Ginsenoside Rh2 Ameliorates Scopolamine-Induced Learning Deficit in Mice

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To understand memory-enhancing effect of red ginseng biotransformed by Bifidobacterium longum H-1 (RGB), which more potently improved scopolamine-induced learning deficit than red ginseng in the preliminary experiment, its main constituents, ginsenosides Rb1, Rg3 and Rh2, were isolated and their memory-enhancing effects investigated in scopolamine-treated mice by using passive avoidance and Y-maze tests. Among them, ginsenoside Rh2 most potently reversed memory impairment caused by scopolamine. Ginsenoside Rh2 also significantly shortened the escape latencies prolonged by scopolamine in the Morris water maze test (p<0.001) and increased the swimming time prolonged by scopolamine within the platform quadrant (p<0.05). The ginsenoside Rh2 (3 µg) reversed scopolamine (10 µg)-induced suppression of long-term potentiation. It recovered field excitatory postsynaptic potential (fEPSP) amplitude potentiation to 152.3±8.7% of the control (p<0.05). Based on these findings, RGB and its main constituent, ginsenoside Rh2, might improve learning deficits. Also the memory-enhancing effects of RGB may be dependent on the content of ginsenoside Rh2.

Key words red ginseng; ginsenoside Rh2; memory; scopolamine; long-term potentiation

Alzheimer’s disease (AD) is a progressive degenerative disease of the brain that is characterized by deterioration of memory and cognitive functions. The characteristic pathological features of the central nervous system in Alzheimer’s disease are the presence of senile plaques, neurofibrillary tangle formation, aberrant oxidative and inflammatory processes, and neurotransmitter disturbances. 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 with fewer side effects for patients with memory impairments.

Ginseng (dried root of Panax ginseng C. A. Meyer, family Araliaceae) is widely used in Asian countries as a traditional medicine for enhancing body strength, recovering physical balance and stimulating metabolic function. In its steamed form, it is called red ginseng. Red ginseng has been proven as a functional food, containing ginsenoside Rh2 that is transformed from ginsenoside Rg3 by Bifidobacterium longum H-1 as the main constituent. Its main constituent ginsenoside Rh2 exhibits potent anti-allergic effect against mast cells, anti-inflammatory activity in microglial cells and anti-ischemic brain injury compared to ginsenoside Rg3. However, the memory-enhancing effect of ginsenoside Rh2 has not been studied thoroughly.

During a herbal medicine screening program for compounds that improved memory impairment, the memory-enhancing effect of red ginseng was significantly increased, when it was transformed by B. longum H-1. Therefore, we isolated the representative constituents ginsenosides Rb1, Rg3 and Rh2, from red ginseng biotransformed by B. longum H-1 (RGB), and investigated its learning and memory ability in mice using a passive avoidance test and the Morris water maze.
MATERIALS AND METHODS

Chemicals  Tarcine (9-amino-1,2,3,4-tetrahydroacridine hydrochloride), (−) scopolamine hydrobromide, were purchased from the Sigma Chemical Co. (U.S.A.). All other materials were obtained from normal commercial sources and were of the highest grade available.

Biotransformation of Red Ginseng Biotransformed by Bifidobacterium longum H-1 (RGB) and Isolation of Its Ginsenosides  The red ginseng extract was prepared based on the previously described procedure. Fresh ginseng (1 kg) was steamed at 98—100 °C for 4 h and dried for 5 h at 60 °C. Then, it was extracted with 60% ethyl alcohol at 70 °C (freeze-dried extract weight, 39 g). Red ginseng extract (30 g) was suspended in water, biotransformed by freeze-dried. It was used as a RGB. The freeze-dried powder was suspended in water, defatted with n-hexane, extracted with n-BuOH (11) twice, and evaporated (dried extract, 5.2 g).

