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Effect of Exercise Training and L-arginine on Oxidative Stress and Left Ventricular Function in the Post-ischemic Failing Rat Heart

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Abstract The aim of the present study was to evaluate the effect of exercise training (ET) and L-arginine on oxidative stress and ventricular function in rat with myocardial infarction (MI). Four weeks after the surgical procedures, 40 Wistar male rats were randomized to the following groups: MI-sedentary (Sed); MI-exercise (Ex); MI-sedentary + L-arginine (Sed + LA); and MI-exercise + L-arginine (Ex + LA); the rats were subjected to aerobic training in the form of treadmill running. Rats in the L-arginine-treated groups drank water containing 4 % L-arginine. Before and after the training program, all subjects underwent resting echocardiography. Catalase (CAT) glutathione peroxidase (GPx), malondialdehyde (MDA) and myeloperoxidase (MPO) were measured. Cardiac output, stroke volume and fractional shortening in Ex and Ex + LA groups significantly increased in comparison with the Sed group. Cardiac systolic function indices in Ex + LA group were significantly greater than Ex group. Also, GPx activity and MDA, respectively, increased and decreased in response to ET, but no change was observed in MPO and CAT. These results suggest that ET increased LV function by decreasing oxidative stress and increasing antioxidant defense system in rats with MI. In addition in response to training, L-arginine appears to have additive effect on cardiac function, but have no effect on oxidative stress indices.

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Introduction

Myocardial infarction (MI) as a prevalent ischemic heart disease is one of the main causes of death from cardiovascular disease [1]. Reduced left ventricular (LV) systolic function is the main characteristic of MI. Previous studies suggest that oxidative stress plays a major role in the pathogenesis of cardiac remodeling and LV dysfunction following MI [2, 3].

Ischemic cardiac tissue is known to generate reactive oxygen species (ROS) and triggers cell dysfunction, lipid peroxidation and DNA mutagenesis and can lead to irreversible cell damage or death [4]. The degree of oxidative stress and myocardial damage and the severity of subsequent LV dysfunction might depend on the imbalance between excess of ROS production and antioxidant defense within the heart [5].

Impaired antioxidant capacity also plays a role in oxidative stress in the infarcted heart. NADPH oxidase, which consists of two subunits $gp91^{phox}$ and $p22^{phox}$, is a major source of O_2^- in the heart [6]. Singal and colleague [7] have shown evidence of the progressive decrease of superoxide dismutase (SOD), catalase and GSHPx activity as well vitamin E levels in the infarcted rat heart, first in the infarcted myocardium followed by the non-infarcted myocardium, while NAD(P)H oxidase expression dramatically increased in the infarcted myocardium [8]. The myocardium may be especially vulnerable to oxidative damage because it has a high VO_2 due to continuous working, and low antioxidant defences [9]. In addition,

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post-mitotic tissues such as the myocardium have a lesser ability to up-regulate antioxidant defences [10].

On the other hand, it has been shown that L-arginine as a precursor nitric oxide (NO) limits cardiac remodeling after an infarction and contributes to the protective effect of ischemia [11, 12]. NO is closely linked to protective properties against oxidative stress agents. NO has various physiological properties including vasodilatation, scavenging superoxide (O_2^-) formation and suppression xanthine oxidase (XO) [13, 14].

In this regard, it has been shown that exercise training (ET) promotes antioxidant capacity and attenuates oxidative stress-mediated tissue damage [15]. In addition, ET improves LV systolic function [6, 16], but it is unknown whether the ET-mediated preservation of the cardiac function is associated with restoration of the local enzymatic radical scavenger system.

Accordingly, the purpose of the present study was to evaluate the protective effect of ET on oxidative stress and LV systolic function in rat with MI. Furthermore, we investigated whether post-MI ET combined with L-arginine supplementation reconstitutes the activity of catalase, and GPx, thereby reducing oxidative stress in the non-infarcted myocardium in rats.

Methods

Animal Characteristics

In individual polycarbonate cages, 6–8-week-old Wistar male rats (initial body mass of 191 ± 24 g) were housed in a temperature-controlled room (22 ± 2 °C) with a 12-h dark/light cycle. The light period began at 7:00 a.m. Rats were provided with standard laboratory chow and water. The study protocols were approved by the Institutional Animal Care and Use Committee at Lorestan University of Medical Science.

