Neuroprotective effects of *Scrophularia buergeriana* extract against glutamate-induced toxicity in SH-SY5Y cells

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Abstract. The aim of this study was to investigate the antioxidant and anti-apoptotic activities, as well as the underlying mechanisms of action, of *Scrophularia buergeriana* (S. buergeriana) extract (SBE) in glutamate-induced SH-SY5Y cell death. The roots of *S. buergeriana* were extracted with 70% ethanol, and standardized SBE was used in this study. To induce cytotoxicity, the SH-SY5Y cells were exposed to glutamate for 3 h, or pre-treated with SBE for 1 h, and subsequently incubated with glutamate for 3 h. The neuroprotective effects were assessed by measuring cell viability and the total glutathione contents using commercial kits. The antioxidant and anti-apoptotic mechanisms of action of SBE were evaluated by western blot analysis. The results confirmed that glutamate-induced toxicity was caused by reactive oxygen species (ROS) production, leading to oxidative stress and DNA damage, thus leading to cell death. However, treatment of the SH-SY5Y cells with SBE significantly increased the viability of the cells exposed to glutamate by upregulating the levels of antioxidant proteins, such as superoxide dismutase (SOD)1, SOD2 and glutathione peroxidase-1 (GPx-1), and directly enhancing the total glutathione contents. Furthermore, SBE attenuated DNA impairment and decreased B-cell lymphoma-2 (Bcl-2)-associated X protein (Bax), cleaved caspase-3 and cleaved poly(adenosine diphosphate (ADP)-ribose) polymerase (PARP) activation. In addition, SBE upregulated Bcl-2 expression via p38 mitogen-activated protein kinases (MAPKs). On the whole, the findings of this study demonstrated that SBE exerts neuroprotective effects against glutamate-induced cell toxicity through its antioxidant and anti-apoptotic activities.

Introduction

Glutamate is known as an excitatory neurotransmitter and plays a crucial role in the plasticity of the central nervous system (CNS) (1,2). When the glutamate concentration is abnormally high, it functions as a neurotoxin and leads to cell death (3). Excessive glutamate levels damage cellular components, including the mitochondria, and promote the generation of reactive oxygen species (ROS) and induce cell death (4). Neuronal cell loss associated with glutamate neurotoxicity is associated with certain neurological diseases, such as stroke or trauma, as well as Alzheimer's, Parkinson's and Huntington's diseases (5-9).

Cells may be protected from oxidative injury by antioxidant systems. Some intracellular ROS may be counteracted by superoxide dismutases (SODs), a group of vital antioxidant enzymes, which include SOD1 and SOD2, as well as glutathione peroxidase (GPx-1) (10,11). Metal-containing SODs play a role in scavenging ROS and facilitate catalytic activity through the use of copper/zinc (Cu/Zn) or manganese (Mn) (12). SOD1 is a Cu/Zn-containing enzyme that is located in cytosolic compartments. Low SOD1 levels are associated with increased ROS levels, which triggers oxidative damage to cellular components, including to DNA, and the functions of SOD1 are protect the CNS from damage. The Mn-containing enzyme, SOD2, is located in the mitochondrial matrix and may protect mitochondrial DNA from oxidative damage (10,12). GPx-1 is a major enzyme that protects the cells from lethal oxidative stress by converting H2O2 to H2O with reduced glutathione (GSH) as a co-factor (13).

Glutathione is a major cellular oxidant that protects cells from free radical damage and exists in both reduced (GSH) and oxidized forms (GSSG). The reduced form is predominant within the cell and represents >99% of total glutathione

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The SH-SY5Y cells were seeded in 96-well plates at a density of 5x10^4 cells/well and incubated for 24 h. The following day, the cells were pre-treated with various concentrations (125, 250 or 500 µg/ml) of SBE for 1 h and then exposed to 100 mM glutamate (Wako, Osaka, Japan) for 3 h with or without SBE. Sample stock solution was prepared (125, 250 and 500 mg/ml) and diluted 1/1000 in the medium to allow the working concentration (125, 250 and 500 µg/ml) to be processed. Following a 3-h incubation period at 37˚C with 5% CO2 conditions, 10 µl cell viability assay reagent (DoGenBio, Seoul, Korea) was added and the cells were incubated at 37˚C with 5% CO2 for a further 4 h. The absorbance was measured at 450 nm using a microplate reader (Tecan, Männedorf, Switzerland) and cell viability was expressed as a percentage relative to that in the control group (100%) with medium and DMSO (Wako) treatment not exposed to glutamate.