The n-BuOH extract (5 g) were subjected to a silica gel column chromatography (6×30 cm) and eluted with a stepwise gradient of CH4Cl2 : methanol (20 : 1→20 : 2→ 20 : 3→20 : 4→20 : 5→20 : 6, each 500 ml) to yield six fractions (FB1—FB6) on the basis of their thin layer chromatography (Silica gel 60F254, Merck Co., Darmstadt, Germany) behaviors. Fractions FB3 (1.1 g), FB4 (1.8 g) and FB5 (0.9 g) underwent further chromatography on silica gel columns (3×25 cm), employing CHCl3 : methanol : H2O (65 : 35 : 10, lower layer), to give a ginsenosides Rh2 (128 mg), Rg3 (85 mg) and Rb1 (95 mg), respectively. These isolated ginsenosides were identified by comparison to authentic standards by instrumental analysis with those of the previously reported literatures.

Ginsenoside Rb1: White needle, mp 197—198 °C (dec.) FAB-MS (m/z): 1110 [M+1]+
Ginsenoside Rg3: White needle, mp 248—250 °C (dec.) FAB-MS (m/z): 786 [M+1]+
Ginsenoside Rh2: White needle, mp 219—221 °C, FAB-MS (m/z): 623 [M+1]+

Animals  All the experiments were carried out using male ICR mice weighing 28—30 g purchased from the Orient Co., Ltd., a branch of Charles River Laboratories (Seoul), according to the guidelines of the Principle of Laboratory Animal Care (NIH publication No. 85—23, revised 1985) and the Animal Care and Use Guidelines of Kyung Hee University, Korea. The mice were housed 5 or 6 per cage, allowed access to water food ad libitum, and maintained at an ambient temperature of 23±1 °C with 60±10% humidity and a 12 h diurnal light cycle (light on 07.30—19.30 h), prior to testing. All behavioral experiments were carried out in a room adjacent to that in which the mice were housed under the same conditions of temperature and humidity and light cycle.

Passive Avoidance Test  Passive avoidance test was carried out in identical illuminated and non-illuminated boxes (Gemini Avoidance System, San Diego, U.S.A.). The illuminated compartment (20×20×20 cm) contained an 100 W bulb, and the floor of 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 acquisition trial, mice were initially placed in the illuminated compartment and the door between the two compartments was opened 10 s later. When 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 were administered red ginseng extract (50 mg/kg, per os (p.o.), RGB (50 mg/kg, p.o.), ginsenosides Rh1, Rg3, Rh2 (10, 20 and/or 40 mg/kg, p.o) or tacrine (10 mg/kg, p.o) as a positive control. Memory impairment was induced by scopolamine treatment (0.9 mg/kg, intraperitoneal (i.p.)) 30 min after the administration of each sample, tacrine, or 10% Tween 80 solution. Control animals were administered 10% Tween 80 solution only. Twenty-four hours after acquisition trial, the mice were again placed in the illuminated compartment for the retention trials. The time taken for a mouse to enter the dark compartment after door opening was measured as latency times in both acquisition and retention trials. If a mouse did not enter the dark compartment within 180 s, it was assumed that the mouse had remembered the single training trial.

Y-Maze Test  The Y-maze is a three-arm horizontal maze (40-cm-long and 3-cm-wide with 12-cm-high walls) in which the arms are symmetrically disposed at 120 ° angles from each other. The maze floor and walls were constructed from dark opaque polyvinyl plastic as has been described previously. Mice were initially placed within one arm, and the sequence (i.e., ABCAB, etc.) and number of arm entries were recorded manually for each mouse over an 8-mm period. An actual alternation was defined as entries into all three arms on consecutive choices (i.e., ABC, CAB, or BCA but not BAB). Maze arms were thoroughly cleaned between tasks to remove residual odors. One hour after the last administration of ginsenoside Rh2, tacrine or vehicle alone, memory impairment was induced by scopolamine treatment (0.9 mg/kg, i.p.). Mice were gently placed in the maze. The percentage of alternations was defined according to the following equation: % alternation=([number of alternations]/(total arm entries−2)]×100. The number of arm entries served as an indicator of locomotor activity.

