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## Garlic Gongjin-Dan Ameliorates Scopolamine-Induced AMNESIA in Mice

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### Abstract

*Gongjin-Dan is a traditional oriental medicine herbal drug that has been used tonics. In the present study, we investigated the effects of the hot water extract of Gongjin-Dan mixed garlic(GGD) against scopolamine-induced memory impairment and its mechanism of action. We used the Y-maze and passive avoidance tests to assess anti-amnesic activities in scopolamine-induced memory impairment mouse model. In this model, GGD(12.5, 25 or 50 mg/kg) significantly ameliorated scopolamine-induced memory impairment. We also explored its mechanism of action by conducting an antioxidant activity assay using 2,2-diphenyl-1-picrylhydrazyl(DPPH) and 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) free and acetylcholinesterase(AChE) activity assay using the mouse whole brain. GGD increased the scavenging activities of free radical DPPH and ABTS, inhibited AChE activity in the brain. Taken together, our findings suggest that GGD exerts an anti-amnesic effect through the free radical scavenging, AChE inhibition, ameliorate of scopolamine-induced memory impairment. The effect of GGD may be useful for treatment of memory impairment in Alzheimer's and its related diseases.*

**[Keywords]** Sport, Garlic Gongjin-Dan, Scopolamine, Memory Impairment, Alzheimer's Diseases

### 1. Introduction

Alzheimer's disease(AD) is a progressive neurodegenerative disorder of the central nervous system associated with cognitive impairment. The pathophysiology of AD includes senile plaques, neurofibrillary tangles[1] and amyloid beta protein deposition[2], increased oxidative stress[3] and neuroinflammation[4], and reduced acetylcholine levels[5]. Many researches have reported that AD patients have severe cholinergic dysfunction in the brain[6], and scopolamine as a muscarinic cholinergic receptor antagonist has been shown to evaluate memory impairments in experimental animals[7][8][9]. Indeed, after administration of scopolamine, the oxidative stress status was altered and impaired cognitive performance on behavioral tests[10][11]. Until now, AD treatment drugs have been used AChE inhibitors such as

tacrine and donepezil(DNPZ)[9]. Nevertheless, there is a growing need for development of new cognitive augmenting agents because of the disadvantages such as gastrointestinal symptoms[12], hepatotoxicity[13] and cardiovascular adverse effect[14].

Garlic Gongjin-Dan(GGD) is the mixture of Gongjin-Dan and aged black garlic extract. Gongjin-Dan is a traditional multi-herb formula that has been used to treat symptoms caused by weak constitution and aged population in Korea and China. A traditional medical books, Dongeuibogam, Donguisusebowon and Bangyakhapyun, documented that Gongjin-Dan can maintain body homeostasis. Many studies have been conducted on the pharmacological effects of Gongjin-Dan, such as anti-amnesic effect[15], anti-fatigue effect[16][17] and immune modulation[18]. Garlic has been used widely medicinal herb

and culinary seasoning in Asia and America. Despite its beneficial effects, raw garlic has unpleasant odour, acrid taste and adverse effects, such as gastrointestinal disorder[19] and hemolytic anemia[20]. Heating treatment has made black garlic to improve the flavor and quality, and further newly form bioactive compounds[21]. Many studies have been reported cardioprotective[22], antioxidant[23] and anti-lipogenic effect[24].

Based on these reports, we hypothesized that GGD might also have memory ameliorating effect. In the present study, we investigated the effects of GGD on scopolamine-induced memory impairment mice. Furthermore, we measured the free radical scavenging and acetylcholinesterase(AChE) activity to identify the mechanism underlying the antioxidant and anti-cholinergic effects of GGD.

## 2. Methods

### 2.1. Preparation

Four medicinal herbs(Angelicae Gigantis Radix, Corni Fructus, Aucklandiae Radix, Ginseng Radix, Rehmanniae Radix Preparata) and one animal-derived material(Cervi Parvum Cornu) were purchased from the Omniherb (Daegu, Korea) and aged black garlic extract was provided from Jeju bio farm(Jeju, Korea). GGD(40 g) was extracted with 1 L distilled water for 2 hours at 100°C, and then their residue was filtered through Whatman No.2 filter paper(Whatman Ltd., England). The extracts were concentrated using a rotary evaporator under vacuum condition, and the residual crude extracts were freeze-dried at -80°C. The GGD were stored at -20°C during test. The yield was 39%.

