Ovariectomy Causes Degeneration of Perivascular Adipose Tissue

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Abstract: Women are more resistant than men to the development of vascular diseases. However, menopause is a factor leading to deterioration of female vascular integrity, and it is reported that the risk of vascular diseases such as atherosclerosis and abdominal aortic aneurysm is increased in postmenopausal women. Although it is suggested that perivascular adipose tissue (PVAT) is deeply involved in the increased risk of vascular disease development, the effect of menopause on PVAT integrity is unknown. In this study, we aimed to elucidate the effect of menopause on PVAT in ovariectomized (OVX) rats. PVAT was divided into 4 regions based on characteristics. Hypertrophy and increased inflammation of adipocytes in the PVAT were observed in the OVX group, but the effects of OVX were different for each region. OVX induced matrix metalloproteinase (MMP)-9 which degrade extracellular matrix such as elastin and collagen fibers in PVAT. Degeneration of the arterial fibers of the thoracic and abdominal aorta were observed in the OVX group. These results indicate that OVX can cause dysfunction of PVAT which can cause degradation of arterial fibers. Appropriate management of PVAT may play an important role in the prevention and treatment of diseases originating from ovarian hypofunction.

Key words: ovariectomy, perivascular adipose tissue, vascular diseases, elastin, collagen, abdominal aortic aneurysm

1 Introduction

Cardiovascular disease (CVD) is a major cause of death worldwide. Sex reportedly affects the incidence and development rate of CVD. Until the age of early 40s, the incidence of CVD in women (1.3/1000 person-years) is lower than that in men (5.7/1000 person-years) of the same age¹. However, when the age of late 60s is reached, this difference almost disappears, with an incidence of 22.1/1000 person-years for women and 26.7/1000 person-years for men¹. The reason for the difference in pathology depending on sex is that estrogen, a female sex hormone, has a regulating effect on blood vessels⁵, maintaining the integrity of blood vessels in premenopausal women. Menopause signals a decline in ovarian function, leading to a rapid decrease in estrogen, increasing the prevalence of vascular disease, and further increasing the risk of vascular disease in women with premature menopause⁴,⁵. Menopause is one of the risk factors for vascular disease, and understanding the vascular pathology characteristics of women is extremely important.

Perivascular adipose tissue (PVAT) is present in adjacent locations around blood vessels. The relationship between blood vessels and PVAT has been overlooked in studies of vascular disease for many years, but PVAT has the ability to cause vasorelaxation and has been shown to regulate vascular function by secreting substances that can be transported to blood vessels⁶. Recent studies have reported that PVAT is deeply involved in vascular disease. It was reported that PVAT dysfunction increases the incidence of atherosclerosis⁷ and that inflated PVAT produces proteases that accumulate around abdominal aortic aneurysms, promoting vascular inflammation⁸,⁹.

Current studies suggest that menopause increases the risk of vascular disease and that PVAT is strongly associated with vascular disease, but the effect of menopause on PVAT is not clear. In this study, we estimated the relationship between ovariectomy (OVX) and PVAT to further understand how decreasing estrogen levels increase the risk of vascular disease.

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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-31-006). Female 3-week-old Sprague-Dawley rats (SHIMIZU Laboratory Supplies Co., Ltd, Kyoto, Japan) were fed freely diet and tap water and kept at 25 ± 1°C with 12 h light/dark cycle. The diet composition is shown in Table S1. In this experiment, the rats were divided into 2 groups: the Control group (n = 6) with Sham treatment and the OVX group (n = 7) with OVX treatment. 1 week later, Sham and OVX treatment were conducted under anesthesia. 7 weeks later, all rats were sacrificed. Experiments were carried out in compliance with the ARRIVE guidelines (http://www.nc3rs.org.uk/page.asp?id=1357).

2.2 Serum triglyceride, total cholesterol and glucose level

At the 7 weeks after the treatment, blood was taken from tail vein under anesthesia (50 mg/kg pentobarbital, i.p.) to determine serum triglyceride, total cholesterol and glucose level. For serum preparation, the blood was centrifuged at 3000 x g for 10 minutes. Then, serum triglyceride, cholesterol and glucose were measured using commercial kits and using the methodology according to the manufacturer instruction.

