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

JONG HEE JEONG\(^1\)\(^*\), MIN-YOUNG NOH\(^2\)\(^*\), JAE-HYEOK CHOI\(^3\)\(^4\), HAIWON LEE\(^1\)\(^5\) and SEUNG HYUN KIM\(^2\)

1Department of Convergences Nanoscience, College of Natural Science, Hanyang University, Seoul 133791;  
2Department of Neurology, College of Medicine, Hanyang University, Seoul 133791, Republic of Korea;  
3School of Materials Science and Engineering, Nanyang Technological University, Singapore 639798;  
4Centre for Biomimetic Sensor Science, Nanyang Technological University, Singapore 637553, Republic of Singapore;  
5Department of Chemistry, College of Natural Science, Hanyang University, Seoul 133070, Republic of Korea

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Abstract. Bamboo salt (BS) and soy sauce (SS) are traditional foods in Asia, which contain antioxidants that have cytoprotective 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 \( \mu \)M). 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, cytochrome c, apoptosis-inducing factor, cleaved caspase-9 and cleaved caspase-3, in all cases. Pretreatment of neuronal cells with BS significantly reduced the levels of ROS generated by \( H_2O_2 \), and increased the levels of phosphorylated AKT and phosphorylated glycogen synthase kinase-3\( \beta \). Furthermore, the observed effects of BSSS could be blocked by administration of 10 \( \mu \)M 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 (\( \geq 9x \)) roasting sun-dried salt (SDS) within a bamboo trunk, sealed by yellow soil, at a temperature \( >1,000\)\(^{\circ}\)C. BS becomes purple following these
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 activity (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$_2$O$_2$)-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$_2$O$_2$, 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 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$_2$O$_2$, 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, 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$_2$O$_2$ (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 2.5x10⁶ cells/cm², 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.

**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 radiomunoprecipitation 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.), 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).

**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 H₂O₂ for assessing neuronal cell viability.** To determine the optimal toxic dose of
H$_2$O$_2$ for assessing neuronal cell viability, rat cortical neurons were treated with 0, 25, 50, 100, 150 or 200 µM H$_2$O$_2$ 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$_2$O$_2$, as ~65% viability is usually deemed appropriate for the study of H$_2$O$_2$-induced neuronal toxicity.

**Determining the optimal concentration of BSSS for the 100 µM H$_2$O$_2$-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$_2$O$_2$-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$_2$O$_2$ 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$_2$O$_2$ treatment group; however, cell viability did not increase above the 1% concentration, as compared with the 100 µM H$_2$O$_2$ treatment group (65.2±3.45% in 100 µM H$_2$O$_2$ 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$_2$O$_2$-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$_2$O$_2$ for 30 min. As a control, cultured neuronal cells were stimulated with 100 µM H$_2$O$_2$ for 30 min, without BSSS pretreatment. TUNEL staining demonstrated that 61.3±3.21% neurons underwent apoptosis following incubation with 100 µM H$_2$O$_2$ for 30 min, whereas pretreatment of neurons with 0.01 and 0.1% BSSS significantly decreased H$_2$O$_2$-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$_2$O$_2$-mediated neuronal cell apoptosis and DNA fragmentation.

**Anti-oxidative effects of BSSS on H$_2$O$_2$-induced neurotoxicity.** To assess H$_2$O$_2$-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$_2$O$_2$ for 30 min. Free radical production significantly increased in the H$_2$O$_2$-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$_2$O$_2$ 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$_2$O$_2$-mediated ROS generation.
BSSS inhibits H$_2$O$_2$-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$_2$O$_2$ treatment alone (P<0.05; Fig. 4). These results suggest that BSSS may exert anti-apoptotic effects that resist H$_2$O$_2$-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-β (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$_2$O$_2$ treated group, 0.55±0.09; combined H$_2$O$_2$ with 0.01% BSSS pretreated group, 0.93±0.03; combined H$_2$O$_2$ with 0.1% BSSS pretreated group, 0.91±0.04; and combined H$_2$O$_2$ 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$_2$O$_2$-mediated cell apoptosis, the mineral content of...
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-

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**Figure 4.** Bamboo salt soy sauce (BSSS) inhibited the expression of hydrogen peroxide (H$_2$O$_2$)-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$_2$O$_2$ 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$_2$O$_2$-treatment only.
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.

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

| Mineral | BSSS | TRSS | BRSS |
|---------|------|------|------|
| K       | 4,300| 2,650| 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| ≤0.001|
| 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.

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.
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 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-

H$_2$O$_2$-induced neuronal cell death, and to demonstrate its underlying protective mechanisms, particularly focusing on the PI3K/Akt mediated signaling pathway. Initially, the optimal H$_2$O$_2$ concentration for studying H$_2$O$_2$-induced cortical neuronal cell toxicity, providing ~65% cell viability, was deduced as 100 µM H$_2$O$_2$. 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$_2$O$_2$-induced neuronal cell death, and reduced apoptotic cell damage and H$_2$O$_2$-induced ROS production. The results of the present study suggested that BSSS had protective efficacy against H$_2$O$_2$-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$_2$O$_2$-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$_2$O$_2$-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).

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 oxidative stress, as compared with conventional BS. It was hypothesized that BSSS may have a superior protective efficacy against oxidative stress, as compared with conventional BS. Therefore, 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.

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.
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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