to yield six subfractions, with subfraction FB5 being the most potent. Five compounds were isolated from FB5, with mangiferin being identified by comparison of spectral data (MS, ¹H- and ¹³C-NMR) with references. Mangiferin inhibited AChE activity in a dose-dependent manner, with an IC₅₀ value of 62.8 μM (Fig. 2).

**Mangiferin Ameliorates Memory Impairment Induced by Scopolamine** We then tested the effect of mangiferin on scopolamine-induced deficits in the passive avoidance task, which is largely dependent on long-term memory. The step-through latency of scopolamine treated mice was significantly shorter than that of vehicle-treated control mice (Fig. 3, p<0.05). Mangiferin increased this scopolamine-induced reduction in step-through latency by 44% (10 mg/kg), 65% (20 mg/kg), and 59% (40 mg/kg). During the acquisition trial, no differences in latencies were observed among the groups. Tacrine (10 mg/kg), as a positive control, restored the step-through latency to 68% of the control group, consistent with previously published data.[15]

In the Morris water maze test, scopolamine treatment produced longer escape latencies in the training trials than the control group (Fig. 4). Mangiferin (20 mg/kg) significantly shortened this latent escape time, as did tacrine. Treatment with mangiferin and tacrine also significantly ameliorated the decreased swimming time in the platform quadrant induced by scopolamine during the probe trial.

**Mangiferin Increases Acetylcholine Level Reduced by Scopolamine in Mice Brain** To understand the mechanism by which mangiferin ameliorated scopolamine-induced memory deficits in mice, we measured the levels of acetylcholine in the hippocampus of the mouse brain (Fig. 5). The treatment with scopolamine significantly reduced acetylcholine levels (p<0.05). However, treatment with mangiferin at a dose of 20 mg/kg recovered the reduced acetylcholine level by 74.9% (p<0.05). This effect was comparable to that of tacrine.

**Mangiferin Inhibits NF-κB Activation in Scopolamine or TNF-α-Stimulated BV-2 Cells** To investigate whether scopolamine, frequently used to prepare memory deficit animal models, could induce proinflammatory cytokines, scopolamine was intraperitoneally injected into mice and the levels of TNF-α in their brain hippocampus was measured. The TNF-α level was significantly increased 1.7-fold, respec-
tively (Fig. 6). However, when mangiferin at doses of 10 and 20 mg/kg was orally administered into scopolamine-stimu-
lated mice, it potently inhibited the effect of scopolamine by 32% and 82%, respectively. Its inhibitory potency was com-
parable to that of tacrine.

We further investigated whether TNF-α or scopolamine could activate transcription factor NF-κB in BV-2 microglial cells (Fig. 7). Scopolamine and TNF-α activated NF-κB in BV-2 cells. Of them, TNF-α more potently activated NF-κB. Mangiferin inhibited NF-κB activation in BV-2 cells stimulated with TNF-α or scopolamine.

**DISCUSSION**

A decrease in cholinergic function, particularly within the basal forebrain can result in a decline in memory and cogni-

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**Fig. 4. Effect of Mangiferin on Escape Latency in the Training Trial (A) and Probe Trial Sessions (B) of the Morris Water Maze Task**

At 60 min before the training trial and probe trial sessions, mangiferin (MF-20, 20 mg/kg, p.o.), tacrine (THA, 10 mg/kg, i.p.) or vehicle (same volume of 10% Tween 80) solution was administered to mice. Memory impairment was induced by scopolamine treatment (1 mg/kg, i.p.). Open diamond, scopolamine treated group; closed triangle, mangiferin (20 mg/kg); open square, THA (10 mg/kg, i.p.); closed diamond, 10% Tween 80 group. Ten different animals were used for each treatment group. Data represent means ± S.E.M. *p < 0.05 versus vehicle control group, **p < 0.05 versus scopolamine-treated group.

**Fig. 5. Effect of Mangiferin on Acetylcholine Level in Scopolamine-stimulated Mice**

Mangiferin (10M10 or 20M20 mg/kg, p.o.), tacrine (THA, 10 mg/kg, i.p.) or vehicle (C, same volume of 10% Tween 80) solution was administered to mice 1 h before scopolamine treatment. Normal group (N) was treated with vehicle alone instead of scopolamine and test agents. Acetylcholine amount in brain hippocampus was deter-

**Fig. 6. Effect of Mangiferin on TNF-α Level in Scopolamine-stimulated Mice**

Mangiferin (10M10 or 20M20 mg/kg, p.o.), tacrine (THA, 10 mg/kg, i.p.) or vehicle (C, same volume of 10% Tween 80) solution was administered to mice 1 h before scopolamine treatment. Normal group (N) was treated with vehicle alone instead of scopolamine and test agents. TNF-α levels in the brain hypothalamus were determined by ELISA. The values indicate the mean ± S.D. (n = 10). *p < 0.05 versus vehicle control group, **p < 0.05 versus scopolamine-treated group.

