Effect of exercise training intensity on mitochondrial dynamics and mitophagy in post myocardial infarction rats

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ABSTRACT: Myocardial infarction (MI) is the most common type of heart disease. According to recent studies, mitochondrial dysfunction has been suggested as a central player in cardiac disease and evidences point out the association of mitochondrial morphology with development of heart diseases. Exercise training plays a protective role against cardiovascular disease. However, the role of exercise training on proteins involved in mitochondrial dynamics and mitophagy system are not well understood. Therefore, the aim of the present study was to investigate these on cardiac mitochondrial dynamic and mitophagy proteins in rats with myocardial infarction. The present study was post-test design experiment with the control group. After MI with ligation of the left anterior descending coronary artery (LAD) and ensuring the creation of MI by echocardiography, male rats were subjected to high intensity interval training (HIIT), moderate (MIIT), low (LIIT), sedentary myocardial infarction (SED-MI) and healthy control groups. After six weeks exercise, the levels of MFN2, DRP1, Parkin, P62 and PGC-1α proteins were measured by ELISA method. Data analysis showed that proteins levels of MFN2, PGC-1α, Parkin and P62 decreased significantly in SED-MI group compared to healthy control while DRP1 protein levels increased significantly (P≤0.05). Also, MFN2 and PGC-1α proteins increased in MIIT group compared with SED-MI group and DRP1 protein levels were significantly decreased (P≤0.05). Moderate-intensity interval training (MIIT) resulted to improve mitochondrial fusion and fusion proteins in rats with myocardial infarction. While high and low intensity interval training (HIIT, LIIT), despite increasing MFN2 and PGC-1α and reducing DRP1, failed to improve fusion and mitochondrial fission.

KEY WORDS: Myocardial infarction, interval exercise training, mitochondrial dynamic, mitophagy and PGC-1α

INTRODUCTION

Cardiovascular diseases are assumed as one of the most important causes of mortality and morbidity in developed countries, particularly myocardial infarction (MI) is the most common heart disease. Identification Research about risk factors, new diagnostic methods and therapeutic have resulted in dramatic improvements in cardiovascular mortality rate. However, changes in lifestyle-related factors and the global obesity epidemic have led to the continuous growth of cardiovascular diseases. Therefore, despite the significant achievements in recent decades, much effort should be done to identify new targets of therapeutic intervention. Several factors have been assumed to induce the change in mitochondrial morphology following ischemia, such as hypoxia-induced inhibition of oxidative phosphorylation, collapse in
Mitochondria have a complex functional morphology with, at basis, a compartmented double-membrane system and a usual tubular shape. However, this organelle presents a great morphological variability, which could be associated with different cellular environments in physiological conditions or pathological stress. In this sense, mitochondria may assume different shapes in various cell types, and this morphological change associated with distribution is generally termed mitochondrial dynamics [3].

Mitochondria dynamic has been well studies in exercise training research, as the majority of the literature on mitochondrial dynamics focused in skeletal muscle [4-9]. Nevertheless, emerging attention has been directed toward the cardiac muscle and the role of mitochondrial dynamics on cardiovascular diseases. Indeed, cardiomyocytes mitochondria occupy more than one-third of the cell volume, and produces more than 30 kg of ATP daily [3], which is crucial for normal heart function. Interestingly, mitochondrial dysfunction has been suggested as a central player in cardiac disease and evidences point out the association of mitochondrial morphology with development of heart diseases [10]. For instance, Schaper et al. observed that myocardial tissue from patients with dilated cardiomyopathy presented mitochondria in large clusters in cytoplasm free of myofibrils and they fall within a wide range of size from very small to very large, as well as the variance in their shape [11]. Similarly, it was shown that mitochondria in the heart failure (HF) were small and fragmented, in human HF [12]. Moreover, this change in mitochondria morphology was associated with mitochondrial fusion protein, suggesting that mitochondria fusion is decreased in HF, impairing mitochondrial function and resulting in the cell loss and downward progression of the HF [12]. In this line, other studies reported the disturbance on mitochondrial size and number in cardiac hypertrophy [13] and cardiac hypoxia [1].

