Effects of Black Soybean on Atherogenic Prevention in Hypercholesterolemic Rabbits and on Adhesion Molecular Expression in Cultured HAECs

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ABSTRACT

The aim of the study was to investigate the effect of black soybean (BS) on the susceptibility of low-density lipoprotein (LDL) in hypercholesterolemic New Zealand white rabbits. Effects of the BS extract (BSE) and its components on monocyte adhesion of human aortic endothelial cells (HAECs), and adhesion molecule were investigated. Rabbits were divided into four groups, including control, 0.5% cholesterol with 20% casein (either with or without 0.5% vitamin E), and BS groups, all fed for 8 weeks. LDL was treated with 10 μM Cu2+ in vitro to determine the LDL lag time, and the vitamin E content of LDL was determined. The thickness of the tunica intima was measured on paraffin sections of thoracic aortas and aortic arches stained with Movat’s pentachrome. HAECs were pretreated with 100 μg/ml of BSE, and 10 μM of genistein, daidzein, cyanidin, and aspirin for 18 h, followed by tumor necrosis factor (TNF)-α (2 ng/ml) for 6 h, after which U937 cell adhesion was determined. Adhesion molecule expression was examined using ELISAs. The LDL lag time in the BS group was similar to that in the vitamin E group, while its lag time was significantly longer than those in the control and casein groups. The ratio of the intimal area/medial area of the aortic arch of the casein group was significantly higher than those in the control, BS, and vitamin E groups. The vitamin E group had the lowest value, and was closest to the control group. The BS group exhibited a significantly decreased atheroma region in the aortic arch compared to the casein group. Pre-incubation with BSE, genistein, daidzein, cyanidin, and aspirin significantly decreased adhesion by U937 monocyteic cells to TNF-α-stimulated HAECs. Genistein, daidzein, cyanidin, and aspirin significantly suppressed the expression of vascular cell adhesion molecule (VCAM)-1. Only genistein and aspirin significantly decreased intracellular adhesion molecule (ICAM)-1 expression compared to TNF-α treatment, while no treatments had any effect on E-selectin expression. BS significantly prolonged the LDL lag time and decreased the atheroma region of the aortic arch in hypercholesterolemic rabbits, thereby exerting an antiatherosclerotic effect. Presumably, the BSE downregulate intracellular redox-dependent signaling pathways in HAECs upon TNF-α stimulation through regulating NF-κB, thereby attenuating the inflammatory response in atherosclerosis. The antiatherogenic and anti-inflammatory effects of BS can be used as a nutraceutical for atherogenesis prevention.

Keywords: Black Soybean; Hypercholesterolemia; Human Aortic Endothelial Cells; Adhesion

1. Introduction

The development of atherosclerosis is positively correlated with increasing levels of cholesterol and low-density lipoprotein (LDL) in blood, and oxidation of LDL in vessel walls is highly related to the development of atherosclerosis [1,2]. Human atherosclerotic plaque contains both oxidized lipids and relatively large amounts of α-tocopherol and ascorbate [3]. Dietary supplementation with
α-tocopherol increases the resistance of LDL subsequently isolated from the blood to oxidation in vitro [4,5]. High intake and elevated plasma levels of vitamin E are associated with low rates of ischemic heart disease in controlled intervention studies [6] and large-scale prospective studies [7,8]. Previous studies showed that the consumption of soy products may lower rates of cardiovascular disease, which was associated with the cholesterol-lowering action of soy and the antioxidant activity of soybean isoflavonoids [9-12]. Genistein and daidzein are the main isoflavone phytoestrogens found in soy products and a number of plants including soybeans, and have been shown to exert estrogen-like [13] and antioxidant actions [14]. High isoflavone aglycone attenuates atherosclerosis development in cholesterol-fed rabbits [15]. Esterified isoflavones can be incorporated into LDL particles, and some of them increase the in vitro oxidation resistance of LDL [16]. Esterified isoflavones contain several antioxidants, such as α-tocopherol, ubiquinol-10, lycopene, β-carotene, and lutein [17], and may be lost during the lag phase period. In addition to the antioxidant potential, genistein prevents endothelial cells from the cytotoxic effects of oxidized (ox)-LDL [11].

