Journal of
Food Biochemistry

Journal of Food Biochemistry ISSN 1745-4514

HEAT-TREATED SOLAR SEA SALT HAS ANTIOXIDANT ACTIVITY *IN VITRO* AND PRODUCES LESS OXIDATIVE STRESS IN RATS COMPARED WITH UNTREATED SOLAR SEA SALT

TIAN-CHENG GAO[1,3]*, JEONG-YONG CHO[1]*, LING-YUN FENG[1], SAORAYA CHANMUANG[1], SUTHIPONG PONGWORN[1], LILY JAISWAL[1], CHUNG-KYOON AUH[2], TONG-KUN PAI[4] and KYUNG-SIK HAM[1,5]

[1]Department of Food Engineering & Solar Salt Research Center,
[2]Department of Biological Science, Mokpo National University, Jeonnam 534-729, Korea
[3]College of Marine Science & Engineering, Tianjin University of Science & Technology; Tianjin Key Laboratory of Marine Resources and Chemistry, Tianjin 300-457, China
[4]Department of Food and Nutrition, Anyang University, Gyonggi, Korea

[5]Corresponding author.
TEL: +82-61-450-2425;
FAX: +82-61-454-1521;
EMAIL: ksham@mokpo.ac.kr

*These authors contributed equally to this study.

Received for Publication December 8, 2014
Accepted for Publication June 15, 2015

doi:10.1111/jfbc.12165

ABSTRACT

We have investigated the *in vitro* and *in vivo* antioxidative activity of solar sea salts(SS) roasted with (BS) or without (RS) bamboo, which are widely consumed as dietary salts in Korea. BS exhibited antioxidative activity in the *in vitro* assays of various radical-scavenging activities and DNA oxidation. RS also scavenged superoxide radicals and inhibited DNA oxidation. However, SS did not exhibit antioxidative activity *in vitro*. Sprague-Dawley rats were orally administered various salts (1.8 g NaCl equivalent/kg) daily for 7 weeks. The rats fed RS and BS exhibited significantly lower levels of lipid peroxidation and a higher total thiol content than the SS group ($P < 0.05$). The RS and BS groups exhibited a lower protein carbonyl content and expression level of nuclear factor-kappaB (NF-κB) p65 than the SS group. These results indicate that roasted solar sea salts (RS and BS) generate less oxidative stress than SS.

PRACTICAL APPLICATIONS

Heat-treated solar sea salt (SS) is a traditional Korean processed salt used as a dietary salt. SS roasted with (BS) or without (RS) bamboo exhibited antioxidative activity *in vitro* and generated less oxidative stress than SS in rats. BS exhibited the highest antioxidative effect *in vitro* and *in vivo*. These results suggest that roasted SSs could be consumed as an alternative dietary salt and could be important in the food and pharmaceutical industries.

INTRODUCTION

Reactive oxygen species (ROS) generated in the body cause oxidative damage (Brieger *et al.* 2012). The oxidation of cell components, such as lipids, proteins, and DNA, has been implicated in various human diseases, including atherosclerosis, cancer and aging (Cooke *et al.* 2003). Antioxidant foods, including cereals, vegetables and fruits, have been reported to mitigate ROS generated in the body (Goodman *et al.* 2011).

Excessive sodium intake generates ROS and causes oxidative stress and inflammation (Banday *et al.* 2007; Zhu *et al.* 2007). In addition, it can cause various health problems, such as hypertension (Cheng *et al.* 2001) and insulin resistance (Ogihara *et al.* 2003). However, the current debate on salt restriction is intensifying because an increasing number of new studies suggest that a low sodium intake leads to an increased risk of health problems, such as cardiovascular disease (O'Donnell *et al.* 2011, 2014; Stolarz-Skrzypek *et al.* 2011; Graudal *et al.* 2012), insulin resistance (Prada *et al.* 2005; Garg *et al.* 2011), increased mortality in type 2 diabetic patients (Ekinci *et al.* 2011) and depression (Goldstein and Leshem 2014). Therefore, the optimum daily dietary sodium intake should be re-evaluated (Heaney 2013). In addition, a good dietary salt that reduces the health-related problems caused by salt is required.

Most dietary salts consumed daily, including refined, purified, rock and solar sea salts (SSs), are mainly composed of NaCl with a low amount of other minerals (Tan *et al.* 2012). However, some SSs contain ample minerals, such as magnesium (Mg), potassium (K) and calcium (Ca) (Tan *et al.* 2012). The intake of salts supplemented with Mg and K has been reported to confer beneficial effects on the treatment of insulin resistance and hypertension (Ogihara *et al.* 2003; Sarkkinen *et al.* 2011). Recently, we have found that the intake of natural SS containing various minerals was associated with less oxidative stress compared with that seen with a mineral-deficient salt containing only NaCl. However, both types of salt did not exhibit antioxidant activity *in vitro* when they were evaluated by measuring the ferric-reducing antioxidant power, 1,1-diphenyl-2-picrylhydrazyl (DPPH)-, 2,2′-azino-bis(3-ethylebenzothiazoline-6-sulphonate)$^{+}$ ($ABTS^{+}$)- and the superoxide anion radical activity (Gao *et al.* 2014). Therefore, particular salts might be expected to serve as a good dietary salt for reducing salt-related health problems even though the same amount of NaCl is consumed daily.

Roasted SSs, a Korean traditional processed salt, have been widely consumed as a dietary salt and as an ingredient in fermented food and toothpaste for hundreds of years (Sohn *et al.* 1991). Roasted SSs are mainly produced with (BS) and without (RS) bamboo. Recently, BS has been reported to provide various benefits, such as antimicrobial (Moon *et al.* 2009), anti-inflammatory (Shin *et al.* 2003), cancer preventive (Zhao *et al.* 2013) and anti-allergy (Kim *et al.* 2012) effects. BS has DPPH radical-scavenging activity (Zhao *et al.* 2012) and inhibits ROS formation in human astrocyte U373MG cells (Om and Jeong 2007). However, the antioxidative activity of RS has not been investigated. In addition, information on the *in vivo* antioxidative activities of roasted SSs (i.e., BS and RS) is unavailable.

Here, we report on the *in vitro* antioxidant activity of roasted SSs (RS and BS) and the effects of those salts on oxidative stress in rats. Compositional changes of SSs during the roasting process were also investigated.

MATERIALS AND METHODS

Materials

SS was obtained from Taepyung Natural Salt Co., Ltd. (Sinan, Korea). RS and BS were provided by the Chung Soo Food Company (Muan, Korea). Briefly, the SS was roasted for 30 min without bamboo at 800C to obtain RS. In the case of BS, the RS was roasted twice with bamboo for 30 min at 800C and then further roasted with bamboo at 1,100C. BS was obtained after removing the bamboo charcoal.

Chemicals

Trolox, 2,4,6-tripyridyltriazine, 2,2′-azobis dihydrochloride, calf thymus DNA, 2,4-dinitrophenylhydrazine (DNPH), 5,5′-dithiobis(2-nitrobenzoic acid) (DNTB), DPPH and malondialdehyde (MDA) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). $ABTS^{+}$ was obtained from Bio Basic, Inc. (Markham, Ontario, Canada), and 1,10-phenanthroline was purchased from Daejung Chemicals, Ltd. (Hwaseong, Korea). Antibody against nuclear factor-kappaB p65 (NF-κB p65) was purchased from Upstate Biotech, Inc. (New York, NY). Rabbit anti-GAPDH polyclonal antibody was obtained from Ab Frontier (Seoul, Korea). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) was obtained from Millipore (Billerica, MA). All reagents used in this study were of analytical grade.