**Fig. 7. Effect of Mangiferin on Transcription Factor NF-κB Activation in Scopolamine or TNF-α-Stimulated BV-2 Cells**

Scopolamine (A) or TNF-α (B) was used to treat BV-2 cells. Mangiferin [50(M50)μM or 100(M100)μM] or tacrine (THA, 10 μM) was administered 1 h after scopolamine (or TNF-α) treatment. NOR was treated with vehicle alone. NF-κB activation (pp65 and p65) was analyzed by immunoblot.
tive function with age.\(^\text{16}\) Scopolamine, an anti-cholinergic agent, reduces synaptic acetylcholine activity without changing acetylcholine concentration, and also influences delta, theta, alpha and beta activity in electroencephalography (EEG), which partially mimics EEG changes in patients with senile dementia or dementia of the Alzheimer type.\(^\text{17}\) Thus, scopolamine treatment represents a good model for the learning and memory changes that occur during CNS aging.

Previous studies suggested that AA might attenuate learning deficits due to brain damage and ageing in rodents.\(^\text{9,18,19}\) Of its constituents, sarsasaponin and timosaponin BII enhanced learning and memory in rats with amyloid β-peptide (25—35)-induced dementia.\(^\text{19}\) Sarsasapogenin also improved learning and memory in scopolamine-treated mice, but did not inhibit AChE or occupy muscarinic acetylcholine receptor binding sites.\(^\text{9}\)

In the preliminary experiment, we found that AA inhibited AChE. We then isolated mangiferin, the main constituent (>2%) of AA, which was found to be an inhibitor of AChE. We thus tested the ability of mangiferin to improve learning and memory deficits in scopolamine-treated memory-deficient mice. Mangiferin improved the step-through latency reduced by scopolamine treatment, with no differences in latency during the acquisition trial, indicating a lack of effect on the general behavior in mice. When the Morris water maze test was used to assess hippocampal-dependent spatial learning and long-term memory,\(^\text{20}\) mangiferin shortened scopolamine-induced increases in escape latency time and showed similar improvements in the quadrant platform. Thus, mangiferin improved long-term memory in scopolamine-induced amnestic mouse models by inhibiting AChE, like AChE inhibitors previously reported.\(^\text{3,11}\) Mangiferin may reverse deficits in hippocampal long-term memory via cholinergic enhancement.

To elucidate the mechanism by which mangiferin ameliorated cholinergic memory deficits, we analyzed the acetylcholine levels in the hippocampus of scopolamine-treated mice. Interestingly, the levels of acetylcholine were significantly reduced in scopolamine-treated mice. However, treatment with mangiferin in scopolamine-treated mice significantly increased the reduced acetylcholine level. Its effects were comparable to that of tacrine, although the inhibitory effect of mangiferin against AChE was weaker than that of tacrine. These results may be due to differences between the absorption of these compounds into the blood or brain, or other factors, such as cholinergic receptor stimulation. In addition, the levels of proinflammatory cytokines, TNF-α but not interleukin (IL)-6 were significantly increased in the hippocampus of scopolamine-treated mice. Thus, scopolamine may reduce the production of acetylcholine and TNF-α in the brain. However, mangiferin treatment in scopolamine-treated mice significantly inhibited the increase of TNF-α. Scopolamine and TNF-α also caused the activation of NF-kB, which is a representative transcription factor for proinflammatory cytokines, in BV-2 microglial cells. Mangiferin inhibited NF-kB activation in BV-2 cells stimulated by scopolamine or TNF-α. The result was supported by previous studies showing that mangiferin exhibits anti-inflammatory effects, scavenges free radicals, and reduces oxidative stress.\(^\text{21—24}\) Mangiferin also rescues neurons from cell death in acute injury and reduces neurological deficits caused by ischemic damage to the brain.\(^\text{25}\) These findings suggest that scopolamine may cause cholinergic neuron injury and inflammation in the brain and that mangiferin may improve memory deficits by acetylcholine esterase inhibition or cholinergic receptor stimulation and inhibition of NF-κB activation. Finally, we suggest that AA and its main constituent, mangiferin, can improve long-term cholinergic memory deficits.

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REFERENCES