中 國 醫 藥 大 學
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碩士論文

運動訓練對卵巢切除之大鼠心臟凋亡以及抗凋亡的影響

Effect of exercise training on cardiac apoptosis and survival pathways in ovariectomized rats

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兩年的研究生活在完成這本論文的同時接近尾聲。做研究是件有趣的事，可以單純的完成實驗；也可以用心的去探討一個主題，而所獲得的可以是熟稔的技術；也可以是解決問題的能力，在此我第一位感謝的人便是我的指導教授 李信達老師，適用的設備、適度的壓力、適時的指導使我成為一位幸運的研究生能夠順利的完成學業。

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摘要

研究資料顯示停經或卵巢切除後的婦女大幅提高了心臟疾病的風險。過去研究發現在卵巢切除的大鼠身上有心肌細胞凋亡的現象，然而對於停經或是卵巢切除後的女性運動對心肌細胞凋亡的影響方面的研究並不多。本篇的目的便在研究雙側卵巢切除後的大鼠經由運動訓練後對其 Fas 蛋白和粒線體依賴性心臟細胞凋亡以及存活和抗凋亡途徑的影響。實驗使用三十三隻大鼠，其中十一隻手術過程中不切除卵巢作爲對照組，另外二十二隻卵巢切除大鼠中再隨機挑選十一隻於休息一周後開始進行十周的運動訓練。在訓練完成後將三十三隻大鼠犧牲取出心臟並測量 heart index、hematoxylin-eosin staining、Western Blotting 以及 positive TUNEL assays。實驗結果卵巢切除組大鼠在心臟重、不正常的心肌組織、Fas 以及粒線體依賴性心臟細胞凋亡途徑表現蛋白以及 TUNEL 陽性細胞，相較於對照組都有明顯的增加；而這些在運動組相較於雙側卵巢切除組則有明顯的下降。藉由本實驗證明了運動可以抑制或是預防卵巢切除所導致的 Fas 蛋白以及粒線體依賴性心臟細胞凋亡，並增進存活途徑的保護作用。由於雌激素與其替代品的補充治療有提高乳癌發生的風險，因此這項發現在停經或是卵巢切除後的婦女藉由運動預防心血管疾病上提供了一個新的治療效果。

Abstract

Background. Cardiac apoptosis were found in ovariectomized rats but very limited information regarding the effects of exercise training on cardiac apoptosis in menopausal or bilateral oophorectomied women was available. The purpose of this study was to evaluate the effects of exercise training on cardiac Fas and mitochondria dependent apoptotic pathways and cardiac survival pathways in ovariectomized rats. **Methods.** Eleven sham-operated rats(Sham) and eleven ovariectomized rats(OVX) at 14 weeks of age were served as negative and positive control. Eleven ovariectomized rats underwent treadmill running exercise 1 hour daily for 10 weeks(OVX-EX). After exercise training or sedentary status, the excised hearts were measured by heart index, hematoxylin-eosin staining, Western Blotting 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 and more cardiac TUNEL-positive apoptotic cells were observed in OVX, but not in Sham. Cardiac Fas ligand, Fas death receptors, Fas-associated death domain (FADD), t-Bid, Bad, Bak, Bax, activated caspase 8, activated caspase 9, and activated caspase 3 in OVX were significantly increased, compared to Sham. OVX-induced protein levels of TNF-alpha, Fas ligand, Fas death receptors, FADD, activated caspase 8, and activated caspase 3 (Fas pathways) became lower in OVX-EX. OVX-induced protein levels of t-Bid, Bad, Bax, Bak, activated caspase 9, and activated caspase 3 (mitochondria pathway) became lower in OVX-EX. Furthermore Cardiac phosphorylated PI3K, phosphorylated AKT, phosphorylated p38, Bcl2 and phosphorylated Bad were significanly increased in OVX-EX, compared to OVX. **Conclusions.** Exercise training suppressed ovariectomy-induced cardiac Fas and mitochondria dependent apoptotic pathways, and furthermore it increase cardiac survival pathways in ovariectomized rat models. The findings may provide one of new therapeutic effect of exercise training on preventing cardiac apoptosis in

menopausal or bilateral oophorectomied women.

Key words: heart, TNF-α, caspases, cell death, Ovariectomy.

CONTEXT

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TABLE AND FIGURE CONTENTS

Part 1.

Effect of exercise training on cardiac Fas and mitochondria dependant apoptosis pathways in ovariectomized rats

Introduction

Exercise has been used as an important approach in management cardiovascular disease[1]. Regular exercise also has benefits for cardiovascular system in postmenopausal women[2]. Evidence shows that menopause or early ovariectomy (oophorectomy) is associated with an increased risk of ischemic heart disease [3; 4]and suggests that female hormone deficiency plays an important pathological role in developing atherosclerotic diseases and deteriorating cardiovascular conditions[5; 6; 7]. In Europe, about 55% of all female deaths are caused by cardiovascular diseases, such as myocardial infarction, heart failure, and sudden cardiac death[8]. A report published in 2006 from 38283 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[9]. 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[10]. Most previous studies regarding cardiovascular diseases in menopausal women and in women with estrogen deficiency always have focused on coronary artery diseases [3; 4; 5; 6; 7]. Cardiac apoptosis may play a certain role in the levels of heart failure. [11; 12; 13].

Apoptosis, a physiological program of cellular death, may contribute to many cardiac disorders[14; 15]. 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[16]. The ‘extrinsic’ Fas receptor-dependent (type I) apoptotic pathway and ‘intrinsic’ mitochondria-dependent (type II) apoptotic pathway were believed to be two of the major pathways directly to trigger cardiac apoptosis[14; 17]. This type I apoptotic

pathway was initiated by binding of Fas ligand to the Fas receptor, which results in clustering of receptors and initiates the extrinsic pathway[17]. Fas receptor oligomerization recruits FADD and pro-caspase 8 to the complex and results in the activation of caspase 8. The activated caspase 8 cleaves pro-caspase 3, which then undergoes autocatalysis to form active caspase 3, a principle effector caspase of apoptosis [17; 18]. Additionally, downstream protein of Fas-dependent apoptotic pathway, caspase-8, can cleave Bcl-2 homology domain 3 (BH3)-interfering domain death agonist (BID). It cleave BID to truncate BID (t-BID), then causes the releasing of mitochondrial cytochrome c, leading to the activation of pro-caspase-9, which can then activate pro-caspase 3 [17; 19].The 'intrinsic' mitochondria-dependent (type II) apoptotic pathway starts from within the cell resulting in the release of a number of pro-apoptotic factors from the intermembrane space of mitochondria[17; 20]. The mitochondria is the main site of action for members of the apoptosis-regulating protein family exemplified by Bcl-2 family, such as Bax, Bak and Bad [21; 22]. 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 [21; 22; 23]. Bad, pro-apoptotic proteins, enhance cytochrome c release from mitochondria [17; 21; 22; 23; 24]. When cytochrome c is released from mitochondria into cytosol, it is responsible for activating caspase-9, which further activates caspase-3 and executes the apoptotic program [17; 22]. In our previous study, the key components of Fas-dependent apoptosis (Fas ligand, Fas death receptors, Fas-associated death domain (FADD), activated caspase 8, and activated caspase 3) and key components of mitochondria-dependent apoptosis (t-Bid, Bax, Bad, Bak, cytosolic cytochrome c, activated caspase 9, and activated caspase 3) were

significantly increased in ovariectomized rats heart.[11]

Cardiac Fas and mitochondria dependent apoptotic mechanism was involved in many pathologic conditions such as hypoxic stress, hypertension, obesity[25; 26; 27; 28]. Long-term 17ß-Estradiol treatment prevents the activation of apoptosis signaling and its downstream effectors in the hearts[29], and also prevent cardiomyocyte apoptosis in animal models of myocardial infarction[30]. However, previous study had proven that losing estrogen cause increasing cardiac fas and mitochondria dependant apoptosis[11]. But the effect of exercise training on cardiac apoptosis in postmenopausal or early oophorectomied women is totally not understood.

The current study was to understand whether exercise training can prevent cardiac Fas and mitochondria dependent apoptosis in ovariectomized rats. We hypothesized that exercise training may prevent the cardiac apoptotic pathways in ovariectomized rats.

Materials and Methods

Animal model

Thirty three 14 weeks 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 2 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 China Medical University, Taichung, Taiwan.

Ovariectomy and Sham operation

All thirty three rats were randomly divided into either sham-operated group, ovariectomized group or ovariectomized rats with exercise group. All animals were conducted by survival surgical procedures with aseptic technique at age of 14 weeks. 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-O 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.

Exercise training

After two weeks from operation Rats in theexercise group were trained by running on a motor-driven treadmill (Model T408E, Diagnostic & Research Instruments Co., Taoyuan, Taiwan) at a speed of 0.2 m/s for 15 min on the first day. This exercise intensity was approximately 60% of predetermined peak oxygen consumption as described in detail previously[31]. On the subsequent days of training, the running time was extended by 10 min/day until a running time of 60 min/day was reached. The training speed was increased 0.05 m/s every 2 weeks. These animals were trained for 5 days/week for 10 weeks. Resting heart rates were measured by a tail-cuff method (Narco Bio-Systems, Houston, Tex., USA) weekly to assess the training effects[32].

To avoid the acute effects of exercise, the animals were sacrificed at least 48 h after training while under general anesthesia with ether inhalation.

Cardiac characteristics

The hearts of sham-operated group(Sham), ovariectomized group(OVX) and ovariectomized rats with exercise group(OVX-EX) were excised and cleaned with PBS (Phosphate buffered saline). The left ventricle were separated and weighed. The right tibias were also separated and tibia lengths were measured by the electronic digital venire caliper to adjust the whole heart weight. The ratios of the total heart weight to body weight, the left ventricle weight 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.

Tissue Extraction

Cardiac tissue extracts were obtained by homogenizing the left ventricle samples

in a lysis buffer at a ratio of 100 mg tissue/1ml buffer. The homogenates were placed on ice and then centrifuged at 12,000 *g* for 40 min. The supernatant was collected and stored at -80°C for further experiments**.**

Electrophoresis and Western Blot

Protein concentration of cardiac tissue extracts was determined by the Lowry protein assay. Protein samples (40μg/lane) were separated on a 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) with a constant voltage of 75 V. Electrophoresed proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, 0.45 μm pore size) with a transfer apparatus (Bio-red, CA, USA). PVDF membranes were incubated in 5% milk in TBS buffer. Primary antibodies including Fas ligand, Fas, TNF-α, TNF receptor 1, FADD, caspase-8, caspase-3 and α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were diluted to 1:500 in antibody binding buffer overnight at 4°C. The immunoblots were washed three times in TBS buffer 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 hour and diluted 500-fold in TBS buffer. The immunoblots were then washed in TBS buffer for 10 min three times. The immunoblotted proteins were visualized using an enhanced chemiluminescence ECL western Blotting luminal Reagent (Santa Cruz) and quantified using a Fujifilm LAS-3000 chemiluminescence detection system (Fuji, Tokyo, Japan).

Hematoxylin-eosin staining (H&E staining) and Terminal Deoxynucleotide Transferase-mediated dUTP Nick End Labeling (TUNEL)

After the hearts were excised, nine of thirty three hearts were soaked in formalin, dehydrated through graded alcohols, and embedded in paraffin wax. In heart tissues, the 0.2-μm thick paraffin sections were cut from paraffin-embedded tissue

blocks. The tissues sections were deparaffinized by immersing in xylene, and rehydrated. For Hematoxylin-eosin staining, the slices were then dyed with hematoxylin and eosin. After gently rinsing with water, each slide was dehydrated through graded alcohols. Finally, they were soaked in xylene twice. Photomicrographs were obtained using Zeiss Axiophot microscopes. For TUNEL assay, the sections were incubated with proteinase K, washed in phosphate-buffered saline, incubated with permeabilisation solution, blocking buffer, and then washed two times with PBS. The terminal deoxynucleotidyl transferase and fluorescein isothiocyanate-dUTP for 60 min at 37 °C from an apoptosis detection kit (Roche Applied Science, Indianapolis, IN, USA) was used for detection. Then added the DAPI (4,6–diamidino-2-phenylindole) 5mins and the nucleus position were fluoresced by blue light at 340 / 380 nm. TUNEL-positive nuclei (fragmented DNA) were fluoresced by bright green light at 450-500 nm. The mean number of TUNEL-positive cells were counted for at least 5-6 separate fields x 2 slices x 3 regions of the left ventricle (upper, middle, lower) excised from 3 rat hearts in each group. All counts were performed by at least two independent individuals in a blinded manner.

Statistical Analysis

The all data of weight index, echocardiography index, protein levels, and the percentage of TUNEL positive cells were compared among the sham-operated group(Sham), ovariectomized group(OVX) and ovariectomized rats with exercise group(OVX-EX) using one-way analysis of variance (ANOVA) with pre-planned contrast comparison. In all cases, $P<0.05$ was considered significant.

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Results

Body weight and cardiac characteristics.

The body weight (BW) of ovariectomized group(OVX) higher than the body weight of sham-operated group(Sham), and after exercise training, the body weight was loss in ovariectomized rats with exercise group(OVX-EX). The whole heart weight (WHW), left ventricular weight (LVW), LVW/BW, WHW/tibia length and LVW/tibia length in OVX were higher than those in Sham. The whole heart weight (WHW), left ventricular weight (LVW), WHW/tibia length and LVW/tibia length in OVX-EX were also higher than those in Sham. And after exercise training, WHW was reduced in OVX-EX (Table 1).

