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RESEARCH NOTE

Mineral-rich Solar Sea Salt Generates Less Oxidative Stress in Rats than Mineral-deficient Salt

Tian-Cheng Gao, Jeong-Yong Cho, Ling-Yun Feng, Saoraya Chanmuang, Sun-Young Park, Chung-Kyoon Auh, Tong-Kun Pai, and Kyung-Sik Ham

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Abstract Certain solar sea salts have a high content of essential minerals other than NaCl. The effects of minerals in sea salts on oxidative stress in rats were investigated. Mineral-rich salt (MRS) and mineral-deficient salt (MDS) did not scavenge DPPH radicals. When MRS and MDS (equivalent to 1.8 g NaCl/kg/day) and a saline control were orally administered to rats for 7 weeks, MRS group rats showed lower levels of plasma lipid oxidation than MDS group rats. MRS group rats exhibited significantly lower levels of protein carbonyls and 8-hydroxy-2'-deoxyguanosine in the liver than MDS group rats. MRS group rats showed lower protein expression of NF-κB p65 in adipose tissue than MDS group rats. Intake of MRS may generate less oxidative stress than intake of MDS.

Keywords: mineral-rich salt, solar sea salt, oxidative stress, peroxidation, oxidation

Tian-Cheng Gao, Jeong-Yong Cho, Ling-Yun Feng, Saoraya Chanmuang, Sun-Young Park, Kyung-Sik Ham (✉)
Department of Food Biotechnology and Solar Salt Research Center, Mokpo National University, Jeonnam 534-729, Korea
Tel: +82-61-450-2425; Fax: +82-61-454-1521
E-mail: ksham@mokpo.ac.kr

Tian-Cheng Gao
College of Marine Science & Engineering, Tianjin University of Science & Technology, Tianjin 300-457, China

Chung-Kyoon Auh
Department of Biological Science, Mokpo National University, Jeonnam 534-729, Korea

Tong-Kun Pai
Department of Food and Nutrition, Anyang University, Gyeonggi 430-714, Korea

Introduction

Reactive oxygen species (ROS) are generated during cellular metabolism and mitochondrial energy production. Excessive production of ROS is associated with oxidative damage to DNA, RNA, proteins, and lipids. Oxidative damage leads to cancer, arthritis, inflammation, and heart disease (1,2). Therefore, much research has focused on antioxidant rich foods, herbs, and medicinal plants that might be used to lower ROS production (3,4).

Salt is essential for human and animal life. It plays an important part in physiological operations, including maintenance of membrane potentials and body fluid balances, by sustaining osmotic pressures and helping the digestion processes through transformation into hydrochloric acid in gastric juice. Salt is also used in food seasonings and food preservation.

The average salt intake in many developed countries is up to 12 g/day, which is about 2x greater than the recommended level (5). It is known that excessive intake of salt causes oxidative stress (6) and the metabolic syndromes of hypertension and insulin resistance (7,8). Table salts used by many people are mostly refined, purified, or rock salt that all lack non-NaCl minerals and contain greater than 99% NaCl. A few solar sea salts produced in some areas of France and Korea have been reported to contain appreciable amounts of potassium (K), magnesium (Mg), and calcium (Ca) (9). Differences in the NaCl and other mineral contents of salts may be due to differences in marine environments and/or production methods.

It is known that several minerals have beneficial effects on a number of diseases. Evidence has been presented that salt intake combined with K and Mg expands blood vessels (10,11), suppresses secretion of aldosterone and rennin,

and restrains the action of angiotensin II (12-14). Thus, mineral-rich solar sea salt (MRS) may be healthier than mineral-deficient salts (MDS). However, the effects of MRS on oxidative stress have not yet been characterized.

In this study, the general composition and mineral contents of MRS were determined. The effects of MRS on antioxidant activities both *in vitro* and *in vivo* were also investigated to explore whether MRS produces less oxidative stress than MDS in rats.

