Isoflavone Ameliorated Oxidative Stress and Vascular Damages in Nicotine-Administered Mice

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Abstract: Nicotine has been linked to the development of abdominal aortic aneurysms. Isoflavones, a group of polyphenolic compounds, reportedly exhibit antioxidant and anti-inflammatory properties and facilitate cardiovascular protection. However, the effects of isoflavone on nicotine-induced abdominal aortic aneurysms have not yet been elucidated. The objective of the current study was to evaluate the inhibitory effect of isoflavone on nicotine-induced weakening of the aortic wall in mouse models. Nicotine reportedly increases the occurrence of abdominal aortic aneurysms by activating endothelin-1 (ET-1), angiotensinogen and the angiotensin II type 1 (AT\(_1\)) receptor, leading to an increase in neutrophil elastase, oxidative stress, and matrix metalloproteinase (MMP)-2 expression, which causes vascular wall weakness and damage. Immunohistological analyses have indicated that isoflavone significantly inhibits the activation of ET-1, angiotensinogen and the AT\(_1\) receptor in nicotine-administered mice. Additionally, isoflavone suppressed elastic fiber destruction and decreased areas positive for MMP-2, neutrophil elastase, and malondialdehyde in the vascular wall of nicotine-administered mice. Considered together, these findings suggest that isoflavone shows potential for preventing vascular wall injury induced by nicotine administration, and that food containing isoflavone may protect against abdominal aortic aneurysms.

Key words: angiotensinogen, endothelin-1, matrix metalloproteinase, neutrophil elastase, oxidative stress

1 Introduction

Abdominal aortic aneurysm (AAA), characterized as a chronic inflammation of the vascular wall coupled with progressive abdominal aorta distention, is considered to be associated with age, male sex, smoking and hypertension. AAA is responsible for high death rates due to ruptures caused by AAA\(^1\). At present, pharmacological treatments for AAA development and rupture are not available. AAA leads to infiltration of the vascular wall by inflammatory cells, such as lymphocytes and macrophages, resulting in the release of inflammatory mediators and free radicals as well as a loss of elastin and collagen in the media and adventitia due to matrix metalloproteinases (MMPs), all of which contribute to a reduction in vascular wall strength\(^2\).

Cigarette smoking has been linked to deaths caused by cardiovascular dysfunction worldwide\(^3\). Cigarette smoking, in particular, is a primary risk factor for AAA and a major cause of death from cardiovascular disease globally\(^4, 5\). Nicotine, the most important chemical in cigarette smoke, is considered to be the leading harmful agent responsible for the occurrence and/or rupture of AAA\(^5\). Many studies have reported that nicotine exerts a potent effect on the induction of AAA development in both human and animal models\(^6-8\). Increased MMP activity, oxidative stress and fiber degradation have been observed in the vascular walls of nicotine administered mice and rats\(^7, 9-12\). Nicotine also enhanced the production of endothelin-1 (ET-1)\(^13\). The overexpression, or increased activity, of ET-1 is reportedly associated with the progression and rupture of AAA\(^14, 15\). Furthermore, nicotine reportedly increased the activation of angiotensin II and the angiotensin II type 1 (AT\(_1\)) receptor\(^16\), leading to an increase in inflammation, oxidative stress, MMPs activity and fiber degradation, which promoted AAA development\(^17\).

The use of therapeutic drugs for treating and preventing AAA progression and rupture is not yet well-established.

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Therefore, characterizing the impact of functional food on the progression of AAA may be of significant importance. Several in vivo studies using models reported that nutrients and food ingredients exerted a beneficial effect on AAA development or rupture \(^{9-12}\). Isoflavones are polyphenolic nonsteroidal compounds found in the legume family, including food crops such as soybeans, green beans, and fava beans. Worldwide production of food items containing isoflavones, such as tofu, bean curd snacks, and soymilk, is on the rise \(^{13}\). A high dietary consumption of foods containing high levels of isoflavone was correlated with a lower incidence of chronic diseases, including coronary heart disease \(^{14}\). Additionally, many reports have suggested that isoflavones may contain antioxidants that scavenge free radicals \(^{15,16}\), are anti-inflammation \(^{17,18}\), and provide cardiovascular protection \(^{19,20}\). However, the effect of isoflavones on vascular injury induced by nicotine remains undetermined. Therefore, the current study investigated the effect of isoflavones on vascular damage inhibition in mouse nicotine-induced models, and whether this effect was implemented via the suppression of ET-1, angiotensinogen and the AT\(_1\) receptor, associated with AAA development.