Cardiac histopathological changes of left ventricle

To understand the myocardial architecture in ovariectomized models with exercise training, we did a histopathological analysis of left ventricular tissue with Hematoxylin-eosin staining in Sham, OVX, and OVX-EX groups. Hearts stained with hematoxylin-eosin showed that the ventricular myocardium in the Sham group showed normal architecture with normal interstitial space. In contrast based on subjective interpretation, the abnormal myocardial architecture and the increased interstitial space were observed in OVX. And the abnormal myocardial architecture and the increased interstitial space became normalized in OVX-EX, compared with OVX(Fig 1).

Upstream components of cardiac Fas receptor dependent apoptotic pathways

To investigate the upstream components of cardiac Fas receptor dependent apoptotic signaling pathways in ovariectomized models with exercise training, the protein levels of Fas, FasL, TNF-α, TNF receptor 1, and FADD were measured in hearts excised from Sham, OVX, and OVX+EX groups. Compared with the Sham

group, the protein levels of Fas ligand, Fas receptor, TNF-α, TNF receptor 1, and FADD were significantly increased in the OVX. The protein levels of Fas ligand, Fas (Fig 2), TNF-α, TNF receptor 1 (Fig 3), and FADD (Fig 4) in the OVX-EX group were significantly lower than those in the OVX group.

Transition from cardiac Fas dependant apoptosis pathways to cardiac mitochondria dependant apoptosis pathways

To identify the transition from cardiac Fas dependant apoptosis pathways to cardiac mitochondria dependant apoptosis pathway in ovariectomized models with exercise training, the protein levels of tBid were measured in the excised hearts of Sham, OVX and OVX-EX groups by Western Blotting. Compared with the Sham group, the protein levels of t-Bid(Fig 5) were significantly increased in the OVX group . And those in the OVX-EX group were significantly lower than those in the OVX group.

Upstream components of cardiac mitochondria dependent apoptotic pathways

To further understand the cardiac Bcl family in mitochondria-dependent apoptotic pathways in ovariectomized models with exercise training, we examined the protein levels of the , Bad, Bak, Bax and cytochrome *c* in the excised hearts of Sham, OVX and OVX-EX groups by Western Blotting. Compared with the Sham group, the protein levels of Bad(Fig 6), Bak, Bax(Fig 7) and cytochrome *c*(Fig 8) were significantly increased in the OVX group . And those in the OVX-EX group were significantly lower than those in the OVX group.

Downstream components of cardiac Fas-dependent and mitochondria-dependent apoptotic pathways

To identify the downstream components of cardiac Fas and mitochondria dependent apoptotic pathways, the protein levels of activated caspase 8, 9 and 3 were

measured in the excised hearts of Sham, OVX and OVX-EX groups by Western Blotting. The activated forms of caspase 8(Fas)(Fig 9), caspase 9(mitochondria)(Fig 10), and caspase 3(Fas and mitochondria)(Fig 11) protein products were increased in the OVX group compared with Sham group, as well as those in the OVX-EX group were lower than those in OVX.

TUNEL-positive apoptotic cells of cardiac tissues

In order to view the apoptotic activity in cardiac tissues, the apoptotic cells and total cells were measured by TUNEL assay and DAPI staining respectively in the hearts excised from the Sham, OVX, and OVX-EX groups. We observed that the left ventricles of the OVX groups stained with TUNEL assay had a greater number of TUNEL-positive cardiac cells than those in the Sham group. Decreased number of TUNEL-positive cardiac cells were found in the OVX-EX group, compared with OVX group (Fig 12).

Discussion

Our main findings can be summarized as follows: (1) The whole heart weight , left ventricular weight, the ratio of left ventricular weight to body weight, the ratio of whole heart weight to tibia length, the ratio of left ventricular weight to tibia length, abnormal myocardial architecture, enlarged interstitial space, cardiac Fas and mitochondria dependent apoptotic pathway and TUNEL-positive apoptotic cells were significantly increased in OVX relative to Sham. (2) Exercise prevents bilateral ovariectomy-induced cardiac Fas receptor dependent apoptotic pathways, the evidence for which is based on the attenuation of TNF-α, Fas ligand, Fas death receptors, Fas-associated death domain (FADD), activated caspase 8, and activated caspase 3 in OVX with exercise group(OVX-EX) relative to more increases in OVX. (3) Exercise prevents bilateral ovariectomy-induced cardiac mitochondria-dependent apoptotic pathway, the evidence for which is based on the attenuation of t-Bid, Bad, Bax and Bak activated caspase 9, and activated caspase 3 in OVX-EX relative to more increases in OVX. Our hypothesis proposed that enhanced cardiac Fas and mitochondria dependent apoptotic pathways after ovariectomy can be prevented via exercise training. (Fig 13)

The ovariectomized rats have the potential to be good models for a menopausal animal model[33], as the changes in biochemical and physiological function are comparable with those seen in menopausal women[34], i.e. decreased levels of progesterone and estrogen[35], increased risk of cardiovascular diseases[36], and enhanced rate of bone loss[37]. The bilateral ovariectomies in the current experimental design not only impact female hormonal system but also impact female systemic physiology. Ovarian hormones include estradiol (or estrogen) and progesterone that are necessary for women's reproduction, cardiovascular protection

and general health.

Post-menopausal women was associated with a 44% increase of the risk of heart failure[9]. Bilateral oophorectomies (or ovariotomies) have long-term negative consequences for heart diseases in post-menopause women[38]. Previous studies suggest that ovariectomy may cause cardiovascular dysfunctions and cardiac apoptosis [11; 39]. Complete oophorectomy from 146 young females in the age range of 15-30 years was found to have been followed by increases in serum cholesterol, serum triglyceride, the incidence of cardiac symptoms, and the frequency of coronary vascular diseases [39]. Very limited information regarding the protective effects of exercise on cardiac apoptosis or heart failure after post-menopause or bilateral oophorectomy in women was available. 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 control [40].

Physical activity is a common lifestyle factor which always recommend for postmenopausal women such as those for chronic heart diseases[1]. The previous has shown that cardiovascular function such as heart rate and vagal tonus were improved after 8 weeks treadmill training in ovariectomized rats' hearts[41]. In the other previous study exercise training by running on a treadmill for 60 min/day, 5 days/week for 10 weeks may increase cardiovascular protective effect via receptor-mediated agonist-stimulated endothelium-derived nitric oxide release in the thoracic aorta, but not in the common carotid artery that increases [32]. Myocardium Oxidative stress decreasing, superoxide dismutase and catalase activities enhancing in ovariectomized rats after 8 weeks exercise training by running on a treadmill[42]. In human study, Regular cardiorespiratory exercise is able to decrease cardiovascular

disease risk factors[43]. Reduction in both systolic and diastolic blood pressure values was found after exercise training which was accompanied by markedly increase of nitrate/nitrite levels in post-menopausal women[44].

In the current study, cardiomyopathic changes, such as abnormal myocardial architecture, enlarged interstitial space and minor cardiac fibrosis, appear to be increased in ovariectomized rats and these changes were attenuated after exercise training by running on a treadmill for 10 weeks. This is the first study to show that exercise training did improve myocardial architecture in ovariectomized animal models. The important finding "Exercise training prevents ovariectomy-induced cardiac Fas and mitochondria dependent apoptotic pathways" was first clarified in the current study. Since cardiac tissues are difficult to be extracted 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 removal of ovaries or decline of female ovarian hormones. If exercise training did suppress cardiac apoptosis in post-menopausal women, the daily physical activity may potentially suppress the possible development of heart failure and sudden cardiac death. Menopausal women should be highly aware of the progressive development in cardiac abnormality and should actively promote heart health by regular exercise.

Our current findings indicate that exercise training may be an important lifestyle modification to prevent ovariectomy-induced cardiac apoptotic pathways after menopause. Exercise training might provide one possible mechanism to interrupt cardiac apoptosis and the development of heart failure in post-menopausal women. Besides, it will raise the further question, whether some physiological responses of exercise, such as decreasing blood pressure, improving automatic tone, mediating cardiac calcium concentration, increasing VO2 max, mediating cardiovascular nitric

oxide release or decreasing myocardium oxidative stress might be beneficial to attenuate cardiac Fas and mitochondria dependent apoptotic pathways when considering possible factors to control or prevent the development of apoptosis related cardiac diseases in post-menopausal women. Of course, further therapeutic or clinical studies in human are required to clarify the effects of treatments or possible molecular mechanisms in post-menopause-related heart abnormalities.

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Table 1. Cardiac characteristics of Sham, OVX group and OVX with exercise training

	Sham	OVX	OVX-EX
	n=8	n=8	n=8
WHW(g)	0.84±0.09	1.00±0.07***	0.93±0.06*#
LVW(g)	0.60±0.07	0.72±0.05***	0.69±0.05**
WHW/BW (g/kg)	2.88±0.24	2.61±0.17**	2.65±0.20*
LVW/BW(g/kg)	2.11±1.10	1.89±0.17*	1.97±0.14
LVW/WHW(g/g)	0.74±0.05	0.71±0.04	0.74±0.04
WHW/TL(g/m)	22.96±2.91	26.39±1.54**	25.58±1.73*
LVW/TL(g/m)	16.90±1.78	18.89±1.57*	19.03±1.42*

Heart weight index among the sham operating rat (Sham), ovariectomized rat (OVX) and ovariectomized rat with exercise training(OVX-EX). WHW: whole heart weight; LVW: left ventricular weight; BW: body weight; TL: tibia length. Values are mean ±SD.; *P＜0.05 and **P< 0.01 compared with Sham; #P＜0.05 compared with OVX.

Fig 1.

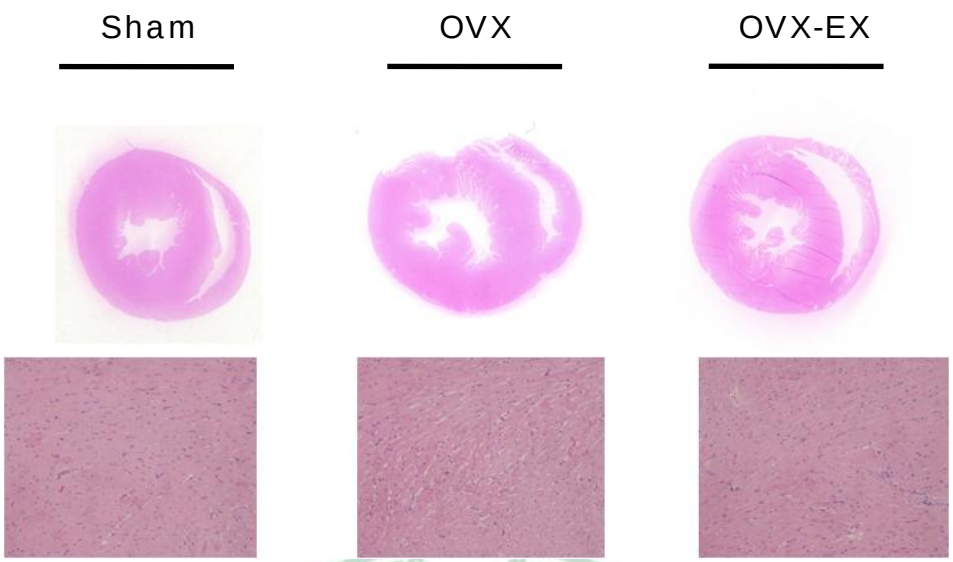


Fig 1. Hematoxylin and eosin staining was analyzed in cardiac sections from left ventricles in sham operating rat (Sham), ovariectomized rat (OVX) and ovariectomized rat with exercise training(OVX-EX). The representative images of cardiac architecture were magnified by 400 times. (n=3 rats × 6 slides in each group).

Fig 2.

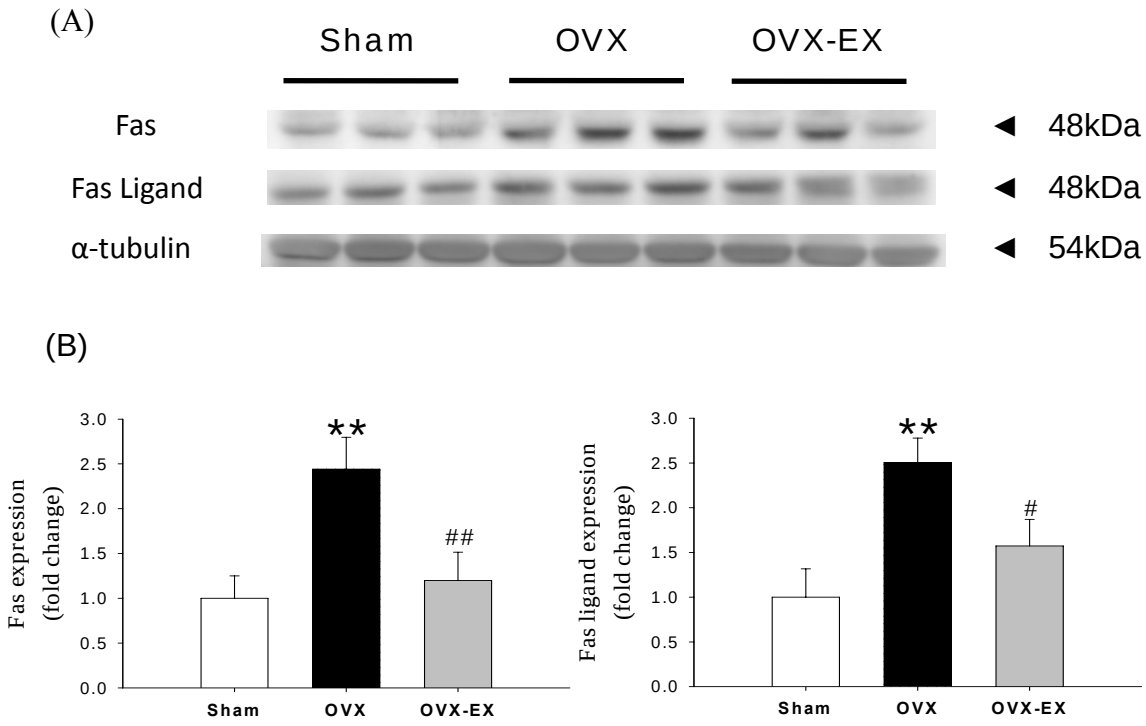


Fig 2. (A) The representative protein products of Fas and Fas ligand extracted from the left ventricles of excised hearts in 3 sham operating rats(Sham), 3 ovariectomized rats (OVX) and 3 ovariectomized rats with exercise training(OVX-EX) were measured by Western Blotting analysis. α-tubulin was use as a internal control. (B) Bars represent the relative fold changes of Fas/α-tubulin and Fas ligand /α-tubulin relative to Sham group and indicate mean values±SD (n=6 in each group). ***P <0.01 compare with Sham.* $^{\#}$P<0.05 and $^{\#\#}$P<0.01 significant differences between OVX group and OVX-EX group.

