Prev. Nutr. Food Sci. 2019;24(2):165-170
https://doi.org/10.3746/pnf.2019.24.2.165
pISSN 2287-1098 · eISSN 2287-8602

Effect of Aged Garlic Ethyl Acetate Extract on Oxidative Stress and Cholinergic Function of Scopolamine-Induced Cognitive Impairment in Mice

Fuyi Li and Mee Ree Kim

Department of Food and Nutrition, Chungnam National University, Daejeon 34134, Korea

ABSTRACT: This study was performed to investigate the effect of aged black garlic ethyl acetate extract on scopolamine-induced cognitive impairment in mice. Aged garlic ethyl acetate extract (BG) was administrated at a dose of 25 or 50 mg/kg in scopolamine-induced mice. Cognitive ability was evaluated using a Morris water maze test and a passive avoidance test. BGs (50 mg/kg) shortened the latency time that was increased by scopolamine and increased the platform crossing numbers that was significantly shortened by scopolamine after 5 days training in the Morris water maze test ($P<0.05$). BG (50 mg/kg) also significantly prolonged the latency time in the passive avoidance test ($P<0.05$). Result from biochemical analysis showed that BG increased levels of glutathione, glutathione peroxidase activity, and glutathione reductase activity, whereas BG significantly inhibited lipid peroxidation ($P<0.05$). BG also attenuated cholinergic degradation through inhibiting acetylcholinesterase activity and increasing choline acetyltransferase activity ($P<0.05$). In conclusion, BG protected against scopolamine-induced cognitive impairment through decreasing oxidative damage and regulating cholinergic function in the brains of mice. BG may therefore be a beneficial food for protecting against neurodegeneration such as Alzheimer's disease.

Keywords: aged garlic, cholinergic enzymes, Morris water maze test, antioxidant enzymes

INTRODUCTION

Alzheimer's disease, the most common type of dementia, is associated with synapse lose and neurodegeneration. Alzheimer's disease leads to memory loss and other cognitive impairments, and is characterized by accumulation of the extracellular deposits of β-amyloid (Aβ) deposits, abnormal formation of intracellular neuro fibrillary tangles, and loss of neurons in the hippocampus (Francis et al., 1999). Several clinical drugs against Alzheimer's disease have been developed, such as donepezil, rivastigmine, and galantamine (Mangialasche et al., 2010), however most of these induce side effects such as diarrhea, nausea, and vomiting (Rogers et al., 2010).

Scopolamine, a cholinergic muscarinic antagonist, induces memory dysfunction in young, healthy people that is similar to that observed in Alzheimer's disease (Stone et al., 1988). Recently, a scopolamine-induced memory impairment mouse model was associated with altered brain oxidative stress (Fan et al., 2005). Therefore, scopolamine-induced memory impairment can be used as an animal model to study Alzheimer's disease (Chen et al., 2008).

Garlic (*Allium sativum*) is a vegetable used in daily life and has been used as a medicinal food ingredient since ancient times in China and Korea (Butt et al., 2009). Aged garlic, which has a soft fruity taste, is popular in Korea. Alliin, one of the unstable compounds in fresh garlic is converted into the stable compound *S*-allyl-L-cysteine; *S*-allyl-L-cysteine is a water-soluble compound that may cross the blood brain barrier and protect against Aβ-induced cell neurotoxicity (Gupta et al., 2007). Aged garlic has also been reported to have many biological and pharmacological effects, such as anti-glycation properties (Ahmad and Ahmed, 2006), anti-obesity effects (Kim et al., 2011), antioxidant properties (Lee et al., 2009; Ide et al., 1999; Kim et al., 2012; You et al., 2011), and hypolipidemic effects (Ide et al., 1999; Dillon et al., 2003). Aged black garlic ethyl acetate extracts (BGs) have also been reported to contain higher total phenolic contents than fresh aged garlic and other aged garlic fractions, which are known to exert multiple benefits (El-Sherbiny

Received 27 March 2019; Accepted 2 May 2019; Published online 30 June 2019

Correspondence to Mee Ree Kim, Tel: +82-42-821-6837, E-mail: mrkim@cnu.ac.kr
Author information: Fuyi Li (Graduate Student), Mee Ree Kim (Professor)

et al., 2003). Our study aimed to explore the protective effect of BGs against scopolamine-induced cognitive impairment in mice.