Morris Water Maze Test  The Morris water maze is a circular pool (90 cm in diameter and 45 cm in height) with a featureless inner surface. The pool was filled to a depth of 30 cm with water containing 500 ml of milk (20±1 °C). The tank was placed in a dimly lit, soundproof test room with various visual cues. The pool was conceptually divided into quadrants. A white platform (6 cm in diameter and 29 cm high) was then placed in one of the pool quadrants and submerged 1 cm below the water surface so that it was invisible at water level. The first experimental day was dedicated to swimming training for 60 s in the absence of the platform. During the four subsequent days the mice were given four trials per day with the platform in place. When a mouse located the platform, it was permitted to remain on it for 10 s. If the mouse did not locate the platform within 60 s, it was placed on the platform for 10 s. The animal was taken to its home cage and was allowed to dry up under an infrared lamp after each trial. The time interval between each trial was 30 s. During each trial, the time taken to find the hidden platform (latency) was recorded using a video camera-based EthoVision System (Nodulus, Wageningen, The Netherlands). For
each training trial, mice were placed in the water facing the pool wall at one of the pool quadrants in a different order each day. One day after the last training trial sessions, mice were subjected to a probe trial session in which the platform was removed from the pool, allowing the mice to swim for 60 s to search for it. A record was kept of the swimming time in the pool quadrant where the platform had previously been placed. Ginsenoside Rh2 (40 mg/kg, p.o.) or tacrine (10 mg/kg, p.o.) as a positive control was given 1 h before the first trial session at every consecutive day. Memory impairment was induced in mice with scopolamine (0.9 mg/kg, i.p.) at 30 min after treatment of the test agent. Control group received 10% Tween 80 solution only.

Electrophysiology ICR mice (male, 25—30 g) were decapitated under pentobarbital anesthesia (50 mg/kg, i.p.). The brain was dissected and the hippocampi were transversely sliced at a thickness of 400 μm by use of a microslicer (VT1000S; Leica, Nussloch, Germany) in a cold low-Na\(^+\) medium (in mM: 230 sucrose, 2 KCl, 1 KH\(_2\)PO\(_4\), 1 MgCl\(_2\), 0.5 CaCl\(_2\), 26 NaHCO\(_3\) and 10 glucose) saturated with 95% O\(_2\) and 5% CO\(_2\). Slices containing the hippocampus were kept in artificial cerebrospinal fluid (ACSF; 120 NaCl, 2 KCl, 1 KH\(_2\)PO\(_4\), 26 NaHCO\(_3\), 2 CaCl\(_2\), 1 MgCl\(_2\) and 10 glucose) saturated with 95% O\(_2\) and 5% CO\(_2\) at room temperature for at least 1 h. The bath was perfused with ACSF at 2 ml/min by the use of a peristaltic pump (MP-1000, EYELA, Tokyo, Japan). Electrical measurements were performed by use of a computer-controlled patch clamp amplifier set on the current clamp mode (MultiClamp 700A; Axon Instruments; Union City, CA, U.S.A.). Glass electrodes (inner diameter; 7—8 mm) filled with ACSF were placed at stratum radiatum. The field excitatory postsynaptic potentials (fEPSPs) were directly stored on a computer equipped with pCLAMP 8.02. To stimulate Schaffer collateral afferents, a glass pipette (inner diameter; 7—8 μm) filled with ACSF was placed around the center of stratum radiatum, and short voltage pulses (100 μs, 5—10 V) were applied at 0.1 Hz using a stimulator (SEN-7203, Nihon Kohden, Tokyo, Japan) equipped with an isolator unit (SS-701J, Nihon Kohden). The distance between stimulating and recording electrodes was 500 to 700 mm. In order to induce long-term potentiation (LTP) of excitatory synaptic transmission, a single tetanic stimulation (100 Hz, 1 s) was applied to stratum radiatum. The amplitudes of fEPSPs were calculated by subtracting the baseline from their peak amplitudes. The magnitude of LTP was calculated as percentage of the averaged field excitatory postsynaptic potential (fEPSP) amplitude value at 50—60 min after tetanic stimulation relative to the control fEPSP amplitudes. All electrophysiological recordings were performed at room temperature (22—25 °C).

