Administration of Isoflavone Attenuates Ovariectomy-induced Degeneration of Aortic Wall

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Abstract: Women are more resistant to vascular diseases; however, the resistance is reduced after menopause. It has been reported that the risk of vascular diseases such as atherosclerosis and abdominal aortic aneurysm is increased in postmenopausal women. Currently, methods to prevent vascular disease in postmenopausal women have not been established. Isoflavones are promising functional food factors that have a chemical structure similar to estrogen. In this study, we investigated the effects of isoflavones on ovariectomized (OVX)-induced degeneration of the aortic wall in mice. Increased destruction of elastic fibers in the thoracic and abdominal aorta was observed in the OVX group, and isoflavones attenuated the destruction of elastic fibers. The positive areas of matrix metalloproteinase (MMP)-2 and MMP-9 in the OVX group were higher than those in the control group. Isoflavones decreased the positive areas of MMP-2 and MMP-9 compared to those in the OVX group. These data suggest that isoflavones have a suppressive effect on OVX-induced degeneration of the aortic wall by inhibiting the increase in MMP-2 and MMP-9.

Key words: ovariectomized, estrogen, abdominal aortic aneurysm, elastic fiber, MMP-2, MMP-9

1 Introduction

The incidence and rate of progression of cardiovascular disease (CVD) vary according to sex. Until the age of early 40s, the incidence of CVD in women (1.3/1000 person per year) was lower than that in men (5.7/1000 person per year) of the same age¹. But by the late 60s, after menopause, the difference almost disappears with 22.1/1000 person per year for women and 26.7/1000 person per year for men⁵. The reason for the difference in the number of patients before and after menopause is that estrogen, the female sex hormone, has a regulating effect on blood vessels and maintains the integrity of blood vessels in women. Menopause causes a decline in ovarian function, leading to a rapid decrease in estrogen, which increases the prevalence of vascular disease and further increases the risk of vascular disease in women who have undergone early menopause. Menopause is a risk factor for vascular disease, and understanding the vascular pathology specific to women is important.

Recently, we reported that menopause-induced dysfunction of the aorta can be associated with the development of abdominal aortic aneurysm (AAA). AAA is characterized by aortic degeneration with progressive dilatation of a portion of the abdominal aorta. Age, smoking history, hypertension, hyperlipidemia, and sex were risk factors for AAA. Many AAA patients are male, and the incidence is approximately four times higher than that of females. However, it has been reported that the rate of progression and rupture of AAA is higher in women than in men. It has been reported that sex hormones are involved in these sex differences, but the detailed mechanism is unknown. In addition, most female patients with AAA are postmenopausal, and it has been reported that female patients with early menopause are more likely to have an enlarged mass diameter. Therefore, it is speculated that the decrease in estrogen production associated with menopause may contribute to the increased growth rate of AAA.

Based on the information mentioned above, we focused on isoflavones which have a chemical structure similar to estrogen and exert estrogenic effects on target organs. Isoflavones are polyphenolic nonsteroidal compounds found in the legume family, including food crops such as soy-
beans, kidney beans, and solanaceous legumes, and the production of isoflavone-containing foods such as tofu and soy milk is increasing worldwide. Because orally ingested isoflavones could be transported to the blood and increase the blood isoflavone levels, consumption of foods containing high amounts of isoflavones has been reported to correlate with a lower incidence of chronic diseases, including coronary heart disease. In addition, isoflavones may contain antioxidants that scavenge free radicals and have anti-inflammatory and vascular protective functions, and are said to be effective in preventing diseases associated with low estrogen levels. However, the effect of isoflavones on the degeneration of the aorta associated with menopause remains unknown. In this study, we evaluated the effects of isoflavone administration on the aortic wall of ovariectomized (OVX) female mice.

