Effect of wild ginseng on scopolamine-induced acetylcholine depletion in the rat hippocampus

Bombi Lee[a], Jongbong Park[a], Sunoh Kwon[a], Moo-Won Park[a,b],
Sang-Muk Oh[d], Mi-Jung Yeom[a], Insop Shim[c], Hye-Jung Lee[a,b]
and Dae-Hyun Hahm[a]

[a]Acupuncture and Meridian Science Research Center, [b]Department of Oriental Medicine, Kyung Hee
University, [c]Department of Integrative Medicine, College of Medicine, The Catholic University of Korea,
Seoul and [d]Department of Biochemistry, College of Medical Science, Konyang University, Daejeon,
Republic of Korea

Abstract

Objectives The ameliorating effects of wild ginseng on learning and memory deficits
were investigated in rats.

Methods Rats were treated daily with wild ginseng or cultivated ginseng for 7 days at
30 min before scopolamine injection (2 mg/kg, i.p.). After inducing cognitive impairment by
the administration of scopolamine, behavioural assessment using the Morris water maze was
performed. Changes in cholinergic system reactivity were also examined by measuring the
immunoreactive neurons of choline acetyltransferase and the reactivity of acetylcholine-
sterase in the hippocampus.

Key findings Scopolamine injection induced impaired performance in the water maze
test and severe cell losses in hippocampal cholinergic neurons, as indicated by decreased
choline acetyltransferase immunoreactivity and increased acetylcholinesterase reactivity.
Daily administration of wild ginseng produced a significant improvement in the escape
latency for finding the platform in the Morris water maze and reduced the loss of
cholinergic immunoreactivity in the hippocampus. The reduced expression of brain-derived
neurotrophic factor mRNA due to the scopolamine injection was recovered to normal
levels by the administration of wild ginseng.

Conclusions Wild ginseng demonstrates a significant neuroprotective effect against
scopolamine-induced neuronal and cognitive impairment.

Keywords central cholinergic system; dementia; learning and memory; neuroprotection;
wild ginseng

Introduction

Alzheimer's disease (AD) is a representative neurodegenerative disease with progressive
loss of memory.[1] A consistent neuropathological occurrence associated with memory loss
is a cholinergic deficit in the central nervous system, and this has been correlated with the
severity of AD.[2–5] The consistent findings in AD patients are impairment in cognitive
performances, such as attention, learning and memory, and loss of cholinergic makers,
including acetylcholine (ACh) and choline acetyltransferase (ChAT).[2,3,5] The cholinergic
approach to treatment of AD involves the recovery of this loss in cholinergic activity by
pharmacological intervention, which leads to the increased cholinergic transmission.[6]

Cognitive deficits are experimentally induced by the chemical modification of selected
brain areas such as the hippocampus, for example by administering compounds such as
scopolamine, which induces reversible cognitive impairment.[7,8] Scopolamine is a non-
selective muscarinic cholinergic receptor antagonist and impairs learning, acquisition and
short-term memory in rodents and humans.[7,9–11] Scopolamine-induced amnesia has been
widely used as an experimental animal model for screening anti-amnesic drugs.[12]

In particular, hippocampal neurons, which are known to play an important role in learning
and memory, are particularly vulnerable to neuronal injury produced by scopolamine-
induced cholinergic activity dysregulation, resulting in deficits in spatial memory tasks[13]
and synaptic plasticity.[14] In fact, it has been well established that scopolamine-induced

Correspondence: Dae-Hyun Hahm,
Acupuncture and Meridian
Science Research Center,
Kyung Hee University, 1,
Hoegi-dong, Dongdaemun-gu,
Seoul 130-701, Korea.
E-mail: dhhahm@khu.ac.kr

lesions in hippocampal cells produce severe deficits in terms of learning and memory in a variety of behavioural tasks.[15] The damage caused by repeated scopolamine-induced cholinergic activity reduction is hypothesized to play a role in reduced hippocampal volume, which is often associated with AD.[16] AD has also been correlated with the loss of cholinergic neurons and decreases in the levels of ACh and ChAT.[17] Lesions in these pathways result in decreased ACh release and thus cause learning and memory dysfunction.[18] Many studies have suggested a relationship between learning and memory functions and the cholinergic system in experimental animals.[19,20] To date, acetylcholinesterase (AChE) inhibitors are the major class of drugs approved for AD, providing symptomatic relief and resulting in improved cognitive function.[21]

Wild ginseng (WG) is ginseng that grows naturally and has not been domestically field-cultivated; it is medicinally more effective and expensive compared with field-cultivated ginseng (CG). In the present study, WG means ginseng grown from seeds undisturbed in the Korean forest even though the seeds were initially scattered by humans. In terms of seeding methods, it is differentiated from truly wild ginseng, the seeds of which arise through the vectors of nature such as birds. Investigators have reported the pharmacological effects of ginseng in the treatment of stroke and vascular dementia.[22] The potent antihypertensive or vasodilator actions of WG in animal models of hypertension have been reported.[23,24] Several studies have also demonstrated that WG or its components has a neuroprotective effect against glutamate-induced cell death,[25] and Ca^{2+}-blocking inhibitory activity.[26] A recent study demonstrated that WG protects hippocampal neurons against AD, suggesting that it is effective in the treatment of vascular dementia.[27] Despite its remarkable therapeutic effects in various disorders, the underlying pharmacological and neurochemical mechanisms of WG, particularly in AD, have not been fully elucidated. There is some evidence that the consumption of WG in old age is associated with better cognitive function.[28]

The aim of the present study was to examine the effects of WG in improving learning and memory in rats exposed to repeated scopolamine-induced cholinergic activity reduction. To elucidate the neural mechanism underlying the memory-enhancing activity of WG, we also examined how these effects are related to the levels of cholinergic markers in the hippocampus.