2.3 Ovariectomy (OVX) and sample collection

In OVX treatment, after opening abdomen, the oviducts were isolated from the peri-ovarian fat and the oviducts were removed with electrocautery. In endpoint of experiment, rats were sacrificed and the abdominal and thoracic aortas with PVAT attached were removed and fixed with 4% PFA in PBS for 1 day (Nacalai Tesque, Kyoto, Japan). Then, aorta and PVAT tissue samples were dehydrated and embedded in paraffin.

2.4 Histological analysis

Isolated aorta and PVAT were sectioned at 10 µm thickness (8 µm thickness allowed us to observe adipocytes more clearly than 10 µm thickness, so we used 8 µm thickness for H&E.) using a microtome and placed on glass slides. They were stained with hematoxylin&eosin (H&E) and Sirius Red EVG. 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. The denatured collagen was measured by using collagen hybridizing peptide (3Helix, Utah, USA). CHP stock solution was added to phosphate-buffered saline (PBS) to 20 μM. The diluted CHP solution was heated at 80°C for 5 minutes, quenched in an ice-water bath for 60 seconds, and the deparaffinized tissue sections were soaked and kept at 4°C overnight. The following day, the sections were washed with PBS and the concentration of streptavidin conjugate was prepared with 1% BSA in PBS to 0.005 mg/ml and incubated for 1 hour.

Quantitative analyses of the immunohistochemical staining were performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The area of positive staining in immunohistochemistry was calculated by binarizing the image into black and white using ImageJ. Sections of the negative control were not subjected to the primary antibodies (Fig. S1).

PVAT was divided into 4 regions based on characteristics (Fig. S2): region 1 outer region, the outer 60 µm of the thoracic PVAT consisting of brown adipose tissue; region 2 central region, inside of the region 1 outer region; region 2, The thoracic PVAT consisting of white adipose tissue; region 3, Abdominal PVAT.

2.5 Immunohistochemical staining

The deparaffinized tissue sections were permeabilized with 1% Triton-X100 in phosphate-buffered saline (PBS) and soaked for 1 hour in 10% oxalic acid to bleach. For antigen retrieval, 0.1% trypsin in PBS was added to the tissue sections for 10 minutes. Then, for blocking endogenous peroxidase, it was soaked in 3% hydrogen peroxide in methanol for 8 minutes. After washing with PBS, the tissue sections were blocked with Blocking One Histo for 30 minutes. The tissue sections with primary antibody were overnighted at 4°C. Antibodies are used for the following proteins: rabbit anti-UCP-1 (1:100; abcam, Tokyo, Japan), rabbit anti-matrix metalloproteinase (MMP)-2 (1:100; GeneTex, Irvine, CA, USA), goat anti-MMP-9 (1:50; Santa Cruz Biotechnology, Dallas, TX, USA), and mouse anti-monocytes/macrophages (MAC387) (1:50; Bio-Rad Laboratories, Hercules, CA, USA). The following day, the sections were washed with PBS and incubated with the appropriate secondary antibody conjugated to HRP for 30 minutes. The DAB kit (Vector Laboratories, Burlingame, CA, USA) was used to detect target proteins.

2.6 Statistical analyses

The experimental data was expressed as the mean ± standard error of mean (S.E.M). The statistical differences were determined using a two-sided Student s t-test. Differences of p-value < 0.05 were considered statistically significant (*p < 0.05, **p < 0.01, ***p < 0.001). Statistical analyses were performed using StatView 5.0 software (SAS Institute, Cary, NC, USA).
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3 Results

3.1 Effects of OVX on body weight, weight of organs and serum parameters

Final body weight, weight of organs, and serum parameters were shown in Table 1. Oviduct weight was significantly lower in the OVX group than in the control group, which showed that OVX was appropriately performed. Weights of adipose tissues and serum parameters were not different between the two groups in this experimental condition.

3.2 Effects of OVX on adipocytes and uncoupling protein 1 in PVAT

In this study, PVAT was divided into 4 regions based on characteristics (Fig. S2). The adipocyte areas in the central and outer regions of region 1 were significantly increased in the OVX group compared to that in the control group (Figs. 1a-1c and 1d-1f, respectively). The adipocyte area in region 2 did not show a significant difference between the two groups (Figs. 1g-1i). The adipocyte area in region 3 was significantly increased in the OVX group compared to that in the control group (Figs. 1j-1l). The uncoupling protein (UCP)-1-positive areas in the central and outer regions of region 1 did not show a significant difference between the two groups (Figs. 1m-1r). The UCP-1-positive area in region 2 did not show a significant difference between the two groups (Figs. 1s-1u). The UCP-1-positive area in region 3 was significantly decreased in the OVX group compared to that in the control group (Figs. 1v-1x).

