

# Anti-Amnesic Effect of Fermented *Ganoderma lucidum* Water Extracts by Lactic Acid Bacteria on Scopolamine-Induced Memory Impairment in Rats

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**ABSTRACT:** This study investigated the anti-amnesic effect of fermented *Ganoderma lucidum* water extracts (GW) on scopolamine-induced memory impairment in rats. GW were fermented by the lactic acid bacterium *Bifidobacterium bifidum* (FGWB), followed by *Lactobacillus sakei* LI033 (FGWBL). To induce amnesia, scopolamine (1 mg/kg) was intraperitoneally injected into rats 30 min before the behavioral tests. Step-through latencies of rats treated with primary fermented extracts (300 mg/kg, FGWB) and secondary fermented extracts (300 mg/kg, FGWBL) were significantly longer than those of rats treated with GW (300 mg/kg) in the retention trial of the multiple trial passive avoidance test. In the Morris water maze task, FGWBL significantly shortened escape latencies in training trials. Furthermore, swimming times within the target zone during the probe trial with FGWBL were significantly higher than the GW and FGWB treatments. In addition, acetylcholinesterase activities were lower in the brains of scopolamine-treated rats treated with FGWBL. These results suggest that FGWBL could be useful to enhance learning memory and cognitive function via cholinergic dysfunction.

**Keywords:** *Ganoderma lucidum*, fermentation, scopolamine, passive avoidance test, Morris water maze test

## INTRODUCTION

Geriatric diseases have recently attracted much attention due to the rapid aging of society, development of medical technology, and improvement of living standards (1). In particular, greater emphasis is now placed on preventing cognitive impairment, which is the initial symptom of dementia and memory impairment due to aging (2). Geriatric diseases refer to cerebral or spinal disorders caused by abnormal neuronal death and are accompanied by impaired cognitive, walking, and motor abilities (3). Lack of acetylcholine due to reduction of hippocampal cholinergic neuronal activity is one of the most important causes of memory impairment (4). Further, although there are many choline acetyltransferase agonists and acetylcholinesterase (AChE) inhibitors for memory enhancement, they are ineffective and controversial due to their serious side effects and toxicity. For this reason, research has tried to identify memory enhancers from natural materials (5,6).

Scopolamine is a muscarinic receptor antagonist that is

frequently used in memory-impaired animals. It inhibits connections between acetylcholine and muscarinic receptors, thereby temporarily blocking information transmission and causing learning and memory impairment (7).

*Ganoderma lucidum* has been used in traditional medicine in Korea, China, Japan, and other countries (8). It contains many physiological active substances, including nucleosides, steroids, alkaloids, proteins, amino acids, minerals, polysaccharides, and triterpenes, which are known as physiologically active substances. Of these, ganoderic acid is rarely generated from the liquid-cultured mycelium of *G. lucidum* and is instead concentrated on the surfaces of fruit bodies (9). In addition, *G. lucidum* has anti-tumor, anti-cancer, anti-inflammatory, anti-virus, anti-oxidative, hepatoprotective, and neuroprotective activities, and it has been shown to improve immunity and memory (10-14).

As traditional fermented foods have been found to possess various health effects, researchers have made efforts to confirm the various physiological activities of

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natural extracts fermented by lactic acid bacteria. These beneficial activities include control of intestinal pathogenic bacteria and intestinal regulation, anti-cancer activity, immune system stimulation, and lowering of blood cholesterol (15,16). In particular, fermentation by lactic acid bacteria can improve taste, flavor, texture, storability, safety, and physiological activity by improving digestion efficiency and synergy between natural substances and lactic acid bacteria (17). However, few studies have analyzed the effects of *G. lucidum* water extracts fermented by lactic acid bacteria in terms of cognitive ability and memory.

This study examined fermentation by lactic acid bacteria as a method to improve the memory-enhancing effects as well as maximize the efficacy of *G. lucidum* water extracts by two-step fermentation with *Bifidobacterium bifidum* and *Lactobacillus sakei*. Morris water maze test and passive avoidance test were performed using scopolamine-induced memory-impaired animals, and behavioral changes and hippocampal AChE activities were measured.