Materials and methods

Animals

Adult male Sprague-Dawley rats (~2 months old, 250–270 g) were purchased from Samtaco Animal Corporation (Osan, Korea). Animals were housed in groups of six rats per cage, under controlled 12-h light/dark cycle and temperature (22–24°C) conditions. Food pellets purchased from a commercial vendor and tap water were provided *ad libitum*, unless otherwise stated. All animals were gently handled in the same way for at least 5 days before the beginning of the experiments. Formal approval to conduct the experimental procedure was obtained from the animal subjects review board of our institution. All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, revised 1996. Efforts were made to minimize the numbers of animals used and to reduce their suffering.

Preparation of methanol extracts of wild ginseng and cultivated ginseng

WG roots (adventitious root culture of *Panax ginseng*) were collected in Chonbuk province in Korea and purchased from Baekjesansam Co. (Jong-Gu, Lee, Jinan-kun, Jinan-up, Yeon-jang-Ri #45-1, Chonbuk, 567-807, Korea). CG (*Panax ginseng*) was purchased from Dongwoodang Pharmacy Co., Ltd (Yeongcheon, Korea). Voucher specimens of WG and CG were deposited at the herbarium located at the College of Oriental Medicine, Kyung Hee University (no. KH-WG01 for WG, no. KH-CG01 for CG). A total of 100 g of WG and CG were cut into small pieces and extracted 3 times with 2 L of 80% methanol by sonication in a reflux condenser for 24 h at room temperature (25 ± 2°C), respectively. The solutions were combined, filtered through Whatman no. 1 filter paper, concentrated using a rotary vacuum evaporator (Rotavapor R-124; Büchi Labortechnik AG, Flawil, Switzerland) under reduced pressure, refrigerated in a recirculating chiller (EYELA CCA-1110; Tokyo Rikakikai Co., Tokyo, Japan) to obtain concentrated extracts, and then lyophilized (EYELA FD-800; Tokyo Rikakikai Co.). The yields of the aqueous phases of WG and CG were 11.6 and 20.6 (w/w), respectively.

Experimental groups

To develop learning and memory deficits, male rats were subcutaneously injected at 2 mg/kg bodyweight with scopolamine hydrobromide (Sigma-Aldrich Co., St Louis, MO, USA) dissolved in physiological saline solution for 7 days (a total of 7 times). Normal animals received saline injections instead of scopolamine as a vehicle control (NOR group). Different rats in an experimental group were subjected to either behavioural testing or immunohistochemistry studies. The rats were randomly divided into seven groups of six individuals as follows: normal group (NOR group), the scopolamine-induced dementia and saline-treated group (DEM group as a sham control), the scopolamine plus 50 mg/kg WG group (WG50 + DEM), the scopolamine plus 100 mg/kg WG group (WG100 + DEM), the scopolamine plus 200 mg/kg WG group (WG200 + DEM), the scopolamine plus 500 mg/kg CG group (CG500 + DEM), and the scopolamine plus 0.2 mg/kg tacrine group (TA + DEM). Tacrine (Sigma-Aldrich Co.), a centrally acting cholinesterase inhibitor, was used as a positive control. The rats were intraperitoneally administered WG or CG for 7 days. At 1 h after administration with WG or CG, all rats except the NOR group received the scopolamine injection and were then subjected to the Morris water maze task with a 30-min interval. The water maze test was performed in the second week after scopolamine injection.

Morris water maze test

The Morris water maze test was performed using a circular pool (painted white internally, 2.0 m in diameter and 0.35 m in height) manufactured in polypropylene. The pool

contained water that was maintained at a temperature of 22 ± 2°C. The water was made opaque by the addition of 1 kg of powdered milk. During testing in the water maze, a platform, 15 cm in diameter, was located 1.5 cm below the water in one of four locations in the pool, approximately 50 cm from the side walls. The pool was surrounded by many cues external to the maze. A video camera was mounted on the ceiling above the pool and was connected to a video recorder and tracking device (S-MART; Pan-Lab, Barcelona, Spain), that permitted online and offline automated tracking of the path taken by the rat. The animals received four trials per session. The rats were trained to locate the hidden escape platform, which remained in a fixed location throughout the testing. The trials lasted a maximum of 180 s and the latency to find the submerged platform was recorded. The animals were tested in this way for 6 days and they then received a 60-s probe trial on the Day 7. For the probe trial, the platform was removed from the pool and then the animal was released from the quadrant opposite to where the platform would have been located. The length of the trial was 60 s, after which the rat was removed from the pool. The proportion of time that the rat spent searching for the platform in the training quadrant, (i.e. the previous location of the platform) was recorded and used as a measure of retention.

