Sulforaphane treatment protects skeletal muscle against damage induced by exhaustive exercise in rats

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Sulforaphane treatment protects skeletal muscle against damage induced by exhaustive exercise in rats. J Appl Physiol 107: 1028–1036, 2009. First published August 27, 2009; doi:10.1152/japplphysiol.00293.2009.—Sulforaphane (SF), one of the most important isothiocyanates in the human diet, present in cruciferous vegetables, is known to have chemopreventive activities in different tissues. No data are available on its effects in the prevention of skeletal muscle damage. In this study, we investigated the potential protective effects of SF treatment on muscle damage and oxidative stress induced by an acute bout of exhaustive exercise in rats. Male Wistar rats were treated with SF (25 mg/kg body wt ip) for 3 days before undergoing an acute exhaustive exercise protocol in a treadmill (+7% slope and 24 m/min). Acute exercise resulted in a significant increase in plasma lactate dehydrogenase (LDH) and creatine phosphokinase (CPK) activities. It also resulted in a significant increase in thiobarbituric acid-reactive substances (TBARS) (35, 49). Moreover, it has been well documented that acute exhaustive exercise causes structural damage to muscle cells as evidenced by an increase in plasma activity of cytosolic enzymes such as lactate dehydrogenase (LDH) and creatine phosphokinase (CPK) (14, 28, 54). These effects allow considering acute exhaustive exercise as an interesting model of oxidative stress and muscle damage.

Oxidative stress plays a major role in the pathophysiology of many diseases such as cardiovascular diseases (56) and cancer (27). To prevent and counteract ROS generation and oxidative stress, many studies have been focused on the identification of natural compounds acting as direct antioxidants with ROS-scavenging properties. However, not all the studies are in agreement in supporting the use of antioxidants to prevent oxidative stress-related diseases (32, 36). Recently, interest has been shifted to the possibility of counteracting oxidative stress by inducing endogenous antioxidant detoxifying enzymes known as phase II enzymes. The induction of these enzymes by molecules known as chemical inducers (6) involves the cis-acting antioxidant-responsive element (ARE), a specific DNA promoter-binding region that is found in the 5′-flanking region of antioxidant genes. The transcription of ARE-driven genes is regulated by nuclear erythroid 2 p45-related factor 2 (Nrf2), a redox-sensitive basic leucine zipper transcription factor that translocates to the nucleus, binds to AREs, and transactivates antioxidant genes (8).

Sulforaphane [SF; 1-isothiocyanate-(4R)-(methylsulfinyl)butane] is a natural isothiocyanate present as glucoraphanin in plants of the Brassicaceae family. According to environmental conditions (e.g., pH, metal ions, and other proteins), chewing, or cutting, glucoraphanin is hydrolyzed by the enzyme myrosinase to a variety of compounds; among them, isothiocyanates are the most common products (5). SF is known to have cancer-preventive activities in different tissues, and this action is due, at least partially, to the induction of phase II enzymes (57).

In this study, we assessed the ability of SF treatment to prevent skeletal muscle damage and oxidative stress in rats undergoing an acute exercise protocol by investigating its capacity to act as a chemical inducer able to activate Nrf2 and to enhance the expression and activity of phase II enzymes, namely, glutathione-S-transferase (GST), glutathione reductase (GR), and NAD(P)H:quinone oxidoreductase 1 (NQO1).

