Cardiac Fas-dependent and mitochondria-dependent apoptosis in ovariectomized rats

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ABSTRACT

Background: Very limited information has been published regarding cardiac apoptosis in menopausal women or in those after bilateral oophorectomy. The purpose of this study was to evaluate whether cardiac Fas-dependent (type I) and mitochondria-dependent (type II) apoptotic pathways are activated in ovariectomized rats.

Methods: Thirty-two female Wistar rats at 6–7 months of age were randomly divided into sham-operated group (Sham) and bilateral ovariectomized group (OVX). Two months after the operation, the cardiac characteristics, myocardial architecture, and two major apoptotic pathways in the excised left ventricle from rats were measured by histopathological analysis, Western blotting and reverse transcription polymerase chain reaction (RT-PCR), and positive TUNEL assays.

Results: The whole heart weight, the left ventricular weight, the ratios of whole heart weight to tibia length, and the ratios of left ventricle to tibia length were significantly increased in OVX relative to Sham. Abnormal myocardial architecture, enlarged interstitial spaces, more minor cardiac fibrosis, and more cardiac TUNEL-positive apoptotic cells were observed in OVX. The key components of Fas-dependent apoptosis (TNF-alpha, Fas ligand, Fas death receptors, Fas-associated death domain (FADD), activated caspase 8, and activated caspase 3) and key components of mitochondria-dependent apoptosis (Bad, Bax, Bax-to-Bcl2 ratio, cytosolic cytochrome c, activated caspase 9, and activated caspase 3) were significantly increased in OVX hearts.

Conclusions: The absence of female ovaries will activate the cardiac Fas-dependent and mitochondria-dependent apoptotic pathways, which might provide one of possible mechanism for developing heart failure in post-menopause women.

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1. Introduction

Evidence shows that menopause or early ovariectomy (oophorectomy) is associated with an increased risk of ischemic heart disease [1,2] and suggests that female hormone deficiency plays an important pathological role in developing atherosclerotic diseases and deteriorating cardiovascular conditions [3–5]. In Europe, about 55% of all female deaths are caused by cardiovascular diseases, such as myocardial infarction, heart failure,
and sudden cardiac death [6]. A report published in 2006 from 38,283 women shows that each 5-year increment in age after menopause is associated with a 44% increase of the risk of heart failure and with a 52% risk of all-cause mortality [7]. Cell apoptosis in terminally differentiated cardiomyocytes is a critical pathological mechanism that causes heart failure. Understanding the process of apoptosis could allow for the development of novel strategies to reverse or attenuate heart failure [8]. Most previous studies regarding cardiovascular diseases in menopausal women and in women with estrogen deficiency always have focused on coronary artery diseases [1–5]. However, very limited information regarding the development of heart failure or cardiac apoptosis in postmenopausal or early ovariectomized women is available.

Apoptosis, a physiological program of cellular death, may contribute to many cardiac disorders [9,10]. The occurrence of apoptosis has been reported to contribute to the loss of cardiomyocytes in cardiomyopathy, and is recognized as a predictor of adverse outcomes in subjects with cardiac diseases or heart failure [11]. The ‘extrinsic’ Fas-dependent (type I) apoptotic pathway is believed to be one of the major pathways that directly triggers cardiac apoptosis [9,12,13]. This pathway is often initiated by Fas ligand or tumor necrosis factor-alpha (TNF-α) [12]. Fas ligand binding followed by Fas-receptor oligomerization leads to the formation of a death-inducing signal complex, starting with recruitment of the Fas-associated death domain (FADD) of the adaptor protein [12]. FADD is known to function as a common signaling conduit in Fas and TNF-α-mediated apoptosis [14]. FADD recruits and aggregates the pro form of caspase-8, which leads to the activation of caspase-8 [13]. The activated caspase 8 cleaves pro-caspase 3, which then undergoes autocatalysis to form active caspase 3, a principle effector caspase of apoptosis [15].