Atherosclerosis is a chronic inflammatory process characterized by increased oxidative stress [18]. The resulting adhesion of monocytes to the vascular endothelium and subsequent migration into the vessel wall are pivotal early events in atherogenesis [19,20]. The interaction between monocytes and vascular endothelial cells may be mediated by adhesion molecules, including vascular cell adhesion molecule (VCAM-1), intercellular adhesion molecule (ICAM)-1, and E-selectin on the surface of the vascular endothelium [21]. The inflammatory cytokine, tumor necrosis factor (TNF)-α, activates nuclear factor (NF)-κB, and activator protein (AP)-1, which are the two major redox-sensitive eukaryotic transcription factors that regulate expressions of adhesion molecules [22-24]. Because activation of NF-κB and AP-1 can be inhibited to various degrees by different antioxidants, endogenous reactive oxygen species (ROS) may play an important role in these redox-sensitive transcription pathways in atherogenesis [18,24]. For example, quercetin, the most abundant flavonoid in the human diet and an excellent free radical-scavenging antioxidant, attenuates expressions of ICAM-1 and E-selectin in human aortic endothelial cells (HAECs) [25]. Several in vitro and in vivo studies showed that the cardioprotective effects of soy isoflavones include improved serum lipid profiles [26,27] and vascular reactivity [28], protection against LDL oxidation [29], modulation of cytokines, and inhibition of platelet aggregation [28]. Soy isoflavones attenuate human monocyte adhesion to endothelial cell-specific protein ICAM-1 (or CD54) by inhibiting the monocyte, CD11a [30].

Black soybean (BS; Glycine max L. Merrilx), like the soybean, is a species in the genus of Glycine, but has a black testa (seed coat). BSs are abundant in natural antioxidants, such as isoflavones, saponins, anthocyanins, and vitamin E [31]. Delphinidin-3-O-β-D-glucoside from the seed coat of BS has strong antioxidant activity in an acidic environment [31]. The seed coats of 60 Chinese BS varieties contain high levels of cyanidin-3-glucoside and antioxidant activity of the oxygen radical absorbance capacity (ORAC) [32,33]. In addition to anthocyanins, the BS seed coat (BSSC) is also a good source of other phenolics, such as condensed tannins and phenolic acids [32,34]. BSs abound in natural antioxidants, and their extracts prolong lag times of Cu-induced LDL oxidation, leading to reduced atheroma formation [35,36]. Furthermore, Kim et al. [37] demonstrated that anthocyanins from the BSSC inhibited TNF-α-induced ICAM-1 and cyclooxygenase (COX)-2 levels through an NF-κB-dependent pathway, and had anti-inflammatory effects on an immortalized epidermal keratinocyte cell line (HaCaT).

The majority of studies showed that soybeans inhibit LDL oxidation both in vitro and ex vivo, and reduce atherosclerosis in cholesterol-fed rabbits [12,15,38]. The effects of BS on atherosclerosis progression of hypercholesterolemic rabbits and monocyte-endothelial cell interactions have not yet been elucidated. The objectives of our study were to determine the effects of BS on atherosclerosis progression and ox-LDL formation in rabbits fed a cholesterol-rich diet to elucidate the mechanism by which BS alleviates atherosclerosis, and provide evidence supporting the use of antioxidants of BS in preventing atherosclerosis. The antiinflammatory effects of BSE and its components, genistein, daidzein, and cyanidin, on TNF-α-induced cell adhesion, and adhesion molecule in an HAEC model were also investigated. The antiatherogenic and antiinflammatory effects of BS can be used as a nutraceutical for atherogenic prevention.

2. Materials and Methods

2.1. Chemicals

Medium 200, low-serum growth supplement (LSGS), fetal bovine serum (FBS), and RPMI-1640 were purchased from Gibco Invitrogen (Carlsbad, CA, USA). RayBio enzyme-linked immunosorbent assay (ELISA) kits were purchased from RayBiotech (Norcross, GA, USA). The 2,7-bis (2-carboxyethyl)-5(6)-carboxyfluorescein acetoxyethyl ester (BCECF-AM) was obtained from Molecular Probes (Eugene, OR, USA). Other chemical reagents were purchased from Sigma (St. Louis, MO, USA).
2.2. Experimental Animal Treatment

Male New Zealand white rabbits were used in the study. The investigation conformed to the Guide for the Care of Laboratory Animals, published by the US National Institute of Health (NIH publication no. 85-23, revised 1996). All institutional and national guidelines for the care and use of laboratory animals were followed. The antiatherogenic effects of BS were evaluated using the following four rabbit groups: control, 0.5% cholesterol with 20% casein, 0.5% cholesterol with 20% casein and 0.5% vitamin E, and BS groups. Each rabbit was housed in a single cage, and fed 100 g daily for 8 weeks. In the week before the study began, animals were acclimatized and received a 100% standard rabbit diet. In the first and second weeks of the study period, animals received a 50% standard rabbit diet and a 50% semi-purified diet. In the third and fourth weeks, animals received a 25% standard rabbit diet and a 75% semi-purified diet. From the fifth study week to the end of the study period, animals received a 100% standard rabbit diet and a 75% semi-purified diet. From the fifth study week to the end of the study period, animals received 100% semi-purified diets containing 0.5% cholesterol and individual proteins. Eight weeks after cholesterol feeding, rabbits were processed for further experiments.