METHODS

Chemicals. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), digitonin, glucose-6-phosphate, sodium piruvate, glucose-6-phosphate dehydrogenase, NADP, NADPH, NADH, FAD, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diaminon
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salt (ABTS), Trolox, DMSO, menadione, dicumarol, 1-chloro-2,4-dinitrobenzene (CDNB), 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB), reduced glutathione (GSH), oxidized glutathione (GSSG), mammalian protease inhibitor mixture, pentobarbital sodium salt, anti-β-actin, and all other chemicals of the highest analytical grade were purchased from Sigma Chemical (St. Louis, MO) unless otherwise stated. Anti-GST-α was purchased from Alpha Diagnostic (San Antonio, TX), anti-GR was purchased from AbFrontier (Seoul, Korea), anti-glutathione peroxidase (GPx)-1 was purchased from LabFrontier (Seoul, Korea), anti-thioredoxin reductase (TR)-1 was purchased from Upstate (Lake Placid, NY), and anti-NQO1 and anti-Nrf2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). D,L-SF (Seoul, Korea), anti-glutathione peroxidase (GPx)-1 was purchased from LabFrontier (UK) by semidry electroblotting (1.5 mA/cm2). Nitrocellulose membranes (Hybond-C, Amersham Biosciences, Buckinghamshire, UK) were exposed to Hyperfilm ECL (Amersham Biosciences, UK). Blots were exposed to ECL Advance reagent for 1–2 min as described in the manufacturer’s protocol (GE Healthcare Biosciences, Buckinghamshire, UK). Blots were exposed to Hyperfilm-ECL (GE Healthcare Bio-Sciences) for 2–5 min and visualized using a Fluor S Multimager and Quantity One 4.1 (Bio-Rad Laboratories, Hercules, CA). The molecular weights of the bands were calculated by a comparison with prestained molecular weight markers (molecular weight range: 6,500–175,000) that were run in parallel with the samples. Semiquantitative analysis of specific immunolabeled bands was performed using a Fluor S image analyzer and Quantity One 4.1 (Bio-Rad Laboratories).

LDH activity assay. LDH activity was assayed in plasma samples by a spectrophotometric method based on the reduction of pyruvate to lactic acid coupled to NADH oxidation as reported by Korzeniewski et al. (31). The decrease in absorbance at 340 nm was monitored at 37°C. Data are reported as U/ml plasma. One unit of LDH activity is defined as the amount of enzyme that catalyzes the reduction of 1.0 µmol of pyruvate to l-lactate per minute at pH 7.0 at 25°C.

CK activity assay. CK activity was assayed in plasma samples by a spectrophotometric method based on the phosphorylation of ADP to ATP coupled to the reduction of NADP+ to NADPH, as catalyzed by glucose-6-phosphate dehydrogenase. The increase in absorbance at 340 nm was monitored at 30°C as reported by Noda et al. (42). Data are reported as U/ml plasma. One unit of CK activity is defined as the amount of enzyme that transfers 1.0 µmol of phosphate from phosphocreatine to ADP per minute at pH 7.4 at 30°C.

TBARS assay. TBARS were measured on muscle homogenates by the spectrophotometric method as reported by Ohkawa et al. (43) with slight modifications. A mixture of 0.1 ml of tissue homogenate, 0.1 ml of 30 g/l SDS, and 4 ml of color reagent containing 7 g/l of thiobarbituric acid was incubated in boiling water for 60 min. After the mixture had cooled, the organic layer was collected after centrifuging at 1,000 g for 10 min at 4°C. Absorbance was measured at 532 nm and compared with a standard curve constructed with known concentrations of malondialdehyde (MDA). Data are reported as nanomoles of MDA per milligram of protein.

Tissue total antioxidant activity. Tissue total antioxidant activity (TAA) was measured in muscle homogenates. Muscle samples were homogenized in 100 mM PBS (pH 7.4) containing 1% EDTA (100 mg/ml), and TAA was evaluated as reported by Re et al. (47). This method is based on the ability of antioxidant molecules in the sample to reduce the radical cation of ABTS, as determined by the decolorization of ABTS++ and measured as the quenching of absorbance at 740 nm. Values obtained for each sample were compared with the concentration-response curve of standard Trolox solutions and are expressed as micromoles of Trolox equivalent per milligram of protein.

