Neuroprotective and antioxidant activities of bamboo salt soy sauce against H_2O_2-induced oxidative stress in rat cortical neurons

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Abstract. Bamboo salt (BS) and soy sauce (SS) are traditional foods in Asia, which contain antioxidants that have cyto-protective effects on the body. The majority of SS products contain high levels of common salt, consumption of which has been associated with numerous detrimental effects on the body. However, BS may be considered a healthier substitute to common salt. The present study hypothesized that SS made from BS, known as bamboo salt soy sauce (BSSS), may possess enhanced cytoprotective properties; this was evaluated using a hydrogen peroxide (H_2O_2)-induced neuronal cell death rat model. Rat neuronal cells were pretreated with various concentrations (0.001, 0.01, 0.1, 1 and 10%) of BSSS, traditional soy sauce (TRSS) and brewed soy sauce (BRSS), and were subsequently exposed to H_2O_2 (100 μM). The viability of neuronal cells, and the occurrence of DNA fragmentation, was subsequently examined. Pretreatment of neuronal cells with TRSS and BRSS reduced cell viability in a concentration-dependent manner, whereas neuronal cells pretreated with BSSS exhibited increased cell viability, as compared with non-treated neuronal cells. Furthermore, neuronal cells pretreated with 0.01% BSSS exhibited the greatest increase in viability. Exposure of neuronal cells to H_2O_2 significantly increased the levels of reactive oxygen species (ROS), B-cell lymphoma 2-associated X protein, poly (ADP-ribose), cleaved poly (ADP-ribose) polymerase, cyto-chrome *c*, apoptosis-inducing factor, cleaved caspase-9 and cleaved caspase-3, in all cases. Pretreatment of neuronal cells with BSSS significantly reduced the levels of ROS generated by H_2O_2, and increased the levels of phosphorylated AKT and phosphorylated glycogen synthase kinase-3β. Furthermore, the observed effects of BSSS could be blocked by administration of 10 μM LY294002, a phosphatidylinositol 3-kinase inhibitor. The results of the present study suggested that BSSS may exert positive neuroprotective effects against H_2O_2-induced cell death by reducing oxidative stress, enhancing survival signaling, and inhibiting death signals.

Introduction

Soy sauce (SS) is a traditional fermented Asian food product consisting of soybeans and salt. Previous studies have demonstrated that SS contains antioxidants (1-4) and exhibits high antioxidant activity *in vitro* and *in vivo* (1,5,6), anti-allergic properties (7), aspirin-like, anti-platelet activity (8), and anti-carcinogenic (2) and anti-microbial activities (9). Furthermore, it has been suggested that SS is able to inhibit serum lipid peroxidation and may exert antioxidant effects that are ~10x more effective than red wine, and ~150x more effective than vitamins E and C (1). Therefore, it has been suggested that SS may have a role in the prevention of various diseases (8,10-13). In spite of the numerous beneficial pharmacological effects of SS, commercially available SS has one shortcoming: It contains a large amount of common salt, which has been shown to raise blood pressure and increase the risk of cardiovascular diseases when consumed in a quantity that exceeds the daily recommended amount (11,14,15). One solution is to reduce the quantity of salt in SS; however, this may negatively affect the taste of the product. An alternative solution may be to replace the common salt with a healthier salt; bamboo salt (BS) is considered to be a good candidate for this.

BS is processed by repeatedly (≤9x) roasting sun-dried salt (SDS) within a bamboo trunk, sealed by yellow soil, at a temperature >1,000˚C. BS becomes purple following these

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roasting procedures. The roasting process is performed within a furnace and is fueled by pinewood and pine resin. Throughout the roasting procedure, >70 essential minerals and micronutrients from bamboo, yellow soil, pinewood and pine resin are amalgamated into the BS via chemical and physical changes (16). BS has a higher concentration of iron, silicon and potassium minerals, as compared with common salt (17). Furthermore, BS is known to have a high medical efficacy for *in vitro* anti-cancer (18), anti-apoptosis (19) and anti-inflammatory activities (20). In addition, BS exerts cytoprotective effects and reduces susceptibility to diverse diseases, including viral infections, dental plaque, diabetes, cardiovascular diseases, and cancer and inflammatory disorders (16,18,20-24).

Bamboo salt soy sauce (BSSS), which contains BS instead of common salt, is produced from fermented small black beans and brine alongside dissolved BS, and is regarded as a healthy and medicinal food in Asia. As both BS and SS have demonstrated cytoprotective roles via antioxidative effects, the present study hypothesized that BSSS may exert greater cytoprotective effects, as compared with regular SS. To the best of our knowledge, the present study is the first to examine the cytoprotective effects of BSSS using a hydrogen peroxide (H_2O_2)-induced neuronal cell death rat model.

Oxidative stress has been widely implicated in neuronal cell death, which in turn has been considered a pathogenic mechanism underlying neurodegenerative disorders (25,26). The production of reactive oxygen species (ROS), and their detoxification, form part of normal physiological processes (27); however, at high concentrations, ROS may promote neuronal dysfunction and cell death. Numerous forms of ROS cause damage to essential cellular components, including lipids, proteins and DNA (28). Furthermore, ROS are able to initiate cell death via necrosis or apoptosis. Therefore, ROS may contribute to neuronal toxicity and be associated with acute and chronic neuropathological conditions. H_2O_2 is commonly used as an exogenous source of ROS. Neuronal cells exposed to H_2O_2 may undergo cell death, with mild oxidative stress causing apoptosis, and severe oxidative stress triggering necrosis (29). Substantial evidence has indicated etiological links between the generation of H_2O_2 and neurodegenerative diseases (30). Therefore, an H_2O_2-induced cytotoxicity model is considered suitable for the study of neurodegeneration induced by oxidative stress (31,32).

The present study evaluated the neuroprotective effects of BSSS, including its ability to reduce levels of oxidative stress, enhance survival signaling, and inhibit death signals, in a H_2O_2-induced rat neuronal cell death model, as compared with two controls: Traditional soy sauce (TRSS) and brewed soy sauce (BRSS). Furthermore, the interactions of salt and minerals in BSSS were analyzed by X-Ray diffraction (XRD), and the mineral compounds were assessed via an inductively coupled plasma-atomic emission spectrometer (ICP-AES), and by ion chromatography.

