Soy Isoflavone Supplementation Alleviates Oxidative Stress and Improves Systolic Blood Pressure in Male Spontaneously Hypertensive Rats

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Summary The aim of this study was to investigate the protective effect of isoflavone against hypertension, via the mitigation of oxidative stress and prevention of nitric oxide (NO, a potent vasodilator) reduction, in spontaneously hypertensive rats (SHR). The 8 wk-old male SHR were divided into two groups, and fed a casein-based high fat diet (120 g fat, 1 g cholesterol/kg diet) for 30 d, either with or without 10 g of soy powder (containing 31.2% of isoflavones)/kg. During the 30-d study period, tail systolic blood pressures (BP) in the control SHR group increased, from 162.4±2.3 to 177.9±5.4 mmHg (p<0.05), while the isoflavone-supplemented group benefited from a clear antihypertensive effect (160.1±1.8 to 160.2±4.9 mmHg). The serum NO and total radical trapping antioxidant potential (TRAP) were elevated in the isoflavone group. The isoflavone group also experienced a significant decrease in oxidative DNA damage in leukocytes, using comet assay. DNA damage correlated positively with incremental BP during the study, and systolic BP at the end of the study (p<0.01). Our results indicate that soy isoflavone has an antihypertensive effect, possibly through the amelioration of oxidative stress, and the augmentation of NO production, in SHR.

Key Words soy-isoflavones, hypertension, nitric oxide, DNA damage, antioxidant

Essential hypertension is one of the most prevalent diseases in the world, and is a major risk factor for cardiovascular disease (1). Recent evidence suggests that oxidative stress, due to overproduction of reactive oxygen species (ROS) such as superoxide radical (O₂⁻·), hydroxyl radical (OH'), may be intimately involved in the pathogenesis and complications of hypertension. Kumar and Das (2) as well as some other research groups (3, 4) have reported increased superoxide anion and hydrogen peroxide production by white blood cells, increased plasma levels of lipid peroxide, and decreased levels of vitamin E, superoxide dismutase (SOD), and nitric oxide in essential hypertensive patients. It has been proposed that a possible mechanism underlying the relationship between oxidative stress and the etiology of essential hypertension involves increases in ROS, especially the superoxide anion. This increase in ROS may result in an inactivation of nitric oxide (NO), and a decrease in its half-life, which can then lead to hypertension, since NO acts as a pivotal molecule in the regulation of vascular tone via the stimulation of vascular smooth muscle cell relaxation and vasodilation (5). Additionally, NO plays an important role in a number of other potent antiatherogenic effects, including the inhibition of leukocyte-endothelial interaction, smooth muscle cell proliferation, and platelet aggregation (6). Many studies have reported positive results of antioxidant treatment in hypertension, via the prevention of NO oxidation (7–9). Vitamin C, alone or in combination with vitamin E, has been shown to accelerate the degradation of S-nitrosogluthathione, increase the rate of NO synthesis, and reduce blood pressure in experimental models of hypertension (7, 8). Chen et al. (9) have also found that vitamins C and E reduce oxidative stress, improve vascular function and structure, and prevent the progression of hypertension in Stroke-Prone SHR, possibly through the modulation of enzyme systems which generate free radicals.

Isoflavones, especially soy-derived isoflavones, are currently receiving heightened attention, due to their reported anti-hypertensive effects (10, 11). Until now, the anti-hypertensive qualities associated with isoflavone have been ascribed mainly to its cholesterol-lowering effect (12). Recent recognitions of soy-isoflavones as free radical quenching agents both in vitro and in vivo (13, 14) have led us to hypothesize that the antioxidant abilities of isoflavones can influence the stability of endothelium-derived vasoactive agents, such as NO, and thus contribute to a decline in blood pressure. The aim of this study was to investigate whether isoflavone supplementation alters the progression of blood-pres
sure by the modulation of NO and relief from oxidative stress in male spontaneously hypertensive rats. In order to assess oxidative stress, we utilized comet assay, a single cell gel electrophoresis (SCGE), which is known to be a sensitive method for the detection of DNA damage (15). DNA damage is known to be one of the most sensitive biological markers for evaluating oxidative stress, representing imbalances between free radical generation and deficiencies in the antioxidant system (16).