## 2 Experimental

### 2.1 Chemical

Paraformaldehyde (PFA) was purchased from Nacalai Tesque (Kyoto, Japan). Primary antibodies specific to matrix metalloproteinase (MMP)-2 were purchased from Thermo Scientific (San Jose, CA, USA). Goat anti-MMP-9 was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Rabbit anti-MMP-12 and rabbit anti-CD68 were purchased from Bioss Antibodies (Woburn, MA, USA). Rabbit anti-malondialdehyde (MDA) and mouse anti-endothelin (ET)-1 were obtained from Abcam (Tokyo, Japan). Rabbit anti-angiotensinogen and rabbit anti-angiotensin II type I (AT\(_1\)) receptor were obtained from Novus Biologicals (CO, USA). Nicotine was obtained from Wako Pure Chemical Industries (Osaka, Japan).

### 2.2 Animals, experimental protocol, and dietary treatment

Thirty-one young, adult male C57BL/6 mice, aged 3 weeks, were obtained from Japan SLC, Inc., Shizuoka, Japan. The mice were randomly placed in separate cages, under controlled conditions of a 12 h light/dark cycle at 25 ± 1°C. Prior approval for all animal experiments was obtained from the Kindai University Animal Care and Use Committee and the trials were carried out according to Kindai University Animal Experimentation Regulations (approval number; KAAG-25-002). Following an adaptation period of 3 days, the animals were randomly divided into 4 groups as follows: control diet and distilled water (C) (n = 7); isoflavone diet and distilled water (I) (n = 8); control diet and 0.5 mg/mL nicotine solution (CN) (n = 8); and isoflavone diet and 0.5 mg/mL nicotine solution (IN) (n = 8). The composition of the diet is shown in Table 1. All animals were euthanized under anesthesia after 15 days of experimentation.

### 2.3 Serum glucose, cholesterol, and triglyceride level

At day 15 of the experiment, blood was drawn from the inferior vena cava under anesthesia (50 mg/kg pentobarbital, i.p.) to determine serum glucose, cholesterol, and triglyceride levels. Blood was centrifuged at 3000 g/min for 10 min for the purpose of serum preparation. Next, serum cholesterol, triglycerides, and glucose were measured using commercial kits, according to manufacturers’ instructions.

### 2.4 Sample collection and histological analyses

The aortas were removed, and fixed in 4% PFA. Briefly, all samples were dehydrated, processed routinely, and embedded in paraffin. Isolated aortas were serially sectioned into 4 μm thick sections using a microtome (PR-50, Yamato Kohki, Japan) and stained with hematoxylin and eosin (H&E), Elastica van Gieson (EVG) and Picrosirius red (PSR). ImageJ software (National Institutes of Health, Bethesda, Maryland, USA) was used for the quantitative analysis of stained sections. The rate of destruction of the elastic lamina configuration was calculated as previously described \(^{19}\).

### 2.5 Immunohistochemical staining

Deparaffinized tissue sections were permeabilized using 0.1% Triton X-100 in phosphate-buffered saline. The sections were then blocked for endogenous peroxidase by soaking in 3% hydrogen peroxide in methanol for 8 min. The sections were blocked for non-specific binding in blocking solution (Nacalai Tesque, INC, Kyoto, Japan) for

| Table 1 | Diet composition. |
|---------|--------------------|
|         | Control diet (g)   | Isoflavone diet (g) |
| Choline chloride | 0.250             | 0.250               |
| Cystine | 0.300              | 0.300               |
| AIN-93 vitamin mix | 1.000             | 1.000               |
| AIN-93G mineral | 3.500              | 3.500               |
| Cellulose | 5.000              | 5.000               |
| Olive oil | 7.000              | 7.000               |
| Sucrose | 10.000             | 10.000              |
| Casein | 20.000             | 20.000              |
| Cornstarch | 52.950            | 52.950              |
| Isoflavone MIX | 0                 | 0.075               |
| Total (g) | 100.000            | 100.075             |

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Table 2  Effect of isoflavone on physiological variables and serum biochemistry.