Measurement of acetylcholinesterase (AchE) activity. To measure AchE activity, the SH-SY5Y cells were treated with 5% all-trans retinoic acid (RA; Sigma-Aldrich, St. Louis, MO, USA) to induce differentiation. The culture medium was replaced with fresh medium containing RA every 2 days until day 6. On day 7, the differentiated cells were pre-treated with SBE for 1 h and the medium was exchanged for that containing glutamate with or without SBE. The cells were further incubated at 37˚C with 5% CO2 conditions for 3 h. The cells were then washed with ice-cold phosphate buffered saline (PBS)3 times, lysed with 0.1 M phosphate buffer (pH 7.5), centrifuged at 2,000 x g for 10 min, and the supernatant was collected. AchE activity was determined using commercial assay kits (Abnova, Taipei, Taiwan) and was calculated as the optical density (OD) at 412 nm per mg protein.

Total glutathione contents. Total intracellular glutathione contents were determined using a commercial assay kit (Cell Biolabs, San Diego, CA, USA) according to the manufacturer's protocol. The cells pre-treated with SBE for 1 h were changed to medium containing glutamate with or without SBE for 3 h. The cells were then washed with ice-cold PBS, lysed using assay reagent and centrifuged at 2,000 x g for 10 min. The supernatant was used to measure the glutathione contents as the sum of GSH and GSSG. The absorbance was measured at 405 nm using a microplate reader (Tecan) and total glutathione contents were expressed as a percentage relative to that in the control group (100%).

4',6-Diamidino-2-phenylindole (DAPI) staining. Glutamate-induced nuclear morphological changes were assessed by DAPI staining (Sigma-Aldrich). Following glutamate treatment with and without sample, cells were washed with ice-cold PBS three times and fixed with 4% paraformaldehyde in PBS for 10 min. Fixed cells were washed with PBS and permeabilized with 0.25% Triton X-100 in PBS for 10 min. After washing again with PBS, the cells were stained with DAPI staining solution for 10 min at room temperature. The stained cells were examined under a fluorescence microscope (x40 magnification;
Nikon, Tokyo, Japan) to confirm the presence of apoptotic cells exhibiting size-reduced nuclei, chromatin condensation, intense fluorescence and nuclear fragmentation. The number of apoptotic cells was expressed as a percentage relative to that in the control group (100%).

Terminal deoxynucleotidyl transferase deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) assay. Glutamate-induced DNA damage was evaluated using a TUNEL assay commercial kit (BioVision, Milpitas, CA, USA) according to the manufacturer's instructions. The SH-SY5Y cells were seeded and incubated for 24 h in 5% CO$_2$ at 37°C. The cells were then pre-treated with various concentrations (125, 250 or 500 µg/ml) of SBE for 1 h and exposed to 100 mM glutamate for 3 h with or without SBE. The treated cells were washed with ice-cold PBS and fixed by the addition of 1% paraformaldehyde in PBS on ice for 15 min. The cells were then treated with 70% ethanol (EtOH) for 30 min on ice and then washed 3 times with wash buffer. A total of 50 µl staining solution contained with FITC-dUTP was added to the washed cells prior to incubation for 60 min at 37°C. After staining, the cells were washed with rinse buffer 3 times and incubated with propidium iodide (PI)/RNase solution in the dark for 30 min at room temperature. The stained cells were analyzed under a fluorescence microscope (x20 magnification; Nikon).

Protein extraction and western blot analysis. The treated SH-SY5Y cells were lysed with radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) and 1 mM PMSE] and 1% protease inhibitor cocktail (Roche, Basel, Switzerland), followed by centrifugation at 2,000 x g for 15 min at 4°C. The supernatant was collected, and the protein concentration was assessed using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific). Equal amounts of protein (20 µg/lane) were separated by 10-15% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with commercial blocking buffer (Thermo Fisher Scientific) and washed 3 times with Tris-buffered saline containing 0.1% Tween-20 (TBST). After washing, the membranes were incubated at 4°C overnight with the following appropriate antibodies: SOD1 (1:1,000; cat. no. sc-515404), SOD2 (1:1,000; cat. no. sc-137254) (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA), GPx-1 (1:1,000; cat. no. 3206), p-p38 (1:1,000; cat. no. 9211), p38 (1:1,000; cat. no. 8690), Bax (1:1,000; cat. no. 2772), Bcl-2 (1:1,000; cat. no. 3498), cleaved caspase-3 (1:1,000; cat. no. 9664), cleaved PARP (1:1,000; cat. no. 5625) and β-actin (1:2,000; cat. no. 8457) (all from Cell Signaling Technology, Danvers, MA, USA) washed 3 times with TBST, and incubated with horseradish peroxidase-conjugated secondary antibodies: goat anti-rabbit (1:1,000; cat. no. SA002), goat anti-mouse (1:1,000; cat. no. SA001) (both from genDEPOT, Katy, TX, USA) for 1 h at room temperature. The membranes were washed 3 times and visualized via enhanced chemiluminescence (ATTO, Tokyo, Japan). Densitometry was performed using Image-Pro Plus software (6.0 version; Media Cybernetics, Inc., USA).

