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Research paper

Effect of Exercise Intensity on Mitophagy-related Proteins and Reactive Oxygen Species in Rats with Myocardial Infarction

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Abstract

Background & Objectives: Myocardial infarction (MI) is a common heart disease in humans, and mitochondrial dysfunction has been implicated in its development. Exercise plays a protective role against the disease; however, the role of reactive oxygen species (ROS) and mitophagy proteins is not well understood. The aim of this study was to investigate the effects of exercise intensity on the levels of mitochondrial proteins and ROS in the heart tissue of male rats' post-MI.

Materials & Methods: We induced MI in 40 male Wistar rats by ligating the left anterior descending artery. Animals were divided into five groups as follows: three exercise groups (high-, moderate- and low-intensity) post-MI, one sedentary group post-MI, and one control group without MI or exercise. The levels of P62 and Parkin (Mitophagy) proteins and ROS were determined in the heart tissue by ELISA and DCFDA methods.

***Results*:** Data analysis showed the levels of free P62 and Parkin proteins decreased significantly in the sedentary group compared to those in the controls ($P \leq 0.05$). The ROS level of cardiac tissue decreased significantly in the moderate-intensity group compared to that in the sedentary group, but it increased compared to that of the control group ($P \leq 0.05$).

Conclusions: The MI decreased P62 and Parkin proteins and increased ROS in the infracted rat heart. Moderate-intensity exercise significantly increased the levels of free

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P62 and Parkin proteins and reduced the ROS in the heart tissue post-MI. The findings support the hypothesis that improvements in the levels of ROS and proteins involved in mitophagy are stimulated by performing moderate-intensity exercises.

Keywords*:* Myocardial Infarction; Mitophagy, Mitochondrial Dysfunction, Exercise intensity, P62 and Parkin Proteins Expression, Reactive Oxygen Species

Abbreviations: PINK1: PTEN induced putative kinase, Parkin: Parkin RBR E3 ubiquitin-protein ligase. P62: Sequestosome 1, ROS: Reactive oxygen species

Introduction

Cardiovascular disease is the leading cause of death in humans worldwide, and myocardial infarction (MI) due to coronary artery disease is the most common condition [1]. Pathologically, MI results in cardiac tissue damage due to myocardial ischemia, leading to biochemical and biophysical changes in the heart tissue and left ventricular failure [2, 3]. The transformed cardiac muscle is characterized by mitochondrial dysfunction, which has been known to be the cause of heart failure [4, 5]. Biologically, the main function of mitochondria is the production of adenosine triphosphate (ATP) through oxidative phosphorylation using the Krebs cycle, electron transport chain, and beta-oxidation of fatty acids. Pathologic changes in mitochondria lead to its dysfunctions, such as loss of oxidative power and antioxidant defense secondary to a rise in the reactive oxygen species (ROS) level. Significant decline in oxidative phosphorylation and ATP generation is convincing evidence that mitochondrial dysfunction precedes MI, primarily due to the defective respiration enzymes in the mitochondria [1, 5-7]. Mitochondrial dysfunction is associated with structural changes including fusions and fissions – two important changes that affect mitochondrial homeostasis [8].

It has been suggested that autophagy plays an important role in mitochondrial quality control [9]. Autophagy is a mechanism for cellular degeneration through the action of lysosomes, which is also important for cell survival during energy stress [10]. Accordingly, mitophagy promotes the digestion of damaged mitochondria by selectively deleting them. Mitochondria play a vital physiological role in protecting the heart muscle from ischemic damages especially the following reperfusion. The pathophysiology of P62, Pink 1, and Parkin is the best-known mechanism of mitochondria in mammalian cells [11,12]. Damage to mitochondria leads to the activation of both mitophagy and apoptosis pathways [12].

In order to prevent cell death, damaged mitochondria are isolated by phagosomes and get destroyed before cell apoptosis or necrosis occurs [13]. The PINK/Parkin route is the most important pathway for the cellular regulation of damaged mitochondria [13]. For this purpose, it has been shown that the damaged mitochondria bound to Parkin protein are gradually digested by autophagy.

However, via detecting a signal transduction adaptor protein (P62/SQSTM1), autophagy provides an important insight into the mitochondrial process. Studies have shown that P62 invokes mitochondria in a Parkin-dependent pattern, and the removal of P62 leads to the inhibition of mitochondria [14,15].