|                      | C            | I            | CN           | IN           |
|----------------------|--------------|--------------|--------------|--------------|
| Initial body weight (g) | 12.92 ± 0.29 | 12.82 ± 0.27 | 12.60 ± 0.30 | 12.36 ± 0.33 |
| Final body weight (g)  | 22.01 ± 0.37 | 21.88 ± 0.24 | 21.26 ± 0.40 | 21.12 ± 0.37 |
| Food intake (g/day)    | 5.81 ± 0.10  | 5.80 ± 0.08  | 5.77 ± 0.12  | 5.71 ± 0.09  |
| Water intake (mL/day)  | 1.75 ± 0.31  | 1.86 ± 0.22  | 1.24 ± 0.40  | 1.21 ± 0.24  |
| Nicotine intake (mg/day) | 0.00 ± 0.00  | 0.00 ± 0.00  | 0.62 ± 0.08  | 0.60 ± 0.09  |
| Isoflavone intake (mg/day) | 0.00 ± 0.00  | 4.35 ± 0.06  | 0.00 ± 0.00  | 4.28 ± 0.02  |
| Serum glucose (mg/dL)  | 299.74 ± 25.55 | 351.21 ± 13.86 | 333.05 ± 10.35 | 342.35 ± 10.56 |
| Serum triglyceride (mg/dL) | 92.00 ± 10.66 | 59.73 ± 8.55  | 83.49 ± 6.20  | 49.12 ± 12.72 |
| Serum total cholesterol (mg/dL) | 150.50 ± 6.98  | 125.15 ± 9.79  | 136.14 ± 5.71  | 103.11 ± 14.52 |

Data are the mean ± S.E.M. Values with different letters are significantly different (*p < 0.05*).

3.2 Effect of dietary isoflavone on vascular wall thickness, collagen fiber, and elastin fiber in mouse nicotine-administered models

Vascular wall thicknesses (Figs. 2a-e) and collagen-positive areas (Figs. 1f-j) were not significantly different between the 4 treatments. The elastin fiber destruction area in the CN group was significantly increased compared to those of groups C and I, whereas the elastin destruction area in the IN group was significantly decreased compared to that in the CN group (*p < 0.05*, Figs. 1k-o).

3.3 Isoflavone downregulated endothelin-1 (ET-1), angiotensinogen, and angiotensin II type 1 (AT1) receptors in mouse nicotine-administered models

Immunohistochemistry was used to detect the expression of ET-1 protein, angiotensinogen, and the AT1 receptor. Immunohistochemical staining revealed that ET-1 expression was significantly increased in both the intima-media and the adventitia of the vascular wall in the CN group (Figs. 2c and e; *p < 0.05*) compared to those of groups C (Figs. 2a and e) and I (Figs. 2b and e). Diet containing isoflavone significantly suppressed ET-1 expression in both the intima-media and the adventitia of the vascular wall in the IN group compared to that of the CN group (*p < 0.05*; Figs. 2d and e). Additionally, nicotine administration caused a significant increase in the expressions of angiotensinogen (*p < 0.05*; Figs. 2h and j) and AT1 receptor (*p < 0.05*; Figs. 2i and k).
Fig. 1 Effect of dietary isoflavone on vascular wall thickness, collagen fiber, and elastin fiber in mouse nicotine-administrated models. (a-d) hematoxylin-eosin staining and (e) vascular wall thickness quantification in the 4 experimental groups. (f-i) Picrosirius red staining and (j) quantification of collagen positive areas in the 4 experimental groups. (k-n) Elastica van Gieson staining and (o) elastic destruction quantification in the 4 experimental groups. Scale bar = 50 \( \mu \)m. Arrows indicate the elastic destruction area. Data are presented as mean ± standard error of the mean (S.E.M). C group \((n = 7)\), I group \((n = 8)\), CN group \((n = 8)\), and IN group \((n = 8)\). Values represented by different letters are significantly different \((p < 0.05)\). C, control diet and distilled water group; I, isoflavone diet and distilled water group; CN, control diet and nicotine solution group; IN, isoflavone diet and nicotine solution group.