**Table 1.** Composition of Garlic Gongjin-Dan.

Name	Ratio (g)
Cervi Parvum Cornu	15g
Angelicae Gigantis Radix	15g
Corni Fructus	15g
Aucklandiae Radix	2g
Ginseng Radix	15g
Rehmanniae Radix Preparata	15g
Aged Black Garlic Extract	90g

### 2.2. 2,2-Diphenyl-1-picrylhydrazyl(DPPH) radical scavenging activity

DPPH free radical scavenging activity was measured according to the method described by Brand-Williams and coworkers[25] with slight modifications. DPPH solution(0.2 mM in ethanol) and sample were prepared in the concentration range of 1 mg/mL and serial diluted in distilled water. Sample solution(100 µL) at various concentrations was mixed with 100 µL of DPPH solution and incubated for 30 min in the dark at room temperature. Absorbance was determined at 570 nm using microplate reader(Tecan, Austria) with ascorbic acid as a positive control. Measurements

were carried out in triplicate. Radical scavenging activity was calculated as follows:

Radical scavenging activity(%)=1-Absorbance of sample/Absorbance of control×100DPPH solution(0.2 mM in ethanol) and sample were prepared in the concentration range of 1 mg/mL and serial diluted in distilled water. Sample solution(100 µL) at various concentrations was mixed with 100 µL of DPPH solution and incubated for 30 min in the dark at room temperature. Absorbance was determined at 570 nm using microplate reader(Tecan, Austria) with ascorbic acid as a positive control. Measurements were carried out in triplicate. Radical scavenging activity

was calculated as follows:

Radical scavenging activity(%)=1-Absorbance of sample/Absorbance of control×100

### 2.3. 2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity

ABTS free radical scavenging activity was measured according to the method described by Re and coworkers[26] with slight modifications. The ABTS radical cations were produced by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate in the dark at room temperature for 16 h. The ABTS radical solution was diluted by ethanol to an absorbance of  $0.7 \pm 0.02$ . Sample solution (100  $\mu$ L) at various concentrations was mixed with 100  $\mu$ L of ABTS radical solution and incubated for 7 min in the dark at room temperature. Absorbance was determined at 734 nm using microplate reader(Tecan, Austria) with ascorbic acid as a positive control. Measurements were carried out in triplicate. Radical scavenging activity was calculated as follows:

Radical scavenging activity (%)=1-Absorbance of sample/Absorbance of control×100

### 2.4. Animals

Male ICR mice(21-23 g) were purchased from Daehan Biolink(Eumseong, Korea) and housed individually in the home cages in a controlled room temperature( $22 \pm 2^\circ\text{C}$ ) and humidity( $50 \pm 5\%$ ) with a 12 h light-dark cycle (lights on at 7 a.m.) and allowed standard food pellets and tap water ad libitum. After acclimatization for 1 week, the mice were randomly divided into seven groups(n =7-8/group): (1)normal group(saline, p.o.); (2) control group[scopolamine(1 mg/kg, i.p.) treatment plus saline, p.o.]; (3)scopolamine + GGD 6.25 mg/kg group [scopolamine treatment plus GGD(6.25 mg/kg, p.o.) treatment group]; (4)scopolamine + GGD 12.5 mg/kg group[scopolamine treatment plus GGD(12.5 mg/kg, p.o.) treatment group]; (5)scopolamine + GGD 25 mg/kg group[scopolamine

treatment plus GGD(25 mg/kg, p.o.) treatment group]; (6)scopolamine + GGD 50 mg/kg group[scopolamine treatment plus GGD (50 mg/kg, p.o.) treatment group]; (7)scopolamine + donepezil group [scopolamine treatment plus donepezil(5 mg/kg, p.o.) treatment group]. Donepezil was used as a positive control. Mice were administered GGD, donepezil or the same volume of saline 1 h before the experiment, and administered of scopolamine or saline 30 min after each drug administration. This protocols were approved by the Institutional Animal Care and Use Committee of Daegu Haany University(Approval number: DHU2018-040).