Materials and methods

Preparation of BSSS, TRSS and BRSS. BSSS was prepared by combining the standard procedure for SS production (33) with a special process involving BS instead of common salt (34). The process for making SS was as follows: Small black beans, which were purchased from a local market in Korea, were cleaned, soaked and cooked for 2 h at atmospheric pressure. Subsequently, small black beans were boiled at 100˚C, crushed in water at 80˚C and molded into a brick shape, following which they were dried for 2 days in the air, suspended by rice straw and fermented for 30-60 days under natural environmental conditions, in order to produce fermented meju. The meju was brined with a ratio of meju:BS:water, 18.4:14.6:67.0. This meju-brine mixture was ripened for 2 months, after which it was separated into liquid and solid phases. The liquid phase was filtered and boiled to produce the SS (33). The TRSS, consisting of large soybeans (Dea-du), and SDS, was purchased from Sinanmade Co. Ltd. (Paju, Republic of Korea). The BRSS used was 'Chungjungwon Yangjo Soy Sauce', consisting of soybeans and purified salt (PS), was produced in Paju, Korea and purchased from the internet market TMON (http://www.ticketmonster.co.kr/home). The BSSS 'HAIWON Jukyeom' was produced ni Gangwon-do (Republic of Korea). BSSS, TRSS and BRSS were filtered through a 0.45 mm filter and maintained at 4˚C, after which they were diluted with culture medium to various concentrations (0.001, 0.01, 0.1, 1 and 10%).

Reagents. Neurobasal media (NBM) and B27 supplement were purchased from Gibco Life Technologies (Carlsbad, CA, USA). H_2O_2, a protein protease inhibitor cocktail, trypan blue solution, insulin, DNase I and LY294002, were obtained from Sigma-Aldrich (St. Louis, MO, USA). Prior to use, these were dissolved in distilled water and further diluted with culture medium to the desired concentrations.

Primary cultures and treatment of rat cortical neurons. All of the procedures for the care and use of the rats were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Hanyang University (Seoul, South Korea). These guidelines follow international guidelines on animal welfare, as well as local and national regulations. Furthermore, the instructions for procedures were approved by the IACUC of Hanyang University (HY-IACUC-12-062A). Every effort was made in order to minimize the number of rats used and their suffering. All of the rats were used only once and none of the experiments were carried out on human materials. The cortical neurons were obtained from the cerebral cortices of fetal Sprague-Dawley rats (16 days gestation; Orient Bio Inc., Gyeonggi, Republic of Korea), following sacrifice in a chamber by 5% CO_2 inhalation. Primary cultures were generated *in vitro* and were suspended in NBM, supplemented with B27 at 37˚C, in an atmosphere containing 5% CO_2. Two days following plating, non-neuronal cells were removed via the addition of 5 μM cytosine arabinoside (Sigma-Aldrich) for 24 h. Only mature cultures (7 days *in vitro*) were used for experiments. The cultures consisted of ~80% primary cortical neurons (35).

In order to examine the effects of BSSS on neuronal cell viability, cortical neurons were pretreated with various concentrations of BSSS (0, 0.001, 0.01, 0.1, 1 and 10%) for 24 h, after which they were washed repeatedly with phosphate-buffered saline (PBS; Gibco Life Technologies). Subsequently, the cortical neuronal cells were exposed to H_2O_2 (0, 25, 50, 100, 150 or 200 μm) for 30 min, and cell viability was evaluated

using Cell Counting kit-8 assays (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) at 2.5x10^6 cells/cm^2, as described previously (35). To compare the effects of different types of soy sauce (BSSS, TRSS and BRSS) on neuronal viability, cortical neurons were pretreated with various concentrations of soy sauce (0, 0.001, 0.01, 0.1, 1 and 10%) for 24 h after being washed repeatedly with PBS. In addition, LY294002, a PI3K inhibitor, was purchased from Sigma-Aldrich to directly block PI3K. Cortical neurons were treated with 10 μM LY294002 as a co-treatment with BSSS for 24 h.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Cells (2.5x10^6 cells/cm^2) were fixed with 4% paraformaldehyde (Sigma-Aldrich) in PBS for 1 h at room temperature. Apoptotic cell death and the inhibition of DNA fragmentation were assessed via TUNEL staining, according to the manufacturer's instructions (Roche Diagnostics Corporation, Indianapolis, IN, USA). Nuclei were counterstained with 4,6-diamidino-2-phenylindole (Sigma-Aldrich). The percentage of TUNEL-positive cells (2.5x10^6 cells/cm^2) was determined according to the total number of cells (36).

Measurement of ROS. The cell-permeable, non-fluorescent compound, H2DCF-DA (Invitrogen Life Technologies, Carlsbad, CA, USA), was used to measure the intracellular concentration of ROS. H2DCF-DA was dissolved in dimethylsulfoxide (Sigma-Aldrich), and diluted with PBS to a final concentration of 10 μM, according to the manufacturer's instructions. Subsequently, 10 μM H2DCF-DA was added, and the cells were incubated for 40 min at 37˚C, after which the cells were returned to pre-warmed growth medium and incubated for a further 10 min at 37˚C. Subsequently, cells were harvested with trypsin (Gibco Life Technologies) and washed once with PBS in preparation for fluorescence intensity determination using flow cytometry (BD FACSCanto; BD Biosciences, San Jose, CA, USA) and the data acquisition program FACSDIVA software (BD Biosciences).