## MATERIALS AND METHODS

### Fermentation of *G. lucidum*

Fermentation of *G. lucidum* was carried out according to the method described by Yang et al. (18). Briefly, water extract of *G. lucidum* (GW) was prepared by soaking 100 g of dry-sliced *G. lucidum* in 2 L of water at 80°C for 3 h. After centrifugation at 15,000 g for 10 min (Beckman Coulter, Fullerton, CA, USA), GW was collected for further examination. GW was adjusted to 1°Brix using a brix meter (Kyoto Electronics Manufacturing, Tokyo, Japan) and then sterilized by autoclaving at 121°C for 15 min (Ilshin Bio Base, Gyeonggi, Korea). Sterilized GW was fermented under an anaerobic system using 2% (v/v) *B. bifidum* KCCM 12096 (Korean Culture Center of Microorganisms, Seoul, Korea) at 37°C for 72 h. Fermented GW was sterilized by autoclaving at 121°C for 15 min (FGWB), followed by fermentation with 2% (v/v) *L. sakei* LI033, which was previously isolated from *kimchi* (19) at 37°C for 24 h. Secondary fermented GW was sterilized by autoclaving at 121°C for 15 min (FGWBL). After centrifugation at 15,000 g for 10 min, the supernatant from each sample was freeze-dried and stored at -20°C prior to use.

### Animals

Male Sprague-Dawley rats weighing 200~250 g each (age: 6 weeks) were purchased from Central Lab. Animal Inc. (Seoul, Korea). The experimental procedure was conducted in compliance with the institutional guidelines of Jeonnam Institute of Natural Resources Research for the care and use of laboratory animals. Rats were housed at 3 or 4 per cage, allowed access to water and food *ad libitum*, and maintained at an ambient temperature of 22±3°C under 50±20% humidity and a 12-h diurnal light cycle (lights on 08:00~20:00 h) prior to testing. The rats were habituated for 6 days before drug administration. All behavioral experiments were carried out in a room adjacent to the housing room under the same ambient conditions.

### Sample group and drug administration

Male Sprague-Dawley rats (200~250 g) were randomly assigned to six groups (seven rats per group): control group; scopolamine group, scopolamine-treated (1 mg/kg) group; donepezil group, donepezil (1 mg/kg) and scopolamine (1 mg/kg) co-treated groups; GW group, GW (100 and 300 mg/kg) and scopolamine (1 mg/kg) co-treated groups; FGWB group, FGWB (100 and 300 mg/kg) and scopolamine (1 mg/kg) co-treated groups; FGWBL group, FGWBL (100 and 300 mg/kg) and scopolamine (1 mg/kg) co-treated groups. After 15 days, rats were orally treated with GW, FGWB, and FGWBL (100 and 300 mg/kg). After 16 days, rats were orally treated with GW, FGWB, FGWBL, or donepezil (1 mg/kg). To induce amnesia, scopolamine (1 mg/kg) was intraperitoneally injected into rats 30 min before starting behavioral tests (Fig. 1). Scopolamine and donepezil were obtained from Sigma-Aldrich (St. Louis, MO, USA). All the reagents used in the present study were of analytical grade.

### Morris water maze test

The Morris water maze (20) involved a circular pool (180 cm in diameter and 75 cm in height) with a featureless inner surface. The pool was filled with water maintained at 21±2°C. The tank was placed in a dimly lit, sound proof test room with various visual cues. The pool was conceptually divided into quadrants. A hidden escape platform was placed into one of the pool quadrants and submerged to 1 cm below the water surface, so it was not visible at water level. Over the next 5 days,