Fig 3.

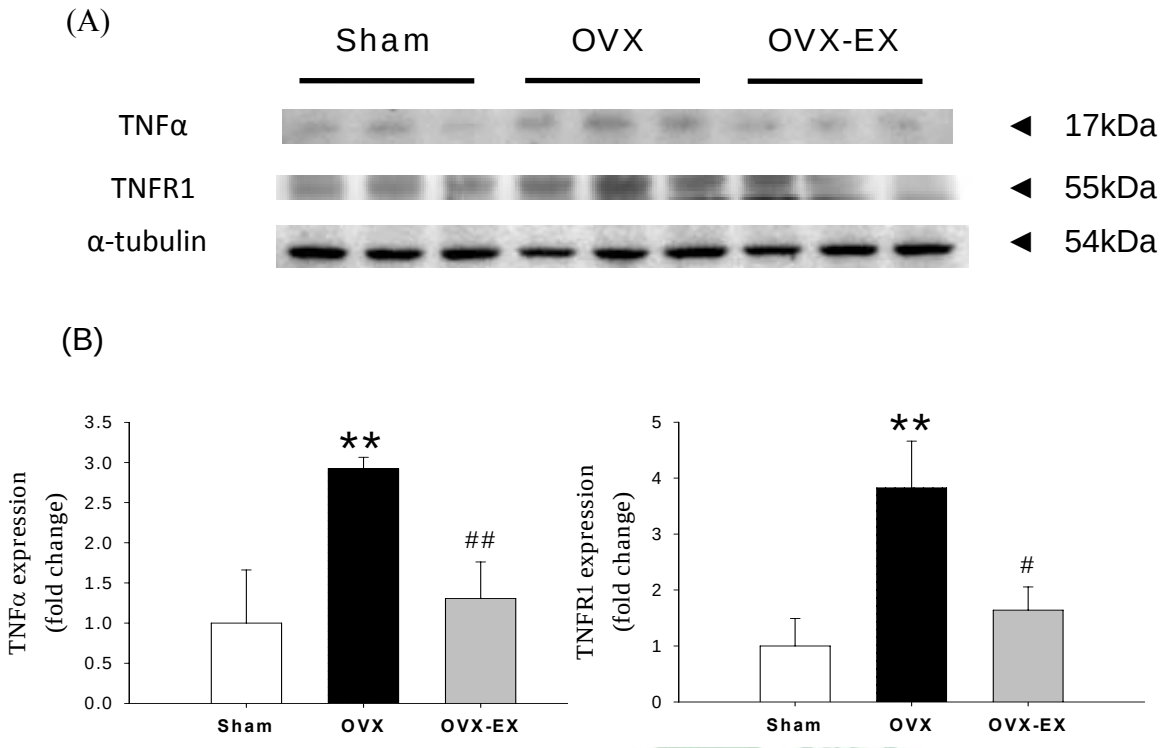
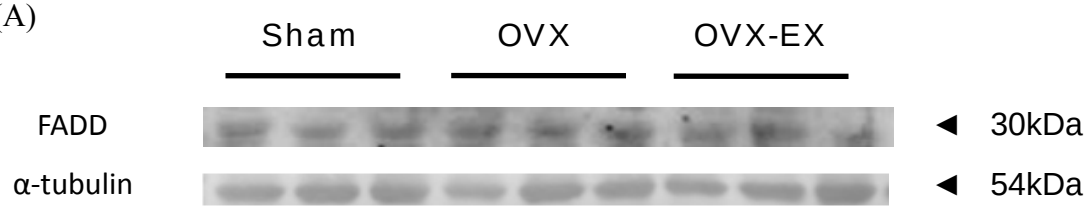


Fig 3. (A) The representative protein products of TNFα and TNFR1 extracted from the left ventricles of excised hearts in 3 sham operating rats(Sham), 3 ovariectomized rats (OVX) and 3 ovariectomized rats with exercise training(OVX-EX) were measured by Western Blotting analysis. α-tubulin was use as a internal control. (B) Bars represent the relative fold changes of TNFα/α-tubulin and TNFR1/α-tubulin relative to Sham group and indicate mean values±SD (n=6 in each group). ***P <0.01 compare with Sham.* $^{\#}$P<0.05 and $^{\#\#}$P<0.01 significant differences between OVX group and OVX-EX group.

Fig 4.

(A)



(B)

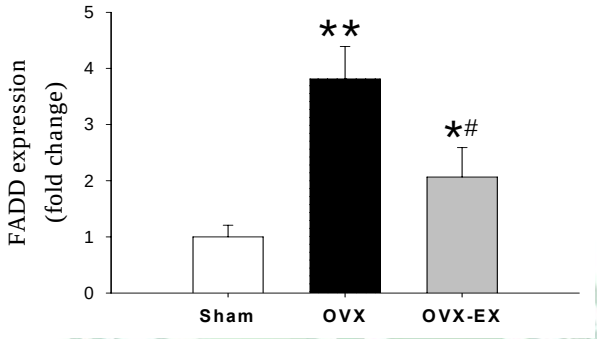


Fig 4. (A) The representative protein products of Fas-associated death domain(FADD) extracted from the left ventricles of excised hearts in 3 sham operating rats(Sham), 3 ovariectomized rats (OVX) and 3 ovariectomized rats with exercise training(OVX-EX) were measured by Western Blotting analysis. α-tubulin was use as a internal control. (B) Bars represent the relative fold changes of FADD/α-tubulin relative to Sham group and indicate mean values±SD (n=6 in each group). **P<0.05 and **P <0.01 compared with Sham.* #P<0.05 significant differences between OVX group and OVX-EX group.

Fig 5.

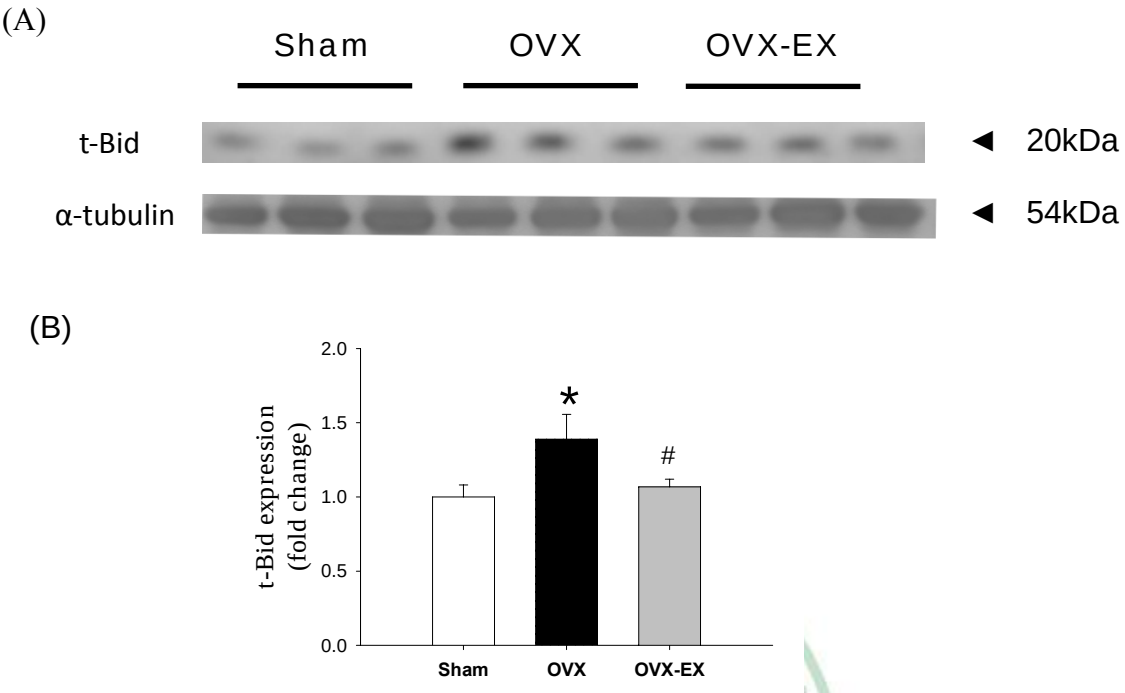
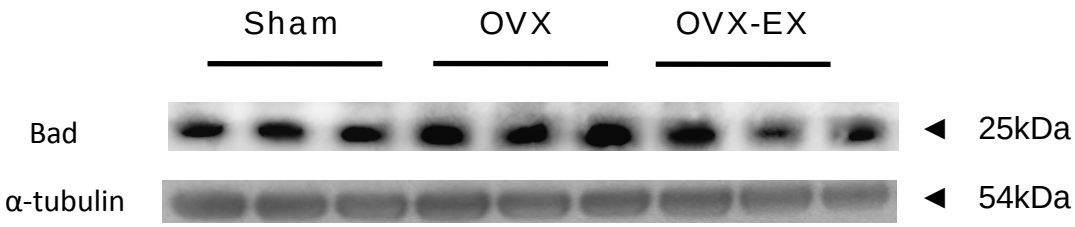


Fig 5. (A) The representative protein products of t-Bid extracted from the left ventricles of excised hearts in 3 sham operating rats(Sham), 3 ovariectomized rats (OVX) and 3 ovariectomized rats with exercise training(OVX-EX) were measured by Western Blotting analysis. α-tubulin was use as a internal control. (B) Bars represent the relative fold changes of t-Bid/α-tubulin relative to Sham group and indicate mean values±SD (n=6 in each group). **P<0.05 compared with Sham.* $^{\#}$P<0.05 significant differences between OVX group and OVX-EX group.

Fig 6.

(A)



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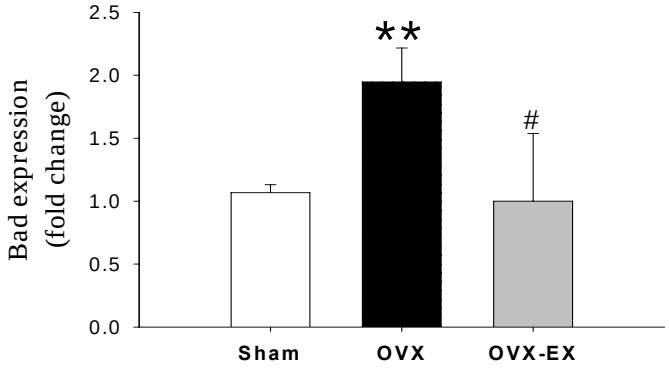


Fig 6. (A) The representative protein products of Bad extracted from the left ventricles of excised hearts in 3 sham operating rat (Sham), 3 ovariectomized rats (OVX) and 3 ovariectomized rats after exercise training(OVX-EX) were measured by Western Blotting analysis. α-tubulin was use as a internal control. (B) Bars represent the relative fold changes of protein quantification relative to Sham group in Bad/α-tubulin and mean values±SD (n=6 in each group). ***P <0.01 compare with Sham.* $^{\#}$P<0.05 significant differences between OVX group and OVX-EX group.

Fig 7.