Western blot analysis. Following all treatments, the cells were harvested, washed twice with PBS and lysed with radioimmunoprecipitation buffer (Sigma-Aldrich), supplemented with phosphatase inhibitor (Sigma-Aldrich). The whole cell lysates were centrifuged at 18,000 x g for 20 min at 4˚C and the supernatant was collected. To obtain subcellular fractions, the Qproteome Cell Compartment kit (Qiagen Sciences, Inc., Germantown, MD, USA) was used. Protein concentrations were determined using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equal amounts (40 μg) of protein were separated by 10% SDS-PAGE (Bio-Rad Laboratories, Inc.) and transferred to nitrocellulose membranes (GE Healthcare Life Sciences, Little Chalfont, UK). The membranes were blocked with 5% skimmed milk and then incubated with specific primary antibodies against phosphorylated (phospho)-AKT (Ser473) (1:1,000; 9271; Cell Signaling Technology, Inc., Danvers, MA, USA), AKT (1:1,000; 9272; Cell Signaling Technology, Inc.), phospho-glycogen synthase kinase (GSK)-3β (Ser9) (1:1,000; sc-11757; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), GSK-3β (1:1,000; sc-9166; Santa Cruz Biotechnology, Inc.), apoptosis-induced factor (AIF; 1:500; 4642; Cell Signaling Technology, Inc.), cytochrome *c* (1:500; sc-514435; Santa Cruz Biotechnology, Inc.), caspase-9 (1:1,000; 9502; Cell Signaling Technology, Inc.), cleaved poly (ADP-ribose) polymerase (PARP; 1:1,000; sc-56196; Santa Cruz Biotechnology, Inc.), B-cell lymphoma-2-associated X protein (BAX; 1:1,000; sc-20067; Santa Cruz Biotechnology, Inc.), PAR (1:500; 4335-MC-100; Trevigen) and caspase-3 (1:1,000; 9662; Cell Signaling Technology, Inc.) at 4˚C overnight. The membranes were washed with Tris-buffered saline containing 0.05% Tween-20 (Gibco Life Technologies), and then further incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit (RPN4301) or anti-mouse (NXA931) secondary antibodies (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) for 2 h at room temperature. The blots were visualized using enhanced chemiluminescence detection (GE Healthcare Bio-Sciences). The western blot results were quantified using an image analyzer (Quantity One-4,2,0; Bio-Rad Laboratories, Inc.). The membranes were also probed with anti-β-actin antibody (1:2,000; sc-47778; Santa Cruz Biotechnology, Inc.), which served as an internal control (35).

Mineral analysis of SS. Element analysis of the minerals in BSSS, TRSS and BRSS samples, was carried out using an ICP-AES (iCAP 6000; Thermo Fisher Scientific, Inc., Cambridge, UK) and analysis of CI content was performed using ion chromatography (Metrohm AG, Herisau, Switzerland). The ion chromatography (Metrohm MIC 7 Advanced, Metrohm AG) was used with a column, Metrosep assup 7 250/4 and a conductivity detector. The eluent was 3.6 mmol/L Na2CO3 and the flow rate was 0.7 mL/min.

XRD analysis. XRD analyses were performed for BSSS, BRSS and TRSS with BS, SDS and PS controls. Shimadzu (X2) (Shimadzu Corporation, Kyoto, Japan), with a 1.0x10 mm copper X-ray tube and vertical type goniometer (185 mm), was used for the XRD analysis. All of the samples were scanned from 10-90˚. BSSS, BRSS and TRSS were dried overnight and heated for 30 min on the 50˚C hot plate. Subsequently, the dried samples were cut using a laser blade cutter, after which the samples underwent XRD analysis. The final particles were less fine than the salt controls, although the sizes of the particles were <1 mm, which is sufficient to see the overall trend in the XRD analysis, even if they were less randomly distributed than those of the salt controls. The salt samples, PS, SDS and BS were ground down in order to make them finer. For quantitative XRD analysis, the software, DIFFRAC. SUITE TOPAZ (Bruker AXS GmbH, Karlsruhe, Germany) was used, and lattice parameters were measured.

Statistical analysis. All statistical analyses were performed using SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean ± standard error of the mean of ≥5 independent experiments. Statistical comparisons between the various treatment groups were performed using Tukey's test following one-way analysis of variance. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Determining the optimal toxic dose of H_2O_2 for assessing neuronal cell viability. To determine the optimal toxic dose of

H_2O_2 for assessing neuronal cell viability, rat cortical neurons were treated with 0, 25, 50, 100, 150 or 200 μm H_2O_2 for 30 min, and the viability of these cells was measured using CCK8 assays. As demonstrated in Fig. 1A, cell viability was gradually reduced in a concentration-dependent manner. Cell viability was 82.9±1.45% at 25 μM, 71.0±1.32% at 50 μM, 65.4±1.09% at 100 μM, 49.4±1.45% at 150 μM and 36.5±2.25% at 200 μM, as compared with the non-treated controls (P<0.01). Based on these data, 100 μM was selected as the optimal toxic dose of H_2O_2, as ~65% viability is usually deemed appropriate for the study of H_2O_2-induced neuronal toxicity.

Determining the optimal concentration of BSSS for the 100 μM H_2O_2-induced neurotoxicity model. To compare the effects of various types of soy sauce (BSSS, TRSS, or BRSS) on neuronal cell viability, rat cortical neuronal cells were treated with various concentrations of soy sauce (0, non-treated group; 0.001, 0.01, 0.1, 1 and 10%) for 24 h and viability was measured. Unlike TRSS and BRSS, BSSS had no detrimental effect on neuronal cell viability at 0, 0.001, 0.01, 0.1, 1 or 10% crude liquid (Fig. 1B). Conversely, TRSS and BRSS had cytotoxic effects that led to cell apoptosis and neuronal cell damage when consumed in excess, whereas BSSS did not exert cytotoxic activity within the same concentration range.

To determine the effect of soy sauce (BSSS, TRSS, or BRSS) on H_2O_2-induced neuronal toxicity, rat cortical neuronal cells were pretreated with various concentrations of soy sauce for 24 h, after which they were treated with 100 μM H_2O_2 for 30 min, and cell viability was determined. Only pretreatment of neuronal cells with BSSS increased cell viability at the 0.001-1% concentration, as compared with the 100 μM H_2O_2 treatment group; however, cell viability did not increase above the 1% concentration, as compared with the 100 μM H_2O_2 treatment group (65.2±3.45% in 100 μM H_2O_2 treatment group; 71.4±2.47% at 0.001% BSSS; 84.3±2.89% at 0.01% BSSS; 77.8±4.38% at 0.1% BSSS; and 72.3±4.91% at 1% BSSS) (P<0.01; Fig. 1C). Based on the viability data, 0.01 and 0.1% BSSS concentrations were associated with maximal cell viability, and were selected as the optimal concentrations for all subsequent experiments (Fig. 1C).

BSSS pretreatment protects cortical neurons from H_2O_2-induced apoptosis. To analyze the rate of apoptosis, TUNEL analysis was performed (Fig. 2). Briefly, cultured neuronal cells were pretreated with BSSS for 24 h, after which they were treated with 100 μM H_2O_2 for 30 min. As a control, cultured neuronal cells were stimulated with 100 μM H_2O_2 for 30 min, without BSSS pretreatment. TUNEL staining demonstrated that 61.3±3.21% neurons underwent apoptosis following incubation with 100 μM H_2O_2 for 30 min, whereas pretreatment of neurons with 0.01 and 0.1% BSSS significantly decreased H_2O_2-mediated apoptosis by ~40% (36.3±2.31% at 0.01% and 38.6±1.52% at 0.1%; P<0.05). These results suggest that BSSS was able to inhibit H_2O_2-mediated neuronal cell apoptosis and DNA fragmentation.