Materials and Methods

Materials and chemicals MRS was obtained from Tae Pyeong Salt Company (Sinan, Korea) and MDS was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Salts were dissolved in distilled water, then filtered through a 0.45 μm membrane filter (Whatman, Maidstone, UK) prior to physicochemical analyses. DPPH, 2,4-dinitrophenylhydrazine (DNPH), 5,5'-dithiobisnitrobenzoic acid (DNTB), malondialdehyde (MDA), and *n*-butanol were obtained from Sigma-Aldrich Co. Antibodies against nuclear factor kappa B p65 (NF-κB p65) were purchased from Upstate Biotech (New York, NY, USA). Rabbit anti-GAPDH polyclonal antibody was obtained from Ab Frontier (Seoul, Korea). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (IgG) was obtained from Millipore (Billerica, MS, USA). All other chemicals were of reagent grade.

Determination of NaCl, moisture, total insoluble solid (TIS), and sulfate (SO_4^{2-}) contents NaCl, moisture, TIS, and SO_4^{2-} contents in salts were determined according to the Food Code of Korea, Food and Drug Administration (KFDA, Osong, Korea) (15). The NaCl content was analyzed according to Mohr's method of precipitation (15). The moisture content was analyzed by comparing weights before and after oven-drying (DS-81-1; Dasol Scientific Co., Ltd., Hwaseong, Korea) at 105ºC. The TIS value was determined using a filtration method (15) with a glass microfiber filter (GF/F 0.7 μm; Whatman, Maidstone, UK). The sulfate (SO_4^{2-}) content was determined using a method based on precipitation of barium chloride (15).

Mineral analysis Salts (5 g) were added to 20 mL of $HNO_3/HClO_4$ (4:1, v/v). After heating at 100ºC, solutions were neutralized using addition of a NH_3 solution, then diluted 500 times using deionized water (15). Samples of this solution were analyzed for the mineral contents using an atomic absorption spectrophotometer (AA-300A; Varian, Palo Alto, CA, USA) and an inductively coupled plasma-atomic emission spectrometer (JY-138 Ultace; Jovin Yvon, Longjumeau, France) (15). All analyses were carried out in triplicate in peak height mode to determine absorbance values.

Assay of DPPH-radical scavenging capabilities The free radical-scavenging capabilities of salts were evaluated using a DPPH radical assay according to the method described by Leong and Shui (16) with slight modification. Briefly, a water solution (50 μL) of each salt was added to a DPPH radical 50% ethanol solution (950 μL; final concentration, 100 mM). The mixture was allowed to stand for 30 min in the dark. The free radical-scavenging activity of each salt was quantified by observing decolonization of DPPH at 517 nm. The DPPH radical-scavenging activities of salts were also determined as a percentage decrease in the absorbance value, compared with the absorbance value determined using a control sample.

Animal studies The study protocol with respect to animal experimentation was approved by the Ethics Committee of Mokpo National University (Mokpo, Korea). Male Sprague-Dawley rats (610±15 g) were purchased from Damool Science (Daejeon, Korea). Rats were housed in a room at constant humidity (55±5%), temperature (25±1ºC), and light cycle (12 h: 06:00 to 18:00). Food and water were provided *ad libitum* throughout the study.

Rats were randomly divided into 3 groups (n=8), which were orally administered a treatment on a daily basis. The control group (CON) received 1 mL of physiological saline (0.045 g NaCl/kg). The MRS group received 1 mL of MRS (equivalent to 1.8 g of NaCl/kg). The MDS group received 1 mL of MDS (equivalent to 1.8 g of NaCl/kg). After treatment administration for 7 weeks, animals were anesthetized using diethyl ether and the abdomen wall was opened. Blood was collected in heparinized tubes from the abdominal aorta. Rat plasma was isolated by centrifugation (1,500×*g*, Micro 17TR; Hanil Science Industrial, Incheon, Korea) at 4ºC for 20 min and was stored at −70ºC until use. In addition, liver and adipose tissues were collected, then immediately frozen in liquid nitrogen and stored at −70ºC until use.

Determination of the protein carbonyls (PCOs) content The PCOs content was measured using the method of Bahramikia *et al.* (17). An amount of 1 mL of a 2.5 N HCl solution containing 10 mM DNPH was added to samples (2.0 mg of protein). The reaction mixture was incubated for 1 h at room temperature and vortex-mixed (LV-100; LaboGene, Seoul, Korea) every 15 min. Then, 1.0 mL of cold trichloroacetic acid (TCA) (10 %, w/v) was added to each reaction mixture, followed by centrifugation (Micro 17TR; Hanil Science Industrial) at 3,000×*g* for 10 min. The protein pellet was washed 3 times with 2.0 mL of ethanol/ethyl acetate (1:1 v/v). The pellets were then

dissolved in 1.5 mL of 6 M guanidine hydrochloride (pH 2.3). After incubation for 10 min at 37°C, the absorbance was measured at 370 nm. The PCOs content was calculated as nmol of carbonyls groups per mg of protein using an extinction coefficient of 2.1×10^4/cm·mL.