Determination of NaCl, Moisture, Total Insoluble Solids (TIS) and Sulfate (SO_4^{2-}) Content

The content of NaCl, moisture, TIS and SO_4^{2-} in the salts was determined using methods according to the Food Code of Korean Food and Drug Administration (KFDA 2005). Mohr's precipitation method was used to determine the NaCl content. The moisture content was measured by comparing the weights before and after oven drying at 105C. The TIS content was measured by using a glass microfiber filter (GF/F 0.7 μm, Whatman, Maidstone, U.K.). The barium chloride precipitation method was used for determining the sulfate (SO_4^{2-}) content.

Determination of Mineral Contents

Salts (5 g) were dissolved in 25 mL of 1.5 N HCl solution, and the solution was passed through a 0.45-μm membrane filter (Whatman) before analysis. All salt samples were analyzed for minerals using an atomic absorption spectrophotometer (AA-300A, Varian, Palo Alto, CA) and an inductively coupled plasma–atomic emission spectrometer (JY-138 Ultace, Jobin-Yvon Horiba, Longjumeau, France). All analyses were undertaken in peak height mode to determine the absorbance values in triplicate.

Determination of the Oxidation–Reduction Potential (ORP)

The salts (1 g) were dissolved in 50 mL of 0.1 M sodium phosphate buffer (pH 7.0). The ORP values of the salt solutions were measured at 25C using an oxidation–reduction potentiometer (RM-20P, Toa Denpa Kogyo Co., Tokyo, Japan). The ORP value is stated in millivolts (mV).

Assay of the DPPH Radical-Scavenging Activity

The free radical-scavenging activities of salts were evaluated using DPPH radicals according to the method of Brand-Williams *et al.* (1995), with slight modification. Briefly, an aqueous solution (50 μL) of each salt was added to a DPPH radical ethanol solution (950 μL, final concentration, 100 μM). The solution was then mixed and allowed to stand for 30 min in the dark. The free radical-scavenging activity of each salt was quantified by observing the decolorization of DPPH at 517 nm. The DPPH radical-scavenging activities of samples were also determined as a percentage decrease compared with the absorbance of a blank.

Determination of the $ABTS^+$ Radical-Scavenging Activity

An $ABTS^+$ radical solution was prepared from the reaction of 7.0 mM ABTS solution and 2.5 mM potassium persulfate (95/5, v/v) for 30 min at 70C in the dark. The $ABTS^+$ radical solution was diluted to an absorbance of 0.7 ± 0.05 at 735 nm by the addition of ethanol. Diluted $ABTS^+$ radical solution (0.98 mL) was added to 20 μL of sample solution. After incubating the mixture for 30 min, the absorbance was measured at 735 nm using a spectrophotometer (HP8542A, GMI, Inc., Ramsey, MN).

Determination of the Ferric Reducing/Antioxidant Power (FRAP)

The ferric ion reducing ability of each salt was measured by the FRAP assay according to the method described by Benzie and Strain (1996), with slight modification. Each salt solution (30 μL) was added to 100 μL of FRAP regent (250 mM sodium acetate buffer [pH 3.6]/10 mM TPTZ solution/20 mM ferric chloride solution = 10:1:1, v/v) and 90 μL of distilled water. The mixture was immediately incubated at room temperature for 4 min in the dark. The absorbance was measured at 593 nm using a spectrophotometer. The ferric ion reducing ability of each sample was expressed as the ferrous sulfate content.

Determination of the Superoxide Anion Radical-Scavenging Activity

The superoxide anion radical-scavenging activity was determined using the lucigenin/arginine/methylglyoxal system according to the method described by Jeong *et al.* (2009). A superoxide anion radical reaction solution (2.0 mM lucigenin [1.0 mL], 1.0 M arginine [0.05 mL], 1.4 μM methylglyoxal [0.05 mL], and phosphate buffer [pH 7.4, 1.0 mL]) was gently mixed in a quartz cuvette. The reaction mixture was incubated for approximately 13 min until chemiluminescence (CL) level reached a plateau on an ultra-weak chemiluminescence analyzer (UWLA, type BPCL-2-KIC, Institute of Biophysics, Academy of Sciences, Beijing, China). The salt solution (0.5 mg/10 μL) was added to the reaction mixture. The superoxide anion radical-scavenging activity of each sample was determined as the percentage decrease in the CL level against a control.

Determination of DNA Oxidation

The antioxidant ability of each salt was evaluated by measuring its inhibitory effects against copper ion/phenanthroline/H_2O_2-induced DNA oxidation according to the method of Ma *et al.* (1999), with slight modification. Briefly, 0.1 mL of DNA (final concentration, 5.0 μg/mL) and 0.1 mL of salt solution (final concentration, 5 mg/mL) were mixed with 0.2 mL of 0.1 M sodium acetate buffer (pH 5.2). The mixture was added to 0.25 mM $CuSO_4$ (0.2 mL) and 0.175 mM 1,10-phenanthroline (0.2 mL). After incubating at 37C for 5 min, the reaction mixture was added to 0.2 mL of H_2O_2. The kinetic curve of CL was immediately recorded by the UWLA.

Animal Studies

The animal experiment was approved by the Ethics Committee of Mokpo National University (Mokpo, Korea). Male Sprague-Dawley rats (8 months old, 610 ± 15 g) were purchased from Damool Science (Daejeon, Korea). The rats were housed in a room at constant humidity ($55 \pm 5\%$), temperature (25 ± 1C) and light cycle (12 h: 06:00–18:00). Food and water were available *ad libitum* throughout the study. The rats were divided into four groups (control, SS, RS and BS), with each group composed of eight rats. Each type of salt (equivalent to 1.8 g NaCl/kg) was orally administered to the animals daily for 7 weeks, except for the control group, which received oral administration of 0.9% saline solution, equivalent to 0.045 g of NaCl/kg. The rats were sacrificed, blood samples were taken, and the liver and adipose tissues were quickly removed. Each liver was cut into small pieces, which were weighed, and homogenized in 1:20 (w/v) 50 mM sodium phosphate buffer (pH 7.4) containing 20 mM ethylenediaminetetraacetic acid and 140 mM KCl. The homogenates were centrifuged at $5{,}000 \times g$ for 15 min at 4C. The protein concentration of supernatants was determined by the Bradford (1976) method using bovine serum γ-globulin as the standard. The remaining samples were frozen in liquid N_2 and stored at −70C until further analyses.

Determination of Lipid Peroxidation

An aliquot (0.25 mL) of plasma was mixed with 1.25 mL of 0.6 M HCl solution containing 20% trichloroacetic acid

(TCA). After pre-incubating for 10 min, 0.75 mL of 1.0 M NaOH solution containing 0.67% thiobarbituric acid was added. The mixture was incubated at 95C for 20 min in a boiling water bath, cooled and partitioned with 2.0 mL of *n*-butanol, shaken and centrifuged at 3,000 × g for 10 min. The absorbance of the upper layer (*n*-butanol) was measured at 532 nm. The calculation was based on a standard curve prepared with different dilutions of MDA.