2.3. Blood Samples and Serum Cholesterol, Triglyceride, and Anthocyanin Assays

Serum cholesterol and triglyceride levels were monitored every 2 weeks throughout the study. Thirty milliliters of blood was drawn from the ear vein of each rabbit, and was collected in sterile microcapillary glass tubes containing 1.5 mg/ml EDTA. Twenty milliliters of blood was also drawn from the heart of each rabbit. Plasma was isolated for the cholesterol, triglyceride, and anthocyanin assays. Serum was isolated for LDL determination. The antiatherogenic effects of BS were evaluated using the following four rabbit groups: control, 0.5% cholesterol with 20% casein, 0.5% cholesterol with 20% casein and 0.5% vitamin E, and BS groups. Each rabbit was housed in a single cage, and fed 100 g daily for 8 weeks. In the week before the study began, animals were acclimatized and received a 100% standard rabbit diet. In the first and second weeks of the study period, animals received a 50% standard rabbit diet and a 50% semi-purified diet. In the third and fourth weeks, animals received a 25% standard rabbit diet and a 75% semi-purified diet. From the fifth study week to the end of the study period, animals received 100% semi-purified diets containing 0.5% cholesterol and individual proteins. Eight weeks after cholesterol feeding, rabbits were processed for further experiments.

2.4. Vessel Samples

An aortic segment was removed from the ascending arch to the diaphragm. The abdominal aorta was then taken from the diaphragm to the iliac trifurcation. Tissue samples (aortic arch and thoracic aorta) were fixed by immersion in 10% formalin fixative overnight, followed by dehydration through a graded ethanol series. Samples were then embedded in paraffin, cut into 5-μm sections, and stained with Movat’s pentachrome stain [42]. The severity of atherosclerosis in the arch and thoracic aorta was determined morphometrically using an LV-2 computerized image analysis system.

2.5. LDL Isolation and Oxidative Susceptibility of the LDL Assay

Thirty milliliters of blood was centrifuged for 10 min at 3000 rpm and 4°C. Fifteen milliliters of serum sample was collected for LDL isolation. Two steps of LDL fractionation (1.006 > d > 1.063 g/ml) were isolated by two-step sequential flotation ultracentrifugation [43]. A Hitachi CP85β ultracentrifuge (Tokyo, Japan) equipped with a P70AT-376 rotor at 127,980 g rpm was used for 16 h (d < 1.063 g/ml) to remove very LDL, and for 20 h (d < 1.063 g/ml) to collect LDL. The isolated LDL fraction from each study animal was separately dialyzed at 4°C against saline buffer containing 0.15 M NaCl and phosphate (pH 7.4) for 22 h before determining the oxidative susceptibility of the LDL assay. Dialyzed LDL was then diluted with saline and incubated with 10 μM copper iron at a final concentration of 0.05 mg cholesterol/ml. The kinetics of LDL oxidation was determined by monitoring the change in absorbance at 234 nm and 25°C with a spectrophotometer (Hitachi U-2000, Tokyo, Japan). The absorbance was recorded every 15 min for less than 5 h. Changes in absorbance at 234 nm against time were divided into three consecutive phases: lag, propagation, and decomposition [44].

2.6. Concentration of α-Tocopherol in LDL

The α-tocopherol content of LDL was determined by a high-performance liquid chromatographic (HPLC) system (Merck, Darmstadt, Germany) [45]. Briefly, 1 ml of α-tocopherol acetate (7.116 μg/ml EtOH with 1% BHT, as an internal standard) was added to 0.25 ml of LDL, followed by adding 50 μl of 12 N HCl to the samples, and extraction with 3 ml of n-hexane (0.25% BHT w/w) twice. The upper hexane layer was collected and dried with nitrogen. The residue was dissolved in 200 μl of methanol, followed by injection of 20 μl for HPLC. The extracts were analyzed by reversed-phase HPLC (Lichopher 100 RP-18, 5 μm, 125 × 4 mm I.D.) eluent with methanol-water (95:5, v/v) at a flow rate of 1.5 ml/min and a 292-nm wavelength.

2.7. BSE Preparation

BS seeds (Tainan no. 3 with a green cotyledon) were obtained from Dr. Tien-Joung Yiu, Tainan District Agricultural Research and Extension Station, Chiayi, Taiwan. Seeds were freeze-dried, ground, and stored at −80°C un-
til being analyzed. One gram of powdered seeds was sonicated for 20 min with 8 ml of 80% methanol containing 2 ml of 6 M HCl, followed by extraction for 24 h at 4°C. After centrifugation (1500 g for 10 min at 4°C), the supernatant was collected as crude extracts containing antioxidants, and the residue was extracted again. The collected supernatant was evaporated and dissolved in 20 ml of 80% methanol for the HPLC assay and HAEC treatment [12].

