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Expression of the Mir-133 and Bcl-2 could be affected by swimming training in the heart of ovariectomized rats

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ABSTRACT

Objective(s): The beneficial and more potent role of exercise to prevent heart apoptosis in ovariectomized rats has been known. The aim of this study was to examine the effects of swimming training on cardiac expression of Bcl-2, and Mir-133 levels and glycogen changes in the myocyte.

Materials and Methods: Forty animals were separated into four groups as control, sham, ovariectomy (OVX) and ovariectomized group with 8 weeks swimming training (OVX.E). Training effects were evaluated by measuring lipid profiles, Bcl-2 and Mir-133 expression levels in the cardiac tissue. Grafts were analyzed by reverse transcription–polymerase chain reaction for Bcl-2 mRNA and Mir-133 and by Western blot for Bcl-2 protein.

Results: Ovariectomy down-regulated Bcl-2 and Mir-133 expression levels in the cardiac tissue, and swimming training up-regulated their expression significantly ($P<0.05$).

Conclusion: Our results showed that regular exercise as a physical replacement therapy could prevent and improve the effects of estrogen deficiency in the cardia.

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Introduction

One of the main serious concerns with the high rate of morbidity and mortality is cardiovascular complications after menopause (1). Premenopausal women are protected from cardiovascular disease in comparison with men of the same age (2). The investigations have shown that the estrogen is responsible for many benefits on the cardiovascular system (3). The occurrence of cardiovascular problems is related to the increase in the OVX induced Bad, Bax, cytochrome C overexpression, and caspase-9 and caspase-3 activation (4). Many studies have explained the ovariectomy-induced cardiac apoptosis through constrain mitochondrial apoptotic pathways in rat models (5). The results of clinical trials have shown that hormone replacement therapy (HRT) such as estrogen therapy raised serious concerns about the use of this treatment in menopausal and postmenopausal women (6) including the increased risks of breast cancer (7), vascular disease and hypertension (8). Some studies have been performed to examine other procedures that have estrogenic effects on the cardiovascular system (9). Therefore, exercise training, as a non-pharmacological way, is a well-known form of preventing or decreasing cardiovascular conflicts without having any side effects (10). Exercise conditioning can reverse or delay the beginning of cardiac failure (11). Moreover, regular exercise has positive impact on preventing the increase of whole heart weight and OVX-induced cardiac cell apoptosis (4).

Numerous studies in the last decade have shown that microRNAs (Mirs), as well as small and non-coding RNAs (18–23 nt) are involved in post-transcriptional silencing of mRNAs by translational repression or cleavage (12), in physiological and pathological form of cardiovascular system (13). Furthermore, the direct association of down or up-regulated Mir-133 along with increased or decreased expression of the target gene expression was also observed in physiological models. Particularly, it has

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been found out that Mir-133 is associated with the cardiac disturbances and apoptosis (14, 15). The mitochondrial pathway of cell death is regulated by upstream of Bcl-2 proteins, and expression of Bcl-2 is regulated by miRNAs (16). It has been shown that Mir-133a had suppressed the expression of apoptotic proteins caspase-8, caspase-9, and caspase-3, and had improved the expression of Bcl-2 (14). In line with the above-cited roles and based on the earlier study detailing the beneficial and more potent role of exercise to prevent heart apoptosis in ovariectomized rats, the present study was aimed to evaluate the effects of regular exercise on the Bcl-2 and Mir-133 expression levels in the heart as replacement for hormone therapy in ovariectomized rats.

Materials and Methods

Animal care

Forty female Wistar rats (weighing 180-220 g) were obtained from the Experimental Animal Research Center, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran. All the rats were kept under controlled conditions (temperature 22-24°C with 12:12 hr light and dark cycle) and received standard chow diet and water *ad libitum* for a week. The study was approved by the Ethics Committee of Tabriz University of Medical Sciences. After one week, rats were divided randomly into 4 groups (n=10) as follows; 1. Control, 2. Sham (rats underwent only surgery without ovariectomy), 3. OVX: (rats underwent bilateral ovariectomy), 4. OVX.E (rats underwent OVX + exercise). Two weeks before beginning the experiment, all the rats except those in sham and control groups underwent a bilateral ovariectomy (17). Ovaries were excised and oviducts were replaced with minimum disruption to surrounding soft tissues.