Determination of the Protein Carbonyl (PCO) Content. Samples containing 2.0 mg of protein were mixed with 100 μL of 2.5 M HCl solution containing 10 mM DNPH. The mixture was incubated for 1 h at room temperature and vortexed every 15 min. The reaction mixture was added to 1.0 mL of cold 10% (w/v) TCA solution, and the solution was centrifuged at 3,000 × g for 10 min. The protein pellet was washed three times with 2.0 mL of ethanol/ethyl acetate (1:1 v/v) and dissolved in 1.5 mL of 6 M guanidine hydrochloride (pH 2.3). After incubating for 10 min at 37C, the absorbance was measured at 370 nm. The results were calculated as nmol of protein carbonyl/mg of protein using an extinction coefficient of 2.1×10^4 cm/mL.

Determination of the Total Thiol Content

The total thiol content in each liver homogenate was measured according to the method of Bahramikia *et al.* (2009). Each liver homogenate (0.3 mL) was mixed in stoppered tubes, with 1.5 mL of 200 mM Tris buffer (pH 8.2) and 0.1 mL of 10 mM DNTB. Absolute methanol was added to the mixture to reach 5.0 mL. After 15 min of reaction, each reaction mixture was centrifuged at 3,000 × g for 15 min at room temperature. The absorbance of each supernatant was measured at 412 nm. The molar extinction coefficient for total thiol at 412 nm was 13,100, and the thiol content is expressed as nmol/mg protein.

Western Blotting Analyses

The lysates (100 μg) in Laemmli sample buffer (Bio-Rad, Hercules, CA) were boiled at 95C for 5 min. Proteins were separated on 12% sodium dodecyl sulfate–polyacrylamide gels and transferred to a polyvinylidene fluoride (PVDF) membrane. The transfer was carried out for 1 h at room temperature at a constant voltage of 150 V. PVDF membranes were subsequently soaked for 1 h in Tris buffer and 0.05% Tween-20 (TBST) blocking buffer containing 3% bovine serum albumin. After blocking, the membranes were washed twice with TBST buffer for 5 min followed by overnight incubation with primary antibodies at 4C. The membranes were subsequently washed twice with TBST buffer for 10 min and then incubated with HRP-conjugated goat anti-rabbit IgG for 1 h. After washing with TBST and TBS buffers, the membranes were transferred to an enhanced chemiluminescence (ECL) solution containing luminol as the substrate, and they were exposed to ECL film for autoradiography. The visualized protein bands were scanned and analyzed with Gene Tools Software (4.01, Syngene, Cambridge, U.K.).

Statistical Analyses

Data were expressed as mean ± standard deviation ($n = 3$ or 8) using the Statistical Package for the Social Sciences (IMB, Armonk, NY) 17.0 package. The values were assessed by one-way analysis of variance, followed by the Tukey–Kramer test. Differences were considered significant at $P < 0.05$.

RESULTS

General Composition and Mineral Content of the Roasted SSs

The general composition and mineral content of the roasted SSs (RS and BS) are shown in Table 1. The NaCl content in BS was 97.5 ± 0.45%, which was higher than those of SS (86.5 ± 1.1%) and RS (94.3 ± 0.89%). SS exhibited a higher moisture (10.9 ± 1.2%) and sulfate (SO_4^{2-}, 2.7 ± 0.01%) content than the roasted salts. Mg, K and Ca were the major minerals in all salts. SS exhibited the highest Mg content. In contrast, BS exhibited the highest amount of K content, followed by Mg and Ca. The Mn, Zn and Cu contents were higher in RS and BS than in SS.

TABLE 1. GENERAL COMPOSITION AND MINERAL CONTENT OF ROASTED SOLAR SEA SALTS

	SS	RS	BS
NaCl (%)	86.5 ± 1.1[a]	94.3 ± 0.89[b]	97.5 ± 0.45[c]
Moisture (%)	10.9 ± 1.2[a]	0.18 ± 0.02[b]	0.13 ± 0.03[b]
TIS (%)	0.03 ± 0.01[a]	0.28 ± 0.03[b]	0.73 ± 0.04[c]
SO_4^{2-} (%)	2.7 ± 0.01[a]	0.92 ± 0.35[b]	0.42 ± 0.02[c]
Minerals (ppm)			
K	3,605 ± 155.1[a]	2,740 ± 65.9[b]	3,849 ± 83.1[a]
Mg	9,926 ± 301.1[c]	9,230 ± 218.1[b]	2,425 ± 95.2[a]
Ca	961 ± 20.1[a]	1,431 ± 20.2[b]	1,422 ± 0.2[b]
Mn	1.3 ± 0.1[a]	6.1 ± 0.6[b]	14.2 ± 0.02[c]
Zn	0.6 ± 0.2[a]	0.9 ± 0.5[b]	3.1 ± 0.2[c]
Cu	0.01 ± 0.0[a]	1.6 ± 0.0[b]	4.1 ± 0.2[c]

Values with different letters in a row are significantly different ($P < 0.05$).

Values are shown as mean ± standard deviation ($n = 3$).

BS, solar sea salt roasted with bamboo; RS, solar sea salt roasted without bamboo; SS, solar sea salt; TIS, total insoluble solids.

In Vitro Antioxidative Activity of the Roasted SSs

ORP Value. The ORP values of roasted salts are shown in Fig. 1A. SS had the highest ORP value (90.0 ± 3.5 mV). The ORP value of RS, which was roasted without bamboo, was 22.0 ± 2.8 mV. The BS, which was roasted repeatedly with bamboo, exhibited the lowest ORP value (−227.0 ± 4.2 mV).

DPPH Radical-Scavenging Activity. The radical-scavenging activity of the various salts at a concentration of 5–10 mg/mL was determined using DPPH radicals. SS and RS did not scavenge DPPH radical at a concentration of 10 mg/mL (Fig. 1B). Interestingly, BS, which was roasted with bamboo, had a DPPH radical-scavenging activity that increased with an increasing salt content. This result agreed with the results for DPPH radical-scavenging activity of BS reported previously (Zhao *et al.* 2012).

$ABTS^+$ Radical-Scavenging Activity. The free radical-scavenging activities of salts were evaluated by using $ABTS^+$ radical. BS exhibited high $ABTS^+$ radical-scavenging activity, and its activity increased depending on the salt concentration (Fig. 1C). The $ABTS^+$ radical-scavenging capability of BS was equivalent to approximately 0.81–1.1 μmol trolox/g salt. However, SS and RS at a concentration of 4 mg/mL did not scavenge $ABTS^+$ radical.