Anti-oxidative effects of BSSS on H_2O_2-induced neurotoxicity. To assess H_2O_2-dependent free radical production in rat cortical neuronal cells, the H2DCF-DA method was used to measure the levels of ROS in neurons treated with 100 μM H_2O_2 for 30 min. Free radical production significantly increased in the H_2O_2-treated cells (16.9±0.81; P<0.05; Fig. 3), although not in the BSSS pretreated cells. Pretreatment with BSSS for 24 h decreased the free radical production following H_2O_2 treatment (8.1±0.31% at 0.01% and 10.6±0.4% at 0.1%; P<0.05). These results indicate that BSSS may have antioxidative effects on H_2O_2-mediated ROS generation.

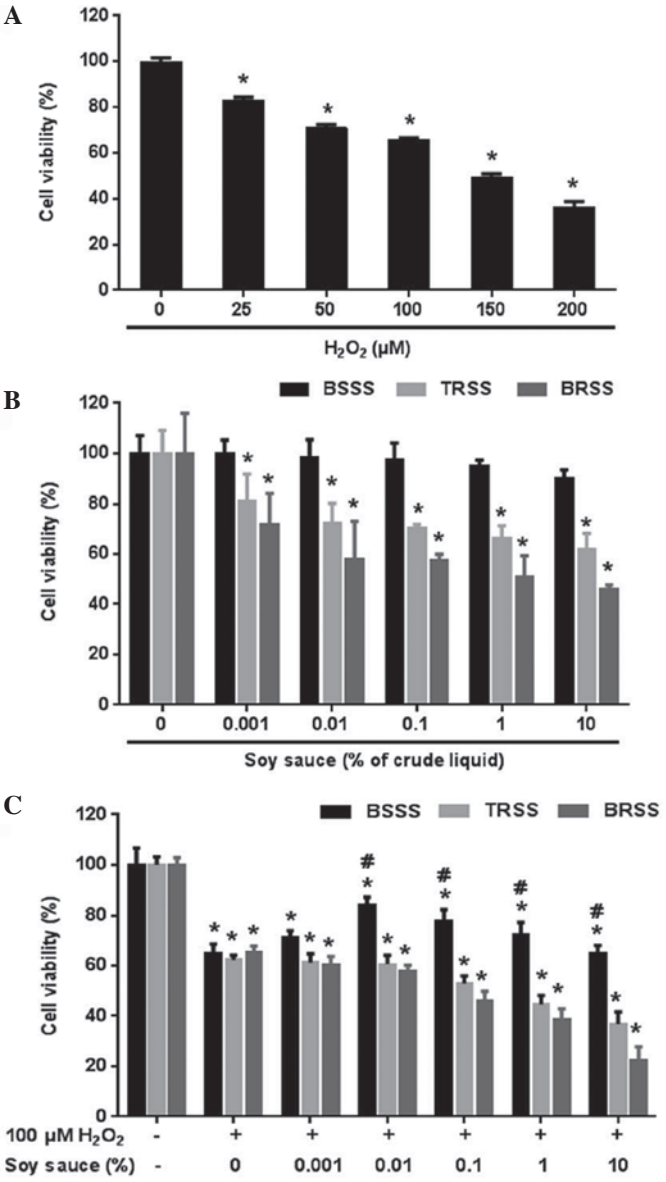

Figure 1. Bamboo salt soy sauce (BSSS) protected rat cortical neuronal cells from hydrogen peroxide (H_2O_2)-induced cell death. (A) To determine the optimal toxic dose of H_2O_2 for assessing neuronal cell viability, rat cortical neurons were treated with various concentrations of H_2O_2 (0, 25, 50, 100, 150, and 200 μM) for 30 min. (B) Comparisons between the various soy sauces in rat cortical neurons. Rat cortical neuronal cells were treated with various concentrations of BSSS, traditional soy sauce (TRSS), or brewed soy sauce (BRSS; 0, 0.001, 0.01, 0.1, 1, and 10%) for 24 h. (C) To estimate the neuroprotective effects of the various soy sauces against 100 μM H_2O_2-mediated cell death, rat cortical neuronal cells were pretreated with various concentrations of BSSS, TRSS, and BRSS (0, 0.001, 0.01, 0.1, 1, and 10%) for 24 h, followed by 100 μM H_2O_2 treatment for 30 min. Data are presented as the mean (% of control) ± standard error of the mean from ≥5 independent experiments. *P<0.05 vs. the non-treated group; #P<0.05 vs. the group treated with 100 μM H_2O_2 only.

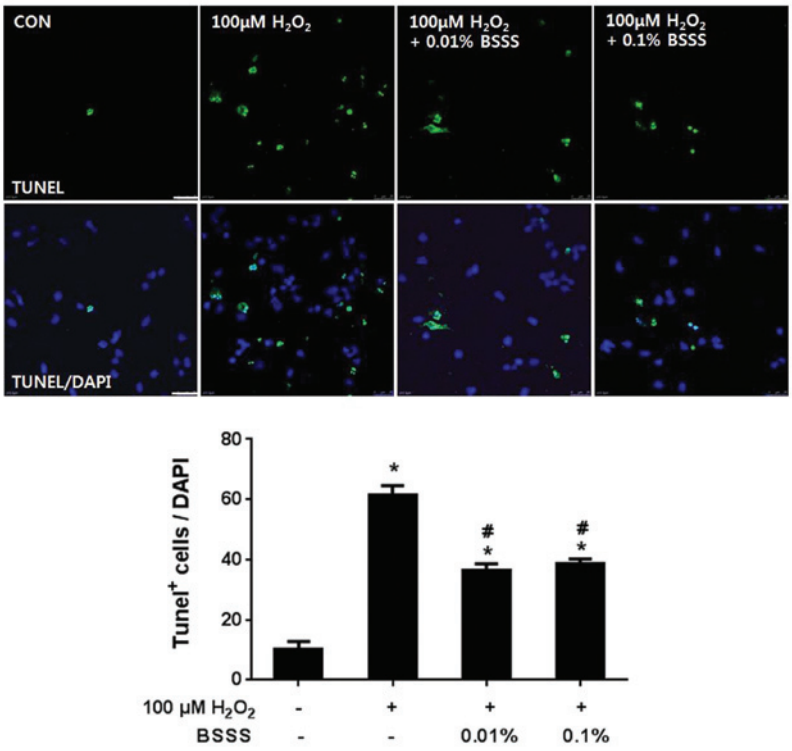

Figure 2. Protective effects of bamboo salt soy sauce (BSSS) on hydrogen peroxide (H_2O_2)-induced neuronal cell apoptosis. The percentage of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells induced by 100 μM H_2O_2-treatment for 30 min decreased when the cells were pretreated with 0.01 or 0.1% BSSS for 24 h. The data are presented as the percentage of TUNEL-positive cells ± standard deviation. *P<0.05 vs. the control (CON) group; #P<0.05 vs. the 100 μM H_2O_2-treatment group. Scale bar, 50 μm; magnification, x200.