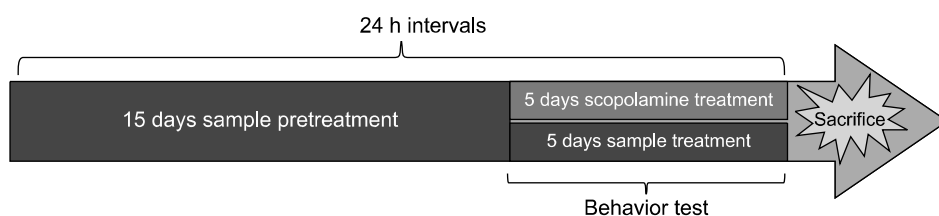


Fig. 1. Scheme of experimental schedule.

rats underwent three trials per day with the platform in place. For each training trial, rats were placed in the water facing the pool wall in each pool quadrant in a different order each day. When a rat located the platform, it was permitted to remain on the platform for 20 s. If the rat did not locate the platform within 45 s, it was placed on the platform for 20 s. The animal was taken to its home cage and then allowed to dry under an infrared lamp after each trial. During each trial, the time required to find the hidden platform (latency) was recorded using a video tracking system (Panlab, Barcelona, Spain). Immediately after the last training session, the rats were subjected to a probe trial session in which the platform was removed from the pool, and the rats were allowed to swim for 90 s to search for it. The swimming time in the pool quadrant where the platform was placed was recorded (21).

#### Passive avoidance test

The passive avoidance test is a well-established experimental procedure used to assess short-term reference memory, which is dependent on cortical and hippocampal circuitries (22,23). The step-through passive avoidance test was performed in identical illuminated and dark chambers (Gemini Avoidance System, San Diego, CA, USA). The illuminated compartment contained a bulb, and the floor of the non-illuminated compartment was composed of stainless steel rods. The two compartments were separated by a guillotine door. For the acquisition trial, the rats were initially placed in the illuminated compartment, after which the door between the two compartments was opened 20 s later. After the rats entered the dark compartment, the door closed automatically, followed by an electrical foot shock (75 V, 0.5 mA, 50 Hz) of 5 s duration delivered through the stainless steel rods. At 8 h after the acquisition trial, the rats were again placed in the illuminated compartment for the retention trials. The time taken for each rat to enter the dark compartment after the door opened was measured as the latency time in both the acquisition and retention trials, with a maximum of 300 s.

#### Rotarod test

The rotarod test was carried out using a rod with a diameter of 8 cm, rotating at a constant speed of 20 rpm (five-lane accelerating rotarod; Jeung DO Bio & Plant, Seoul, Korea). For the training trials, the rats were placed on the rotarod at 20 rpm for about 10 min per day before beginning each experiment. After 2 days, the experiment measured the fall time using the same conditions.

#### Vertical pole test

To assess the equilibrium function, the vertical pole test

was carried out according to the method of Ogawa et al. (24). Rats were placed on a vertical pole and the fall time from the pole was measured. Rats were habituated to the task over two trials per day for 2 days. On test day (third day), three measurements were taken over five trials per rat.

#### AChE activity in brains of rats

Samples of brains were homogenized in 100 mM phosphate buffer and centrifuged at 2,400 g for 15 min at 4°C. AChE activity was measured spectrophotometrically at 410 nm using supernatants according to the method of Ellman et al. (25).