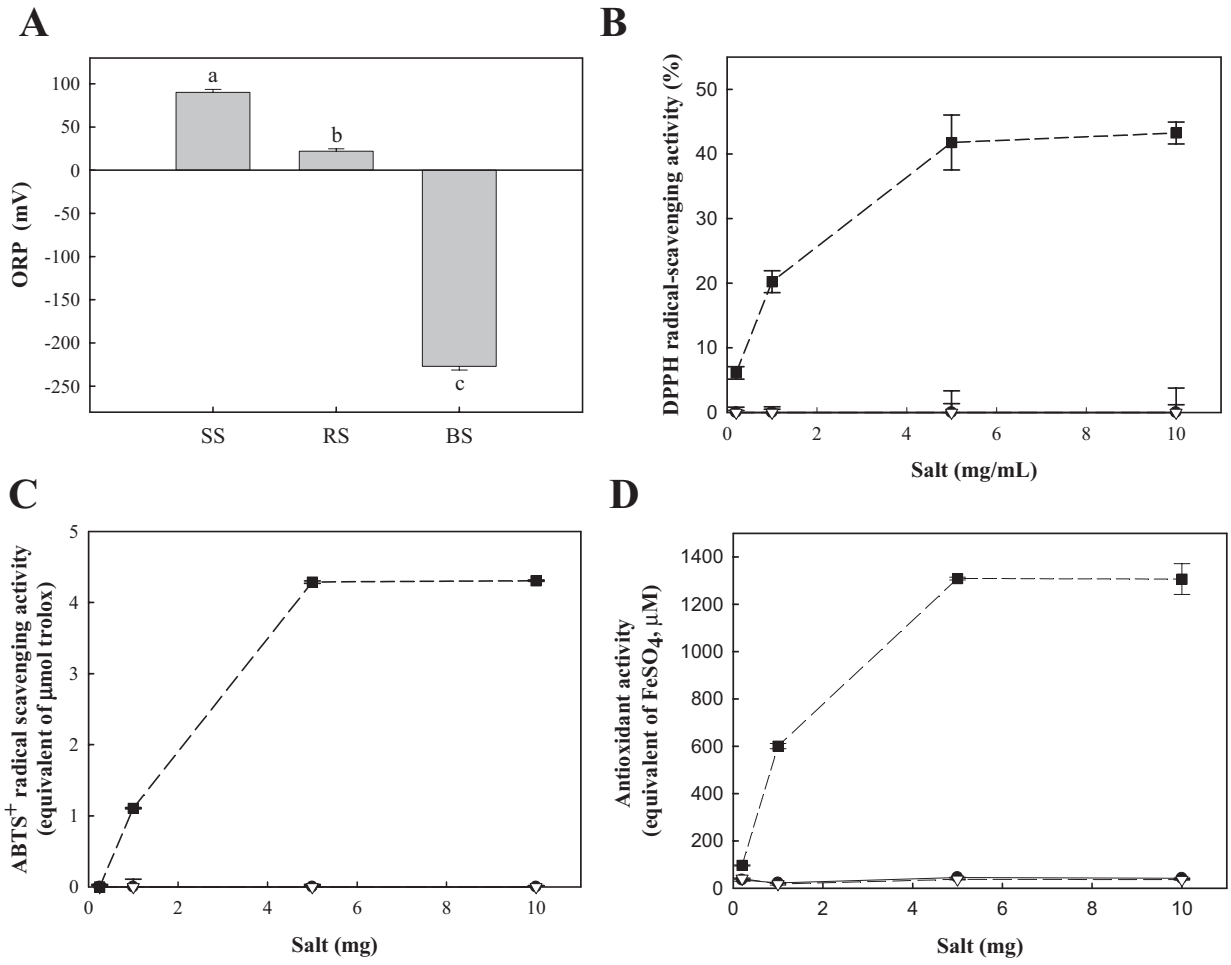

FIG. 1. ORP VALUE (A), DPPH (B) AND $ABTS^+$ (C) RADICAL-SCAVENGING ACTIVITIES, AND FERRIC REDUCING ANTIOXIDANT POWER (D) OF ROASTED SOLAR SEA SALTS

●, SS (solar sea salt); ▽, RS (solar sea salt roasted without bamboo); ■, BS (solar sea salt roasted with bamboo). Values are shown as mean ± standard deviation ($n = 3$). Values with different letters on bars are significantly different ($P < 0.05$). $ABTS^+$, 2,2′-azino-bis(3-ethylebenzothiazoline-6-sulphonate) $^+$; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ORP, oxidation–reduction potential.

Ferric Reducing Antioxidant Power (FRAP). The FRAP capacities of salts were measured by a ferric-tripyridyltriazine reducing assay. BS exhibited a FRAP capacity of 96.6–1,306.8 μmol $FeSO_4$ equivalent/g, which was consistent with the results of DPPH and $ABTS^+$ radical-scavenging activity assays. However, SS and RS did not exhibit FRAP capacity (Fig. 1D).

Superoxide Radical-Scavenging Activity. The superoxide anion radical-scavenging activity of the salts was evaluated using a lucigenin/arginine/methylglyoxal system to generate superoxide anion radical-based CL. When the salt was added three times to a superoxide anion radical solution after CL reached a plateau, the addition of the salts decreased CL levels (Fig. 2A–C). This result suggests that the salt has a superoxide anion radical-scavenging activity. However, a different degree of decrement was observed in the CL level depending on the salts added. The superoxide radical-scavenging activities of BS, RS and SS were 86.5, 65.3 and 24.0%, respectively.

Protective Effects of BS and RS against DNA Oxidation

The antioxidative activities of the salts were also determined using a copper ion/phenanthroline/H_2O_2-induced DNA oxidation system (Fig. 3). When DNA was oxidized by the addition of copper ion/phenanthroline/H_2O_2 solution, the intensity of the CL emission was increased. The addition of BS and RS in a copper ion/phenanthroline/H_2O_2 solution reduced the CL intensity, indicating that BS and RS inhibited DNA oxidation. In particular, the weak CL intensity of BS was more apparent than that of RS. However, the pattern and intensity of the CL emission after SS treatment was