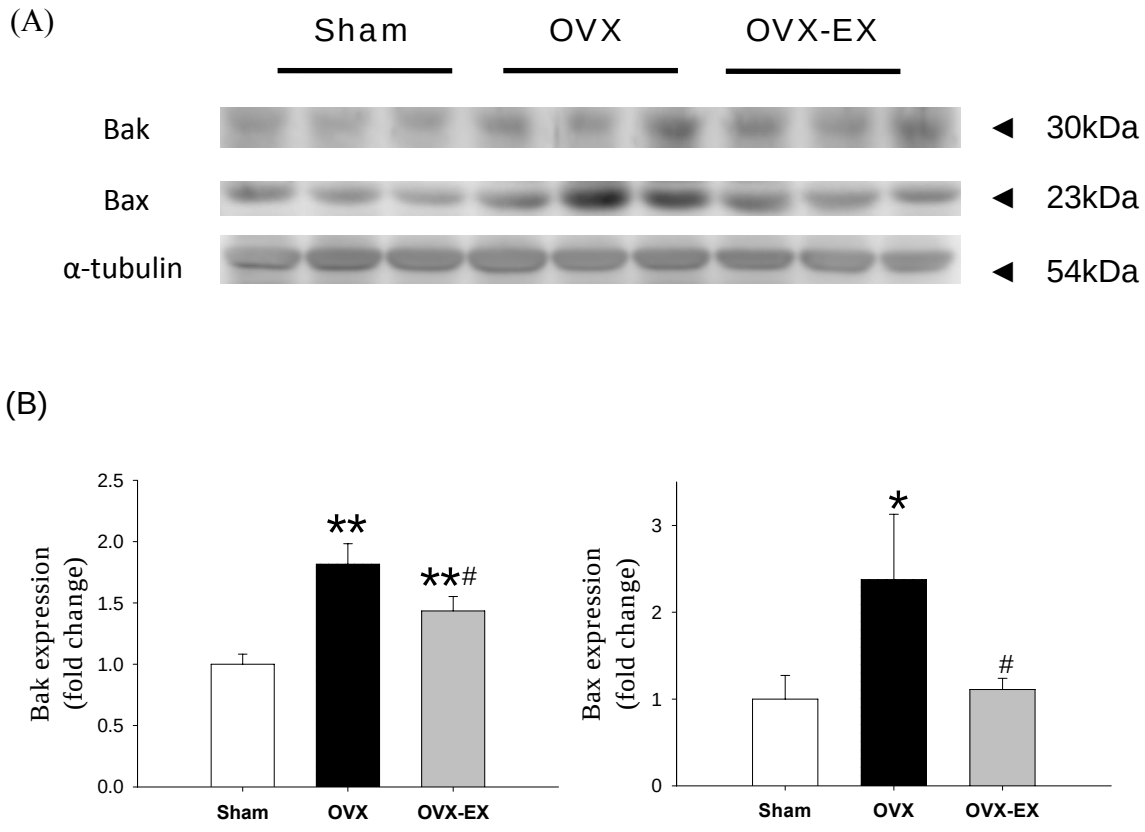
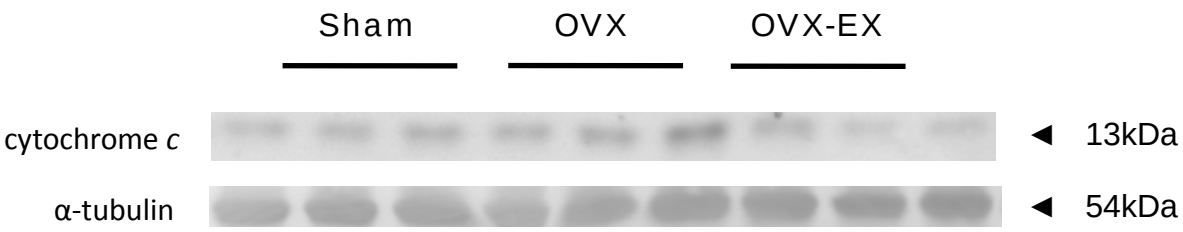


Fig 7. (A) The representative protein products of Bak and Bax extracted from the left ventricles of excised hearts in 3 sham operating rats(Sham), 3 ovariectomized rats (OVX) and 3 ovariectomized rats with exercise training(OVX-EX) were measured by Western Blotting analysis. α-tubulin was use as a internal control. (B) Bars represent the relative fold changes of Bak/α-tubulin and Bax/α-tubulin relative to Sham group and indicate mean values±SD (n=6 in each group). **P <0.05 and **P < 0.01 compare with Sham.* #P<0.05 significant differences between OVX group and OVX-EX group.

Fig 8.

(A)



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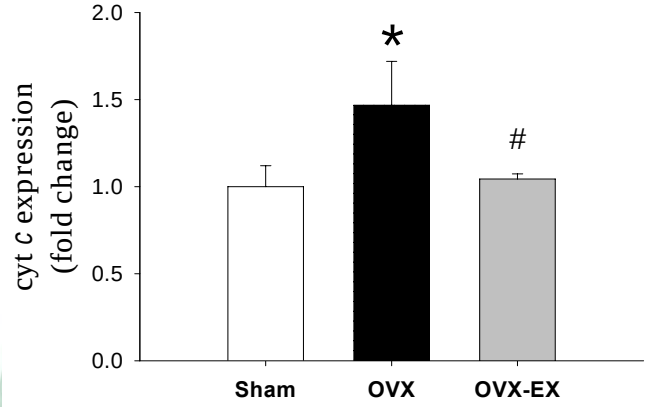


Fig 8. (A) The representative protein products of cytochrome *c* extracted from the left ventricles of excised hearts in 3 sham operating rat (Sham), 3 ovariectomized rats (OVX) and 3 ovariectomized rats after exercise training(OVX-EX) were measured by Western Blotting analysis. α-tubulin was use as a internal control. (B) Bars represent the relative fold changes of protein quantification relative to Sham group in cytochrome *c* /α-tubulin and mean values±SD (n=6 in each group). **P ＜0.05 compare with Sham.* #P<0.05 significant differences between OVX group and OVX-EX group.

Fig 9.

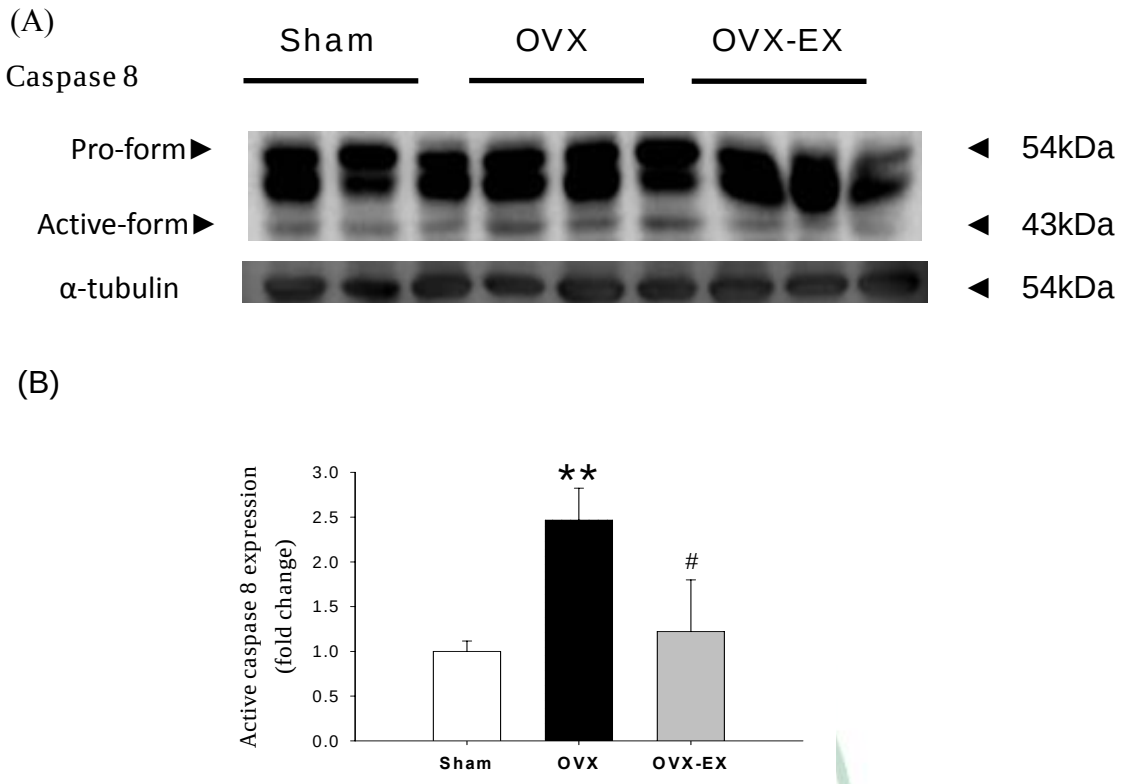
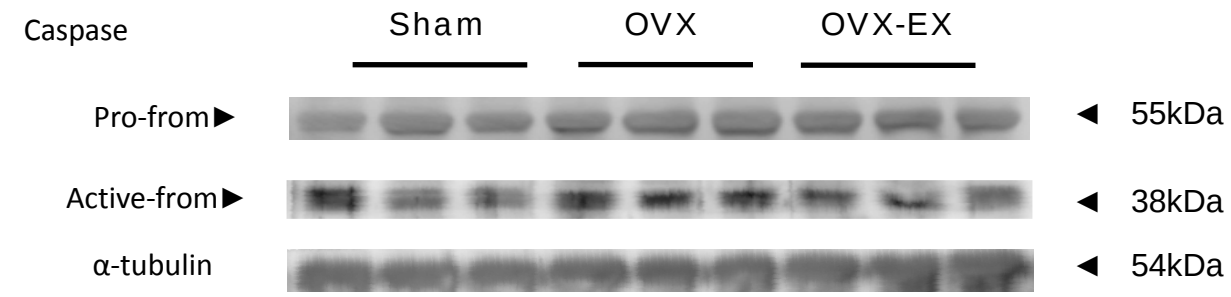


Fig 9. (A) The representative protein products of pro-form and active-form caspase 8 extracted from the left ventricles of excised hearts in 3 sham operating rats(Sham), 3 ovariectomized rats (OVX) and 3 ovariectomized rats with exercise training(OVX-EX) were measured by Western Blotting analysis. α-tubulin was use as a internal control. (B) Bars represent the relative fold changes of caspase 8 active-form/α-tubulin relative to Sham group and indicate mean values±SD (n=6 in each group). ***P <0.01* significant differences between OVX group and *Sham group.* $^{\#}$P<0.05 compared with OVX group.

Fig 10.

(A)



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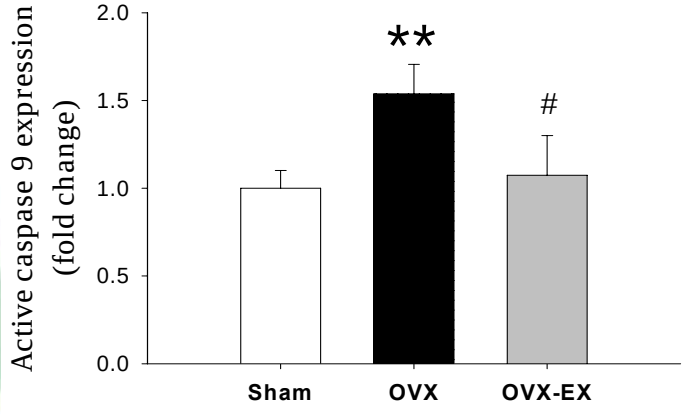

Fig 10. (A) The representative protein products of pro-form and active-form caspase 9 extracted from the left ventricles of excised hearts in 3 sham operating rat (Sham), 3 ovariectomized rats (OVX) and 3 ovariectomized rats after exercise training(OVX-EX) were measured by Western Blotting analysis. α-tubulin was use as a internal control. (B) Bars represent the relative fold changes of protein quantification relative to Sham group in caspase 9 active-form /α-tubulin and mean values±SD (n=6 in each group). ***P <0.01 compare with Sham.* $^{\#}$P<0.05 significant differences between OVX group and OVX-EX group.

Fig 11.

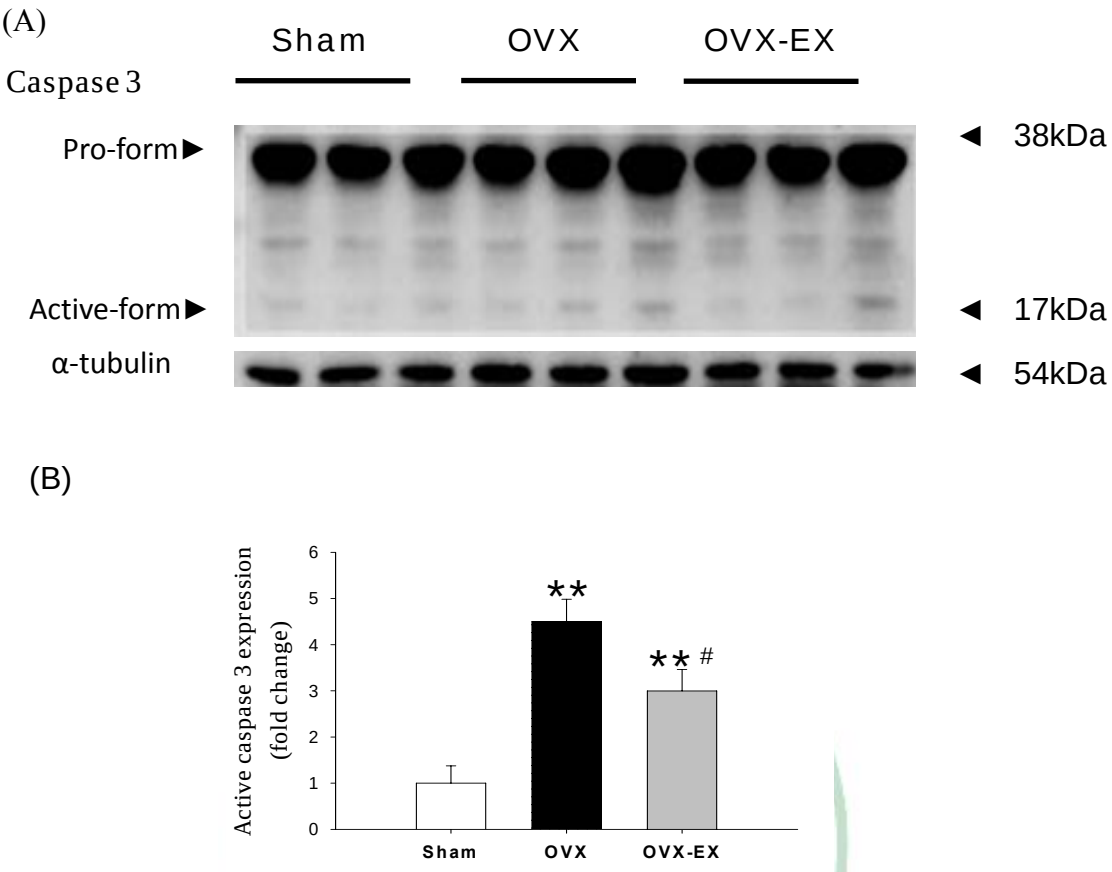

Fig 11. (A) The representative protein products of pro-form and active-form caspase 3 extracted from the left ventricles of excised hearts in 3 sham operating rat (Sham), 3 ovariectomized rats (OVX) and 3 ovariectomized rats after exercise training(OVX-EX) were measured by Western Blotting analysis. α-tubulin was use as a internal control. (B) Bars represent the relative fold changes of protein quantification relative to Sham group in caspase 3 active-form/α-tubulin and mean values±SD (n=6 in each group). ***P < 0.01 compare with Sham. **P < 0.01 compared with Sham and* #P<0.05 had significant differences between OVX group and OVX-EX group.