### 2.5. Y-maze test

This test was assessed to examine the effects of GGD on short-term and working memory. The Y-maze equipment has three arms(40 cm long×3 cm wide×12 cm high) which are disposed at  $120^\circ$  angles from each other(labeled A, B, and C). The mice were individually placed at the end of one arm, and the test period was 8 min. During the period, the sequence(e.g., ABCCAB) and number of arm entries for each mouse was manually recorded. An actual alternation was considered as entries into all three arms with consecutive choices(e.g., ABC, BCA, or CAB, but not ABA). The percentage of alternation(%) for each mouse was calculated as the ratio of the actual-to-possible alternations, as shown in following equation: percentage alternation=(number of alterations)/(total arm entries-2)×100. The total number of arm entries was used as an indicator of locomotor activity. Each arm was thoroughly cleaned with ethanol between each test to remove odors and residues.

### 2.6. Passive avoidance test

The test was used to measure the effects of GGD on long-term and learning memory. The passive avoidance apparatus consists of two compartments, one lighted and one dark were separated by a guillotine door. The two

chambers were equipped with stainless steel bars for electric shock. During the acquisition trial, test mice were gently placed in the light chamber, and the guillotine door was opened 10 sec later and latency to enter the dark chamber was recorded as step-through latency. After the mice entering the dark chamber, the door was closed and an electric foot shock(0.5 mA, 3 sec) was applied. The retention trial was conducted 24h after the acquisition trial by returning the mouse to the light chamber, and the time to enter the dark chamber after door opening was manually recorded again without electric foot shock for 300 sec as cut-off.

### 2.7. Acetylcholinesterase activity

The AchE activity was determined on the basis of degradation of acetylthiocholine iodide into a product that binds to 5,5-dithio-bis-2-nitrobenzoic acid(DTNB) and turns yellow using the acetylthiocholine iodide-based colorimetric method according to the method of Ellman et al.[27] with some modifications. Sample and donepezil were dissolved and serial dilute. Mouse whole brain was homogenized in the 100 mM phosphate buffer(pH 8.0) using homogenizer and centrifuged at 12,500 rpm for 20 min at 4°C. The supernatant was collected and used as an enzyme source. Phosphate buffer(144  $\mu$ L, 100 mM, pH 8.0) was mixed with 22  $\mu$ L of buffered Ellman's reagent(10 mM DTNB and 15 mM sodium bicarbonate), 1.1  $\mu$ L substrate(75 mM acetylthiocholine iodide solution), 10  $\mu$ L sample solution and enzyme source at room temperature for 10 min. Thereafter, the mixture was stopped by adding 4.4  $\mu$ L of a neostigmine solution(100 mM). The AchE activity was measured at a wavelength of 405 nm using microplate reader(Tecan, Austria).

### 2.8. Statistical analysis

All data are expressed as the means $\pm$ standard error of mean(SEM). The statistical significance of the differences between the groups

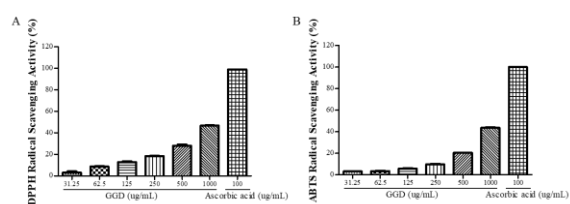
were analyzed using one-way analysis of variance(ANOVA) followed by Newman-Keuls test in GraphPad Prism(version 5.03). Differences were considered statistically significant if p value less than 0.05.

## 3. Results

### 3.1. Effects of GGD on the antioxidant activity

Antioxidant activity of GGD was evaluated by free radical scavenging assay <Figure 1>. DPPH free radical scavenging of GGD was 3.0 $\pm$ 1.2, 8.7 $\pm$ 0.3, 12.7 $\pm$ 0.7, 18.3 $\pm$ 0.3, 28.0 $\pm$ 1.0, 46.7 $\pm$ 0.3 at a concentration range of 31.25, 62.5, 125, 250, 500 and 1000  $\mu$ g/mL <Figure 1A>. Ascorbic acid showed 99.0 $\pm$ 0.0 inhibition at 100  $\mu$ g/mL against DPPH. ABTS free radical scavenging of GGD was 3.0 $\pm$ 0.0, 3.3 $\pm$ 0.3, 5.7 $\pm$ 0.3, 9.7 $\pm$ 0.3, 20.3 $\pm$ 0.3, 43.7 $\pm$ 0.3 at a concentration range of 31.25, 62.5, 125, 250, 500 and 1000  $\mu$ g/mL <Figure 1B>. Ascorbic acid showed 100.0 $\pm$ 0.0 inhibition at 100  $\mu$ g/mL against ABTS. GGD was revealed concentration dependent response. Ascorbic acid was used as a positive control.