Exercise training protocol

After two weeks of recovery and repairing of scars, animals were familiarized with swimming pool (5-20 min/day) for 5 consecutive days. After the habituation, the exercised rats performed swimming exercise in 6 consecutive days (60 min/day) for 8 weeks. Control rats did not perform swimming. Exercised rats were studied 24 hr after their last exercise session. This protocol has been used previously and is effective for promoting cardiovascular adaptation (18).

Biochemical measurements

Lipid profile was assessed by using a commercial diagnostic kit (Randox (UK)) in accordance with the manufacturer's instructions.

Histological evaluation

The cardiac tissues were fixed in 10% buffered-formalin solution, dehydrated in ascending grades of ethanol, cleared in xylol and embedded in paraffin. Sections of 5 µm were taken, stained with periodic acid Schiff (PAS), and examined under light microscope (Olympus BH-2, Tokyo, Japan) in a blinded manner by a pathologist. Cardiac tissue was examined for changes of glycogen in the sarcoplasm of cardiomyocytes.

Molecular analysis

RNA isolation and the cDNA synthesis

Rats were deadened and their hearts were separated at the end of 8 weeks. Total RNA including mRNA and MicroRNA were extracted from the left ventricle of hearts using RNX-Plus solution kit (Fermentase, Cinagen Co Iran) and mir-amp kit (parsgenome Co Iran) respectively in accordance with the manufacturer's instructions (using chloroform layer separation followed by treatment with isopropanol and ethanol). RNA quantity and A260/280 ratio were measured using the Nano Drop 1000 (Thermo Scientific, Waltham, and Mass), and gel electrophoresis with GelRed (Biotium, Hayward, California) were used to evaluate the integrity of the samples. The Bcl-2 and Mir-133 genes expression were quantitatively assessed by real-time polymerase chain reaction. Primers' sequences for each gene were demonstrated in Table 1. The amount of PCR products was normalized to that for the housekeeping gene -3-phosphate dehydrogenase (GAPDH) mRNA samples and Mir-191 for Mir samples (internal control).

For synthesis of cDNA in mRNA sample, 1 µl of total RNA was reverse transcribed by Revert Aid M-MuLV reverse transcriptase (1 µl), DNase I (1 µl) and random hexamer primers (1 µl), dNTPS (2 µl), and RiboLock RNase-inhibitor (0.25 µl), for 10 min at 25 °C, followed by 60 min at 42°C in a final volume of 20 µl. The reaction was terminated by heating at 70 °C for 5 min. In addition, synthesis of cDNA from mir-RNA sample was performed according to mir-amp kit (parsgenome Co. Iran).

Real-time quantitative PCR

A master mix of 25 µl containing 12.5 µl SYBR Green PCR Master Mix (Jena Bioscience, Germany), 1 µl forward primer, 1 µl reverse primer, and 8.5 µl water was prepared to carry out real-time PCR. Two microliters of reverse transcribed cDNA were then added to the PCR master mix to achieve a final volume of 25 µl. Furthermore, to check the accuracy of amplifications, we included a negative control in each run by eliminating the cDNA sample in the tube.

The PCR protocol was used on the real-time PCR machine (Rotor-Gene 3000) in three steps including: 1- initial denaturation (10 min at 95 °C); 2- a three-step amplification program (15 sec at 95 °C followed by 30 sec at 60 °C for Mir-133 and 30 sec at 58 °C for Bcl-2 gene; and 30 sec at 72 °C) repeated 40 times; and step 3-melting curve analysis (1 cycle: 72 to

Table 1. The primers sequences for each of genes in different studied groups

Genes	Accession number	Primers Sequence [a]
Bcl-2	NM_016993.1	F: F: CGGGAGAACAGGGTATGA R: CAGGCTGGAAGGAGAAGAT
GAPDH	NM_017008.4	F: TGCCGCCTGGAGAAACCTGC R: TGAGAGCAATGCCAGCCCCA
		Target sequence[b]
miR-133	MIMAT0017124	AGCUGGUAAAAUGGAACCAAAU
miR-191a	MIMAT0000866	CAACGGAAUCCCAAAAGCAGCUG

a Sequences were derived from NCBI (www.ncbi.nlm.nih.gov)
b Sequences were derived from miRBase (www.mirbase.org)

