Ginseng berry aqueous extract prevents scopolamine-induced memory impairment in mice

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Abstract. Ginseng berry exhibits a diverse range of pharmacological activities. The present study aimed to examine the neuroprotective effects of ginseng berry aqueous extract (GBE) against oxidative stress and to assess the impact of GBE on memory impairment in mice. In HT-22 cells, GBE pretreatment significantly inhibited glutamate- and hydrogen peroxide-mediated cytotoxicity in a concentration-dependent manner, while treatment with up to 100 μg/ml GBE alone did not change cell viability. In a murine model of scopolamine (SCP)-induced memory impairment, results from the passive avoidance test and the Morris water maze test indicated that GBE administration for 4 weeks prolonged step-through latency time and shortened escape latency time, suggesting that GBE can attenuate deficits in long-term memory induced by SCP. Additionally, GBE prevented SCP-induced reductions in acetylcholine by decreasing acetylcholinesterase activity and upregulating choline acetyltransferase mRNA levels in the hippocampus. GBE mitigated SCP-mediated mRNA decreases in brain-derived neurotrophic factor levels and its associated signaling molecules. Furthermore, GBE administration significantly suppressed malondialdehyde production and increased glutathione levels, catalase activity and superoxide dismutase activity in SCP-induced memory impaired mice. Therefore, the results of the current study indicated that ginseng berry may be a potential candidate for treating or preventing memory deficits that are associated with neurodegenerative disorders.

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Abbreviations: ACh, acetylcholine; AChE, acetylcholine esterase; AD, Alzheimer's disease; BDNF, brain derived neurotropic factor; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; CAT, catalase; ChAT, choline acetyltransferase; CREB, cAMP response element binding protein; ERK, extracellular signal-regulated kinase; GBE, ginseng berry extract; GSH, glutathione; H_2O_2, hydrogen peroxide; HPLC, high-performance liquid chromatography; Nrf2, nuclear factor-E2 related factor 2; PI3K, phosphoinositide 3-kinase; SCP, scopolamine; SD, standard deviation; SOD, superoxide dismutase; TrkB, tropomyosin receptor kinase B

Key words: acetylcholine, antioxidant, brain-derived neurotrophic factor, ginseng berry aqueous extract, scopolamine-induced memory impairment

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that accounts for 60-70% of patients with dementia (1). While the neuropathological mechanisms of AD remain mostly unknown, they are associated with the accumulation of neurofibrillary tangles and senile plaques that accelerate oxidative stress and decrease cholinergic activity in the brain, which in turn impair memory and cognitive function (2,3). Thus, restoration of cholinergic function in the brain has been suggested to be a standard strategy for delaying the progression of the disease (3,4). Several acetylcholinesterase (AChE) inhibitors have been approved to manage the symptoms of mild AD and are associated with various adverse effects, including nausea, diarrhea, anorexia, vomiting, and hepatic toxicity (3,5). Moreover, no medication has been approved to treat patients with memory deficits. Therefore, traditional herbs may be a valuable resource for treating memory impairment with fewer side effects.

In East Asia, including Korea, root of ginseng (*Panax ginseng* Meyer) has been prescribed for centuries to tonify '*qi*'. It is also used to treat wide range of diseases. Although the bioactive compounds found in this root are also distributed in other parts of ginseng (i.e., berry, stem, leaf, and flower), the berry has often been regarded as a 'useless by-product' and discarded during the process of ginseng root production. However, accumulated evidence suggests that ginseng berry contains high levels of ginsenosides (6,7) and has more potent pharmacological activities than the root (6,8,9). In particular, it has been reported that the ginseng berry has beneficial effects in decreasing blood glucose (9), sensitizing insulin signaling (10), inhibiting adipogenesis (11), reducing blood coagulation (12), enhancing blood circula-

tion (6,8), ameliorating cisplatin-induced nephrotoxicity (13) and acetaminophen-induced hepatotoxicity (14), and inhibiting dextran sodium sulfate-induced colitis (15). Despite its potential beneficial effects on diverse diseases, the effects of ginseng berry against amnesia remain poorly understood.

Thus, the present study aimed to examine the *in vitro* neuroprotective effects of ginseng berry aqueous extract (GBE) against oxidative stress and to explore the *in vivo* anti-amnesic effects of GBE in a murine model of scopolamine (SCP)-induced memory impairment. Tacrine (9-amino-1,2,3,4-tetrahydroacridine hydrochloride) is a prototypic cholinesterase inhibitor that increases cholinergic transmission at synapses (3). Tacrine has been shown to improve cognitive function in experimental animal models of AD (16,17). To evaluate the therapeutic effects of GBE, tacrine was used as a positive reference drug. Furthermore, the effects of GBE on the cholinergic nervous system, mRNA expression of memory-related genes, and antioxidant activities were further examined to understand its role in memory impairment.

Materials and methods

Quantification of ginsenoside Re. GBE were prepared and supplied from Aribio Co., Ltd., as previously reported (12). Ginsenoside Re was purchased from ChemFaces (Wuhan, China). Ginsenoside Re concentration in GBE was quantified using high-performance liquid chromatography (HPLC) (Agilent 1100, Agilent Technologies) equipped with reversed phase column (Capcell Pak C18 UG120, 4.6x250 mm, 5 μm; Shiseido) and diode array detector system (Agilent Technologies). GBE was dissolved in 60% of methanol, and ginsenoside Re in 100% of methanol. GBE and ginsenoside Re were eluted using 20-30% acetonitrile gradient solution containing 0.01% of phosphoric acid. Ginsenoside Re was detected at the wavelength of 203 nm. Ginsenoside Re in GBE was quantified according to peak area and retention time.