MATERIALS AND METHODS

Animals

4-week old male ICR mice were obtained from Raonbio Company (Yongin, Korea). Mice were maintained at a constant temperature (23.0±2.0°C) and humidity (55±10%), with a 12-h light/dark cycle, and were fed a chow-free diet and water. Mice were weighed once per week. All animal experiments were conducted in compliance with "Guide for Care and Use of laboratory Animals" of the National Institutes of Health Guidelines (Approved No.: CNU-00449).

Preparation of aged garlic extract

Aged black garlic (4 kg) was extracted with 5 L of 80% methanol using ultrasonic agitation. Samples were filtered and the remaining garlic was extracted with 70% methanol; the ethyl acetate layer was obtained by separating the supernatant fraction and was concentrated. The ethyl acetate layer was used in this study.

Treatments

Mice were randomly divided in 6 groups (each of 8 animals), as followed: I, CON (normal, 0.9% saline); II, SCO (scopolamine 2 mg/kg, intraperitoneal injection); III, TAC (scopolamine 2 mg/kg+tacrin 10 mg/kg, orally); IV, BG50 (aged garlic extract 50 mg/kg, orally); V, SBG25 (scopolamine 2 mg/kg+aged garlic extract 25 mg/kg, orally; VI, SBG50 (scopolamine 2 mg/kg+aged garlic extract 50 mg/kg, orally). Scopolamine (scopolamine hydrobromide, Sigma-Aldrich Co., St. Louis, MO, USA) was dissolved in saline at 2 mg/kg and was injected 30 min before the water maze task and the passive avoidance test. Tacrin (9-amino-1,2,3,4-tetrahydroacridine hydrochloride hydrate, Sigma-Aldrich Co.) was dissolved in saline and administrated 1 h before Morris water maze task 30 min before scopolamine injection.

Morris water maze task

The Morris water maze test is a behavioral test used to evaluate the effect of BG on cognitive enhancement. A black circular pool with a diameter of 150 cm and a depth of 60 cm was filled with water at 23±1°C and the pool was split into 4 quadrants. On first day of trial, mice were inserted into one of the water pool guadrants without a platform for 2 min for free swimming as adaptation training. During the next 4 days of training, a platform (10 cm diameter) was placed 1 cm below the water surface in one quadrant of the pool. Mice were inserted into each quadrant of the pool were trained to find the platform; the time to find the platform was recorded. When the mice reached the platform, they could stay for 20 s. If the mice did not find the platform within 120 s, the mice were placed on the platform for 10 s to remember. On the last day of the trial, platform was hidden; the time taken for the mice to first pass the platform and the number of times the mice crossed the platform were recorded. After each trial, mice were dried with a towel. Mice were administrated with scopolamine 30 min before the trail and with tacrin and BG 1 h before the trial.

Passive avoidance test

The passive avoidance box is composed of a light and dark chamber. Mice was first placed into the light chamber for 1 min for adaption with the door shut; the door was then opened and the time taken by the mice to return to the dark chamber was recorded. After mice ventured the dark chamber, they were given an electric shock in the foot of 0.5 mA for 5 s, and the door was shut. Mice were returned to the dark chamber after 300 s if they did not venture back on their own. After 24 h, mice were placed into the light chamber again; the time taken for mice to return to the dark chamber was recorded. Mice were administrated 2 mg/kg scopolamine 30 min before being placed in the light chamber. The passive avoidance was conducted over 2 consecutive days.

Biochemical analyses

***Brain tissue preparation*:** Whole mouse brain was homogenized with 9 volumes of homogenization buffer (12.5 mM sodium phosphate buffer pH 7.0, 400 mM NaCl), and centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was used as source of enzyme for the assay. The supernatant was stored as −70°C before use.