Western immunoblot analysis. Muscle samples (100 mg) were placed in 1 ml of cold homogenization buffer (20 mM HEPES, 2 mM EDTA, 0.1% CHAPS, and 1% Triton X-100 containing mammalian protease inhibitor mixture, 1:100 dilution). Samples were boiled at 98°C for 3 min in boiling buffer [62.5 mM Tris (pH 6.8) containing 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.0025% bromophenol blue]. Boiled samples were run on 8% SDS-polyacrylamide gels (20 µg/lane), and proteins were transferred to nitrocellulose membranes (Hybond-C, Amersham Biosciences, Buckinghamshire, UK) by semidry electroblotting (1.5 mA/cm2). Nitrocellulose membranes were then incubated in blocking buffer [Tris-buffered saline (TBS) supplemented with 0.05% (vol/vol) Tween 20; TTBs] containing 5% (wt/vol) skimmed milk powder for 60 min at room temperature followed by three 5-min washes in TTBs. Blots were then incubated with anti-GST-α, anti-GR, anti-GPx, anti-TR, anti-NQO1, anti-Nrf2, and anti-β-actin as internal normalizers in TTBs containing 5% (wt/vol) skimmed milk powder (antibody buffer) overnight at 4°C. Membranes were washed three times for 5 min in TTBs and then incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:2,000 dilution) in antibody buffer for 60 min. Finally, blots were washed three times for 5 min in TTBs and exposed to ECL Advance reagent for 1–2 min as described in the manufacturer’s protocol (GE Healthcare Biosciences, Buckinghamshire, UK). Blots were exposed to Hyperfilm-ECL (GE Healthcare Bio-Sciences) for 2–5 min and visualized using a Fluor S Multimager and Quantity One 4.1 (Bio-Rad Laboratories, Hercules, CA). The molecular weights of the bands were calculated by a comparison with prestained molecular weight markers (molecular weight range: 6,500–175,000) that were run in parallel with the samples. Semiquantitative analysis of specific immunolabeled bands was performed using a Fluor S image analyzer and Quantity One 4.1 (Bio-Rad Laboratories).
**GST activity assay.** GST activity was assayed using CDNB according to the procedure of Habig et al. (18). Briefly, muscle samples were lysed with 100 mM PBS (pH 7.4), 1% EDTA, and mammalian protease inhibitor mixture (1:100 dilution) (100 mg/ml). Muscle homogenates (20 μl) were added to 980 μl of reaction mix [100 mM phosphate buffer (pH 6.5) with 1 mM EDTA, 2 mM GSH, and 2 mM CDNB], and absorbance was read at 340 nm at 30-s intervals over 5 min. GST activity is expressed as micromoles per minute per milligram of protein.

**GR activity assay.** GR activity was measured according to the method of Smith et al. (52). Briefly, muscle samples were lysed with 100 mM PBS (pH 7.4), 1% EDTA, and mammalian protease inhibitor mixture (1:100 dilution) (100 mg/ml). Muscle homogenates (50 μl) were mixed with 150 μl of 100 mM phosphate buffer (pH 7.5) containing 1 mM EDTA, 250 μl of 3 mM DTNB, 50 μl of 2 mM NADPH, and 500 μl of 2 mM GSSG solution. The increase in absorbance at 412 nm was monitored spectrophotometrically at 25°C. GR activity is expressed as milliunits per milligram of protein. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the reduction of 1.0 μmol of DTNB to 5'-thiobisnitrobenzoic acid (TNB) per minute at 25°C at pH 7.5.

**GPx activity assay.** GPx activity was assayed spectrophotometrically according to the method described by Flohe et al. (12), which is based on the reduction of GSSG coupled to the oxidation of NADPH. Briefly, muscle samples were lysed with 100 mM PBS (pH 7.4), 1% EDTA, and mammalian protease inhibitor mixture (1:100 dilution) (100 mg/ml). The decrease in absorbance at 340 nm was monitored spectrophotometrically at 25°C. GPx activity in the cells is expressed as nanomoles per minute per milligram of protein (nmU/mg protein). One unit of GPx activity is defined as the amount of enzyme that catalyzes the reduction of 1 μmol of NADPH per minute.

**NQO1 activity assay.** NQO1 enzymatic activity was measured according to the procedure of Prochaska and Santamaria (44). Briefly, muscle samples were lysed with 0.8% digitonin and 2 mM EDTA (100 mg/ml). Muscle homogenates (50 μl) were added to 200 μl of reaction mixture containing 0.025 mM Tris·HCl, 0.67 mg/ml BSA, 0.01% Tween 20, 5 μM FAD, 1 mM glucose-6-phosphate, 30 μM NADP, 1 U/ml yeast glucose-6-phosphate dehydrogenase, 0.3 mg/ml MTT, and 50 μM menadione in a 96-well plate. Plates were then scanned at 610 nm with a microplate spectrophotometer VICTOR3 V Multilabel Counter (Perkin Elmer). NQO1 activity is expressed as nanomoles per minute per milligram of protein.