Surgical Preparation

Rats were initially anesthetized with administration of sodium thiopental (60 mg/kg body weight, i.p.), intubated via tracheotomy and placed on a rodent respirator (Small Animal Ventilator, Model 683, Harvard Apparatus, 15 mL/kg stroke volume and 60–70 breaths/min). Rats were given heparin (200 IU/kg, iv), and then, the heart of each was exposed through a left thoracotomy between the fifth and sixth ribs (1.5 cm in diameter), and the pericardium was opened. The heart was exteriorized, and ligation of the left anterior descending (LAD) coronary artery (approximately 2 mm below the left atrium) was performed using 6-0 polyethylene thread. Proximal LAD artery ligation in a rat model creates a reproducibly large lateral wall infarction. A standard limb lead-II electrocardiogram (ECG) was continuously monitored and recorded throughout the experiment, using a computerized data acquisition system (ML750 Power Lab/4sp, ADInstruments). Proper ligation of the LAD was confirmed by ST elevation and increase in R-wave amplitude in ECG. Muscle and skin incisions were closed with separate purse-string silk sutures (size 4-0), and the lungs were fully expanded. Body temperature was measured by rectal thermometer and maintained at 37 ± 1 °C. To ensure complete healing of the infarct zone, all rats recovered in their cages for 4 week after the operation before beginning the exercise program. During this time period, 12 rats died before randomization to ET and were excluded from subsequent analysis.

Doppler Echocardiography

Doppler echocardiographic measurements were taken on the day before the initiation of post-MI ET (4 weeks post-MI), and after 10 weeks of ET (14 weeks post-MI). The procedures were performed by an observer blinded to the groups to which the animals had been assigned. A Sonos 5500 equipment (Philips Medical Systems, Andover, MA, USA) with a 12-MHz transducer was used in a depth between 2 and 3 cm. After having their thorax shaved, under anesthesia by sodium thiopental (50 mg/kg body weight, i.p.), the animals were placed in the left lateral position. The images were recorded on VHS videotapes, and the final result was obtained from the mean of three different cardiac cycles [17]. We measured the resting ECHO parameters such as LV end-diastolic dimension (LVEDd), LV end-systolic dimension (LVESd), LV end-diastolic volume (LVEDV) and LV end-systolic volume (LVESV). LV fractional shortening (FS), LV ejection fraction (EF), stroke volume (SV) and cardiac output (CO) were calculated as FS (%) = [(LVEDd − LVESd)/LVEDd] × 100, EF = (LVEDV − LVESV)/LVEDV, SV = (LVEDV–LVESV) and CO = (Heart rate × Stroke volume), respectively.

Experimental Groups and Exercise Training

Echocardiography was performed on the surviving rats 4 week after surgery. Rats were matched by cardiac functions and randomly assigned to the following experimental groups: MI-sedentary ($n = 10$, Sed); MI-exercise ($n = 10$, Ex); MI-sedentary + L-arginine ($n = 10$, Sed + LA); and MI-exercise + L-arginine ($n = 10$, Ex + LA). The rats assigned to the exercise group started exercising at 4 week post-MI using a motorized rodent treadmill, while the Sed rats remained sedentary throughout the experiment period. To allow gradual adaptation to

exercise stress, training was initiated at 10 m/min, 5° incline for 10 min per session. The speed and duration were gradually increased to 17 m/min and 50 min per session and maintained constant throughout the experiment. Animals were encouraged to run by means of a shock bar positioned at the bottom of the treadmill lane. In order to limit the stress, when rats failed to stay off the shock bar, the system was switched off and exercise was stopped for 5 min. After this resting period, animals restarted to run till they reached an overall daily exercising time of 50 min. The exercise intensity was moderate and 55–60 % of maximal oxygen consumption [18]. The ET was performed 5 days per week for 10 weeks. The determination of treadmill speed and exercise duration was based on the previous studies [18, 19].