3.3 Effects of OVX on MMPs and macrophages/monocytes in PVAT

The MMP-2-positive areas in the central and outer regions of region 1 did not show a significant difference between the two groups (Figs. 2a-2f). The MMP-2-positive areas in regions 2 and 3 did not show a significant difference between the two groups (Figs. 2g-2l, respectively). The MMP-9-positive area was significantly increased in the OVX group compared to that in the control group in the central region (Figs. 3a-3c), but there was no significant difference between the two groups in the outer region (Figs. 3d-3f) of region 1. The MMP-9-positive areas in regions 2 and 3 were significantly increased in the OVX group compared to that in the control group (Figs. 3g-3l and 3m-3l, respectively). The macrophage/monocyte-positive areas in the central and outer regions of region 1 were significantly increased in the OVX group compared to that in the control group (Figs. 4a-4c and 4d-4f, respectively). The macrophage/monocyte-positive area did not show a significant difference between the two groups in region 2 (Figs. 4g-4i) but was significantly increased in the OVX group compared to that in the control group in region 3 (Figs. 4j-4l).

3.4 Effects of OVX on elastic and collagen fibers, matrix metalloproteinases, and macrophages/monocytes in the thoracic aortic wall

The destruction of elastin was significantly increased in the OVX group compared to that in the control group (Figs. 5a-5c). The collagen-positive area was significantly decreased in the OVX group compared to the control group.

| Table 1 | body weight, weight of organs and serum parameters. |
|---------|---------------------------------------------------|
|         | Control               | OVX                  |
| Initial body weight (g) | 41.1 ± 0.56           | 42.13 ± 1.08         |
| Final body weight (g)   | 252.35 ± 2.9          | 307.87 ± 14.62*     |
| Food intake (Kcal/day)  | 80.07 ± 0.49          | 92.88 ± 2.72**      |
| Oviduct weight (g)      | 0.57 ± 0.1            | 0.04 ± 0***         |
| Periovarian fat (g)     | 5.64 ± 0.34           | 5.44 ± 0.5          |
| Mesenteric fat (g)      | 2.91 ± 0.23           | 3.91 ± 0.69         |
| Brown fat weight (g)    | 0.35 ± 0.03           | 0.26 ± 0.03         |
| Serum triglyceride (mg/dL) | 61.7 ± 9.9           | 51.4 ± 3.45         |
| Serum total cholesterol (mg/dL) | 80.9 ± 12.12 | 83 ± 5.41 |
| Serum glucose (mg/dL)   | 124.3 ± 6.19          | 135.5 ± 6.08        |

Data are expressed as the mean ± S.E. *p<0.05, **p<0.01, ***p<0.001 vs control, Student’s t-test.
Fig. 1  Effect of OVX on adipocyte area and UCP-1 in the PVAT.
Represented images of hematoxylin-eosin staining (a, b, d, e, g, h, i, k) and adipocyte area (c, f, i, l) of the PVAT. Represented images of UCP-1 immunostaining (m, n, p, q, s, t, v, w) and UCP-1 positive area (o, r, u, x) of the PVAT. (c, i, l, o) Control group (n = 6), OVX group (n = 7). (f, r, u) Control group (n = 4), OVX group (n = 7). (x) Control group (n = 5), OVX group (n = 5). Scale bar = 100 µm. Data are expressed as the mean ± S.E. *p < 0.05, ***p < 0.001 vs control, Student’s t-test.

(Figs. 5d-5f). The denatured collagen-positive area was significantly increased in the OVX group compared to that in the control group (Figs. 5g-5i). The matrix metalloproteinase (MMP)-2-positive area did not show a significant difference between the two groups (Figs. 5j-5l). The MMP-9-positive area was significantly increased in the OVX group compared to the control group (Figs. 5m-5o). The macrophage/monocyte-positive area did not show significant differences between the two groups (Figs. 5p-5r).

3.5 Effects of OVX on elastic and collagen fibers, MMPs, and macrophages/monocytes in the abdominal aortic wall

The destruction of elastin was significantly increased in the OVX group compared to that in the control group (Figs. 6a-6c). The collagen-positive area did not show a significant difference between the two groups (Figs. 6d-6f). The denatured collagen-positive area was significantly increased in the OVX group compared to the control group (Figs. 6g-6i). The MMP-2-positive area did not show a significant difference between the two groups (Figs. 6j-6l). The MMP-9-positive area was significantly increased in the OVX group compared to the control group (Figs. 6m-6o). The macrophage/monocyte-positive area was significantly increased in the OVX group compared to the control group (Figs. 6p-6r).