***Acetylcholinesterase (AChE) and choline acetyltransferase (ChAT) activities assays*:** AChE activity assay was performed using an acetylthiocholine iodide substrate based colorimetric method, as described by Ellman method. Briefly, brain supernatant was mixed with 0.1 M phosphate buffer, 10 mM Ellman's reagents, and 75 mM acetylthiochloride iodide, and was measured by spectrophotometer at 410 nm over 5 min with intervals of 1 min. ChAT activity was assayed using a kit (Elabscience Biotechnology Inc., Houston, TX, USA), following the manufacturer's instruction.

***Glutathione (GSH) activity*:** GSH activity was determined by mixing with 0.1 M potassium phosphate buffer, 10 mM 5,5'-dithiobis(2-nitrobenzoic acid), 5 nM nicotinamide adenine dinucleotide phosphate hydrate (NADPH); the reaction was equilibrated for 1 min before 1 unit of glutathione reductase (GR) was added and the absorbance was measured at 412 nm using spectrophotometer. GSH (0.04 mM) was used for the standard curve.

***Glutathione peroxidase (GPx) activity*:** GPx was determined in reacting containing with 0.1 M sodium phosphate buffer, 5 mM NADPH, and 100 mM GSH. One unit of GR and 100 mM cumene-OOH were added for 3 min and the absorbance was measured at 340 nm using a spectrophotometer.

***GR activity*:** GR activity was determined in reactions containing with 0.1 M phosphate buffer, 1.0 M glutathione disulfide, and 5 mM NADPH; the absorbance was measured at 340 nm using a spectrophotometer.

***Lipid peroxidation determination*:** Thiobarbituric acid (TBA) method was carried out in reactions containing 8.1 % sodium dodecyl sulfate, 20% acetic acid, and 0.75% TBA. Reactions were placed in a water bath at 100°C for 30 min, centrifuged at 10,000 rpm for 15 min, and the absorbance of the supernatant measured at 532 nm using a spectrophotometer. Malondialdehyde (MDA) was used for the standard curve.

***Protein concentration*:** Protein concentration were measured using Bradford assays (Bio-Rad Laboratories, Hercules, CA, USA). Mouse brain supernatants were mixed with 1:5 diluted dye reagent, incubated at room temperature for 10 min, and measured the abosorbance at 595 nm using a spectrophotometer within 1 h. Bovine serum albumin (1 mg/mL) was used for the standard curve at 0.2～1.0 mg/mL.

Statistical analysis

Results were expressed as mean±standard error (SE). All statistical analysis was performed using IBM SPSS 24 software (IBM Corp., Armonk, NY, USA). Differences between groups were calculated using one-way ANOVA followed by LSD post hoc tests. $P<0.05$ was used to signify statistically significant differences.

RESULTS

Effect of BG on spatial memory in Morris water maze

The Morris water maze test was performed to determine if aged garlic extracts attenuate spatial learning and memory. Mice in the SCO group showed memory impairment during 5 days of training, whereas mice treated with aged garlic extract for 4 weeks showed enhanced memory impairment during the 5 days of training (Fig. 1A). Mice treated with aged garlic extract (BG50, SBG25, and SBG 50) showed significantly lowered escape latency times compared with mice in the SCO group on the retention day ($P<0.05$); mice receiving aged garlic extracts (SBG 50) also had lowered escaped latency times than those receiving tacrin (Fig. 1B). Mice treated with aged garlic extract (SBG50) also showed enhanced memory impairment, with significantly increased platform crossings compared with those in the SCO group ($P<0.01$) (Fig. 1C).