In addition, the mitotic process plays a significant role through targeting the proteins that regulate mitochondrial dynamics; hence, preventing heart myocardial damage due to the ischemia. Although the association of the mitochondria with the development of MI is not fully clear, they seem to play an effective role in regulating the post-MI pathologies [16]. Despite a better understanding of MI, pathologic processes and use of various drugs in recent decades, mortality from MI is still high [16,17]. Therefore, new strategies are required to prevent ischemic and reperfusion injuries and to improve the survival rate after MI. It is well accepted that prescribing regular exercises has a positive effect on most bodily systems and reduces the risk of chronic conditions, such as cardiovascular disease [18]. Cardiovascular adaptation to regular exercise has caused a rise in the mitochondrial respiratory enzymes, which support improved cardiac muscle contraction. However, our understanding of the underlying mechanisms is very limited currently [19, 20].

Mitophagy is a new issue recently introduced in the field of sports and health research; however, there is little evidence about the impact of exercising on mitophagy. Many of the benefits of sports activities overlap the protective functions of autoimmune pathways, and so many of the benefits may be due to the activation of mitophagy. It has been indicated that eight-week exercise and acute training significantly reduce P62 levels in rat brain cells followed by an increase in mitochondrial autophagy [21]. In addition, it has been reported that four weeks of moderate-intensity aerobic exercise increase PGC-1α levels in Sprague rats and facilitate mitochondrial autophagy by converting LC3-I into LC3-II and other related factors. Based on the activation of the PGC-1a pathway, exercise training seems to improve mitochondrial quality and decreases the incidence of pathologies [22]. Nevertheless, it is now clear that in cardiovascular disease, the relevant markers are changing in cardiac myocytes, and it seems that exercise training has a beneficial role in the improvement of heart function following MI, reflecting the role of mitophagy in this process [23]. The present study was conducted to investigate the effects of three exercise intensities on the expression of P62 and Parkin proteins in the cardiac muscle of rats with induced MI.

Methods & Materials

Setting: The research methodology was based on a pre- and post-experiment design. This study received the approval of the Ethics Committee of Rajaie Cardiovascular, Medical and Research Center, Tehran, Iran (Certificate#:

RHC.AC.IR.REC.1395.40). Fifty-five 16-week-year-old Wistar male rats with weight range of 320g (±20g) were purchased from the Iranian Pasteur Institute, Tehran, Iran. They were transferred to the animal house at the Experimental Research Center of Rajaie Hospital and were kept according to the policy of the Iranian Society for the Protection of Animals used for scientific and laboratory experiments. All rats were kept in 4-way cages under controlled environmental conditions at 22°, under a dark-light cycle of 12:12 hours, relative humidity of 50%, with free access to water and rat food. Figure 1 represents the study flow chart.

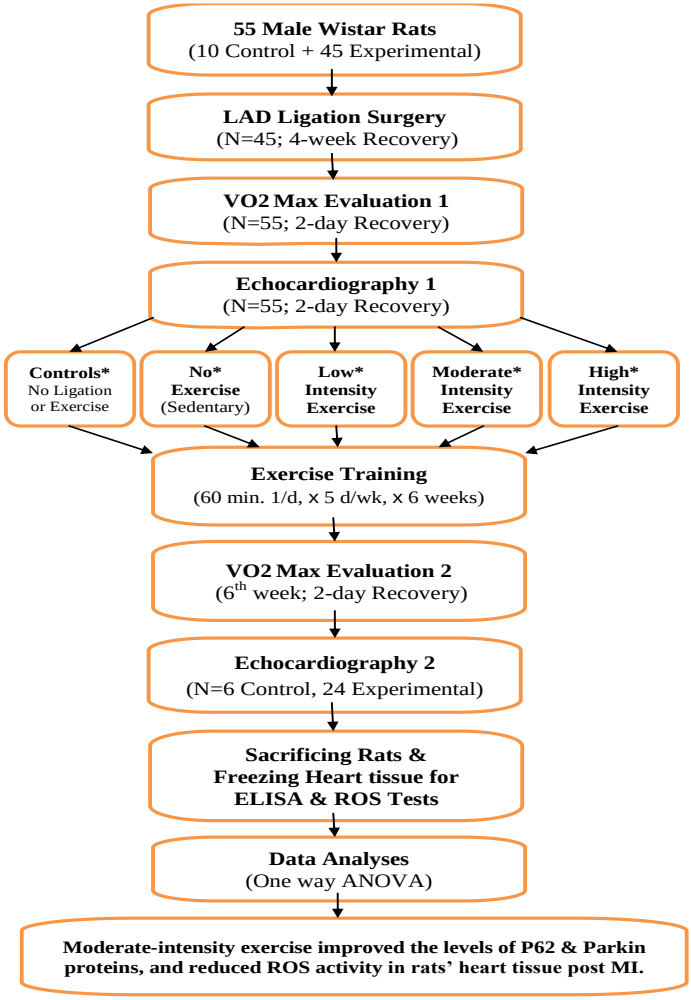

Figure 1: Study Flow Chart
* = Group of 6 comparable rats. LAD = Left anterior descending artery. ROS = Reactive oxygen species.