2 Materials and Methods

2.1 Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee and were conducted according to the Kindai University Animal Experimentation Regulations (Approval number: KAAG-31-007). Female 7-week-old mice (SHIMIZU Laboratory Supplies Co., Ltd, Kyoto, Japan) were fed a free diet and tap water and kept at 25 ± 1°C with a 12 h light/dark cycle. The diet composition is shown in Table 1. In this experiment, the mice were divided into three groups: the control group (C), OVX group (O), and OVX and isoflavone group (OI). During the experiment, the C and O groups were administered the control diet, and the OI group was administrated an isoflavone diet. One week later, C group underwent sham surgery without ovary removal, and the O and OI groups underwent OVX treatment under anesthesia. Five weeks later, all the mice were sacrificed.

2.2 Serum glucose, triglyceride and total cholesterol levels

Five weeks after the sham or OVX treatment, blood was collected from the abdominal vein cava under anesthesia (50 mg/kg pentobarbital, i.p.) to determine serum glucose, triglyceride, and total cholesterol levels. For serum preparation, blood was centrifuged at 3000 × g for 10 min. Then, serum glucose, triglyceride, and total cholesterol were measured using commercial kits (Wako Pure Chemical Industries, Osaka, Japan) and using the methodology according to the manufacturer’s instruction.

2.3 OVX and sample collection

After opening the abdomen, the oviducts were isolated from the peri-ovarian fat, and the ovaries were removed with electrocautery. At the end of the experiment, mice were sacrificed, and the abdominal and thoracic aortas were removed and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 d (Nacalai Tesque, Kyoto, Japan). The aorta samples were dehydrated and embedded in paraffin.

2.4 Histological analysis

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) and Elastic van Gieson (EVG) stains. EVG staining was performed using Sirius red solution instead of fuchsin acid solution with a slight modification to the previously reported method. The deparaffinized tissue sections were soaked in resorcine-fuchsin solution (Nacalai Tesque, Kyoto, Japan) for 30 min. After rinsing in tap water, sections were stained for 10 min with a 1:1 mixture of Weigert’s iron hematoxylin solution I (1% hematoxylin in ethanol) : solution II (2% ferric chloride in 0.25% HCl). After decolorizing in 1% hydrochloric acid alcohol, the sections were stained with 1% Sirius red solution diluted 1:20 in van Gieson picric acid solution (Wako Pure Chemical industries) for 15 min. Dehydrated with ethanol, permeated with xylene, and covered with a lipid-soluble mounting medium and glass cover slips. The destruction rate of the wavy configuration of the elastic lamina was calculated by dividing the area of destruction (indicated by flattening and fragmentation of the elastic lamina) by the entire area of elastic lamina as previously described. Collagen fibers were observed using a polarizing microscope, and the positive area of collagen was calculated by binarizing the image into black and red using software (National Institutes of Health, Bethesda, MD, USA).

2.5 Immunohistochemical staining

The deparaffinized tissue sections were permeabilized

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with 1% Triton-X100 in PBS and soaked for 1 h in 10% oxalic acid for bleaching. For antigen retrieval, 0.1% trypsin in PBS was added to the tissue sections for 10 min. Then, to block endogenous peroxidase, it was soaked in 3% hydrogen peroxide in methanol for 8 min. After washing with PBS, the tissue sections were blocked with Blocking One Histo (Nacalai Tesque, Kyoto, Japan) for 30 min. The tissue sections were incubated with the primary antibody overnight at 4 °C. The following antibodies were used: rabbit anti-matrix metalloproteinase (MMP)-2 (1:100; GeneTex, Irvine, CA, USA) and goat anti-MMP-9 (1:50; Santa Cruz Biotechnology, Dallas, TX). The following day, the sections were washed with PBS and incubated with the appropriate secondary antibody conjugated to HRP for 30 min. A DAB kit (Vector Laboratories, Burlingame, CA, USA) was used to detect the target proteins. The area of positive staining in immunohistochemistry was calculated by binarizing the images into black and white images using ImageJ.

2.6 Statistical analyses

The experimental data are expressed as the mean ± standard error of the mean (SEM). Statistical differences were determined using the Fisher PLSD.