Statistics Values are expressed as means±S.E.M. For the passive avoidance test, data were analyzed by a Kruskal–Wallis non-parametric ANOVA test. If the results were significant, each treatment group was compared by Tukey’s post hoc test. Statistical significance was set at \(p<0.05\).

RESULTS

In a preliminary study, when red ginseng was biotransformed by *Bifidobacterium longum* H-1, its memory-enhancing effect was significantly increased in the passive avoidance test (Fig. 2A). RGB (100 mg/kg) increased the scopolamine-induced reduction in step-through latency by 33%. Therefore, we isolated the main constituents, ginsenosides Rb1, Rg3 and Rh2, to evaluate its effect in scopolamine-treated memory-deficient mice during the passive avoidance test (Fig. 2B). The step-through latency of scopolamine treated mice was significantly shorter than that of vehicle-treated control mice (\(p<0.05\)). Among these ginsenosides, ginsenoside Rh2 most potently improved the memory deficits of the animals tested (\(p<0.05\)).

![Fig. 2. Effect of Red Ginseng (RG), Red Ginseng Biotransformed by *Bifidobacterium* H-1 (RGB), and Ginsenosides Rb1, Rg3 and Rh2 on Scopolamine-Induced Memory Deficits in the Passive Avoidance Test](image)
Scopolamine-induced deficits during the passive avoidance test are dependent on long-term memory. When the effect of ginsenoside Rh2 in scopolamine-induced deficits was tested using the passive avoidance test (Fig. 2C), ginsenoside Rh2 increased the scopolamine-induced reduction in step-through latency by 44% (10 mg/kg), 49% (20 mg/kg), and 64% (40 mg/kg). During the acquisition trial, no differences in latencies were observed among the groups studied. Tacrine (10 mg/kg), as a positive control, restored the step-through latency to 61% of the control group and this was consistent with previously published data.

Effect of ginsenoside Rh2 on memory impairment induced by scopolamine in the Y-maze test was also investigated (Fig. 3). The spontaneous alteration of scopolamine-treated mice was significantly lower than that of mice treated with vehicle alone. Ginsenoside Rh2 (40 mg/kg, p.o.) significantly reversed the lowered spontaneous alteration induced by scopolamine (p<0.05). Its potency was comparable to that of tacrine (10 mg/kg). However, the number of arm entries between the groups treated with and without ginsenoside Rh2 was not significantly different (data not shown).

The effects of ginsenoside Rh2 (40 mg/kg, p.o.) on spatial learning were evaluated using the Morris water maze test (Fig. 4A). The scopolamine-treated group exhibited longer escape latencies throughout the training days than did the control group (p<0.01). Ginsenoside Rh2 significantly shortened the escape latencies prolonged by scopolamine treatment (p<0.01). Tacrine, a positive agent, also significantly reduced escape latencies (p<0.01). On the day following the final day of training trial sessions, swimming times within the platform quadrant for the scopolamine-treated group were significantly lower than those of the vehicle-treated normal group animals (Fig. 4B, p<0.05). Moreover, the swimming time shortened by scopolamine within the platform quadrant was significantly increased by ginsenoside Rh2 or tacrine (Fig. 4B, p<0.05).