Animal Grouping

The goal was to select a sample that was homogeneous for its data on VO2 max and the markers of mitochondrial autophagy, P62, and Parkin proteins. For this purpose, an *initial* followed by a *final* grouping strategy was planned. Of the 55 Wistar rats, 10 cases were assigned into the initial control group, which did not undergo surgery or exercise training. The 45 rats in the initial experimental group were subjected to ligation of the left anterior descending (LAD) artery. All of the five groups were allowed 4 weeks of recovery (see below). All rats were then tested for VO2 Max and echocardiography, and the data were used to assign the final control and experimental groups. Out of the 10 initial control rats, six ones were found with the closest VO2 Max and echocardiography data. These were assigned as the final control group. Similarly, 24 rats were selected based on the VO2 Max and echocardiography data being closest to those in the final control group. These were randomly assigned into four experimental groups consisting of one "*no-exercise*", and three "*low-*, *moderate-* and *high-intensity exercise*" groups (Fig. 1). See details of the exercise protocols below.

Myocardial Infarction

To induce MI in rats, the LAD arteries were completely sutured [24] as described below. After adaptation to the new environment, each rat underwent anesthesia with ketamine (75 mg/kg) and xylazine (10 mg/kg), the chest hair was shaved, and the animal was intubated under controlled ventilation [25]. The left side of the chest was horizontally incised to a depth of 4-5 cm, and the cardiac surface was fully exposed, the LAD was visualized and completely ligated by suturing. After the LAD ligation, the surgical site was closed by stitching. After the surgery, the rats remained under assisted ventilation until they normally woke up and started breathing [25]. The rats were prepared for echocardiography one week after the surgery. All animal surgeries were performed by an expert veterinarian at the Experimental Research Center of Rajaie Cardiac Hospital in Tehran, Iran.

Echocardiography

After surgery and blocking, the LAD ligated rats were placed on separate shelves for four weeks. After 4 weeks of recovery, rats were anesthetized at the surgical ward and underwent echocardiography on the 4th and 10th weeks post-surgery. The procedure was done by an echocardiography specialist at the Radiology Department of Shahid Rajaie Hospital. The ejection fraction (EF) and fractional shortening (FS) of the rat hearts were measured. The rats with FS less than 35% that had MI were selected for this study. The EF and fractional shortening were calculated according to the following formulas [26].

- EF: (LVDd2-LVDs2)/LVDd2,
- FS: ((LVDd-LVDs)/LVDd)100
- LVDd: left ventricular diameter at the end of diastole
- LVDs: left ventricular diameter at the end of systole

Familiarization with Exercise

After animal grouping (section 2.2), the exercise groups met for running on the treadmill for one week with five 10-15-minute sessions per week at the speed of 8-10 m/min. After the familiarization period, the actual exercise protocol was performed for each group to measure VO2 Max and the maximum running speed of rats to exhaustion was determined. Then, the rats in each group performed their specific exercise training protocols. The control and no-exercise groups did not participate in any of the exercise protocols.

Low-Intensity Exercise Protocol

consisted of ten cycles of running on a treadmill for a total of 60 minutes. Each cycle included four minutes at an intensity of 55-60% VO2 Max and two minutes of active recovery at an intensity of 45 to 50% VO2 Max. These sessions were conducted three days a week for 6 weeks [27].

Moderate-Intensity Exercise Protocol

comprised ten cycles of running on the treadmill for a total of 60 minutes. Each cycle included four minutes at an intensity of 65-70% VO2 Max and two minutes of active recovery at an intensity of 50 to 60% VO2 Max. These sessions were done three days a week for 6 weeks [28].

High-Intensity Exercise Protocol

included ten cycles of running on the treadmill for a total of 60 minutes. Each cycle consisted of four minutes at an intensity of 85-90% VO2 Max and two minutes of active recovery at an intensity of 50 to 60% VO2 Max. These sessions were carried out three days a week for 6 weeks [29].