**Figure 1.** Effects of the GGD on antioxidant activity.



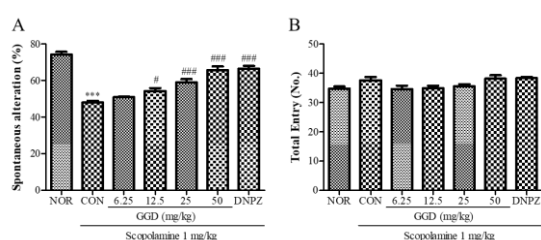
In vitro antioxidant activity assay was performed as described in the Methods section. Antioxidant activity of GGD was assessed by a free radical scavenging assay for DPPH(A) and ABTS(B). Ascorbic acid was used as a positive control. Data represent the mean  $\pm$  SEM of three independent experiments.

### 3.2. Effects of GGD on the Y-maze test

The results of the Y-maze test are shown in <Figure 2>. Scopolamine administration significantly reduced spontaneous alterations

compared with the normal group ( $p < 0.001$ ). This alternation was significantly recovered by GGD (12.5, 25, and 50 mg/kg, p.o.) and donepezil (5 mg/kg, p.o.) treatments compared with the control group <Figure 2A>. The difference in the total numbers of arm entries among all the treatment groups was not significant <Figure 2B>. This result implies that GGD did not affect the locomotor activity in mice.

**Figure 2.** Effects of GGD on scopolamine-induced memory deficits in Y-maze test in mice.

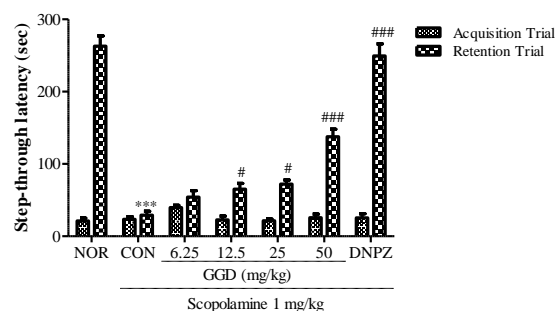


The results from the spontaneous alternation (A) and total entry (B) are presented. GGD or donepezil (DNPZ; 5 mg/kg, p.o.) as positive control were orally administered to mice 1 h prior to the Y-maze tests. After 30 min, the mice were injected with 1 mg/kg scopolamine (i.p.) and tested in the Y-maze. Data represent the mean  $\pm$  SEM. ( $n = 7-8$ /group) (\*\* $p < 0.001$  vs. the NOR group; # $p < 0.05$ , ### $p < 0.001$  vs. CON group).

### 3.3. Effects of GGD on the passive avoidance test

The results of the passive avoidance test are shown in Fig.3. Scopolamine administration significantly shortened the step-through latency compared with the normal group ( $p < 0.001$ ). GGD (12.5, 25 and 50 mg/kg, p.o.) and donepezil (5 mg/kg, p.o.) treatments significantly recovered the control group. There was no difference in step-through latency among the all groups during the acquisition trial.

**Figure 3.** Effects of the GGD on scopolamine-induced memory deficits in the passive avoidance test in mice.

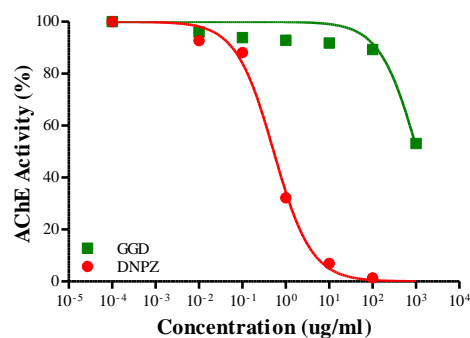


Results from the acquisition trial and retention trial are presented. GGD or donepezil (DNPZ; 5 mg/kg, p.o.) as positive control were orally administered to mice 1 h prior to the passive avoidance tests. After 30 min, the mice were injected with 1 mg/kg scopolamine (i.p.) and tested in the passive avoidance. Normal group (NOR) received vehicle solution (0.9% saline). Donepezil (DNPZ) was used as a positive control. Data represent the mean  $\pm$  SEM. ( $n = 7-8$ /group) (\*\* $p < 0.001$  vs. the NOR group; # $p < 0.05$ , ### $p < 0.001$  vs. CON group).