Determination of the 8-hydroxy-2'-deoxyguanosine (8-OHdG) content Cellular DNA from liver tissue (sample weight, 400 mg) was extracted using a commercial kit (Easy-DNA kit; Invitrogen, Carlsbad, CA, USA). Samples were treated with 20 μL of ribonuclease A (2 mg/mL) at a final concentration of 40 μg/mL at 37°C for 30 min. The content of 8-OHdG was determined using an 8-OHdG ELISA Kit (OXIS International, Beverly Hills, CA, USA) and the absorbance was measured at 450 nm. The 8-OHdG standards (OXIS International) used for the assay ranged from 0.5 to 200 ng/mL. The concentration of 8-OHdG in test samples was interpolated from the standard curve using log transformation.

Determination of the tribarbituric acid reactive substance (TBARS) content Determination of the lipid peroxidate content was performed according to the method of Ledwozyw *et al.* (18). An aliquot (0.25 mL) of plasma was mixed with 1.25 mL of 20% TCA in 0.6 M HCl. After 10 min, 0.75 mL of 0.67% thiobarbituric acid in 1 M NaOH was added. The mixture was incubated for 20 min in a boiling water bath, then 2.0 mL of *n*-butanol was added after cooling. The mixture was shaken and centrifuged (Micro 17TR; Hanil Science Industrial) at 3,000×*g* for 10 min. The absorbance of the upper layer was measured at 532 nm. Calculation was based on a standard curve prepared using different dilutions of MDA.

Determination of NF-κB p65 using western blotting Rat adipose tissue lysates (20 μL) were boiled at 95°C for 5 min in a Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) to allow for protein denaturation. Proteins (50 μg) were placed on a stacking gel and separated on 10% sodium dodecyl sulfate-polyacrylamide, then transferred to a semi-dry blotting system using polyvinylidene fluoride (PVDF) membranes. Transfer was carried out for 1 h at room temperature at a constant voltage of 150 V. PVDF membranes were subsequently soaked in a Tris-buffer-saline plus 0.05% Tween-20 (TBST) blocking buffer containing 3% bovine serum albumin in a plastic container for 1 h. After blocking, membranes were washed with TBST (twice for 5 min each) incubated with primary antibodies, and gently rocked at 4°C overnight. Membranes were subsequently washed in TBST (twice for 10 min each) then incubated with HRP-conjugated goat anti-rabbit IgG for 1 h. After incubation, membranes were washed with TBST and TBS (twice for 10 min each) prior to visualization. Membranes were immersed in an enhanced chemiluminescence (ECL) solution containing luminol as a substrate, then were exposed to ECL film using the autoradiogram method. Visualized protein bands were scanned and analyzed using GeneTool software (version 4.01; Syngene, Cambridge, UK).

Determination of the protein content Each liver and adipose tissue sample was cut into small pieces, weighed, and homogenized in volumes (1:20, w/v) of 50 mM sodium phosphate buffer (pH 7.4) containing 20 mM EDTA and 140 mM KCl. Homogenates were centrifuged (Micro 17TR; Hanil Science Industrial) at 5,000×*g* for 15 min at 4°C, then the protein concentration of the supernatant was determined using Bradford's method (19) with bovine serum γ-globulin as a standard.

Statistical analysis Animal study data are expressed as mean±standard deviation (SD) and were analyzed using the Statistical Package for Social Sciences (SPSS; IBM, Armonk, NY, USA) 19.0 programs. Statistical differences were measured using a one-way ANOVA, followed by Duncan's multiple comparison test. Significant differences were defined at $p<0.05$.

