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Soy Products Fermented with Sprouted Garlic Have Increased Neuroprotective Activities and Restore Cognitive Functions

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Abstract Enhanced antioxidant activities of sprouted garlic over garlic were considered. The popular Korean traditional fermented soybean product cheonggukjang (CGJ) was prepared as normal CGJ and prepared with fermentation of a mixture of cooked soybeans and sprouted garlic. Different varieties were investigated for anti-oxidative and protective activities against oxidative stress in neuronal cells. Normal CGJ was compared with CGJ prepared with garlic and sprouted garlic for anti-oxidative and neuroprotective activities and protection of cognitive function. CGJ prepared with sprouted garlic during fermentation exhibited higher anti-oxidative and neuroprotective activities in a mouse hippocampal model than the normal fermented soy product with enhanced cognitive function in the mouse model. Sprouted garlic can be used to improve the health benefits of fermented soy products.

Keywords: cheonggukjang, sprouted garlic, antioxidant activity, cognitive function, neuroprotection

Introduction

Soybeans have received attention because of potential health benefits, including preventive effects against cardiovascular disease, post-menopausal complications, and sex hormone-dependent cancers. The fermented soy products cheonggukjang (CGJ) and doenjang prepared using cooked soybeans followed by fermentation with species of *Bacillus* or *Aspergillus* have also shown diverse bioactive functions of anti-oxidative activity, hypo-allergenicity, insulin sensitization, and hepatoprotection (1-5). Enzymes produced from some *Aspergillus* strains used for making soybean paste (doenjang) can increase the biological activity of other foodstuffs, like onions, in which quercetin glycosides are converted by enzymatic action into aglycone, a more bioactive form.

Garlic (*Allium sativum*) is also widely recognized as having medicinal functions, including lowering of cholesterol levels, decreasing blood pressure, and decreasing risks of chronic diseases (6). Health benefits of garlic have been attributed to a strong antioxidant action (7,8), an ability to boost immune responsiveness (9), and stimulation of prostanoid production (10,11). Furthermore, the metabolite profile of garlic is altered by aging, sprouting, and fermentation, leading to changes in biological activity. The antioxidant activity of garlic was increased by sprouting, possibly due to modification and/or synthesis of sulfur-containing compounds, and enhanced synthesis of flavonoids (12). In addition, garlic components suppress growth of the harmful bacterium *Bacillus cereus* (13), which can cause contamination during preparation, storage, transportation, and handling of CGJ. While health benefits of soybeans and garlic have been reported independently (1-6), few studies have reported combination effects (14).

Asian people have been eating fermented soy products containing minced garlic as a seasoning for a long time. Therefore, this study was conducted to examine whether fermentation of soybeans with garlic or garlic products synergistically enhances antioxidant and neuroprotective activities of CGJ.

Materials and Methods

Chemicals and reagents 2,4,6-Tripyridyl-s-triazine (TPTZ), ferric chloride, 7'-dichlorofluorescein diacetate (DCFDA), dihydroethidium (DHE), and butylated hydroxytoluene (BHT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trichloroacetic acid was supplied from Kanto Chemical Co. (Tokyo, Japan). All other reagents were of American Chemical Society (ACS) grade.

Animal experiments Male 5 week old C57BL/6J mice were obtained from Daehan Biolink (Eumsung, Korea) and were housed in

plastic cages at a constant temperature of 22±2°C and 50% humidity under a 12 h light/dark cycle. Animals were freely provided a standard diet (Chow; Purina, St. Louis, MO, USA) and water. After an adaptation period of a week, mice were randomized by weight into 5 groups (8-9 mice per group) and fed experimental diets prepared based on the American Institute of Nutrition (AIN)-76A formulation with *i.p*. injection of D-galactose at 200 mg/kg of body weight (BW) every other day for 6 weeks during an experimental period. Experimental groups were (1) a group fed a normal AIN-6 diet without D-galactose injection, (2) a group fed a normal AIN-6 diet with D-galactose injection, (3) a group fed a diet containing 3% CGJ powder with D-galactose injection, (4) a group fed a diet containing 3% CGJ powder prepared via fermentation of raw garlic with D-galactose injection, and (5) a group fed a diet containing 3% CGJ powder prepared via fermentation of sprouted garlic with D-galactose injection.

Ethics statement All animal care and handling, including surgical procedures, was approved by the Animal Ethics Committee of Kyungpook National University (IRB permission number KNU 2014-01). All efforts were made to minimize suffering. Care administered to animals was in accordance with guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health (15).

Preparation of CGJ Daewon variety soybeans were soaked in distilled water for 24 h at room temperature (25°C), then autoclaved (MLS-3781; Sanyo, Osaka, Japan) at 121°C for 20 min. After cooling to 50°C, cooked soybeans were mixed with raw garlic or sprouted garlic (10%, w/w), followed by inoculation with the following 3 Bacillus species; *Bacillus subtilis* HCD02, *Bacillus amyloliquefaciens* EMD17, *Bacillus amyloliquefaciens* MJ1-4. Then the mixture was fermented for 72 h at 37°C. The finished CGJ product was freeze-dried (FD 5505; Ilsin Bio Base, Dongducheon, Korea) and was subjected to extraction via shaking, followed by keeping in 10 volumes of 80% ethanol for 24 h with shaking in a homogenizing stirrer (HS-50A; Daehan Scientific Co., Ltd., Wonju, Korea) at 150 rpm (CGJ extract).