2.8. Concentrations of Isoflavones and Cyanidin in BSs

Daidzein and genistein in the purified mixtures were individually determined by HPLC. One milliliter of the methanolic extract of the acid hydrolysate was filtered through a 0.45-µm filter prior to a 20-µl injection into a C18 reversed-phase column (Asteica silica-based, 110 Å, ODS, 25 cm × 4.6 mm, 5-µm particle size, Sigma-Aldrich, St. Louis, MO, USA), and eluted for 45 min using a ternary TSP Thermo Separation Products Pump (Agilent 1100 G1311A Quat Pump SpectraLab Scientific, Markham, Ontario, Canada). Solvents containing methanol-water (30%:70%; v/v) with 1% formic acid, 100% methanol, and aqueous 10% (v/v) acetic acid were used for the HPLC. The solvent gradient was allowed to equilibrate for 15 min (at a flow rate of 1 ml/min), and monitored at 528 nm using a Waters Photodiode Array Detector (Waters, Milford, MA, USA). Results are expressed as daidzein and genistein weight equivalents [46].

2.9. Cell Culture and Treatment

HAECs were grown in Medium 200 supplemented with 1% low-serum growth supplement and 10% FBS in an atmosphere of 95% air and 5% CO2 at 37°C in plastic flasks as described by Vielma et al. [47]. The U937 human monocyte cell line was grown in suspension culture in RPMI-1640 containing 10% FBS and 1% of an antibiotic-antimycotic mixture. After incubation with BSE, genistein, daidzein, cyanidin, aspirin, and TNF-α, cell viability was assessed using a 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Mitochondrial dehydrogenase activity, by the reduction of MTT in active mitochondria to purple formazan, was used to determine cell survival in a colorimetric assay. Cell viability was calculated according to the formula: Cell viability = (absorbance sample tested/absorbance medium only) × 100%.

2.10. Cell Adhesion Assay

To explore the effects of BSE and its components, genistein, daidzein, cyanidin, and aspirin, on endothelial cell-monocyte interactions, the adherence of U937 cells to TNF-α-activated HAECs was examined under static conditions. HAECs were grown to confluence in 24-well plates. HAECs were then pretreated with 100 µg/ml of BSE, and 10 µM of genistein, daidzein, cyanidin, and aspirin for 18 h, followed by stimulation with TNF-α (2 ng/ml) for 6 h [48,49]. Adhesion assays of McCrohon et al. [50] were performed with minor modifications. Briefly, U937 cells were labeled with 10 µmol/l of the fluorescent dye, 2,7-bis(2-carboxyethyl)-5(6)-carboxy-fluorescein acetoxyethyl ester at 37°C for 1 h in RPMI-1640 medium, and subsequently washed by centrifugation. Confluent HAECs in 24-well plates were incubated with labeled U937 cells (10⁶ cells/ml) at 37°C for 1 h. Non-adherent monocytes were removed, and plates were gently washed twice with PBS. Numbers of adherent monocytes were determined by counting four fields per 100-fold high-power-fields using fluorescence microscopy (Nikon, Tokyo, Japan) and photographed. Four randomly chosen high-power fields were counted per well. Experiments were performed in duplicate and repeated at least 3 times independently.

2.11. ELISA

The effects of BSE, genistein, daidzein, cyanidin, and aspirin on the HAEC surface expressions of VCAM-1, ICAM-1, and E-selectin were analyzed with an ELISA using RayBio ELISA kits. Briefly, HAECs cultured to 95% confluence in 24-well microplates were incubated for 18 h during a 6-h TNF-α activation period. Monolayers were washed 3 times with cool phosphate-buffered saline (PBS), and cells were lysed with 1 ml of Celytic reagent, vortexed, incubated on ice for 30 min, and centrifuged at 12,000 g for 30 min at 4°C. Aliquots (100 µl) of the supernatant were frozen in liquid nitrogen, and stored at −70°C until later use. The ICAM-1, VCAM-1, and E-selectin present in an aliquot were captured by an immobilized antibody after overnight incubation at 4°C. Wells were washed 4 times with 0.1% Tween-20 in PBS, and 100 µl of 1-fold biotinylated primary antibody (specific for ICAM-1, VCAM-1, and E-selectin) was added for 1 h at room temperature. After washing, 100 µl of HRP-conjugated streptavidin was added to the wells for 45 min. After washing, 100 µl of a 3,3′,5,5′-tetramethylbenzidine substrate solution was added to the wells for 30 min in the dark. Finally, 50 µl of 2 M sulfuric acid was added to the cells, and the intensity of the color was measured at 450 nm using a spectrophotometer.
means was analyzed by the least significant difference (LSD) test.