The ‘intrinsic’ mitochondria-dependent (type II) apoptotic pathway starts from within the cell and results in the release of a number of pro-apoptotic factors from the intermembrane space of mitochondria [12,13]. The mitochondria is the main site of action for members of the apoptosis-regulating protein family exemplified by Bcl-2 family, such as Bcl-2, BNIP3 (Bcl-2/adenovirus E1B 19 kDa interacting protein 3), and Bad (Bcl-2 antagonist of cell death) [12]. Commitment to apoptosis is typically governed by opposing factions of the Bcl-2 family, including pro-apoptotic versus anti-apoptotic family members [16]. Pro-apoptotic and anti-apoptotic Bcl-2 family members can homodimerize or heterodimerize to each other, and appear to interact with and neutralize each other, so that the relative balance of these effectors strongly influences cytochrome c release and cell fate [17]. Bcl-2, an anti-apoptotic protein, prevents cytochrome c release, whereas Bax (Bcl-2-associated X protein) and Bad, pro-apoptotic proteins, enhance cytochrome c release from mitochondria [12]. When cytochrome c is released from mitochondria into the cytosol, it is responsible for activating caspase-9, which further activates caspase-3 and executes the apoptotic program [18]. In addition, caspase-8 can cleave Bid (Bcl-2 homology domain 3 (BH3) interacting domain death agonist). Cleaved Bid then causes the release of mitochondrial cytochrome c, leading to the activation of pro-caspase-9, which can then activate pro-caspase-3 [12,19,20]. The Bcl-2 family member BID is a key components of main intracellular signaling from Fas to mitochondrial apoptotic pathway [12,19]. Cardiac Fas-dependent and mitochondria-dependent apoptotic mechanism were involved in many pathologic conditions such as hypoxic stress, hypertension, obesity [21–24]. Long-term 17β-estradiol treatment prevents the activation of apoptosis signaling and its downstream effectors in the hearts [25], and also prevent cardiomyocyte apoptosis in animal models of myocardial infarction [26]. However, the cardiac Fas-dependent and mitochondria-dependent apoptotic mechanism in ovariectomized animal models, in bilateral oophorectomized women or in post-menopause women remains uncertain.

The current study was to understand whether cardiac Fas-dependent and mitochondria-dependent apoptosis in ovariectomized rats were determined from the exercised cardiac tissues in Sham and ovariectomized (OVX) rats. We hypothesized that the absence of female ovaries in ovariectomized rats may lead to more activated cardiac Fas-dependent and mitochondria-dependent apoptosis.

2. Materials and methods

2.1. Animals

Thirty-six 5–6-month-old female Wistar rats were purchased from National Laboratory Animal Center, ROC. Ambient temperature was maintained at 25 °C and the animals were kept on an artificial 12 h light–dark cycle. The light period began at 7:00 a.m. Rats were provided with standard laboratory chow (Lab Diet 5001; PMI Nutrition International Inc., Brentwood, MO, USA) and water ad libitum. Each animal was handled, 15 min/day, on two consecutive days prior to the experiment. All experimental procedures were performed according to the NIH Guide for the Care and Use of Laboratory Animals and all protocols were approved by the Institutional Animal Care and Use Committee of Chung Shan Medical University, Taichung, Taiwan.

2.2. Ovariectomy and Sham operation

All 32 rats were randomly divided into either Sham-operated group or ovariectomized group. All animals were conducted by survival surgical procedures with aseptic technique at age of 6–7 months. After anesthetized with intramuscular injection of ketamine (100 mg/kg), the lumbar dorsum was shaved bilaterally and the exposed skin was cleaned with a 75% alcohol wipe followed by a 10% povidone–iodine scrub. For each ovary, a 2-cm dorsal flank incision penetrating the abdominal cavity was made. After the par ovarian fatty tissues were identified and retracted, the ovarian arteries were ligated and the bilateral ovaries were removed. The wound was then closed using 4–0 sterile suture and each rat was injected with Penicillin-G procaine (0.2 ml, 20,000 IU, IM). The Sham-operated group underwent the same surgical procedure except for the removal of the ovaries. After OVX or Sham operation, the rats were kept individually in plastic cages (25 × 41 × 19 cm) for recovery for about 10 days, and then grouped back to their home cages.