MATERIALS AND METHODS

Spontaneously hypertensive rats (SHR) from the strain developed by Okamoto et al. (17) were obtained from the Korean Food and Drug Administration, and were bred in the Department of Food and Nutrition of Hamnam University. Animals were housed and cared for in accordance with the Guide for Care and Use of Laboratory Animals (Department of Health, Education and Welfare, 1985). Male SHRs (8 wk old, 215.2 ± 23.1 g) were divided into two groups, and fed experimental diets for 30 d. Experimental diets were high-fat, high-cholesterol diets, based on 20% casein (120 g lard and 1 g cholesterol/kg diet), as shown in Table 1. The control group (n = 10) of rats received a diet bereft of isoflavone, whereas the experimental group (n = 10) of rats received a diet fortified with 10 g/kg of soy isoflavone powder (containing 31.2% isoflavones, Tokiwa Phytochemical Co., Japan). Contents of isoflavones in the soy powder included 4.0% genistein glycosides, 15.3% daidzein glycosides, and 11.9% glycinein glycosides.

Animals were monitored daily for general health, and body weights were recorded every week for the duration of the study. At the end of the experimental period, the rats were anesthetized with ethyl ether and blood was collected from the abdominal artery in tubes. Serum was obtained from the blood samples by centrifugation (1,500 rpm for 30 min) and stored at −80°C until further analysis.

Tail systolic blood pressure was determined with a pulse transducer (Narco Bio-system, USA) and a physiograph. While each rat was kept in a restrainer, its tail artery was warmed for 20 min on a 37–40°C plate, and blood pressure was measured three times during a 10–15-min period.

NO determination was carried out by Griess assay (18). Serum proteins were removed by ultrafiltration with 10 kD micron at 4°C, 130,000 rpm. Nitrate in the serum was reduced with nitrate reductase and β-NADPH for 3 h, and β-NADPH was removed by 10 min incubation with 10 U/mL L-GDH, 80 mM α-ketoglutaric acid and 1 M NH₄Cl. The amount of nitrite was measured by adding 150 μL of Griess reagent (Promega, USA). The absorbance of the chromophores formed was read at 540 nm.

Total radical trapping antioxidant potential (TRAP) was measured by a modification of the photometric method, initially described by Rice-Evans and Miller (19). This method for measuring antioxidant activity is based on antioxidant-mediated inhibition of the absorbance of the radical cation of 2,2'-azinobis (3-ethylben-

<table>
<thead>
<tr>
<th>Table 1. Diet composition (g/100 g).</th>
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<tr>
<td>Control diet</td>
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<tr>
<td>Corn starch</td>
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<td>Mineral mix¹</td>
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<td>Vitamin mix²</td>
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<tr>
<td>i-Cysteine</td>
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<td>Choline bitartrate</td>
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<td>Butylated hydroxy toluene</td>
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<tr>
<td>Cholesterol</td>
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<td>Soy powder³</td>
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¹ AIN 76 mineral mixture contained (in g/kg of mixture): calcium phosphate, dibasic 500; sodium chloride 74; potassium citrate, monohydrate 220; potassium sulfate 52; magnesium oxide 24; manganese carbonate (43–48% Mn) 3.5; ferric citrate (16–17% Fe) 6; zinc carbonate (70% ZnO) 1.6; cupric carbonate (53–55% Cu) 0.3; potassium iodate 0.01; sodium selenite 0.01; chromium potassium sulfate 0.55; sucrose, finely powdered 118.03.