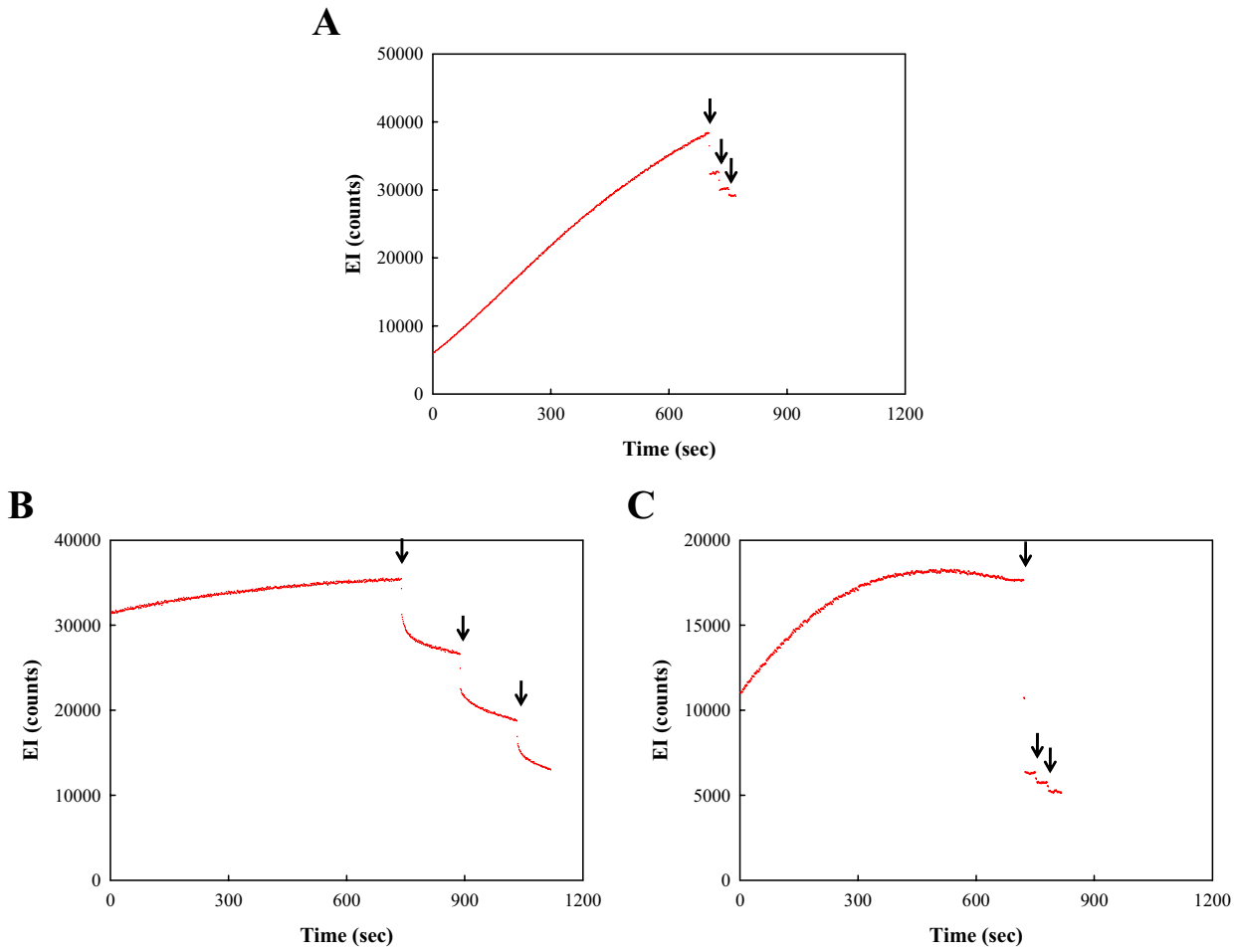

FIG. 2. SUPEROXIDE RADICAL-SCAVENGING ACTIVITIES OF SS (SOLAR SEA SALT) (A), RS (SOLAR SEA SALT ROASTED WITHOUT BAMBOO) (B) AND BS (SOLAR SEA SALT ROASTED WITH BAMBOO) (C)
The arrow indicates the addition of 5.0 mg salt solution in lucigenin/arginine/methylglyoxal-induced superoxide anion radical solution.

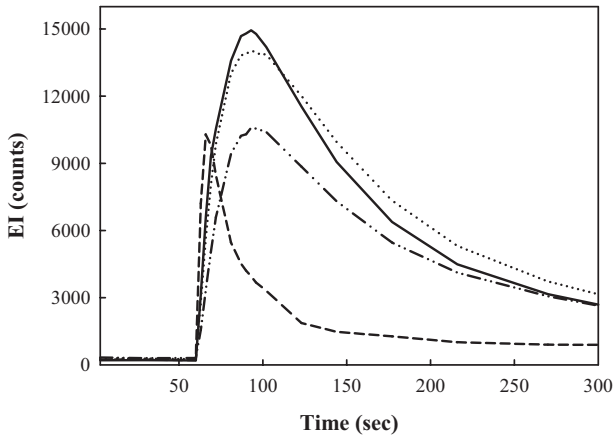

FIG. 3. INHIBITORY EFFECTS OF SS, RS AND BS AGAINST COPPER ION/PHENANTHROLINE/H_2O_2-INDUCED DNA OXIDATION
—, control; ····, SS (solar sea salt); ··—, RS (solar sea salt roasted without bamboo); – –, BS (solar sea salt roasted with bamboo).

similar to that of the control, indicating that SS did not inhibit DNA oxidation.

Effects of the Roasted Salts on Oxidative Stress in Rats

The salts were orally administered to SD rats (equivalent to 1.8 g NaCl/kg) for 7 weeks. Lipid peroxidation in plasma was assessed by measuring MDA levels. The MDA level in the plasma of rats treated with the roasted salts (RS and BS) was significantly lower than that of the SS group ($P < 0.05$) (Fig. 4A). In addition, the BS group (BS was roasted with bamboo) exhibited a significantly lower MDA level than RS (RS was roasted without bamboo). Interestingly, the BS group had a significantly lower MDA level than the control group. The RS and SS groups exhibited significantly higher