In addition to the mitochondrial dynamic, a growing body of evidence suggests that mitochondrial autophagy plays an essential role in the regulation of optimal performance of mitochondria population, which is commonly referred as mitochondrial quality control. Autophagy is a mechanism of cell destruction by lysosomes and particularly important for cell survival during energy stress, degradation of misfolded proteins, dysfunctional cytoplasmic organelles, as well as dysfunctional mitochondria, which is known as mitophagy [14]. Therefore, mitophagy is also essential to the maintenance of cellular metabolism and homeostasis. Indeed, it has been shown that the mitophagy is involved in the pathogenesis of cardiovascular disease, diabetes, inflammatory disorders, and cancer [14]. Therefore, it seems that clarifying the mechanisms involved in the regulation of mitochondrial function and fusion process, fission and mitophagy with exercise training intensity can provide a critical understanding about the role of regular exercise on improving of myocardial infarction and could be of high importance in the context of clinical settings. Therefore the purpose of this study was to investigate of exercise training intensity effect on mitochondrial dynamics and mitophagy system in infarcted rats.

**METHODS**

**Study design and animals**

A total of 60 adult male Wistar rats (320g ±20g ~16 weeks of age) were used with accessibility of food and water in ad libitum. All animal procedures were performed based on the ethics committee of Rajaie Cardiovascular Medical and Research Center (RHC.AC.IR.REC.1395.40) and were cared for according to the guiding principles for the Care and Use of Animals on the basis of the Helsinki Declaration.

**Procedure of coronary artery ligation and echocardiographic measurements**

To anesthetize the rats Ketamine (75 mg/kg) and Xylazine (5 mg/kg) was combined and injected with a 16-gauge intravenous catheter orally intubated. With oxygen the positive pressure ventilation was preserved utilizing a Harvard rodent ventilator (Model 683, USA). To disinfect the surgical area betadine/alcohol wiping was utilized and the left anterior descending coronary artery (LAD) was ligated utilizing a 6-0 prolene (Ethicon Inc., USA) suture. [15]. At the end of operation, to recover the rats they were placed on the heating pad until anesthesia, warm sterile saline (0.5-1 mL, SC), Flunixin (1-2 mg/kg IM) and Cefazolin (25 mg/kg IM) were injected. By utilizing analgesia and hemodynamic monitoring for 24 h postoperative care was resumed [16, 17].

A VIVID, with a 12-MHz electronic, transducer seven-dimension system (General Electric-Vingmed Ultrasound, Horten Norway) were

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1 reactive oxygen species
utilized to echocardiography. Both four weeks after the surgery and at the end of the study protocols Echocardiographic examination was performed. To evaluate parameters such as the left ventricular end-diastolic diameter (LVEDD), the posterior wall thickness in systolic (PWTs), the left ventricular end-systolic diameter (LVESD) and the posterior wall thickness in diastole (PWTd) echocardiography was used. To calculate the LV ejection fraction (EF) and fractional shortening (FS) the following formulas were applied [18, 19]

\[
\%EF = \frac{(LVEDD^2 - LVESD^2)}{(LVEDD^2)} \times 100
\]

\[
\%FS = \frac{(LVEDD - LVESD)}{LVEDD} \times 100
\]

In this study the high degree myocardial damage rats which developed MI (FS ≤ 35%) based on echocardiographic result were included [20].

### Training protocol

Thirty - five rats which met the inclusion criteria which survived a MI out of sixty MI-operated rats were randomly set to the low intensity training (low intensity training (LIT, n= 6), moderate intensity training (MIT, n = 6), and high intensity training (HIT, n=6), MI-Sedentary (MI-Sed, n = 6)) and Control (CNTRL, n = 6). By walking at a slow pace (5m/min) for 5 min/day, five days per week the MI-Trained groups familiarized with a motorized. Following this step, the rats rested for two days before maximal oxygen uptake (VO2 max). To measure VO2 max each maximal rat’s running exercise test was utilized [21]. Control and MI-Sed animals did not received ET while LIT, MIT, and HIT animals were exercised according to the following table[22, 23]. Running intensities increased to 0.02 m/s per week by considering previous studies [21,22]. During the exercise training, no treadmill slope were applied [23].

