The Seed Coat Extract of Black Soybean Decreases Nicotine-Induced Vascular Fiber Degradation by Suppressing Matrix Metalloproteinase 2 Expression

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Summary Abdominal aortic aneurysm (AAA) is a vascular disease characterized by weakening of vascular walls and progressive dilation of the abdominal aorta. Nicotine, the main component of tobacco, is reportedly associated with the development and rupture of AAA. It is desirable to attenuate the destructive effect of nicotine on vascular walls, using dietary food components. However, effective methods for preventing AAA progression using dietary food components remain unestablished. This study focuses on proanthocyanidins, known for their potent antioxidant activity. We speculated that proanthocyanidins can suppress nicotine-induced weakening of vascular walls. To estimate the effect of black soybean seed coat extract (BSSCE), rich in proanthocyanidins, on nicotine-induced weakening of the aortic wall, mice were divided into four groups: the control diet and distilled water group (named C), BSSCE solution diet and distilled water group (named B), control diet and 0.5 mg/mL nicotine solution group (named CN), and BSSCE solution diet and 0.5 mg/mL nicotine solution group (named BN). Nicotine-induced degradation of elastin and collagen fibers were significantly suppressed in BN group. The positive areas for matrix metalloproteinase (MMP)-2 and oxidative stress in BN group were significantly decreased compared to those in CN group. These results suggest that proanthocyanidins-rich BSSCE can prevent the weakening of the aortic wall via inhibiting MMP-2 upregulation.

Key Words abdominal aortic aneurysm, proanthocyanidins, black soybean seed coat extract, smoking, passive smoking, elastin fiber, collagen fiber

Abdominal aortic aneurysm (AAA) is a lethal disease characterized by abdominal aorta expansion due to blood vessel wall weakening. Risk factors for AAA development include hypertension, age, smoking, and being a male (1). The vascular wall weakening and increase in the diameter of the aneurysm progress asymptotically, which eventually results in sudden AAA rupture with strong pain. Once the abdominal aorta ruptures, more than half of AAA patients suddenly die due to hemorrhaging. AAA patients have a high mortality rate of approximately 50% even if they undergo emergency surgery (2). Presently, endovascular stent graft placement and prosthetic graft replacement are applied for preventing the progression and rupture of aneurysms. However, these surgical treatments are for patients with an aneurysm size ≤5.5 cm (3). Despite the fact that small diameter aneurysms might rupture, patients with small AAA have no treatment options. It is therefore necessary to prevent the development and rupture of AAA, using drugs or dietary foods.

AAA pathophysiology is characterized by the infiltration of immune cells and destruction of the extracellular matrix, including elastin and collagen fibers in the vascular wall (4). In a normal aorta, elastin and collagen fibers support blood pressure and maintain a constant aortic diameter. Immune cells such as macrophages, and inflammatory mediators such as monocyte chemotactic protein-1 (MCP-1) and interleukin-6 (5), are responsible for the pathological events in AAA. As a result, increased levels of matrix metalloproteinases (MMPs) degrade elastin and collagen fibers, weakening and expanding the vascular wall (6).

Smoking is reportedly closely associated with AAA development and rupture (7, 8), and reportedly increases the expansion rate of the aortic diameter by 20–25% (9). In fact, most AAA patients reportedly have a smoking history (10), and the AAA rupture-induced mortality risk in smokers is higher than in non-smokers (7). The Incidence risk of AAA increases with the number of years of smoking (8). It has been suggested that nicotine, a main component of tobacco, is strongly associated with AAA development and rupture (11). Wang et al. previously reported that nicotine could induce AAA formation by a mechanism that is similar to that of the action of angiotensin II (Ang II) administered to
apoprotein E deficient (ApoE−/−) mice (12). Nicotine or Ang II causes activator protein (AP)-2a phosphorylation by activating AMP-activated protein kinase (AMPK)-α2 in vascular smooth muscle cells, causing abnormal MMP-2 expression and extracellular matrix degradation (12). In addition, nicotine-induced AAA formation is reportedly associated with microRNA-21 expression (13).