Fig. 2 Isoflavone downregulated endothelin-1 (ET-1), angiotensinogen and the angiotensin II type 1 (AT_{1}) receptor in mouse nicotine-administrated models. (a-d) ET-1 immunostaining and (e) quantification of ET-1 positive areas of the vascular wall in the 4 experimental groups. (f-i) angiotensinogen immunostaining and (j) quantification of angiotensinogen positive areas of the vascular wall in the 4 experimental groups. (k-n) AT_{1} receptor immunostaining and (o) quantification of AT_{1} receptor positive areas of the vascular wall in the 4 experimental groups. Scale bar = 50 \( \mu \)m. Data are presented as the mean ± standard error of the mean (S.E.M). C group \((n = 7)\), I group \((n = 8)\), CN group \((n = 8)\), and IN group \((n = 8)\). Values represented by different letters are significantly different \((p < 0.05)\). C, control diet and distilled water group; I, isoflavone diet and distilled water group; CN, control diet and nicotine solution group; IN, isoflavone diet and nicotine solution group.
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0.05; Figs. 2m and o) in both the intima-media and the adventitia of the vascular wall in the CN group compared to those of the C and I groups. Interestingly, administering isoflavone significantly inhibited angiotensinogen (p < 0.05; Figs. 2i and j) and the AT₁ receptor (p < 0.05; Figs. 2n and o) expression in both the intima-media and the adventitia of the vascular wall in the IN group compared to that in the CN group. These results indicated that nicotine enhanced the activity of angiotensinogen, AT₁ receptor, and ET-1, all of which play a crucial role in the development of vascular damage. Significantly, administering of isoflavone inhibited vascular damage induced by nicotine via the downregulation of angiotensinogen, AT₁ receptor, and ET-1 protein expression.

3.4 The effect of dietary isoflavone on MMP-2, MMP-9, and MMP-12 in mouse nicotine-administered model

MMP-2 protein expression was significantly upregulated in the intima-media of the vascular wall of the CN group (p < 0.05; Figs. 3c and e) compared to that of the C and I groups. Administering of isoflavone significantly decreased MMP-2 expression in the intima-media of the vascular wall in the IN group (p < 0.05; Figs. 3d and e) compared to that of the CN group. However, no significant differences were detected in the expression of MMP-9 and MMP-12 between the 4 treatment groups (p > 0.05, Figs. 3f-o).

3.5 The effect of dietary isoflavone on CD68, neutrophil elastase, and malondialdehyde (MDA) in mouse nicotine-administered models

Protein expression of CD68 and neutrophil elastase were evaluated via immunohistochemistry. There was no statistical difference in CD68 expression between groups (Figs. 4a-e). In contrast to CD68, the expression of neutrophil elastase was significantly upregulated in the adventitia of the vascular wall in the CN group (p < 0.05; Figs. 4h and j) compared to that of C and I groups. Isoflavone significantly decreased the expression of neutrophil elastase in the adventitia of the vascular wall in the IN group compared to that in the CN group (p < 0.05; Figs. 4i and j). Furthermore, we measured MDA which reflects oxidative stress changes. Immunohistochemical staining revealed that MDA accumulation was significantly increased in the adventitia of the vascular wall in the CN group (p < 0.05; Figs. 4m and o) compared to that of C and I groups. Isoflavone significantly inhibited the increase in MDA accumulation in the IN group (p < 0.05; Figs. 4n and o) compared to that of the CN group.

4 Discussion

Cigarette smoking is a powerful risk factor for vascular diseases, including abdominal aortic aneurysm (AAA) [3, 4]. Isoflavones, are known to prevent cardiovascular diseases...
by exerting therapeutic effects through which these compounds are able to prevent or ameliorate vascular damage via lipid profile improvement, antioxidant activity, vascular relaxation and platelet aggregation inhibition. The current study investigated the effects of dietary isoflavone on vascular injury in mouse nicotine-administered models. Several studies have reported that cigarette smoking enhanced endothelin-1 (ET-1) production and upregulated arterial expression of the angiotensin II type 1 (AT1) receptor, resulting in the development of vascular diseases. Moreover, the levels of angiotensinogen and AT1 receptor protein expression were upregulated in human AAAs compared to those in the aortas of healthy and atherosclerosis individuals. Hence, lowering of ET-1, angiotensinogen and the AT1 receptor levels may possibly be used as a key to detect the inhibition of vascular injury and AAA occurrence. Several recent reports have indicated that isoflavone may also protect the vascular cell system. Our results demonstrated that dietary isoflavone had significantly decreased the expression of angiotensinogen and the AT1 receptor in nicotine induced mice. Additionally, isoflavone administration caused a reduction in ET-1 expression in mice with nicotine induced vascular damage, demonstrating the effect of isoflavone on ET-1 expression. Our findings, suggest that inhibition of vascular damage in nicotine-induced mice by isoflavone may be due to its suppressive effect on ET-1, angiotensinogen and the AT1 receptor. Our study also revealed the vascular protective properties of isoflavone which also substantiated studies by Beavers et al., describing the vascular protective activities of isoflavone in humans. However, the mechanism underlying isoflavone induced inhibition of ET-1, angiotensinogen and the AT1 receptor may need further clarification.