2.3. Cardiac characteristics

After 2 months of ovariectomized or Sham operation, all 32 rats were weighed and decapitated at age of 8–9 months. The hearts of eight Sham and eight OVX animals were harvested in formalin and further analyzed by hematoxylin–eosin, Masson trichrome staining, DAPI staining and TUNEL assay as well as Western blotting and RT-PCR. The hearts of animals were excited, cleaned, and distilled H2O. The left and right atrium and ventricle were separated and weighed. The right tibias were also separated and tibia length was measured by the electronic digital Vernier caliper for correcting the whole heart weight (WHW). The ratios of the whole heart weight to body weight (BW), the ratios of the left ventricular weight (LVW) to body weight, the left ventricle weight to the whole heart weight, the whole heart weight to tibia length, and the left ventricle weight to tibia length were calculated.
2.4. Hematoxylin–eosin and Masson trichrome staining

The hearts from animals were excised and were soaked in formalin and covered with wax.Slides were prepared by deparaffinization. They were passed through a series of graded alcohols (100%, 95% and 75%), 15 min for each. The slides were then dyed with hematoxylin and eosin or Masson trichrome. After gently rinsing with water, each slide was then soaked with 85% alcohol, 100% alcohol I and alcohol II for 15 min each. At the end, they were soaked with xylene I and xylene II. Photomicrographs were obtained using Zeiss Axiophot microscopes.

2.5. Tissue extraction

Cardiac tissue extracts were obtained by homogenizing the left ventricle samples in a lysis buffer (20 mM Tris, 2 mM EDTA, 50 mM 2-mercaptoethanol, 10% glycerol, pH 7.4, proteinase inhibitor cocktail (Roche), phosphatase inhibitor cocktail (sigma)) at a ratio of 100 mg tissue/1 ml buffer for 1 min. The homogenates were placed on ice for 10 min and then centrifuged at 12,000 × g for 40 min twice. The supernatant was collected and stored at −70 °C for further experiments.

2.6. Separation of cytosolic and mitochondrial fractions

To detect cytosolic cytochrome c, tissues were suspended in a buffer (50 mM Tris (pH 7.5), 0.5 M NaCl, 1.0 mM EDTA (pH 7.5), 10% glycerol and proteinase inhibitor cocktail tablet (Roche) for 3 min on ice), homogenized by 40 strokes in a Dounce homogenizer, and centrifuged at 12,000 × g for 15 min. The supernatant was the cytosol fraction, and the pellet was resuspended in lysis buffer as the membrane fraction.

2.7. Electrophoresis and Western blot

The tissue extract samples were prepared as described by homogenizing with buffer. Sodiumdodecyl sulfate-polyacrylamide gel electrophoresis was done with 10% polyacrylamide gels. The samples were electrophoresed at 140 V for 3.5 h and equilibrated for 15 min in 25 mM Tris–HCl, pH 8.3, containing 192 mM glycine and 20% (V/V) methanol. Electrophoresed proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, 0.45 μm pore size) with a Bio-rad Scientific Instruments Transphor Unit at 100 V for 2 h. PVDF membranes were incubated at room temperature for 1 h in blocking buffer containing 100 mM Tris–HCl, pH 7.5, 0.9% (w/v) NaCl, 0.1% (v/v) Tween-20 overnight. Incubations were performed at 4 °C overnight. The immunoblots were washed three times in TBS buffer (Tris–Base, NaCl, Tween-20, pH 7.4) for 10 min and then immersed in the second antibody solution containing goat anti-mouse IgG-HRP, goat anti-rabbit IgG-HRP, or donkey anti-goat IgG-HRP (Santa Cruz) for 1 h and diluted 500-fold in TBS buffer. The immunoblots were then washed in blotting buffer for 10 min three times. The immunoblotted proteins were visualized by using an enhanced chemiluminescence ECL Western blotting luminal Reagent (Santa Cruz, CA, USA) and quantified using a Fujifilm LAS-3000 chemiluminescence detection system (Tokyo, Japan).

