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Limb Blood Flow Restriction Plus Mild Aerobic Exercise Training Protects the Heart Against Isoproterenol-Induced Cardiac Injury in Old Rats: Role of GSK-3β

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Abstract
The present study was conducted to evaluate the effect of blood flow restriction (BFR) training on cardiac resistance to isoproterenol (ISO) induced heart injury in old rats and examined the hypothesis that BFR training may interfere with age-associated impairment of mitochondria by the inhibitory phosphorylation of GSK-3β at Ser9. Old male Wistar rats were divided into the following six groups: CTL (control), ISO (isoproterenol-treated), Sh + ISO (sham-operated plus ISO), BFR + ISO (blood flow restriction plus ISO), Sh-Ex + ISO (sham-operated subjected to exercise and ISO), and BFR-Ex + ISO (blood flow restriction along with exercise and ISO). 10 weeks of exercise training was considered. Then, cardiac injury was induced and physiological, histological, and biochemical parameters were recorded and assessed. Compared to CTL group, isoproterenol administration significantly reduced the systolic arterial pressure (SAP), left-ventricular systolic pressure (LVSP), and ± dp/dt max ($P < 0.05$). BFR training improved these parameters in the way that BFR-Ex + ISO group had higher SAP, LVSP and ± dp/dt max ($P < 0.05$) and lower LVEDP (left-ventricular end diastolic pressure) ($P < 0.01$) than untrained and Sh-Ex + ISO groups. The pS9-GSK-3β and pS9-GSK-3β/GSK-3β ratio were increased in the BFR-Ex + ISO group compared to CTL, ISO, Sh + ISO, and BFR + ISO groups ($P < 0.05$). The level of plasma cardiac Troponin-I and the severity of the injuries were significantly reduced in BFR-Ex + ISO group versus other cardiac damaged groups. In conclusion, our findings clearly confirmed the cardio-protective effect of BFR training against ISO-induced myocardial injury. Increased phosphorylated GSK-3β and angiogenesis in this model of exercise justify the resistance of old hearts facing stressful situations.

Keywords Myocardial injury · Cardiac performance · Aging · BFR training · GSK-3β

Introduction

Oxidative stress [1], impaired angiogenesis [2], reduced cellular cardio-protective reserves [1], and mitochondrial dysfunction [3] are of major inevitable age-related complications in humans and animals hearts. These physiological changes, independent of other clinical variables, decrease the aged heart capacity in response and adaptation to mechanical and oxidative stresses and increase the myocardial susceptibility to ischemia so that recovery of the aged hearts subjected to ischemia has been proved to be worse

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than that of young hearts [4]. Mitochondrial permeability transition pore (mPTP) is a nonspecific high conductance channel in the inner mitochondrial membrane [3]. Through the aging process, increased oxidative stress [5] and high concentration of intracellular Ca^{2+} [3] create the favorable conditions for the opening of these pores and irreversible opening of mPTP has been suggested as an important factor in the mitochondrial dysfunction and degenerative processes associated with aging [4].

Glycogen synthase kinase-3β (GSK-3β) is a 47-kDa multi-functional kinase that is mainly expressed in the brain and heart and is regulated by inhibitory phosphorylation on Ser9 [6]. Inhibition of GSK-3β, which prevents the mitochondrial permeability transition pore (mPTP) opening, has been suggested as a cardio-protective mechanism induced by preconditioning [7], opioids [8], erythropoietin [9], bradykinin [10], and PKCδ inhibition [11]. It has been declared that direct inhibition of GSK-3β rise the mPTP-ROS threshold and decrease infarction size which has triggered out the focus on GSK-3β inhibitors as potential tools in obtaining cardioprotection in clinical settings [12].

As an important part of a healthy lifestyle, physical exercise, particularly endurance training, improves the life quality of aged people. Recently, mitochondria are considered as interesting candidates to explain the exercise-induced cardioprotection against several stressful events [13]. The PI3K/AKT/GSK-3β signaling pathway has been identified as a major regulator of cell growth, metabolism, survival, and angiogenesis [12], and induces cardioprotection during both pre- and post-conditioning [14, 15]. Exercise-mediated restoration of this pro-survival signaling pathways is involved in progression of left-ventricular hypertrophy [16], and is well known to target mitochondria by protecting against mPTP opening and ultimately from cell death.

The results of animal and human studies have demonstrated that combination of blood flow restriction along with low-intensity resistance exercise (BFR training) induce muscle hypertrophy, improve perfusion conditions, strength, and oxygen delivery of skeletal muscles in aging subjects [17]. BFR training increases the hypertrophy index and muscle-specific kinase (MuSK) in both slow and fast muscles and leads to a significant increase in PGC-1α in aged rats [18]. Using of moderate to high loads of exercise is often not feasible in old populations. Therefore, low-intensity training along with blood flow restriction as a rehabilitation tool for aging populations is becoming popular. However, little is known about the effect of this kind of exercise on aged hearts subjected to ischemia. In the present study, we evaluated the heart resistance to isoproterenol-induced cardiac injury in old rats which pre-treated with BFR plus mild aerobic exercise training. We also examined the hypothesis that the effect of this model of exercise may be mediated through phosphorylation of GSK-3β at Ser9.