Cell viability assay. HT-22 cells, a murine normal hippocampal neuronal cell line, were obtained from Millipore and maintained at 37˚C with 5% CO_2. After HT-22 cells ($1x10^4$ cells/well) were grown for 24 h, the cells were treated with GBE (0.01-100 μg/ml) for 72 h to examine the effect of GBE on the viability of HT-22 cells. In another experiments, GBE-pretreated HT-22 cells (0.01-100 μg/ml, 0.5 h) were subsequently exposed to 5 mM of glutamate or 500 μM of hydrogen peroxide (H_2O_2) for 12 h. Cell viability was measured at wavelength of 450 nm using EZ-Cytox cell viability assay kit (Daeil Labservice) and automated microplate reader (VersaMax™; Molecular Devices). The cell viability was calculated as relative to the untreated control cells.

Animal husbandry and treatment. C57BL/6NCrljOri mice (n=120; male, 18-21 g, 6 weeks old) were supplied from Orient Bio, Inc., maintained with a supply of filtered pathogen-free air, and provided with standard rodent chow (Purinafeed) and water *ad libitum* at standard condition (temperature, 20-25˚C; light/dark cycle, 12/12 h; relative humidity, 40-45%). After 1 week of acclimatization, mice were divided into 6 groups (n=20/group); vehicle, SCP, SCP + tacrine 10 mg/kg, SCP + GBE 400 mg/kg, SCP + GBE 200 mg/kg, and SCP + GBE 100 mg/kg. GBE or tacrine dissolving in distilled water was administered orally once a day for 28 days. To induce memory impairment, 1 mg/kg of SCP dissolving in sterilized saline was intraperitoneally injected three times 1 h after GBE (or tacrine) administration on days 6, 13 and 27. Instead of GBE and SCP, vehicle group was given an equal volume of distilled water and saline. And SCP group administered distilled water to induce same stresses.

Measurement of body weight. To reduce the differences from feeding, all mice were fasted for 12 h (water was not) at initiation of first GBE (or tacrine) administration. Body weight was measured on days -1, 0, 1, 13, 20, 27 and 28 using an automatic electronic balance (XB320M; Precisa Instrument).

Passive Avoidance Test. The step-through passive avoidance test (n=10/group) was conducted, as previously described (16,18). After the last SCP injection, each mouse was placed in the light/noise compartment 30 min for training mice. Light and noise was applied until the mouse escaped into the neighboring grid floor compartment. An electric shock (3.0 mA) for 3 sec was applied to the grid floor. A retention test was performed 24 h after training test using the same condition, and the moving time from the light/noise compartment to the grid floor was recorded as a step-through latency time.

Morris water maze test. The Morris water maze test (n=10/group) was carried out using separated animals from the passive avoidance test, as previously reported (19). For training, each mouse was placed in circular pool (diameter, 100 cm; depth, 27 cm; temperature, 22˚C) 30 min after the last SCP injection, and began to find the submerged escape platform (diameter, 10 cm) in one of the pool quadrants. When the mouse was successfully placed on the platform, it was allowed to remain on the platform for 10 sec. However, if the mouse was failed to find the platform within 150 sec, the mouse was placed on the platform for 10 sec. A retention test was performed 24 h after training test, and the escape latency time from the water to the escape platform was recorded using a video tracking system (Smart junior).

Isolation of total RNA and RT-PCR. After the passive avoidance test, the mouse was anesthetized under inhalation anesthesia with 2-3% of isoflurane (Hana Pharm. Co.), euthanized by cervical dislocation, and the hippocampus (n=10/group) was collected. Total RNA was isolated using Trizol reagent (Invitrogen), and then reacted with recombinant DNase I (Ambion) to remove contaminated DNA. cDNA was synthesized using oligo-dT_{16} primer and High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). RT-PCR was carried out using an ABI Step One Plus Sequence Detection System (Applied Biosystems). Gene specific primer pairs were used as followed: Choline acetyltransferase (ChAT) forward 5'-CTTGGATGGTCCAGGCAC-3', backward 5'-GTCATA CCAACGATTCGCTCC-3'; brain-derived neurotrophic factor (BDNF) forward 5'-GACAAGGCAACTTGGCCTAC-3 ', backward 5'-CTGTCACACACGCTCAGCTC-3'; phosphoinositide 3-kinase (PI3K) forward 5'-TCCAAATACCAG CAGGATCA-3', backward 5'-ATGCTTCGATAGCCGTTC TT-3'; Akt forward 5'-TACTCATTCCAGACCCACGA-3',

backward 5'-GAGGTTCTCCAGCTTCAGGT-3'; extracellular signal-regulated kinase 1 (ERK1) forward 5'-TGGCTTTCT GACGGAGTATG-3', backward 5'-GGTCCAGGTAGTGCT TGC-3'; ERK2 forward 5'-CCTCAAGCCTTCCAACCTC-3', backward 5'-GCCCACAGACCAAATATCAATG-3'; cAMP response element binding protein (CREB) forward 5'-TAC CCAGGGAGGAGCAATAC-3', backward 5'-GAGGCAGCT TGAACAACAAC-3'; Ca^{2+}/calmodulin-dependent protein kinase (CaMK) IV forward 5'-AAATCAGCCTGGTCCTTG AG-3', backward 5'-GAAGCATTTGCGGTGCACGATG-3'; β-actin forward 5'-GCTGAGAGGGAAATCGTGCGT-3', backward 5'-TCTGGTTTGAGGTCACGATG-3'. Expression level of β-actin mRNA was used as endogenous control, and the relative expression level of specific gene was calculated by $2^{-\Delta\Delta Cq}$ (20).

Quantification of acetylcholine (ACh) and measurement of AChE activity. The hippocampus (n=10/group) was homogenized in ice-cold 0.01 M Tris-HCl (pH 7.4) using bead beater (Taco™ Prep; GeneResearch Biotechnology Corp.) and ultrasonic cell disruptor (KS-750; Madell Technology Corp.), and then centrifuged at 12,000 x g for 15 min. ACh concentration and AChE activity in the hippocampal homogenates were measured using an Amplex Red ACh/AChE assay kit (Invitrogen), according to manufacturer's instruction. Fluorescence intensities at 560 nm (excitation wavelength) and 590 nm (emission wavelength) were detected using an automated microplate reader (Infinite 200 Pro; Tecan).