Matrix metalloproteinases (MMPs), especially MMP-2, MMP-9, and MMP-12, have been involved in extracellular matrix degradation, which leads to pathological changes in vascular wall structure and the subsequent development of AAA. In the current study, nicotine increased MMP-2 expression and elastin destruction in mouse vascular walls, which contributed to vascular injury. Interestingly, isoflavone reduced MMP-2 expression and decreased the destruction of elastin in nicotine-induced mice. Thus, isoflavone evidently inhibits both MMP-2 overproduction and elastin destruction, thereby exerting a protective effect against the progression of vascular injury. The capacity of isoflavone to suppress MMP-2 expression may be associated with the inhibition of p44/42 mitogen-activated protein kinases (MAPK) pathways and nuclear factor kappa B (NF-κB) activities.

Activated polymorphonuclear leukocytes (PMNs) play an important role in vascular disease. The role of PMNs may be related to their ability to generate and release proteo-
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Fig. 5  Proposed inhibitory activity of isoflavone against nicotine-induced vascular damage.

lytic enzymes and reactive oxygen species (ROS), which may directly promote vascular damage. Elastase perfusion into the aorta reportedly stimulated the development of aneurysms in several animal AAA models. Processes underlying the occurrence of aneurysms in animal models have generally been linked to elastase-induced inflammatory responses, which cause degradation of extracellular matrix proteins. Findings of the current study confirmed that nicotine significantly increased neutrophil elastase expression in mouse vascular walls. This indicated that nicotine-induced augmentation of neutrophil elastase leads to vascular destruction and subsequently AAA occurrence. Notably, our results indicated that dietary isoflavone significantly downregulated neutrophil elastase expression, which was substantiated by the results of Kim et al., illustrating the inhibitory effect of isoflavone on neutrophil elastase in vitro. Furthermore, recent investigations have focused on oxidant stress, wherein cigarette smoking augments the generation of ROS, including superoxide radicals, hydrogen peroxide, and hydroxy radicals. Accumulation of ROS in the vascular wall directly promotes cellular damage and may directly, or indirectly, harm extracellular matrix elements, by enhancing degradation of matrix mediator. The current study found that nicotine significantly upregulated malondialdehyde (MDA) levels in the vascular wall of mice. Moreover, dietary isoflavones significantly suppressed MDA levels in the vascular wall adventitia of nicotine-induced mice. This result was consistent with that of a previous finding which observed significantly decreased MDA levels following the administering of soy isoflavones to humans. In addition, in vitro studies have reported that isoflavones act as potent free-radical scavengers of superoxide anion radicals, hydroxyl radicals, 2,2-diphenyl-1-picrylhydrazyl radicals, and hydrogen peroxide. Thus, isoflavones were associated with high antioxidant activities that react with radical species and terminate chain reactions of free radicals, thereby suppressing oxidative stress in vascular walls.

5 Conclusions
The current study investigated whether isoflavone suppresses vascular damage by inhibiting the activation of ET-1, angiotensinogen, and the AT1 receptor, which downregulates MMP-2, neutrophil elastase, and MDA levels (Fig. 5). Limitation of this experimental model is lack of the formation of AAA and the appearance of adventitial adipocyte. Further investigations are needed to clarify the mechanisms underlying the role of isoflavone in the above process. Furthermore, human studies are still needed to obtain detailed information in regard to human related applications. Findings of the current study suggest that development of isoflavone containing foods may ensure protection from vascular disease.

Conflict of Interest
There are no conflicts of interests to declare.

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