4 Discussion
In this study, we evaluated the effects of OVX on PVAT. Hypertrophy and increased inflammation of adipocytes in the PVAT were observed in the OVX group. The effects of OVX on 4 regions of PVAT were different. Hypertrophy of adipocytes and infiltration of macrophages/monocytes were observed in region 1. In addition, the increased number of adipocytes was observed in outer region of region 1 (Fig. S3). Hypertrophy of adipocytes and infiltration of macrophages/monocytes were not observed in region 2. Hypertrophy of adipocytes, infiltration of macrophages/monocytes, and decreased level of UCP-1 was observed in region 3. Pathological analysis of the aortic wall of the thoracic aorta showed that OVX degraded elastic fibers and collagen fibers. Miyamoto et al. reported that OVX induced degradation of vascular fibers in the abdominal aorta33. Johnston reported that OVX resulted in de-
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**Fig. 2** Effects of OVX on the MMP-2 in the PVAT.
Represented images of MMP-2 immunostaining (a, b, d, e, g, h, i, k) and MMP-2 positive area (c, f, i, l) of the PVAT. (c) Control group (n = 6), OVX group (n = 7). (f, l) Control group (n = 4), OVX group (n = 7). (i) Control group (n = 3), OVX group (n = 6). Scale bar = 100 µm. Data are expressed as the mean ± S.E. *p < 0.05 vs control, Student’s t-test.

**Fig. 3** Effects of OVX on the MMP-9 in the PVAT.
Represented images of MMP-9 immunostaining (a, b, d, e, g, h, i, k) and MMP-9 positive area (c, f, i, l) of the PVAT. (c) Control group (n = 6), OVX group (n = 6). (f) Control group (n = 6), OVX group (n = 5). (i) Control group (n = 5), OVX group (n = 4). (l) Control group (n = 6), OVX group (n = 7). Scale bar = 100 µm. Data are expressed as the mean ± S.E. *p < 0.05, **p < 0.01 vs control, Student’s t-test.
Fig. 4  Effects of OVX on the macrophages/monocytes in the PVAT. Represented images of macrophages/monocytes immunostaining (a, b, d, e, g, h, i, k) and macrophages/monocytes positive area (c, f, i, l) of the PVAT. (c, l) Control group (n = 6), OVX group (n = 7). (f) Control group (n = 5), OVX group (n = 6). (i) Control group (n = 6), OVX group (n = 6). Scale bar = 50 µm. Data are expressed as the mean ± S.E. *p < 0.05, **p < 0.01 vs control, Student’s t-test.

Fig. 5  Effect of OVX on fibers in the thoracic vascular wall. Represented images of elastin fiber (a, b), destruction rate of elastin of the thoracic aorta (c), represented images of collagen fiber (d, e), collagen positive area (f), represented denatured collagen staining (g, h), and denatured collagen positive area (i) in the thoracic aorta. Represented images of immunostaining for MMP-2 (j, k), MMP-2 positive area (l), represented images of immunostaining for MMP-9 (m, n), MMP-9 positive area (o), represented images of immunostaining for macrophages/monocytes (p, q), and macrophages/monocytes positive area (r) in the thoracic aorta. (c, f, i, o, r) Control group (n = 6), OVX group (n = 7). (i) Control group (n = 6), OVX group (n = 5). Scale bar = 100 µm. Data are expressed as the mean ± S.E. *p < 0.05 vs control, Student’s t-test.
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Abdominal aorta

| Control | OVX |
|---------|-----|
| EVG     |     |
| PSR     |     |
| Denatured collagen | |

Fig. 6 Effect of OVX on fibers in the abdominal vascular wall.
Represented images of elastin fiber (a, b), destruction rate of elastin of the thoracic aorta (c), represented images of collagen fiber (d, e), collagen positive area (f), represented denatured collagen staining (g, h), and denatured collagen positive area (i) in the abdominal aorta. Represented images of immunostaining for MMP-2 (j, k), MMP-2 positive area (l), represented images of immunostaining for MMP-9 (m, n), MMP-9 positive area (o), represented images of immunostaining for macrophages/monocytes (p, q), and macrophages/monocytes positive area (r) in the abdominal aorta. (c, f, l, o, r) Control group (n = 6), OVX group (n = 7). (i) Control group (n = 4), OVX group (n = 7).
Scale bar = 100 µm. Data are expressed as the mean ± S.E. *p < 0.05, **p < 0.001 vs control, Student’s t-test.