Measurement of antioxidant activities in the cerebral cortex. After mouse that had performed the Morris water maze test was euthanized by cervical dislocation under inhalation anesthesia (2-3% of isoflurane), the cerebral cortex (n=10/group) was collected, homogenized with a buffer consisting of 10 mM sucrose, 10 mM Tris-HCl (pH 7.4), and 0.1 M ethylenediaminetetraacetic acid, and then clarified by centrifugation. Protein concentration of tissue homogenates was determined using bovine serum albumin as a standard protein. Lipid peroxidation was measured at 525 nm of wavelength by quantifying concentration of malondialdehyde using a thiobarbituric acid, and was expressed as malondialdehyde (ng) per tissue (g). After homogenates were precipitated by 25% of trichloroacetic acid, reduced glutathione (GSH) contents in resulting supernatants were determined at 412 nm of wavelength using a 2-nitrobenzoic acid, and were expressed as nM/mg protein. Decomposition of H_2O_2 by tissue homogenates was measured at 240 nm. One unit of catalase (CAT) activity was defined as the amount of enzyme required to degrade 1 nM of H_2O_2 for 1 min. After homogenates were reacted with nitroblue tetrazolium to form formazan, absorbance at 560 nm was measured for determining superoxide dismutase (SOD) activity. One unit of SOD activity was defined as the amount of enzyme that can reduce initial absorbance of nitroblue tetrazolium by 50% for 1 min. Specific activity was expressed as U/mg protein.

Statistical analysis. All numerical values are expressed as the mean ± standard deviation (SD) (n=6 for *in vitro* assay; n=10 or 20 for *in vivo* assay). One-way ANOVA was used to assess the significance among experimental groups, followed by Tukey's honest significance difference or Dunnett's T3 as post hoc analysis. P values less than 0.05 were considered as statistical differences of significance.

Results

GBE prevents oxidative stress-mediated cytotoxicity in HT-22 cells. Ginsenoside Re is one of the most abundant ginsenosides found in the ginseng berry (12,21). Before investigating the beneficial effects of GBE, we quantified the concentration of ginsenoside Re in GBE using HPLC under optimized conditions. HPLC analysis indicated that the GBE used in this study contained 29.13±0.15 mg/g of ginsenoside Re (Fig. 1A). Next, we examined whether GBE exerted cytotoxic effects against HT-22 cells. Using a cell viability assay, no statistical differences were found when HT-22 cells were exposed to up to 100 μg/ml of GBE for 72 h (Fig. 1B). To explore the cytoprotective effects of GBE, GBE-pretreated HT-22 cells were continuously exposed to 5 mM of glutamate or 500 μM of H_2O_2, representative oxidative stressors of neuronal cells, for 12 h. As expected, treatment with glutamate or H_2O_2 alone significantly decreased the cell viability of HT-22 cells ($P<0.01$; Fig. 1C and D). However, pretreatment with GBE tended to increase cell viability in a concentration-dependent manner. Compared with glutamate-treated cells, a significant difference in cell viability was observed in cells pretreated with 0.1-100 μg/ml of GBE ($P<0.05$, 0.01 μg/ml GBE; $P<0.01$, 1-100 μg/ml GBE; Fig. 1C). Relative cell viability after treatment with glutamate, glutamate + 0.01 μg/ml GBE, glutamate + 0.1 μg/ml GBE, glutamate + 1 μg/ml GBE, glutamate + 10 μg/ml GBE, and glutamate + 100 μg/ml GBE was 43.0±4.4, 43.2±8.0, 55.0±5.5, 62.0±6.3, 66.5±5.7 and 69.5±7.2% of control cells, respectively. In addition, 1-100 μg/ml of GBE significantly reduced H_2O_2-mediated cytotoxicity in HT-22 cells ($P<0.01$; Fig. 1D). Relative cell viability after treatment with H_2O_2, H_2O_2 + 0.01 μg/ml GBE, H_2O_2 + 0.1 μg/ml GBE, H_2O_2 + 1 μg/ml GBE, H_2O_2 + 10 μg/ml GBE, and H_2O_2 + 100 μg/ml GBE was 42.3±3.4, 42.5±1.1, 52.7±8.4, 60.3±8.0, 63.7±7.6 and 65.0±4.9% of control cells, respectively. These results suggest that GBE can protect neuronal cells from oxidative stress *in vitro*.

GBE mitigates SCP-induced memory impairment in mice. The hippocampus is involved in cognitive function including memory, learning, and emotion. To expand the findings that GBE can protect hippocampal neuronal cells from oxidative stress *in vitro*, we tested its effects *in vivo* using a SCP-induced memory impairment murine model. C57BL/6NCrljOri mice (6 weeks old, male) were orally administered one of three dosages of GBE (100-400 mg/kg) or tacrine (10 mg/kg; positive control) once per day for 28 days (i.e., initial GBE administration on day 0). To induce memory impairment, SCP was intraperitoneally injected three times, 1 h after GBE administration on days 6, 13, and 27 (Fig. 2A). All mice experienced a decrease in body weight on day 0 due to an overnight fasting period of 12 h, and body weight gradually increased during the 28-days of the experimental period. There were no significant differences in body weight among the experimental groups (Fig. 2B). Two behavior tests were conducted to investigate the neuroprotective effects associated with the hippocampus. In the retention trial, the passive avoidance test indicated that SCP decreased the step-through latency time compared with the vehicle-treated control group