Based on these facts, inhibiting nicotine intake from active or passive smoking is one of the most important methods for preventing AAA development and/or rupture. Our previous study revealed that nicotine administration to mice weakened their vascular walls by vascular fiber degradation through increased MMP activity (14, 15). The administration of fish oil, rich in eicosapentaenoic acid, to nicotine-administered mice, suppressed elastin fiber degradation by suppressing nicotine-induced MMP activation and oxidative stress (15). Further, sesamin- and sesamolin-rich sesame extract, and DNA-rich salmon milt extract, also suppressed nicotine-induced vascular fibers destruction (16, 17). Therefore, well-balanced diets must contain a variety of AAA-preventing functional food components. In this study, the focus was on black soybean seed coat extract (BSSCE). It contains significant amounts of proanthocyanidins, a type of polyphenol, possessing strong anti-oxidative effects (18, 19). The content of proanthocyanidins in black soy bean are higher than those in grape, apple, and persimmon (19). Proanthocyanidins can be distributed in animal body (20). This study reveals how proanthocyanidin-rich BSSCE is effective for preventing vascular wall degradation in nicotine-administered mice.

MATERIALS AND METHODS

Materials. Nicotine was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), and dissolved in distilled water to obtain a nicotine solution. BSSCE solution was acquired from Otsuka Foods Company (Osaka, Japan).

Animals. The Kindai University Animal Care and Use Committee approved all animal experiments, which were performed according to the University’s Animal Experimentation Regulations (Approval number: KAAG-25-002). A humidity-controlled room was used to house 3-wk-old male C57BL/6j mice (Japan SLC, Inc., Shizuoka, Japan) with a 12-h light and 12-h dark cycle. Food and water were then provided ad libitum, and the temperature of the room was maintained at 25±1°C. After habituation for 7 d, the mice were divided into 4 groups: the control diet and distilled water group (named C), BSSCE solution diet and distilled water group (named B), control diet and 0.5 mg/mL nicotine solution group (named CN), and BSSCE solution diet and 0.5 mg/mL nicotine solution group (named BN). Each diet was administered to mice in the C (n=9), the B (n=10), the CN (n=10), and BN (n=9) groups. Table 1A shows the diet composition for the different groups, while Table 1B gives a summary of the proanthocyanidin contents. At 52 d after administration, all mice were sacrificed with suffering minimized by the use of medetomidine, midazolam, and butorphanol as anesthesia.

Histological analyses. The abdominal aortas of mice in all groups were isolated and embedded in paraffin from Sakura Finetek Japan (Tokyo, Japan), as previously described (14). Cross-sections of the isolated aortas of thickness 4 μm were prepared using a microtome from Yamato Kohki Industrial (Saitama, Japan), and then mounted on glass slides. The aortic walls were stained with hematoxylin–eosin (HE), Elastica van Gieson (EVG), Picrosirius red (PSR), and immunohistochemical stains. Quantitative analyses of the histological stains were performed using ImageJ software from National Institutes of Health (Bethesda, Maryland, USA). The destruction rates of the wavy configuration of the elastic lamina were calculated as previously described (15).

Immunohistochemical staining. The tissue sections were deparaffinized, and then immunohistochemical staining was performed as previously described (14). The histological results of the aortic walls were assessed after staining using the following antibodies: rabbit anti-MMP-2 (1:100; Thermo Scientific, San Jose, CA, USA), goat anti-MMP-9 (1:100; Santa Cruz Biotechnology, Dallas, TX, USA), rabbit anti-MMP-12 (1:100; Bioss Antibodies, Woburn, MA, USA), mouse anti-malondialdehyde (MDA) (1:100; Abcam, Tokyo, Japan), rabbit anti-monocyte chemotactic protein-1 (MCP-1) (1:100; Bioss Antibodies), and rabbit anti-CD68 (1:100; Bioss