<table>
<thead>
<tr>
<th>Exercise Protocol</th>
<th>Speed</th>
<th>Time(min)</th>
<th>Active rest</th>
<th>Times a week</th>
<th>Interval(min)</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low intensity interval training</td>
<td>55-60% VO2max</td>
<td>60</td>
<td>2min with 45-50% VO2max</td>
<td>5</td>
<td>4</td>
<td>6 week</td>
</tr>
<tr>
<td>Moderate intensity interval training</td>
<td>65-70% VO2max</td>
<td>60</td>
<td>2min with 50-60% VO2max</td>
<td>5</td>
<td>4</td>
<td>6 week</td>
</tr>
<tr>
<td>High intensity interval training</td>
<td>85-90 VO2max</td>
<td>60</td>
<td>2min with 50-60% VO2max</td>
<td>5</td>
<td>4</td>
<td>6 week</td>
</tr>
</tbody>
</table>

### Myocardial mitochondria preparations

Mitochondria were isolated using the tissue around of the infarcted areas. In order to prepare the Myocardial mitochondria for extraction of proteins, the infarcted hearts were cut into pieces. The residual blood was rinsed with medium I (0.25 mmol/L sucrose, 3.0 mmol/L HEPES, 0.5 mmol/L EDTA, pH 7.4), having detached the connective tissue. Then, samples were centrifuged in medium I (20 mL, 1200xg, 2 min; 50 mL, 1200xg, 2 min; 600xg, 5 min) and the resulting supernatant was homogenized again (10.000xg, 10 min). A manual homogenizer of Teflon-type was used to homogenize the resulted precipitation, adding 5 mL of medium II (0.25 mmol/L sucrose, 2.0 mmol/L HEPES, 0.5 mmol/L EDTA, pH 7.4).

The samples were then re-homogenized at 10.000xg for 10 min to attain the Mitochondria, contained in the final precipitation.

It should be noted that the whole aforementioned process were performed on ice and a BCA™ Protein Assay Kit (sc-202389, Santa Cruz Biotechnology, USA), with bovine serum albumin were employed at room temperature to determine the mitochondrial protein concentration [24].

### ELISA1 analysis

Microtiter plates were coated with target protein diluted in 50 mM carbonate buffer at pH 9.0. Optimal concentrations determined by titration, but for purified antigens 100 µl per well at 1 µg/ml is usually sufficient. Samples were incubated overnight at 4° C and covered with parafilm. Plates washed three times with PBS containing 0.05% Tween-20. We added 200 µl/well of blocking buffer (PBS containing 3% BSA) to block non-specific protein binding, then samples were incubated for 2 hours at room temperature. Then plates rinsed three times with PBS containing 0.05% Tween-20, then test antibody samples (SANTA CRUZ

1 Enzyme-linked immunosorbent assay
biotechnology, USA) and controls at 100 µl/well diluted in PBST were added. Antibodies serially diluted for determining titer or diluted to previously determined working concentration for antigen quantitation. Samples incubated 1 hour at room temperature.

Plates washed three times with PBS containing 0.05% Tween-20, in order to remove excess liquid as previously explained.

100 µl/well of alkaline phosphatase conjugated secondary antibody Monoclonal Secondary Antibodies (SANTA CRUZ biotechnology, USA), diluted to 1:100–1:1000 in PBST was added. Optimal antibody concentration is determined by titration. Samples incubated 1 hour at room temperature. Plates washed three times with PBS containing 0.05% Tween-20, then the liquid in wells has been removed. Wells washed once with diethanolamine buffer (10 mM diethanolamine, 0.5 mM MgCl2 (pH 9.5) and liquid has been removed. Diluted substrate in diethanolamine buffer to a final concentration of 1 mg/ml. Add 100 µl/well allowed to develop for 10–20 minutes or until positive control reaches an OD 405/490 of about 1.0, then reaction by adding 100 µl of 0.1mM EDTA, pH 7.5 stopped. Plates have been read on microtiter plate reader at OD 405/490.