² AIN 76 vitamin mixture contained (in g/kg of mixture): thiamine HCl 0.6; riboflavin 0.6; pyridoxine HCl 0.7; niacin 3; D-calcium pantothenate 1.6; folic acid 0.2; D-biotin 0.02; cyanocobalamin (vitamin B12) 0.001; dry vitamin A palmitate (500.000 U/d) 0.8; dry vitamin E acetate (500.000 U/d) 10; vitamin D₃ tritration (400.000 U/g) 0.25; menadione sodium bisulfite complex 0.15; sucrose finely powdered 981.08.

³ Soy powder contained 31.2% of isoflavones. Contents of isoflavones in the soy powder were 4.0% of genistein glycosides, 15.3% of daidzein glycosides, and 11.9% of genistin glycosides.

zothiazoline 6-sulfonate) (ABTS⁺). The ABTS⁺ radical cation is formed by the interaction of ABTS⁺ (150 μM) with the ferrylmyoglobin radical species, generated by the activation of metmyoglobin (2.5 μM) with H₂O₂ (75 μM). Ten microliters of sample/buffer/Trolox-standard was added to tubes containing 400 μL of PBS buffer, 20 μL of metmyoglobin and 400 μL of ABTS, and mixed by vortexing. The reaction was started by the addition of 170 μL of H₂O₂. After 6 min of incubation, absorbance was measured at 734 nm, using a spectrophotometer. Values were expressed as TEAC (Trolox equivalent antioxidant capacity), defined as the mM concentration of the Trolox antioxidant capacity of a calibration curve.

The alkaline comet assay was conducted according to the protocols established by Singh et al. (20), with little modification. Five microliters of whole blood was mixed with 75 μL of 0.7% low melting agarose (LMA) and distributed onto slides coated with 0.5% normal melting agarose (NMA). After solidification of the agarose, slides were covered with another 75 μL LMA and then immersed in a jar containing pH 10 cold lysing solution, consisting of 2.5 M NaCl, 100 mM EDTA,
10 mM Tris, and 1% sodium laurylsarcosine; 1% Triton X-100 and 10% DMSO were freshly added to the solution, which was then stored in the refrigerator for 1 h. After lysis, the slides were placed in a horizontal electrophoresis tank (Threeshine Co., Ltd., Korea). The slides were covered with a fresh alkaline buffer (300 mM NaOH, 10 mM Na2EDTA, pH 13.0), and maintained at 4°C for 40 min. Electrophoresis of the DNA was accomplished by applying an electric current of 25 V/300±3 mA for 20 min at 4°C. The slides were washed three times with neutralizing buffer (0.4 M Tris, pH 7.5), for 5 min at 4°C, and then were treated with ethanol for another 5 min. All steps following the lysis treatment were undertaken in darkness in order to prevent additional DNA damage. Fifty microliters of ethidium bromide (20 μg/mL) was added to each slide, which was then analyzed using a fluorescence microscope (LEICA DMLB, Germany). Images of 100 randomly selected cells (50 cells from each of two replicate slides) were analyzed from each subject. Measurements were made by image analysis (Kinetic Imaging, Komet 4.0, UK), determining tail moment (TM, calculated as the percentage of DNA in the tail times tail length).

Data were analyzed using the SPSS* package for Windows (ver. 10). Values were expressed as mean±SE, unless otherwise stated. Statistical differences between the control and isoflavone-supplemented group were considered significant at p<0.05 by Student's t-test. The comparison of tail systolic blood pressure measured before and after the trial was performed by a paired t-test, at a significance level of 0.05. Evaluations of the associations of other factors with DNA damage parameters were performed using Pearson's correlation.

RESULTS

Blood pressure

Figure 1 illustrates the absolute values for systolic blood pressure in SHR male rats fed control or soy isoflavone diets. After the 30-d study period, tail systolic blood pressures increased significantly, from 162.4±2.3 to 177.9±5.4 mmHg (p<0.05) in the control group. Conversely, isoflavone supplementation prevented blood pressure elevation (160.1±1.8 to 160.2±4.9 mmHg). Blood pressures were significantly lower in the isoflavone-supplemented group, compared with the control group at the end of the study (p<0.05). Body weight did not differ significantly between groups throughout the duration of the experiment (data not shown).