Results and Discussion

General composition of MRS and MDS The general composition of MRS is shown in Table 1. The NaCl content of MRS was 86.95±0.89% and the moisture content was 11.4±0.19%. The sulfate (SO_4^{2-}) content of MRS was 2.7±0.01%. The mineral contents of MRS and MDS are shown in Table 2. The Mg, K, and Ca contents of MRS were relatively large. For MRS, Mg was present in the largest amount, followed by K and Ca. The contents of Mg, K, and Ca in MRS were 9,629.0±218.1 ppm, 2,764.2±65.9 ppm, and 1,365.4±20.2 ppm, respectively. The Sr content of MRS (62.0±0.8 ppm) was higher than other minor minerals.

DPPH radical-scavenging capabilities of MRS and MDS The antioxidative activities of MRS and MDS at different concentrations (0-10 mg) were evaluated using the DPPH radical. Both MRS and MDS did not scavenge DPPH radicals (data not shown). This pattern was similar to results for the DPPH radical-scavenging activity of MDS. Consequently, there was no *in vitro* antioxidative activity for either MRS or MDS on DPPH radicals.

Effects of MRS and MDS on oxidative stress in rats Rats were orally administered daily either MRS or MDS at a concentration of 1.8 g NaCl/kg for 7 weeks, then

Table 1. General composition of mineral-rich solar salt (MRS) and mineral-deficient salt (MDS)[1)]

Salt	General composition (%)			
	NaCl	Moisture	TIS	SO_4^{2-}
MRS	86.95±0.89	11.4±0.19	0.03±0.01	2.7±0.01
MDS	99.9±0.06	ND	0.01±0.001	ND

[1)]Values are mean±SD of three experiments. ND, not detected; TIS, total insoluble solid

Table 2. Mineral contents of MRS and MDS[1)]

Mineral	Content (ppm)	
	MRS	MDS
K	2764.2±65.9	17.4±1.1
Mg	9629.0±218.1	ND
Ca	1365.4±20.2	ND
Sr	62.0±0.8	1.0±0.1
Fe	4.9±1.7	ND
Mn	5.3±0.6	0.01±0.02
Zn	0.5±0.5	0.4±0.2
Cu	0.1±0.0	ND

[1)]Values are mean±SD of three experiments. ND, not detected

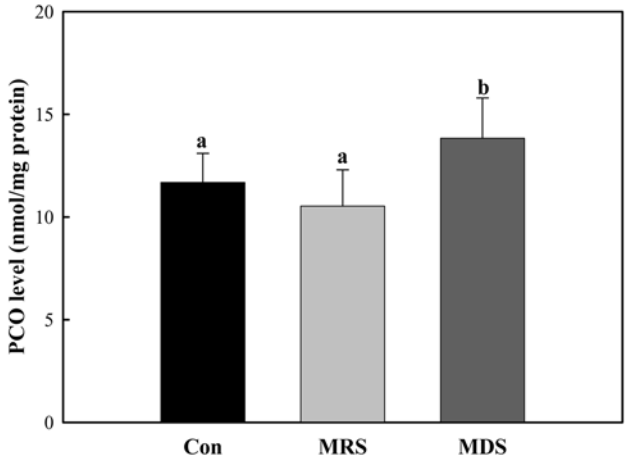

Fig. 1. PCOs content in the liver of rats orally administered MRS and MDS for 7 weeks. The protein carbonyl (PCOs) content in rat livers was determined using the colorimetric method with guanidine hydrochloride. Values are mean±SD (n=7 or 8). [a,b]Results with different letters on bars are significantly different (p<0.05). Con, control group; MRS, mineral-rich solar salt group; MDS, mineral-deficient salt group

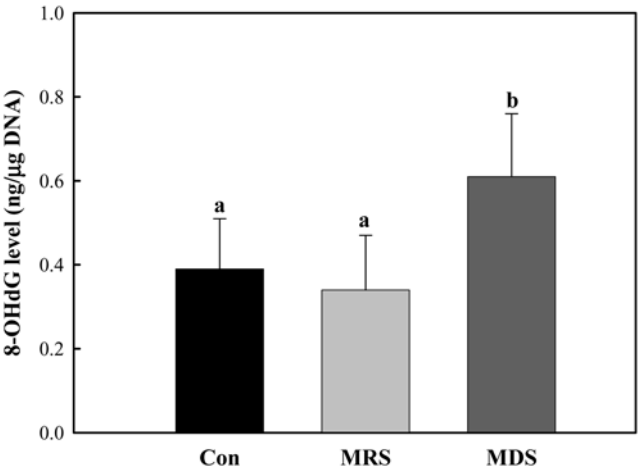

Fig. 2. DNA oxidation in the liver of rats orally administered MRS and MDS for 7 weeks. The 8-Hydroxy-2-deoxyguanosine (8-OHdG) content, as a biomarker of DNA oxidation in the rat liver, was determined using an 8-OHdG ELISA Kit. Values are mean±SD (n=7 or 8). [a,b]Results with different letters on bars are significantly different (p<0.05). Con, control group; MRS, mineral-rich solar salt group; MDS, mineral-deficient salt group