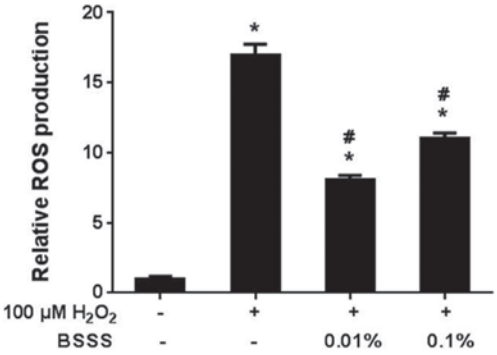

Figure 3. Effects of bamboo salt soy sauce (BSSS) on reactive oxygen species (ROS) production in hydrogen peroxide (H_2O_2)-induced neuronal cell death. To assess whether BSSS attenuated H_2O_2-induced ROS generation, rat cortical neuronal cells were pretreated with various concentrations (0.01 or 0.1%) of BSSS for 24 h, followed by 100 μM H_2O_2-treatment for 30 min. Data are presented as the mean ± standard deviation from five independent experiments. *P<0.01 vs. the non-treated group; #P<0.01 vs. the group treated with 100 μM H_2O_2 only.

BSSS inhibits H_2O_2-mediated neuronal cell death by regulating intracellular signaling protein expression. To confirm the effects of BSSS on intracellular signaling pathways, the expression levels of BAX, poly (ADP-ribose) (PAR), PARP, cleaved PARP, cytosolic cytochrome *c*, cytosolic AIF, caspase-9 (total/cleaved), caspase-3 (total/cleaved), Akt (total/phosphorylated) and GSK3-β (total/phosphorylated), were measured. The immunoreactivities (IRs) of BAX (Fig. 4A), PAR (Fig. 4B), cleaved PARP (Fig. 4C), cytosolic cytochrome *c* (Fig. 4D), cytosolic AIF (Fig. 4E) and cleaved caspase-9/cleaved caspase-3 (Fig. 4F) were significantly decreased following pretreatment with BSSS, as compared with following 100 μM H_2O_2 treatment alone (P<0.05; Fig. 4). These results suggest that BSSS may exert anti-apoptotic effects that resist H_2O_2-induced cytotoxic damage, including inhibiting BAX and PAR activities, and decreasing the levels of cleaved PARP, cytosolic cytochrome *c*, cytosolic AIF, cleaved caspase-9 and cleaved caspase-3.

Pretreatment with BSSS significantly increased the IRs of phospho-Akt (Ser473) and phospho-GSK-3β (Ser9) (Fig. 5A and B). In addition, whether the neuroprotective effects of BSSS were associated with the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, was examined by co-administering 10 μM LY294002, a PI3K inhibitor, with BSSS for 24 h. As compared with the BSSS treatment group, the IR ratio of phospho-Akt decreased in the 10 μM LY294002 pretreated group (only H_2O_2 treated group, 0.55±0.09; combined H_2O_2 with 0.01% BSSS pretreated group, 0.93±0.03; combined H_2O_2 with 0.1% BSSS pretreated group, 0.91±0.04; and combined H_2O_2 with 0.1% BSSS and 10 μM LY294002 pretreated group, 0.72±0.05; P<0.05; Fig. 5A). These results suggest that BSSS-mediated neuroprotective effects were partially prohibited by the presence of the PI3K inhibitor (LY294002), thus indicating that the neuroprotective effects of BSSS were at least partially mediated via the PI3K/Akt signaling pathway.

Mineral analysis. In an effort to elucidate the mechanism by which BSSS enhanced neuronal cell viability and inhibited H_2O_2-mediated cell apoptosis, the mineral content of