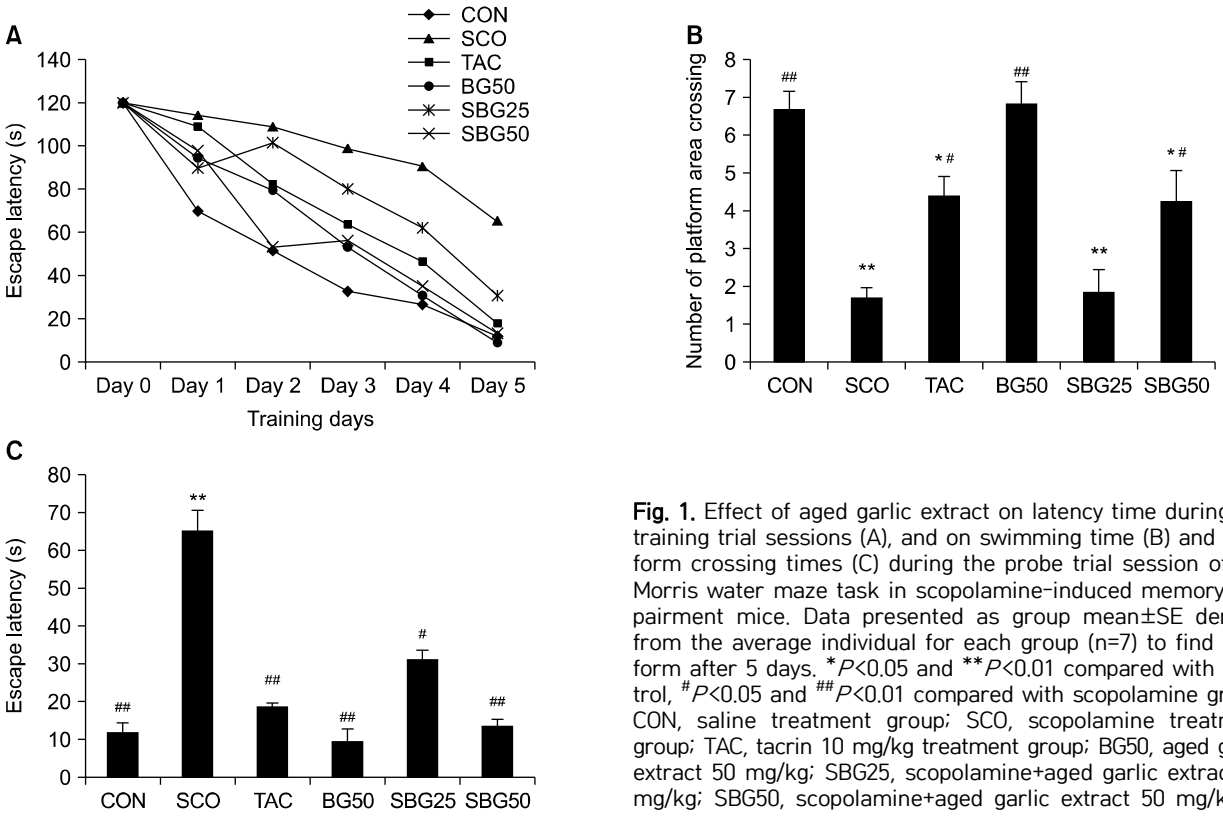

Fig. 1. Effect of aged garlic extract on latency time during the training trial sessions (A), and on swimming time (B) and platform crossing times (C) during the probe trial session of the Morris water maze task in scopolamine-induced memory impairment mice. Data presented as group mean±SE derived from the average individual for each group (n=7) to find platform after 5 days. $^{*}P<0.05$ and $^{**}P<0.01$ compared with control, $^{\#}P<0.05$ and $^{\#\#}P<0.01$ compared with scopolamine group. CON, saline treatment group; SCO, scopolamine treatment group; TAC, tacrin 10 mg/kg treatment group; BG50, aged garlic extract 50 mg/kg; SBG25, scopolamine+aged garlic extract 25 mg/kg; SBG50, scopolamine+aged garlic extract 50 mg/kg.

Effect of BG on the passive avoidance task

The latency time of the scopolamine-induced cognitive impaired group was significantly shorter than that of the control group (Fig. 2). After 4 weeks of treatment with aged garlic extract, mice in the aged grarlic extract (SBG 50) and tacrin groups showed significantly longer latency times than mice in the scopolamine-induced group.