Statistical analysis. Data are expressed as the means ± standard error of the mean (SEM) and were analyzed using SPSS Statistics 22.0 software (SPSS Inc., Chicago, IL, USA). Different treatment groups were compared using the Student's t-test and one-way analysis of variance (ANOVA) followed by multiple comparisons correction using Dunnett's post-hoc test using Origin 7.0 software (OriginLab, Northampton, MA, USA). Differences were considered statistically significant or highly significant at values of P<0.05 and P<0.01, respectively.

Results

Effects of SBE on glutamate-induced cell death and cytotoxicity in SH-SY5Y cells. To investigate the protective effects on SBE against the glutamate-induced death of SH-SY5Y cells, we measured cell viability. To select the appropriate concentration of glutamate, the SH-SY5Y cells were exposed to various concentrations of glutamate (12.5-100 mM) for 3 h and cell viability was measured using a commercial kit. (B) SH-SY5Y cells were pre-treated with SBE (125-500 µg/ml) for 1 h and then exposed to 100 mM glutamate with or without SBE for 3 h, before measuring cell viability. Cell viability was calculated as a percentage of that in the control group (100%) and the results are expressed as the means ± standard error of the mean (SEM) of independent experiments (n=3). *P<0.05 and **P<0.01 compared with the group exposed to glutamate only; ###P<0.01 compared with the control (untreated) group.
concentrations of glutamate for 3 h. As shown in Fig. 1, exposure to 12.5-100 mM glutamate dose-dependently decreased cell viability: Cell viability decreased by 50% following 3 h of exposure to 100 mM glutamate compared to the group not exposed to glutamate. Conversely, the viability of the cells pre-treated with SBE for 1 h and exposed to glutamate increased by 18, 27 and 36% compared to that of the cells exposed to glutamate only and not treated with SBE.

**Inhibitory effects of SBE on AchE activity in glutamate-exposed SH-SY5Y cells.** To confirm the neuroprotective effects of SBE, AchE activity was investigated in the SH-SY5Y cells with glutamate-induced neurotoxicity. As shown in Fig. 2A, AchE activity in the glutamate-exposed group was significantly higher than that in the control group. However, co-treatment with SBE dose-dependently decreased AchE activity. AchE activity in the groups treated with 250 and 500 µg/ml SBE was reduced by 9.4 and 18.5%, respectively, compared to that in the group exposed to glutamate only.

**Effects of SBE on total glutathione content in the glutamate-induced apoptosis of SH-SY5Y cells.** To evaluate the antioxidant effects of SBE, we measured the total glutathione content in the glutamate-exposed SH-SY5Y cells. As expected, and as shown in Fig. 2B, exposure to glutamate induced oxidative stress and markedly decreased the total glutathione contents in the cells compared to that in the control cells. However, the total glutathione contents in the SBE-treated cells were recovered in a dose-dependent manner. The total glutathione contents in the groups treated with 125, 250 and 500 µg/ml SBE were increased by 9.3, 17.1 and 21.5%, respectively, compared to those in the group exposed to glutamate only; these results provide evidence of the antioxidant effects of SBE.

**SBE treatment attenuates the glutamate-induced apoptosis of SH-SY5Y cells.** To observe the nuclear morphological changes following exposure to glutamate, the cells were stained with DAPI. As shown in Fig. 3A, the control cells exhibited regular oval shapes, whereas the glutamate-exposed cells displayed nuclear condensation and DNA fragmentation, and were unevenly stained. However, the number of DAPI-positive cells in the SBE-treated groups was significantly lower than that in the group not treated with SBE, and the glutamate-induced nuclear morphological changes were attenuated. Furthermore, we examined the anti-apoptotic effects of SBE on glutamate-induced cell death by confirming DNA fragmentation by TUNEL assay using PI and fluorescein isothiocyanate (FITC)-dUTP double staining. PI stains each cell including normal and apoptotic cells, whereas FITC-dUTP stains only apoptotic cells with DNA fragmentation. As shown in Fig. 3B, there were more FITC-dUTP-stained cells in the glutamate-exposed group than in the control group. However, the number of stained cells in the SBE-treated group decreased, indicating that SBE reduced glutamate-induced DNA fragmentation.