Fig. 1. Representative histopathological analysis of cardiac tissue sections with (A) hematoxylin and eosin staining and with (B) Masson trichrome staining (fibrosis: blue color) in sham-operated rats (Sham) and ovariectomized rats (OVX). The images of myocardial architecture were magnified by 100 times. Bars present the percentage of fibrotic area relative to total area (8 rats × 30 scope field count in each group). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 1
Cardiac characteristics of Sham and OVX group.

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>OVX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of animals</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Body weight (BW), g</td>
<td>289±12</td>
<td>346±27**</td>
</tr>
<tr>
<td>Whole heart weight (WHW), g</td>
<td>0.78±0.09</td>
<td>0.92±0.08*</td>
</tr>
<tr>
<td>Left ventricle weight (LVW), g</td>
<td>0.55±0.05</td>
<td>0.64±0.05*</td>
</tr>
<tr>
<td>WHW/BW × 10³</td>
<td>2.71±0.30</td>
<td>2.66±0.08</td>
</tr>
<tr>
<td>LVW/BW × 10³</td>
<td>1.90±0.12</td>
<td>1.36±0.09</td>
</tr>
<tr>
<td>LVW/WHW</td>
<td>0.70±0.04</td>
<td>0.69±0.02</td>
</tr>
<tr>
<td>WHW/tibia length (mm) × 10³</td>
<td>20.53±0.02</td>
<td>22.89±0.02*</td>
</tr>
<tr>
<td>LVW/tibia length (mm) × 10³</td>
<td>14.39±0.01</td>
<td>15.99±0.02**</td>
</tr>
</tbody>
</table>

Values are means ± S.D. BW, body weight; WHW, whole heart weight; LVW, left ventricular weight. Values are means ± S.D. Sham, sham-operated rats; OVX, ovariectomized rats.

* P < 0.05.
** P < 0.01 significant differences between Sham group and OVX group.

2.8. RNA extraction

Total RNA was extracted by the Ultraspec RNA Isolation System (Biotex Laboratories, Inc., Houston, TX, USA) according to the manufacturer’s instructions. Each heart was thoroughly homogenized in 1 ml Ultraspec reagent/100 mg tissue with a polytron homogenizer. The homogenates were washed with 70% ethanol by gentle vortexing. RNA precip-
Fig. 2. (A) The representative protein products of Fas ligand and (B) the representative gene expressions of tumor necrosis factor-alpha (TNF-α) extracted from the left ventricles of excised hearts in sham-operated rats (Sham) and ovariectomized rats (OVX) were measured by Western blotting analysis and RT-PCR, respectively. (C and D) Bars represent the relative protein quantification of Fas ligand on the basis of β-tubulin and the relative mRNA quantification of TNF-α on the basis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and indicate mean values ± S.D. (n=6 in each group). **P<0.01, significant differences between Sham and OVX group.

iates were then collected by centrifugation at 12,000 × g and dried under vacuum for 5–10 min before dissolving in 50 μl diethylpyrocarbonate-treated water, and then incubated at 55–60 °C for 10–15 min.

2.9. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was reverse transcribed and then amplified by the polymerase chain reaction using a Super Script Preamplification System for first strand cDNA Synthesis and Taq DNA polymerase (Life Technologies, Rockville, MD, USA). RT-PCR products (45 μl) were separated on a 1.25% agarose gel (Life Technologies). Amplimers were synthesized by MdBio, Inc. based on cDNA sequences from Gen Bank. The rat GAPDH was used as an internal standard. The following rat primers were used: Rat TNF-α forward primer: TCGAG TGACA AGCCC GTAG; Rat TNF-α reverse primer: CAGAG CAATG ACTCC AAAGT AGAC; Rat Fas forward primer: CTCCG AGAGT TTAAA GCTGA GG; Rat Fas reverse primer: GGAGA ATCGC AGTAG AAGTC TGG; Rat GAPDH forward primer-GGGTG TGAAC CACGA GAAAT; Rat GAPDH reverse primer: CCACA GTCTT CTGAG TGGCA. Densitometric analysis of immunoblots and PCR was performed by AlphaImager 2200 digital imaging system (Digital Imaging System, San Leandro, CA, USA).