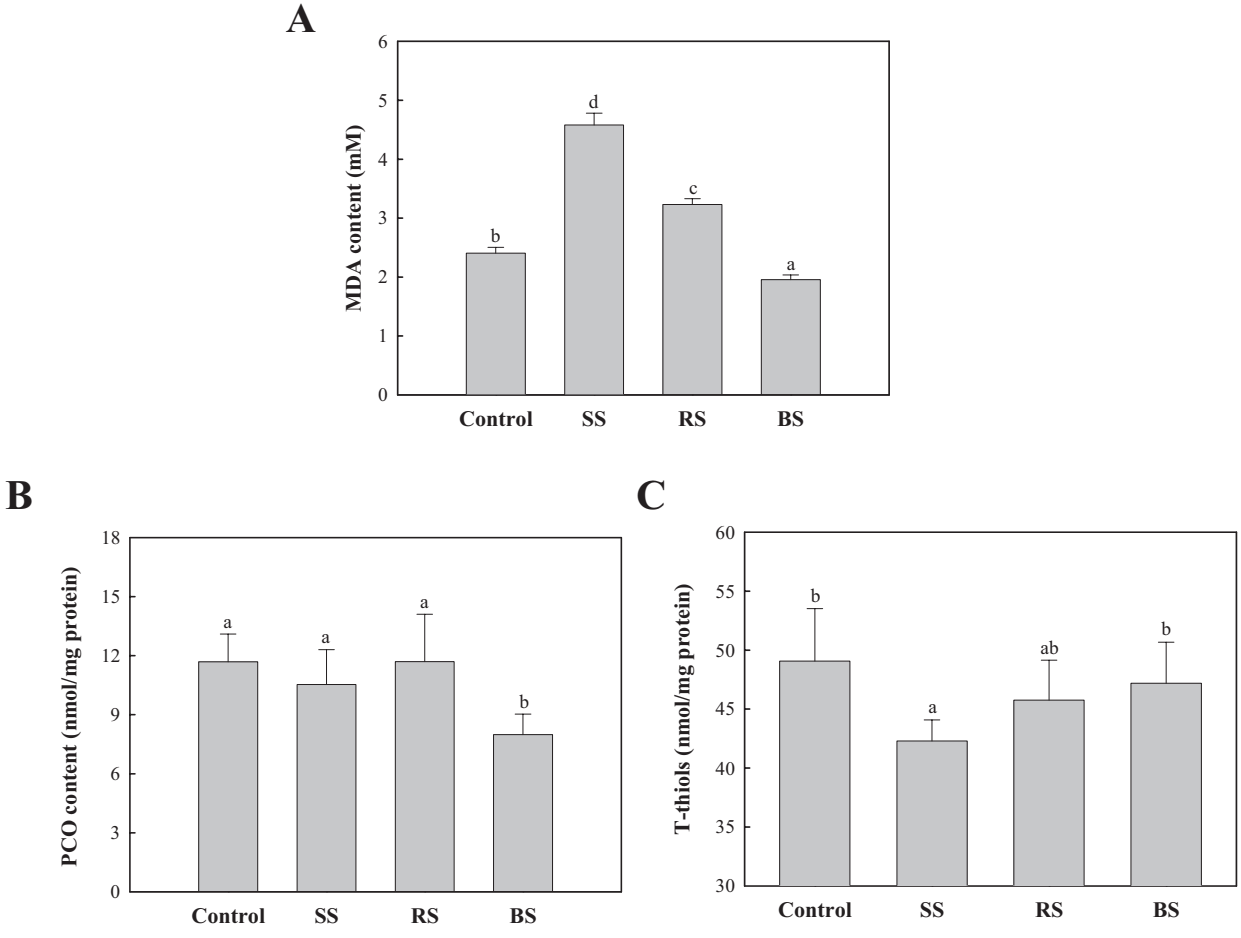

FIG. 4. MALONDIALDEHYDE (MDA) (A), PROTEIN CARBONYL (PCO) (B) AND TOTAL THIOL (C) CONTENT IN RATS ORALLY ADMINISTERED ROASTED SOLAR SEA SALTS DAILY FOR 7 WEEKS
Values are shown as mean ± standard deviation ($n = 8$). Values with different letters on bars are significantly different ($P < 0.05$). Control, 0.9% saline solution; SS, solar sea salt; RS, solar sea salt roasted without bamboo; BS, solar sea salt roasted with bamboo.

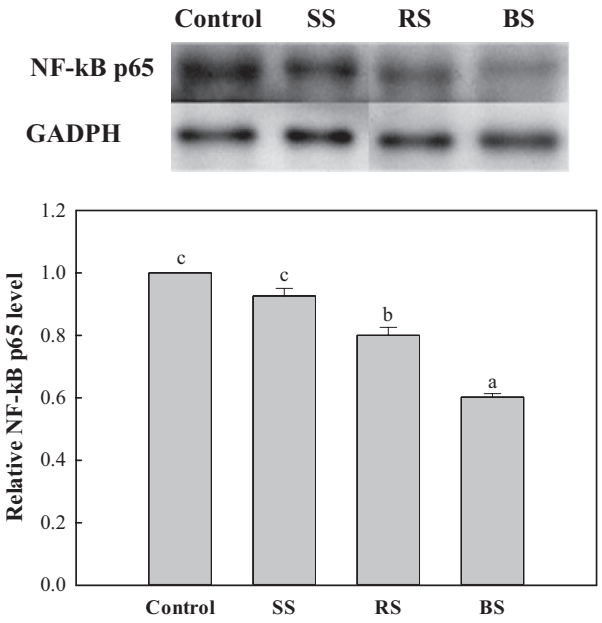

FIG. 5. EXPRESSION LEVEL OF NUCLEAR FACTOR-KAPPAB (NF-κB) p65 IN THE ADIPOSE TISSUE OF RATS ORALLY ADMINISTERED ROASTED SOLAR SEA SALTS DAILY FOR 7 WEEKS
Values are shown as mean ± standard deviation ($n = 8$). Values with different letters on bars are significantly different ($P < 0.05$). Control, 0.9% saline solution; SS, solar sea salt; RS, solar sea salt roasted without bamboo; BS, solar sea salt roasted with bamboo.

MDA levels than the control group ($P < 0.05$) (Fig. 4A). The oxidative damage of proteins was analyzed by measuring the PCO content (Fig. 4B). The PCO content in the SS and RS groups was highly similar to that in the control group. The BS group exhibited the lowest PCO level ($P < 0.05$), indicating that the lowest level of protein oxidation occurred in the BS group. In contrast, all salt diet groups exhibited a lower total thiol content than the control group (Fig. 4C). In particular, the SS group exhibited the lowest total thiol content, which suggested that a considerable amount of thiol groups were used for oxidation. The RS and BS groups had a lower total thiol content than the control group, although the difference was not statistically significant ($P < 0.05$). As shown in Fig. 5, the groups treated with the roasted SSs exhibited a significantly lower NF-κB p65 expression level when compared with the control and SS groups ($P < 0.05$). The BS group had the lowest NF-κB expression level followed by the RS group.

DISCUSSION

We have reported that mineral-rich SS lowered oxidative stress compared with the table salts that were mineral-deficient when the same amounts of NaCl were given *in vivo* (Gao *et al.* 2014). Reduced oxidative stress by mineral-rich salts is likely due to the various minerals contained in the salt. The mineral content in the roasted salts varied depending on the roasting conditions, such as temperature, roasting time and roasting number. When compared with SS, the roasted SSs (RS and BS) exhibited higher NaCl and TIS contents but a lower sulfate content (Table 1). Higher content of NaCl and TIS in RS and BS was likely due to the reduction of the moisture content and the formation of insoluble minerals, such as CaO and MgO, respectively, during roasting. Sulfate contents in RS and BS compared with that in SS were lower, which could be due to the formation of other sulfur compounds (e.g., Na_2S, MgS) from the sulfate of SS during roasting. In fact, some metal sulfides, such as Na_2S and MgS, could be detected in BS (data not shown). The Mg content in BS was significantly lower compared with that in RS and SS. This could be explained by the possibility of the formation of insoluble Mg compounds, such as MgO and MgS, which have a high melting temperature and could be precipitated during the preparation of BS and were thus not present in the BS final product. In contrast, K appeared to be increased in BS compared with RS, which could be due to the transfer of them from the bamboo during roasting.

Our previous study found that SS containing various minerals (e.g., Mg, K, Ca) did not show *in vitro* antioxidant activity (Gao *et al.* 2014). BS, which was roasted with bamboo, was found to show DPPH radical-scavenging activity (Zhao *et al.* 2012) and the inhibition of ROS formation in human astrocyte U373MG cells (Om and Jeong 2007). These results implied that roasting and bamboo may be essential factors generating antioxidative compounds. However, it was unclear whether antioxidative compounds could be produced by roasting SS without bamboo. Therefore, to reveal the potential role of bamboo in the formation of antioxidative compounds, we manufactured RS and BS and evaluated their *in vitro* antioxidative activity. Interestingly, in this study, the roasted SSs (RS and BS) were found to have *in vitro* antioxidative activity, although the activity was different in each. RS, which was roasted without bamboo, scavenged superoxide radicals and inhibited DNA oxidation (Figs. 2 and 3) but did not scavenge DPPH and $ABTS^+$ radicals. However, RS exhibited a lower antioxidative activity in all *in vitro* assays than BS, which was roasted three times with bamboo (Figs. 1–3). These results strongly indicate that roasting plays an important role in enhancing the antioxidative activity. In addition, bamboo may be an essential factor for more effectively enhancing the antioxidative activity. Although antioxidative compound(s) contained in the roasted SSs are not fully understood, antioxidative compound(s) appear to be produced from chemical changes of minerals in SS during the roasting process.