**TR activity assay.** TR activity was assayed following the reduction of DTNB to TNB using a procedure adapted from Holmgren and Bjornstedt (21). Briefly, muscle samples were lysed with 100 mM PBS (pH 7.4), 1% EDTA, and mammalian protease inhibitor mixture (1:100 dilution) (100 mg/ml). Muscle homogenates (10 μl) were added to 990 μl of reaction mix [0.25 mM DTNB, 0.24 mM NADPH, 10 μM yeast glutathione peroxidase, 0.024 U/ml, 2 U/ml mammalian protease inhibitor mixture (1:100 dilution). Muscle homogenates (10 μl)] were added to 200 μl of 2 mM GSSG solution. The increase in absorbance at 412 nm was monitored spectrophotometrically at 25°C. TR activity is expressed as milliunits per milligram of protein. One TR unit will cause an increase in absorbance at 412 nm of 1.0 per minute per milliliter (when measured in a noncoupled assay containing DTNB alone) at pH 7.0 at 25°C.

**Protein concentration.** Protein concentrations were determined by the Bio-Rad Bradford protein assay.

**Statistics.** All values are reported as means ± SD of 8 animals. To check whether difference were statistically significant, given the small sample sizes and the independence of samples, we adopted the Kruskal-Wallis nonparametric test, which is statistically informative despite the small number of subjects in each group.

The Kruskal-Wallis test is based on the null hypothesis that all the subpopulations have the same distribution function. To test for potential significant differences between the groups, the Mann-Whitney test was performed (Prism 5, GraphPad Software, San Diego, CA). Values of P < 0.05 were considered as statistically significant.
RESULTS

Figures 1 and 2 show the plasma LDH and CPK activities of rats subjected to acute physical exercise in the absence or presence of SF treatment. LDH and CPK activities were significantly higher in the E group than in the C, S and ES groups. Interestingly, the plasma LDH and CPK activities of the ES group was comparable with that of the C group, as evidenced by the ability of SF to counteract acute exhaustive exercise-induced muscle damage.

The end products of lipid peroxidation were assessed through the levels of TBARS, a well-known biomarker of lipid peroxidation and oxidative stress (15). Figure 3 shows TBARS levels in vastus lateralis homogenates of rats subjected to acute physical exercise. TBARS levels were significantly higher in the E group, and the levels of the S and ES groups were comparable with those of the C group.

TAA is shown in Fig. 4. In the E group, acute exhaustive exercise significantly decreased the TAA activity level with respect to the C group. SF treatment was able to significantly increase TAA activity in animals in the S group and to maintain the TAA activity of animals in the ES group at the same level of the C group.

The histological analysis of rat skeletal muscles is shown in Fig. 5. The histological section of the rat skeletal muscle in the C group showed no signs of sufferance and damage to the myofibrillar structure (Fig. 5A). After exhaustive exercise, an increased amount of mononuclear cells in the interstitium of the muscle was detectable (Fig. 5B), and moderate myofibrillar disruptions in some areas were observed (Fig. 5B, inset).

The skeletal muscle section from the S group showed a well-preserved morphology in which normal myofibers were observed with nuclei localized in the periphery of the myofibers and vessel sections were seen in the endomysium (Fig. 5C). The section of the skeletal muscle from the ES group showed no deep changes compared with samples from the C and SF groups. The myofibers were regularly organized, and nuclei and section vessels were well detectable (Fig. 5D).

The effects of acute exhaustive exercise and SF treatment on protein expression and the activity of GST, GR, GPx, TR, and NQO1 are shown in Figs. 6–10. Exercise did not influence GST protein expression and activity, whereas SF treatment significantly induced GST in both the S and ES groups compared with the C group (Fig. 6). Similarly, GR expression and activity were not influenced by exercise, whereas SF treatment
caused a significant increase of GR protein expression and activity in both the S and ES groups compared with the C group (Fig. 7). GPx and TR protein expression and activity (Figs. 8 and 9) did not significantly change in exercised animals and were not modified by SF treatment. Acute exhaustive exercise caused a marked decrease in NQO1 expression and activity compared with the C group. SF treatment was able to counteract the effect of acute exhaustive exercise on NQO1 expression and activity (Fig. 10).