Materials and Methods

Animals

Male Wistar 22–24 months rats obtained from Tehran University, Tehran, Iran. They were housed in a 22–24 °C environment on a 12-h light-dark cycle with free access to water and food. Animals were randomly divided into six groups of 14 members: normal group without any treatment and cardiac injury (CTL) and five ISO-treated groups subjected to isoproterenol-induced cardiac injury. The ISO-treated groups which received isoproterenol consist of: sham-operated treated with isoproterenol (Sh + ISO), blood flow restriction group subjected to isoproterenol (BFR + ISO), sham-operated plus exercise (Sh-Ex + ISO), and blood flow restriction along with exercise (BFR-Ex + ISO) received isoproterenol. These six groups are divided into two separate subgroups for histomorphological and molecular studies. As we reported in details previously [18, 19], blood flow restriction was created by the bilateral partial closing of the femoral arteries so that the hind limbs of animal have normal blood flow at rest condition and are encounter with blood flow restriction during exercise activity. In this method 0.014-inch diameter steel wire was embedded on the femoral artery and it was tightly knitted using a silk stitch thread (4-0); then the wire was pulled out and incision sites was sutured. Sham-operated rats underwent the same surgery to blood flow restricted rats without closing the artery.

Chemical Reagents

Chemical materials were prepared as sodium thiopental from Sandoz, Austria, Ketamine and Xylazine from Alfasan Company, Holland, isoproterenol from Sigma, USA. cTnI assay kit was purchased from monobind, USA. Monoclonal primary antibodies against GSK-3β and the secondary antibody were prepared from Santa Cruz Biotechnology, USA, Phospho-GSK-3β (Ser9) from Cell Signaling Technology, USA, and PVDF membrane and Enhanced chemiluminescence (ECL) detection kits from Roche, Germany.

Experimental Protocols

Animals were submitted to a low-intensity training protocol for 10 weeks. The training was started on treadmill in 15-min sessions daily at speeds of 7.5 m/min and 0% grade after a week of recovery of animals undergoing surgery. The speed and duration of exercise gradually increased so that at the onset of the last week, the animals ran at a speed of 15 m/min, 60 min per session while maintaining a 0% grade [18–20]. All animals in training groups underwent a

pre-adaptation to the training protocol and equipment for 5 days and control rats were placed on the treadmill at least 20 min in each session. 48 h after the last bout of exercise, experimental myocardial infarction was induced by injection of 85 mg/kg isoproterenol dissolved in normal saline and injected subcutaneously daily for two consecutive days [20]. After receiving the second dose of isoproterenol (3 h later), blood samples were collected from all animals, centrifuged and stored at − 20 °C until troponin-I was measured by related Kit.

Hemodynamic Measurement and Heart Monitoring

24 h after the second dose of isoproterenol, animals from all groups were weighed and then anesthetized with i.p. injection of sodium thiopental (50 mg/kg). The trachea was cannulated and animals were allowed to breath spontaneously throughout the experiment. Two polyethylene catheter (PE-50) filled with heparin saline (15 units/ml) were inserted into the right and left carotid artery and the former one was advanced into the left ventricle (LV) through the aortic valve [21] to evaluate the left-ventricular performance and arterial blood pressure recordings, respectively. After a 10-min period of stabilization left-ventricular end diastolic pressure (LVEDP), the maximal positive and negative rate of changes in left-ventricular pressure (dP/dt max and dP/dt min), as indices of cardiac contractility and cardiac relaxation velocity, respectively, left-ventricular systolic pressure (LVSP) and systolic and diastolic blood pressures (SAP, DAP) were recorded on an 8-channel Power lab Physiograph system (ADInstruments, Australia). The mean arterial pressure (MAP) was calculated by ''MAP = DAP + (SAP − DAP)/3 formula,'' where DAP is the diastolic arterial pressure and SAP is the systolic arterial pressure. Throughout the experiment, the body temperature was maintained at 37 °C by a thermostat connecting to a metal plate placed in the center of the surgical table, designed in the Department of Physiology, Kerman University of Medical Sciences.

Western Blotting

Western blot method was used to measure the cardiac amount of GSK-3β and Phosphorylated Ser9-GSK-3β. At the end of the experiment, after the hemodynamic measurement, the hearts of animals were removed rapidly; about 30–50 mg of the hearts apex of all groups was separated, homogenized in radioimmuno-precipitation assay buffer (RIPA buffer), and centrifuged at 14,000 rpm for 20 min at 4 °C. Total protein concentration was determined by Bradford method (Bio-Rod Laboratories, Muenchen, Germany). Equal amounts of proteins (60 µg total protein/well) were electrophoresed on a 12% SDS-polyacrylamide gels and transferred onto a PVDF membrane (Bio-Rad Laboratories, USA).The membranes were incubated in 25 ml of blocking buffer for 1 h at room temperature, Washed three times for 5 min and with 15 ml of TBS-T(tris-buffered saline, 0.1% Tween 20) then incubated overnight at 4 °C with monoclonal primary antibody [22] against GSK-3β (1:500 dilution, Santa Cruz Biotechnology, USA), Phospho-GSK-3β (Ser9) (1:500 dilution, Cell Signaling—Technology, USA) and GAPDH (1:1000, Santa Cruz Biotechnology, USA). After washing in TBS-T at room temperature (3 × /10 min and 3 × 5 min), the membranes were incubated with a polyclonal peroxidase-conjugated secondary antibody (1:1000 dilution) with gentle agitation for 1 h at room temperature. Finally, proteins bands were visualized by enhanced chemiluminescence (ECL) method according to the manufacturer's recommendation (Bio-Rad Laboratories, USA) [23].