oxidative stress-related factors were measured. Formation of PCOs in the livers of MDS group was significantly (p<0.05) increased (1.18× higher) after administration of MDS for 7 weeks, compared with CON group (Fig. 1). Rats in the MRS group showed a significantly lower PCOs level, compared with rats of the MDS group (p<0.05).

It is known that oxidative damage produces 8-OHdG from DNA. Formation of 8-OHdG in the DNA of rat liver was induced by administration of MDS for 7 weeks. The 8-OHdG content in MDS group livers was significantly higher than MRS group livers (p<0.05), indicating that more DNA damage occurred in MDS group rats (Fig. 2). This result is consistent with results of the PCOs contents in rat livers.

Plasma MDA levels in the MRS and MDS group rats were significantly higher than in Con group rats (p<0.05) (Fig. 3). Plasma MDA level in the MRS group rats also was significantly lower than in MDS group rats (p<0.05).

Oxidative stress promotes inflammation by activating redox sensitive NF-κB which, in turn, triggers generation of proinflammatory cytokines, chemokines, and adhesion molecules. Therefore, NF-κB has been used as a marker of oxidative stress. NF-κB expression levels were determined via measurement of the amount of NF-κB p65 component using western blotting. NF-κB expression levels were lower in rats of the Con and MRS groups than in MDS group rats, and the relative intensity of NF-κB p65 expression in MRS group rats was similar to the intensity for rats in the Con group (Fig. 4A, 4B). These results indicate that MDS group rats had higher oxidative stress levels than rats in other groups.

Recently, several studies have reported that a Mg deficiency can lead to oxidative injury in the post-ischemic myocardium (20,21). A few studies (22,23) also have reported a link between low K and high Na levels in cells with an increased risk of cancer. Therefore, Mg and K rich MRS can reduce DNA damage via metabolic processing in rats although no antioxidant capacity was observed *in vitro*. In this study, the antioxidant activities of MRS and MDS were evaluated using various *in vitro* antioxidant assays for measurement of the ferric reducing antioxidant power,

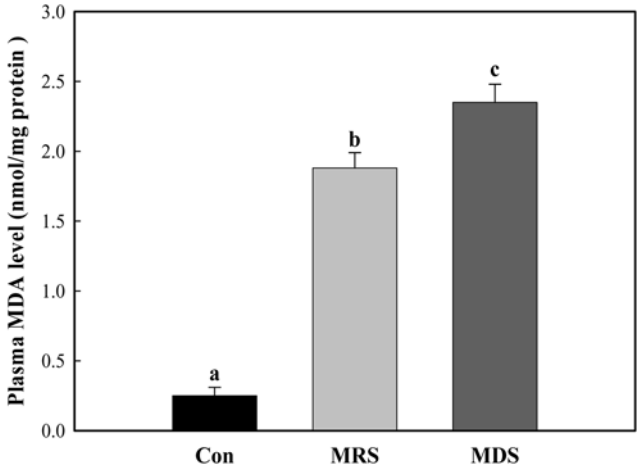

Fig. 3. Lipid peroxidation in the plasma of rats orally administered MRS and MDS for 7 weeks. The MDA content in rat plasma was determined using the TBARS method. Values are mean±SD (n=7 or 8). [a,b]Results with different letters on bars are significantly different (p<0.05). Con, control group; MRS, mineral-rich solar salt group; MDS, mineral-deficient salt group