Determination of total phenolic contents Total phenolic contents were determined using Folin-Ciocalteu reagent (16). Briefly, 100 μL of a CGJ extract was mixed with 50 μL of a sodium bicarbonate solution (10%, w/v), followed by addition of 15 μL of Folin-Ciocalteu reagent diluted 5 times with distilled water. After 5 min at room temperature, the mixture was transferred to a Tecan 96 well microplate (Tecan, Männedorf, Switzerland) and the absorbance at 593 nm was measured using a Tecan microplate reader. Results were expressed as garlic acid equivalents.

Measurement of antioxidant activities Ferric reducing potential of plasma (FRAP) values of CGJ extract and serum prepared from mice in experimental groups were measured using a redox-linked colorimetric method (17). The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was analyzed following a previous method with minor modification (18). CGJ extract at 100 μg/mL were investigated for peroxyl radical scavenging activities using an $ORAC_{ROO\cdot}$ (ORAC) assay system carried out using a Tecan fluorescence microplate reader (Infinite 200; Tecan) with fluorescent filters at an excitation wavelength of 485 nm and an emission filter of 535 nm. In the final assay mixture, 91.4 nM fluorescein was used as a target of free radical attack of 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) (final concentration of 11.4 mM) as a peroxyl radical generator in a peroxyl radical scavenging capacity (ORAC) assay (19). Trolox at 1 μM, used as a control, was prepared fresh daily. The fluorescence of fluorescein was recorded (Infinite; Tecan) every 2 min after AAPH was added. All fluorescence measurements were expressed relative to an initial reading. Final results were calculated based on a difference in areas under the fluorescence decay curve of a blank and CGJ extract. All values were expressed as μmol of Trolox equivalents (TE). One ORAC unit was equivalent to the net protection area provided by 1 μM of Trolox.

Assessment of intracellular reactive oxygen species (ROS) The intracellular ROS level was determined as previously reported (20). HT22 cells (2×10^3 cells/well) were seeded in a 96 well black culture plate and cultured for 24 h. After attachment to the culture plate, cells were incubated in a CO_2 incubator (MCO-19-AIC; Sanyo) in the presence of 5 mM glutamate and CGJ for 6 h. Cells were then washed with phosphate buffered saline (PBS), incubated for 30 min with DCFDA (MCO-19-AIC; Sanyo), dissolved in DMEM at a final concentration of 50 μM, then washed with PBS. The fluorescence generated due to interaction of ROS with dichlorofluorescein (DCF) was observed and photographed under a fluorescence microscope (Eclipse 80i; Nikon, Tokyo, Japan). Intracellular levels of the superoxide anion ($\cdot O_2$) were monitored using dihydroethidium (DHE) staining, which formed oxyethidium that, in turn, interacted with nucleic acids to emit a bright red color that was qualitatively detected under fluorescent microscopy (Eclipse 80i) (21). The experimental procedure was the same as described for the DCF assay. The fluorescence probe used was 10 μM DHE and fluorescence was measured after 1 h with excitation at 485 nm and emission at 535 nm using a Tecan microplate reader. The change in fluorescence was calculated as: $[(F_{30\ min}-F_{0\ min})/F_{0\ min}]\times100$.

Assessment of intracellular glutathione levels Reduced glutathione was stained using a monochlorobimane (mBCl) molecular probe as described previously with slight modification (3). Briefly, HT22 cells were seeded at a density of 3×10^4 cells per well onto a 24 well plate coated with 0.1% gelatin. The next day, cells were treated with CGJ extracts plus glutamate for 6 h. Then, cells were incubated (MCO-19-AIC; Sanyo) with 20 μM mBCl for 1 h, followed by quick rinsing with PBS. Fluorescence was analyzed using a fluorescence microscope (ECLIPS 80i).

Neuroprotective activity HT22 mouse hippocampal neuronal and

human neuroblastoma SHSY5Y cells were routinely maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 100 units/mL penicillin and 100 μg/mL streptomycin in a humidified CO_2 incubator (MCO-19-AIC) at 37°C and 5% CO_2/95% air.

Cells were seeded in a 96 well culture dish in DMEM supplemented with 10% FBS at a density of 2×10^3 cells and 5×10^3 per well for HT22 and SHSY5Y cells, respectively. The next day, garlic-containing CGJ and 5 mM of glutamate, were added to cells (22). After culturing cells for 24 h, the cell survival rate was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay (23). Cell viability was recorded as a percentage relative to an untreated control.

Measurement of NAD(P)H: quinone oxidopreductase 1 (NQO1)-induction activity in HT22 cells and mouse tissues After 24 h of treatment with 100 μg/mL CGJ extract cultured HT22 cells were washed once with cold PBS and harvested using a cell scraper. Cold lysis buffer (20 mM Tris-HCl, 145 mM NaCl, 10%, glycerol, 5 mM EDTA, 1% Tritonx100, and 0.5% Nonidet) was added for preparation of a cell suspension. After standing for 40 min on ice, the suspension was centrifuged at 12,000×*g* for 15 min at 4°C. Mouse tissues including brain, stomach, kidney, lung, small intestine, and large intestine were incubated in a cooling lysis buffer (20 mM Tris-HCl, 145 mM NaCl, 10%, glycerol, 5 mM EDTA, 1% Triton x-100, and 0.5% Nonidet) with 40 μL/mL protease inhibitor (Roche Diagnostics, Mannheim, Germany), then homogenized for 30 s using a Kontes KT 50 Micro ultrasonic cell disruptor. Tissue homogenates were kept on ice for 40 min, followed by centrifugation (1730 MR; Gyrozen, Daejeon, Korea) at 12,000×*g* for 10 min at 4°C, followed by transfer to Eppendorf tubes stored at −20°C. The, supernatants were collected, and protein concentrations was determined in triplicate using a Lowry assay (24). The NQO1 activity was measured using a spectrophotometric assay in which the rate of reduction of 2,6-dichlorophenolindophenol was monitored at 600 nm (11,12). Specific activities of enzymes were normalized to the protein concentration, which was determined in duplicate following the Lowry method (13). All values were expressed as a mean± standard deviation (SD).