2.10. DAPI staining and terminal deoxynucleotide transferase-mediated dUTP nick end labeling (TUNEL)

After the hearts were excised, the hearts were soaked formalin, dehydrated through graded alcohols, and embedded in paraffin wax. In heart tissues, the 3–μm thick paraffin sections were cut from paraffin-embedded tissue blocks. The sections were deparaffinized in xylene, rehydrated, and incubated in phosphate-buffered saline with 2% H2O2 to inactive endogenous peroxidases. Next, the sections were incubated with proteinase K (20 μg/ml), washed in phosphate-buffered saline, and incubated with terminal deoxynucleotidyl transferase and fluorescein isothiocyanate-dUTP for 60 min at 37 °C using an apoptosis detection kit (Roche Applied Science, Indianapolis, IN, USA). Next, after washing PBS twice, the sections were also stained with 4',6-diamidine-2-phenylindole dihydrochloride (DAPI, Sigma) for 5 min to detect cell nucleus by UV light microscopic observations (blue). TUNEL-positive nuclei (fragmented DNA) fluoresced bright green at 450–500 nm, whereas DAPI-positive nuclei (intact DNA) fluoresced blue at 360 nm. The mean number of TUNEL-positive and DAPI-labeled cells were counted for at least 5–6 separate fields × 2 slides × 3 LV regions (upper, middle, lower) excised from rat hearts in each group. All counts were performed by at least 2 independent individuals in a blinded manner.

2.11. Statistical analysis

The body weight, the heart weight index, the densitometric analysis of immunoblots, % of cardiac fibrosis, % of TUNEL-positive apoptotic activity of rats heart between Sham and OVX groups was compared by Student’s t-test for two independent samples. In all cases, a difference at P<0.05 was considered statistically significant.

3. Results

3.1. Body weight and cardiac characteristics

Rats weighed in OVX about 20% more than age-matched Sham animals (Table 1). Whole heart weight, left ventricular weight, the ratio of whole heart weight to tibia length, and the ratio of left ventricular weight to tibia length were significantly higher in OVX group than those in Sham group, whereas the ratio of whole heart weight to body weight (WHW/BW), the ratio of left ventricular weight to body weight (LVW/BW), and the ratio of left ventricular weight to whole heart weight (LVW/WHW) were not changed (Table 1).
Fig. 3. (A) The representative gene expressions of Fas receptor and (B) the representative protein products of Fas receptor and Fas-associated death domain (FADD) extracted from the left ventricles of excised hearts in sham-operated rats (Sham) and ovariectomized rats (OVX) were measured by RT-PCR and Western blotting analysis, respectively. (D–F) Bars represent the relative mRNA quantification of Fas receptor on the basis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or the relative protein quantification of Fas receptor and FADD on the basis of α-tubulin, and indicate mean values ± S.D. (n = 6 in each group). **P < 0.01, significant differences between Sham and OVX group.

3.2. Cardiac histopathological changes

To understand the myocardial architecture and cardiac fibrosis of the increased whole heart weight in OVX rats, we did a histopathological analysis of ventricular tissue of Sham and OVX groups with hematoxylin–eosin staining and Masson trichrome staining. Hearts stained with hematoxylin–eosin showed that the ventricular myocardium in the Sham group showed normal architecture with normal interstitial space. In contrast, the abnormal myocardial architecture and the increased interstitial space was observed in OVX, in 100× magnification images (Fig. 1A). Besides, hearts stained with Masson trichrome showed minor fibrosis and increased collagen deposition in OVX in 100× magnification images (Fig. 1B) as well as the average % of quantified cardiac fibrosis were significantly increased in the OVX group compared with the Sham group (Fig. 1C).