| Table 1. Diet composition. |
|-----------------------------|
|                             | Control diet (g) | BSSCE solution diet (g) |
| Choline chloride            | 0.24             | 0.24                   |
| Methionine                  | 0.36             | 0.36                   |
| AIN-93 vitamin mix          | 1.20             | 1.20                   |
| AIN-93G mineral             | 4.20             | 4.20                   |
| Cellulose                   | 6.00             | 6.00                   |
| Sucrose                     | 12.00            | 12.00                  |
| Lard                        | 24.00            | 24.00                  |
| Casein                      | 26.64            | 26.64                  |
| Cornstarch                  | 45.36            | 45.36                  |
| Vehicle control             | 120              | 0                      |
| BSSCE solution¹             | 0                | 120                    |
| Total (g)                   | 240.00           | 240.00                 |

¹ BSSCE solution contains 6% BSSCE.

| Table 1A. BSSCE composition |
|-----------------------------|
|                             | Amount of compound (mg/100 g) |
| Catechin                    | 1.6                         |
| Epicatechin                 | 44.2                        |
| Procyanidin B2              | 29.7                        |
| Procyanidin C1              | 13.6                        |
| Total (g)                   | 89.1                        |
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Antibodies). Statistical analyses. The values are expressed as mean ± standard error of the mean (SE). Statistical differences were determined using the Tukey–Kramer test. A p-value < 0.05 was considered statistically significant. The Stat View 5.0 software (SAS Institute, Cary, USA) was used to carry out statistical analyses.

RESULTS

Effect of BSSCE on body weight, food intake, and serum parameters

Final body weight (g) was not significantly different between the 4 groups (Table 2). The average food intake (g) significantly increased in the B group compared with the C group (Table 2). The average water intake (g) was...
not significantly different between the 4 groups (Table 2). There were trends for lower levels of serum glucose and triglyceride in the B group compared with the C group. Serum glucose and triglyceride levels significantly decreased in the BN group compared with the CN group (Table 2). Total cholesterol level was not significantly different between the 4 groups (Table 2).

**Effect of BSSCE on elastin and collagen fiber degradation**

The vascular structure is maintained by elastin and collagen fibers. Because the degradations of these fibers caused the dysfunction of vascular wall, we estimate the effects of BSSCE on elastin and collagen fibers. The thickness of the aortic wall was not significantly different between the 4 groups (Fig. 1a–d, and m). Figure 1e–h shows representative pictures of elastin fibers. The ratio of elastin fiber destruction significantly increased in the CN group compared with the C and B groups (Fig. 1n), while it was significantly suppressed in the BN group compared with the CN group (Fig. 1n). In order to measure the positive areas and the density of collagen fibers, PSR staining (Fig. 1i–l) was used. The collagen positive areas significantly decreased in the CN group compared with the C group (Fig. 1o), while they were significantly increased in the BN group compared with the CN group (Fig. 1o).

**Effect of BSSCE on MMPs**

To investigate the preventive mechanisms of BSSCE on elastin and collagen fibers, we estimated the levels of MMP-2, MMP-9, and MMP-12 in aortic layers (intima, media, and adventitia). Because intima was difficult to distinguish from media, we separate the aortic region into two regions, intima-media and adventitia. Immunohistochemical examination revealed MMP-2, MMP-9, and MMP-12 levels in each group (Fig. 2a–l). The MMP-2-positive area in the intima-media of the CN group significantly increased compared with those of the C and B groups (Fig. 2m), and significantly decreased in the BN group compared with the CN group (Fig. 2m). The difference in the MMP-2-positive area in the adventitia was not significantly different between the 4 groups (Fig. 2m). The differences in the MMP-9-positive area in the intima-media and adventitia were not significantly different between the 4 groups (Fig. 2n). The MMP-12-positive area in the intima-media tended to decrease in the BN group compared with the CN group (Fig. 2o), and the difference in the positive area for MMP-12 in the adventitia was not significantly different between the 4 groups (Fig. 2o).
Effect of BSSCE on the positive areas for CD68 positive macrophage/monocyte, MCP-1, and MDA in the vascular wall