L-arginine Treatment

Subjects in the L-arginine-treated groups drank water containing 4 % (w/v) L-arginine (A5006, Sigma-Aldrich, USA). The average L-arginine intake, estimated from water intake, was 5.2 g/kg/day. Because the rats were housed one per cage, the water intake per individual rat was estimated from the water intake per cage (approximately 30–50 mL) [20]. To prevent L-arginine precipitation, the water was changed every day. The determination of L-arginine dosage was based on the previous studies, which demonstrated positive effects on heart without symptoms of side effect [20].

Tissue Processing and Homogenate Preparation

The rats were weighed, anaesthetized and killed with an overdose of sodium thiopental 48 h after the program training (in order to eliminate results that caused by effects of last acute exercise session, the rats were killed 48 h after the last session and in this period they were receiving L-arginine supplementation). The heart was quickly removed, washed with ice-cold saline, blotted and weighed. After the atria and great blood vessels were trimmed, the non-infarcted myocardium of LV was cut and quickly frozen in liquid N_2. Duration of the process was <2 min. Cardiac homogenates were prepared at 4 °C. In brief, fifty milligrams of non-infarcted myocardium of LV was homogenized on ice in 1 mL of ice-cold lysis buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 20 mM HEPES, 20 % glycerol, 0.1 % Triton X-100, 1 mM dithiothreitol, pH 7.4). The homogenates were centrifuged at 1000 rpm for 10 min at 4 °C and stored at −80 °C for later use.

Infarct Size Measurement

Hearts were excised and washed in cold 1 × PBS. The proximal region of the heart (above the LAD ligation) was removed, and the distal region was subsequently sliced into 3–4-mm cross sections and incubated at 37 °C for 20 min using 1 % 2,3,5-triphenyltetrazolium chloride (TTC) in 0.1 M phosphate buffer (pH 7.4). The slices were fixed in 10 % formaldehyde overnight and digitally photographed the following day. Viable myocardium was stained red by TTC, whereas the areas of non-viable tissue appeared pale pink and/or white. Planimetric analysis was performed using NIH ImageJ software (http://rsb.info.nih.gov/ij/) in a blinded fashion. Infarct size of each slice was determined by dividing the TTC precipitate-excluded area by the total heart slice area and averaged for each heart. Conventional histological TTC staining as was done in this study has previously been used to determine infarct size in models of permanent occlusion [21].

Antioxidant Enzymatic Activities

The enzymatic activities of glutathione peroxidase (GPx) and catalase were measured in the non-infarcted myocardium of LV. GPx activity was determined using a RANSEL kit (Randox labs.), according to the method of Paglia and Valentine [22]. GPx catalyzes the oxidation of glutathione (at a concentration of 4 mmol/L) by cumene hydroperoxide. In the presence of glutathione reductase (at a concentration ≥0.5 U/L) and 0.28 mmol/L of NADPH, the oxidized glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to NAD+. The decrease in absorbance at 340 nm was measured by Hitachi U-2000 spectrophotometer. Tissue GPx activity was expressed as U/mg protein. Also, catalase activity (CAT) was measured as previously described by Aebi [23]. The decomposition of H_2O_2 was followed directly by the decrease in absorbance at 240 nm and 20 °C. Previously, myocardial homogenate aliquots were centrifuged at 1000*g* and 4 °C for 10 min. The adequate amount of supernatants (60 µL equivalent to 1.5 mg tissue wet weight) was added to a reaction mixture that contained 0.002 % Triton X-100, 0.1 mmol EDTA, 0.5 m potassium phosphate buffer, pH 7.0, and 15 mmol H_2O_2 in 1 mL final volume. Activity was calculated with the initial 30-s decomposition rate.

Oxidative Stress Assessment

Quantification of lipid peroxidation is essential to assess the role of oxidative injury. Lipid peroxidation results in the formation of malondialdehyde (MDA) that can then be used to indicate the levels of lipid peroxidation in cardiac tissue. Lipid peroxidation products were assessed by measuring MDA level according to the method of Niehaus and Samuelsson [24]. The level of MDA in the supernatant was determined spectrophotometrically by measuring