Antioxidant response element (ARE) luciferase reporter assays The pGL4.37 [luc2P/ARE/Hygromycin] vector (Promega, Fitchburg, WI, USA) contains 4 copies of an antioxidant response element (ARE) that drives transcription of the luciferase reporter gene. Cultured HT22 and SHSY5Y cells were transfected with pARE-Luc using Lipofectamine 2000 following manufacturer instructions. The luciferase reporter contains a hygromycin-resistant region that allows selection of pGL4.37 vector-transfected cells. HT22-ARE and SHSY5Y-ARE are stable cell lines established after 5 subculture passages. Cells were grown in DMEM supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 100 μM hygromycin at 37°C in 5% CO_2 incubator (MCO-19-AIC; Sanyo). Cells were subcultured at a 1:5 ratio every 2 days.

For the reporter assay, transfected cells were incubated in a 12 well plate at a density of 1×10^5 cells /well in the presence of CGJ extracts for 24 h for the ARE reporter assay. After incubation, cultured cells were washed with ice-cold PBS twice, then harvested in reporter lysis buffer. Each CGJ extract was centrifuged (1730MR; Gyrozen) at 12,000×*g* for 2 min at 4°C. Thirty μL of supernatant was used for analysis of ARE activity using a TD/20/20 luminometer (Turner Designs, Sunnyvale, CA, USA).

Blood parameter measurements in mice FRAP values of CGJ extract were measured using a redox-linked colorimetric method (17) in which a ferric-2,4,6-tripyridyl-*s*-triazine (Fe III-TPTZ) complex is reduced to a blue colored ferrous ion. Thirty μL of FRAP working reagent containing 300 mM acetate buffer (pH 3.6) and 10 mM Fe(III)-TPTZ in 40 mM HCl and 20 mM $FeCl_3\cdot6H_2O$ in ratios of 10:1:1 was mixed with 7 μL of a CGJ extract or a standard, and 170 μL of distilled water in a 96 well microplate. After incubation for 10 min at room temperature, the absorbance at 593 nm was measured using an ELISA reader (Tecan Sunrise Microplate Reader; ReTiSoft Inc., Mississauga, Canada). Alpha-Tocopherol and Trolox were used as positive controls (25).

8-Hydroxy-2'-deoxyguanosine (8-OH-dG) has been most commonly used as a biomarker for DNA damage. Collected whole blood was allowed to stand at room temperature for 30 min, centrifuged (1730MR; Gyrozen) at 3,000×*g* for 15 min, then used for quantification of 8-OH-dG using a DNA damage ELISA kit (Enzo Life Sciences International Inc, Plymouth Meeting, PA, USA) following manufacturer instructions.

The serum level of malondialdehyde (MDA) was determined using an assay kit (OxiSelect™ TBARS assay kit; Cell Biolabs Inc., San Diego, CA, USA) based on the method of Mihara and Uchiyama (26). Briefly, 0.5 mL of each tissue homogenate was mixed with 3 mL of an H_3PO_4 solution (1 %, v/v), followed by addition of 1 mL of a thiobarbituric acid (TBA) solution (0.67 %, w/v). The mixture was incubated at 95°C in a water bath for 45 min. The colored complex was extracted into *n*-butanol, and the absorption at 532 nm was measured in a Jasco UV/Visible spectrophotometer (V530; Jasco, Tokyo, Japan) using tetramethoxypropane as a standard. MDA levels were expressed as nmol per mg of protein. The serum 4-HNE concentration was assayed using an OxiSelect™ HNE Adduct Competitive ELISA Kit (Cell Biolabs Inc.)

Lipid peroxidation inhibition assay Assay of lipid peroxidation was carried out as described previously (27). The brain from 6 week old mice was dissected and homogenized with 9 parts of PBS at 4°C using a Kontes KT 50 Micro ultrasonic cell disruptor. The homogenate was centrifuged (1730MR; Gyrogen) at 10,000×*g* for 5 min and the supernatant was used for an *in vitro* lipid peroxidation assay.

Animal behavioral testing Y-maze testing was conducted to study mouse working memory based on assessment of spontaneous

alternation (4). Prior to Y-maze and water maze testing, mice were administrated 5 different experimental diets and treated with D-galactose for 3 weeks as described above. Y-maze testing was performed as a single 8 min test in which each mouse was placed in an arm. The total number of arm entries and the order of entries were recorded. Spontaneous alternations were defied as consecutive triplets of different arm choices. The percentage of alternations was defined as: % Alternation=[(Number of alternations)/(Total arm entries−2)]×100.

Morris water maze testing was performed as described previously with modification (28). A Morris water maze consists of 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 22±1°C water containing stationary posts. The tank was placed in a dimly lit, sound-proof testing room with 4 visual cues. A white platform 6 cm in diameter and 29 cm high was then placed in a pool quadrant. The first experimental day was dedicated to swimming training for 60 s in the absence of the platform. During 4 subsequent days, mice were allowed 4 trials per session per day with the platform in place. When the platform was located, a mouse was permitted to rest there for 10 s. If the mouse did not locate the platform within 60 s, the mouse was placed on the platform for 10 s. The mouse was then placed in the home cage and was allowed to dry after each trial. The time interval between trial sessions was 30 s. Swimming time in the pool quadrant where the platform had previously been placed was recorded. Memory impairment was induced in mice treated with D-galactose at 200 mg/kg by *i.p.* injection twice a week while consuming an experimental diet. A control group received a saline injection.