To investigate the suppressive mechanisms of BSSCE on MMP-2, we estimated the levels of monocyte/macrophage (CD68 positive cell), MCP-1, and oxidative stress (MDA). Immunohistochemical examination elucidated CD68, MCP-1, and MDA levels in each group (Fig. 3a–l). The positive area for CD68 and MCP-1-positive areas in the intima-media and adventitia were not significantly different between the 4 groups (Fig. 3m, n), while the MDA-positive area in the intima-media and adventitia significantly increased in the CN group compared with those in the C and B groups (Fig. 3o). Further, the MDA-positive area in the intima-media significantly decreased in the BN group compared with the CN group (Fig. 3o), while that in the adventitia tended to decrease in the BN group compared with the CN group (Fig. 3o).

DISCUSSION

In this study, we evaluated the effect of proanthocyanidin-rich BSSCE on nicotine-induced destruction of vascular wall fibers. Histological analyses revealed that elastin and collagen fiber degradations were significantly suppressed by BSSCE. Immunohistochemical analyses revealed that BSSCE suppressed the increase of MMP-2 positive areas as well as the increase in oxidative stress in the vascular wall.

The levels of serum glucose and triglyceride in the BSSCE-fed groups were lower than those in control groups. Proanthocyanidin B2 reportedly decreased postprandial blood glucose level by inhibiting α-glucosidase activity (21). In addition, Proanthocyanidin B2 reportedly decreased serum triglyceride levels by activating SIRT1 (22). The decreased effects of BSSCE can be partly attributed to the activity of proanthocyanidin B2 in BSSCE.

Proanthocyanidins have a wide variety of structures based on varied molecular modifications and transformations (23). Amongst them, oligomeric proanthocyanidins express strong biological activity against oxidative stress (24). Recent studies showed that proanthocyanidins have suppressive oxidative stress in obstructive jaundice, and liver and lung tissue damage (25). Nicotine reportedly increased MMP-2 expression via oxidative stress due to increased reactive oxygen species (ROS) levels in the vascular wall of Apoe−/− mice.
and human vascular smooth muscle cells (VSMCs) (12). Our results suggest that the anti-oxidative effects of proanthocyanidins can contribute to the suppression of nicotine-induced oxidative stress. Reports hold that proanthocyanidin-rich BSSCE can suppress ROS-induced oxidative stress resulting from the presence of nicotine in the vascular wall, which might suppress elastin fiber degradation by the activation of MMP-2.

Proanthocyanidins are used as a dentinal cross-linking reagent because of the potentiation effect of the mechanical strength of collagen fibers due to adding new cross-linking to collagen fibers (26). As a cross-linking reagent, they can also contribute to the suppression of nicotine-induced collagen fiber destruction by modifying the structure of vascular wall collagen fibers. In this study, proanthocyanidin-rich BSSCE administration could modify collagen cross-linking structure and inhibit nicotine-enhanced collagen fiber destruction. However, the detailed mechanisms underlying the suppression of nicotine-induced collagen fiber degradation remain unknown and need to be elucidated.

The estimated intakes were: catechin: 3.2 (µg/d), epicatechin: 88.4 (µg/d), procyanidin B2: 59.4 (µg/d), and procyanidin C1: 27.2 (µg/d). These values were lower than those of other functional food factors in previous study (15–17, 27). BSSCE may be effective functional food factors for high risk populations such as passive smoker.

Disclosure of state of COI

H.M. and MM. are employees of Otsukafoods Co., Ltd. All the other authors declared no competing interests.

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