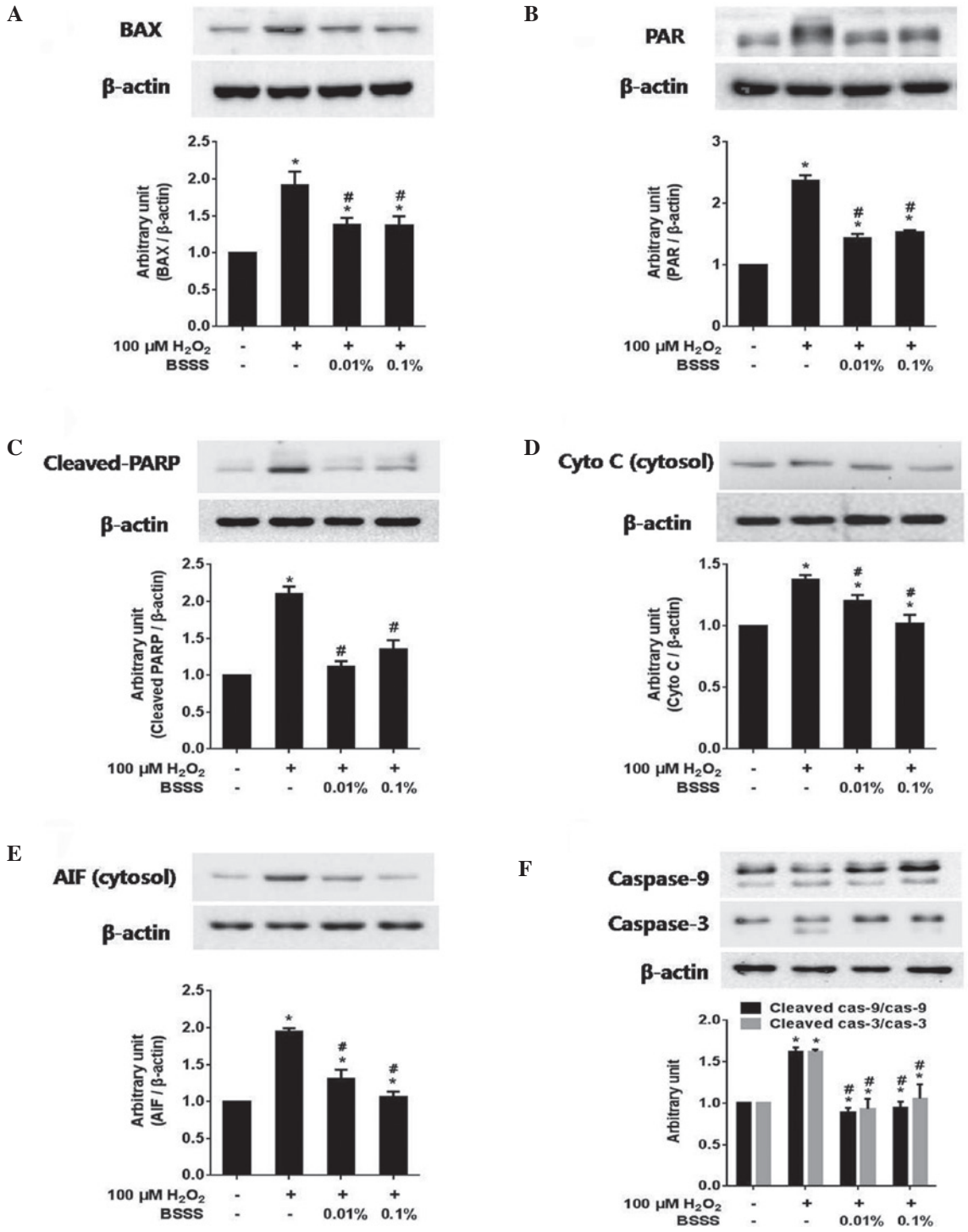

Figure 4. Bamboo salt soy sauce (BSSS) inhibited the expression of hydrogen peroxide (H_2O_2)-mediated cell death-associated intracellular signaling protein expression in rat cortical neurons. Immunoreactivities (IRs) of the cell death signal proteins in neuronal cells following BSSS pretreatment were assessed by western blotting. Representative enhanced chemiluminescence radiographs of the immunoblots demonstrate that pretreatment with BSSS decreased the IRs of (A) B-cell lymphoma-2-associated X (BAX) protein, (B) poly (ADP-ribose) (PAR), (C) cleaved PAR polymerase (PARP), (D) cytosolic cytochrome *c*, (E) cytosolic apoptosis-inducing factor (AIF), (F) cleaved caspase-9 and cleaved caspase-3, as compared with 100 μM H_2O_2 treatment alone. Data are expressed as a ratio of the simultaneously assayed control group's value. *P<0.05 vs. the control group; $^{\#}$P<0.05 vs. the 100 μM H_2O_2-treatment only.

BSSS, as compared with TRSS and BRSS, was analyzed. SS contains indispensable minerals, including K, Ca, Mg, S, Fe, P, Rb, Mo, V, Au, Pt, Ge and Se (Table I). BSSS was shown to contain higher levels of potassium, as compared with TRSS and BRSS. Furthermore, BSSS had unique elements, including Mo, V, Au, and Se, in higher quantities than either TRSS or BRSS. These results suggest that the unique mineral content of BSSS may contribute to its neuroprotective activity.

XRD analysis. In the present study, XRD analyses were performed in order to evaluate the salt and mineral interactions occurring in the BSSS and to assess whether they resembled those formed in BS alone. In addition, XRD was used to deter-

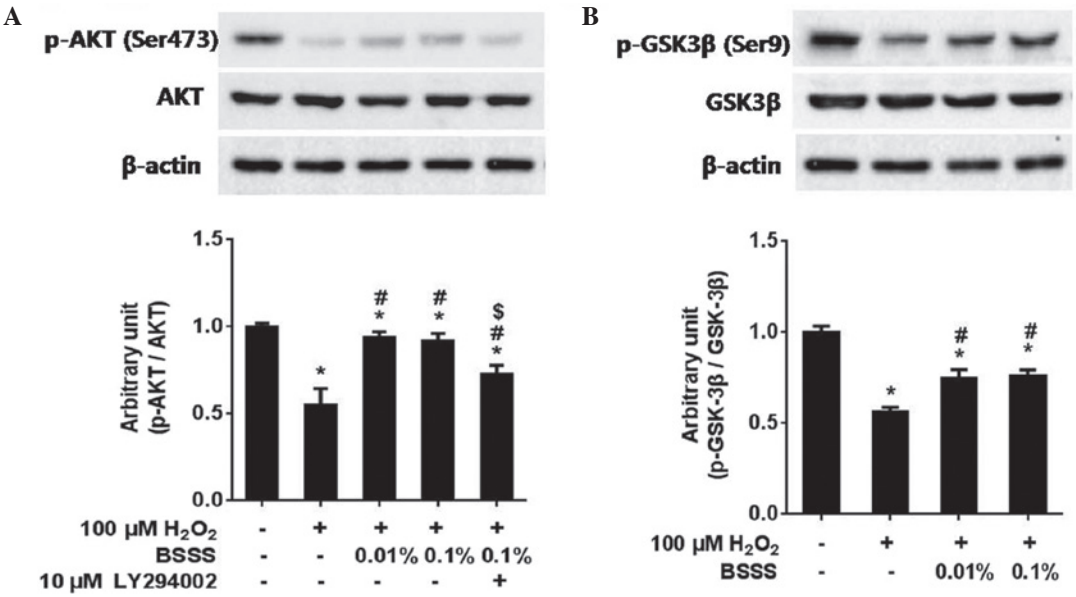

Figure 5. Neuroprotective effects of bamboo salt soy sauce (BSSS) on cortical neurons via AKT and glycogen synthase kinase (GSK)-3β in a hydrogen peroxide (H_2O_2)-induced neurotoxicity model. Immunoreactivities (IRs) of the cell survival-associated proteins in neuronal cells following BSSS pretreatment were assessed by western blotting. Representative enhanced chemiluminescence radiographs of the immunoblots demonstrate that combined treatment with BSSS increased the IRs of (A) phosphorylated (p)-AKT (B) and p-GSK-3β, as compared with 100 μM H_2O_2-induced neuronal damage alone. Pretreatment with a specific phosphatidylinositol 3-kinase (PI3K) inhibitor, 10 μM LY294002 for 24 h blocked the neuroprotective effects of BSSS. Data are expressed as a ratio of the simultaneously assayed control group's value. *P<0.05 vs. the control group; #P<0.05 vs. the 100 μM H_2O_2-treatment only group.