Nrf2 protein expression is shown in Fig. 11. As expected, SF treatment was able to significantly induce Nrf2, and this induction was maintained in the presence of exhaustive exercise (ES group).

DISCUSSION

In this study, we demonstrated that SF treatment is able to counteract muscle damage induced by acute exercise in rats through the modulation of a scope of detoxifying phase II enzymes. The practice of regular, moderate physical activity improves health conditions and maintains skeletal muscle and bone trophism as well as stimulates cellular antioxidant defenses (16, 24). In contrast, when physical exercise is acute and exhaustive, it becomes a harmful source of oxidative stress and a cause of muscle and bone injuries. Many authors have investigated the possibility of counteracting oxidative stress-induced muscle damage during exhaustive exercise by administering antioxidant molecules acting as ROS scavengers, but the overall results are conflicting. Liu et al. (33) checked this topic by treating rats with lycopene before submitting them to an exhaustive exercise protocol and concluded that lycopene treatment does not prevent the release of muscle damage biomarkers such as LDH and CPK. Recently, Mastaloudis et al. (34), in a human study, reached similar results after administering vitamins C and E to ultramarathon runners. Differently, in a previous study, Jackson et al. (22) found that vitamin E shows a protective role against damage caused by physical exercise.

In the last years, SF, an isothiocyanate derived from broccoli, has gained great attention mainly for its chemopreventive activity (39). Although it has been demonstrated that SF is a strong inducer of endogenous antioxidants and phase II enzymes in various cells and tissues, the inducibility of the cellular defenses in skeletal muscle cells has not yet been studied.
In our study, LDH and CPK activities, two well-known biomarkers of tissue damage (11, 53) that significantly increase after an exhaustive exercise protocol, were significantly reduced by SF treatment, evidencing a protection of muscle tissue against exhaustive exercise-induced damage. Similarly, TBARS, a well-known biomarker of lipid peroxidation and oxidative stress (3), is known to increase both at plasma and tissue levels after an exhaustive exercise bout (29, 45). In this study, the TBARS increase induced by the exhaustive exercise protocol was significantly reduced by SF treatment, evidencing a protection of muscle tissue against exhaustive exercise-induced oxidative stress.

TAA is a complex trait reflecting the homeostasis of redox metabolism, which is highly relevant to nearly all oxidative stress-related processes, including aging, neurodegenerative disorders, atherosclerosis, carcinogenesis, and inflammatory and immunological reactions (55). It is affected by the relative contributions of each antioxidant species and the stress of oxidative free radicals. The production and consumption (by free radicals) of each specific antioxidant will affect the total antioxidant capacity. So, TAA provides an indication of the tissue antioxidant capacity (10). Our data show that acute exhaustive exercise significantly reduced TAA, which could be ascribed to the strong activation of prooxidant enzymes, such as xanthine oxidase, during this kind of exercise, leading to ROS generation and to the depletion of the antioxidant pool (53). SF treatment caused a significant increase of TAA and was also able to counteract the decrease in tissue antioxidant capacity due to exhaustive exercise.

To confirm biochemical data, we also performed histological experiments on muscle samples. As previously described by Armstrong et al. (4), the histological analysis clearly demonstrated that exhaustive exercise caused myofibrillar damage. SF treatment was able to prevent these muscle lesions, preserving myofibrillar organization.

SF is known to increase the cellular antioxidant capacity through the induction of enzymes such as heme oxygenase-1 (23) and NQO1 (50, 60). Moreover, SF is known to induce enzymes involved in the GSH redox cycle, such as GR and GST, in many cells and tissues (25, 38, 61), but, to our knowledge, no studies have investigated the role of SF treatment on skeletal muscle. In the present work, neither GST nor GR expression and activity were affected by exercise in the vastus lateralis muscle, in agreement with the results of recent studies demonstrating that GR is not affected immediately after an exercise bout (2) in rats and that GST is not influenced by physical exercise (17). However, SF treatment induced GST expression and activity.