3 Results

3.1 Effects of OVX and isoflavones on body weight, food intake, weight of adipose tissue and weight of organs

The average body weight was significantly higher in the OI group than in the C group on days 26, 42, and 43 (Fig. 1a). No significant differences in food intake were observed among the groups (Fig. 1b). Oviduct weight was significantly lower in OVX treated groups (O and OI) than in the sham treated group (Fig. 1c), indicating that OVX treatment was appropriate. There were no significant differences in oviduct peripheral fat weight, brown fat weight, liver weight and kidney weight among all groups.

Fig. 1 Effects of OVX and Isoflavone diet on body weight, food intake, weight of adipose tissue and weight of organs. Average body weight during the experiment (a), average food intake during the experiment (b). Weight of adipose tissue and organs collected during dissection; Oviduct weight (c), Oviduct peripheral weight (d), Brown fat weight (e), Liver weight (f), Kidney weight (g). Weight of adipose tissue and organs are calculated based on 100 g body weight. Data are presented as the mean ± S.E. Values represented by different letters and * are significantly different (p < 0.05). C, Sham treatment and Control diet; O, OVX treatment and Control diet; OI, OVX treatment and Isoflavone diet.
3.2 Effects of OVX and Isoflavone diet on serum parameters

Serum glucose and total cholesterol levels were significantly higher in the OI group than in the C group (Figs. 2a, 2c). There was no significant difference among the groups (Fig. 2b).

3.3 Effects of OVX and Isoflavone diet on elastic and collagen fibers in the thoracic aortic wall

In the thoracic aorta, there was no significant difference in the thickness of the vascular wall between all groups (Figs. 3a-3d). The destruction rate of elastin was significantly higher in the O group than the C group, and was significantly lower in the OI group than the O group (Figs. 3e-3h). The collagen-positive area was significantly higher in O and OI groups than in C group (Figs. 3i-3l).

3.4 Effects of OVX and Isoflavone diet on elastic and collagen fibers in abdominal aortic wall

In the abdominal aorta, there was no significant difference in the thickness of the vascular wall between all groups (Figs. 4a-4d). The destruction rate of elastin was significantly higher in O group than in C and OI groups (Figs. 4e-4h). There was no significant difference in the collagen-positive area between the groups (Figs. 4i-4l).

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**Fig. 2** Effects of OVX and Isoflavone diet on serum parameters.
Serum glucose (a), serum triglyceride (b), and serum total cholesterol (c) levels. Data are presented as the mean ± S.E. Values represented by different letters are significantly different (p < 0.05). C, Sham treatment and Control diet; O, OVX treatment and Control diet; OI, OVX treatment and Isoflavone diet.

**Fig. 3** Effects of OVX and Isoflavone diet on elastic and collagen fibers in thoracic aortic wall.
Represented images of hematoxylin-eosin staining (a-c), and quantification of thickness of vascular wall (d) in the thoracic aorta. Represented images of elastin fiber (e-g), and destruction rate of elastin of the thoracic aorta (h). Represented images of collagen fiber (i-k), and quantification of collagen positive area (l) in the thoracic aorta. C group (n = 5), O group (n = 5), OI group (n = 5). Scale bar = 100 μm. Data are presented as the mean ± S.E. Values represented by different letters are significantly different (p < 0.05). C, Sham treatment and Control diet; O, OVX treatment and Control diet; OI, OVX treatment and Isoflavone diet.
3.5 Effects of OVX and Isoflavone diet on MMP-2 and MMP-9 in thoracic aorta wall
In the MMP-2 positive area, there was no significant difference between all groups in the adventitia and intima (Figs. 5a-5d); however, O group tended to be higher than C group (Fig. 5d). In the MMP-9 positive area, there was no significant difference in adventitia (Figs. 5e-5h). In the intima-media, the MMP-9 positive area in O group was significantly increased compared to that in C group (Figs. 5e-5h).