3. Results

3.1. Levels of Plasma Cholesterol and Triglycerides

In the control group, the plasma cholesterol and triglyceride concentrations before the experiment were 0.72 ± 0.18 and 1.60 ± 0.33 g/l, respectively (n = 6) (unpublished data). Concentrations of plasma cholesterol (0.40 ± 0.06 g/l) and triglycerides (1.64 ± 0.10 g/l) in the control group did not change significantly during the 8-week feeding period (Table 1). In the treatment groups, plasma cholesterol and triglyceride concentrations before the experiment in the casein (n = 6), BS (n = 5), and vitamin E (n = 6) groups were 0.75 ± 0.42 and 1.72 ± 0.16, 0.77 ± 0.16 and 1.55 ± 1.11, and 0.54 ± 0.13 and 1.62 ± 0.88 g/l, respectively (unpublished data). In the treatment groups, in the 8 weeks after feeding cholesterol-containing chow to the casein, BS, and vitamin E groups, concentrations of plasma cholesterol significantly increased to 8.35 ± 6.26, 14.42 ± 4.30, and 10.60 ± 2.75 g/l, respectively (Table 1). After cholesterol feeding in the casein, BS, and vitamin E groups for 8 weeks, levels of plasma triglycerides in the treatment groups were 2.27 ± 0.84, 2.47 ± 1.00, and 3.32 ± 1.05 g/l, respectively (Table 1).

3.2. BS Attenuated the Atheroma Area and Oxidative Susceptibility of LDL in New Zealand White Rabbits

The ratio of the intimal to medial area in the aortic arch of the casein group (0.49% ± 0.29%) was significantly higher than those in the control (0.09% ± 0.02%), BS (0.25% ± 0.13%), and vitamin E groups (0.05% ± 0.03%) (Figure 1(a)). The atheroma region in the aortic arch of the BS group decreased 49% compared to the casein group. In addition, the ratio of the intimal to the medial area in the thoracic aorta of the casein group (0.56% ± 0.44%) was significantly higher than those in the control (0.10% ± 0.03%), vitamin E (0.11% ± 0.09%), and BS groups (0.23% ± 0.17%) (Figure 1(b)). The atheroma region of the thoracic aorta of the BS group decreased 58.9% compared to the casein group, while atheroma formation in the thoracic aorta in the vitamin E group was prevented and was close to the control level. Figure 2 demonstrates that the lag time of LDL in the BS group (319.6 ± 100.5 min) was similar to that in the vitamin E group (286.7 ± 97.0 min), which was significantly longer than those in the control (185.8 ± 61.6 min) and casein groups (187.2 ± 73.9 min).

3.3. Vitamin E Content in LDL and Anthocyanin in Plasma of New Zealand White Rabbits

The LDL vitamin E content in the vitamin E group (134.78 ± 87.67 nmole/mg LDL-cholesterol) was significantly higher than those in the control (58.77 ± 37.72 nmole/mg LDL-cholesterol), casein (30.23 ± 19.07 nmole/mg LDL-cholesterol), and BS groups (57.95 ± 68.94 nmole/mg LDL-cholesterol) (Table 2). The level of plasma anthocyanin in the BS group (2.10 ± 0.08 units/ml) was significantly higher than those in the control (0.29 ± 0.08 units/ml) (Table 2).

3.4. Concentrations of Isoflavones and Cyanidin in the BSs

Table 3 illustrates contents of genistein, daidzein, and cyanidin in BSs were greater than those of dry weight, respectively. Individual concentrations were equivalent to 0.074, 0.12, and 0.039 g/g of dry weight, respectively. Concentrations in the HAECs model (unpublished data).

Table 2. Vitamin E content in low-density lipoprotein (LDL) and anthocyanin in plasma of New Zealand white rabbits fed various 0.5% cholesterol-supplemented diets for 8 weeks.

<table>
<thead>
<tr>
<th>Group</th>
<th>Vitamin E (nmole /mg LDL-cholesterol)</th>
<th>Anthocyanin (unit/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>58.77 ± 37.72</td>
<td>0.22 ± 0.19</td>
</tr>
<tr>
<td>Casein</td>
<td>30.23 ± 19.07</td>
<td>0.21 ± 0.10</td>
</tr>
<tr>
<td>Black soybean</td>
<td>57.95 ± 68.94</td>
<td>2.10 ± 0.77</td>
</tr>
<tr>
<td>Casein-vit. E</td>
<td>134.78 ± 87.67</td>
<td>0.29 ± 0.08</td>
</tr>
</tbody>
</table>