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and GR expression and activity in vastus lateralis homogenates.

Neither the exhaustive exercise protocol nor SF treatment affected GPx activity. GPx is a selenium-dependent enzyme. A recent study (7) compared, in an endothelial cell line, the possibility of inducing GSH-related enzymes and thioredoxin (Trx) by treating cells with SF or selenium. In agreement with our findings, the authors (7) demonstrated that only selenium treatment induced GPx, whereas SF had no effect. The Trx system, composed of Trx, TR, and NADPH as a cofactor, is a ubiquitous thiol oxidoreductase system that contributes to the regulation of the cellular redox status (21). Moreover, Trx is also involved in the regulation of the activity of various intracellular molecules including transcription factors (51). To date, only one study (19) has evaluated the effect of an exhaustive exercise on TR activity in rats, concluding that exhaustive exercise does not affect lung TR activity. Our data are in agreement with this previous report and indicate that the exercise protocol did not affect TR activity. Recent research (30) demonstrated that SF treatment is able to induce TR at the retinal level in mice, but no studies have investigated the relationship between SF treatment and TR induction in skeletal muscles. SF treatment did not induce TR, so further studies are needed to better elucidate whether a higher SF dose could enhance the expression and activity of this enzyme.

NQO1, a cytosolic flavoprotein, acts as an antioxidant enzyme protecting cells against the toxicity of quinones by catalyzing the two-electron reduction of these compounds and generating the corresponding hydroquinone. NQO1 also catalyzes the regeneration of oxidized vitamin E, contributing to the maintenance of these important antioxidants (41). It has been demonstrated that NQO1 is strongly induced by SF in different tissues, such as the rat bladder, animal and human mammalian tissues (9, 61), and cell lines, such as bronchial epithelial cells, rat aortic smooth muscle cells, and cardiac fibroblasts (48, 62, 63). To our knowledge, no studies have evaluated whether physical exercise affects NQO1 activity. In our research, exhaustive exercise strongly reduced NQO1 expression and activity in vastus lateralis muscle. SF treatment was able to increase NQO1 expression and activity in sedentary rats and prevented NQO1 downregulation in rats undergoing the exercise protocol, suggesting a strong implication of NQO1 in the protection of skeletal muscles from oxidative stress-induced muscle damage.

The cumulative effects of SF treatment reported in this study suggest that SF contributes to enhance the antioxidant capacity of muscle tissue by inducing important antioxidant and phase II enzymes. Phase II enzymes are induced partially through the ARE-Nrf2 pathway in many tissues and cells (58, 62, 63). Under basal conditions, Nrf2 is sequestered in the cytoplasm by a cytosolic repressor, Kelch-like ECH-associated protein 1 (Keap1) (37). Keap1 has been recently characterized as a Cullin 3-dependent ubiquitination substrate adaptor protein (13, 59), so Nrf2 is not only physically sequestered in the cytoplasm but also constantly degraded. Chemical inducers...
cause Nrf2 release from Keap1 repression, so that Nrf2 translocates to the nucleus and recognizes and binds to a cis-acting enhancer called ARE (37). In our research, Nrf2 expression was strongly induced by SF, and this induction was also maintained after the exhaustive exercise protocol, suggesting that the induction of antioxidant phase II enzymes is, at least partly, due to the ability of SF to induce Nrf2. Nrf2 phosphorylation by protein kinases such as Akt kinase, ERK, JNK, and PKC influences the activation of the Nrf2/ARE pathway (20, 40). Further studies are in progress to better elucidate the mechanism underpinning Nrf2 induction and activation by SF.

In conclusion, in this study, we clearly demonstrated that SF acts as an indirect antioxidant in skeletal muscle by increasing GR, GST, and NQO1 expression and activity in vastus lateralis muscle. These effects could explain the overall protection of SF against muscle damage during exhaustive exercise. Since the 25 mg/kg body wt dose used in this study is not easily achievable through diet by the consumption of SF-rich foods, it is still yet not possible to suggest a SF clinical application. Further studies are needed to define whether SF oral administration through dietary supplements is also able to exert similar protective effects. We are now evaluating, in a rat model, this possibility through the dietary administration of SF-enriched extracts.

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