Serum NO concentration and TRAP

Levels of NO serum concentration are shown in Fig. 2. NO concentration was significantly elevated in the isoflavone group, compared to the control group, a difference of 40% (48.4±8.9 vs 29.8±2.0 μM, p<0.05). This result was associated with significantly increased serum TRAP in the isoflavone-supplemented group (1.24±0.03 μM), compared with the control group (1.15±0.03 μM), as shown in Fig. 3.

Leukocytic DNA damage

The effects of isoflavone treatment on DNA damage are presented in Fig. 4. DNA damage was expressed by tail moment, which is defined as the migration of DNA from the nucleus, multiplied by its intensity in the tail. The isoflavone group exhibited significantly less DNA damage measured in peripheral white blood cells than was detected in the control group (4.1±0.2 vs 6.1±0.6, p<0.01). DNA damage was positively corre-
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![Graph](image)

**Fig. 4.** Effect of isoflavone supplementation on concentration of DNA damage (tail moment) in SHRs. *** Statistically significant at \( p<0.001 \) by Student's t-test.

![Graph](image)

**Fig. 5.** Relationship between DNA damage and increment of tail systolic blood pressure. \( r \): Pearson's correlation coefficient.

![Graph](image)

**Fig. 6.** Relationship between DNA damage and final tail systolic blood pressure. \( r \): Pearson's correlation coefficient.

lated with the increment of blood pressure during the study \( r=0.654, \ p=0.003 \), Fig. 5), and with systolic blood pressure at the end of the study \( r=0.615, \ p=0.007 \), Fig. 6).

**DISCUSSION**

The SHR is a common model of human essential hypertension, in which blood pressure rises sharply after about 4–5 wk of age, accompanied by hypernoradrenergic innervation of blood vessels and vascular hypertrophy (21). In addition to this, Suzuki et al. (22) reported that the level of microvascular oxidative stress in SHR was significantly above that displayed in its Wistar Kyoto (WKY) normotensive control strain. The SHR model allows the study of the entire period of hypertension development often not possible to study in humans. In the present study, we used this SHR model to assess the antihypertensive effect of isoflavone supplementation. Isoflavones, the major phytoestrogens found in the soybean, have recently attracted a great deal of public attention, due to a variety of health beneficial effects, including the regulation of plasma lipids, cell growth, and an increase in bone density (12, 23, 24). Recently, several soy or isoflavone studies have shown them to exhibit protective effects against hypertension in both animal models and human intervention studies (10, 25–27). Nevala et al. (10) reported that 20% soy protein supplementation of standard rat chow for 5 wk decreased systolic blood pressure by ~20 mmHg in male SHR, compared to the control group, which was fed the casein diet. It was also discovered by Martin et al. (25) that 8 wk on a 19% soy meal diet resulted in a decrease in mean arterial pressure (MAP) of ~14 mmHg in ovariectomized female SHR, which was statistically significant compared with the casein-fed control group. In accordance with these studies, we have observed that a 1% soy isoflavone supplementation to a high fat, high cholesterol diet for 30 d could prevent an increase in systolic blood pressure. In fact, it was possible to induce a significant reduction in blood pressure, by ~18 mmHg in male isoflavone-fed SHRs, compared to the control animals, whose blood pressure rose significantly over the same period. It is interesting to note that our study showed a similar blood pressure lowering effect with a much lower concentration of soy isoflavone. However, this can, perhaps, be explained by the different isoflavone contents in the studies. Although they did not specify how much isoflavone was actually given to the animals, it can be roughly assumed that the soy protein source (Supro 670) used by Nevala et al. contains 1.27 mg of genistein and 0.42 mg of daidzein per 1 g (24.13 and 7.98 mg, respectively, when calculated per 100 g diet) (28), and that the soy meal used by Martin et al. contains 0.68 mg of genistein and 0.58 mg of daidzein per 1 g (13.6 and 11.6 mg, respectively, when calculated per 100 g diet) (29). The soy powder in the present study was identified to contain 40 mg of genistein, 153 mg of daidzein and 119 mg glycine/100 g diet, which is several times higher than the other soy sources. The concentration of soy isoflavone appears to be a determining factor since Rivas et al. (27), showed that the greater amount of isoflavones in soy milk (80 mg genistein + 63 mg daidzein/d) lowered diastolic blood pressure in subjects with mild-to-moderate essential hypertension (~16 mmHg), while low isoflavonoid-containing tablets (30 mg genistein, 1 mg daidzein,
16 mg biochanin A and 8 mg formononetin/d) had no hypotensive action in essential hypertensive patients (26).