Estimation of Cardiac Troponin-I

The serum level of cTnI was estimated by enzyme-linked immune sorbent assay (ELISA), using standard kit, monobind technology, USA.

Histological Study

At the end of experiments, animals were considered for histological assessment. They were sacrificed under deep sodium thiopental (50 mg/kg i.p.) anesthesia. The hearts were removed. The left ventricle (LV) was separated, washed with cold saline, fixed in 10% buffered formalin, and embedded in paraffin. The LV was divided into 8–12 isotropic uniform random (IUR) sections generated using orientator method [24]. Sections of 5 µm thickness were cut and half of them stained with hematoxylin and eosin (H&E). Sections were examined by pathologist blinded to animal groups and pathological indices include: interstitial edema, myofibrillar degeneration and necrosis, leukocyte infiltration, and inflammatory process were considered. The observer examined at least ten fields in each slide and graded them for severity of changes using scale scores (4) severe (necrosis with diffuse inflammatory process), (3) moderate (extensive myofibrillar degeneration and/or diffuse inflammatory process), (2) mild (small multifocal degeneration with slight degree of inflammatory process), (1) minimum (focal myocytes damage), and (0) no change [25]. The other half of slides stained with Heidenhain's AZAN trichrome and were used for quantitative stereological evaluations.

Estimation of the Volume of the Vessels

The volume densities of the vessels ($Vv_{(vess)}$) were estimated using the point-counting method [26]. Briefly, a grid of points was superimposed upon the images of the ventricle sections viewed on the monitor. The right upper corner of

each cross-point was considered as a point. Then, the volume density "Vv(structure/ref)" of vessels was obtained using the following formula:

$Vv(structure/ref) = P(structure)/P(ref)$ where "P(structure)" and "P(ref)" represented the total number of the points hitting the structures (capillaries) and the left ventricle sections, respectively. The total volume of vessels was estimated by multiplying the volume density of vessels in the last ventricular volume.

Estimation of the Length Density of the Vessels

The length density of the vessels was estimated using an unbiased counting frame and calculated using the following formula:

$L_V(vessels/heart) = 2\Sigma Q/(\Sigma P \times a/f)$ where "ΣQ" denoted the total number of the vessel profiles counted per heart and "ΣP" and "a/f" were the total number of the counted frame and the area of the counting frame (2877.25 μm2), respectively [24].

Statistical Analysis

The values are expressed as mean ± standard error of the mean. Data analysis was performed by SPSS, version 20 (SPSS Inc., Chicago, IL, USA). One-way ANOVA followed by post hoc Tukey test was used to compare the quantitative data. Comparisons of histopathological findings were carried out using the non parametric Kruskal–Wallis and pairwise differences by the Mann–Whitney *U* test [25]. *P* value < 0.05 was considered as statistically significant.

Result

Blood Pressure, Heart Rate and Cardiac Contractility Indices

Despite some degree reduction, there was no significant alteration in HR (heart rate), DAP (diastolic arterial pressure) and MAP (mean arterial pressure) following to isoproterenol-induced cardiac injury in ISO, Sh + ISO and BFR + ISO groups in comparison with CTL group. However, cardiac injury was associated with significant decrease in SAP and LVSP ($P < 0.01$) and significant increase in left-ventricular end diastolic pressure (LVEDP) in ISO, Sh + ISO and BFR + ISO groups versus CTL group ($P < 0.05$). On the other hand, in the cardiac injury condition, the exercise trained groups showed more stability in above pressures with no significant difference between these parameters in exercise groups and CTL groups (Table 1; Fig. 1a). This positive effect was more prominent in BFR-Ex + ISO groups which cardiac and blood pressures were completely maintained in levels of CTL group and significantly were different with ISO, Sh + ISO and BFR + ISO groups ($P < 0.01$). Interestingly, the level of LVEDP was reduced in BFR-Ex + ISO when compared with Sh-Ex + ISO group ($P < 0.05$, Fig. 1b). Myocardial injury caused significant reduction in values of contractility (+ dp/dt max) and relaxation (− dp/dt max) indices in ISO, Sh + ISO and BFR + ISO groups in comparison with CTL group ($P < 0.05$). Blood flow restriction along with exercise adjusted these two parameters in the way that the BFR trained rats had markedly greater + dp/dt max and − dp/dt max values ($P < 0.01$) in comparison to ISO-treated untrained and sham-operated-trained rats (Fig. 1b).