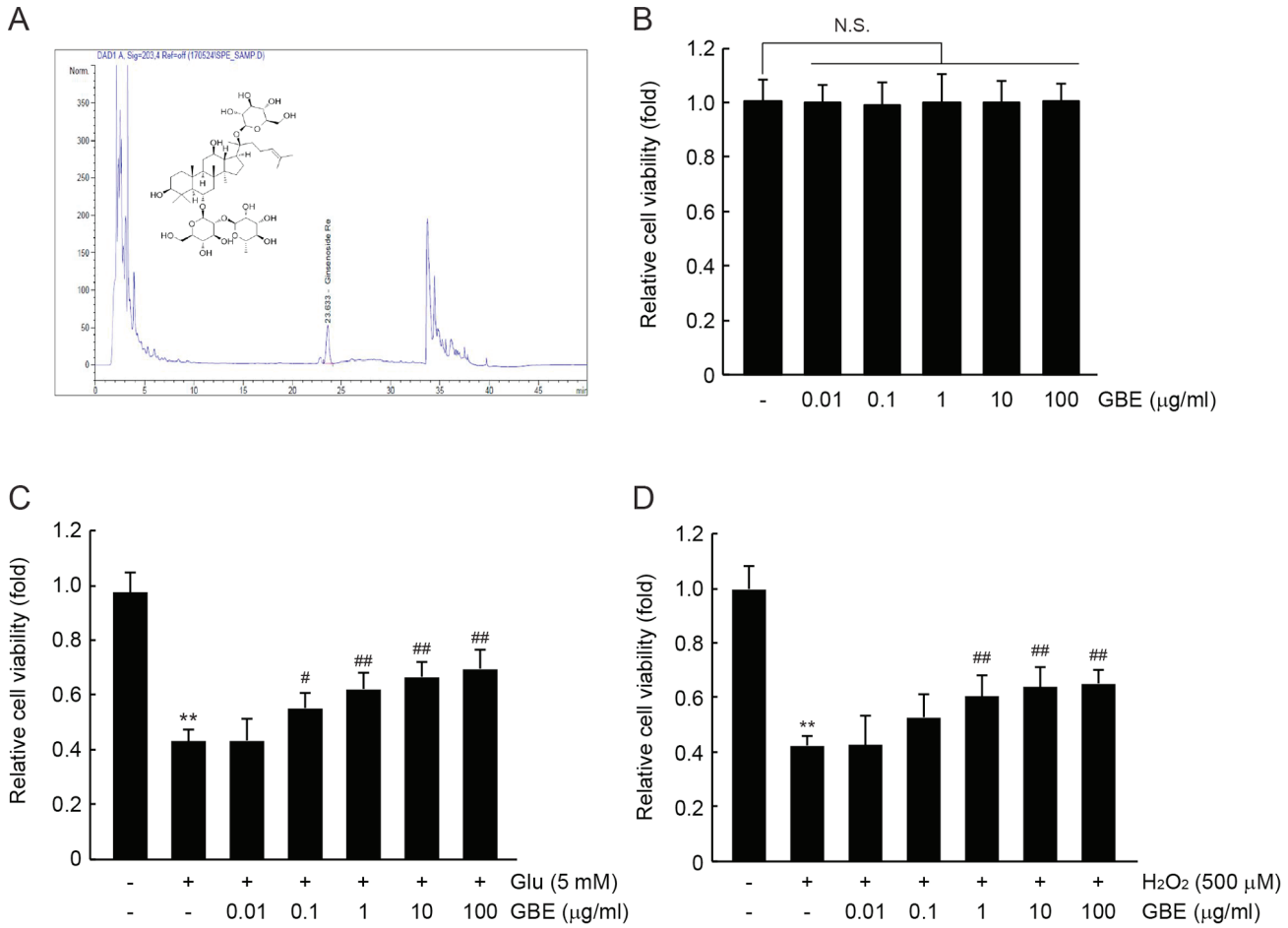

Figure 1. GBE protects HT-22 cells from oxidative stress. (A) Ginsenoside Re in GBE was quantified using a high-performance liquid chromatography. (B) Cell viability assay. HT-22 cells were treated with GBE (0.01-100 μg/ml) for 72 h. Relative cell viability was determined using the EZ-Cytox cell viability assay kit. (C, D) After HT-22 cells were pretreated with GBE (0.01-100 μg/ml) for 0.5 h, the cells were further exposed to (C) 5 mM glutamate or (D) 500 μM H_2O_2 for 12 h. All values are expressed as the mean ± SD of 6 independent experiments. **P<0.01 vs. vehicle-treated control; $^{\#\#}$P<0.01, $^{\#}$P<0.05 vs. glutamate- or H_2O_2-treated cells. GBE, ginseng berry aqueous extract; Glu, glutamate; N.S., not significant; SD, standard deviation.

(P<0.01). However, administration of all three dosages of GBE or tacrine significantly prevented the decrease in step-through latency time by SCP (P<0.01). Specifically, the increase in step-through latency time in mice treated with 400 mg/kg of GBE was significantly higher than in mice treated with tacrine (P<0.05; Fig. 2C). Similarly, SCP injection resulted in increased latency time in the Morris water maze (P<0.01), and treatment with the three dosages of GBE significantly decreased the escape time (P<0.01). There were no statistical differences of latency time between mice administered GBE vs. tacrine (Fig. 2D). Collectively, these results suggest that GBE can improve cognitive function in mice with SCP-induced memory impairment.

GBE enhances cholinergic nervous system functioning in the hippocampus. To investigate whether GBE prevents memory impairment by enhancing cholinergic signaling in the hippocampus, we measured mRNA levels of ChAT, which produces ACh from acetyl-CoA and choline. As expected, SCP injection significantly decreased ChAT mRNA levels (P<0.01). However, all three dosages of GBE or tacrine administration prevented this reduction in hippocampal ChAT mRNA (P<0.01). Specifically, ChAT mRNA levels in mice treated with 400 mg/kg of GBE were higher than those in mice treated with 10 mg/kg of tacrine (P<0.05; Fig. 3, left). In addition, GBE administration significantly alleviated the reduction of Ach by SCP in the hippocampus (P<0.01; Fig. 3, middle). Moreover, the increase in hippocampal AChE activity after SCP injection was significantly decreased following GBE administration with all three dosages (P<0.01; Fig. 3, right). ACh concentration and AChE activity after treatment with the three dosages of GBE were comparable to those after treatment with tacrine.