3.3. Upstream components of cardiac Fas receptor-dependent apoptotic pathways

To investigate the upstream components of cardiac Fas-dependent apoptotic signaling pathways in OVX, the protein levels or gene expression of Fas ligand, TNF-α, Fas receptor and FADD in the excised hearts of Sham and OVX groups were examined by Western blotting or RT-PCR. Compared with the Sham group, the protein levels of Fas ligand and the gene expression of TNF-α significantly (P < 0.01) rose in the OVX group (Fig. 2). Besides, the gene expression of Fas receptor and the protein levels of Fas receptor and FADD were significantly (P < 0.01) increased in the OVX group (Fig. 3).

3.4. Main intracellular molecule signaling mediator from Fas to mitochondrial pathway

To investigate the cardiac Bid cleavage, a mediator that connects the Fas death receptor to the mitochondrial apoptosis pathway in the OVX, we examined the protein levels of Bid of Sham and OVX groups by Western blotting. The protein level of Bid was significantly (P < 0.01) decreased in the OVX group, compared with Sham group (Fig. 4).

3.5. Upstream components of cardiac mitochondria-dependent apoptotic pathways

To further understand the cardiac Bcl-2 family in mitochondria-dependent apoptotic pathway in OVX, we examined the protein
levels of the Bcl-2 family (Bad, Bax, Bcl-2) and cytosolic cytochrome c (cytochrome c release from mitochondria) in the excised hearts of Sham and OVX groups by Western blotting. Mitochondrial related pro-apoptotic proteins of Bad and Bax were significantly increased in OVX, whereas anti-apoptotic proteins Bcl-2 were not changed in OVX, compared with Sham group (Fig. 5). Furthermore, the ratio of Bax to Bcl-2 was significantly increased in OVX (Fig. 5E). The protein level of cytochrome c in the cytosolic fraction was significantly ($P < 0.01$) increased in the OVX group, compared with Sham group (Fig. 6).

### 3.6. Downstream components of cardiac Fas-dependent and mitochondria-dependent apoptotic pathways

To identify the downstream components of cardiac Fas (caspase 8 and 3) and mitochondria (caspase 9 and 3)-dependent apoptotic
Fig. 7. (A) The representative protein products of activated caspase 8, caspase 9, and caspase 3 extracted from the left ventricles of excised hearts in sham-operated rats (Sham) and ovariectomized rats (OVX) were measured by Western blotting analysis. (C) Bars represent the relative protein quantification of caspases on the basis of α-tubulin and indicate mean values ± S.D. (n = 6 in each group). **P < 0.01, significant differences between Sham and OVX group.

pathways, the protein levels of activated caspase 8, 9 and 3 were measured in the excised hearts of Sham and OVX groups by Western blotting. The activated forms of caspase 8, 9, and 3 protein products were increased in the OVX group, compared with Sham group (Fig. 7).

3.7. TUNEL-positive apoptotic cells of cardiac tissues

To reconfirm the cardiac apoptotic activity in OVX, we examined the apoptosis-positive cardiac cells in the excised hearts of Sham and OVX groups by TUNEL assay. We found that left ventricle stained with TUNEL assay showed increased TUNEL-positive cardiac cells in the OVX group, compared with Sham group from 400× magnification images (Fig. 8).