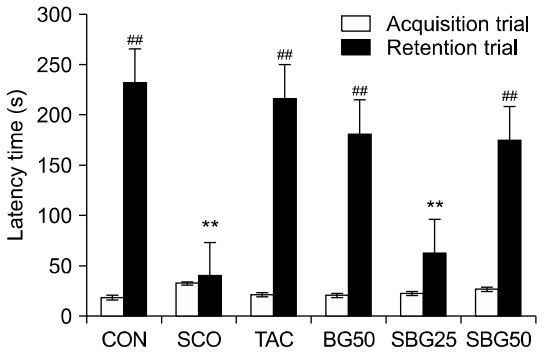

Fig. 2. Effect of aged garlic extract on latency time of passive avoidance test in scopolamine-induced memory impairment mice. Data measured by the latency time in sec required to enter the dark chamber. Data presented as group mean±SE derived from the average individual for each group (n=7) to find platform after 5 days. $^{**}P<0.01$ compared with control and $^{\#\#}P<0.01$ compared with scopolamine group. CON, saline treatment group; SCO, scopolamine treatment group; TAC, tacrin 10 mg/kg treatment group; BG50, aged garlic extract 50 mg/kg; SBG25, scopolamine+aged garlic extract 25 mg/kg; SBG50, scopolamine+aged garlic extract 50 mg/kg).

Effect of BG on the antioxidant capacity of brain tissue in mice

We also investigated lipid peroxidation, GPx, GSH, and GR activities in brain tissue to determine whether aged garlic extract effects on oxidative stress induced by scopolamine. Levels of MDA are shown in Fig. 3A. Increased levels of MDA were observed in mice in the SCO group compared with control group; after administration of aged garlic extract for 4 weeks (SBG50 group), levels of MDA were decreased verses the SCO group. Scopolamine treatment significantly inhibited GR activity, compared with that of mice in the control group (Fig. 3B). SBG50 administration significantly increased GR activity, whereas no significant difference were observed between mice in the SBG25 group and the SCO group. Moreover, GPx activity was also inhibited by scopolamine treatment compared to mice in the control group; this was significantly attenuated following treatment with either tacrin or 50 mg/kg aged garlic extract; no significantly differences between mice in the SCO group and those receiving 25 mg/kg aged garlic extracts were observed (Fig.