**STATISTICAL RESULTS**

Weight changes, Ejection fraction (EF), and Fractional shortening (FS) among research groups at different stages of the study are presented in Table 1.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MI-SED</th>
<th>LIIT</th>
<th>MIIT</th>
<th>HIIT</th>
<th>Pre-test weight (gr)</th>
<th>EF</th>
<th>FS</th>
</tr>
</thead>
<tbody>
<tr>
<td>weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-test</td>
<td>340.00±30.80</td>
<td>349.00±41.28</td>
<td>355.16±32.80</td>
<td>346.83±29.26</td>
<td>373.33±31.65</td>
<td>Pre-test</td>
<td>50.14±7.48</td>
<td>39.24±3.53</td>
</tr>
<tr>
<td>Post-test</td>
<td>375.33±30.17</td>
<td>380.50±37.20</td>
<td>382.50±3193</td>
<td>363.33±26.28</td>
<td>350.00±29.68</td>
<td>Post-test</td>
<td>51.05±5.1</td>
<td>51.05±5.10</td>
</tr>
<tr>
<td></td>
<td>75.65±4.10</td>
<td>51.05±9.59</td>
<td>48.43±9.69</td>
<td>52.23±5.51</td>
<td>50.14±7.48</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>74.96±3.90</td>
<td>51.00±8.51</td>
<td>55.43±13.16</td>
<td>66.3±9.65</td>
<td>56.23±9.13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>39.24±3.53</td>
<td>51.76±5.67</td>
<td>20.78±5.20</td>
<td>25.41±4.36</td>
<td>22.59±4.70</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>38.76±3.47</td>
<td>22.83±5.13</td>
<td>24.19±6.25</td>
<td>32.77±6.24</td>
<td>25.60±5.75</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

The expression levels of mitochondrial proteins, mfn21, DRP12, Parkin and P623 were determined by ELISA. (1) Mfn2; (2) DRP1; (3) Parkin; (4) P62 and (5) PGC1α. The significance was calculated using one-way ANOVA followed by Bonferroni’s post-hoc test.

MI-SED: sham operation; MI: myocardial infarction; HIIT: myocardial Infarcted rats subjected to High intensity interval training. MIIT: myocardial Infarcted rats subjected to Moderate intensity interval training. MIIT: myocardial Infarcted rats subjected to Low intensity interval training. Control: None operated healthy rats.

1 Mitofusin 2
2 Dynamin-related protein 1
3 called Sequestosome 1 (SQSTM1), is an ubiquitin-binding scaffold protein
Figure 1. Mfn2 Protein levels changes in research groups, significant differences between MIIT and MI-SED groups *P < 0.05, also between Control and MI-SED ***P < 0.001.

Figure 2. Drp1 Protein level changes in research groups, significant differences between Trained groups and MI-SED **P < 0.01 also between Control and MI-SED ***P < 0.001.
Figure 3. Parkin Protein level changes in research groups, significant differences between Control and MI-SED groups *P < 0.05

Figure 4. P62 Protein level changes in research groups, significant differences between Control and MI-SED groups ***P < 0.001
DISCUSSION

The findings of the present study showed that protein level of MFN2, PGC-1α, Parkin and p62 significantly decreased in SED-MI group compared to healthy control while DRP1 protein levels significantly increased. Also, protein level of MFN2 and PGC-1α significantly increased (while protein level of DRP1 significantly decreased in MIIT group compared with SED-MI group). Exercise training leads to increase of intracellular production of ROS and energy regulating molecules such as ADP or AMP, free phosphate groups (Pi) and calcium ions (Ca²⁺), as they are considered as effective signaling transducers. Such substances can activate protein kinases including Protein kinase B (PKB), AMP-activated protein kinase (AMPK), p38 mitogen-activated kinase (p38-MAPK), calcium/calmodulin-dependent protein kinases (CaMK), Akt and Unc-51 Like Autophagy Activating Kinase 1(ULK1), also known as Jun kinase (JNK/SAPK) and extracellular signal-regulated kinase (ERK). Both mitochondrial dynamics and mitophagy system can be regulated by the above listed proteins [25, 26]. Generally, exercise training is known to contribute to muscle metabolic adaptations including improvement of the mitochondrial function. This is mainly because some important pathways of mitochondrial biogenesis, dynamic and mitophagy cause to maintain or change the mitochondrial function, as they drive the signaling cascades, influencing the phenotypic changes of the mitochondria. Therefore, exercise training, calorie restriction (CR) and antioxidant supplementation are known as the therapeutic applications to mitigate the mitochondrial dysfunction, employing the above multiple mechanisms [5]. In fact, many studies have widely reported the increase in mitochondrial biogenesis signals and their performance in response to the intensity and duration of exercise and more importantly is that these changes were not limited to a particular form of exercise [27-29]. It has been shown that exercise training improves mitochondrial dynamics and quality through enhanced mitophagy system. Evidence showed that mitophagy is regulated by AMPK, therefore, when mitochondria are disrupted, AMPK by increasing receptor p62/sequestosome triggers mitophagy system in two ways: First, by inhibiting mTOR through localizing to the lysosome, because mTOR through the phosphorylation inactivates the. If ULK1 is disabled, it will inhibits autophagy and mitophagy happens. Secondly, AMPK causing a progress in mitophagy through direct effect on ULK1. Exercise may cause mitophagy through AMPK-ULK1 signaling pathways in skeletal muscle. But this hypothesis still is not agreed how the mitophagy system could recognize disrupted mitochondria in response to exercise. The phosphatase and tensin homolog (PTEN)-induced putative kinase (PINK4)/Parkin cascade is one of the most well-known ways of targeting the impaired mitochondria by mitophagy process. Mitochondrial damage causes the loss of membrane potential of the mitochondria, resulting in accumulation of PINK1 on the translocase.