#### Statistical analysis

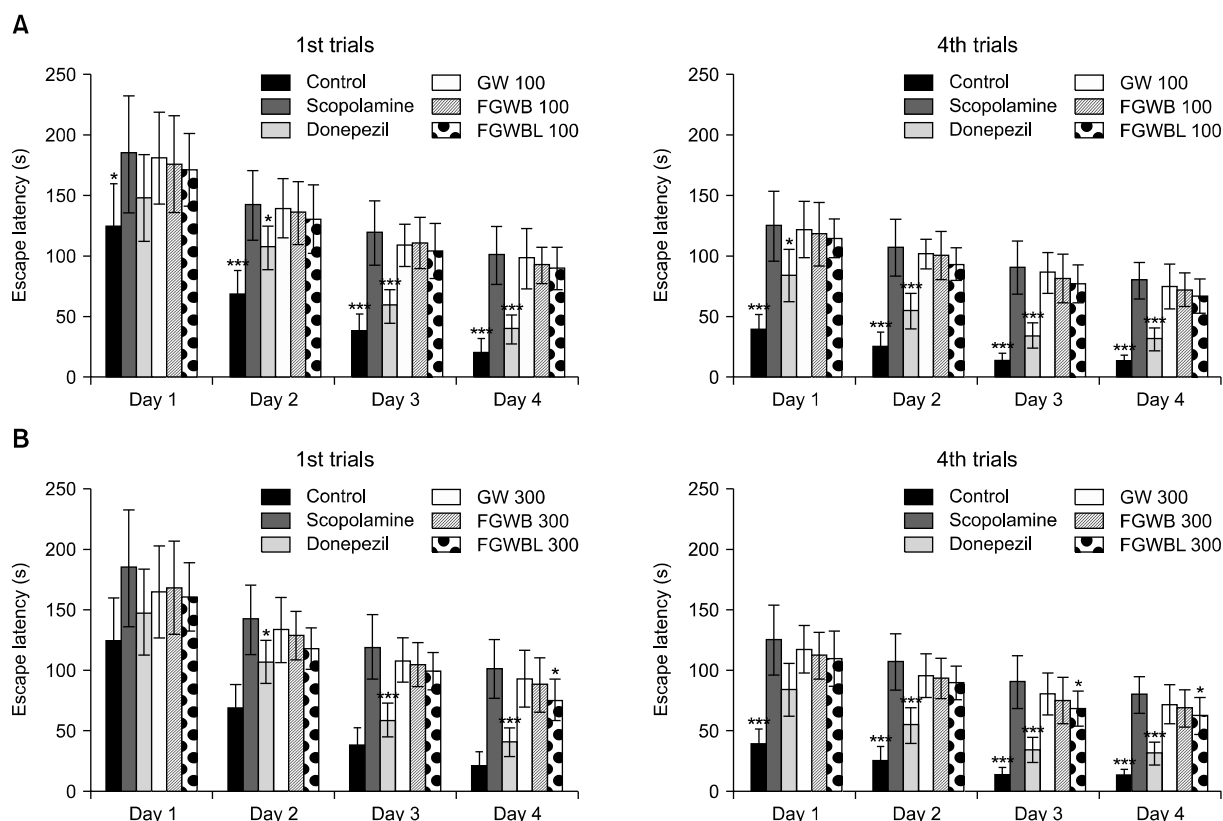
Data were expressed as mean±SD (standard deviation), and statistical significance was analyzed using Student's *t*-test and one-way analysis of variance (ANOVA), followed by the least significant difference (LSD) post hoc test using SPSS for windows version 17.0 (SPSS Inc., Chicago, IL, USA). The values were considered to be significant when *P*-value was <0.05.

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## RESULTS AND DISCUSSION

#### Effectiveness of fermented *G. lucidum* water extracts in recovering memory in the Morris water maze test

The Morris water maze test was performed to determine the efficacy of fermented *G. lucidum* water extracts in improving spatial memory despite scopolamine-induced memory and cognitive impairment (Fig. 2). Of the high-concentration experimental groups (300 mg/kg), the FGWBL group (75.44±16.95 s) showed a significantly reduced escape time compared to the scopolamine group (100.82±24.29 s) (Fig. 2B, 1st trial). For short-term memory, the 300 mg/kg of FGWBL group (61.94±15.09 s) also showed a significantly reduced escape time compared to the scopolamine group (79.89±15.06 s) (Fig. 2B, 4th trial). After 5 days of training, the escape platform was removed and the probe test was performed to measure swimming time in the quadrant where the platform was placed. The scopolamine group (19.53±5.82 s) was incapable of normal learning since scopolamine administration made it difficult for the rats to remember in which quadrant the platform was placed, resulting in significantly reduced swimming time compared to the control group (48.67±5.58 s) (Fig. 3). In contrast, the 300 mg/kg of GW group (24.79±4.82 s), FGWB group (23.53±4.53 s), and FGWBL group (27.62±6.06 s) showed significantly increased swimming times in the target quadrant compared to the scopolamine group. These results suggest that fermentation of *G. lucidum* increased cognitive enhancing activity.