Table 1 Cardiovascular indices in different animal groups

Groups	SAP (mm Hg)	DAP (mm Hg)	MAP (mm Hg)	LVSP (mm Hg)	Heart rate (beat/min)
CTL	147 ± 12	101 ± 8	116 ± 7.6	152 ± 10	353 ± 14
ISO	110 ± 11[b]	80 ± 7.8	94 ± 8	119 ± 11[b]	321 ± 13
Sh + ISO	113 ± 7[b]	83 ± 10.7	93 ± 10	122 ± 7[b]	347 ± 26
BFR + ISO	111 ± 6[b]	81 ± 11	92 ± 10	118 ± 10[b]	350 ± 15
Sh-Ex + ISO	122 ± 6	91.6 ± 5.5	101 ± 4	132 ± 6	357 ± 18
BFR-Ex + ISO	146 ± 10[a]	104 ± 11	118 ± 6	155 ± 8[a]	350 ± 10

The values are expressed as means ± SEM, $n = 7$

n number of animals, *SAP* systolic arterial pressure, *DAP* diastolic arterial pressure, *MAP* mean arterial pressure, *LVSP* left ventricular systolic pressure (mm Hg), *HR* heart rate, *CTL* control group, *ISO* group with isoproterenol-induced cardiac injury, *Sh + ISO* sham group with cardiac injury, *BFR + ISO* blood flow restricted group with cardiac injury, *Sh-Ex + ISO* sham group subjected to exercise training for 10 weeks before cardiac injury, *BFR-Ex + ISO* blood flow restricted group subjected to exercise training for 10 weeks before cardiac injury

[a] $P < 0.05$ versus ISO, Sh + ISO, and BFR + ISO

[b] $P < 0.01$ versus CTL

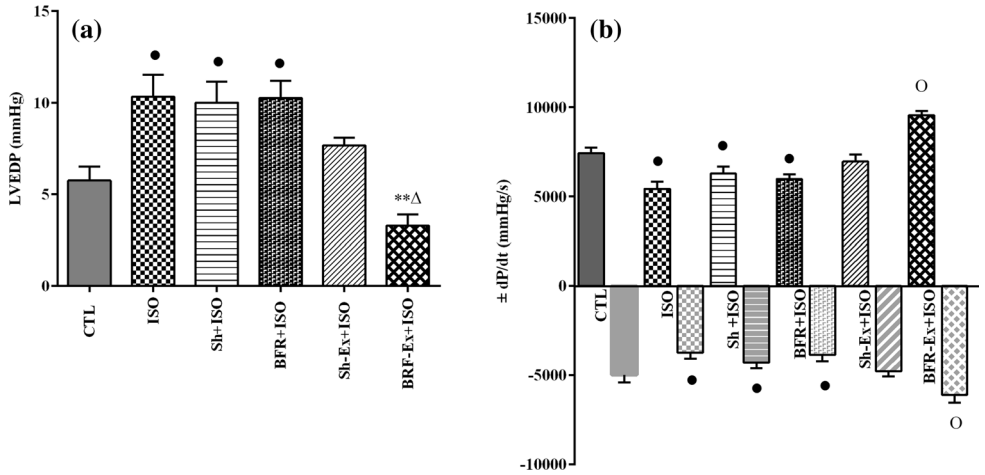

Fig. 1 **a** Left-ventricular end diastolic pressure (LVEDP) and **b** positive dP/dt max (+dP/dt max) and negative dP/dt max (−dP/dt max). The values are expressed as means ± SEM, $n = 6$. *n* number of animals, *CTL* control group, *ISO* group with isoproterenol-induced cardiac injury, *Sh + ISO* sham group with cardiac injury, *BFR + ISO* blood flow restricted group with cardiac injury, *Sh-Ex + ISO* sham group subjected to exercise training for 10 weeks before cardiac injury, *BFR-Ex + ISO* blood flow restricted group subjected to exercise training for 10 weeks before cardiac injury. (Filled circle) $P < 0.05$ versus CTL, $^{**}P < 0.01$ versus ISO, Sh + ISO, BFR + ISO, (triangle) $P < 0.05$ versus Sh-Ex + ISO, (open circle) $P < 0.05$ versus ISO, Sh + ISO, BFR + ISO, and Sh-Ex + ISO

GSK-3β and Phosphorylated Ser^{9}-GSK-3β (pS9-GSK-3β) Proteins Expression

The expression of cardiac GSK-3β, pS9-GSK-3β, and pS9-GSK-3β/GSK-3β ratio in different animal groups has been shown in Fig. 2. There was no difference in GSK-3β levels between all groups. However, the pS9-GSK-3β and pS9-GSK-3β/GSK-3β ratio increased in the BFR-Ex + ISO group compared to CTL, ISO, Sh + ISO, and BFR + ISO ($P < 0.05$) groups (Fig. 2).

Plasma Cardiac Troponin-I Levels

ISO-induced cardiac injury was associated with a significant increase in plasma cardiac troponin-I levels in all untrained animal groups ($P < 0.01$) and with a lower degree in Sh-Ex + ISO group ($P < 0.05$) versus CTL group. However, combination of low-intensity endurance exercise along with blood flow restriction decreased cTnI significantly in comparison to the untrained ISO-treated groups ($P < 0.01$), as there was no significant difference between levels of Troponin-I in BFR-Ex + ISO and CTL groups (Fig. 3).