All values are presented as the mean ± S.D. of n = 6 (except for the black soybean group, n = 5). Values with different superscripts significantly differ at p < 0.05.
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Figure 1. Effects of black soybean on aortic atherosclerosis lesions in atherosclerotic rabbits. A: Statistical data showing the atherosclerotic area ratio in the aortic arch (a) and thoracic aorta (b). All values are expressed as the mean ± S.D. of n = 6 (except for the black soybean group, n = 5). Values with different superscript letters significantly differ from each other. B: Representative photos showing a cross-section of an atherosclerotic lesion indicative of a thickened intima. Arrows indicate the thickened intima. Images were generated using a standard light microscope at 20× magnification. Rabbits were fed one of the following diets: control diet; casein plus high-cholesterol diet; black soybean plus high-cholesterol diet; and vitamin E plus high-cholesterol diet. (A)-(D) Aortic arch sections were stained with H&E. The intimal area significantly decreased in the soybean and vitamin E treatment groups. (E)-(H) Thoracic aorta sections were stained with H&E. The intimal area significantly decreased in the vitamin E treatment group. Stenosis was significantly reduced in aortic arch sections of the black soybean and vitamin E treatment groups. Due to the death of one rabbit, there were 5 rabbits in the black soybean group, and there were 6 rabbits per group in the other three groups.

Table 3. Contents of the main compounds of black soybean from the crude methanolic extract.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Contents (μg/g of DW extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genistein</td>
<td>198.7 ± 35.2</td>
</tr>
<tr>
<td>Daidzein</td>
<td>342.0 ± 16.6</td>
</tr>
<tr>
<td>Cyanidin</td>
<td>141.5 ± 1.8</td>
</tr>
</tbody>
</table>

Values are the mean of three triplicate determinations.

Figure 2. Effect of feeding various 0.5% cholesterol-supplemented diets for 8 weeks on the lag phase of low-density lipoprotein oxidation in New Zealand white rabbits.

3.5. Effects of BSE and its Components on Cell Viability

The effects of BSE and its components on cell cytotoxicity were determined by an MTT assay. HAECs were incubated with BSE (50 - 200 μg/ml) and its components (5 - 40 μM) for 18 h at various concentrations. Cyanidin at a concentration of >20 μM showed damage to the viability of HAECs, but none of the other treatments had significant effects on the viability of cells, which had cell viabilities of >90% (unpublished data). Therefore, concentrations of 10 μM of genistein, daidzein, cyanidin and 100 μg/ml of the BSE were chosen for the study.
3.6. BSE and its Components Reduced TNF-α-Induced Adhesion of Monocytes to HAECs

To determine the effect of BSE and its components on the adhesion of U937 cells to endothelial cells, HAECs were pretreated with 100 μg/ml of BSE and 10 μM of genistein, daidzein, cyanidin, and aspirin for 18 h, followed by stimulation with TNF-α (2 ng/l) for 6 h. Fluorescence-labeled monocytic U937 cells were added to HAECs and allowed to adhere for 2 h. The percentage cell adhesion was evaluated by quantification with the BCECF-AM staining method. However, BSE and its components (independently used to treat HAECs) did not adhere to U937 cells. Adhesion of U937 cells to TNF-α was stimulated in HAECs which increased by about 14-fold higher than that of the control group (Figure 3). Adhesion was markedly decreased by treatment with BSE and its components, which showed significantly decreased adhesion of U937 monocytic cells to TNF-α-stimulated HAECs by 98.2% with BS, 97.2% with genistein, 98.8% with daidzein, 93.1% with cyanidin, and 90.9% with aspirin (Figure 3). An examination of the cytotoxicity of BSE and its components on endothelial cells using an MTT assay indicated that BSE and its components had no adverse effect on cell viability (>90% cell viability, unpublished data). Therefore, the inhibition of monocyte adhesion to endothelial cells in the presence of BSE and its components was not the result of cytotoxicity.

3.7. BSE and Its Components Attenuated TNF-α-Induced CAM Protein Expression

Because the expression of CAMs by endothelial cells is a prerequisite for adhesion of monocytes, effects of BS on TNF-α-induced expressions of ICAM-1, VCAM-1, and E-selectin were investigated. Results from Figures 4-6 demonstrate that ICAM-1, VCAM-1, and E-selectin were expressed at low levels on unstimulated endothelial cells. There was >2-fold increases in the expressions upon stimulation with TNF-α in VCAM-1. Pretreatment of endothelial cells with BS, genistein, daidzein, cyanidin, and aspirin significantly inhibited TNF-α-induced VCAM-1 expression levels by 47%, 56.8%, 59%, 38.9%, and 42.7%, respectively (Figure 4). Moreover, genistein, daidzein and aspirin significantly decreased ICAM-1 expression by 33.4%, 17.1% and 41.7%, respectively (Figure 5), while no treatments had any effect on E-selectin (Figure 6).