**Fig. 2.** Effects of GW, FGWB, and FGWBL on scopolamine-induced memory impairment in the Morris water maze test. (A) the effects of the low-concentration experimental groups (100 mg/kg) on scopolamine-impaired memory in rats on the first and fourth trials of the Morris water maze test over 4 days. (B) the effects of the high-concentration experimental groups (300 mg/kg) on scopolamine-impaired memory in rats on the first and fourth trials of the Morris water maze test over 4 days. Data are expressed as the mean $\pm$ SD (n=7). \*\*\* $P$ <0.001, \*\* $P$ <0.01, \* $P$ <0.05, significantly different from the scopolamine group. Statistical significance was tested by unpaired Student's  $t$ -test. Scopolamine, scopolamine group; Donepezil, scopolamine plus donepezil group; GW, scopolamine plus *G. lucidum* water extracts group; FGWB, scopolamine plus GW fermented with *B. bifidum*; FGWBL, scopolamine plus GW fermented with *B. bifidum* and *L. sakei* LI033.

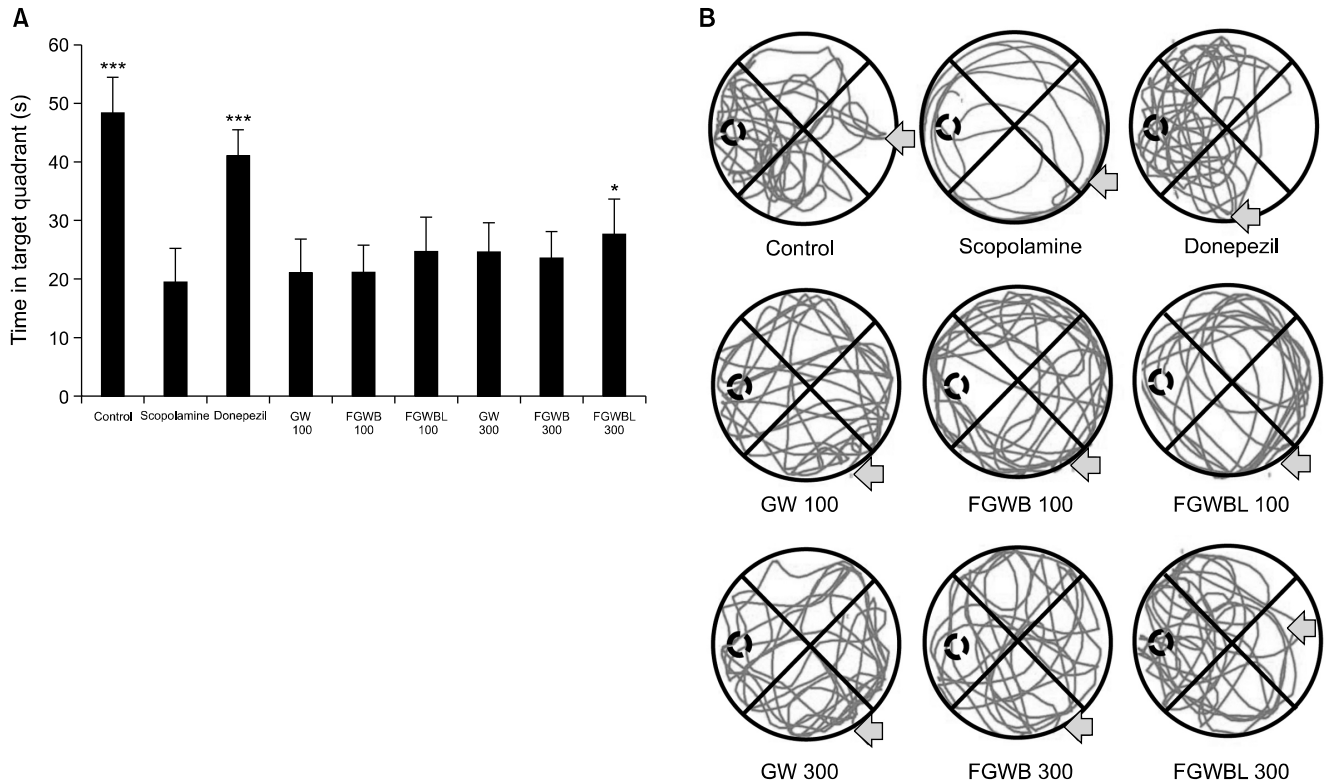
### Effectiveness of fermented *G. lucidum* water extracts in recovering memory in the passive avoidance test