thiobarbituric acid-reactive substances with a maximum absorbance at 532 nm. Briefly, 0.5 mL of the sample was mixed with 3 mL of 1 % phosphoric acid and 1 mL of 0.6 % TBA solution. The mixture was heated in a boiling water bath for 45 min and cooled to the room temperature. Then, 4 mL of *n*-Butanol was added, and mixture was vortexed and centrifuged at $1000\times g$ for 10 min. The absorbance of butanol phase (supernatant) was measured at 532 nm. Tissue MDA content was expressed as nmol/mg protein. Furthermore, myeloperoxidase (MPO) activities of tissue samples were determined as a marker enzyme for measuring neutrophil accumulated in tissue samples, because correlates closely with the number of neutrophils present in the tissue [25]. MPO is a known marker of a powerful ROS releaser [26]. MPO activity was determined using the method described by Huang et al. [27]. 50 μL of diluted sample was added to 1 mL of mixed substrate containing 3 mmol/L hydrogen peroxide dissolved in 3,3′,3,5′-tetramethylbenzidine (R&D Systems, Minneapolis, MN, USA). MPO activity was measured at 37 °C on a spectrophotometer at 655 nm for 3 min. One unit of MPO activity was defined arbitrarily as the amount of enzyme necessary to catalyze an increase in absorbance of 1.0 at 655 nm/min. MPO activity was expressed as nmol/mg protein for specific activity.

Statistical Analysis

In order to analyze data, the SPSS statistical software version 16 was used. Results were analyzed by a one-way analysis of variance (ANOVA) across the different experimental conditions. When a significant F value was found by ANOVA, Tukey HSD post-test was performed to demonstrate differences between the means. The minimal level of significance was adopted $p < 0.05$, and data expressed as the mean ± SEM.

Results

General Characteristics

General characteristics of animals at the end of the 14 weeks of the protocol are shown in Table 1. Body weight, heart weight and heart weight to body weight ratio were not significant differences in ET groups compared with the corresponding sedentary groups with or without drug treatments.

Echocardiographic Data

As shown in Table 2, there were no significant differences among the four experimental groups in LVEDd, LVESd and LVEDV after 10 weeks of ET. The LVESV was significantly decreased by exercise intervention when compared with the Sed groups. In addition, LV ejection fraction (LVEF) increased by ET in comparison with the sedentary groups. Moreover, the LVESV and LVEF were similar in Ex and Ex + LA groups.

On the other hand, ET significantly increased FS, SV and CO compared to the sedentary infarcted groups, while FS, SV and CO were significantly higher in the Ex + LA group rather than Ex group.

Furthermore, infarct size insignificantly decreased in response to 10-week moderate ET. The difference between Ex and Ex + LA was not significant.

Antioxidant Enzyme Activities

Myocardial catalase and GPx activities were examined in the non-infarcted myocardium in experimental groups. As shown in Table-3, GPx activity was significantly increased in the Ex and Ex + LA groups compared with the Sed groups (Sed, Sed + LA), whereas it was not significant difference in the Ex and Ex + LA groups. We did not find any significant difference among all the groups with respect to the catalase activities.

Oxidative Stress Assessment

Lipid peroxidation was quantified to assess the extent of oxidative stress after 10 weeks of ET and/or drug treatments. Table 3 illustrates that MDA levels were significantly lower in the Ex and Ex + LA groups ($p < 0.001$) compared with the Sed and Sed + LA groups. In this study, we did not find any significant difference in MPO among all the infarcted groups.

Discussion

Cardiac adaptation in response to intrinsic or external stress involves a complex process of chamber remodeling and myocyte molecular modifications. The mechanisms underlying myocardial remodeling are undoubtedly multifactorial and are the subject of intense investigation. Growing evidence highlights oxidative and nitrosative stresses as important mechanisms for this maladaptation. A significant body of experimental and clinical data point to important roles of increased ROS production both in adverse remodeling and its precursor conditions [3].