95 °C with temperature transition rate 1 °C/sec for 5 sec). All runs were performed in duplicates. Real-time quantification was monitored by measuring the increase in fluorescence caused by binding of the SYBR Green dye to double-stranded DNA at the end of each amplification cycle. The relative amount of mRNA for each target gene was calculated based on its threshold cycle (Ct) compared to the Ct of the housekeeping (reference) gene (GAPDH). The relative quantification was performed by $2^{-\Delta\Delta Ct}$ method as follows. The specificity of PCR reactions was verified by generation of a melting curve analysis followed by gel electrophoresis, stained with GelRed (Biotium, Hayward, California).

Western blot analysis

The level of Bcl-2 was measured by Western blotting. In brief, for western blotting, the proteins in SDS-gels were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore). The transfer was carried out at about 90 mA for 2 hr. Thereafter, the membranes were blocked in 3% skim milk buffer containing 0.1% Tween- 20 for 2 hr and then incubated with primary antibodies against the Bcl-2 and β-actin (all antibodies from santa cruse, USA) overnight at 4 °C on a shaker. After 4 x 5 min washes with Tris buffer saline containing 0.1% Tween-20, membranes were incubated with horseradish peroxidase conjugated (HRP) secondary antibody (santa cruse, USA) for 1 hr at room temperature on a shaker. The membranes were then rinsed at least 3 x 5 min with washing buffer before detection. Blots were then developed using the enhanced chemiluminescence (ECL) method. Following incubation with the ECL reagents, the membranes were exposed to an X-ray hyperfilm inside a hypercassette in darkroom and then the chemiluminescence of antibody binding was visualized by a visualizing machine. The intensity of protein bands in the blots was digitally quantified using densitometric analysis. To analyze the amount of Bcl-2, the expression of β-actin was calculated and expressed in arbitrary unit (AU).

Data analysis and statistics

Data analysis was performed with one-way ANOVA. The *post hoc* Tukey test was applied to make a comparison between groups. All values were expressed as means±SEM. *P*-values of less than 0.05 were considered as statistically significant.

Results

Because of no statistically significant difference among control and sham animal groups, we only discussed about sham group.

Body and heart weight

As shown in Table 2, the OVX and OVX.E rats had a significantly higher body weight after 8 weeks experiment than the Sham rats ($P<0.05$). In addition, heart weight (HW) in the OVX rats were higher (no significantly) than those in the sham rats, and statistically significant differences were observed between the OVX rats that had exercised compared to the OVX and sham group ($P<0.05$). Table 2 also shows that the HW to BW ratio was significantly increased in the rats in OVX and OVX.E groups compared to Sham rats, also HW/BW ratio in exercise treated group was significantly higher than that in the OVX groups ($P<0.05$).

Table 2. Effects of ovariectomy and swimming training on body and heart weight in different studied groups

	SHAM	OVX	OVX.E
BW final (g)	274.4±3.76	320.7±5.33*	313.5±4.41**
HW (mg)	857.7±15.5	872±17.1	1088±18.2#
HW/ BW (mg/g)	3.12 ±0.04	2.72±0.06*	3.47±0.08**

BW: body weight; HW: heart weight and HW/BW: heart and body weight ratio; OVX: ovariectomized group, OVX.E: ovariectomized with 8-weeks swimming group. Data are expressed as mean±SEM
*$P<0,05$ vs Sham & OVX.E; **$P<0,05$ vs Sham; #$P<0,05$ vs Sham &OVX

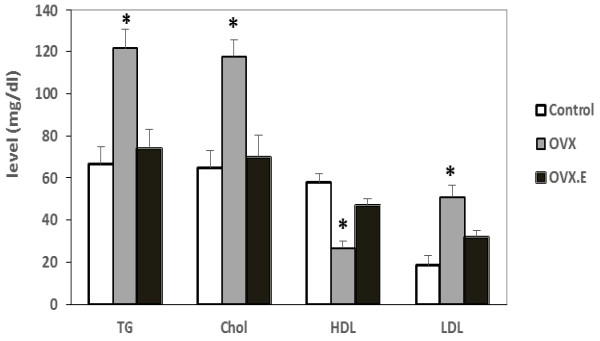

Figure 1. Plasma lipid profiles in different studied groups
OVX: ovariectomized group, OVX.E: ovariectomized with 8-weeks swimming group
* Significant difference compared with Sham & OVX.E groups (P<0.05). Data are expressed as mean±SEM