Choline acetyltransferase and acetylcholinesterase immunohistochemistry

For immunohistochemistry studies, the animals were deeply anesthetized with 80 mg/kg sodium pentobarbital by intraperitoneal injection and then perfused through the ascending aorta with normal saline (0.9%), followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). The brains were removed, post-fixed overnight and cryoprotected in a 20% sucrose solution. Coronal sections, 30-μm thick, were cut through the hippocampus using a cryostat (CM, 1850; Leica Microsystem, Co., Nussloch, Germany). The sections were obtained according to the rat atlas of Paxinos and Watson.[29] The sections were immunostained for ChAT or AChE expression using the avidin–biotin–peroxidase method. Briefly, the sections were rinsed three times for 5 min each in PBS, and then incubated with rabbit anti-ChAT antibody (1 : 2000 dilution; Cambridge Research Biochemicals Co., Wilmington, DE, USA), or goat anti-AChE antibody (1 : 200 dilution; Santa Cruz Biotechnology Inc., CA, USA) in PBS containing 0.3% Triton X-100 (PBST) for 72 h at 4°C, respectively. The sections were washed for 5 min in PBS and then incubated for 120 min at room temperature with biotinylated goat anti-rabbit IgG secondary antibody (for the anti-ChAT antibody) or biotinylated sheep anti-goat IgG secondary antibody (for the anti-AChE antibody), respectively. Both secondary antibodies were obtained from Vector Laboratories Co. (Burlingame, CA, USA) and diluted 1 : 200 in PBST containing 2% normal goat serum. To visualize immunoreactivity, the sections were incubated for 90 min in avidin–biotin complex reagent (Vectastain Elite; Vector Laboratories Co.), washed 3 times for 5 min in PBS, and incubated in a solution containing 3,3′-diaminobenzidine (Sigma-Aldrich Co.) and 0.01% H$_2$O$_2$ for 1 min. Finally, the tissues were washed in PBS, followed by a brief rinse in

distilled water, and individually mounted onto slides. Slides were allowed to air dry and then a coverslip was applied. Images were captured using the AxioVision 3.0 imaging system (Zeiss Co., Oberkochen, Germany) and processed using Adobe Photoshop (Adobe Systems Inc., San Jose, CA, USA). The sections were viewed at 200 × magnification and the number of cells within 100 × 100-mm grids was counted by an observer blinded to the experimental groups. The cells from the hippocampal areas were obtained according to the stereotactic atlas of Paxinos and Watson.[29] The cells within the hippocampal areas were counted in three sections for each rat.

Total RNA preparation and RT-PCR analysis

Brain hippocampus was isolated from eight rats in each group. Total mRNA was extracted from the 50–100-mg hippocampus according to the instructions of the TRIzol kit (Invitrogen, CA, USA). The expression levels of brain-derived neurotrophic factor (BDNF) and TrkB mRNAs were determined by reverse transcription–polymerase chain reaction (RT-PCR). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), used as an internal control, was co-amplified with BDNF and TrkB. The primers were designed by Gene Fisher 2.0 (http://bibiserv.techfak.uni-bielefeld.de/genefisher2/) according to the serial number from Genbank as follows: TrkB (347 bp): 5′-TGG GAC GTT GGG AAT TTG GTT-3′ (sense) and 5′-CAG CCG TGG TAC TCC GTG TG-3′ (antisense); BDNF (153bp): 5′-CAG GGG CAT AGA CAA AAG-3′ (sense) and 5′-CTT CCC CTT TTA ATG GTC-3′ (antisense); GAPDH (409bp): 5′-CCC ACG GCA AGT TCA ACG G-3′ (sense) and 5′-CTT TCC AGA GGG GCC ATC CA-3′ (antisense). The PCR products were observed after electrophoresis on 1.2% agarose gel and the density of each band was analysed using an image analysing system (i-Max; CoreBio System Co., Ltd, Seoul, Korea). The expression levels of mRNA were determined by calculating the density ratio of each band of BDNF or TrkB relative to GAPDH.

HPLC analysis

For the preparation of samples for HPLC analysis, a given amount (1/10 of total extracts) of WG or CG extracts was dissolved in 20 ml distilled water. The samples were filtered through a syringe filter and injected onto the HPLC column equipped with a 2489 UV/vis detector (Waters Co., MA, USA) and mobile phase was delivered by two pumps. A 10-μl sample was injected using an autosampler. Ginsenosides in WG and CG extract were analysed using Waters Empower software (Waters Co.). For the analysis of ginsenosides using HPLC-UV, the mobile phase constituents were acetonitrile (A) and water (B) with 0.001% formic acid. The mobile phase was filtered and ultrasonically processed to remove gas bubbles before use. The gradient programme was a linear gradient from A to B (22 : 78) to (26 : 74) over 8 min, then A to B (35 : 65) to 25 min, followed by A to B (40 : 60) to 60 min. The column was a Waters Atlantis C18 column, 100 × 2.1 mm, 3.5 μm (Waters Co.) and the flow rate was set to 1.5 ml/min. The sample injection volume was 10 μl. The column temperature was set to 35°C and the wavelength of the detector was set to 203 nm. The ginsenosides were separated along an acetonitrile/water gradient. Standard concentrations of ginsenosides were 100, 300, 500 or 1000 μg/ml.

Statistical analysis

The experimental results were expressed as the mean ± SEM. The behavioural data were calculated and analysed by repeated measures analysis of variance using SPSS (version 13.0; SPSS Inc., Chicago, IL, USA). The statistical significance of the differences among groups was further analysed using Tukey's post-hoc test. Immunohistochemical data were also analysed by one-way analysis of variance followed by Tukey's post-hoc test. In all analyses, $P < 0.05$ was considered significant.