This study, to the best of our knowledge, is the first to investigate the effects of ET and L-arginine supplementation on MDA and MPO in MI subjects. In the present study, we demonstrated that ET after MI improves LV systolic function by increasing GPx activity and decreases

Table 1 General characteristics

	Sed	Sed + LA	Ex	Ex + LA
N	10	10	10	10
Age at start of experiment (week)	6–8	6–8	6–8	6–8
Heart rate (bpm)	370 ± 35	392 ± 31	364 ± 41	357 ± 19
Heart weight (g)	1.06 ± 0.14	1.35 ± 0.17	1.28 ± 0.21	1.03 ± 0.20
Body weight (g)	356 ± 41	352 ± 24	321 ± 19	332 ± 38
Heart weight/body weight (g/g) × 10^{-3}	2.97 ± 0.12	3.61 ± 0.23	3.72 ± 0.7	3.1 ± 0.47

Values are expressed as mean ± SEM, heart rate, body weight and heart weight at killing

bpm beats per min

Table 2 Doppler echocardiographic assessment of left ventricular geometry and function

	Sed	Sed + LA	Ex	Ex + LA
LVEDd (mm)	10.74 ± 0.8	11.36 ± 0.3	10.08 ± 0.23	10.36 ± 0.41
LVESd (mm)	9.35 ± 0.13	9.76 ± 0.21	8.25 ± 0.17	8.01 ± 0.18
LVEDV (μL)	650 ± 68	745 ± 43	564 ± 48	609 ± 61
LVESV (μL)	463 ± 51	529 ± 83	370 ± 61*	348 ± 44*
EF (%)	30 ± 3	28 ± 5	36 ± 4*	38 ± 7*
FS (%)	12 ± 2	14 ± 4	17 ± 4*	24 ± 5*#
SV (μL)	178 ± 24	182 ± 39	194 ± 41*	254 ± 42*#
CO (mL/min)	62 ± 09	66 ± 18	71 ± 11*	84 ± 21*#
Infarct size (%)	33 ± 7	31 ± 7	30 ± 3	27 ± 4

Values are mean ± SEM

LVEDd left ventricular end-diastolic dimension, *LVESd* left ventricular end-systolic dimension, *LVEDV* left ventricular end-diastolic volume, *LVESV* left ventricular end-systolic volume, *EF* left ventricular ejection fraction, *FS* fractional shortening, *SV* stroke volume, *CO* cardiac output

* Significant difference from the Sed groups at the level of $p < 0.05$

Significant difference from the Ex group at the level of $p < 0.05$

Table 3 Antioxidant and oxidative stress indices

	Sed	Sed + LA	Ex	Ex + LA
Antioxidant defense systems				
GPx (U/mg protein)	214 ± 34	210 ± 22	233 ± 15*	250 ± 27*
CAT(U/mg protein)	8.2 ± 2.4	7.4 ± 2.0	5.5 ± 4.1	7.4 ± 0.2
Oxidative stress indices				
MDA (nmol/mg protein)	85.19 ± 9.06	76.64 ± 15.89	52.2 ± 11.5*	46.47 ± 12.3*
MPO (nmol/mg protein)	7.7 ± 2.6	6.9 ± 4	11.2 ± 1.5	19 ± 11.3

Values are mean ± SEM

GPX Glutathione peroxidase, *CAT* Catalase, *MDA* Malondialdehyde, *MPO* Myeloperoxidase

* Significant difference between the trained and their sedentary groups ($p < 0.05$)

MDA. Data from this study provide further insights into the molecular mechanisms underlying the improvement in LV remodeling produced by ET in patients with MI.

There are several major findings in this study. First of all, post-MI exercise training with or without L-arginine significantly increased GPx activities. After that, ET with or without L-arginine treatment significantly down-regulated MDA and decreased lipid peroxidation. Then, L-arginine treatment by itself may not have a beneficial effect on oxidative stress indicators. Moreover, post-MI ET with or without L-arginine treatment significantly increased LV systolic function. Finally, concomitant of L-arginine treatment with ET appears to have additive effect on systolic function. These results show that ET increased cardiac GPx levels and decreased MDA levels, and infarct size, indicating the beneficial effects of the training against oxidative injuries to the heart.

These results were in agreement with the findings of Xu et al. [6] which reported increasing in cardiac function in

response to 8-week ET and L-arginine [2 % (w/v)] supplementation in MI rat. In addition, Xu et al. showed that L-arginine alone did not affect LV function and antioxidant indices. But the results of this study were not in agreement with Lin et al. [13] that showed L-arginine supplementation [2 % (w/v)] can prevent elevations of XO and MPO activities in the lung and favorably influence pulmonary antioxidant defense systems after exhaustive exercise.