**SBE inhibits glutamate-induced oxidative stress by regulating antioxidant enzyme expression in SH-SY5Y cells.** To examine the antioxidant mechanisms of action of SBE, we examined the expression levels of antioxidant proteins, such as SOD1, SOD2, and GPx-1 in the SH-SY5Y cells undergoing glutamate-induced oxidative stress. As shown in Fig. 4, SOD1, SOD2 and GPx-1 protein expression decreased following exposure to glutamate compared to that in the control cells. However, treatment with 125, 250 and 500 µg/ml SBE increased SOD1 protein expression by 1.17-, 2.14- and 2.08-fold, respectively, compared to the group exposed to glutamate only and not treated with SBE. Furthermore, treatment with 125, 250 and 500 µg/ml SBE increased SOD2 protein expression compared to that in the cells exposed to glutamate only and not treated with SBE. GPx-1 protein expression was significantly decreased by 9.5%, 19.6% and 24.2% compared to that in the group exposed to glutamate only and not treated with SBE.
increased by SBE treatment compared to that in the control group. On the whole, these results demonstrate that SBE reduces glutamate-induced oxidative stress via the regulation of SOD1, SOD2 and GPx-1 protein expression, and enhances the expression of antioxidant enzymes.

SBE attenuates the glutamate-induced activation of p38 MAPKs and apoptotic pathways in SH-SY5Y cells. To examine the anti-apoptotic mechanisms of action of SBE, we assessed the expression of apoptotic proteins associated with p38 MAPKs, such as B-cell lymphoma-2 (Bcl-2), Bcl-2-associated X protein (Bax), cleaved caspase-3 and cleaved poly(adenosine diphosphate (ADP)-ribose) polymerase (PARP) in glutamate-exposed SH-SY5Y cells by western blot analysis. As shown in Fig. 5, the levels of phosphorylated (p)-p38, Bax, cleaved caspase-3 and cleaved PARP were upregulated in the glutamate-exposed group compared to those in the control group. By contrast, Bcl-2 expression was downregulated in the glutamate-exposed cells compared to that in the control cells. However, treatment with 250 and 500 µg/ml SBE decreased the p-p38 protein expression levels compared to those in the cells exposed to glutamate only and not treated with SBE. Furthermore, treatment with SBE decreased Bax protein expression by 11, 12 and 46% in the cells treated with 125, 250 and 500 µg/ml SBE, and treatment with 500 µg/ml SBE increased Bcl-2 expression compared to that in the cells exposed to glutamate only and not treated with SBE. SBE treatment at 125, 250 and 500 µg/ml also dose-dependently decreased glutamate-induced cleaved caspase-3 protein expression by 19, 50 and 67%, respectively, compared to that in the cells exposed to glutamate only and not treated with SBE. Cleaved PARP protein expression was also significantly lower in the SBE-treated group compared to that in the cells exposed to glutamate only and not treated with SBE.

Treatment with SBE significantly decreased p-p38, Bax, cleaved caspase-3 and cleaved PARP expression, and upregulated Bcl-2 expression. The relevant mechanisms of action of SBE in this study are summarized in Fig. 6.
Glutamate is an excitatory amino acid neurotransmitter. It is involved in several physiological processes and mediates brain function, including cognition, memory and learning (28,29). However, glutamate induces oxidative stress and neurotoxicity, and may lead to the development of various neurodegenerative diseases when present at high concentrations. Oxidative stress is considered to be a key factor that leads to neuronal loss and death (30). In glutamate-induced cell death, glutamate plays a role in the inhibition of glutathione synthesis and depletion, which induces the excessive production of ROS, resulting in oxidative stress (31,32). The accumulation of excessive ROS leads to structural and functional changes in the mitochondria and activates cell death pathways (32).

SB is a black plant with a strong scent, whose roots are used in oriental medicine. The roots of SB have been reported to possess anti-allergic (33), immune-protective (34) and antioxidant activities (35), and compounds isolated from SB roots such as MCA have been reported to have neuro-protective effects. Therefore, we hypothesized that SBE may also inhibit the apoptotic process by exerting antioxidant effects.