Table I. Mineral contents of various soy sauces, analyzed with an inductively coupled plasma-atomic emission spectrometer.

Mineral	BSSS	TRSS	BRSS
K	4,300	2650	3,160
Ca	119	83.3	277
Mg	740	1,290	533
Fe	19.12	125	17.1
S	657	997	330
P	218	75.8	1,230
Rb	6.81	2.08	5.55
Mo	9.30	≤0.1	≤0.1
V	0.0109	<0.0001	<0.0001
Au	0.024	≤0.0001	≤0.0001
Pt	≤0.001	≤0.001	≤0.0001
Ge	<0.002	<0.002	<0.002
Se	0.0106	0.0024	≤0.0001

All values presented as parts per million. BSSS, bamboo salt soy sauce; TRSS, traditional soy sauce; BRSS, brewed soy sauce.

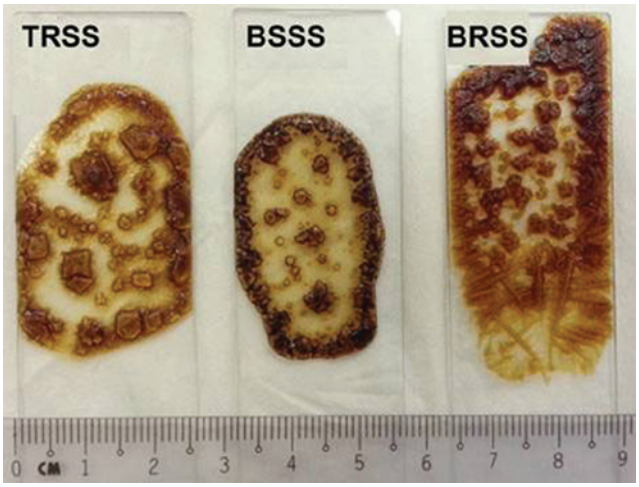

Figure 6. Bulk crystals from dried samples of traditional soy sauce (TRSS), bamboo salt soy sauce (BSSS) and brewed soy sauce (BRSS). X-ray diffraction (XRD) samples from TRSS, BSSS and BRSS on glass substrate show various size distributions of bulk crystals.

mine whether the interactions in BS could be distinguished from those in SDS or PS.

During the preparation of dried XRD samples, it was observed that the BSSS crystals were more regular, as compared with those formed by TRSS, and were clearer than those formed by BRSS (Fig. 6). This may be due to the well-homogenized distribution of the minerals within the BS used to produce the BSSS.

The main peaks of the XRD analysis output corresponded to PS peaks, although there were slight shifts of angles (Fig. 7A) and lattice constants (Fig. 7B), as compared with the PS control. PS peaks coincided with those noted by Cherginets *et al* (37). These results of the analysis demonstrated that the major PS structures were retained in all samples. However, the slight shifts of angles and lattice constants showed that certain minerals were replaced or amalgamated together in PS structures. Notably, BSSS had a similar shift of peak (inserts of Fig. 7A) and lattice constant (Fig. 7B), as compared with the BS. BS had a greater peak shift from the PS peak, as compared with SDS. Therefore, XRD analysis demonstrated that BSSS could retain not only minerals from BS but also the same amalgamation of minerals with salt to produce near-identical crystals, which are important for retaining the benefits of BS.

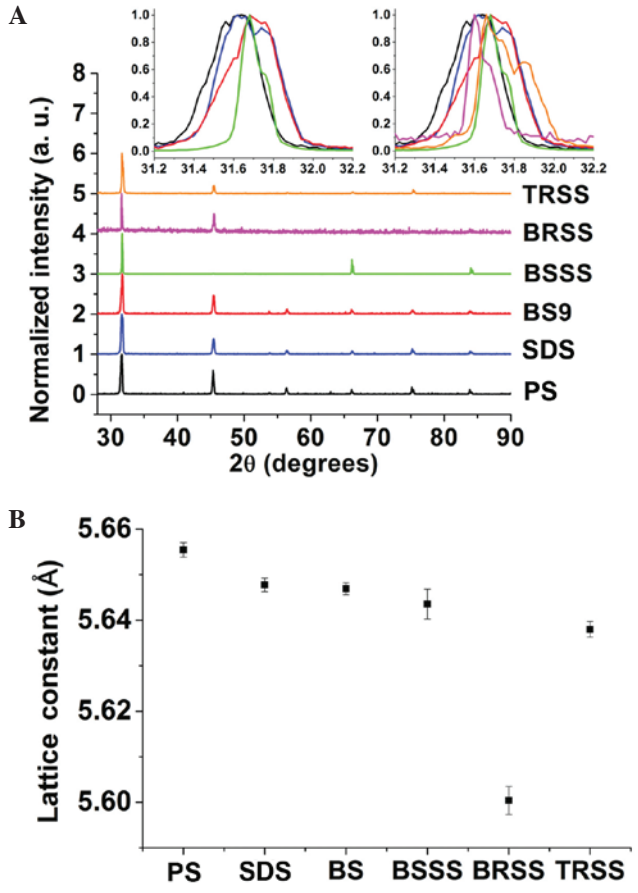

Figure 7. (A) X-ray diffraction (XRD) analysis for bamboo salt soy sauce (BSSS), brewed soy sauce (BRSS) and traditional soy sauce (TRSS) with bamboo salt (BS), sun-dried salt (SDS) and purified salt (PS) controls. From 31.2 to 32.2˚, it was magnified on the top two inserts with two kinds of combinations. The left is the comparison of BSSS to controls BS, SDS and PS. The right shows all samples and controls. (B) Lattice constants from XRD analysis for BSSS, BRSS and TRSS with PS, SDS and BS controls.

Discussion

In spite of the numerous reported pharmacological benefits of SS, commercial SS contains common salt, which has been associated with raised blood pressure, and an increased risk of cardiovascular diseases and stroke when consumed at higher levels than the daily recommended amount. In order to overcome this shortcoming of SS, BSSS was prepared by replacing common salt with BS during the manufacturing process. In the present study, BSSS exhibited pharmacological efficacy without the side effects of common salt, as well as retaining the desired salty taste of SS (8,10-13).