The mechanism underlying the hypotensive effect of soy powder in our study may be related to a NO-enhancing vasodilator function. We observed a significant increase in serum NO levels upon isoflavone supplementation. Similar modulation of NO was observed with other studies. Sobey et al. (30) have demonstrated that short-term administration of daidzein modulates cerebral arterial reactivity in male rats, by enhancing the rates of synthesis and release of endothelium-derived NO. The 6 mo genistein supplementation improves flow-mediated endothelium-dependent vasodilation, mediated by an increased ratio of plasma NO to endothelin (the major constrictor factor) in healthy postmenopausal women (31). Endothelium-derived NO acts as a potent vasodilator, thus protecting vessel walls against the development of hypertension and other cardiovascular pathology (32). Relative deficiencies in NO are known to be associated with oxidative stress, particularly the increased production of superoxide anion (O$_2^-$), which can attenuate the biological activity of NO, thus exacerbating hypertension in human and in some models of experimental hypertension (e.g. SHR). Many studies support the notion that antioxidant treatment is effective in hypertension, via the prevention of NO oxidation (33). Our assumption, namely that increased serum NO could be induced by the antioxidant effects of isoflavone, was confirmed by our results, which demonstrated significant increases in serum TRAP in the isoflavone-supplemented group. Ruiz-Larrea et al. (34) reported that isoflavones exhibit free radical-scavenging activity. It has also been reported that genistein enhanced the activity of antioxidant enzymes in mice (35), and also that it significantly inhibits phorbol ester-type tumor promoter-induced H$_2$O$_2$ formation, both in vitro and in vivo, by inhibiting the formation of reactive oxygen species (36). Furthermore, the consumption of isoflavones significantly increases the lag time of LDL oxidation in humans (37). The free radical-scavenging properties of isoflavone are dependent on its polyphenolic structures, which function as donors of hydrogen atoms to deleterious oxy-radicals (38).

In this study, increased serum total antioxidant status was also linked to decreased leukocytic DNA damage in the isoflavone-supplemented group. Increased DNA damage, which is known to be the most significant consequence of oxidative stress, has been observed both in hypertensive subjects and stroke-prone hypertensive rats (39, 40). In relation to these results, we observed that DNA damage correlated positively with incremental blood pressure during the study, and with systolic blood pressure at the end of the study. Inoue and Kawanishi (41) have demonstrated that superoxide radical reacts with the NO to form peroxynitrite, and that peroxynitrite in turn induces DNA damage through an active intermediate, the reactivity of which is similar to the hydroxyl radical (OH'). On the other hand, Yen and Lai (42) found that the antioxidant activity of isoflavones and soy-based food (e.g. soybeans, soy milk, tofu, etc.) can provide protection against peroxynitrite-mediated DNA damage in intact cells and in plasmid DNA, and can also protect against peroxynitrite-induced LDL oxidation. Mizutani et al. (43) also reported that daidzein and genistein attenuate oxidative DNA damage in the vascular smooth muscle cells of stroke-prone spontaneously hypertensive rats.

In conclusion, this study demonstrated that soy powder, rich in isoflavones, exerted effects to counter the development of hypertension in male SHR. This effect may be the result of improved endothelial NO bioavailability exerted by the antioxidant effects of isoflavone as demonstrated by the increase in serum total antioxidant status and decreased leukocytic DNA damage.

Acknowledgments

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REFERENCES

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