Biochemical analysis

The plasma lipid profiles are shown in Figure 1. Cholesterol, triglyceride and low-density lipoprotein (LDL) levels were markedly higher in the OVX groups than the OVX.E and Sham groups (P<0.05). However, high-density lipoproteins (HDL) in the OVX.E and Sham groups was significantly higher from that in the OVX group (P<0.05).

Histological results

In the control and sham animal groups, a constant and homogeneous granule of glycogen in the sarcoplasm of cardiomyocytes was markedly evident (Figure 2a). However, fragmentation and irregular accumulation of glycogen granules (black marker) were observed in the OVX compared with the sham animal group (Figure 2b). Swimming training decreased fragmentation of glycogen granules in the OVX.E compared with OVX animal group in the sarcoplasm of cardiomyocytes (Figure 2c).

Effects of the Mir-133 expression on the heart

The expression level of heart Mir-133 is presented in Figure 3. Mir-133 expression level was significantly low in the heart of the OVX rats, in comparison to that of the sham group (P<0.05). Eight-weeks swimming treatment showed a high expression of Mir-133 in the heart of OVX.E animals group as compared to that of the OVX group (P<0.05). However, significant difference was found in Mir-133 expression in the heart of rats with 8-weeks swimming treatment compared to sham groups.

Effects of exercise on the expression of heart Bcl-2

The expression level of heart Bcl-2 is presented in Figure 4. Bcl-2 expression level was found to be significantly lower in the heart of the OVX rats than in the sham group (P<0.05). Treatment with 8-weeks exercise showed a high level of expression of Bcl-2 in the heart of the exercise ovariectomized group, as compared to that of the OVX group(P<0.05).

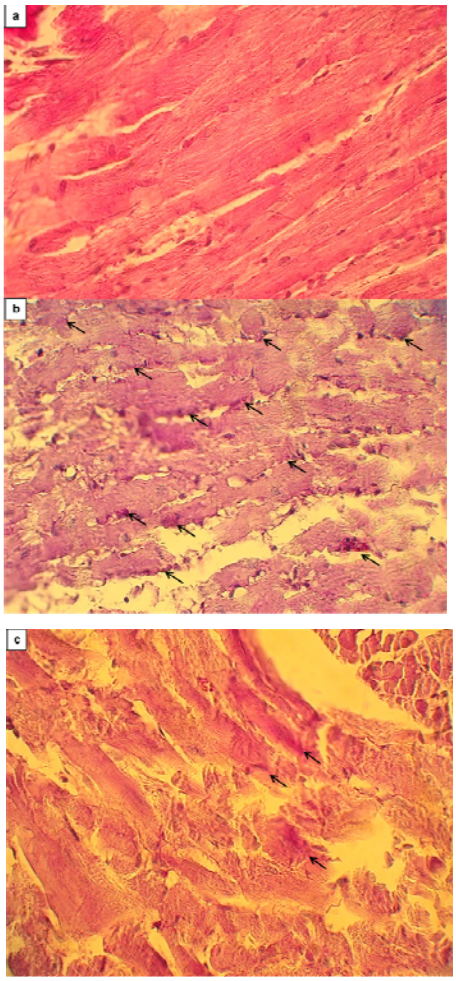

Figure 2. Histological evaluation of myocardium that stained by periodic acid Schiff (PAS) and examined by a light microscope. Transverse sections of hearts showed different storage of glycogen in the hearts of sham (A), OVX (B) and OVX.E (C) animal groups. OVX: ovariectomized group, OVX.E: ovariectomized with 8-weeks swimming group