GBE increases mRNA levels of memory-related genes in the hippocampus. BDNF is a representative neurotrophic factor associated with formation and storage of memory that activates CREB through transducing cellular signaling involving PI3K, Akt, ERK1, ERK2, and CaMK VI (22-24). To examine the effects of GBE on mRNA levels of memory-related genes, RT-PCR analysis was conducted using hippocampal RNAs. SCP significantly decreased mRNA levels of BDNF, PI3K, Akt, ERK1, ERK2, CaMK VI, and CREB (P<0.01; Table I). Administration of 200 and 400 mg/kg of GBE prevented the reduction of mRNA levels of all genes related to memory in the hippocampus (PI3K in 200 mg/kg GBE: P<0.05; P<0.01 for the others), while treatment with 100 mg/kg of GBE only

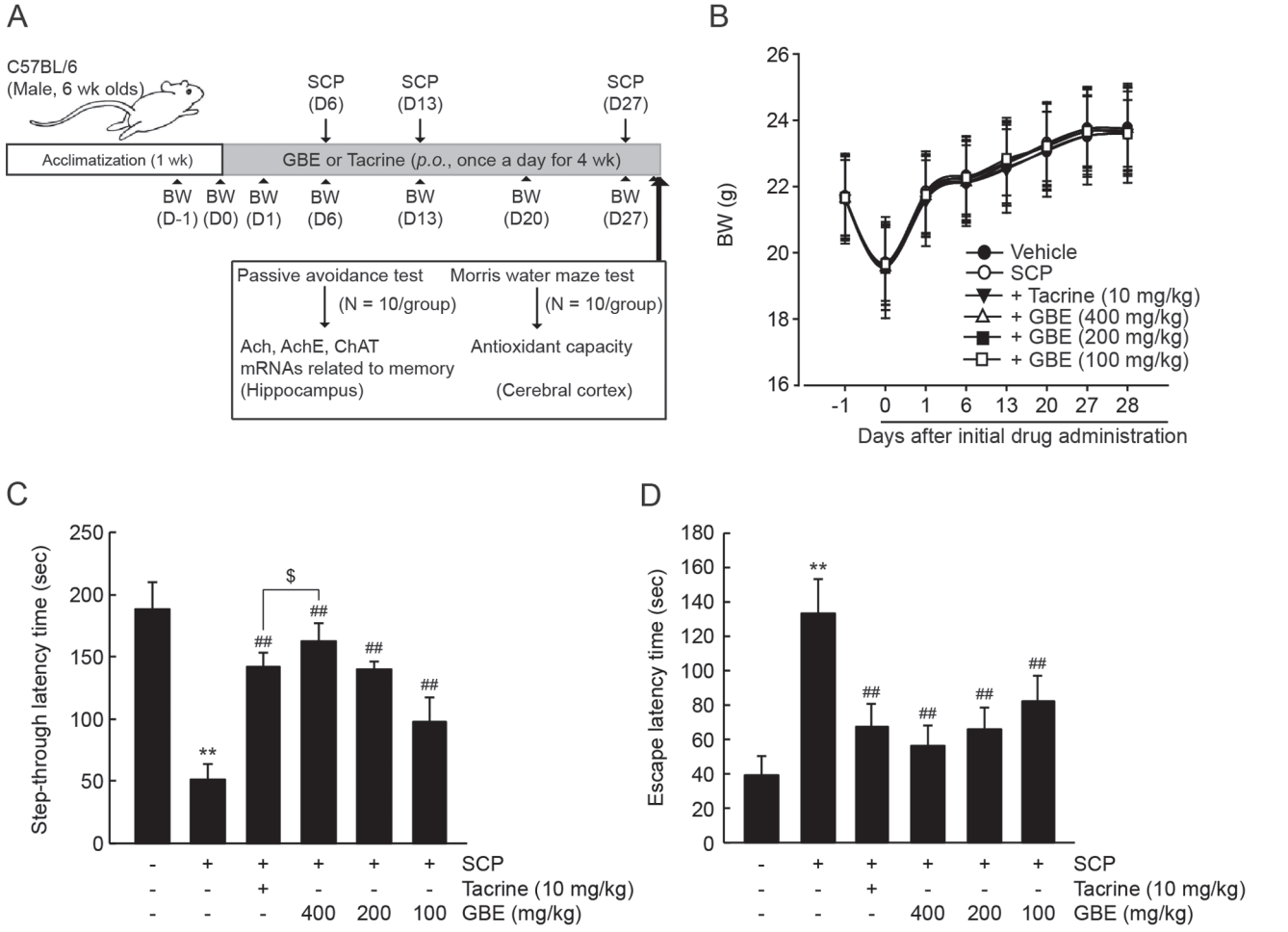

Figure 2. GBE prevents SCP-induced memory impairment in mice. (A) *In vivo* experimental designs for drug treatment, behavior testing, and biochemical analysis; 100-400 mg/kg of GBE or 10 mg/kg of tacrine was orally administered once per day for 4 weeks, and SCP was injected three times 1 h after GBE/tacrine administration on days 6, 13 and 27. (B) Mouse BW. All mice were fasted for 12 h before the first GBE/tacrine administration. BW (n=20/group) was measured on days -1, 0, 1, 13, 20, 27 and 28 after the initial drug administration. (C) Passive avoidance test. The time from the light/noise compartment to the electric grid floor for each individual mouse was considered a measure of step-through latency time (n=10/group). (D) Morris water maze test. The time from the water to the submerged platform for each individual mouse considered a measure of escape latency time (n=10/group). All values are expressed as the mean ± SD. **P<0.01 vs. vehicle-treated group; $^{\#\#}$P<0.01 vs. SCP-injected group; $^{\$}$P<0.05 vs. tacrine-treated group). BW, body weight; GBE, ginseng berry aqueous extract; SCP, scopolamine; SD, standard deviation.

changed BDNF, Akt, and ERK1 mRNA levels compared with the SCP-injected group (Akt: P<0.05; BDNF and ERK1: P<0.01). Moreover, the mRNA levels of PI3K, Akt, and ERK2 in the 400 mg/kg GBE group were significantly higher than those in the tacrine group (PI3K: P<0.05; Akt and ERK2: P<0.01; Table I).