Figure 5. PGC1α protein level changes in research groups, significant differences between MIIT and MI-SED groups, **P < 0.01

Also ***P < 0.001 between Control and MI-SED groups

1 Peroxisome proliferator-activated receptor gamma coactivator 1-α
2 Adenosine Monophosphate-Activated Protein Kinase
3 Mammalian Target of Rapamycin
4 PTEN-induced putative kinase 1
of the OMM1 complexes, which leads to recruitment of Parkin and accumulation of P62. The damaged mitochondria is finally degraded via mitophagy process, employing the autophagosome [26]. Moreover, endurance training increases vagal tone that these nerves could be impaired in heart failure, acetylcholine as vague nerve mediators can reduce oxidative response, activate the vague nerve inhibits dynamic perturbations as well. Disruption of calcium cycle and reduced calcium -calmodulin dependent kinase (CaMK) can reduce the expression of PGC-1α, the aerobic interval training with regulation of calcium and increased expression of CaMK, could have beneficial effects on PGC-1α [24]. Tao et al. has demonstrated three-week swimming significantly reduced infarcted area as well as autophagic and apoptotic activity in Rats heart. Furthermore, by increasing the expression of mtDNA2 levels mitochondrial biogenesis were increased. According to the researchers, PGC-1α acts as a key regulator of mitochondrial biology could be affected by exercise training and has a vital role in cardiomyocyte metabolic control and cardiovascular disease. Therefore, they observed that the PGC-1α expression and related downstream proteins such as TFAM3 and NRF2B24 significantly increased in response to swimming [30]. It has been suggested that the protective effects of exercise training due to improvement of glucose and fat metabolism is associated with increased mitochondrial biogenesis with activation of PGC-1α. Increased apoptosis and autophagy may be a sign of cardiomyocytes reduction that is caused by infarction. Accordingly, another way of protective activity of exercise training against myocardial infarction could be reduced apoptotic activity. Reduction in mitochondrial function and biogenesis after myocardial infarction is the main and dominant cause of heart failure development. It is postulated that PGC-1α could be used as a potential marker for heart recovery capacity after infarction. Finally, exercise with Beta-3 adrenergic receptor stimulation and activation of the signaling pathway of nitric oxide could also help protect against ischemia-reperfusion injury [30]. Generally, despite the lack of ROS measurements, it seems that interval exercise with different intensities results in the improvement and regeneration of MI-mediated mitochondrial function by changing the expression of mitochondrial dynamics and mitophagy proteins, which the role of intermediate intensity training was more obvious. In conclusion, we highlighted intermediate-intensity interval training leads to improved mitochondrial fusion and fusion and mitophagy proteins in rats with myocardial infarction. Exercise training is the best and easiest intervention for most cardiovascular disease in which mitochondrial network dynamics play a key role. However, more clinical trials and studies addressing the mechanisms of action of mitochondrial dynamics and mitophagy regarding impact of exercise training are needed to consider in clinical practice. Some approaches for quantification and evaluation of mitochondrial dynamics and mitophagy in living muscle cell, especially in cardiomyocyte should be launched. It is explicit, there is much more to be learned about the mechanisms responsible for exercise-induced cardioprotection against CAD.

**CONFLICT OF INTERESTS STATEMENTS**

The authors declare no conflict of interest.

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1 outer mitochondrial membrane  
2 Mitochondrial DeoxyriboNucleic Acid  
3 transcription factor A  
4 nuclear respiratory factor beta2
REFERENCES