Histological Findings

On histopathological examination, isoproterenol induced moderate to severe cardiac damage so that about 90% of the animals' hearts in ISO-treated groups showed widespread discontinuity with adjacent myofibrils, interstitial edema, and leukocyte infiltration and only about 10% of the hearts were associated with mild lesions (Fig. 4b–d) as compared to the normal group (Fig. 4a).The severity of the injuries was attenuated in Sh-Ex + ISO (Fig. 4e) but was significantly reduced in BFR-Ex + ISO group in such a way that the tissue sections from the BFR training group showed some infiltration of leukocyte and discontinuity with adjacent myofibrils but the morphology of cardiac muscle fibers was relatively well preserved compared to ISO, Sh + ISO and BFR + ISO groups ($P < 0.05$) (Fig. 4f; Table 2).

The results of the quantitative stereological evaluations of vessels are shown in Fig. 5. In the presence of limb-blood flow restriction in accordance with low-intensity exercise the volume density of the vessels was more than that of all other untrained groups ($P < 0.05$). The length density of the vessel with the diameter of < 8 μm in the BFR-Ex + ISO rats was significantly higher than that of CTL, ISO, Sh + ISO, and BFR + ISO groups ($P < 0.05$).

Discussion

In this study we used animal model to evaluate the effect of low-intensity resistance exercise program along with blood flow restriction (BFR training) on resistance of aged hearts against experimental damage. The results of our study indicated that pre-exposure to low-intensity exercise along with blood flow restriction protects the cardiac performance, maintains the blood pressure and attenuates the severity of cardiac damage following to ISO-induced myocardial injury.

It is confirmed that high doses of isoproterenol, a non-selective beta adrenergic agonist, through the increase of cytosolic calcium, positive chronotropic and inotropic effects

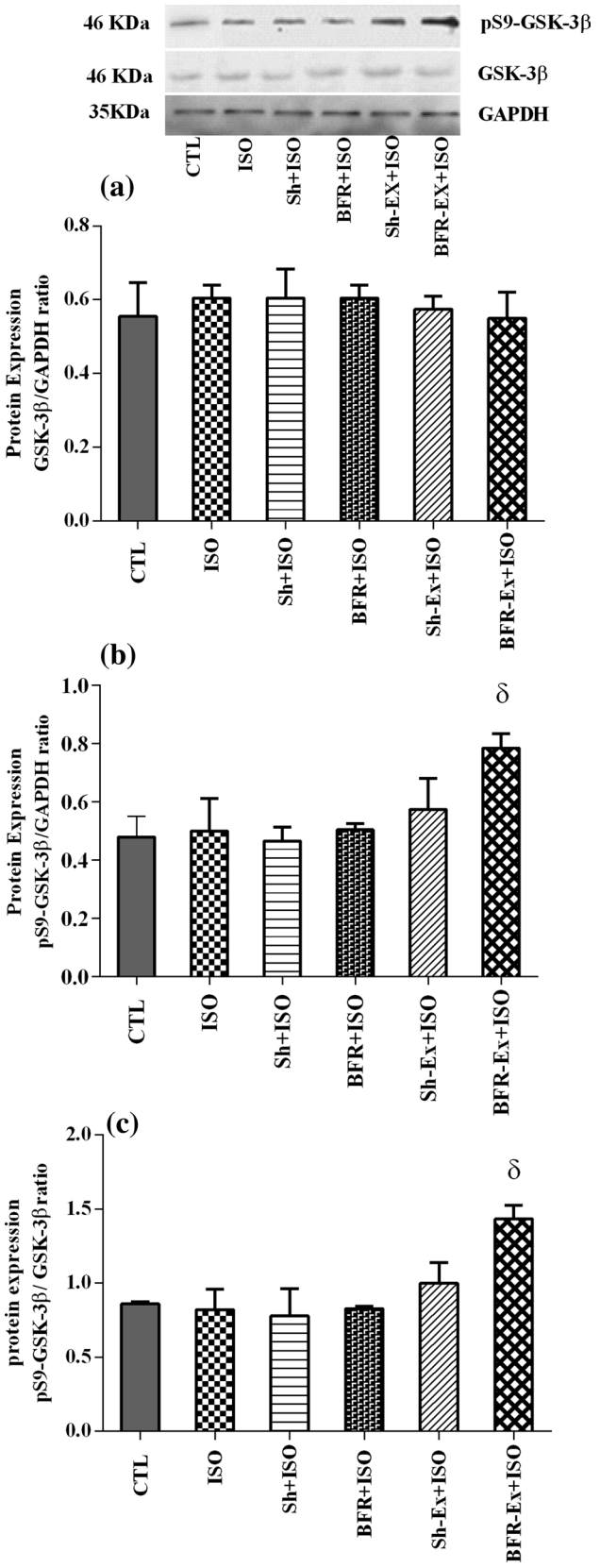

Fig. 2 The expression of cardiac GSK-3β (**a**), pS9-GSK-3β (**b**) ▶ proteins and pS9-GSK-3β/ GSK-3β ratio (**c**) in different experimental groups at the end of the study. The values are expressed as means ± SEM, $n = 6$. *n* number of animals, *CTL* control group, *ISO* group with isoproterenol-induced cardiac injury, *Sh+ISO* sham group with cardiac injury, *BFR+ISO* blood flow restricted group with cardiac injury, *Sh-Ex+ISO* sham group subjected to exercise training for 10 weeks before cardiac injury, *BFR-Ex+ISO* blood flow restricted group subjected to exercise training for 10 weeks before cardiac injury. $\delta P < 0.05$ versus CTL, ISO, Sh + ISO, and BFR + ISO