SH-SY5Y neuroblastoma cells have been used as an excitotoxic in vitro model following exposure to high concentrations of exogenous glutamate. SH-SY5Y cells may be useful in exploring excitatory amino acid-induced processes as they...
express both ionotropic and metabotropic receptors (29,36). Therefore, in this study, we examined whether treatment with SBE would exert antioxidant effects and prevent the apoptosis of SH-SY5Y cells induced by glutamate-induced cytotoxicity.

The present study demonstrated that glutamate reduced cell viability, whereas SBE treatment significantly inhibited cell death caused by glutamate as a measurement of cell viability. Cell viability is used as an indication of cell death. To examine the neuroprotective effects of SBE, we also assessed AchE activity. AchE hydrolyzes acetylcholine to choline and acetate and is an essential enzyme responsible for the inactivation of the neurotransmitter acetylcholine. It maintains normal function of the nervous system. However, the problems in synaptic integrity, neurite outgrowth and neurodevelopment are observed, and apoptosis is induced when AchE concentrations are high (37). Therefore, it is important to consider low AchE levels as a marker of neurotoxicity. In this study, we confirmed that glutamate caused cytotoxicity by increasing AchE expression, although SBE treatment inhibited AchE activity compared to that in the SH-SY5Y cells exposed to glutamate only, consistent with the results from a previous study (23). To confirm the antioxidant activity of SBE, we evaluated the total glutathione contents. Glutathione is known as a neuronal antioxidant essential for the removal of ROS and the reduction of oxidative stress. Excessive glutamate indicates the depletion of glutathione, which is caused by inhibiting cysteine uptake (38). In this study, glutamate treatment significantly decrease the glutathione contents. However, SBE treatment upregulated the glutathione contents compared to those in the cells exposed to glutamate only and not treated with SBE. Furthermore, we evaluated the expression of antioxidant enzymes, such as SOD1, SOD2 and GPx-1, which scavenge free radicals (39). SOD converts superoxide radicals (O$_2^-$) to H$_2$O$_2$, which is converted to H$_2$O by GPx in the cytosol (40). These results suggest that the SBE protects against oxidative neuronal damage by exerting antioxidant effects.

ROS causes DNA damage and nuclear fragmentation, and leads to abortive apoptosis that can be detected by TUNEL assay and DAPI staining (41). Therefore, in this study, we investigated whether SBE inhibits oxidative stress-induced DNA impairment and cell death. The findings of this study demonstrated that SBE attenuated glutamate-induced cell death by inhibiting nuclear condensation, and DNA fragmentation and degradation.

In this study, we also demonstrated that SBE significantly inhibited the glutamate-induced activation of caspase-related proteins. Caspase is known as an apoptotic effector and is regulated by pro- and antiapoptotic Bcl-2 family proteins. Glutamate-induced ROS production affects mitochondrial function by releasing caspase activators (42). Previous studies have reported that p38 kinases induce apoptosis and increase caspase-3 expression, thus leading to PARP activation (43,44). In this study, we confirmed that exposure to glutamate induced an increase in the levels of p-p38, Bax, cleaved caspase-3 and cleaved PARP, and induced a decrease in Bcl-2 levels by promoting the apoptotic pathway (45,46). The relevant mechanisms of action of SBE in this study are summarized in Fig. 6.

Taken together, the results of the present study demonstrate that SBE exerts neuroprotective effects against glutamate-induced toxicity in SH-SY5Y cells by removing ROS and reducing oxidative stress through the increased expression of antioxidant enzymes and the inhibition of DNA impairment. SBE also displayed anti-apoptotic activity by downregulating Bax, cleaved caspase-3 and cleaved PARP expression via the inhibition of p38 MAPKs. Thus, the findings of this study suggest that SBE may be used as a functional
food for attenuating memory impairment through its antioxidant and anti-apoptotic activities.

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors’ contributions

HJL analyzed the experimental data and optimized the SH-SY5Y cell cultures. DM and BNI performed the data processing and quality control assessment. DAS contributed to the conception and design of the study, provided critical comments and revised the manuscript. AT and SHY designed the project, and contributed to the analysis of the data and finalization of the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

DAS is the Editor-in-Chief for the journal, but had no personal involvement in the reviewing process, or any influence in terms of adjudicating on the final decision, for this article. The other authors declare that they have no competing interests.

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