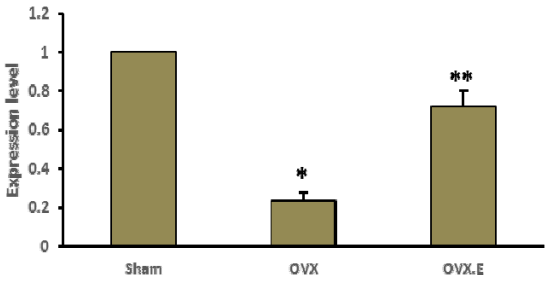

Figure 3. Level of Mir-133 expression in heart of the OVX rats after treatment with swimming training
OVX: ovariectomized group, OVX.E: ovariectomized with 8-weeks swimming group
* Significant difference compare with Sham group (P<0.05)
** Significant difference compare with Sham & OVX groups (P<0.05). Data are expressed as mean ± SEM

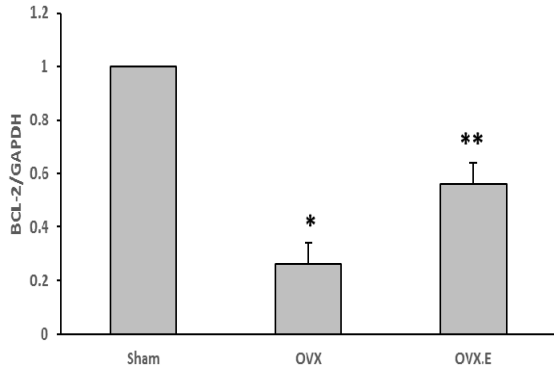

Figure 4. Level of Bcl-2 expression in the heart of OVX rats after swimming training

OVX: ovariectomized group, OVX.E: ovariectomized with 8-weeks swimming group
* Significant difference compare with Sham group (P<0.05)
** Significant difference compare with Sham & OVX groups (P<0.05).Data are expressed as mean±SEM

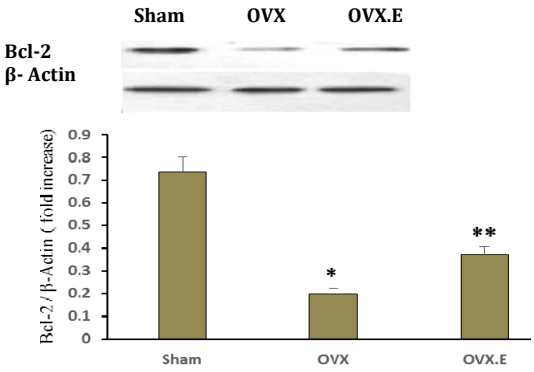

Figure 5. Western blot analysis of Bcl-2 in myocytes of heart

OVX: ovariectomized group, OVX.E: ovariectomized with 8-weeks swimming group
* Significant difference compare with Sham group (P<0.05)
** Significant difference compare with Sham & OVX groups (P<0.05). Data are expressed as mean±SEM

Nevertheless, there was significant difference in Bcl-2 expression level in the heart of the rats with 8-weeks exercise in comparison to that of the sham group (P<0.05).

Levels of Bcl-2 protein in heart tissue

The level of Bcl-2 protein determined by Western blot is presented in Figure 5. Bcl-2 levels significantly decreased in myocytes of heart of OVX compared to sham animal group (P<0.05). On the other hands, Bcl-2 levels were significantly increased in the OVX.E in compration to OVX animal group (P<0.05).

Discussion

In postmenopausal women, the increased predominance of abnormalities, which are notable to be related to estrogen deficit provoke cardiac failure, has been extensively known (14). In this study, we aimed to investigate Mir-133 and Bcl-2 cardiac gene expression patterns at early stages of ovarian hormone loss in a postmenopausal animal model. We also investigated the exercise as a possible modality for reducing ovariectomy-induced changes in these cardiac expressions. We hypothesized that ovariectomy would cause a pathological cardiac gene expression pattern and apoptosis, and that swimming training would leaded to upregulation of antiapoptotic biomarkers. Our major findings were as follows: 1) in comparison with the sham surgery rats, down-regulated cardiac expression of the Mir-133 gene in ovariectomized rats was associated with anti-apoptosis, which may be related to increased Bcl-2 expression, 2) exercise increased the expression of anti-apoptotic-associated genes such as Mir-133 and Bcl-2, 3) exercise decreased fragmentation of glycogen granules in the OVX.E compared with OVX animal group in the sarcoplasm of cardiomyocytes.