Statistical analysis Data were analyzed using an analysis of variance (ANOVA), followed by Duncan's multiple range test using SPSS software (SPSS Inc., Chicago, IL, USA). The significance level was set at $p<0.05$.

Results and Discussion

I*n vitro* antioxidant activity using CGJ prepared with different garlic products CGJ preparations of fermented cooked soybeans with garlic and sprouted garlic were evaluated for antioxidant activities based on DPPH and ABTS radical scavenging activities, and FRAP and ORAC assays. Addition of garlic or sprouted garlic significantly ($p<0.05$) increased the anti-oxidative activity of CGJ, compared with controls (Table 1). In contrast, no significant ($p>0.05$) differences in ABTS+ radical scavenging activity, ORAC values, total phenolic contents, or inhibitory activities against lipid peroxidation were observed among CGJ, garlic-added CGJ, and sprouted garlic-added CGJ. Addition of garlic or sprouted garlic during CGJ preparation did not affect *in vitro* antioxidant parameters because garlic and sprouted garlic do not contain direct phenolic antioxidants. Rather, they are more likely to exert antioxidant activities inside cells or *in vivo* due to sulfur-containing compounds that can induce expression of the intracellular antioxidant enzymes NQO1 and heme oxygenase 1 (HO-1).

Garlic supplementation significantly ($p<0.05$) inhibited glutamate-induced intracellular ROS generation, compared with normal CGJ in mouse hippocampal HT22 cells (Fig. 1A). The intracellular level of reduced glutathione was significantly ($p<0.05$) reduced by glutamate treatment, compared with controls. This reduction was partially restored by CGJ extracts prepared with and without garlic and sprouted garlic extracts, although there were no significance differences (Fig. 1B).

Neuroprotective effects of CGJ prepared with different garlic products The protective effects of CGJ prepared with garlic or sprouted garlic against glutamate-induced growth inhibition were investigated using mouse hippocampal HT22 and human neuroblast-like SHSY5Y cells (Fig. 2A). HT22 cell growth was significantly ($p<0.05$) inhibited by both 5 and 20 mM glutamate, compared with controls. Extracts of garlic-containing CGJ attenuated cell growth inhibition to different degrees, depending on the CGJ product (Fig. 2A). CGJ prepared with fermentation of soybeans and sprouted garlic showed significantly ($p<0.05$) higher levels of protective effects against glutamate-induced cell growth retardation than both normal and garlic-containing CGJ. However, no significant ($p>0.05$) protective effects against glutamate-induced cytotoxicity were observed for normal and garlic-containing CGJ extracts in SHSY5Y cells.

Regulation of an antioxidant enzyme and transcription by CGJ prepared with different garlic products Effects of CGJ prepared with garlic or sprouted garlic on activity of the NQO1 enzyme, an antioxidant biomarker enzyme, and on transcriptional regulation of antioxidant enzymes in HT22, HT22-ARE, and SHSY5Y-ARE cells were evaluated. NQO1 enzyme activity was significantly ($p<0.05$) increased in ethanol extracts of CGJ prepared both with and without garlic products (Fig. 2B). However, there was no significant ($p>0.05$) difference in enzyme induction between normal CGJ and garlic-containing products (Fig. 2B).

A reporter assay, which was used to measure the transcriptional activation ability of CGJ, revealed that CGJ extracts enhanced transcription of antioxidant enzyme genes containing the ARE sequence in the promoter region in both HT22 cell lines, but not in the SHSY5Y cell line (Fig. 2C). In particular, CGJ prepared with sprouted garlic led to the highest level of reporter gene expression.

Effect of CGJ prepared with garlic on blood parameters of mice challenged with D-galactose The protective effect of CGJ prepared with different garlic products against systemic oxidative stress induced by D-galactose was evaluated based on measurement of blood parameters associated with oxidative damage. FRAP values and serum levels of 4-HNE and 8-OH-dG were not significantly ($p>0.05$) different among different varieties of CGJ (Fig. 3A, 3B, and 3C). The serum level of MDA measured using TBARS was significantly ($p<0.05$)

Table 1. *In vitro* antioxidant activities of extracts from different CGJ products

	CGJ[1)]	GC	SGC
DPPH (% Inhibition)	57.8±2.4[b]	66.5±3.4[a2)]	65.1±1.6[a]
FRAP (mM)	445.4±43.6[b]	532.0±37.5[a]	547.0±15.2[a]
$ABTS^+$ (% Inhibition)	87.8±5.2	89.1±6.4[NS]	89.3±2.2
Total Phenolics (GAE mg/g)	44.4±5.9	38.8±5.5[NS]	34.7±3.8
ORAC (TE, mM)	4552.0±307.7	4995.5±1265.9[NS]	5596.6±1046.7
TBARS (% control)	240.2±5.7	236.4±5.9[NS]	235.1±6.7

[1)]CGJ, normal cheonggukjang; GC, cheonggukjang prepared with garlic; SGC, cheonggukjang prepared with sprouted garlic.
[2)]Values not sharing the same superscript are significantly different from each other (p<0.05). NS=not significant among values in the same row.