The present work showed that L-arginine treatment by itself was not beneficial, but in combination with Ex it might be advantageous. There is a trend for the increase in GPx and decrease in MDA in Ex + LA group when compared with Ex group even though it did not reach the statistical significance. We think with a longer time of treatment and larger number of rats these values could reach the statistical significance.

Earlier studies have shown that only certain cardiac antioxidant enzyme activities were enhanced in trained animals [28–30]. The literature is ambiguous regarding which myocardial antioxidants are elevated after ET [10, 31]. These variations in the cardiac antioxidant enzyme adaptation to training may be due to differences in mode, intensity and duration of the training.

The benefit of regular physical activity has been reported to improve cardiovascular functions in patients with chronic heart failure and other cardiovascular diseases. However, the exact mechanism in the regulation of cardiovascular functions by physical training is not well studied [30].

Exercise seems to increase the oxygen consumption rate by 10–20-folds and may release the endogenous factors such as cytokines, tumor necrosis factor (TNF), corticosteroid hormones and adenosine. These factors are known to regulate the antioxidant enzyme activities [30, 32]. ROS both low and high concentrations can cause damage to cellular function. ROS, which include superoxide anion (O_2^-), H_2O_2, and hydroxyl radical (OH), serve as signaling molecules in low concentrations [33]. However, these agents elicit harmful effects when produced in excess [6, 34]. Exercise training maintains the level of ROS between these two. Although exercise-induced ROS formation may be harmful to cellular function, but previous study showed that exercise-generated free radicals seem to increase the activity of antioxidant enzymes in the heart [35]. It is likely that redox reaction during exercise activates nuclear factor NF-κB and thereby induces transcription of antioxidant enzymes in the heart [30, 36, 37]. The role of NF-κB has also been reported in the induction of antioxidant enzyme expression through NO signaling during ischemic of the cardiovascular system [30, 38]. Proposed mechanisms to explain the cardioprotective effects of exercise include alterations in coronary circulation, expression of endoplasmic reticulum stress proteins, increased cyclooxygenase-2 activity, induction of myocardial heat shock proteins, and elevation of ATP-sensitive potassium channels on both the sarcolemmal and the mitochondrial inner membranes. Although we did not investigate the effects of ET and L-arginine supplementations on heat shock proteins (HSPs) in the present study, but Petry and coworkers demonstrated that exercise-induced HSP70 response may modulate autophagy by regulating mTOR/Akt pathway and block signaling pathways associated with protein degradation [39, 40]. Moreover, it seems possible that other, yet to be defined, mechanisms of exercise-induced cardioprotection may also exist [31]. Necrosis, apoptosis and autophagy are all implicated in myocardial cell loss during ischemic heart disease. NO can limit the deleterious effects of cardiac remodeling after MI. In this paper, result showed that the improvement of systolic function in response to ET in Ex + LA group was more in comparison with Ex group, while oxidative stress indices (MDA, MPO) and antioxidant defense systems (GPx, Cat) were not different between Ex and Ex + LA group. These effects of the L-arginine are related to increase in vascular endothelial growth factor (VEGF) and capillary density, leading to enhanced blood flow and vasorelaxation, and attenuate myocardial fibrosis and cardiac stiffness by NO production [6, 30]. In this regard, at the myofilament level, de Waard et al. [41] showed L-arginine/tetrahydrobiopterin supplementation increased Fmax, myofilament Ca^{2+} sensitivity and phosphorylation of myofilament proteins myosin binding protein-C, troponin-T and myosin light chain-2. Furthermore, L-arginine/tetrahydrobiopterin treatment resulted in a significant increase in protein levels of SERCA2a and the SERCA2a to phospholamban ratio.

In summary, this study demonstrates that ET after MI is likely playing an important role in cardiac remodeling by up-regulating the cardiac antioxidant defense system and inhibiting lipid peroxidation. These improvements, in turn, attenuate myocardial infarct size slightly and improve post-MI cardiac systolic function. In addition in response to training, L-arginine appears to have additive effect on cardiac function, but have no effect on oxidative stress indices.

Conflict of interest The authors declare that there are no conflicts of interests or financial contributions.

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