4. Discussion

4.1. BS Alleviated Atheroma and Ox-LDL Formation through Its Antioxidative Efficacy of Isoflavones, Vitamin E, and Anthocyanins

The underlying mechanism of the protective effect of BS on the development of atherosclerosis was investigated in the present study. It was proposed that hyperlipidemia is a well-established risk factor for atherosclerosis, and drugs with a hypolipidemic effect are used clinically to inhibit atherosclerosis formation and progression [51]. Thus, the effect of BS on serum lipid profiles of rabbits was observed in this study. Results from Table 1 show
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Figure 4. Effect of black soybean extract and its components on human aortic endothelial cell (HAEC) expression of vascular cell adhesion molecule (VCAM)-1. Data are presented as the mean ± S.D. of three experiments, **p < 0.05.

Figure 5. Effects of black soybean extract and its components on human aortic endothelial cell (HAEC) expression of intercellular adhesion molecule (ICAM)-1. Data are presented as the mean ± S.D. of three experiments, **p < 0.05.

Figure 6. Effects of black soybean extract and its components on human aortic endothelial cell (HAEC) expression of E-selectin. Data are presented as the mean ± S.D. of three experiments.

that BS had no influence on the serum lipid profiles compared to the cholesterol-fed casein group, suggesting that actions other than the lipid-lowering effect of BS were responsible for the amelioration of atherosclerosis in rabbits. In addition, vitamin E inhibited atherosclerosis, which perhaps was through a significant reduction in the LDL oxidization ability. BS significantly decreased atherosclerotic plaque formation only in the aortic arch region, and tended to reduce atherosclerotic plaque formation in the thoracic aorta region, although it significantly reduced the LDL oxidization ability. In other studies, soy protein significantly decreased atherosclerosis at the aortic arch and thoracic aorta of rabbits, and soy protein isolate affected both the prevalence of atherosclerosis and the plaque size [10]. The alcohol extract of soy protein significantly lowered LDL cholesterol concentrations [52]. Both high (1%) and low (0.33%) isoflavone aglycone extracts without soy protein significantly decreased aortic arch lesions by 36.9% and 26.3%, respectively, and reduced cholesteryl ester hydroperoxide levels in LDL and the aortic arch [15]. In our study, BS significantly decreased the aortic arch lesion by 49%, which was better than the high (1%) isoflavone aglycone group. The thoracic aorta lesion in the BS group decreased 58.9%, similar to 1% isoflavone aglycones which reduced the thoracic aorta lesion in cholesterol-fed rabbits by 57.3% [15]. Antioxidative actions of isoflavones inhibit the oxidation of LDL, thereby exerting an antiatherosclerotic effect.

α-Tocopherol administration in rabbits may have directly diminished lesion formation by preventing radical-mediated injury and inflammatory reactions in the endothelium and aortic intima. This possibility is consistent with a report which showed that vitamin E directly diminished U-937 monocyte adhesion to inflammatory cytokine-stimulated endothelial cells in tissue culture [53]. α-Tocopherol as the sole lipophilic antioxidant in moderately hypercholesterolemic hamsters substantially suppressed vascular oxidative stress and atherogenesis [54]. The vitamin E group in our study also demonstrated inhibition of LDL oxidation and suppression of aortic lesion formation (Figures 1 and 2).

The isoflavone aglycones, genistein, and daidzein, have scavenging activities [55] and antioxidant properties [55, 56]. Isoflavone phytoestrogens act as antioxidants, providing increased oxidation resistance for lipoproteins [12]. However, due to their relative hydrophilicity, these isoflavones are incorporated into LDL to only a small extent, corresponding to approximately one isoflavone molecule for each 500 LDL molecules (≤0.33 molecules/LDL particle), and the oxidation resistance of particles showed no change. Genistein and daidzein were then converted into fat-soluble derivatives by esterification with fatty acids to facilitate their incorporation into LDL. Oleic acid esters
were incorporated most effectively, reaching a concentration of 2.19 molecules per LDL particle, and resulting in significantly increased oxidation resistance in vitro [16]. Isoflavones may protect lipoproteins against oxidation, because they are converted to lipophilic derivatives and incorporated into lipoprotein particles [57].

Consumption of antioxidant flavonoids in tea, fruits, and vegetables, lycopene in tomato products, and vitamin E as a supplement is associated with a reduced risk of coronary heart disease [58,59]. Both vitamin E and black tea significantly increased the lag phase of hyper-cholesterolemic rabbits; however, neither treatment had any effect on the formation of atherosclerotic lesions [60]. The lag phase had no significant association with the extent of atherosclerosis. Yang et al. [61] reported that salvianolic acid (Sal B), a water-soluble antioxidant obtained from a Chinese medicinal herb, reduced Cu$^{2+}$-induced LDL oxidation, lipid deposition in the thoracic aorta, intimal thickness of the aortic arch and thoracic aorta, and neointimal formation in the abdominal aorta. In our study, the content of anthocyanin in BS was 119.63 ± 35.2 and 342.0 ± 16.6 μg/g dry weight, respectively (Table 2), while the level of anthocyanin in plasma was 2.1 ± 0.77 units/mL (unpublished data), which might be one of the mechanisms contributing to the amelioration of atherosclerosis in hyperlipidemic rabbits. BS alleviates atherosclerosis, and it provides evidence to support the use of antioxidants to prevent atherosclerosis.