The present study hypothesized that BSSS may limit the side effects of SS, which includes high levels of common salt, and would increase the potential pharmacological efficacy of SS. Among the numerous advantages of SS and BS, the present study focused on the reports that SS contains high levels of antioxidants (1-4), and exhibits a high total antioxidant activity (1,5), as well as the anti-apoptotic effects reported for BS (19). Therefore, it was hypothesized that BSSS may have a superior protective efficacy against oxidative stress, as compared with conventional SS, due to the replacement of common salt with BS.

The present study aimed to evaluate whether BSSS had unique neuroprotective effects in the prevention of H_2O_2-induced neuronal cell death, and to demonstrate its underlying protective mechanisms, particularly focusing on the PI3K/Akt mediated signaling pathway. Initially, the optimal H_2O_2 concentration for studying H_2O_2-induced cortical neuronal cell toxicity, providing ~65% cell viability, was deduced as 100 μM H_2O_2. In addition, it was determined that BSSS did not have direct toxic effects on cell viability at any concentration, from 0.001 to 10%, as compared with TRSS and BRSS. Furthermore, it was demonstrated that only BSSS pretreatment exerted cytoprotective effects against 100 μM H_2O_2-induced neuronal cell death, and reduced apoptotic cell damage and H_2O_2-induced ROS production. The results of the present study suggested that BSSS had protective efficacy against H_2O_2-induced oxidative stress, suppressed cell dysfunction in cortical neuronal cells and induced antioxidative effects. A previous study supported the involvement of BSSS in the inhibition of ROS production, and demonstrated its antioxidative activities (38).

In order to understand the protective mechanism underlying the BSSS-mediated prevention of oxidative stress, the present study particularly focused on the PI3K/Akt pathway. The PI3K/Akt pathway has been demonstrated to have an important role in cell survival (39). Phospho-Akt directly affects GSK-3β activity via phosphorylation at Ser9, and GSK-3β activation via phospho-Akt inhibition may induce the mitochondrial cell death pathway, which is associated with cytochrome *c* release from the mitochondria and activation of caspase-3 (40). Numerous studies have demonstrated that H_2O_2-induced neuronal cell death is associated with the PI3K/Akt pathway (41,42); and this prompted the present study to hypothesize that BSSS-mediated Akt activation may be associated with the protective effects of BSSS against H_2O_2-induced cytotoxicity.

In order to validate this hypothesis, western blotting was used to demonstrate the ability of BSSS to attenuate cell death-related signals, and enhance survival signals through the PI3K-Akt pathway. BSSS was able to increase the levels of the extrinsic growth factors, Akt and GSK3β, which generate an anti-apoptotic response and promote cell survival through their ability to promote phosphorylation and inactivate apoptotic factors (43). Conversely, BSSS was able to downregulate components of the intrinsic pathway, decreasing the levels of apoptosis signaling molecules, including BAX, caspase-9, caspase-3, cytochrome *c*, PAR, cleaved PARP, and AIF (44). Furthermore, the present study demonstrated that the protective effects of BSSS were attenuated following treatment of the cells with LY294002, a PI3K inhibitor. The results of the present study supported the hypothesis that activation of the PI3K/Akt pathway may be associated with the protective effects of BSSS.

In an effort to elucidate why BSSS enhanced neuronal cell viability and inhibited apoptosis, the mineral contents of BSSS, TRSS, and BRSS, were analyzed. BSSS contains 39 categories of minerals indispensable for human functioning, including K, Ca, Mg, S, Fe, P, Rb, Mo, V, Au, Pt, Ge and Se. Among them, the levels of K, Ca, P, Rb, Mo, V, Au, and Se in BSSS, were higher, as compared with those of TRSS and BRSS. These various mineral ions have crucial roles in cellular functions, including cell proliferation, energy metabolism, protein and DNA synthesis, cytoskeleton activa-

tion, and ROS scavenging activities (19). In particular, at high concentrations in BSSS, the additional potassium may have antioxidant activities by inhibiting ROS over-production in salt-sensitive hypertension, and thereby preventing cardiovascular damage (45). Furthermore, intracellular potassium may influence the efficacy and polarity of synaptic transmission in neurons (46). Selenium has been shown to protect against glutamate toxicity, hypoxia and ischemic brain damage, and has been associated with mitochondrial function (47). Vanadium is known for its antioxidant activity, supposedly forming well-defined complexes with antioxidants, including glutathione or superoxide dismutase (48,49). In addition, the formation of vanadium complexes on triglycerides may confer a positive antioxidant effect by inhibiting lipid peroxidation, which prevents the production of ROS (50). Molybdenum deficiency results in neurological damage in humans, which is most apparent in untreatable seizures and various brain dysmorphisms (51). The results of the present study suggested that a combination of various beneficial mineral ions in BSSS may act synergistically in the neuronal cell to protect against H_2O_2-induced oxidative stress.

The present study demonstrated that BSSS, which was produced using BS instead of common salt, was non-toxic to rat neuronal cells when administered at a concentration up to 10%, and may have potential neuroprotective effects, including the prevention of apoptosis through inhibition of cell toxicity caused by H_2O_2-induced oxidative stress. The findings of the present study clearly distinguish BSSS from conventional SS products, including TRSS and BRSS, which were shown to be toxic at high concentrations and were unable to confer protection against oxidative stress. Further *in vitro* and *in vivo* studies are required, in order to confirm and understand the antioxidant activity of BSSS.

In conclusion, the present study demonstrated that the neuroprotective effects of BSSS against H_2O_2-induced oxidative stress conditions in a rat cortical neuronal cell model were related not only to anti-apoptotic and ROS-scavenging activities, but also to the activation of the PI3K/AKT pathway, which was verified using the PI3K inhibitor, LY294002. Conversely, the general SS products, TRSS and BRSS, did not demonstrate such neuroprotective activities. Therefore, considering the only difference between BSSS and SS was the use of BS instead of common salt in the production process, it may be hypothesized that it was the unique mineral composition of BSSS that contributed to the neuroprotective effect of BSSS on H_2O_2-induced oxidative stress.

Following the results of the present study, future endeavors should include identifying the active ingredients of BSSS and studying the neuroprotective effects of BSSS *in vivo*. Future studies may contribute to the prevention and treatment of brain diseases or aging processes, including Alzheimer's disease, which is closely associated with neuronal cell death (52).

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