Results

Effect of wild ginseng in the Morris water maze test

The effect of WG treatment on the swimming time to reach the submerged platform is shown in Figure 1a. The NOR group rapidly learned the location of the submerged platform and thus reached it within 20 s on Day 6 of the trials. The WG-treated rats (50, 100 and 200 mg/kg) also showed a dose-dependent reduction in the escape latency throughout the training period compared with the DEM group, in which the escape latency markedly increased due to the artificial impairment of learning and memory. Repeated measures analysis of variance test on the training data showed that the escape latency significantly differed among the groups when the times were averaged over all the sessions ($F(6,42) = 21.789$, $P < 0.001$). During the experiment, the latency to escape diminished over the time ($F(5,210) = 375.122$, $P < 0.001$), however there was no significant interaction between experimental groups and time ($F(30,210) = 0.948$, $P = 0.549$). The Tukey's post-hoc test revealed that the WG50 + DEM ($P < 0.05$ on Day 6), WG100 + DEM ($P < 0.01$ on Day 6), WG200 + DEM ($P < 0.01$ on Days 5 and 6) and CG500 + DEM groups ($P < 0.01$ on Day 6) showed a significant reduction in the swimming latency time compared with that of the control group (Figure 1a). To examine the spatial memory of rats, analysis of the performance in the probe trial by comparing the percentage of time spent swimming to the platform is shown in Figure 1b. The time spent around the platform was significantly different among the groups ($F(6,48) = 8.113$, $P < 0.001$), and the WG- or CG-treated groups spent more time around the platform than the sham control DEM group ($P < 0.01$ for the DEM group and $P < 0.01$ for the WG200 + DEM group). In the present study, scopolamine injection severely impaired spatial cognition in the water maze task and WG treatment attenuated the scopolamine-induced deficit in learning and memory in the water maze task. It also indicated that the recovery of escape latency (or swimming latency time) in the WG200 + DEM group was better than that in the CG500 + DEM group and almost compatible with the TA + DEM group.

Effect of wild ginseng on the central cholinergic system choline acetyltransferase immunohistochemistry

After performing the behavioural tasks, the rat brains were analysed to determine the extent of neuronal cell loss using immunohistochemistry. The results of the ChAT immunoreactivity analysis in the CA1 area are shown in Figures 2 and 3. The brains of the DEM group showed significant neuronal cell
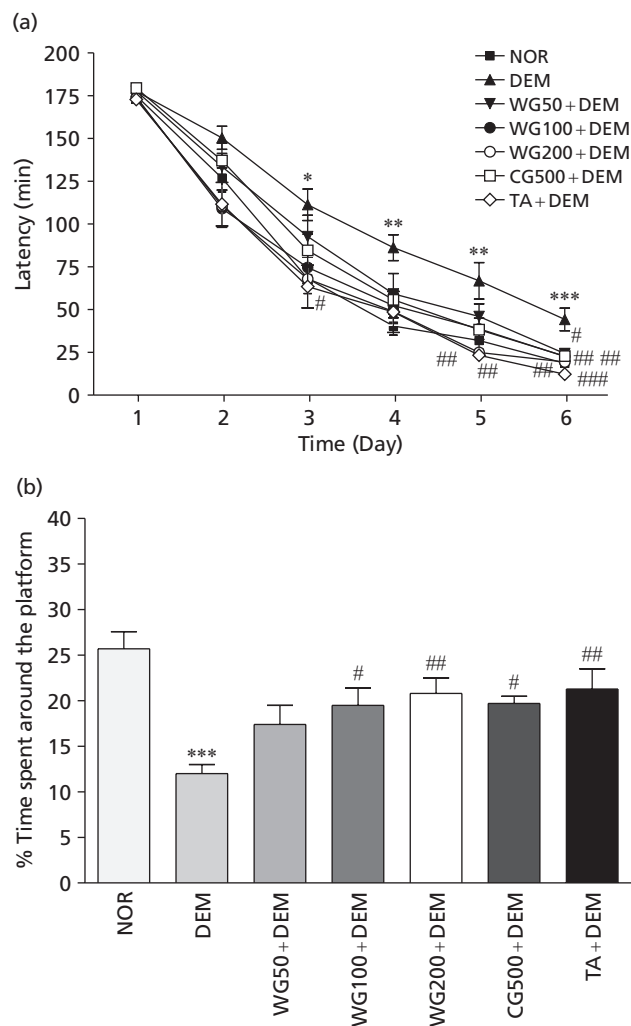

Figure 1 Effect of wild ginseng in the Morris water maze test. Time to escape (latency) during acquisition trials (a) and time spent around the platform (b) during the Morris water maze test. NOR, normal animals received saline instead of scopolamine as a vehicle control. DEM, scopolamine-induced dementia and saline-treated group. WG50 + DEM, WG100 + DEM, WG200 + DEM, scopolamine plus 50, 100 and 200 mg/kg wild ginseng, respectively. CG500 + DEM, scopolamine plus 500 mg/kg cultivated ginseng. TA + DEM, scopolamine plus 0.2 mg/kg tacrine. Data were analysed using repeated measures analysis of variance followed by Tukey's post-hoc test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the NOR group. $^\#P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with the DEM group. Vertical bars indicate SE.