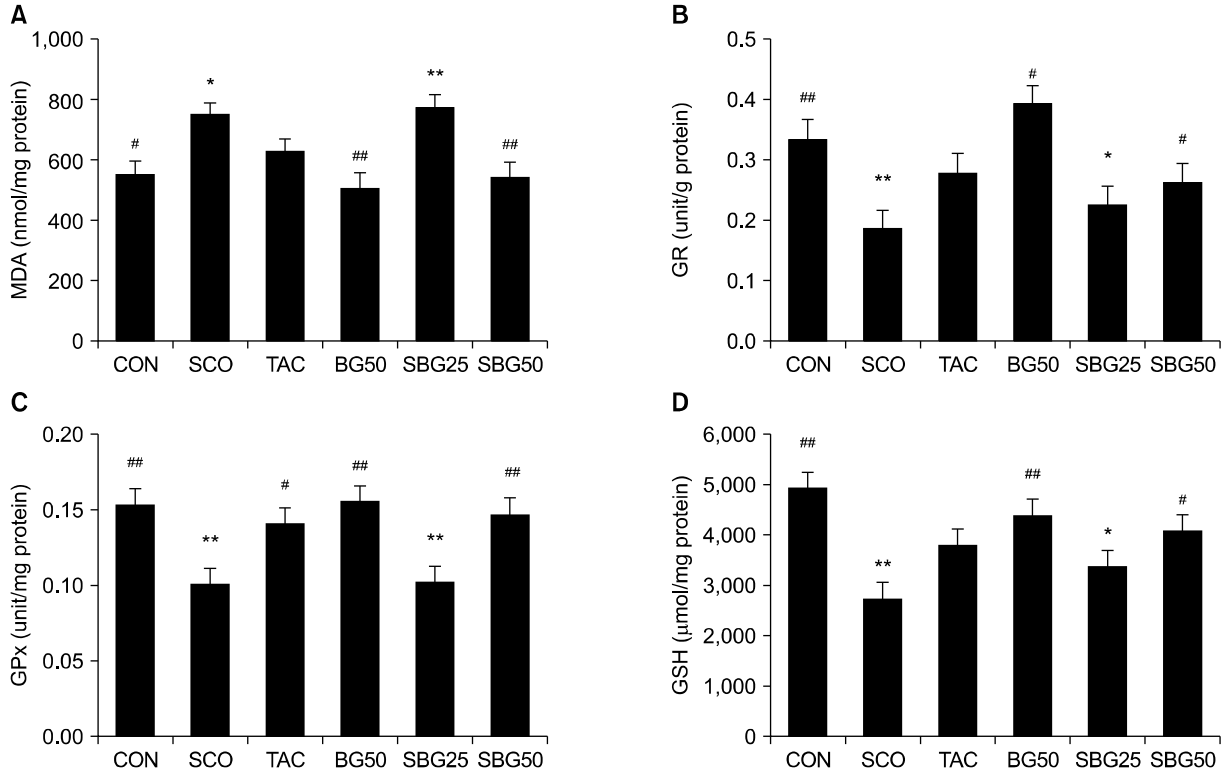

Fig. 3. Effect of aged garlic extract on malondialdehyde (MDA) level (A), glutathione reductase (GR) (B), glutathione peroxidase (GPx) activity (C), and glutathione (GSH) level (D) of brain in scopolamine-induced memory impairment mice. Data presented as group mean±SE derived from the average individual for each group (n=6) to find platform after 5 days. $^{*}P<0.05$ and $^{**}P<0.01$ compared with control, $^{\#}P<0.05$ and $^{\#\#}P<0.01$ compared with scopolamine group. CON, saline treatment group; SCO, scopolamine treatment group; TAC, tacrin 10 mg/kg treatment group; BG50, aged garlic extract 50 mg/kg; SBG25, scopolamine+aged garlic extract 25 mg/kg; SBG50, scopolamine+aged garlic extract 50 mg/kg.

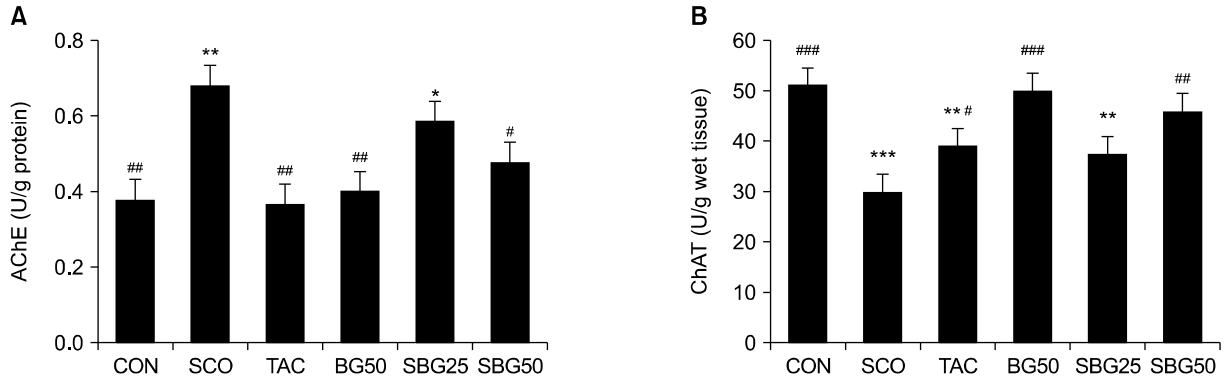

Fig. 4. Effect of aged garlic extract on acetylcholinesterase (AChE) activity (A) and choline acetyltransferase (ChAT) activity (B) in scopolamine-induced memory impairment mice. Data presented as group mean±SE derived from the average individual for each group (n=6) to find platform after 5 days. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with control, $^{\#}P<0.05$, $^{\#\#}P<0.01$, and $^{\#\#\#}P<0.001$ compared with scopolamine group. CON, saline treatment group; SCO, scopolamine treatment group; TAC, tacrin 10 mg/kg treatment group; BG50, aged garlic extract 50 mg/kg; SBG25, scopolamine+aged garlic extract 25 mg/kg; SBG50, scopolamine+aged garlic extract 50 mg/kg.

3C).

Levels of GSH were also measured in brain tissue to determine the effect of aged garlic extract on oxidative damage. Aged garlic extract (SBG50) decreased levels of GSH in brain tissue after 4 weeks of treatment (Fig. 3D). These results suggest that aged garlic extract may attenuate oxidative damage induced by scopolamine in the brain tissue.