4.2. BSE and Its Components Block Adhesion Molecule-Endothelial Cell Interactions

Adhesion of monocytes to the vascular endothelium and subsequent migration into the vessel wall are early events in atherogenesis [20]. Proinflammatory cytokines, including TNF-α, interferon (IFN)-γ, IL-1, IL-8, monocyte chemoattractant protein (MCP)-1, macrophage colony-stimulating factor (M-CSF), cyclooxygenase (COX)-2, nitric oxide synthase (NOS), and CD40, enhanced expressions of surface expression adhesion molecules, such as ICAM-1, VCAM-1, and E-selectin [63,64]. Dietary polyphenols, such as catechin and quercetin, significantly reduce binding of monocytes to HAEcs [65,66], which is similar to the results of the present study. Figures 4 and 5 illustrate that the reduced cellular adhesion may have been due to inhibition of CAM expression as genistein and daidzein reduced both VCAM-1 and ICAM-1 expressions, and BSE and cyanidin only reduced VCAM-1 expression. Genistein significantly inhibited IL-1- and TNF-α-induced upregulation of neutrophils and monocyte adherence [67]. Induction of ICAM-1, VCAM-1, and E-selectin surface expressions by TNF was reduced by the protein tyrosine kinase inhibitor, genistein, suggesting that specific phosphorylation following protein tyrosine kinase activation may be required for NF-kB mobilization and induction of VCAM-1 and endothelial leucocyte adhesion molecule 1 (ELAM-1) by TNF [68]. Similar results were also observed in different studies [69,70]. These findings reveal that tyrosine-phosphorylated proteins may regulate leukocyte adherence and CAM expression in the endothelium [71]. Recently, Chinta et al. [72] demonstrated that daidzein significantly suppressed the production of the proinflammatory factors, nitric oxide and interleukin 6 (IL-6), and reduced ROS production, p38 MAPK phosphorylation, and NF-kB activation. Endothelial cells treated with the soy isoflavone, genistein, inhibited monocyte adhesion, suggesting that the atheroprotective effect of soy isoflavones can be mediated by regulating endothelial cell functions [73]. In addition, modulation of interactions between inflammatory factors, such as IL-6 and endothelial cells may be a plausible mechanism explaining the beneficial effects of soy-based diets. Nagarajan et al. [30] also showed that pre-exposure to soy isoflavones inhibited monocyte adhesion to endothelial cells through the endothelial ICAM (CD54). This inhibition by isoflavones led to less production of inflammatory cytokines, such as IL-6 and IL-8, by monocytes. The mechanism by which soy isoflavones block monocyte adhesion was through inhibition of CD11a (integrins) affinity to CD54.

Many groups have identified a number of small mo-
lecules from natural sources and several plant extracts that block nuclear accumulation of NF-κB and abrogate TNF-α-induced expressions of ICAM-1, VCAM-1, and E-selectin by endothelial cells [74,75]. In addition, several reports showed that natural products with antioxidant activity inhibit the TNF-α-induced activation of redox-sensitive NF-κB [76-78]. Kim et al. [34] reported that anthocyanidin reduced ICAM-1 and VCAM-1 expressions through inhibiting the nuclear translocation of NF-κB. Furthermore, Kim et al. [37] demonstrated that anthocyanins from BSSC inhibited TNF-α-induced ICAM-1 and COX-2 levels through an NF-κB-dependent pathway, and had anti-inflammatory effects on the HaCaT cell line. It is possible that cyanidin and isoflavones influenced inhibitory proteins of nuclear factor-kB (IkBα) and of IkB kinase α (IKKα) activity as suggested by Garcia-Mediavilla et al. [79] and Min et al. [80].

5. Conclusion
BS had no effect on serum lipid profiles. Suppression of LDL oxidation by BS was through attenuation of oxidative stress, which contributes, at least in part, to the amelioration of atherosclerosis. Presumably, the atheroprotective effect of BS diets is mediated by blocking adhesion molecular-endothelial cell interactions through an NF-κB-dependent pathway. These results show the therapeutic potential of BS as an atheroprotective and anti-inflammatory agent for the use in cytokine-induced vascular disorders, including atherosclerosis.

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REFERENCES
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