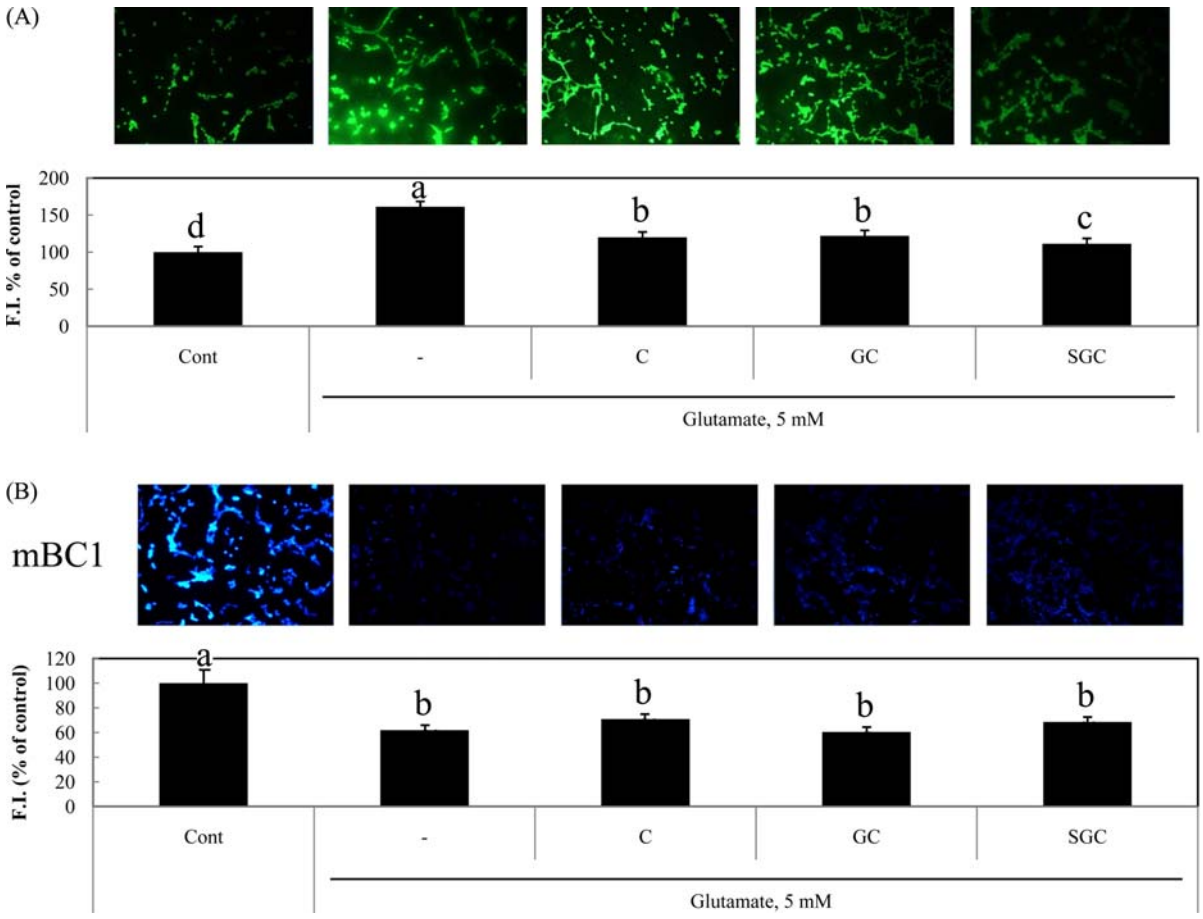

Fig. 1. Effect of CGJ extracts on intracellular ROS (A) and reduced glutathione (B) levels. Cont, negative control; C, normal CGJ; GC, CGJ prepared with garlic; SGC, CGJ prepared with sprouted garlic. Values not sharing the same letter are significantly different from each other (p<0.05). NS=not significant.

lower in mice fed CGJ prepared with sprouted garlic than for normal CGJ or garlic-containing products (Fig. 3D).

Although constituents responsible for antioxidant and neuroprotective effects of sprouted garlic-CGJ were not identified, biological activities of CGJ could be associated with flavonoids, including quercetin, produced in the process of CGJ preparation, which consisted of sprouting garlic and fermentation of a mixture of cooked soybeans and sprouted garlic. Previous study showed that sprouting of garlic causes accumulation of high levels of flavonoids without any change in the total phenolic content (12). Furthermore, transformation of quercetin glycosides into aglycones during fermentation was reported to enhance neuroprotective effects (29). Isoflavone glycosides present in soybeans are known to be transformed into aglycones, which are more bioavailable and electrophilic than glycosides, by β-glycosidase produced by *B. subtilis* during preparation of CGJ (30). However, CGJ prepared with garlic or sprouted garlic apparently contained fewer flavonoids than normal CGJ, although there were no significant differences (Table 1). Analysis of metabolites showed that garlic or sprouted garlic in CGJ inhibited conversion of soybean isoflavone glycosides into aglycones, resulting in an increased ratio of glycosides/aglycones and an apparent decrease in the flavonoid content (data not shown).