loss in the hippocampal CA1 area, compared with those of the NOR group ($P < 0.01$). Comparison of the numbers of ChAT-immunoreactive neurons using one-way analysis of variance revealed a significant difference among groups ($F(6,127) = 3.618$, $P < 0.01$). Tukey's post-hoc test showed that the number of ChAT neurons increased significantly in the CA1 area in the WG200 + DEM group ($P < 0.05$) compared with the DEM group (Figure 3). It was indicated that the ChAT immunoreactivity of the WG200 + DEM group was equal to that of the CG500 + DEM group and compatible with that of the TA + DEM group.
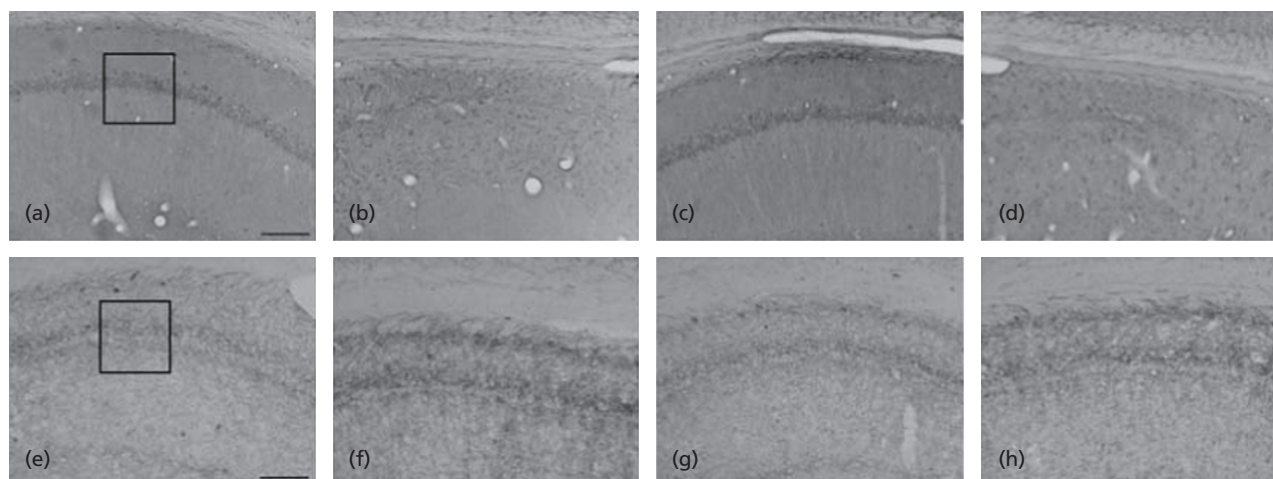
Figure 2 Effect of wild ginseng on the central cholinergic system choline acetyltransferase immunohistochemistry. Representative photographs showing the distribution of acetylcholinesterase (AChE) and choline acetyltransferase (ChAT) reactive cells in the hippocampus of NOR-ChAT (a), DEM-ChAT (b), WG200 + DEM-ChAT (c), CG500 + DEM-ChAT (d), NOR-AChE (e), DEM-AChE (f), WG200 + DEM-AChE (g) and CG500 + DEM-AChE (h) groups. NOR, normal animals received saline instead of scopolamine as a vehicle control. DEM, scopolamine-induced dementia and saline-treated group. WG200 + DEM, scopolamine plus 200 mg/kg wild ginseng. CG500 + DEM, scopolamine plus 500 mg/kg cultivated ginseng. Sections were coronally cut at 30 μm and the scale bar represents 50 μm.
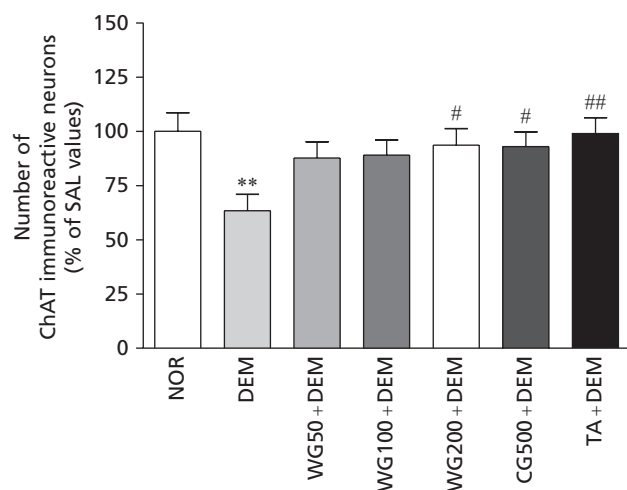

Figure 3 Percentage of choline acetyltransferase immunostained nuclei in different hippocampal areas after the Morris water maze task. Immunohistochemical data were analysed via separate one-way analysis of variance followed by Tukey's post-hoc test. NOR, normal animals received saline (SAL) instead of scopolamine as a vehicle control. DEM, scopolamine-induced dementia and saline-treated group. WG50 + DEM, WG100 + DEM, WG200 + DEM, scopolamine plus 50, 100 and 200 mg/ kg wild ginseng, respectively. CG500 + DEM, scopolamine plus 500 mg/ kg cultivated ginseng. TA + DEM, scopolamine plus 0.2 mg/kg tacrine. $^{**}P < 0.01$ compared with the NOR group; $^{#}P < 0.05$, $^{##}P < 0.01$ compared with the DEM group. Vertical bars indicate SE.
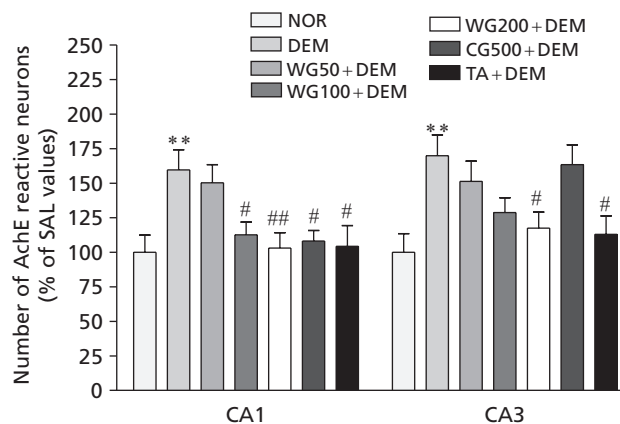

Figure 4 Percentage density of acetylcholinesterase stained nuclei in different hippocampal areas after the Morris water maze task. Immunohistochemical data were analysed via separate one-way analysis of variance followed by Tukey's post-hoc test. NOR, normal animals received saline (SAL) instead of scopolamine as a vehicle control. DEM, scopolamine-induced dementia and saline-treated group. WG50 + DEM, WG100 + DEM, WG200 + DEM, scopolamine plus 50, 100 and 200 mg/kg wild ginseng, respectively. CG500 + DEM, scopolamine plus 500 mg/kg cultivated ginseng. TA + DEM, scopolamine plus 0.2 mg/kg tacrine. $^{**}P < 0.01$ compared with the NOR group; $^{#}P < 0.05$, $^{##}P < 0.01$ compared with the DEM group. Vertical bars indicate SE.