Effect of BG on AChE activity and ChAT activity

A significant increase in AChE activity was observed in brain tissue of mice in the SCO group compared with the control group (Fig. 4A). Treatment with tarcrin for SBG50 significantly inhibited AChE activity compared to mice in the SCO group. No differences in AChE activity were observed between mice in the SBG25 and SCO groups. However, scopolamine significantly inhibited ChAT activity in mice compared with those in the control group ($P<0.001$). A significant increase in ChAT activity was alse observed in mice following administration of BG (50 mg/kg) for 3 weeks compared with those treated with scopolamine (Fig. 4B).

DISCUSSION

In this study, we examined the impact of BG on the behavioral and biochemical functions of scopolamine-induced cognitive impaired mice.

A previous study showed that garlic extracts effect Aβ-induced cytoxicity in neuron-like PC12 cells (Zhou et al., 2016); however, in this study, oxidative and cholinergic effects were not investigated. The scopolamine-induced mouse model was used in the present study because it is a well-known animal model of experimental Alzheimer's disease (Kim et al., 2012) and provides a good and relevant example of cholinergic dysfunction; scopolamine induces memory impairment, decreases the activities of anti-oxidative enzymes such as GPx, and increases levels of lipid peroxidant, MDA, in the brain (Budzynska et al., 2015). In previous studies, long term administration of scopolamine has been shown to interfere with neurons in the dentate gyrus region of the mouse hippocampus (Chauhan, 2006). In another study, aged garlic extracts were shown to reduce cerebral plaques and Aβ-species through increased α-cleaved amyloid precursor protein α, and to reduce conformational changes in tau phosphorylation involved in glycogen synthase kinase-3β but not Cdk5 in transgenic model Tg2576 (Venkatesan et al., 2016).

Reactive oxygen species (ROS)-induced oxidative stress is a critical factor in development of neurodegenerative diseases and ROS may contribute to increased Aβ-induced apoptosis *in vitro* (Peng et al., 2002). Overproduction of ROS and reduction of the antioxidant enzyme (GPx) were demonstrated in the current study after scopolamine administration.

Acetylcholine is an important neurotransmitter synthesized by ChAT. AChE is an enzyme that degrades acetylcholine into acetate and choline and accelerates Aβ accumulation in brain, both of which are correlated with memory impairment (Araujo et al., 2005). AChE inhibitors, such as rivastigmine and donepezil, are the most common Alzheimer's disease treatments. ChAT function to synthesize actylcholine and is a key indicator of cholinergic neuronal function in the nervous system which is functioning to. Our present study is limited by the fact that we were unable to obtain the ChAT at a sufficient purity for a structural analysis.

In conclusion, in this study we evaluated the effect of BGs on scopolamine-induced cognitive impairment in mice. Our results showed that BGs protect against oxidative stress induced by scopolamine through elevating levels of GSH, GPx, and GR activities, and decreasing lev-

els of lipid peroxidation. BGs also protected against progression of scopolamine-induced cognitive impairment through regulating cholinergic function by enhancing ChAT activity and inhibiting AChE activity. In conclusion, aged garlic may be a potent functional food resource for preventing neurodegenerative diseases such as Alzheimer's disease.

ACKNOWLEDGEMENTS

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2017R1D1A3B03027867).

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

REFERENCES

Ahmad MS, Ahmed N. Antiglycation properties of aged garlic extract: possible role in prevention of diabetic complications. J Nutr. 2006. 136:796S-799S.

Araujo JA, Studzinski CM, Milgram NW. Further evidence for the cholinergic hypothesis of aging and dementia from the canine model of aging. Prog Neuropsychopharmacol Biol Psychiatry. 2005. 29:411-422.

Budzynska B, Boguszewska-Czubara A, Kruk-Slomka M, Skalicka-Wozniak K, Michalak A, Musik I, et al. Effects of imperatorin on scopolamine-induced cognitive impairment and oxidative stress in mice. Psychopharmacology. 2015. 232:931-942.

Butt MS, Sultan MT, Butt MS, Iqbal J. Garlic: nature's protection against physiological threats. Crit Rev Food Sci Nutr. 2009. 49:538-551.