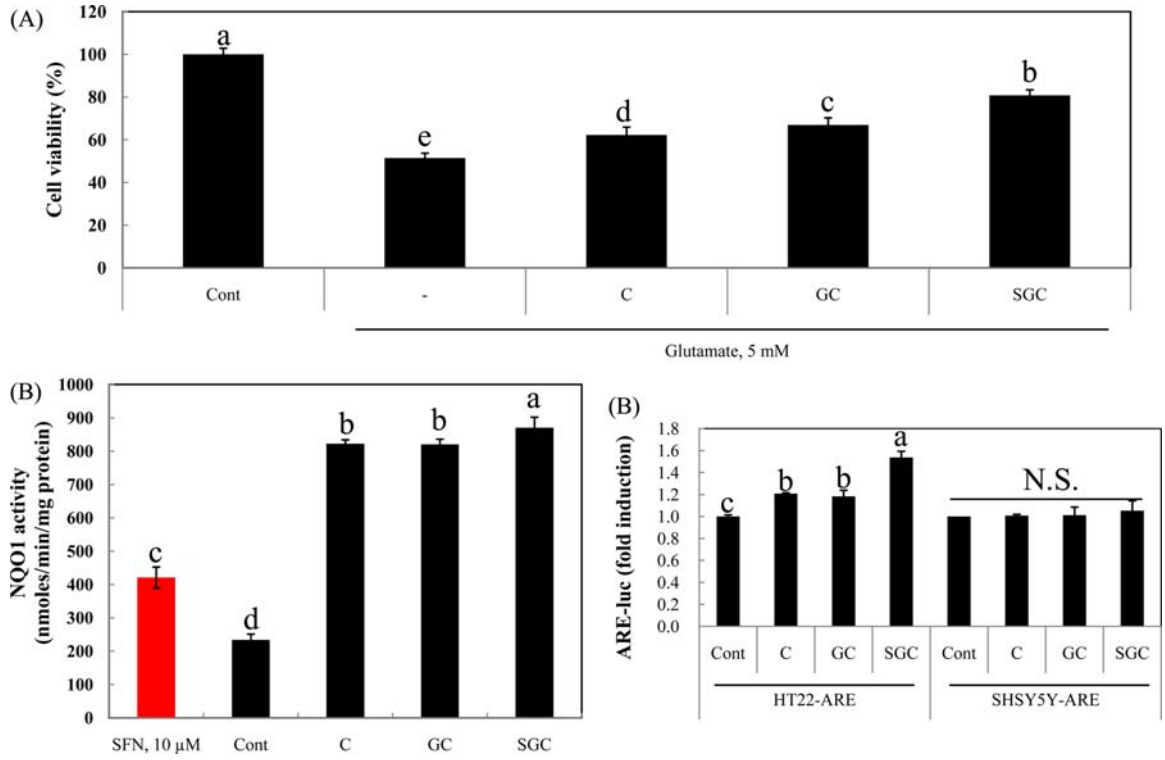

Fig. 2. Effect of CGJ extracts on glutamate-induced cytotoxicity (A), NQO1 enzyme activity (B),and reporter activity (C) in HT22 and transfectant cells. Values shown in bars are mean values of triplicate measurements±SD. Bars not a sharing common letter are significantly different from each other at $p<0.05$.

Effects of CGJ prepared with different garlic products on behavioral impairment of mice challenged with D-galactose Intake of CGJ made from cooked soybeans and different garlic products reduced the frequency of behavioral abnormalities induced by D-galactose injection in mice (Fig. 4A). Y-maze testing showed that mice treated with D-galactose exhibited a significantly ($p<0.05$) reduced willingness to explore new environments, while administration of CGJ extracts restored typical behavioral patterns. In particular, CGJ prepared with sprouted garlic completely restored the propensity of mice to explore new environments whereas normal CGJ and raw garlic-containing products exerted moderate effects.

Water maze testing showed that administration of sprouted garlic-containing CGJ improved spatial learning and memory of mice impaired using D-galactose treatment (Fig. 4B).

D-Galactose is known to cause oxidative stress in the mouse brain and, ultimately, to result in neurodegeneration and cognitive dysfunction (31). D-Galactose treatment caused cognitive dysfunction in mice, in particular, an unwillingness to explore new environments. All CGJ products attenuated cognitive impairment caused by D-galactose, while CGJ made from sprouted garlic completely restored the willingness to explore new environments to the level of untreated control mice. Learning and memory function damaged by D-galactose treatment were also restored, and even improved, above the level for untreated control mice after feeding with garlic-containing CGJ (Fig. 4B). Although it was not clear how CGJ, or CGJ prepared using sprouted garlic, protected against D-galactose-induced cognitive impairment in mice, it is possible that CGJ extracts exerted a protective action via induction of antioxidant enzymes, resulting in induction of NQO1 enzyme activity in the brain, liver, kidney, and large intestine.

Effects of CGJ prepared with different garlic products on NQO1 activity in mice tissues After consumption of a diet containing CGJ powder for 1 week, mice were sacrificed and the brain, liver, kidney, small intestine, large intestine, and stomach were collected and assayed for antioxidant NQO1 enzyme activities. Administration of CGJ prepared with sprouted garlic caused significant ($p<0.05$) induction of NQO1 enzyme activity in the brain, liver, kidney, and large intestine, compared with controls (Fig. 5). However, NQO1 activities in the small intestine and stomach were not affected by normal or garlic-containing CGJ extracts.

CGJ and garlic are popular food ingredients in Korean cuisine and numerous studies have reported health benefits, including antioxidant and anti-inflammatory activities, and prevention of coronary heart disease, cancer, atherosclerosis, and diabetes (30,32,33). Fermentation of soybeans with species of *Bacillus* during CGJ preparation transforms glycoside forms of the isoflavone phytochemical into aglycones, leading to increased *in vitro* antioxidant activities and bioavailability (34). In addition, numerous chemical reactions can produce diverse bioactive compounds that are not present in raw soybeans. Natto