which in turn increases the workload and oxygen demand of myocardial muscle and also oxidative stress, metabolism alteration, and coronary insufficiency induces the global heart ischemia and infarction like lesions in rodent hearts which associated with reduction in cardiac performance and enhancement of cTnI [21, 27]. Therefore, it is used as a standard experimental model, to evaluate cardio-protective and destructive role of various agents.

Similar to the results of present study, previous studies demonstrated that high doses of isoproterenol can induce reduction in blood pressure [28], LVSP, positive and negative dP/dt max and increase the LVEDP [21, 29]. Maintenance of blood pressure, improvement of + dp/dt and − dp/dt max as important indices of myocardial contractility and cardiac relaxation velocity, respectively, along with reduction in left-ventricular end diastolic pressure and cTnI in BFR training animals following to isoproterenol injection, implies that low endurance exercise along with limbs blood flow restriction increases the cardiac resistance and decreases the cardiac destruction encountering stressful conditions.

In the other part, findings revealed that severity of myocardial injury was significantly less in animals submitted to BFR training than that of other ISO-treated animals.

Previously, without injury induction, we examined the structural changes of the heart quantitatively in the same animal model using the stereological methods [30]. Our findings demonstrated that animals submitted to BFR training had higher level of myocyte and vessels and lower level of connective tissue. Such beneficial reconstructions as increasing volume and length density of vessels following to BFR training was also confirmed in the present study which can justify a part of the increase in cardiac resistance to injury as we observed. Increasing the heart vascularization improves the blood supply and hence the oxygen requirement of the heart in conditions of cardiovascular stress, including exposure to high isoproterenol doses, thus prevents ischemia and heart damage. In addition, human studies revealed that physical aerobic exercise activity plus blood flow restriction leads to an increase in heart stroke volume, maximal oxygen uptake (VO_2max) [31, 32] maximal minute ventilation (VE max) [32], time to exhaustion [31, 33], and hence aerobic fitness and muscular strength [34]. These physiological benefits come from alteration in cellular signaling machine

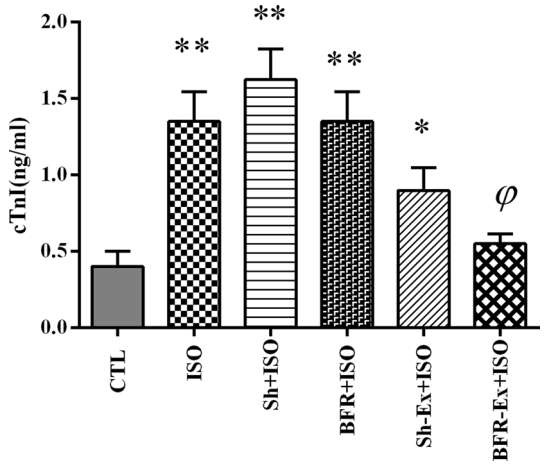

Fig. 3 Plasma cardiac troponin-I (cTnI) levels in experimental groups. The results are presented as mean ± SEM, $n = 6$. *n* number of animals, *CTL* control group, *ISO* group with isoproterenol-induced cardiac injury, *Sh + ISO* sham group with cardiac injury, *BFR + ISO* blood flow restricted group with cardiac injury, *Sh-Ex + ISO* sham group subjected to exercise training for 10 weeks before cardiac injury, *BFR-Ex + ISO* blood flow restricted group subjected to exercise training for 10 weeks before cardiac injury. $^{**}P < 0.01$ versus CTL, $^{*}P < 0.05$ versus CTL, $\varphi P < 0.01$ versus ISO, Sh + ISO, and BFR + ISO

following to BFR exercise training. We showed that BFR training induces expression of VEGF, an angiogenesis-stimulating signaling protein, and its receptors, Flt-1 and kdr, in the heart [30] and also peroxisome proliferator activated receptor gamma coactivator 1-alpha (PGC1-α), a pivotal protein for control of metabolism, mitochondrial biogenesis and function, in both fast and slow skeletal muscles [17] of aging rats. In consistent with previous reports regarding alteration in cellular signaling pathways, we observed that the value of pS9-GSK-3β and pS9-GSK-3β/GSK-3β ratio significantly increase in BFR training group.