Fig 12.

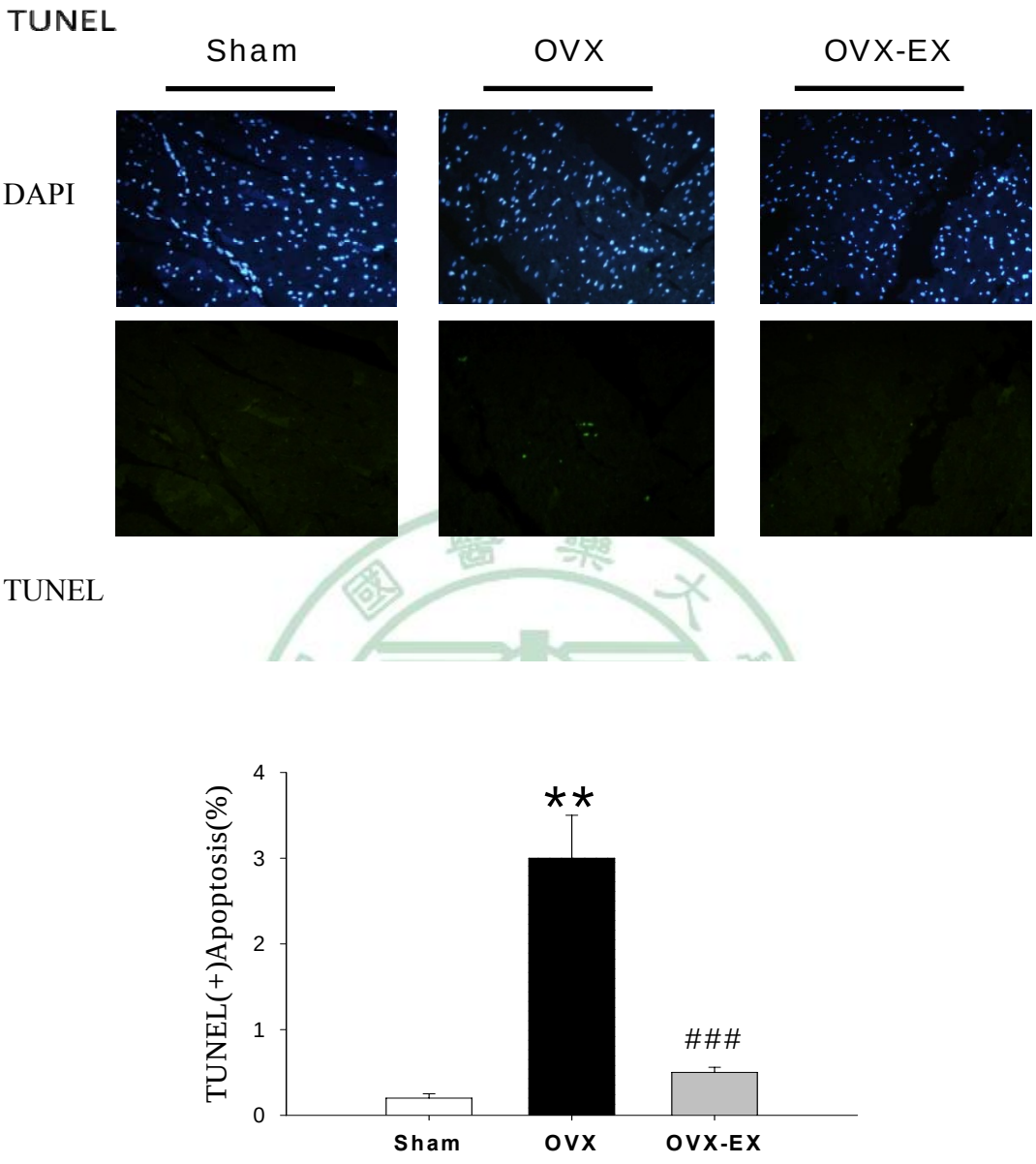

Fig 12. (A) Representative stained apoptotic cells of cardiac sections from left ventricle in sham operating rat (Sham), ovariectomized rats (OVX) and ovariectomized rats after exercise training(OVX-EX) by staining with DAPI staining(upper panels) and TUNEL assay with dark background (lower panels, green spots). The images were magnified by 400 times(n=3 rats × 6 slide in each group). (B) Bars present the percentage of TUNEL positive cells relative to total cells. **P<0.01, significant differences from Sham group. ##P<0.01 significant differences between OVX group and OVX-EX group.

Fig 13.

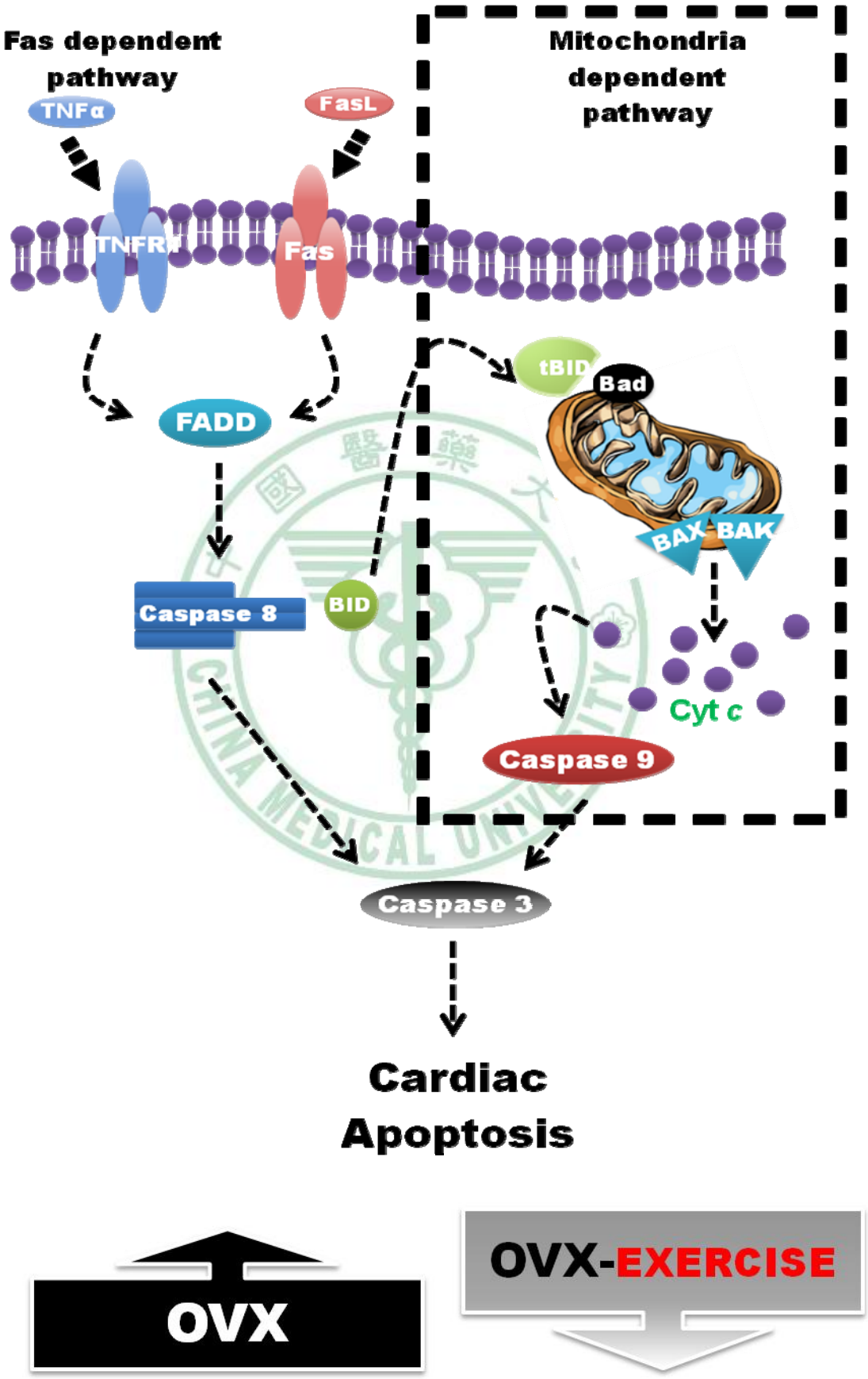
Fas dependent pathway
Mitochondria dependent pathway
TNFα
FasL
TNFRI
Fas
FADD
tBID
Bad
BAX
BAK
Caspase 8
BID
Cyt c
Caspase 9
Caspase 3
Cardiac Apoptosis
OVX
OVX-EXERCISE

Fig 13. The cardiac Fas receptor-dependent apoptotic pathway(TNFα, TNFR1, Fas ligand, Fas, activated caspase-8, and activated caspase-3) and The cardiac mitochondria-dependent apoptotic pathway(tBid, Bax, Bax, Bad, activated caspase-9, and activated caspase-3) were decreased after exercise training compared with the OVX group. Our findings imply that exercise training could be one of possible therapeutic approaches to prevent delirious cardiac apoptosis in postmenopausal or bilateral ovariectomized women.

Part 2.

Effect of exercise training on cardiac survival pathways in ovariectomized rats

Introduction

Exercise has been used as an important approach in management cardiovascular disease[1]. Regular exercise also has benefits for cardiovascular system in postmenopausal women[2]. Evidence shows that menopause or early ovariectomy (oophorectomy) is associated with an increased risk of ischemic heart disease [3; 4]and suggests that female hormone deficiency plays an important pathological role in developing atherosclerotic diseases and deteriorating cardiovascular conditions[5; 6; 7]. In Europe, about 55% of all female deaths are caused by cardiovascular diseases, such as myocardial infarction, heart failure, and sudden cardiac death[8]. A report published in 2006 from 38283 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[9]. 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[10]. Most previous studies regarding cardiovascular diseases in menopausal women and in women with estrogen deficiency always have focused on coronary artery diseases [3; 4; 5; 6; 7]. Cardiac apoptosis may play a certain role in the levels of heart failure. [11; 12; 13]

Apoptosis, a physiological program of cellular death, may contribute to many cardiac disorders[14; 15]. 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[16].

The insulin-like growth factor (IGF) system efficiently signals to cells to grow, differentiate, and survive. IGF-1 is a major survival factor in serum and prevents apoptosis in a number of cell types[45]. When it activates phosphatidylinositol

3-kinase (PI3K) and serine/threonine kinase Akt pathway, it will attenuate the apoptotic activity[46]. Akt is a cell survival downstream growth factors, it enhances the survival of cells by blocking the function of proapoptotic proteins and processes. Phosphatidylinositol 3-kinase (PI_3K) and serine/threonine kinase Akt pathway phosphorylates p38 was considered a cardiac anti-apoptosis mechanism[47]. Akt negatively regulates the function or expression of several Bcl-2 family, which exert anti-apoptotic effects by binding to and inactivating pro-survival Bcl-2 family members. For instance, Akt directly phosphorylates and inhibits the BH3-only protein BAD [48; 49]. Survival factors stimulate Akt-mediated phosphorylation of BAD on S136 (p-BAD), which triggers release of BAD from its target proteins [50]. p-BAD is an anti-apoptotic protein, prevents cytochrome c release and apoptotic activity. Pro-apoptotic and anti-apoptotic Bcl2 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. Bcl-2 and Bcl-xL, anti-apoptotic proteins, may stabilize the mitochondrial membrane and prevent the activation of downstream apoptotic signaling [51].

Cardiac apoptotic mechanism was involved in many pathologic conditions such as hypoxic stress, hypertension, obesity[25; 26; 27; 28].Long-term 17ß-Estradiol treatment prevents the activation of apoptosis signaling and its downstream effectors in the hearts[29], and also prevent cardiomyocyte apoptosis in animal models of myocardial infarction[30]. However, previous study had proven that losing estrogen cause increasing cardiac Fas and mitochondria dependant apoptosis[11]. But the effect of exercise training on cardiac apoptosis in postmenopausal women is totally not understood.

The current study was to understand whether exercise training can enhance

survival pathways to prevent cardiac apoptosis in ovariectomized rats. We hypothesized that exercise training may enhance cardiac survival pathways and prevent apoptosis.

Materials and Methods

Animal model

Thirty three 14 weeks 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 2 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 China Medical University, Taichung, Taiwan.

Ovariectomy and Sham operation

All thirty three rats were randomly divided into either sham-operated group, ovariectomized group or ovariectomized rats with exercise 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-O 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.