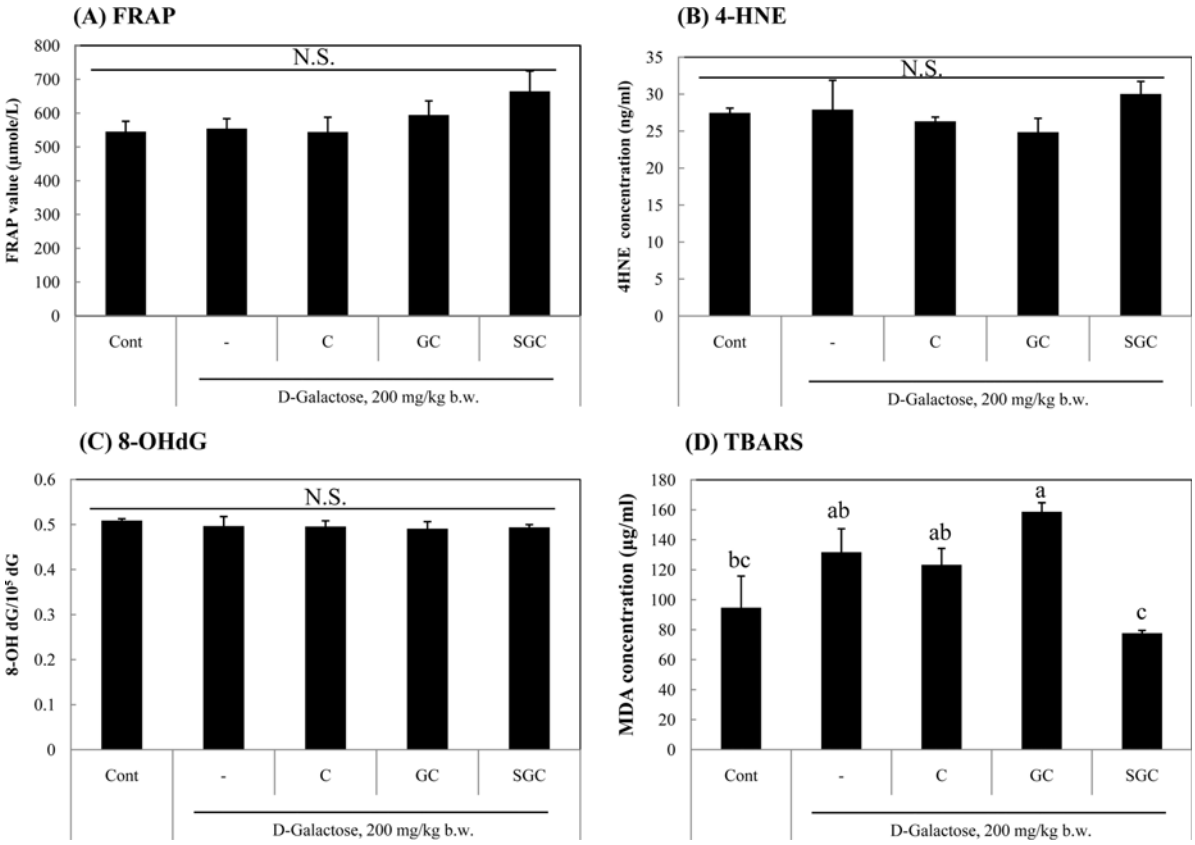

Fig. 3. Effect of administration of CGJ extracts on blood parameters in mice. Values not sharing the same letter are significantly different from each other at $p<0.05$. NS=not significant.

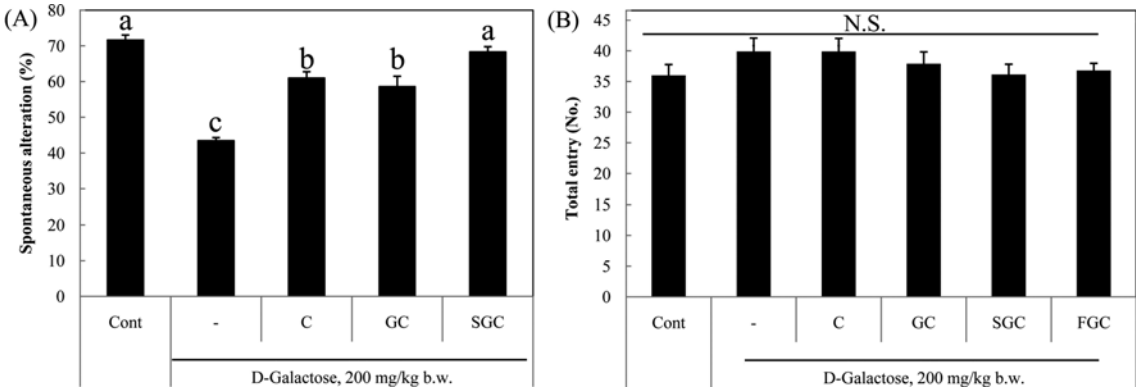

Fig. 4. Improvement of cognitive function due to CGJ extracts in mice. C57BL/6J mice were administered CGJ-containing diets for 6 weeks (A) with and (B) without D-galactose and subjected to Y-maze testing. Values not sharing the same letter are significantly different from each other at $p<0.05$. NS=not significant.

kinase and poly-γ-glutamate, well-known compounds produced during the process of CGJ preparation, were reported to have anti-thrombotic and moisturizing properties, respectively (35,36). CGJ in Korea has been prepared not only with soybeans alone, but also as a combination of soybeans with garlic. Addition of garlic during preparation of CGJ confers numerous benefits on the fermented product, including growth inhibition of unwanted bacteria, masking of the unattractive flavor of CGJ during cooking, and shelf life extension of fermented soy products (37-39).

This study was conducted to address whether CGJ prepared using fermentation of soybeans with garlic or sprouted garlic could enhance antioxidant and neuroprotective activities and, thereby, provide better protection than normal CGJ against cognitive impairment induced using ROS challenge. Previous study confirmed the superior antioxidant activity of sprouted garlic over normal garlic (12). Therefore, comparison of the biological activity of CGJ supplemented using sprouted garlic with CGJ prepared using normal garlic was performed.