4. Discussion

4.1. Major findings

Our main findings can be summarized as follows: (1) the whole heart weight, left ventricular weight, the ratio of whole heart weight to tibia length and the ratio of left ventricle to tibia length were significantly increased in the OVX group relative to the Sham group. (2) Abnormal myocardial architecture, enlarged interstitial space, more minor cardiac fibrosis, and more TUNEL-positive apoptotic cells were observed in OVX group than in the Sham group. (3) The cardiac Fas-dependent apoptotic pathway was significantly more activated in OVX group than in the Sham group. The evidence for this is based on increases in TNF-alpha, Fas ligand, Fas death receptors, FADD, activated caspase-8, and activated caspase-3. (4) The cardiac mitochondria-dependent apoptotic pathway was significantly more activated in the OVX group than in the Sham group. The evidence for this is based on increases in Bad, Bax, Bax/Bcl-2, cytosolic cytochrome c, activated caspase-9, and activated caspase-3. Our current findings support our hypothesis that cardiac Fas-dependent and mitochondria-dependent apoptotic pathways might be activated in the absence of female ovaries (Fig. 9).

4.2. Experimental design

Concerning ovariectomized animal models, middle-aged animals for most of the species considered (mice, rats, dogs, rabbits, pigs, and sheep) have the potential to be good models for the effect of ovariectomy and estrogen replacement on associated bone and cardiovascular changes [27]. The bilateral ovariectomies not only impact the female hormonal system but also impact multiple physiologic system. Ovarian hormones, including estradiol (or estrogen) and progesterone, are necessary for women’s reproduction and general health. The ovarian hormones interact with hypothalamus–pituitary–adrenal axis, other hormones, neuromodulators, or neurotransmitters [28]. For example, a disruption in the hypothalamic–pituitary–gonadal axis was found after menopause and partially contributed to the aging process [28]. Therefore, in the current experimental design, we have to add a note of caution that any effect on cardiomyopathic changes noted after ovariectomy cannot be isolated to one specific factor, such as estrogen-deficiency, but may be affected directly or indirectly by various factors, such as estradiol, progesterone, oxidative stresses, hypothalamus–pituitary–adrenal axis, or unclear interacting factors other than female ovarian hormones.

4.3. Cardiac function and cardiomyopathic changes after ovariectomy

The risk of heart failure was associated with a 44% increase and the incident of heart failure was associated with a fourfold increase in post-menopausal women relative to the corresponding reference group [7]. Bilateral oophorectomy (or ovariectomy) have long-term negative consequences [29]; furthermore, it has been shown that the mortality rate is higher in oophorec-
tomized women <45 years than in referent women [30]. Previous studies suggest that ovariectomy may cause some cardiovascular remodeling and dysfunction. Complete oophorectomy in 146 young females ranging in age from 15 to 30 years resulted in an increase in serum cholesterol and triglycerides, an increased incidence of cardiac symptoms and a significant increase in the frequency of coronary vascular diseases [31]. In one study, premenopausal women who underwent bilateral oophorectomy had significantly increased heart rates for up to 1 month after surgery [32]. In another study, bilateral oophorectomy did not induce any significant modifications in the ECG parameters of the postmenopausal women, whereas in the premenopausal women, a significant increment in mean duration of the T wave, a significant decrease in its amplitude and significant reduction in some ST depression were observed [33]. Ovariectomized rats were previously reported to develop more extensive cardiac remodeling than intact females, characterized by significantly greater left ventricular hypertrophy and a substantial increase in left ventricular dilatation relative to controls [34]. The underlying mechanism of developing heart failure in post-menopausal women or oophorectomized women is still uncertain. In the current study, cardiomyopathic changes, such as cardiac hypertrophy, abnormal myocardial architecture, enlarged interstitial space, abnormal myocardial architecture, and minor cardiac fibrosis were increased in ovariectomized rats. The cardiomyopathic changes found in the current animal models may provide one mechanism to clarify how cardiac morphologic deliteration occurs in menopausal or ovariectomized women.

4.4. Cardiac apoptosis after ovariectomy

The activated Fas receptor-dependent and mitochondrial-dependent apoptotic pathway in hearts has been clearly reported after ovariectomy in the current study. All key components of Fas receptor-dependent apoptotic pathway, such as Fas ligand, Fas receptor, FADD, activated caspase-8, and activated caspase 3, as well as all key components of mitochondria-dependent apoptotic pathway in hearts have been clearly reported after ovariectomy in the current study.