Exercise training

After two weeks from operation Rats in theexercise group were trained by running on a motor-driven treadmill (Model T408E, Diagnostic & Research Instruments Co., Taoyuan, Taiwan) at a speed of 0.2 m/s for 15 min on the first day. This exercise intensity was approximately 60% of predetermined peak oxygen consumption as described in detail previously[31]. On the subsequent days of training, the running time was extended by 10 min/day until a running time of 60 min/day was reached. The training speed was increased 0.05 m/s every 2 weeks. These animals were trained for 5 days/week for 10 weeks. Resting heart rates were measured by a tail-cuff method (Narco Bio-Systems, Houston, Tex., USA) weekly to assess the training effects[32].

To avoid the acute effects of exercise, the animals were sacrificed at least 48 h after training while under general anesthesia with ether inhalation.

Cardiac characteristics

The hearts of sham-operated group, ovariectomized group and ovariectomized rats with exercise group were excised and cleaned with PBS (Phosphate buffered saline). The left ventricle were separated and weighed. The right tibias were also separated and tibia lengths were measured by the electronic digital venire caliper to adjust the whole heart weight. The ratios of the total heart weight to body weight, the left ventricle weight 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.

Tissue Extraction

Cardiac tissue extracts were obtained by homogenizing the left ventricle samples

in a lysis buffer at a ratio of 100 mg tissue/1ml buffer. The homogenates were placed on ice and then centrifuged at 12,000 *g* for 40 min. The supernatant was collected and stored at -80°C for further experiments**.**

Electrophoresis and Western Blot

Protein concentration of cardiac tissue extracts was determined by the Lowry protein assay. Protein samples (40μg/lane) were separated on a 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) with a constant voltage of 75 V. Electrophoresed proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, 0.45 μm pore size) with a transfer apparatus (Bio-red). PVDF membranes were incubated in 5% milk in TBS buffer for 1 hour. Primary antibodies including IGF1, IGF1R, phophorylated PI3K, PI3K, phophorylated AKT, AKT, phophorylated P38, P38, Bcl2, phophorylated Bad(Santa Cruz Biotechnology, Santa Cruz, CA, USA) were diluted to 1:500 in antibody binding buffer overnight at 4°C. The immunoblots were washed three times in TBS buffer 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 hour and diluted 500-fold in TBS buffer. The immunoblots were then washed in TBS buffer for 10 min three times. The immunoblotted proteins were visualized 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).

Hematoxylin-eosin staining (H&E staining) and Terminal Deoxynucleotide Transferase-mediated dUTP Nick End Labeling (TUNEL)

After the hearts were excised, nine of thirty three hearts were soaked in formalin, dehydrated through graded alcohols, and embedded in paraffin wax. In heart

tissues, the 0.2-μm thick paraffin sections were cut from paraffin-embedded tissue blocks. The tissues sections were deparaffinized by immersing in xylene, and rehydrated. For Hematoxylin-eosin staining, the slices were then dyed with hematoxylin and eosin. After gently rinsing with water, each slide was dehydrated through graded alcohols. Finally, they were soaked in xylene twice. Photomicrographs were obtained using Zeiss Axiophot microscopes. For TUNEL assay, the sections were incubated with proteinase K, washed in phosphate-buffered saline, incubated with permeabilisation solution, blocking buffer, and then washed two times with PBS. The terminal deoxynucleotidyl transferase and fluorescein isothiocyanate-dUTP for 60 min at 37 °C from an apoptosis detection kit (Roche Applied Science, Indianapolis, IN, USA) was used for detection. Then added the DAPI (4,6–diamidino-2-phenylindole) 5mins and the nucleus position were fluoresced by blue light at 340 / 380 nm. TUNEL-positive nuclei (fragmented DNA) were fluoresced by bright green light at 450-500 nm. The mean number of TUNEL-positive cells were counted for at least 5-6 separate fields x 2 slices x 3 regions of the left ventricle (upper, middle, lower) excised from six rat hearts in each group. All counts were performed by at least two independent individuals in a blinded manner.

Statistical Analysis

The all data of weight index, echocardiography index, protein levels, and the percentage of TUNEL positive cells were compared among the sham-operated group, ovariectomized group and ovariectomized rats with exercise group using one-way analysis of variance (ANOVA) with pre-planned contrast comparison. In all cases, $P<0.05$ was considered significant.

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Results

Body weight and cardiac characteristics.

The body weight (BW) of ovariectomized group higher than the body weight of sham-operated group, and after exercise training, the body weight was loss in ovariectomized rats with exercise group. The whole heart weight (WHW), left ventricular weight (LVW), LVW/BW, WHW/tibia length and LVW/tibia length in ovariectomized group were higher than those in sham-operated group. The whole heart weight (WHW), left ventricular weight (LVW), WHW/tibia and LVW/tibia in ovariectomized rats with exercise group were also higher than those in sham-operated group. And after exercise training, the ovariectomized rats with exercise group was reduced in WHW(Table 1).

Cardiac IGF1-R/PI3K/AKT survival pathway

To investigate the components of Cardiac IGF1R/PI3K/AKT survival pathway in ovariectomized rats after exercise training, we measured the pro-survival relative protein expression in hearts excised from Sham, OVX, and OVX-EX groups. Compared with the OVX group, the protein levels of IGF1, IGF1R(Fig 2), p-PI3K(Fig 3), p-Akt(Fig 4) and p-p38(Fig 5) were significantly increased in the OVX-EX group. There were no significantly different on protein levels of PI3K(Fig 3), AKT(Fig 4), p38(Fig 5) in three groups.

Cardiac pro-survival Bcl-2 family associated pathway

In order to identify the components of Cardiac pro-survival Bcl-2 family associated pathway in hypertensive rats after exercise training. The pro-survival protein expression level of Bcl-2, and p-BAD were measured by Western blotting in the hearts excised from Sham, OVX, and OVX-EX groups. The protein level of Bcl-2 (Fig 6) and p-BAD (Fig 7) in the OVX-EX group were significantly higher than those

in the OVX group.

TUNEL-positive apoptotic cells of cardiac tissues

In order to view the apoptotic activity in cardiac tissues, the apoptotic cells and total cells were measured by TUNEL assay and DAPI staining respectively in the hearts excised from the Sham, OVX, and OVX-EX groups. Viewing images magnified 400 X, we observed that the left ventricles of the OVX groups stained with TUNEL assay had a greater number of TUNEL-positive cardiac cells than those in the Sham group whereas the number of TUNEL-positive cardiac cells was similar between Sham and OVX-EX group. Decreased in number of TUNEL-positive cardiac cells were found in the OVX-EX group, compared with OVX group (Fig 8).

Discussion

Our main findings can be summarized as follows: (1) Exercise prevent the abnormal cardiac changes via decreased the whole heart weight , left ventricular weight, the ratio of left ventricular weight to body weight, the ratio of whole heart weight to tibia length, the ratio of left ventricular weight to tibia length, abnormal myocardial architecture, enlarged interstitial space and TUNEL-positive apoptotic cells which significantly increased in OVX relative to Sham. (2) The activity of the cardiac survival pathway in ovariectomized rats was significantly increased after exercise training, the evidence for which is based on increases in IGF1, IGF1R, PI3K, p-PI3K, Akt, p-Akt, p-p38, p-BAD and Bcl-2 compared with the OVX. Our hypothesis proposed that cardiac survival pathways after ovariectomy can be induced via physical exercise training. (Fig 9)

The ovariectomized rats were suggest to be menopausal animal model[33], as well as the similar changes of biochemical and physiological function in menopausal women[34]. The bilateral ovariectomies in the current experimental design not only impact female hormonal system but also impact female systemic physiology. Ovarian hormones include estradiol (or estrogen) and progesterone that are necessary for women's reproduction, cardiovascular protection and general health.

Post-menopausal women was associated with a 44% increase of the risk of heart failure[9]. Bilateral oophorectomies (or ovariotomies) have long-term negative consequences for heart diseases in post-menopause women[38]. Previous studies suggest that ovariectomy may cause cardiovascular dysfunctions and cardiac apoptosis [11; 39]. Very limited information regarding the protective effects of exercise on cardiac apoptosis or heart failure after post-menopause or bilateral oophorectomy in women was available. 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 control [40].

Estrogen addition and hormonal replacement were used to protect cardiovascular system and other body mechanisms in post-menopausal or oophorectomied women[52; 53]. Estrogen protect myocardium via estrogen receptor β activate PI3K/AKT and P38 cardiac survival pathways[47; 54]. But estrogen and estrogen substitutes using will increase the breast cancer risk[52; 55; 56].

Physical activity is a common Lifestyle Factor which always recommend for postmenopausal women such as those for chronic heart diseases[1]. The previous has shown exercise enhance several protective effects in ovariectomized rats' hearts[32; 41; 42]. In human study, there was previous research showed regular cardiorespiratory exercise is able to decrease cardiovascular disease risk factors[43; 44]. Furthermore, regular exercise also be able to reduce breast cancer risk[57].

In the current study, cardiomyopathic changes, such as abnormal myocardial architecture, enlarged interstitial space and minor cardiac fibrosis, appear to be increased in ovariectomized rats and these changes were attenuated after exercise training by running on a treadmill for 10 weeks.

The increasing of p-PI3K and p-AKT in OVX group might cause by the compensatory effects. It might induced the other protect mechanism when myocardium suffered stress in OVX groups. But the protein levels of p-PI3K and p-AKT in OVX-EX were significant higher than OVX, which proved the capability of exercise enhancing PI3K/AKT survival pathway in ovariectomized rats.

The important finding "Exercise training induced cardiac survival pathways" was first clarified in the current study. Since cardiac tissues are difficult to be

extracted 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 removal of ovaries or decline of female ovarian hormones. If exercise training did suppress cardiac apoptosis in post-menopausal women, the daily physical activity may potentially suppress the possible development of heart failure and sudden cardiac death. Furthermore exercise enhanced IGF1/IGF1R, PI3K/AKT and P38 cardiac survival pathways, which were similar to estrogen and estrogen substitutes cardiac protective effects. Although estrogen and estrogen substitutes may increase breast cancer risk, exercise or exercise combined therapy should be considered as better ways for post-menopausal or oophorectomied women.

Our current findings indicate that exercise training may be an important lifestyle modification to prevent ovariectomy-induced cardiac apoptotic pathways after menopause. Exercise training might provide one possible mechanism to interrupt cardiac apoptosis and the development of heart failure in post-menopausal women. Besides, it will raise the further question, whether some physiological responses of exercise, such as decreasing blood pressure, improving sympathetic tone, mediating cardiac calcium concentration, reducing oxidative stress and increasing VO2 max might be beneficial to induce cardiac survival pathways when considering possible factors to control or prevent the development of apoptosis related cardiac diseases in post-menopausal women. Of course, further therapeutic or clinical studies in human are required to clarify the effects of treatments or possible molecular mechanisms in post-menopause-related heart abnormalities.

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Table 1. Cardiac characteristics of Sham op, OVX group and OVX with exercise training

	Sham op	OVX	OVX-EX
	n=8	n=8	n=8
WHW(g)	0.84±0.09	1.00±0.07**	0.93±0.06*#
LVW(g)	0.60±0.07	0.72±0.05**	0.69±0.05**
WHW/BW (g/kg)	2.88±0.24	2.61±0.17**	2.65±0.20*
LVW/BW(g/kg)	2.11±1.10	1.89±0.17*	1.97±0.14
LVW/WHW(g/g)	0.74±0.05	0.71±0.04	0.74±0.04
WHW/TL(g/m)	22.96±2.91	26.39±1.54**	25.58±1.73*
LVW/TL(g/m)	16.90±1.78	18.89±1.57*	19.03±1.42*

Heart weight index among the sham operating rat (Sham), ovariectomized rat (OVX) and ovariectomized rat with exercise training(OVX-EX). WHW: whole heart weight; LVW: left ventricular weight; BW: body weight; TL: tibia length. Values are mean ±SD.; * and ** means $P < 0.05$ and $p < 0.01$ compared with Sham; # means $P < 0.05$ compared with OVX.

Fig 1.

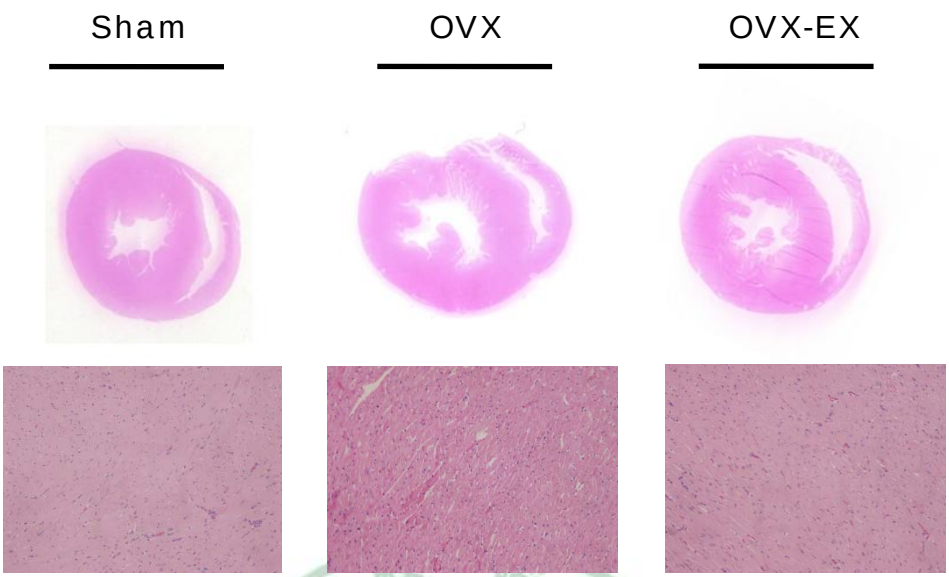


Fig 1. Hematoxylin and eosin staining was analyzed in cardiac sections from left ventricles in sham operating rat (Sham), ovariectomized rat (OVX) and ovariectomized rat with exercise training(OVX-EX). The representative images of cardiac architecture were magnified by 400 times. (n=3 in each group).