To clarify the memory-enhancing effect of ginsenoside Rh2, we measured its effect on scopolamine-induced suppression of long term potentiation (LTP) in the hippocampal CA1 area in mice by use of an extracellular field recording method (Fig. 5). A brief tetanic stimulation (100 Hz, 1 s)
elicited a long-lasting increase in fEPSP amplitude, where the fEPSP amplitude at 50—60 min after tetanic stimulation was potentiated to 167.4±9.9% of the control. In the presence of 10 µM scopolamine, the fEPSP amplitude potentiation was reduced to 123.4±7.2% of the control (n=6). However, ginsenoside Rh2 reversed scopolamine-induced suppression of LTP. In the presence of both 10 µM scopolamine and 3 µM ginsenoside Rh2, the fEPSP amplitude potentiation was recovered to 152.3±8.7% of the control (n=6, p<0.05, Student unpaired t-test).

DISCUSSION

A decrease in cholinergic function, particularly within the basal forebrain can result in a decline in memory and cognitive function with age.20 Scopolamine, an anti-cholinergic drug, caused memory impairments in healthy young humans that paralleled the memory impairments seen in nondemented drug-free elderly.20 Many studies indicate that scopolamine is increasingly disruptive with increasing age and declining cognitive status.21,22 This sensitivity pattern is consistent with the hypothesis that cholinergic tone decreases with increasing age and dementia. Thus, scopolamine treatment represents a good model for the learning and memory changes that occur during central nervous system (CNS) aging, despite Aβ-treated or transgenic animal models.20 Many attempts have been made to use medicinal plants to reverse cognitive deficits that occur due to the current few approved medicines use for the treatment of patients with memory impairment.

Ginsengs are effective in the attenuation of learning deficits due to brain damage and aging in humans and animals.23—25) Their saponins, ginsenosides Rh1, Rg1, Rg3, Rg5, Re and Rk1, improve learning ability in animals.26—30) Among these saponins, ginsenosides Rh1 and Rg3 showed strong neuroprotective effects, which may contribute to improving learning and memory. Ginsenoside Rg3 prevents memory impairment in mice that are given electroconvulsive shocks. Ginsenosides Rg5, Rk1, Re and Rg1 were shown to improve scopolamine-induced learning deficits. Ginsenoside Rg2 improves the cyropeptide-induced recognition deficits in rats. Furthermore, ginsenosides Rb1 and Rg3 potently improves ischemic brain injury as well as scopolamine-induced learning deficits.13,28—30)

However, upon oral administration, these ginsenosides are metabolized to compound K or ginsenoside Rh2 by intestinal microflora and then absorbed into the bloodstream, respectively.12,31—33) Ginsenoside Rg3 is particularly significant as it is the main constituent of red ginseng. Upon oral administration of red ginseng, its main constituent, ginsenoside Rg3, may be metabolized to ginsenoside Rh2.12 The absorption of metabolites, such as ginsenoside Rh2 and compound K, may be dependent on the metabolic activity of intestinal flora on ginsenosides such as Rg3 and Rb1. Furthermore, these metabolites have demonstrated potent pharmacological effects, such as anti-inflammatory, anti-ischemic and neuroprotective effects.12,13,17) Therefore, RGB, containing ginsenoside Rh2 as the main constituent,12 is expected to exert beneficial Rh2-dependent pharmacological effects in vivo. The ginsenoside Rh2 in RGB is transformed from ginsenoside Rg3 by B. longum H-1.12,19) Nevertheless, the memory-enhancing effect of ginsenoside Rh2 has not been thoroughly studied.

In the present study, the memory-enhancing effect of RGB was found to be more potent than that of red ginseng. Ginsenoside Rh2 exhibited more potent memory-enhancing effects than ginsenoside Rg3 or Rb1. This may have been attributable to differences in their absorption capacities. The ginsenoside Rh2 not only showed potent memory-enhancing effects by passive avoidance, Y-maze and Morris water maze tests, but also recovered scopolamine-induced reduction of LTP in the hippocampal CA1 area. This indicated that muscarnic ACh receptors were closely related to the induction of hippocampal LTP, as reported previously.34

Based on these findings, RGB and its main constituent, ginsenoside Rh2, might improve learning deficits. Also the memory-enhancing effects of RGB may be dependent on the content of ginsenoside Rh2.

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