The passive avoidance test was performed to determine the efficacy of fermented *G. lucidum* water extracts in improving short-term memory despite scopolamine-induced memory and cognitive impairment (Fig. 4). The scopolamine group (131.39 $\pm$ 41.57 s) showed reduced learning and memory and stayed in the bright space for a significantly shorter time period compared to the control group (295.29 $\pm$ 50.28 s). In contrast, the 300 mg/kg of GW group (167.73 $\pm$ 28.50 s), FGWB group (174.63 $\pm$ 22.38 s), and 300 mg/kg of FGWBL group (177.73 $\pm$ 27.50 s) stayed in the bright space for significantly longer time periods compared to the scopolamine group, suggesting improvement of scopolamine-induced memory and learning. Yoo et al. (26) previously used a senescence-accelerated mouse model to investigate the efficacy of *G. lucidum* water extracts in improving memory and oxidative stress. Yuan et al. (27) used an animal model of Alzheimer's disease to examine the efficacy of *G. lucidum* polysaccharides in improving memory and spatial recognition ability. Their results showed that *G. lucidum* water extracts can be used as functional in-

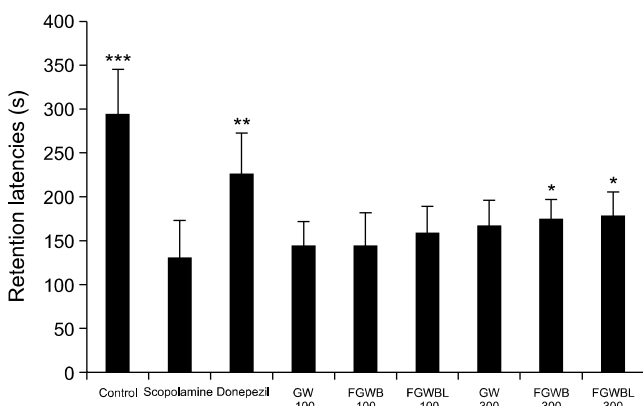
gredients to improve memory. In this study, FGWB was secondarily fermented with lactic acid bacteria separated from *kimchi* in Imsil in order to resolve polymer polysaccharides from *G. lucidum* water extracts into less differentiated polysaccharides and obtain functional ingredients, including polysaccharides, triterpenes, alkaloid, and diverse vitamins (28). Therefore, FGWB and FGWBL improve scopolamine-induced memory and cognitive function by preventing brain cell death or by stimulating secretion of memory-related neurotransmitters such as acetylcholine and glutamate.

### Effectiveness of fermented *G. lucidum* water extracts in improving motor ability deficit

The rotarod test and the vertical pole test were carried out to determine the efficacy of fermented *G. lucidum* water extracts in improving scopolamine-induced motor coordination and behavioral disorders (Fig. 5). Most degenerative diseases such as memory impairment and dementia are accompanied by reduction of motor ability, including sense of balance and coordination (29). The rotarod test measures the animal's ability to maintain its balance on a rolling cylinder, and it is widely used to as-



**Fig. 3.** Effects of GW, FGWB, and FGWBL on scopolamine-induced impairment of memory acquisition and retention in the probe trials. (A) effects of GW, FGWB, and FGWBL on probe trial sessions of the Morris water maze test. Cumulative time in the target quadrant of the pool in the 90 s probe trial is shown. (B) typical trace of swimming patterns on probe trial sessions of the Morris water maze test. Data are expressed as the mean±SD (n=7). \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , \* $P < 0.05$ , significantly different from the scopolamine-treated group. Statistical significance was tested by unpaired Student's  $t$ -test. Groups are the same as in Fig. 2.