3.6 Effects of OVX and Isoflavone diet on MMP-2 and MMP-9 in abdominal aorta wall
In the adventitia, MMP-2 and MMP-9 positive areas were not significantly different between all groups (Figs. 6a-6d). On the other hand, MMP-2 and MMP-9 positive areas in the intima-media of O group were significantly higher than those in C group (Figs. 6a-6h). MMP-2 and MMP-9 positive areas in the adventitia were not significantly different between all groups (Figs. 6a-6d). On the other hand, MMP-2 and MMP-9 positive areas in the intima-media of O group were significantly higher than those in C group (Figs. 6a-6h).

Fig. 4 Effects of OVX and Isoflavone diet on elastic and collagen fibers in abdominal aortic wall.
Represented images of hematoxylin-eosin staining (a-c), and quantification of thickness of vascular wall (d) in the abdominal aorta. Represented images of elastin fiber (e-g), and destruction rate of elastin of the thoracic aorta (h). Represented images of collagen fiber (i-k), and quantification of collagen positive area (l) in the abdominal aorta. C group (n = 5), O group (n = 5), OI group (n = 5). Scale bar = 100 μm. Data are presented as the mean ± S.E. Values represented by different letters are significantly different (p < 0.05). C, Sham treatment and Control diet; O, OVX treatment and Control diet; OI, OVX treatment and Isoflavone diet.

Fig. 5 Effects of OVX and Isoflavone diet on MMP-2 and MMP-9 in thoracic aorta wall.
Represented images of MMP-2 immunostaining (a-c), and quantification of MMP-2 positive area in adventitia and intima-media (d) in the thoracic aorta. Represented images of MMP-9 immunostaining in adventitia (e-g), and quantification of MMP-9 positive area in adventitia and intima-media (i) in the thoracic aorta. C group (n = 5), O group (n = 5), OI group (n = 5). Scale bar = 30 μm. Data are presented as the mean ± S.E. Values represented by different letters are significantly different (p < 0.05). C, Sham treatment and Control diet; O, OVX treatment and Control diet; OI, OVX treatment and Isoflavone diet.
areas in OI group in intima-media were significantly lower than those in O group (Figs. 6a-6h).

4 Discussion

The average body weight was higher in the OI group than in the C and O groups. It has been reported that OVX treatment increases body weight\(^5\), and isoflavone has fat-burning and anti-obesity effects. In this experiment, body weight increased, but there was no significant difference in fat or organ weight, suggesting that the subjects were not obese or ill.

In serum parameters, blood glucose and total cholesterol levels were higher in the OI group than in the C and O groups. Isoflavone has been reported to lower blood glucose levels in diabetic rats\(^22\), but the mice used in this experiment were not diabetic and had not fasted before dissection, which may have caused the difference.

OVX treatment has been reported to induce degradation of aortic fibers in the thoracic and abdominal aorta\(^6\). It has also been reported that OVX treatment decreases the levels of smooth muscle cells and elastic fibers in the mouse aortic wall\(^23\), which is consistent with the results obtained in this study. In addition, the destruction rate of elastin in both the thoracic and abdominal aorta was significantly lower in group OI than in group O (Figs. 3h, 4h). These results suggest that the destruction rate of elastic fibers in the thoracic and abdominal aorta induced by OVX treatment may be inhibited by isoflavone administration.

To investigate how the destruction of elastic fibers is induced, immunohistochemical staining was performed to observe MMP-2 and MMP-9, which are involved in the destruction of aortic fiber components. In the thoracic aorta, the MMP-2 positive area in O group tended to be higher than that in C group for both the intima-media and adventitia. In the thoracic aorta, the MMP-9 positive area in the adventitia tended to be lower in OI group than in O group, and in the intima-media was significantly increased in O group compared to C group. In the abdominal aorta, the MMP-2 and MMP-9 positive areas in the intima-media were significantly higher in O group higher in C group, and there was no significant difference between the C group and the OI group. MMP-2 is mainly derived from smooth muscle cells and fibroblasts, while MMP-9 is mainly derived from macrophages\(^24\). Both MMP-2 and MMP-9 are known to degrade fibrous components of the vascular wall\(^25\). In O group, both the thoracic aorta and abdominal aorta showed destruction of elastic fibers and increased positive areas of MMP-2 and MMP-9 in the intima-media. However, in the OI group treated with isoflavone, no increase in MMP-2 and MMP-9 positive areas in the intima media of the thoracic and abdominal aorta were observed, suggesting that suppressive mechanisms are associated with a common pathway between MMP-2 and MMP-9.