Increasing evidence has suggested that mitochondria are the main target of many favorable adaptations induced by physical exercise that increase the cardiac ability to tolerate the harmful effects of the deleterious conditions [35]. In addition to the lower free radical production, reduced levels of mitochondrial-mediated apoptotic cell death markers, and induction of HSP (heat shock protein), exercise can elevate the threshold for opening of the mPTP [28], a mitochondrial event that is considered as a key point in organ degeneration. It has been demonstrated that exercise restores the cardiac mitochondrial dysfunction [13], increase antioxidant capacity and attenuate DOX-induced decreased tolerance to MPTP opening [35]. GSK-3β is a down-stream target

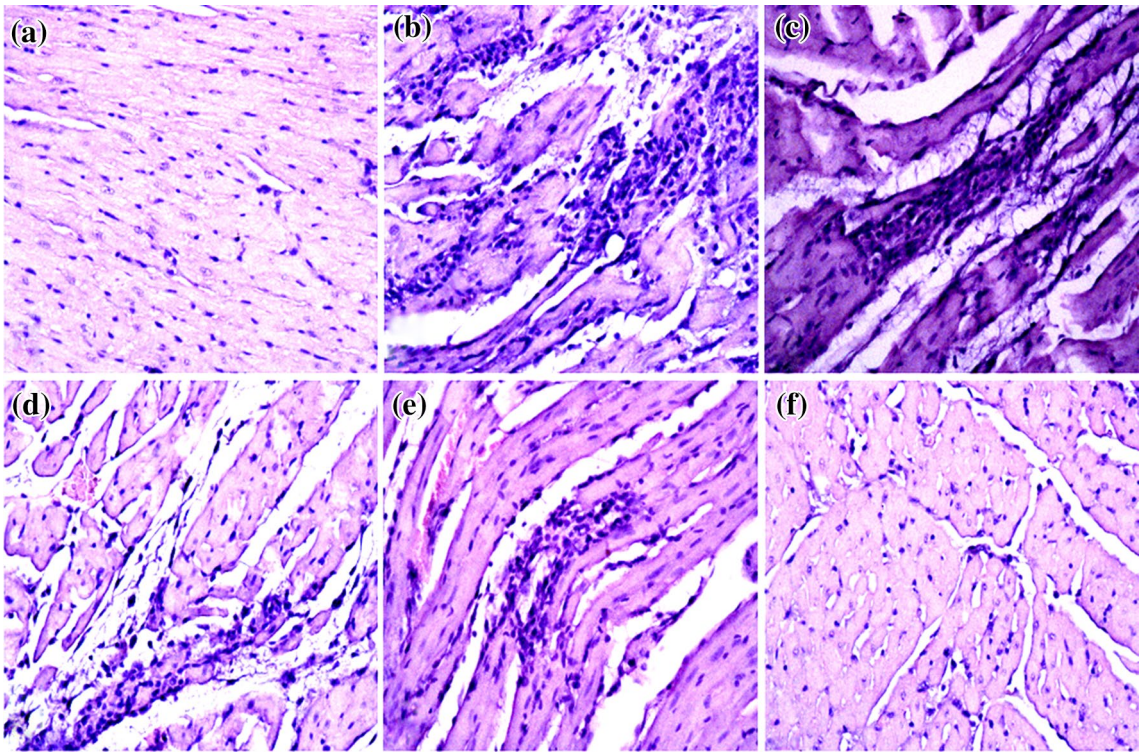

Fig. 4 Histological changes of the heart in the normal and ISO-treated groups. **a** CTL, **b** ISO, **c** sham + ISO, **d** BFR + ISO, **e** Sh-Ex + ISO, **f** BFR-Ex + ISO. Heart tissues were stained with hematoxylin and eosin and visualized under light microscope. *CTL* control group, *ISO* group with isoproterenol-induced cardiac injury, *Sh + ISO* sham group with cardiac injury, *BFR + ISO* blood flow restricted group with cardiac injury, *Sh-Ex + ISO* sham group subjected to exercise training for 10 weeks before cardiac injury, *BFR-Ex + ISO* blood flow restricted group subjected to exercise training for 10 weeks before cardiac injury

Table 2 Histopathological scores and animal number with different degrees of injury in each group

Groups	Myocardial pathology scores					
	No change	Minimum	Mild	Moderate	Sever	Mean
CTL	7	0	0	0	0	0
ISO	0	0	0	3	4	3.57•
Sh + ISO	0	0	1	2	4	3.42•
BFR + ISO	0	0	0	3	4	3.57•
Sh-Ex + ISO	0	0	2	2	3	3.14•
BFR-Ex + ISO	0	2	2	2	1	2.28•*

$n = 7$

n number of animals, *CTL* control group, *ISO* group with isoproterenol-induced cardiac injury, *Sh + ISO* sham group with cardiac injury, *BFR + ISO* blood flow restricted group with cardiac injury, *Sh-Ex + ISO* sham group subjected to exercise training for 10 weeks before cardiac injury, *BFR-Ex + ISO* blood flow restricted group subjected to exercise training for 10 weeks before cardiac injury. (0) no change, (1) minimum (focal myocytes damage), (2) mild (small multifocal degeneration with slight degree of inflammatory process), (3) moderate (extensive myofibrillar degeneration and/or diffuse inflammatory process), and (4) sever (necrosis with diffuse inflammatory process)