Disturbance in the regulation of Mir-133 expression has been reported in some situations including during menopause (19). Moreover, the regulation of microRNAs by estrogen and other sex hormones has also been proven (20). In the cardiovascular field, MiRNAs were considered to play important roles in the process of myocardial function (21). The direct relation of Mir-133 regulation along with the increased or decreased expression of the target gene expression has been observed in physiological and pathological models (22, 23). Mir-133, given to its therapeutic application in the heart, played as a key regulator of cardiac hypertrophy (24). It has been argued that Mir-133 exerted cardioprotective effects on the disturbances and apoptosis (25). Overexpression of Mir-133a in cardiac cells caused the down-regulation of caspase-9 and caspase-3 in the presence of H2O2 (26). Overexpression of Mir-133a also reduced reactive oxygen species (ROS) and malondialdehyde content, and increased superoxide dismutase (SOD) activity and Glutathione peroxidase (GPx) levels, protecting cardiomyocytes from apoptosis (27). Mir-133a suppressed the expression of apoptotic proteins and improved the expression of Bcl-2 (14). Bcl-2, an anti-apoptotic protein, regulated apoptotic signaling by preventing cytochrome C release and inhibiting downstream activation of caspase (28). It also played a central role in the delivery of apoptotic signals (29). In the present study, Mir-133 and Bcl-2were markedly down-regulated in cardiomyocytes from the heart of OVX rats in comparison to the sham group. Although the cardioprotective role of Mir-133 in the menopause is not well known, we speculate that the down-regulation of Mir-133 and Bcl-2 may decrease proliferation and induce apoptosis of cardiomyocytes in the OVX rats.

However, in the present study we investigated the regulatory effects of exercise on the expression of Mir-133 and Bcl-2 in the myocyte of OVX rats. Despite many studies about benefits of the different protocols of exercise on the heart, the effect of exercise on expression of Mir-133 and Bcl-2 in the heart of ovariectomized rats has not been studied. In the present study, Bcl-2 expression was significantly higher in the exercise group than in the OVX group. Recent studies have shown that the expression of muscle-specific MiRNAs (myomirs), such as Mir-1, Mir-133a/b, and Mir-206 is modulated by the essential amino acid ingestion and the endurance exercise training (30). More important , Mir-1 and Mir-133 expression was consistently down-regulated both in pathological and physiological hypertrophy as demonstrated in mice subjected to transverse aortic constriction (TAC), protein kinase B (PKB as known AKt) overexpressing transgenic mice or exercise-trained wild-type mice, respectively (31). Exercise training enhances cardiac insulin-like growth factor 1 receptor/ phosphoinositide 3-kinase/ protein kinase B (PKB as known AKt) (IGFI-R/PI3K/Akt) and Bcl-2 family, which are associated with pro-survival pathways. This provides one of the new beneficial effects for exercise training in diabetes (32). Fas-dependent and mitochondria-dependent apoptosis in hearts are prevented by exercise (33). In the vein of above-cited roles and based on the earlier study detailing the beneficial and more potent role of exercise to prevent heart apoptosis in ovariectomized rats, the present study was planned to evaluate the effects of regular exercise on the Bcl-2 and Mir-133 expression levels in the heart as replacement therapy in ovariectomized rats. In our study, the expression levels of Mir-133 and Bcl-2 in OVX group rats were significantly increased after swimming training in two months. Also, effect of exercise on improving lipid profiles and decreased glycogen granules in the cardiac myocardiocyte helped to prevent from cardiac failure. Although the direct effect of exercise on the expression of Mir-133 and Bcl-2 has not been investigated, our result also provides new clues that exercise may promote estrogen deficiency induced apoptosis via up-regulation of Mir-133 and Bcl-2 genes in the cardiac myocyte. Moreover, swimming improved Mir-133 and Bcl-2 expression that may prevent apoptosis via increasing anti-apoptotic proteins.

Conclusion

Our findings provided the evidence that Mir-133-mediated expression of Bcl-2 participates in estrogen deficiency induced damages of cardiac apoptosis, and highlights a new insight into molecular mechanism of post-menopause related arrhythmia at the microRNAs level. Also, these results suggested that the exercise has a protective effect on the heart against apoptosis in the ovariectomized rats. The results of this study support the potential therapeutic value of exercise in improving cardiac function after menopause.

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Conflict of interest

The authors report no conflict of interest.

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