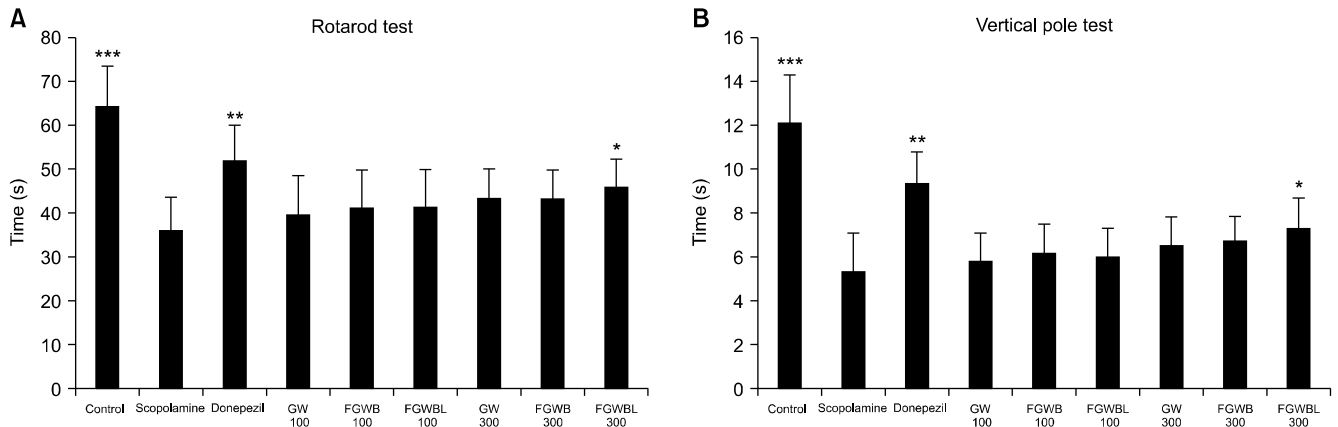
**Fig. 4.** Effects of GW, FGWB, and FGWBL on scopolamine-induced memory impairment in the passive avoidance test. Data are expressed as the mean±SD (n=7). \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , \* $P < 0.05$ , significantly different from the scopolamine-treated group. Statistical significance was tested by unpaired Student's  $t$ -test. Groups are the same as in Fig. 2.

sess motor ability of an animal with degenerative brain disease (30). In the rotarod test, the scopolamine group stayed on the cylinder for a significantly shorter period of time ( $54.65 \pm 6.18$  s) than the control group ( $65.27 \pm 5.64$  s) (Fig. 5A). In contrast, the 300 mg/kg of GW group ( $43.31 \pm 6.61$  s), FGWB group ( $43.15 \pm 6.27$  s), and FGWBL group ( $45.99 \pm 6.20$  s) stayed on the cylinder for significantly longer time periods than the scopolamine