In our proposed hypothesis, cardiac Fas-dependent and mitochondria-dependent apoptotic pathways will be activated in ovariectomized rats (OVX) compared with Sham. Cardiac Fas-dependent apoptotic pathway appears to be increased due to increases in TNF-α, Fas ligand, Fas receptor, FADD, activated caspase-8, and activated caspase-3. Cardiac mitochondria-dependent apoptotic pathway appears to be increased in OVX due to increases in Bad, Bax, Bax/Bcl-2, cytochrome c release, activated caspase-9, and activated caspase-3. Up arrows and down arrows on the right side represent increases and decreases, respectively.
dependent, such as Bad, Bax, cytosolic cytochrome c, activated caspase-9, and activated caspase-3, from upstream cascade to downstream cascade consistently show pro-apoptotic effects in the hearts excised from ovariectomized rat. Therefore, our findings strongly suggest that the absence of female ovaries will activate the cardiac Fas-dependent and mitochondria-dependent apoptotic pathways, which might lead to cardiac apoptosis.

Cardiac apoptosis in ovariectomized rats may be cussed directly or indirectly by various factors, such as decrease in estrogen-related anti-apoptosis [26], increase in weight gain [23,24], increase in oxidative stress [35], decrease in vascular perfusion [36,37], or other factors. One study shows that estrogen replacement reduced cardiomyocyte apoptosis in animal models of myocardial infarction via estrogen receptor and phospho-inositol-3 kinase-Akt-dependent pathways [26], which may indirectly support our finding in ovariectomy-induced cardiac apoptosis. After ovariectomy, body weight in the O VX group is significantly higher than that in the Sham group. Previous study show that the increased body weight in ovariectomized rats might be associated with decreases in mean level of central and peripheral leptin receptors, which makes transmission of signals to suppress the amount of food intake difficult, thus leading to an increase in body weight [38]. Since obesity or weight gain itself leads to activated cardiac Fas-dependent and mitochondria-dependent apoptotic pathways [23,24], it is still unclear whether the increased cardiac apoptotic activity is partially due to deleterious factor of weight gain after ovariectomy. Estrogen deficiency is associated with decreased nitric oxide production, which could be responsible for an increased oxidative stress and blood pressure in ovariectomized rats [35]. Estradiol hormone replacement has been shown to exert a beneficial effect on myocardial perfusion in menopause in part by upregulating nitric oxide synthase in the coronary vasculature [36,37]. Therefore, it is still questionable whether ovariectomized rats decrease cardiac vascular perfusion and thus lead to cardiac apoptosis. Further studies are required to answer the underlying causes of apoptosis in ovariectomized rats.

5. Hypothesized clinical application

Since cardiac tissues are difficult to extract from menopausal women hearts, the current ovariectomized animal model should provide an important mechanism for explaining the apoptosis-related cardiac diseases in women with the absence of ovaries or decline in female ovarian hormones. If cardiac apoptosis occurs in post-menopausal women, the long-lasting and extensive cardiac apoptosis may progressively lead to heart failure and sudden cardiac death. Since the absence of ovaries will enhance cardiac apoptosis, menopausal women should be highly aware of the progressive development of cardiac abnormalities, such as heart failure. Our current finding that cardiac mitochondria-dependent and Fas-dependent apoptotic pathways were activated after ovariectomy might provide one possible mechanism to explain the development of heart failure in post-menopausal women. Furthermore, it will raise the further question, whether hormonal replacement therapy, certain phyto-hormone therapy, or anti-apoptotic therapy might be beneficial to attenuate cardiac Fas-dependent and/or mitochondria-dependent apoptotic pathway when considering possible therapeutic agents to control or prevent the development of apoptosis related cardiac diseases in post-menopausal women. Of course, further therapeutic or clinical studies are required to clarify the effects of treatments or possible mechanisms in post-menopause-related heart abnormalities.

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