Effect of wild ginseng on the acetylcholinesterase histochemistry

The density of AChE fibres in the CA1 area of the rat hippocampus in the sham control DEM group was markedly higher than those in the NOR group, as shown in Figure 4. The density of the AChE neurons in the CA1 area was 8.83 ± 0.73 ($100.0 \pm 6.40\%$) in the NOR group, 14.10 ± 1.29 ($159.57 \pm 14.63\%$) in the sham control DEM group, 13.28 ± 1.16 ($150.31 \pm 13.13\%$) in the WG50 + DEM group, 9.94 ± 0.83 ($112.58 \pm 9.35\%$) in the WG100 + DEM group, 9.11 ± 0.97 ($103.14 \pm 10.94\%$) in the WG200 + DEM group and 9.56 ± 0.67 ($108.18 \pm 7.61\%$) in the CG500 + DEM group ($F(6,127) = 5.100$, $P < 0.001$). Tukey's post-hoc test showed that the density of the AChE reactive neurons in the

hippocampus was significantly reduced in the WG100 + DEM group ($P < 0.05$) and the WG200 + DEM group ($P < 0.01$) compared with the DEM group (in the CA1 region).

The density of the AChE fibres in the CA3 region of the hippocampus in the sham control DEM group was also higher than in the NOR group. The density of the AChE neurons in the CA3 region was 6.39 ± 0.28 ($100.0 \pm 10.48\%$) in the NOR group, 10.86 ± 0.97 ($169.94 \pm 15.13\%$) in the sham control DEM group, 9.67 ± 0.94 ($151.30 \pm 14.65\%$) in the WG50 + DEM group, 8.22 ± 0.69 ($128.70 \pm 10.78\%$) in the WG100 + DEM group, 7.50 ± 0.75 ($117.39 \pm 11.75\%$) in the WG200 + DEM group and 10.44 ± 0.92 ($163.48 \pm 14.33\%$) in the CG500 + DEM group ($F(6,127) = 4.700$, $P < 0.001$). Tukey's post-hoc test showed that the density of the AChE reactive neurons was significantly reduced only in the WG200 + DEM group ($P < 0.05$). In the CG500 + DEM group, the increased intensity of AChE neurons in the CA3 region of the DEM group was not reduced as in WG- or TA-treated groups.

Effect of wild ginseng on mRNA expression levels of BDNF and TrkB in the hippocampus

In order to investigate the effect of WG on the expression of neurotrophic factors in the rat hippocampus injured by scopolamine injection, the mRNA expression of BDNF and its neuronal receptor TrkB was analysed using RT-PCR (Figure 5). The mRNA expression levels of BDNF and TrkB were normalized against GAPDH as an internal control. While the mRNA level of BDNF in the DEM group significantly decreased compared with that in the NOR
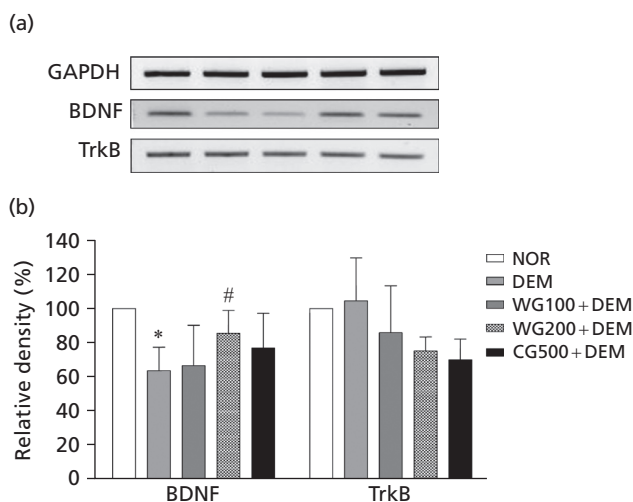
group ($P < 0.05$), there was no significant difference in the level of TrkB mRNA between the two groups ($P = 0.058$), as shown in Figure 5. While the mRNA level of BDNF in the WG200 + DEM group significantly increased compared with that in the DEM group ($P < 0.05$), there was no significant difference in the levels of TrkB mRNA in the hippocampus between the WG-treated group and the CG-treated group when compared with the control DEM group.

HPLC analysis of methanol extracts of wild ginseng and cultivated ginseng

Quantitative determination of the seven ginsenosides (Rg1, Re, Rf, Rb1, Rc, Rb2 and Rd) in the methanol extracts of WG and CG was performed to identify the chemical difference between two types of ginseng. It was found that CG extract contained 2.31, 0.95, 7.33, 3.75, 1.83 and 1.26% of ginsenosides Rg1 + Re, Rf, Rb1, Rc, Rb2 and Rd, respectively, whereas WG contained 9.5, 3.43, 0.63, 18.66, 7.95 and 2.59%, respectively (Figure 6; Table 1). With regard to the seven ginsenosides analysed in this study, WG contained greater quantities than CG. It was determined that WG included 4 times more ginsenoside Rg1 and Re, 3 times more Rf, 6 times more Rb1 and Rc, and 2 times more Rd than CG.