Fig 2.

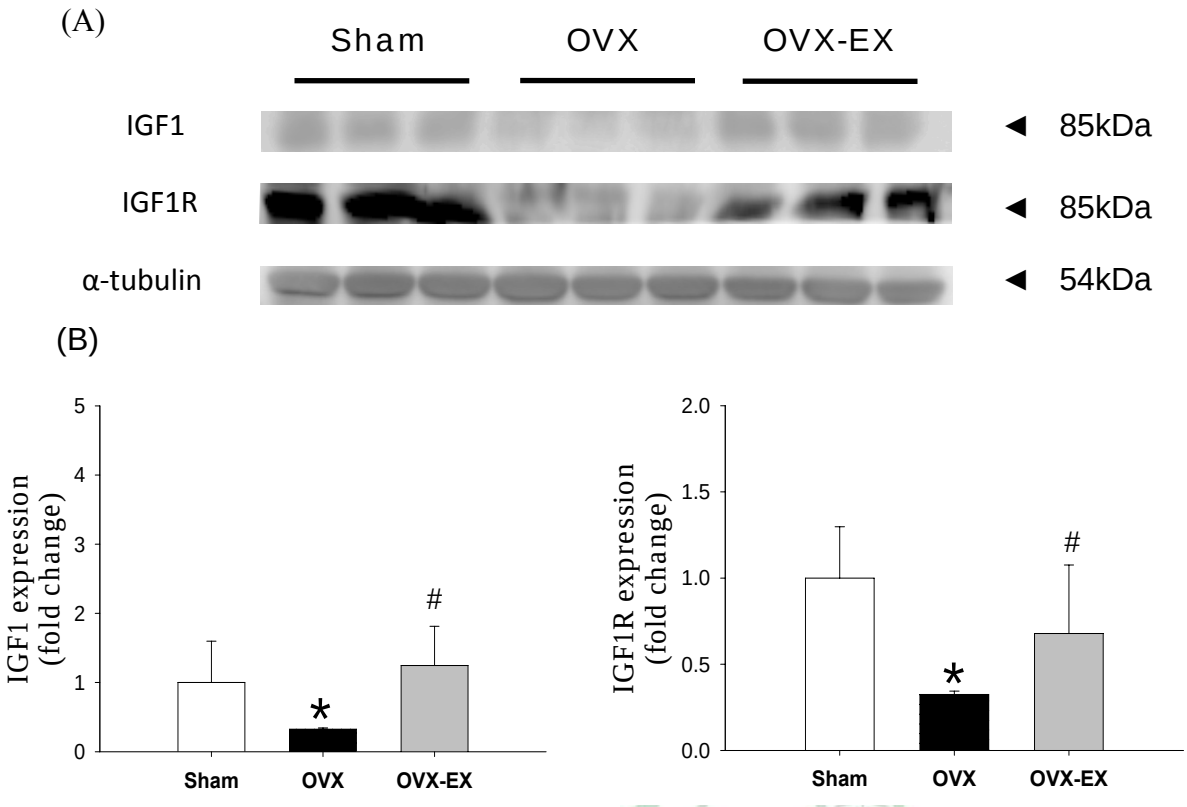


Fig 2. (A) The representative protein products of insulin-like growth factor one (IGF1) and IGF receptor (IGF1R) extracted from the left ventricles of excised hearts in 3 sham operating rats(Sham), 3 ovariectomized rats (OVX) and 3 ovariectomized rats with exercise (OVX-EX) were measured by Western Blotting analysis. α-tubulin was use as a internal control. (B) Bars represent the relative fold changes of IGF1/α-tubulin and IGF1R/α-tubulin relative to Sham group and indicate mean values±SD (n=6 in each group). * *means* $P<0.05$ *compare with Sham.* # means $P<0.05$ significant differences between OVX group and OVX-EX group.

Fig 3.

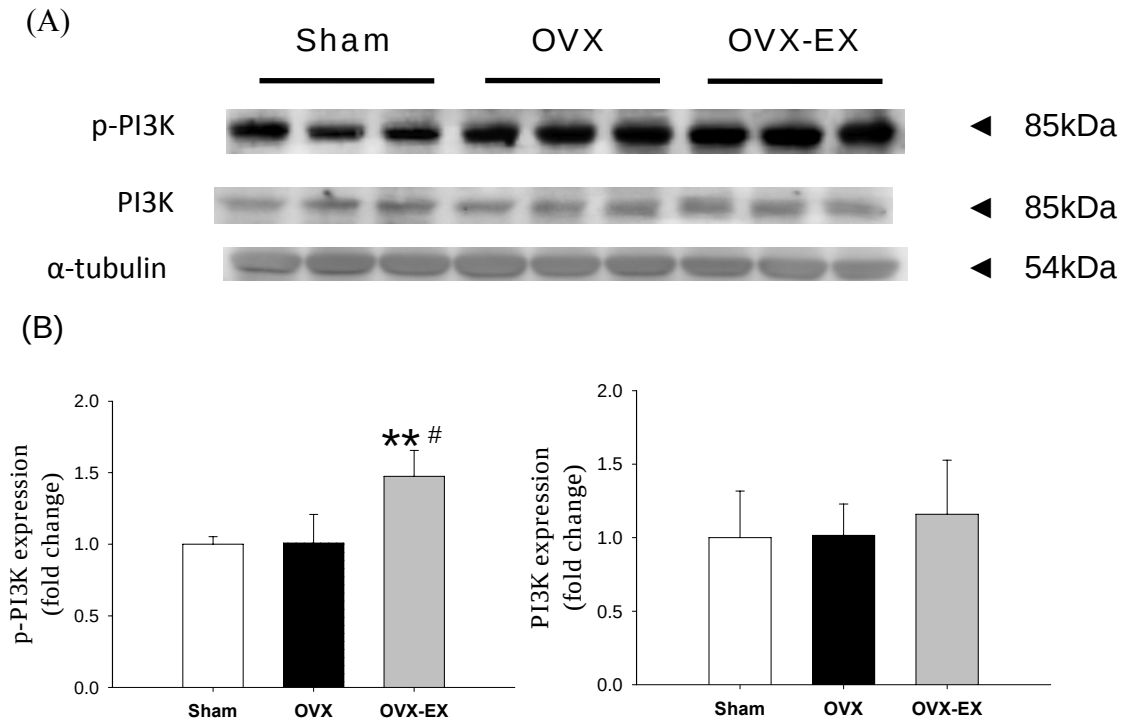


Fig 3. (A) The representative protein products of phophorylated Phosphatidylinositol 3-kinasesand(p-PI3K) and Phosphatidylinositol 3-kinasesand(PI3K) extracted from the left ventricles of excised hearts in 3 sham operating rats(Sham), 3 ovariectomized rats (OVX) and 3 ovariectomized rats with exercise (OVX-EX) were measured by Western Blotting analysis. α-tubulin was use as a internal control. (B) Bars represent the relative fold changes of p-PI3K/α-tubulin and PI3K/α-tubulin relative to Sham group and indicate mean values±SD (n=6 in each group). ** *means P <0.01 compare with Sham.* # means P<0.05 significant differences between OVX group and OVX-EX group.

Fig 4.

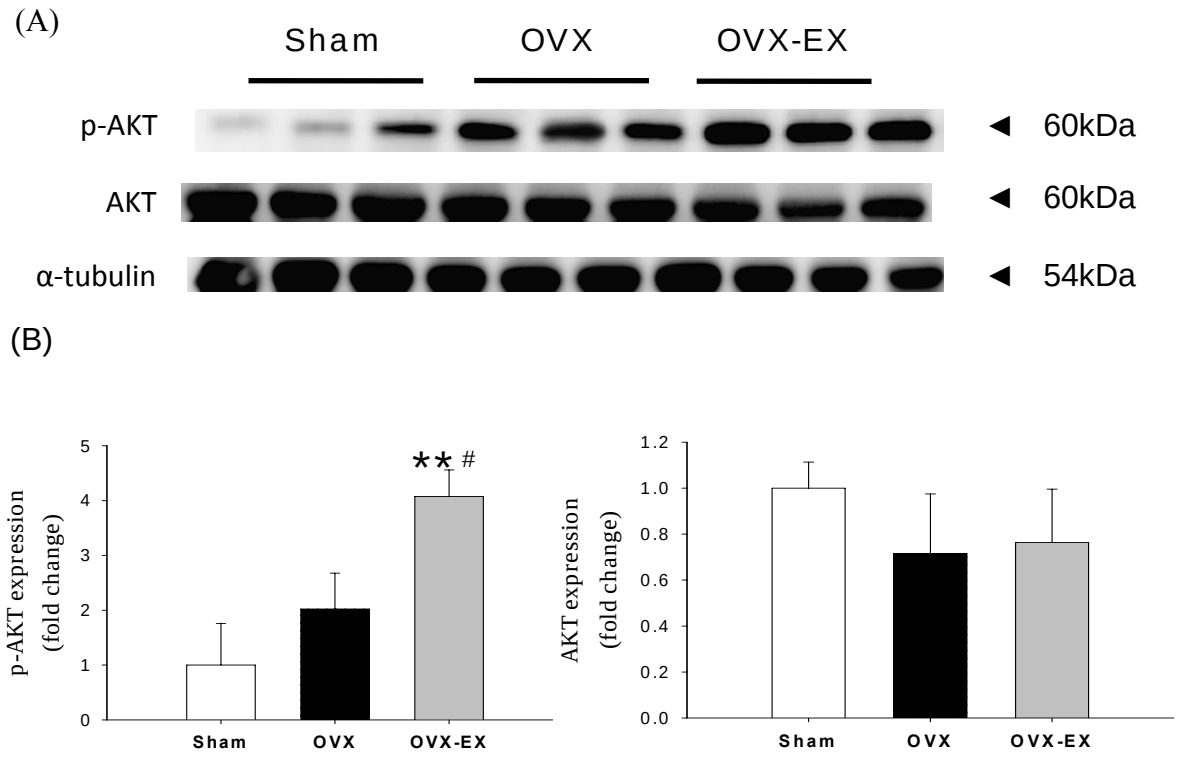


Fig 4. (A) The representative protein products of phophorylated AKT(p-AKT) and AKT extracted from the left ventricles of excised hearts in 3 sham operating rats(Sham), 3 ovariectomized rats (OVX) and 3 ovariectomized rats with exercise (OVX-EX) were measured by Western Blotting analysis. α-tubulin was use as a internal control. (B) Bars represent the relative fold changes of p-AKT/α-tubulin and AKT/α-tubulin relative to Sham group and indicate mean values±SD (n=6 in each group). ** *means* $P < 0.01$ *compare with Sham.* # means P<0.05 significant differences between OVX group and OVX-EX group.

Fig 5.

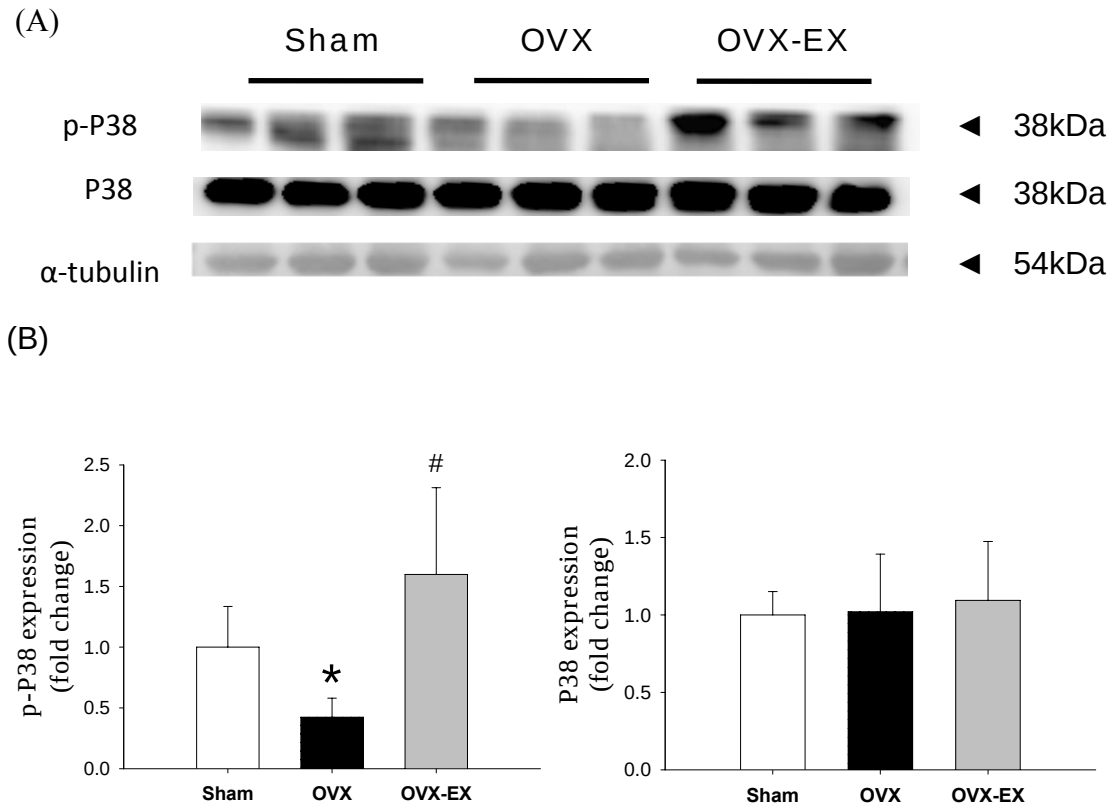
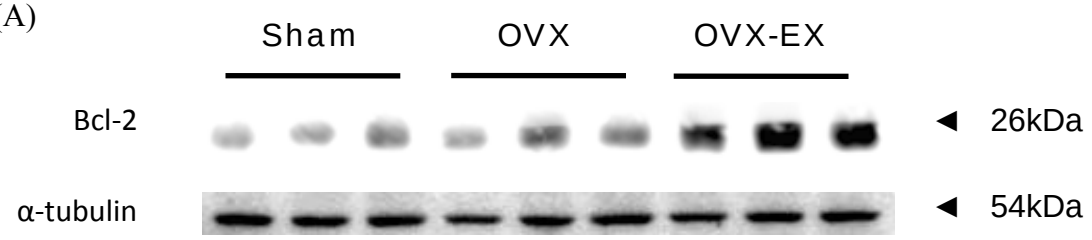


Fig 5. (A) The representative protein products of phophorylated P38(p-P38) and P38 extracted from the left ventricles of excised hearts in 3 sham operating rats(Sham), 3 ovariectomized rats (OVX) and 3 ovariectomized rats with exercise (OVX-EX) were measured by Western Blotting analysis. α-tubulin was use as a internal control. (B) Bars represent the relative fold changes of p=P38/α-tubulin and P38/α-tubulin relative to Sham group and indicate mean values±SD (n=6 in each group). * *means P <0.05 compare with Sham.* # means P<0.05 significant differences between OVX group and OVX-EX group.