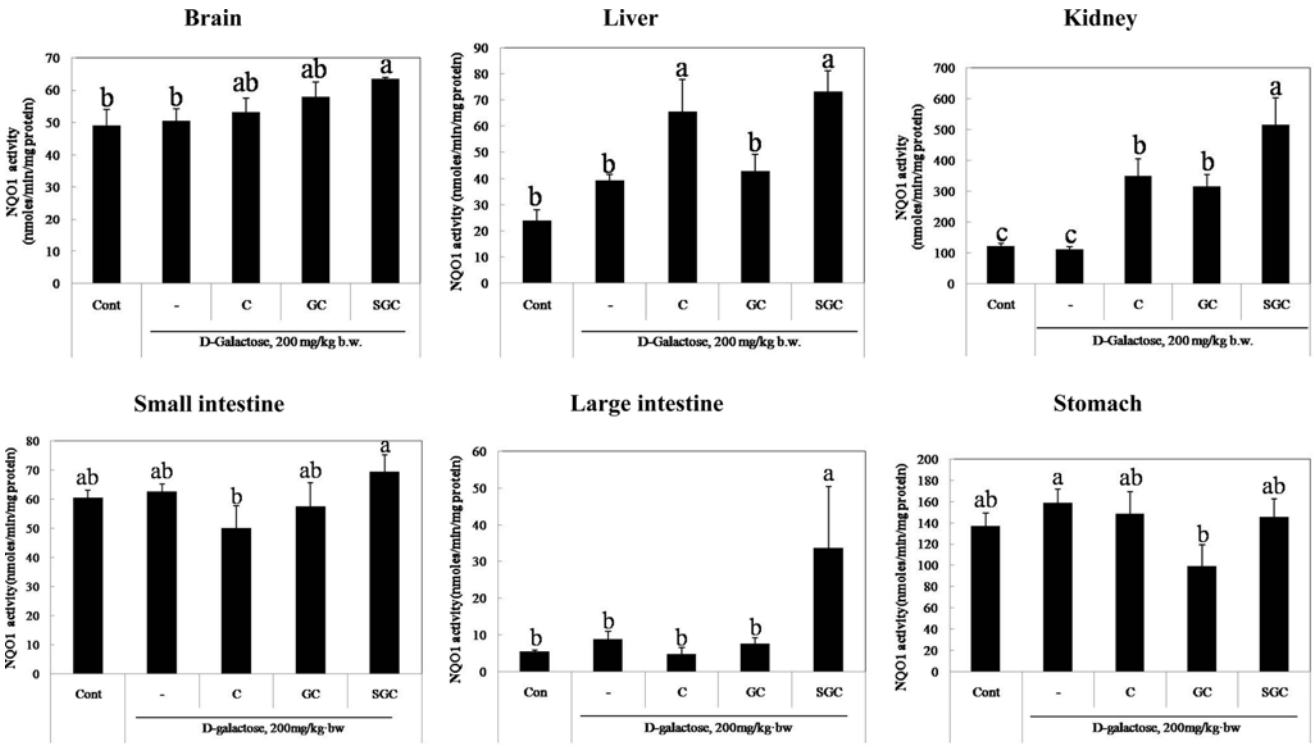

Fig. 5. Effect of CGJ extracts on NQO1 activity in mouse tissues. Liver, kidney, brain, stomach, small intestine, and large intestine tissues of C57BL/6J mice administered CGJ-containing diets for 6 weeks with and without D-galactose treatment were subjected to NQO1 enzyme assays. Values not sharing the same letter are significantly different from each other at $p<0.05$. NS=not significant.

In vitro antioxidant activity assays demonstrated that CGJ prepared with sprouted garlic had a higher activity than plain CGJ and CGJ prepared using raw garlic, and showed the highest level of neuro-protective effects among CGJ varieties tested. Furthermore, the activity of the NQO1 enzyme, which is an antioxidant biomarker enzyme, was highly induced by CGJ-sprouted garlic in HT22 cells. Antioxidant enzymes, including NQO1, are known to be regulated by endogenous and exogenous factors, such as electrophiles and reactive oxygen species, in cells (40-43). Further, regulation was reported to occur at the transcriptional level involving an antioxidant response element (ARE) sequence in the promoter region of antioxidant enzyme genes.

The transcriptional factor Nrf2 is present in a heterodimeric form with Keap1 in the cytosol of unstressed normal cells. When released upon exposure to electrophilic compounds or oxidative stress, Nrf2 migrates into the nucleus and binds to the ARE region of the promoter, leading to transcriptional activation of inducible antioxidant enzymes, including NQO1, glutathione reductase, HO-1, and gamma glutamylcysteine ligase. CGJ prepared with sprouted garlic increased the reporter luciferase activity, suggesting that this variety of CGJ has greater potential for activation of transcription of inducible antioxidant enzymes than other kinds of CGJ. Consistent with these results, CGJ prepared with sprouted garlic significantly ($p<0.05$) suppressed the lipid peroxidation (serum MDA level) caused by D-galactose injection in mice, compared with controls (Fig. 3D). However, other oxidative damage markers, such as serum levels of FRAP, 8-OH-dG, 4-HNE, were not significantly ($p>0.05$) different among mice of all experimental groups, suggesting that these blood parameters were not sensitive enough to respond to either D-galactose or CGJ in mice.

In conclusion, CGJ prepared using fermented soybeans and sprouted garlic with the following three Bacillus strains such as *Bacillus subtilis* HCD02, *Bacillus amyloliquefaciens* EMD17, and *Bacillus amyloliquefaciens* MJ1-4 prevented D-galactose-induced cognitive dysfunction in mice, probably due to an enhanced antioxidative activity.

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