GBE increases antioxidant activity in the cerebral cortex. To investigate whether GBE increases antioxidant capacity *in vivo*, we first measured the level of malondialdehyde (a marker of lipid peroxidation) in the cerebral cortex. SCP injection significantly increased the level of malondialdehyde (P<0.01), showing that SCP promoted lipid peroxidation in the brain. By contrast, administration of all three doses of GBE or tacrine significantly reduced lipid peroxidation (P<0.01). The decrease in lipid peroxidation after treatment with the three doses of GBE was comparable to that by tacrine (Fig. 4A). In addition, SCP decreased GSH levels and CAT and SOD activities in the cerebral cortex (P<0.01), and administration of GBE or tacrine significantly prevented the decreases in GSH levels and CAT and SOD activity (CAT activity in tacrine: P<0.05; P<0.01 for the others). In contrast, the decrease in CAT activity was not prevented in the group following administration with 100 mg/kg of GBE. GSH and SOD activity levels in mice treated with 400 mg/kg of GBE were higher than those in mice treated with tacrine (P<0.05; Fig. 4B-D).

Discussion

It has been reported that ginseng root, its enriched fractions, and isolated bioactive compounds can attenuate SCP-induced memory impairment in experimental animals (16,18,25-30). Protection of the cholinergic nervous system via antioxidant and anti-inflammatory actions is associated with the pharmacological effect of ginseng root in animals with SCP-induced amnesia (16,25,26,28). However, the effects of ginseng berries on memory impairment remain poorly understood. Although similar beneficial effects to those of ginseng root have been reported in other parts of the *P. ginseng* plant (6,8,9), certain bioactive compounds are selectively distributed throughout

Table I. The effect of GBE administration on mRNA levels related to memory in SCP-induced memory-impaired mice.

Group	BDNF	PI3K	Akt	ERK1	ERK2	CaMK IV	CREB
Vehicle	1.01±0.13	1.01±0.10	1.00±0.11	0.99±0.07	1.02±0.09	1.01±0.14	0.99±0.12
SCP	0.37±0.10[a]	0.30±0.11[a]	0.38±0.08[a]	0.30±0.09[a]	0.28±0.09[a]	0.31±0.12[a]	0.33±0.09[a]
+ Tacrine 10 mg/kg	0.63±0.13[b]	0.46±0.10[c]	0.60±0.10[b]	0.53±0.06[b]	0.53±0.10[b]	0.51±0.08[b]	0.55±0.13[b]
+ GBE 400 mg/kg	0.75±0.11[b]	0.67±0.14[b,e]	0.80±0.12[b,d]	0.71±0.15[b]	0.72±0.12[b,d]	0.62±0.13[b]	0.69±0.14[b]
+ GBE 200 mg/kg	0.65±0.11[b]	0.47±0.13[c]	0.60±0.13[b]	0.54±0.07[b]	0.52±0.10[b]	0.50±0.07[b]	0.54±0.09[b]
+ GBE 100 mg/kg	0.56±0.08[b]	0.42±0.08	0.55±0.11[c]	0.48±0.07[b]	0.39±0.08	0.44±0.05	0.46±0.10

All values are expressed as the mean ± SD of 10 mice. [a]P<0.01 vs. vehicle-treated group; [b]P<0.01, [c]P<0.05 vs. SCP-injected group; [d]P<0.01, [e]P<0.05 vs. tacrine-treated group. BDNF, brain-derived neurotrophic factor; CaMK, Ca^{2+}/calmodulin-dependent protein kinase; CREB, cAMP response element binding protein; ERK, extracellular-regulated protein kinase; GBE, ginseng berry aqueous extract; PI3K, phosphoinositide 3-kinase; SCP, scopolamine; SD, standard deviation.

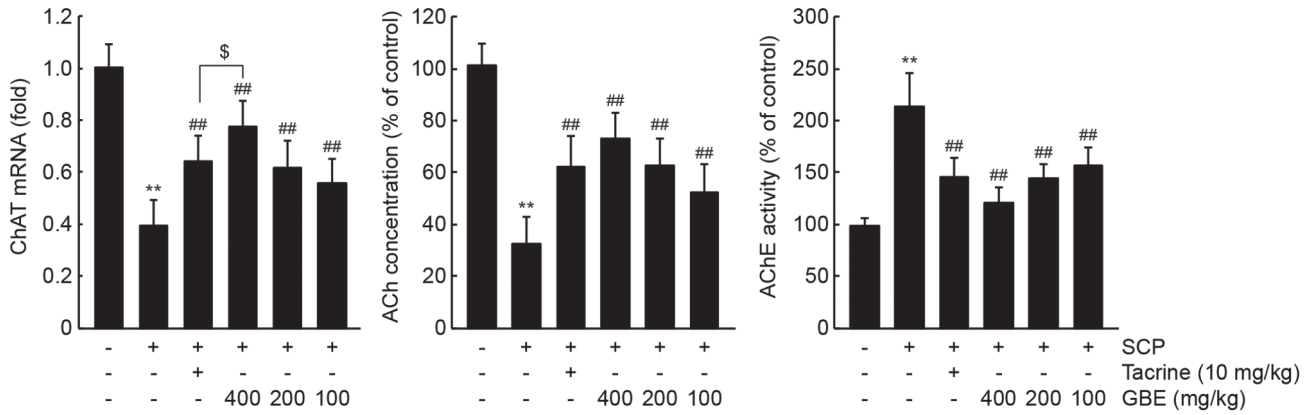

Figure 3. GBE enhances cholinergic nervous system activity in the hippocampus of SCP-injected mice. ChAT mRNA levels (left), ACh concentrations (middle), and AChE activities (right) in the hippocampus were determined from mice that underwent the passive avoidance test. All values are expressed as the mean ± SD of 10 mice. [**]P<0.01 vs. vehicle-treated group; [##]P<0.01 vs. SCP-injected group; [$]P<0.05 vs. tacrine-treated group. ACh, acetylcholine; AChE, acethylcholinesterase; ChAT, choline acetyltransferase; GBE, ginseng berry aqueous extract; SCP, scopolamine; SD, standard deviation.

P. ginseng (21). Before investigating the anti-amnesic effect of GBE, we performed an HPLC analysis to assess the quality of GBE and found that GBE used in the present study contained 29.13±0.15 mg/g of ginsenoside Re.