Fig 6.

(A)



(B)

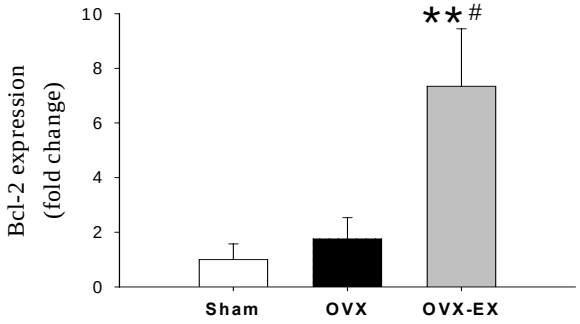
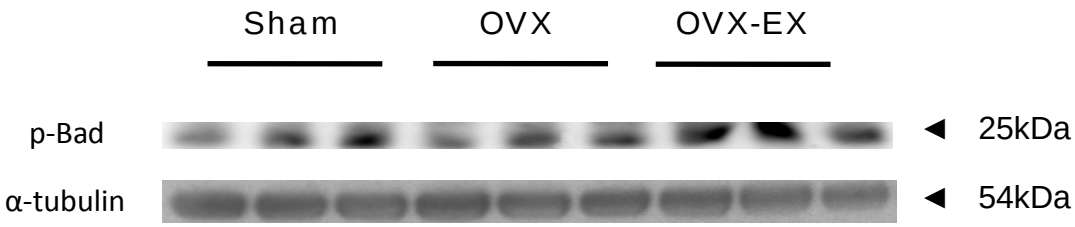


Fig 6. (A) The representative protein products of Bcl-2 extracted from the left ventricles of excised hearts in 3 sham operating rats(Sham), 3 ovariectomized rats (OVX) and 3 ovariectomized rats with exercise (OVX-EX) were measured by Western Blotting analysis. α-tubulin was use as a internal control. (B) Bars represent the relative fold changes of Bcl-2/α-tubulin relative to Sham group and indicate mean values±SD (n=6 in each group). ** *means P <0.01 compare with Sham.* # means P<0.05 significant differences between OVX group and OVX-EX group.

Fig 7.

(A)



(B)

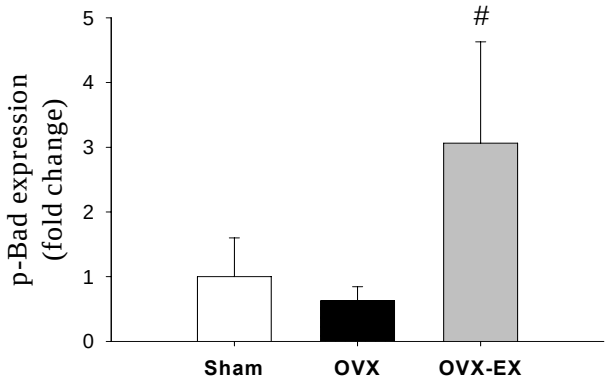
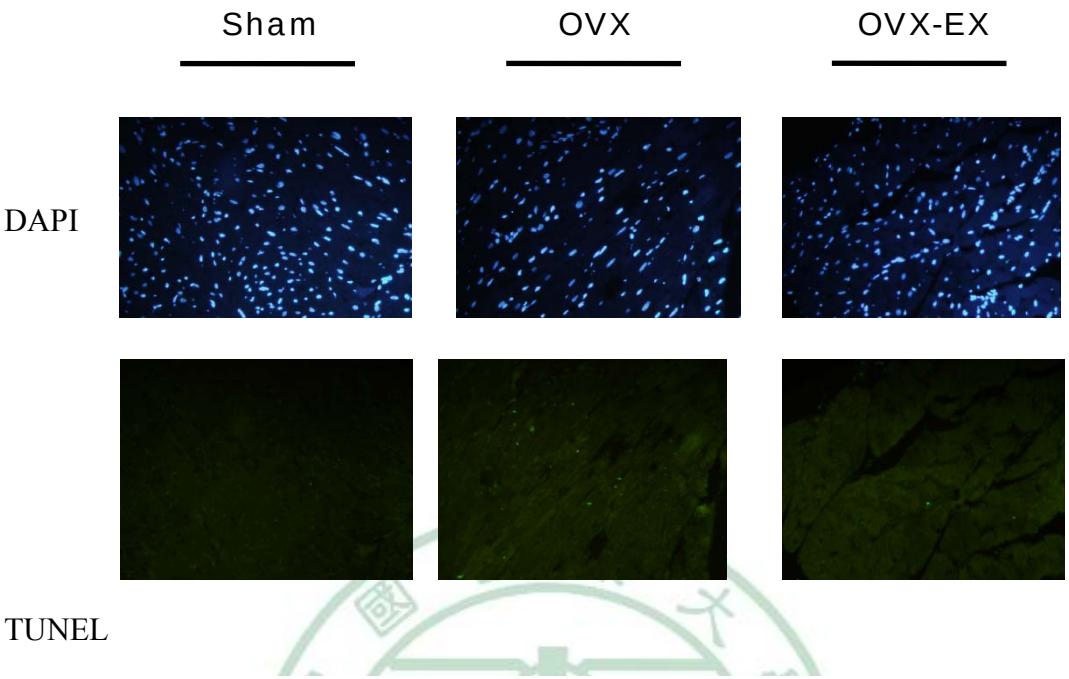


Fig 7. (A) The representative protein products of p-Bad extracted from the left ventricles of excised hearts in 3 sham operating rat (Sham), 3 ovariectomized rats (OVX) and 3 ovariectomized rats after exercise (OVX-EX) were measured by Western Blotting analysis. α-tubulin was use as a internal control. (B) Bars represent the relative fold changes of protein quantification relative to Sham group in p-Bad/α-tubulin and mean values±SD (n=6 in each group). # means $P<0.05$ significant differences between OVX group and OVX-EX group.

Fig 8.



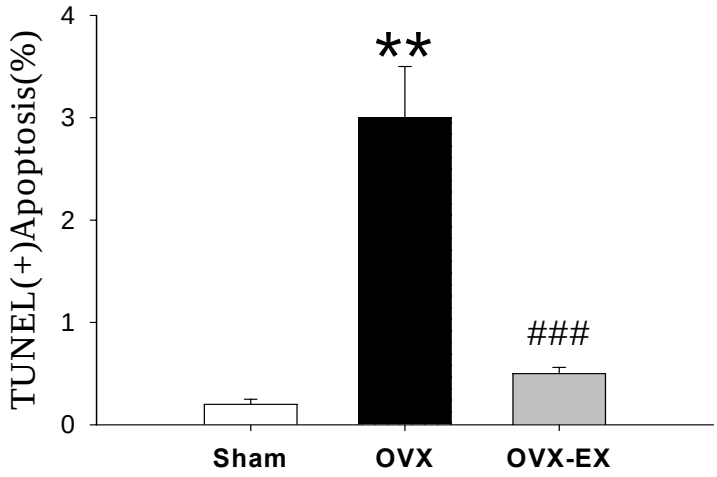


Fig 8. (A) Representative stained apoptotic cells of cardiac sections from left ventricle in sham operating rat (Sham), ovariectomized rats (OVX) and ovariectomized rats after exercise (OVX-EX) by staining with DAPI staining(upper panels) and TUNEL assay with dark background (lower panels, green spots). The relative background tissues were shown in left panels. The images were magnified by 400 times. (B) Bars present the percentage of TUNEL positive cells relative to total cells. ** means $P<0.01$, significant differences from Sham group. ## means $P<0.01$ significant differences between OVX group and OVX-EX group.

Fig 9.

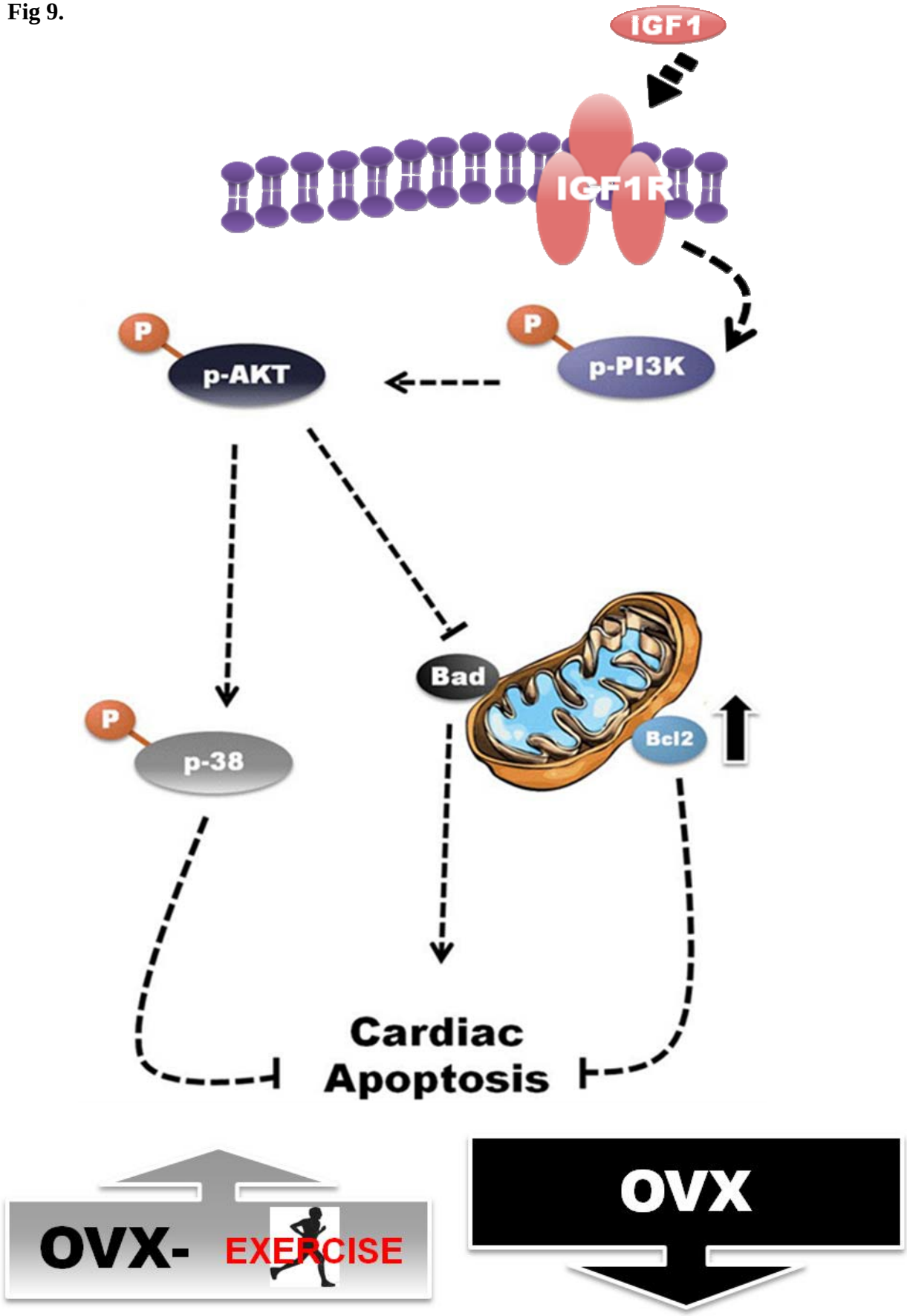

IGF1
IGF1R
P
p-AKT
P
p-PI3K
P
p-38
Bad
Bcl2
Cardiac
Apoptosis
OVX-
EXERCISE
OVX

Fig 9. The cardiac survival pathways(p-PI3K, p-AKt, p-P38, p-P38, p-Bad and Bcl2 were increased after exercise training compared with the OVX group. Our findings imply that exercise training could be one of possible therapeutic approaches to enhance cardiac survival pathway and prevent cardiac apoptosis in postmenopausal or bilateral ovariectomized women.