Discussion

The results demonstrate that repeated scopolamine-induced dementia produced severe deficits in the performance of cognitive function tests, with corresponding signs of neuro-degeneration in the brain, including decreased ChAT and

Figure 5 Effect of wild ginseng on mRNA expression levels of brain-derived neurotrophic factor and TrkB in the hippocampus. PCR bands (a) and their relative intensities (b) of brain-derived neurotrophic factor (BDNF) and TrkB in the hippocampus of rats that received repeated scopolamine injection. Data were analysed via separate one-way analysis of variance followed by Tukey's post-hoc test. NOR, normal animals received saline instead of scopolamine as a vehicle control. DEM, scopolamine-induced dementia and saline-treated group. WG100 + DEM, WG200 + DEM, scopolamine plus 100 and 200 mg/kg wild ginseng, respectively. CG500 + DEM, scopolamine plus 500 mg/kg cultivated ginseng. $^{*}P < 0.05$ compared with the NOR group; $^{\#}P < 0.05$ compared with the DEM group. Vertical bars indicate SE.
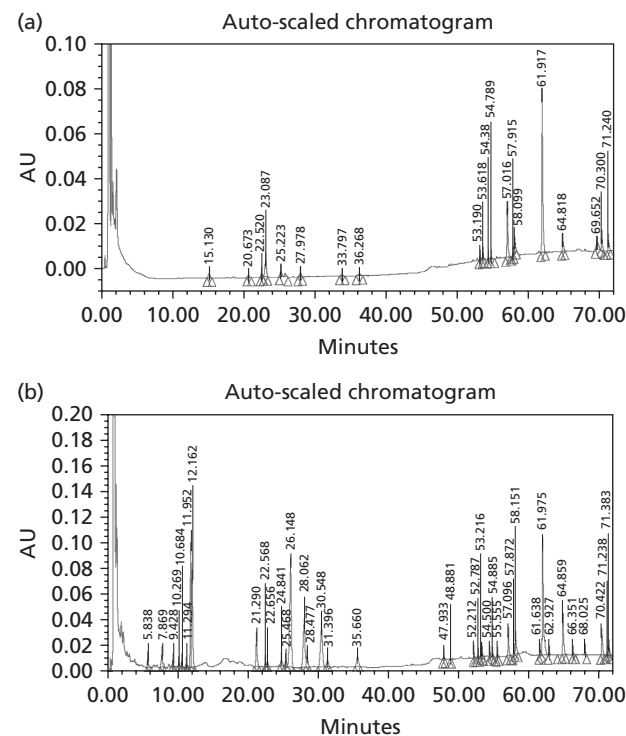

Figure 6 HPLC chromatograms of methanol extracts of cultivated and wild ginseng. Cultivated ginseng (a) and wild ginseng (b).

Table 1 Distribution of ginsenosides in cultivated and wild ginseng

	% content						
	Rg1 + Re	Rf	Rb1	Rc	Rb2	Rd	Total
Cultivated ginseng	2.31	0.95	7.33	3.75	1.83	1.26	17.43
Wild ginseng	9.5	3.43	0.63	18.66	7.95	2.19	42.36

increased AChE activity in the hippocampus. Our results showed that pretreatment with WG improved learning and memory retention in the Morris water maze test, and also increased ChAT and decreased AChE immunoreactivity in the hippocampal areas of scopolamine-induced dementia male rats. This suggests that WG alleviated the deficits in spatial learning capability induced by repeated scopolamine-induced dementia and prevented cell loss in the hippocampal region.

The muscarinic antagonist scopolamine, when administered to animals, is capable of transiently producing some of the deficits in the processes of learning acquisition and short-term memory that are considered as characteristic of AD.[30–32] The well replicated amnesic effect of scopolamine is interpreted as a principal consequence of a blockade of post-synaptic muscarinic M1 transmission, which leads to disruption of the function of the hippocampus in the working memory. Accordingly, in the present study, we observed cholinergic dysfunction in the rats with amnesia induced by scopolamine. The results from the Morris water maze test ameliorated the scopolamine-induced deficits in learning and memory. It is also well established that activity of cholinergic neurons in the hippocampal terminals or acetylcholine content was decreased by scopolamine.[33–35]

We performed a pilot dose–response experiment with WG (50, 100 or 200 mg/kg) and found that 200 mg/kg was most effective in inhibiting repeated scopolamine-induced dementia effects, including memory deficits in the Morris water maze test and the release of ACh. The dose chosen for this study has been used in other animal studies.[36] Pretreatment with WG increased cholinergic immunoreactivity previously reduced by exposure to scopolamine in the hippocampus. In the hippocampal CA1 area, pretreatment with WG prevented the induction of AChE immunoreactivity in a dose dependent manner and it may be highly correlated with the behavioural results. In the hippocampal CA3 area, only 200 mg/kg WG significantly decreased the immunoreactivity and there was no significant effect on cholinergic activity at 50 mg/kg. It is not clear why the highest dose of WG only produced a larger decrease in AChE immunoreactivity and not at other lower doses in the CA3 area. It is possible that the CA1 is a more active site than the CA3 in response to treatment with WG. This result suggests that the pharmacological target site of WG action may be the hippocampal CA1 area. This suggestion is supported by previous studies demonstrating that acetylcholine release is different from the subfields of the hippocampus CA1 and CA3. A higher concentration was measured in the CA1 than the CA3.[37] The other possibility is that certain components of WG inhibited the central cholinergic system so strongly that the stimulating effect of WG was not observed in this experiment. Further studies are necessary to examine the individual effects of WG components on scopolamine-induced learning and memory, and cholinergic systems.