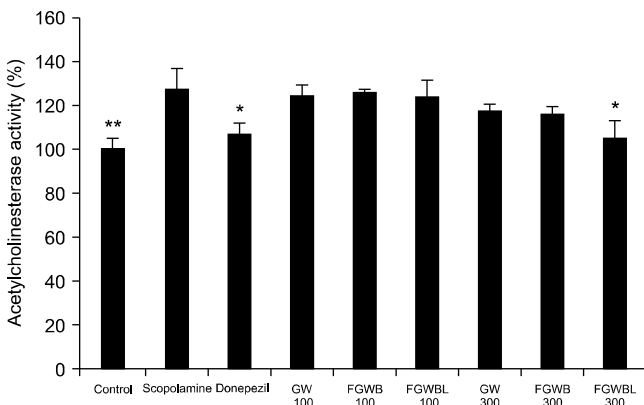
group. In the vertical pole test for measuring grip and motor coordination ability, the length of time on a slanted pole was significantly shorter in the scopolamine group ( $5.35 \pm 1.73$  s) but significantly longer in the 300 mg/kg of GW group ( $6.53 \pm 1.29$  s), FGWB group ( $6.72 \pm 1.09$  s), and FGWBL group ( $7.34 \pm 1.31$  s) compared to the control group ( $12.13 \pm 2.27$  s) (Fig. 5B). In particular, oil from *G. lucidum* spores administered to an animal with Parkinson's disease accompanied by brain neuronal death was reportedly effective in improving motor ability and neuroprotective activity (31). Based on these results, FGWBL administration can be effective in recovering sense of balance, coordination, and grip strength in an animal with scopolamine-induced memory and cognitive impairment.

**Effects of fermented *G. lucidum* water extracts on AChE activity in brain tissues**

Lack of acetylcholine due to malfunction of cholinergic nervous system is known as one of the most important causes of memory impairment. Dementia patients with neuronal damage generate only a small amount of acetylcholine even though AChE, which helps break down acetylcholine, keeps functioning. This results in abnormal neurotransmission and pathological phenomena, such as learning disorders, memory deficits, and cogni-



**Fig. 5.** Effects of GW, FGWB, and FGWBL on scopolamine-induced behavioral deficits. (A) effects of GW, FGWB, and FGWBL on scopolamine-induced motor coordination and balance deficits in the rotarod test. (B) effects of GW, FGWB, and FGWBL on scopolamine-induced sensorimotor deficits in the vertical pole test. Data are expressed as the mean $\pm$ SD (n=7). \*\*\* $P$ <0.001, \*\* $P$ <0.01, \* $P$ <0.05 significantly different from scopolamine group. Statistical significance was tested by unpaired Student's  $t$ -test. Groups are the same as in Fig. 2.



**Fig. 6.** Effects of GW, FGWB, and FGWBL on AChE activity in scopolamine-injected rats. Data are expressed as the mean $\pm$ SD (n=3). \*\*\* $P$ <0.001, \*\* $P$ <0.01, \* $P$ <0.05, significantly different from the scopolamine-treated group. Statistical significance was tested by unpaired Student's  $t$ -test. Groups are the same as in Fig. 2.

tive impairment (32). Research is currently being conducted on natural materials capable of AChE inhibition and improving cognitive functions (33,34). When AChE activity in brain tissues was examined to determine the efficacy of fermented *G. lucidum* water extracts in improving memory despite scopolamine-induced memory and cognitive impairment (Fig. 6), the scopolamine group showed significantly increased AChE activity. In contrast, the GW, FGWB, and FGWBL groups (300 mg/kg) showed reduced AChE activities in brain tissues. In particular, the FGWBL group showed significantly reduced AChE activity compared to the scopolamine group. *G. lucidum* contains numerous physiologically active substances such as polysaccharides, triterpenes, nucleoside, steroids, and alkaloids. Lee et al. (35) reported that lanostane triterpenes separated from fruit bodies of *G. lucidum* are excellent inhibitors of AChE. In particular, *G. lucidum* triterpenoids were shown to improve learning

and memory dysfunction in a rat model of Alzheimer's disease by increasing the acetylcholine content in the brain (36). Further, *G. lucidum* water extracts inhibit AChE activity in brain tissues and thus prevent reduction of acetylcholine levels, resulting in the protection of brain tissues from cerebral ischemia, vascular dementia, as well as Alzheimer's dementia (37). Therefore, triterpenes and many other useful substances contained in *G. lucidum* water extracts fermented by lactic acid bacteria can be effective in inhibiting AChE activity in brain tissues via the cholinergic nervous system in scopolamine-induced rats. Further research is needed to determine the exact mechanism of AChE inhibition.

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## AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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