Increased levels of smooth muscle cells (SMCs) and elastin fibers in the mouse aortic wall[10]. Our results on aortic fibers are consistent with those of previous studies.

Recent studies indicate that dysfunctional PVAT transmits inflammatory signals from the outside to the inside of adjacent blood vessels[11], and PVAT dysfunction is considered to lead to the incidence and progression of vascular disease. Usually, PVAT is reported to secrete adiponectin and prevent atherosclerosis[12]. However, attenuation of the anti-contractile effect of local hypoxia and inflammation of PVAT is caused by impaired adiponectin secretion and increased production of vasoconstrictors such as superoxide, angiotensin II, and aldosterone[13, 14]. Vascular dysfunction is caused by increased secretion of inflammatory adipokines that induce insulin resistance, which is also a risk factor for endothelial dysfunction[15]. In human aortas with atherosclerosis, inflammatory cells are reported to densely accumulate in PVAT adjacent to the vessel adventitia, and PVAT is suggested to have the potential to promote vascular inflammation[16, 17]. OVX reportedly promotes the incidence and progression of abdominal aortic aneurysms[3]. The OVX-induced increase in abdominal aortic aneurysm incidence may be partly associated with OVX-induced dysfunction of abdominal PVAT.

Our results showing changes in MMP-9 and macrophage/monocyte-positive areas after OVX indicate that PVAT degeneration is induced by OVX. Increased infiltration of macrophages into the PVAT and/or the increased hypertrophic adipocytes can be associated with increased MMP-9 levels in PVAT. Hypertrophy of adipocytes reportedly causes local hypoxia and infiltration of macrophages and immune cells into adipose tissue and contributes to enhanced MMP expression that may damage the aortic wall[9, 18]. In addition, hypertrophic adipocytes themselves secrete MMP-2 and MMP-9 and directly cause chronic inflammation[19]. In PVAT, macrophages and adipocytes may induce an increase in MMPs levels and promote adipose tissue dysfunction due to OVX. These data suggest that OVX-induced hypertrophic adipocytes in the PVAT of both the thoracic and abdominal regions resulted in increased inflammation of the PVAT. MMP-2 in all regions of PVAT were not changed by OVX.

This study shows OVX can cause degeneration of PVAT. Destruction of the fiber component of the aortic wall was also observed in the thoracic and abdominal aortas, suggesting a possible relationship between OVX-induced dysfunction of PVAT and destruction of vascular fibers. Decrease in 17β-estradiol levels was observed in serum of OVX female rats[3]. Decreased estrogen leads to various symptoms such as metabolic syndrome and hypertension, which cause PVAT dysfunction, and leads to vascular fiber destruction, vascular dysfunction, and attenuation of the anti-contractile effect of blood vessels[20–22]. Estrogen receptor (ER)α and ERβ are expressed in adipose tissue[23, 24].

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ERα signaling is play an important role in the maintenance of adipose tissue function\textsuperscript{15, 30}. 17β-estradiol decreases triglycerides accumulation in adipocytes via ERα\textsuperscript{30}. Estrogen may act directly on PVAT function through ER signaling pathways. In addition, estrogen receptors are present in vascular SMCs and mediate the vasculoprotective effects of estrogen by suppressing the oxidative stress\textsuperscript{30}. Estrogen may also act directly on SMCs. Estrogen may play an important role in protecting PVAT and aortic wall against inflammatory damage. Isoflavones which exhibit estrogen-like properties significantly downregulated neutrophil elastase expression\textsuperscript{29, 30}. Neutrophil elastase, which is released by activated neutrophils, plays a role in the vascular wall injury and aneurysm development\textsuperscript{31}. Elastin degradation due to OVX may involve neutrophil elastase activity as well as MMPs. However, the detailed mechanisms of vascular fibers degradation by OVX have not been clarified, and further studies are needed. Appropriate management of PVAT may play an important role in the prevention and treatment of diseases originating from ovarian hypofunction. A limitation of this study is the lack of data that estimate the relationship between estrogen levels and PVAT dysfunction.

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Conflict of Interest

The authors declare no conflict of interest.

Supporting Information

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