Our results showed that WG treatment significantly modulated ChAT and AChE expression. ACh is another important factor that may affect learning and memory. In chronic dementia, re-uptake of ACh decreases in neurons of the frontoparietal cortex and CA1 region of the hippocampus, and changes in AChE activity occur.[38] It was evident that AChE activity significantly increased in the hippocampus following repeated scopolamine-induced dementia in the present study. Although there have been very few reports elucidating the changes in the cholinergic system in experimental dementia, some studies showed a significant reduction in the levels of brain acetylcholine following experimental dementia.[39] On the other hand, the role of acetylcholine in learning and memory processes has been well documented in many reports.[40] The disruption of basal forebrain cholinergic projections to the hippocampus and other limbic structures impairs the functions of learning and memory.[18–20,41] Thus, the results of the present and other studies suggest that the reduced cholinergic transmission might, at least in part, be responsible for the cognitive deficits in the repeated scopolamine-induced dementia in rats. Because the central cholinergic system is important in the regulation of cognitive function, decreased ACh levels may contribute to the observed impairment of learning and memory during chronic dementia. ChAT and AChE belong to a family of enzymatic proteins that are expressed in cholinergic neurons. ChAT is responsible for the biosynthesis of ACh and is required for cholinergic neurotransmission in the central and peripheral nervous systems. Because ACh is rapidly hydrolysed by AChE, the duration of ACh action in the synaptic cleft is dependent upon AChE activity.[42] The present study demonstrated that pretreatment with WG protected the rats from spatial working memory deficits and attenuated the decrease in ChAT and the increase in AChE immunoreactive neurons in the hippocampus, which is a particularly vulnerable region of the brain.[43] We found that repeated scopolamine-induced dementia caused a reduction in ChAT activity in the hippocampus and significantly increased the density of AChE-immunoreactive neurons in the hippocampal CA1 and CA3 regions. It is likely that the observed improvement in learning and memory in the Morris water maze test was associated with the attenuation of hippocampal cell loss in WG-treated animals. Treatment with WG produced a significant increase in the expression of cholinergic markers such as ChAT and AChE in the hippocampus, compared with the DEM group.

We demonstrated markedly decreased expression of BDNF mRNA in rat hippocampus tissue following repeated scopolamine-induced dementia. Pretreatment with WG significantly increased the expression level of BDNF mRNA in the hippocampus. Our studies have shown the altered BNDF expression in the hippocampus with dementia, which might be attributed to the reduction of cholinergic activity. Several

studies have suggested an association of hippocampal BDNF expression with memory performance, particularly in the water maze test.[44,45] Scopolamine-induced dementia has been shown to reduce learning and memory in the water maze test and BNDF expression in the hippocampus.[46] In genetically modified mice with reduced expression of BDNF either in the forebrain[47] or in the hippocampus,[48] deficits in water maze learning were found. Finally, direct infusion of BDNF into the hippocampus prevented impaired spatial memory performance in rats that had been subjected to repeated scopolamine-induced dementia.[49] These studies strongly suggest a direct correlation between the reduced expression of BNDF in the hippocampus and impaired cognition, which explains some of the results in the present study.

We verified the quantitative differences of seven ginsenosides between WG and CG using HPLC analysis. It was observed that all the ginsenosides analysed in this study were included in greater quantities in WG compared with CG, and in the cases of Rb1 and Rg1, WG included 4 to 6 times more than CG. In previous studies, investigators have reported that the ginsenosides Rb1, Rb2 and Rc play a role in inducing antimetastatic or anticarcinogenic activity.[50] In addition, ginsenoside Rg1 has potential neurotrophic and neuroprotective activities.[51] Our HPLC data showed that the differences in ginsenoside profiles of WG and CG are distinct, suggesting that some ginsenosides are responsible for the significant effect of neuroprotection against scopolamine-induced neuronal and cognitive impairment. Other more potent ginsenosides in the WG extract may have similar neuroprotective effects against scopolamine-induced neuronal and cognitive impairment. Further work is needed to identify the ginsenosides in the WG extract that contribute to its effective neuroprotective activity.

It has been reported that the application of herbal medicines or their pharmacological components in the treatment of Alzheimer-type dementia patients causes an improvement in memory-related behaviour.[52,53] However, there is little scientific evidence for the effectiveness of herbal medicines and there have been few systematic trials screening the pharmaceutical components of herbs. WG has a long history of use in Oriental medicine for treating various diseases including dementia. Its therapeutic efficacy has been confirmed by clinical studies in the Dong-Eu-Bo-Gam (a compendium of Korean traditional medicines complied by Her Jun). WG has long been included in many herbal combinations for the treatment of AD and vascular dementia.[54] Many studies have also demonstrated that WG is one of the most widely used herbs in Korean traditional medicine and many studies have been performed on its neuroprotective effects.[43] Several ginsenoside mixtures also showed a cognition enhancing effect in rats with experimentally impaired memory due to alcohol.[54]

The present study showed that the administration of WG extract improved impaired memory and caused a concomitant decrease in cholinergic activity of scopolamine-treated rats. The methanol extract of WG might have ameliorated the scopolamine-induced decrease of memory retention in the Morris water maze test by enhancing acetylcholine release and ChAT activity in hippocampal neurons. Pretreatment with WG improved performance in tests examining spatial learning and

memory. Moreover, WG treatment significantly restored the changes in number of ChAT neurons and the density of AChE-immunoreactive fibres in the hippocampus which were induced by the scopolamine injection. Similar results were observed with regard to the expression pattern of BDNF mRNA.

Conclusions

It was demonstrated that repeated scopolamine-induced dementia produces learning and memory deficits in rats during the Morris water maze test, with associated degeneration of cholinergic neurons. Pretreatment with WG attenuated these scopolamine-induced dementia effects, as indicated by improved cognitive function during behavioural tests and increases in the density of cholinergic neurons. Therefore, WG appears to be a good candidate for further investigations, which may ultimately result in a clinical treatment for dementia.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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