There are two estrogen receptor (ER) types, \(\alpha\) and \(\beta\). ER\(\alpha\) is expressed in the uterus, ovaries, and hypothalamus, whereas ER\(\beta\) is specifically expressed in the ovaries. ER\(\alpha\) and ER\(\beta\) play different roles in the immune system, skeletal system, central nervous system, and cardiovascular system\(^24\), \(^26\), \(^27\), \(^28\), and are expressed in the human vascular smooth muscle of the coronary arteries and aorta. ER\(\alpha\) and ER\(\beta\) are expressed in endothelial and smooth muscle cells in the aortic wall\(^27\), \(^28\), and ERs are necessary for estrogen-mediated vasoprotection\(^29\). ER\(\beta\) expression is more common in females\(^30\). Both ER\(\alpha\) and ER\(\beta\) are expressed in macrophages\(^31\). It has been suggested that one of the mechanisms by which estrogen replacement therapy reduces the risk of cardiovascular disease in menopausal women is by improving vascular reactivity\(^32\). To suppres-

\[\text{Fig. 6} \quad \text{Effects of OVX and Isoflavone diet on MMP-2 and MMP-9 in abdominal aorta wall.} \]

Represented images of MMP-2 immunostaining (a-c), and quantification of MMP-2 positive area in adventitia and intima-media (d) in the thoracic aorta. Represented images of MMP-9 immunostaining in adventitia (e-g), and quantification of MMP-9 positive area in adventitia and intima-media (i) in the thoracic aorta. C group (n = 5), O group (n = 5), OI group (n = 5). (a-c, e-g) Scale bar = 30 \(\mu\)m. Data are presented as the mean ± S.E. Values represented by different letters are significantly different (\(p < 0.05\)). C, Sham treatment and Control diet; O, OVX treatment and Control diet; OI, OVX treatment and Isoflavone diet.
sive effect of isoflavones may be involved in ERs. There are several types of the isoflavones, and isoflavone used in this experiment contain more than 50% genistein. It has been suggested that genistein acts through estrogen receptors\(^\text{\textsuperscript{51}}\), and it has been reported that genistein have multiple cellular effects, including the inhibition of MARK pathway\(^\text{\textsuperscript{34}}\) and NF-κB activation\(^\text{\textsuperscript{35}}\). It has also been reported to inhibit the expression of MMP-2 and MMP-9 by being involved in the inhibition of MARK pathway and/or NF-κB activity\(^\text{\textsuperscript{36}}\). Therefore, we speculate that genistein in the isoflavones suppressed the destruction of elastic fibers by inhibiting the increase of MMP-2 and MMP-9 in the inner tunica media of the aortas through its involvement in the inhibition of the MARK pathway and NF-κB activity. It can be inferred that the inhibition of MMP-2 and MMP-9 in the medial tunica media of the respective aortas suppressed elastic fiber destruction.

In conclusion, we demonstrated the suppressive effect of isoflavones on OVX-induced degeneration of the aortic walls. However, it should be noted weight gain and increased levels of serum glucose and total cholesterol were observed in the OI group. In addition, adverse effects such as excessive estrogen in the body due to excessive estrogen intake have been reported to cause cervical cancer\(^\text{\textsuperscript{37}}\). Further research is needed to determine the optimal conditions that do not adversely affect health and lifespan.

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References


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