High salt intake has been reported to induce oxidative stress in rats (Banday *et al.* 2007; Zhu *et al.* 2007). In this study, to compare the effects of the roasted SSs on oxidative stress in an animal model, rats were orally dosed daily for 7 weeks with high amount (1.8 g NaCl/kg/day) of salts (SS, RS and BS). The amount of 1.8 g NaCl/kg/day was selected because many scientists who studied effects of salt on health used diets containing 4–8% NaCl and 1.8 g NaCl/kg/day was similar to the salt intake when a diet containing 4% NaCl would be fed if we assume that rats (body weight ~500 g) eat 20–25 g of diet per day. Oral administration of NaCl was chosen to reduce the fluctuation of salt intake that could come from the unequal diet consumption, although oral injection could give more stress to the animal. However, the amount of salt used in this study was much higher than the salt intake (Coelho *et al.* 2006) that is known to be optimum. Therefore, it is necessary to compare effects of various salts on the generation of oxidative stress at the level of salt intake that is known to be optimum. MDA is a representative marker of lipid peroxidation (Adibhatla and Hatcher 2010). Excessive MDA also induces the oxidization of protein thiols and the incorporation of carbonyl groups into polypeptide chains (Traverso *et al.* 2004). Therefore, PCO and the total thiol content have generally been used as biomarkers for protein oxidation (Dalle-Donne *et al.* 2003; Eaton 2006). In this study, we found differences in the oxidative stress caused by SS and roasted SSs in animal experiments (Fig. 4). The rats fed with BS exhibited the lowest level of MDA in the plasma and PCO in the liver (Figs. 4A and 4B). In addition, the rats fed with the roasted SSs exhibited a higher content of total thiols in the liver, which are known to be involved in protecting against oxidative stress, than the rats fed with the SS (Fig. 4C). RS suppressed the increasing levels of oxidative stress-related biomarkers induced by a high salt intake more efficiently than SS. However, the RS treatment exhibited higher levels of oxidative stress-related biomarkers than BS. Oxidative stress is also associated with inflammation by activating NF-κB via tumor necrosis factor alpha (TNF-α) (Vaziri 2008). In this study, we found that the rats fed with the roasted SSs exhibited a lower level of NF-κB expression in adipose tissue than the rats fed with the SS and BS showed the lowest level of NF-κB expression (Fig. 5). This result was also correlated well with the results of the oxidative stress. It was already reported that BS had anti-inflammatory activity via suppressing TNF-α, interleukin (IL)-1β and IL-6 secretion induced by NaCl in human mast cell line (HMC-1) (Shin *et al.* 2003). Therefore, our results confirm in animal model that BS has an anti-inflammatory activity, although more biomarkers for inflammation need to be studied *in vivo*. The levels of oxidative stress- and inflammation-related biomarkers were closely correlated with the *in vitro* antioxidative activity of the salts. These results indicate that the roasted SSs reduce oxidative stress and inflammation induced by a high salt intake. In particular, BS may act as an excellent antioxidant in biological systems.

Many studies reported that Mg and K improve hypertension and insulin resistance. Total mineral level decreased most significantly in BS and especially reduction of Mg level was the most significant. Even though BS had the lowest level of minerals, it showed the highest anti-oxidative effects *in vitro* and *in vivo* among the salts used in this study. It appeared that there was no relationship between minerals and antioxidant capacity of salts. We previously reported that mineral-rich salt generates less oxidative stress compared with mineral-deficient salt. Therefore, there must be some compounds in BS that could overcome the lack of minerals in BS and explain the antioxidative activity of BS. Investigation on antioxidative compounds in BS is now in progress.

CONCLUSION

In this study, we demonstrated that roasted SSs (BS and RS) had *in vitro* antioxidative activity and generated less oxidative stress than SS in rats. Therefore, the roasted SSs (BS and RS) are expected to be excellent dietary salts that can reduce oxidative stress in the body induced by excessive sodium intake. In that case, minerals were likely responsible for the reduction of oxidative stress. However, the major mineral content in RS and BS appeared to be decreased compared with that in SS. Therefore, the roasted SSs seem to contain antioxidative compounds that are likely produced during the roasting process. Moreover, in this study, BS was found to have a higher antioxidative activity *in vitro* and to efficiently suppress oxidative stress induced by a high salt diet in an animal model compared with RS. Therefore, bamboo may play an important role in the formation of antioxidative compounds while roasting the SS, as we discussed previously. Further studies on the role of bamboo and antioxidative compounds in RS and BS need to be done.

ACKNOWLEDGMENTS

This work was supported by Grant 20130290 to the Solar Salt Research Center of Mokpo National University from the Ministry of Oceans and Fisheries of Korea and Grant B0009747 to the Solar Salt and Halophyte Program from the Ministry of Knowledge Economy of Korea.

REFERENCES

ADIBHATLA, R.M. and HATCHER, J.F. 2010. Lipid oxidation and peroxidation in CNS health and disease: From molecular mechanisms to therapeutic opportunities. Antioxid. Redox Signal. *12*, 125–169.

BAHRAMIKIA, S., ARDESTANI, A. and YAZDANPARAST, R. 2009. Protective effects of four Iranian medicinal plants against free radical-mediated protein oxidation. Food Chem. *115*, 37–42.

BANDAY, A.A., MUHAMMAD, A.B., FAZILI, F.R. and LOKHANDWALA, M. 2007. Mechanisms of oxidative stress-induced increase in salt sensitivity and development of hypertension in Sprague-Dawley rats. Hypertension *49*, 664–671.

BENZIE, I.F.F. and STRAIN, J.J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. Anal. Biochem. *239*, 70–76.

BRADFORD, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. *72*, 248–254.

BRAND-WILLIAMS, W., CUVELIER, M.E. and BERSET, C. 1995. Use of a free radical method to evaluate antioxidant activity. LWT – Food Sci. Technol. *28*, 25–30.

BRIEGER, K., SCHIAVONE, S., MILLER, F.J. and KRAUSE, K.H. 2012. Reactive oxygen species: From health to disease. Swiss Med. Wkly. *142*, w13659.

CHENG, Z.J., VASKONEN, T., TIKKANEN, I., NURMINEN, K. and RUSKOAHO, H. 2001. Endothelial dysfunction and salt-sensitive hypertension in spontaneously diabetic Goto-Kakizaki rats. Hypertension *37*, 433–439.

COELHO, M.S., PASSADORE, M.D., GASPARETTI, A.L., BIBANCOS, T., PRADA, O.P., FURUKAWA, L.L., FURUKAWA, L.N.S., FUKUI, R.T., CASARINI, D.E., SAAD, M.J.A. *et al*. 2006. High- or low-salt diet from weaning to adulthood: Effect on body weight, food intake and energy balance in rats. Nutr. Metab. Cardiovasc. Dis. *16*, 148–155.

COOKE, M.S., EVANS, M.D., DIZDAROGLU, M. and LUNEC, J. 2003. Oxidative DNA damage: Mechanisms, mutation, and disease. FASEB J. *17*, 1195–1214.

DALLE-DONNE, I., ROSSI, R., GIUSTARINI, D., MILZANI, A. and COLOMBO, R. 2003. Protein carbonyl groups as biomarkers of oxidative stress. Clin. Chim. Acta *329*, 23–38.

EATON, P. 2006. Protein thiol oxidation in health and disease: Techniques for measuring disulfides and related modifications in complex protein mixtures. Free Radic. Biol. Med. *40*, 1889–1899.

EKINCI, E.I., CHEONG, K., CLARKE, S., MACISAAC, R.J., THOMAS, M.C., JERUMS, G. and MORAN, J.L. 2011. Dietary salt intake and mortality in patients with type 2 diabetes. Diabetes Care *34*, 703–709.