•$P < 0.001$ versus CTL, *$P < 0.05$ versus ISO, Sh + ISO, and BFR + ISO

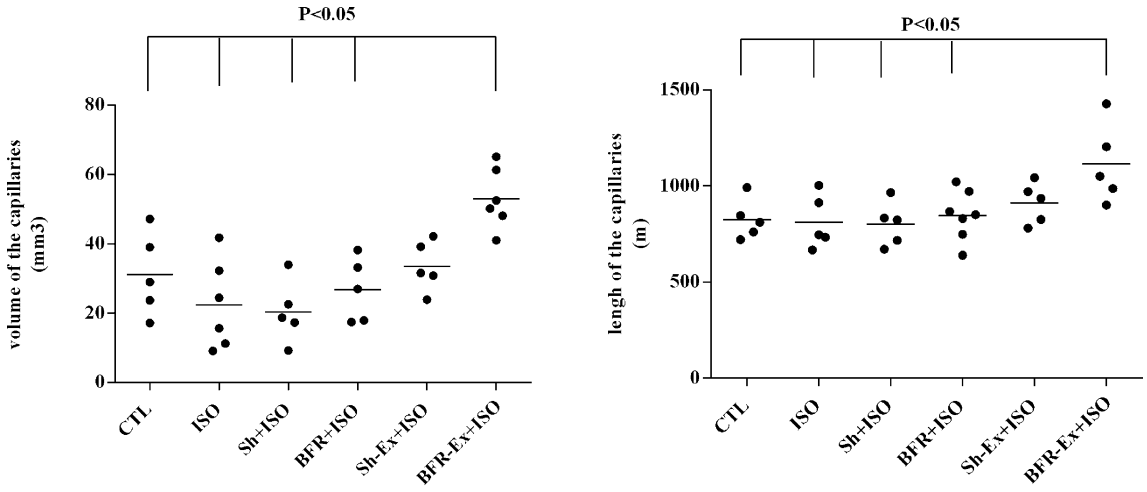

Fig. 5 The scatter plots of the quantified volume (mm^3) (**a**) and length (m) (**b**) of capillaries in the left ventricle of different animal groups. Each round (filled circle) represents an animal and the horizontal bar indicates the mean value of each parameter in every group, $n = 5–6$. *n* number of animals, *CTL* control group, *ISO* group with isoproterenol-induced cardiac injury, *Sh + ISO* sham group with cardiac injury, *BFR + ISO* blood flow restricted group with cardiac injury, *Sh-Ex + ISO* sham group subjected to exercise training for 10 weeks before cardiac injury, *BFR-Ex + ISO* blood flow restricted group subjected to exercise training for 10 weeks before cardiac injury. Significant level has been reported above the figures

of Akt and activated Akt inhibits GSK-3β by inducing its phosphorylation [36]. Regular exercise triggered activation of the reperfusion ischemia salvage kinase (RISK) pathway through phosphorylation of its components, i.e., Akt, ERK ½ and their common downstreams, p70S6K and GSK-3β [37]. Ozaki et al. demonstrated that 8 weeks of resistance training was associated with significant increase in phosphorylated Akt and GSK-3β [16]. It is indicated that phosphorylated GSK-3β translocates to the mitochondria, binds to an mPTP subunit protein, and increases the threshold of mPTP opening [38]. The significant role of mPTP has been demonstrated during cardioprotection elicited by ischemic and pharmacological pre- and post-conditioning [15, 39]. Following to ischemia–reperfusion [40] and high doses of isoproterenol [41], opening of the mitochondrial permeability transition pore (mPTP) is one of the main mechanisms of cell necrosis induced by intracellular calcium overload. Irreversible mPTP opening eradicates mitochondrial membrane potential and its ATP production and consequently leads to cell death [40]. As mentioned above, phosphorylated GSK-3β mitigates the mPTP opening and helps to maintain the mitochondrial membrane potential and function. Interestingly, phosphatases limit the efficacy of some cardio-protective strategies such as pre- or post-conditioning

during aging [37]. Perhaps superimposing exercise on the BFR increases the relative ischemic burden in the hind limb; thereby increasing release of mediators from skeletal muscles may reinforce the RISK pathway in the heart and protects it through phosphorylated GSK-3β when challenged with isoproterenol. However, confirmation of this idea requires further studies.

In conclusion, based on physiological, histological and biochemical findings, this study clearly demonstrated the cardio-protective effect of low-intensity exercise along with blood flow restriction against ISO-induced myocardial injury. Beneficial reconstructions such as angiogenesis in one hand and improving age-related dysfunction of mitochondria through increasing phosphorylated GSK-3β on the other hand may contribute to cardio-protective effect of BFR training. More studies are needed to extend this finding to old people who suffer from some degrees of motor disabilities and heart insufficiency. If the safety of this method can be approved in human, it will potentially be a strategy and approach to rehabilitate of heart in elderly.

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Compliance with Ethical Standards

Conflict of interest The authors declare there is no conflict of interests in this study.

Ethical Approval All applicable national guidelines for the care and use of animals were followed. The study was approved by the Ethical Committee in Research of the Kerman University of medical science, Kerman, Iran (permission No IR.KMU.REC.1394.495) and the experimental protocol was conducted in accordance with the guidelines of that university for conducting animal studies.

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