Lee *et al* (2017) reported that 29 of the 58 ginsenosides previously tested are found in ginseng berry methanolic extract (21). Ginsenosides F1 and Rg4 are found only in the berry at low concentrations, while ginsenoside Re, Rg2, F4, Rg5, malonylated Rd, malonylated Rb2, malonylated Rc, and malonylated Rb1 are concentrated in berry compared to other parts of the ginseng plant (21). Moreover, previous results from different groups have shown that ginsenoside Re, Rf, Rb1, Rc, Rb2, and Rd are contained in ginseng berry aqueous extract (12), which is the identical to the extract used in this study. Ginsenoside Re is found to be the most abundant ginsenoside in ginseng berry (12,21). Although there is no direct evidence that ginsenoside Re ameliorates SCP-induced amnesia, an accumulation of evidence suggests that ginsenoside Re enhances cognitive function through the reduction of amyloid β (31,32). Additionally, other ginsenosides found in GBE have been reported to exhibit neuroprotective activities (33-35). Therefore, ginsenoside Re and other unidentified compounds in GBE are thought to contribute cooperatively to mitigating cognitive deficits caused by SCP. Further studies are needed to identify the major compounds involved in GBE-mediated neuroprotection.

In the present study, SCP (1 mg/kg) was intraperitoneally injected three times in mice to induce memory loss. SCP is a nonselective antagonist of the muscarinic ACh receptor, which reduces cholinergic transmission in the central nervous system and causes cognitive dysfunction, including long-term memory loss (36). Therefore, SCP is one of the most extensively used neurotoxins to elucidate possible therapeutic agents for modulating memory impairment (16,18,26-30). To study the neuroprotective effects of GBE related to memory, we conducted two behavioral tests after SCP injection. The passive avoidance test has been performed to investigate non-spatial long-term memory after an aversive experience (37). In parallel with previous reports (16,18,26,29), SCP significantly decreased the step-through latency time. However, GBE administration significantly prolonged the step-through latency time in a dose-dependent manner. The Morris water maze test is another behavioral test used to assess long-term and spatial memory in the hippocampus (19,38). In the present study, SCP injection significantly increased the latency time to the escape platform in the water maze, whereas GBE mitigated

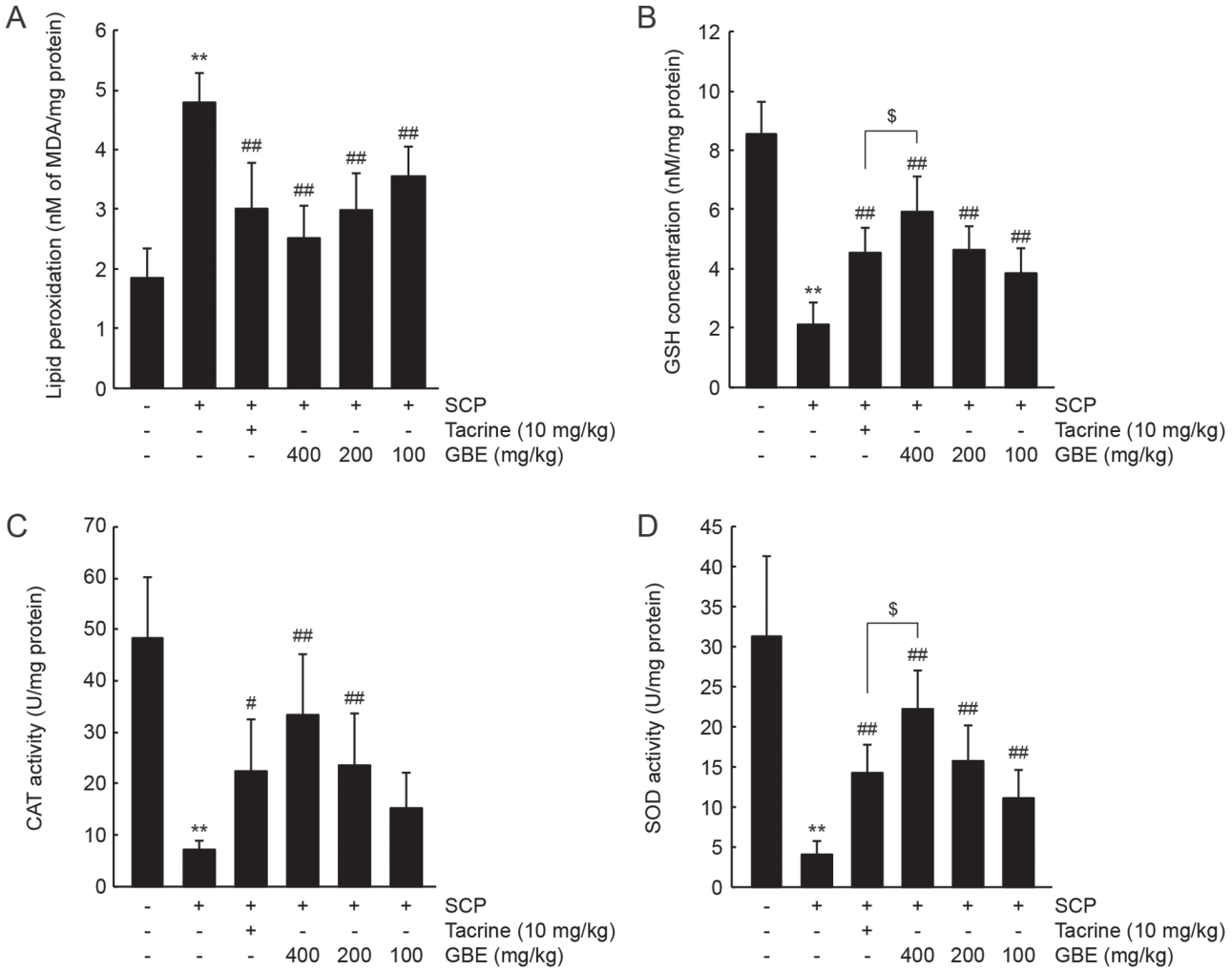

Figure 4. GBE increases antioxidant activity in the SCP-injected mice. (A) Lipid peroxidation, (B) GSH concentration, and (C) CAT and (D) SOD activities in the cerebral cortex were determined from mice that had performed the Morris water maze test. All values are expressed as the mean ± SD of 10 mice. **P<0.01 vs. vehicle-treated group; $^{\#\#}$P<0.01, $^{\#}$P<0.05 vs. SCP-injected group; $^{\$}$P<0.05 vs. tacrine-treated group. CAT, catalase; GBE, ginseng berry aqueous extract; GSH, glutathione; MDA, malondialdehyde; SCP, scopolamine; SD, standard deviation; SOD, superoxide dismutase.

the SCP-induced spatial memory impairment as shown by a decreased escape time.