### 3.4. The effect of GGD on AChE activity

AChE inhibition activity of GGD was investigated to cholinergic antagonistic effect in the brain <Figure 4>. GGD has shown concentration dependent manner with an IC<sub>50</sub> value of 1088  $\mu$ g/mL. Donepezil, a positive control, has shown an IC<sub>50</sub> value of 0.5  $\mu$ g/mL.

**Figure 4.** Effects of the GGD on AChE activity. Ex vivo AChE activity assay was performed as described in the methods section.



Donepezil (DNPZ) was used as a positive control. Data represent the mean  $\pm$  SEM of three independent experiments.

## 4. Discussion

The present study provided that GGD, a modified Gongjin-Dan, has the anti-amnesia effects on scopolamine-induced memory impairment in mice. The results demonstrated that GGD administration alleviated the cognitive deficits induced by scopolamine in both Y-maze and passive avoidance tests. And its action may be related to scavenge free radical and inhibited AChE activity.

Oxidative stress is considered to play a significant role in the onset and development of various diseases[28][29]. In AD, oxidative stress is caused to produce neuronal changes in the brain, especially hippocampus[30][31]. In previous studies, scopolamine increased oxidative stress in the hippocampus by a number of mechanisms, including decreasing superoxide dismutase(SOD), glutathione peroxidase(GPX) and catalase(CAT) levels[32][33][34]. GGD increased the radical scavenging activity for DPPH and ABTS in a dose-dependent manner. These results confirmed that GGD had the anti-oxidative effects.

In this study, two types of memory tests were utilized to assess the effects of GGD on scopolamine-induced amnesia in mice. The Y-maze test was carried out to evaluate the hippocampus-dependent short-term and spatial working memory. Our current results demonstrated that scopolamine significantly decreased the percentage of spontaneous alteration in Y-maze test, which was consistent with the previous studies. GGD administration was showed to improve the decreased memory in the scopolamine-treated mice. There were no significant differences in the total number of entries in all groups, which indicated that GGD did not affect the locomotor activity. The passive avoidance test was conducted to evaluate the long-term and learning memory. These results exposed that scopolamine significantly reduced the step-through latency in the retention trial, which

was consistent with the previous studies. GGD administration was revealed to increase the reduced memory in the scopolamine-treated mice. There were no significant differences in the latency in all groups during the acquisition trial, which implied that GGD did not influence the locomotor and exploratory activity. These results of the behavioral studies confirmed that GGD had the anti-amnesia effects against scopolamine-induced memory impairments.

The cholinergic neurotransmitter is known to play the most important role in cognitive function. Acetylcholine is synthesized from choline and acetyl-CoA by choline acetyltransferase, which is in turn catalyzed into acetate and choline by AChE after its release[5]. It is known that scopolamine increased AChE activity leading to memory loss[35]. AChE activity is elucidated the underlying mechanism of effect of GGD. Our present findings showed that GGD reduced the AChE activity in the whole brain. These results confirmed that GGD reduced the AChE activity.

## 5. Conclusion

The present study provides evidence that GGD ameliorates the scopolamine-induced cognitive impairment and memory deficits by scavenging free radicals and inhibiting AChE activity. All these results suggested that GGD may be a potential candidate agent for the treatment of some neurodegenerative diseases such as AD.

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## Research field

- Involvement of Histaminergic System in the Anxiolytic-like Activities of Morus Alba Leaves in Mice, *Biol Pharm Bull*, 36(11) (2013).
- The Enhancing Effect of Aubang Gahl Soo on the Hippocampal Synaptic Plasticity and Memory through Enhancing Cholinergic System in Mice, *Journal of Ethnopharmacology*, 224 (2018).

## Major career

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- Effect of 12-week Senior Qigong Training on Blood Pressure and Blood Lipid Concentration, *International Journal of Sport*, 3(1) (2018).

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- Effect of 12-week Senior Qigong Training on Blood Pressure and Blood Lipid Concentration, *International Journal of Sport*, 3(1) (2018).

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