Chauhan NB. Effect of aged garlic extract on APP processing and tau phosphorylation in Alzheimer's transgenic model Tg2576. J Ethnopharmacol. 2006. 108:385-394.

Chen J, Long Y, Han M, Wang T, Chen Q, Wang R. Water-soluble derivative of propolis mitigates scopolamine-induced learning and memory impairment in mice. Pharmacol Biochem Behav. 2008. 90:441-446.

Dillon SA, Burmi RS, Lowe GM, Billington D, Rahman K. Antioxidant properties of aged garlic extract: an *in vitro* study incorporating human low density lipoprotein. Life Sci. 2003. 72: 1583-1594.

El-Sherbiny DA, Khalifa AE, Attia AS, Eldenshary EEDS. *Hypericum perforatum* extract demonstrates antioxidant properties against elevated rat brain oxidative status induced by amnestic dose of scopolamine. Pharmacol Biochem Behav. 2003. 76:525-533.

Fan Y, Hu J, Li J, Yang Z, Xin X, Wang J, et al. Effect of acidic oligosaccharide sugar chain on scopolamine-induced memory impairment in rats and its related mechanisms. Neurosci Lett. 2005. 374:222-226.

Francis PT, Palmer AM, Snape M, Wilcock GK. The cholinergic hypothesis of Alzheimer's disease: a review of progress. J Neurol Neurosurg Psychiatry. 1999. 66:137-147.

Gupta VB, Rao KSJ. Anti-amyloidogenic activity of *S*-allyl-L-cysteine and its activity to destabilize Alzheimer's β-amyloid fibrils *in vitro*. Neurosci Lett. 2007. 429:75-80.

Ide N, Lau BHS. Aged garlic extract attenuates intracellular oxidative stress. Phytomedicine. 1999. 6:125-131.

Kim I, Kim JY, Hwang YJ, Hwang KA, Om AS, Kim JH, et al. The beneficial effects of aged black garlic extract on obesity and hyperlipidemia in rats fed a high-fat diet. J Med Plant Res. 2011. 5:3159-3168.

Kim JH, Nam SH, Rico CW, Kang MY. A comparative study on the antioxidative and anti-allergic activities of fresh and aged black garlic extracts. Int J Food Sci Technol. 2012. 47:1176-1182.

Lee YM, Gweon OC, Seo YJ, Im J, Kang MJ, Kim MJ, et al. Antioxidant effect of garlic and aged black garlic in animal model of type 2 diabetes mellitus. Nutr Res Pract. 2009. 3:156-161.

Mangialasche F, Solomon A, Winblad B, Mecocci P, Kivipelto M. Alzheimer's disease: clinical trials and drug development. Lancet Neurol. 2010. 9:702-716.

Peng Q, Buz'Zard AR, Lau BHS. Neuroprotective effect of garlic compounds in amyloid-β peptide-induced apoptosis *in vitro*. Med Sci Monit. 2002. 8:BR328-BR337.

Rogers SL, Farlow MR, Doody RS, Mohs R, Friedhoff LT; Donepezil Study Group. A 24-week, double-blind, placebo-controlled trial of donepezil in patients with Alzheimer's disease. Neurology. 1998. 50:136-145.

Stone WS, Croul CE, Gold PE. Attenuation of scopolamine-induced amnesia in mice. Psychopharmacology. 1988. 96:417-420.

Venkatesan R, Subedi L, Yeo EJ, Kim SY. Lactucopicrin ameliorates oxidative stress mediated by scopolamine-induced neurotoxicity through activation of the NRF2 pathway. Neurochem Int. 2016. 99:133-146.

You BR, Kim HR, Kim MJ, Kim MR. Comparison of the quality characteristics and antioxidant activities of the commercial black garlic and lab-prepared fermented and aged black garlic. J Korean Soc Food Sci Nutr. 2011. 40:366-371.

Zhou M, Xue Y, Sun S, Wen M, Li Z, Xu J, et al. Effects of different fatty acids composition of phosphatidylcholine on brain function of dementia mice induced by scopolamine. Lipids Health Dis. 2016. 15:135.