ACh, which is synthesized by ChAT, is a critical neurotransmitter for regulating cognitive function. In addition, memory loss by SCP is closely correlated with an increase in AChE (an ACh-hydrolyzing enzyme in the cholinergic synaptic cleft) activity and a subsequent reduction in ACh in the hippocampus (26,27). To confirm whether GBE restores long-term memory via the cholinergic nervous system, we further measured hippocampal levels of ACh and its metabolizing enzymes. SCP increased AChE activity and decreased ACh and ChAT mRNA levels in the hippocampus, which was consistent with previous reports (26,27). However, GBE administration significantly reduced these changes. Therefore, these results suggest that GBE attenuates SCP-induced memory deficit by enhancing cholinergic signaling in the hippocampus.

BDNF is a neurotrophic factor that contributes to neuronal plasticity and synaptic transmission (23). BDNF enhances memory function by promoting the formation of long-term potentiation and by up-regulating the sensitivity of N-methyl-D-aspartate receptors in the hippocampus (22,39,40). Binding of BDNF to tropomyosin receptor kinase B (TrkB) facilitates dimerization and phosphorylation of TrkB tyrosine residues, and recruits diverse Src homology 2 adaptor molecules, including phospholipase C, insulin receptor substrate, and Shc (23,41). Phospholipase C activates Ca^{2+}-dependent calmodulin kinase by increasing intracellular Ca^{2+} levels. In addition, insulin receptor substrate and Shc activate the phosphoinositide 3-kinase-dependent Akt and Ras/Raf/MEK-dependent ERK signaling pathways (23). Specifically, the BDNF signaling cascades regulate CREB, which is an essential transcription factor necessary for late-stage of long-term potentiation and long-term memory formation in the hippocampus (42,43). The present results demonstrated that SCP significantly decreased BDNF mRNA levels and its related signaling molecules, and the administration of GBE prevented these reductions in the hippocampus. Although GBE may restore long-term memory by upregulating the expression of BDNF-related signaling molecules in the hippocampus, further studies on the covalent modifications (e.g., phosphorylation) of signaling molecules are needed to establish the role of the GBE in signaling pathway related to memory.

We also showed that GBE protected HT-22 cells from glutamate- and H_2O_2-induced cytotoxicity. Because of its

low endogenous antioxidant capacity, an abundance of poly-unsaturated fatty acids, and high oxygen consumption rate, the brain is one of the most susceptible organs to oxidative stress (44,45). Therefore, oxidative stress is important in the progression of neurodegenerative diseases, including AD (44). It has been reported that ginseng protects the brain from oxidative stress via activation of nuclear factor-E2 related factor 2 (Nrf2) (16,25,46). Moreover, genetic deletion of Nrf2 abolishes the protective effect of compound K (a major aglycosylated metabolite of protopanaxadiol-type ginsenosides) against SCP-induced amnesia (16). In the resting state, Nrf2 resides in the cytoplasm by binding to Kelch-like ECH-associated protein 1 and is autonomously degraded by the ubiquitin-dependent proteasome system. Antioxidants as well as oxidative stress disturb protein-protein interaction between Nrf2 and Kelch-like ECH-associated protein 1, and translocate Nrf2 into the nucleus. Nuclear Nrf2 heterodimerizes with Jun or small Maf and subsequently binds to the antioxidant response elements of many antioxidant genes, including γ-glutamate cysteine ligase (a rate-limiting enzyme of GSH synthesis), CAT, or SOD (47). In this study, SCP injection increased lipid peroxidation, decreased GSH levels, and reduced CAT and SOD activity, which is in agreement with previous observations that SCP provokes oxidative stress in the brain (26,48). GBE administration inhibited lipid peroxidation significantly and elevated the levels of GSH while also elevating CAT and SOD activities in a concentration-dependent manner. Therefore, these results suggest that GBE may attenuate SCP-induced memory impairment, potentially by protecting neurons from oxidative stress. However, further studies investigating the detailed cellular mechanisms involved, including Nrf2 activation, are warranted.

In this study, we compared the pharmacological effect of GBE with tacrine. Although some statistical results (e.g., step-through latency time, GSH level, SOD activity, and ChAT, PI-3K, Akt, and ERK2 mRNA levels) showed that GBE (400 mg/kg) was more potent than tacrine, most of the anti-amnesic effects of GBE (200-400 mg/kg) were comparable to those of tacrine (10 mg/kg). In conclusion, the results of this study suggest that GBE alleviates SCP-induced memory impairment by restoring the cholinergic nervous system, mRNA expression of BDNF-related signaling molecules, and antioxidant capacity. Therefore, ginseng berry may be a promising complementary medicine for managing various neurodegenerative disorders with memory impairment.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SKK designed the study. JRH, YSC, JKK and SKK conducted the research. JRH, IJC and SKK analyzed the data. JRH, IJC and SKK co-wrote the manuscript. IJC and SKK had primary responsibility for the final content. All authors approved the final draft of the manuscript.

Ethics approval and consent to participate

Animal experiments were conducted according to the national regulations regarding the use and welfare of laboratory animals and were approved by the Institutional Animal Care and Use Committee in Daegu Haany University (approval no. DHU2017-081).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing of interests.

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