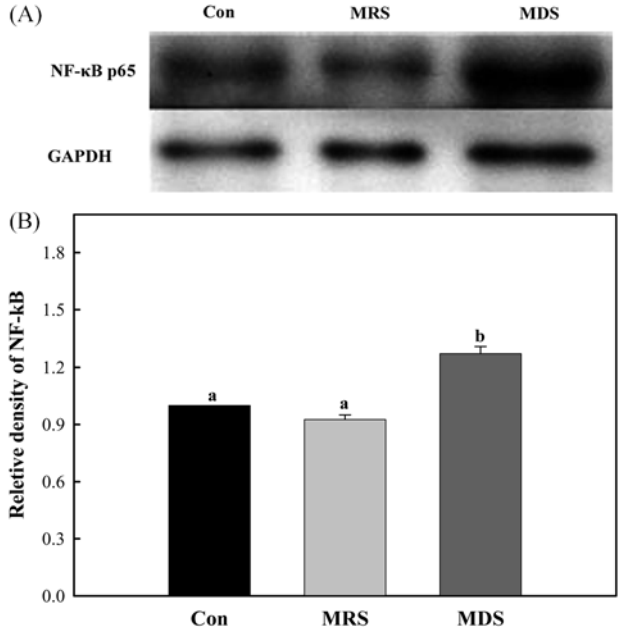

Fig. 4. Protein expression (A) and relative density (B) of NF-κB p65 in adipose tissue of rats orally administered MRS and MDS for 7 weeks. The expression level of NF-κB p65 in adipose tissue of rats was determined using western blotting. Values are mean±SD (n=7 or 8). [a,b]Results with different letters on bars are significantly different (p<0.05). Con, control group; MRS, mineral-rich solar salt group; MDS, mineral-deficient salt group

DPPH, 2,2-azino-bis (3-ethylebnzthiazoline-6-sulphonate), and superoxide anion radical scavenging capabilities. However, neither MRS nor MDS showed any antioxidant capacity (data not shown). However, differences in the antioxidant activities of MRS and MDS were observed using animal models.

MDA, one of the end products of lipid peroxidation, is commonly used as a marker of oxidative stress and antioxidant conditions in diseased patients. MDA is one of the final products of peroxidation of polyunsaturated fatty acids in cells. Overproduction of MDA causes secondary oxidative damage to proteins by oxidizing protein thiols, incorporating carbonyl groups into polypeptide chains, and by causing crosslinking between protein molecules (24). These processes lead to impairment of protein functions. In addition, determinations of the PCOs and 8-OHdG contents in urine have been widely used to assess oxidative protein modification (25) and oxidative DNA damage (26). MRS rats showed reduced levels of MDA, PCOs, and 8-OHdG, compared to MDS rats. MRS also showed a lower level of NF-κB expression, in agreement with other results for lipid peroxidation, protein oxidation, and oxidative DNA damage. Results of this study indicate that MRS generates less oxidative stress than MDS.

Several studies have reported that dietary salt is associated with increased oxidative stress in rats (27,28), and that oxidative stress is associated with salt-dependent hypertensive nephrosclerosis in Dahl salt-sensitive rats (29). In the previous studies cited herein related to oxidative stress (27-29), MDS was used as a salt treatment. A high salt intake has been shown to increase ROS generation and decrease expression of superoxide dismutase genes, resulting in oxidative stress in rats (6). A high salt diet has been shown to induce aggravated oxidative stress in animal models of hypertension (30). In this study, rats were orally administrated a large amount of salt (1.8 g NaCl/kg/day), which is probably much higher than a normal diet salt level (31), in order to explore the effects of oxidative stress between MRS and MDS over a short period of time,. As a next step, the effects of salts on oxidative stress at a physiological level should be investigated to determine chronic effects.

Minerals that are contained in MRS might play a role in the reduced level of oxidative stress observed in MRS group rats. K, Mg, and Ca are the main minerals in MRS, although other minerals are present in small amounts. It has been reported that Mg, K, and other minerals have beneficial effects on diabetes and hypertension (11,32). Therefore, reduced levels of oxidative stress in MRS rats may be due to Mg and K. However, it is possible that the balanced mineral composition of MRS is an important factor in reduction of oxidative stress in MRS group rats rather than the influence Mg and/or K. To expand this work, an investigation into the mechanism of how MRS reduces oxidative stress in comparison with MDS is needed. This is the first report that MRS produces less oxidative stress than MDS. It will be important to study whether the reduced level of oxidative stress for MRS is

due to an increased level of antioxidants, or reduced generation of reactive oxygen species.

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