GAO, T.C., CHO, J.Y., FENG, L.Y., CHANMUANG, S., PARK, S.Y., AUH, C.K., PAI, T.K. and HAM, K.S. 2014. Mineral-rich solar sea salt generates less oxidative stress in rats than mineral-deficient salt. Food Sci. Biotechnol. *23*, 951–956.

GARG, R., WILLIAMS, G.H., HURWITS, S., BROWN, N.J., HOPKINS, P.N. and ADLER, G.K. 2011. Low-salt diet increases insulin resistance in healthy subjects. Metabolism *60*, 965–968.

GOLDSTEIN, P. and LESHEM, M. 2014. Dietary sodium, added salt, and serum sodium associations with growth and depression in the U.S. general population. Appetite *79*, 83–90.

GOODMAN, M., BOSTICK, R.M., KUCUK, O. and JONES, D.P. 2011. Clinical trials of antioxidants as cancer prevention agents: Past, present, and future. Free Radic. Biol. Med. *51*, 1068–1084.

GRAUDAL, N.A., HUBECK-GRAUDAL, T. and JÜRGENS, G. 2012. Effects of low-sodium diet versus high-sodium diet on blood pressure, renin, aldosterone, catecholamines, cholesterol, and triglyceride (Cochrane review). Am. J. Hypertens. *25*, 1–15.

HEANEY, R.P. 2013. Sodium: How and how not to set a nutrient intake recommendation. Am. J. Hypertens. *26*, 1194–1197.

JEONG, I.Y., LEE, H.J., PARK, Y.D., JIN, C.H., CHOI, D.S. and BYUN, M.W. 2009. Effects of gamma irradiation on total polyphenols, radical scavenging activities and decolourization of *Nelumbo nucifera* extracts. Radic. Phys. Chem. *78*, 575–577.

KIM, K.Y., NAM, S.Y., SHIN, T.K., PARK, K.Y., JEONG, H.J. and KIM, H.M. 2012. Bamboo salt reduces allergic responses by modulating the caspase-1 activation in an OVA-induced allergic rhinitis mouse model. Food Chem. Toxicol. *50*, 3480–3488.

KOREAN FOOD AND DRUG ADMINISTRATION (KFDA). 2005. Food Standard Codex. Korean Foods Industry Association, Seoul, Korea.

MA, W.J., CAO, E.H. and QIN, J.F. 1999. The involvement of singlet oxygen in copper-phenanthroline/H_2O_2-induced DNA base damage: A chemiluminescent study. Redox Rep. *4*, 271–276.

MOON, J.H., SHIN, H.A., RHA, Y.A. and OM, A.S. 2009. The intrinsic antimicrobial activity of bamboo salt against *Salmonella enteritidis*. Mol. Cell Toxicol. *5*, 323–327.

O'DONNELL, M., MENTE, A., RANGARAJAN, S., MCQUEEN, M.J., WANG, X., LIU, L., YAN, H., LEE, S.F., MONY, P., DEVANATH, A. *et al*. 2014. Urinary sodium and potassium excretion, mortality, and cardiovascular events. New Engl. J. Med. *371*, 612–623.

O'DONNELL, M.J., YUSUF, S., MENTE, A., GAO, P., MANN, J.F., TEO, K., MCQUEEN, M., SLEIGHT, P., SHARMA, A.M., DANS, A. *et al*. 2011. Urinary sodium and potassium excretion and risk of cardiovascular events. J. Am. Med. Assoc. *306*, 2229–2238.

OGIHARA, T., ASANO, T. and FUJITA, T. 2003. Contribution of salt intake to insulin resistance associated with hypertension. Life Sci. *73*, 509–523.

OM, A.S. and JEONG, J.H. 2007. Bamboo salts have antioxidant activity and inhibit ROS formation in human astrocyte U373MG cells. Cancer Prev. Res. *12*, 225–230.

PRADA, P.O., COELHO, M.S., ZECCHIN, H.G., DOLNIKOFF, M.S., GASPARETTI, A.L., FURUKAWA, N.S., SAAD, M.J. and HEIMANN, J.C. 2005. Low salt intake modulates insulin signaling, JNK activity and IRS-1Ser307 phosphorylation in rat tissues. J. Endocrinol. *185*, 429–437.

SARKKINEN, E.S., KASTARINEN, M.J., NISKANEN, T.H., KARJALAINEN, P.H., VENÄLÄINEN, T.M., UDANI, J.K. and NISKANEN, L.K. 2011. Feasibility and antihypertensive effect of replacing regular salt with mineral salt-rich in magnesium and potassium- in subjects with mildly elevated blood pressure. Nutr. J. *10*, 88–97.

SHIN, H.Y., LEE, E.H., KIM, C.Y., SHIN, T.Y., KIM, S.D., LEE, K.N., HONG, S.H. and KIM, H.M. 2003. Anti-inflammatory activity of Korean folk medicine purple bamboo salt. Immunopharmacol. Immunotoxicol. *25*, 377–384.

SOHN, W.S., YOO, Y.C. and KIM, C.Y. 1991. The effect of NaCl and bamboo salt on the growth of various oral bacteria. J. Korean Acad. Oral Health *15*, 255–268.

STOLARZ-SKRZYPEK, K., KUZNETSOVA, T., THIJS, L., TIKHONOFF, V., SEIDLEROVÁ, J., RICHART, T., JIN, Y., OLSZANECKA, A., MALYUTINA, S., CASIGLIA, E. *et al.* 2011. Fatal and nonfatal outcomes, incidence of hypertension, and blood pressure changes in relation to urinary sodium excretion. JAMA *305*, 1777–1785.

TAN, M.M., CUI, S., YOO, J., HAN, S.H., HAM, K.S., NAM, S.H. and LEE, Y. 2012. Feasibility of laser-induced breakdown spectroscopy (LIBS) for classification of sea salts. Appl. Spectrosc. *66*, 262–271.

TRAVERSO, N., MENINI, S., MAINERI, E.P., PATRIARCA, S., ODETTI, P., COTTALASSO, D., MARINARI, U.M. and PRONZATO, M.A. 2004. Malondialdehyde, a lipoperoxidation-derived aldehyde, can bring about secondary oxidative damage to proteins. J. Gerontol. A. Biol. Sci. Med. Sci. *59*, 890–895.

VAZIRI, N.D. 2008. Causal link between oxidative stress, inflammation, and hypertension. Iran J. Kidney Dis. *2*, 1–10.

ZHAO, X., JUNG, O.S. and PARK, K.Y. 2012. Alkaline and antioxidant effects of bamboo salt. J. Korean Soc. Food Sci. Nutr. *41*, 1301–1304.

ZHAO, X., DENG, X., PARK, K.Y., QUI, L. and PANG, L. 2013. Purple bamboo salt has anticancer activity in TCA8113 cells *in vitro* and preventive effects on buccal mucosa cancer in mice *in vivo*. Exp. Ther. Med. *5*, 549–554.

ZHU, J.X., HUANG, T.J. and LOMBARD, J.H. 2007. Effect of high-salt diet on vascular relaxation and oxidative stress in mesenteric resistance arteries. J. Vasc. Res. *44*, 382–390.