Tissue Sample Collections

To collect the cardiac tissue samples, all rats from the five groups were anesthetized 48 hours after the last exercise session with a combination of ketamine (75 mg/kg) and xylazine (10 mg/kg) intraperitoneally. After ensuring adequate anesthesia, the rat chest was opened, and a blood sample was taken directly from the animal's heart. Then, the heart was carefully removed, and the left ventricle was washed in normal saline and transferred immediately to the microtubes, immersed in liquid nitrogen, and transferred to a freezer at -80°C for further processing.

Measurement of P62 and Parkin Proteins Contents

The direct ELISA was used to determine the concentrations of P62 and Parkin proteins as follows: 100µl of the desired protein was added to 100ng/100µl, and the plate was refrigerated overnight at 4°C. The next day, the plates were washed three times with phosphate-buffered saline (PBS), and each time, 250µg of PBS was added to the well for 30 seconds. The next step was blocking, then, thoroughly rinsed with PBS three times, each time, 250µl from the PBS was poured into the well and held for about 30 seconds. In each well, 100µl of the initial antibody at a concentration of (e.g., 1/15000) is dispensed at room temperature for one hour.

The next step is rinsing three times with PBS, and each time we added 250µlPBS into the well and waited for about 30 seconds, and then washed. To each well, we added 100µl of the secondary antibody at a concentration (e.g., 1/15000) for one hour at room temperature. Next, the plate was rinsed with PBS five times, 250µl of PBS was kept per well for 30 seconds, and then washed it. At this point, 100µl tetramethylbenzidine (TMB) substrate was dispensed into the wells for 10-20 minutes, and if the staining was adequate to stop the reaction. At this stage, 100µl of stop solution was added to each well, and the plate was read by ELISA reader [30].

Measurement of ROS Content

The ROS was measured using 2′,7′-dichlorofluorescein diacetate (DCFDA) fluorescence assay. The method is based on the transformation of nonfluorescent DCFDA to the highly fluorescent DCF in the presence of ROS. Tissue samples were homogenized in 0.05% Trypsin-EDTA (Invitrogen; USA) and incubated for 30 min at 37°C. after centrifugation (5 min at 14,000 g), pellets were incubated in DCFDA dissolved in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, USA) at a final concentration of 5µM for 30 min at 37°C. Samples were centrifuged for 5 min at 14,000 g, and pellets were resuspended in 200µl of lysis buffer and incubated for 10 min at 4°C under constant agitation. After that, the samples were centrifuged at 14,000 g for 5 min, and supernatants were transferred into plates. The DCF was determined by spectrophotometry, using a fluorescence plate reader at 485nm excitation/530 nm emission. Data were normalized to protein content, and the results were expressed as a fold change relative to the controls [31].

Statistical Analysis

The data were analyzed by SPSS 20 at the significance level of ≤ 0.05. After the data were normalized by the Kolmogorov-Smirnov test, the one-way ANOVA and Bonferroni post hoc test were used to delineate the significance of the variables in the animal groups.

Results

Changes in body weight, EF, and FS among the animal groups at different stages of the study are presented in Table 1.

Table 1- Mean and Standard Deviation of Rats' Weight (g), EF, and FS During the Study

Control	Exercise Intensity				Parameter	
	None	Low	Moderate	High		
340.00±30.80	349.00±41.28	355.16±32.80	346.83±29.26	373.33±31.65	Pre-test	weight (g)
375.33±30.17	380.50±37.20	382.50±31.93	363.33±26.28	350.00±29.68	Post-test	
75.65±4.10	51.05±9.59	48.43±9.69	52.23±5.51	50.14±7.48	Pre-test	EF
74.96±3.90	51.00±8.51	55.43±13.16	66.3±9.65	56.23±9.13	Post-test	
39.24±3.53	22.76±5.67	20.78±5.20	25.41±4.36	22.59±4.70	Pre-test	FS
38.76±3.47	22.83±5.13	24.19±6.25	32.77±6.24	25.60±5.75	Post-test	

The results of ANOVA showed that there was a significant difference in EF and FS values in the pretest (4 weeks postoperative) ($P<0.05$). Based on the Bonferroni test, EF and FS values in MI groups were significantly lower than those in the healthy controls ($P<0.05$). In order to compare the animal groups after 6 weeks of training, the differences between the pre- and post-tests for each group were analyzed using ANOVA test. The results indicated that there were significant differences among the groups ($P<0.05$). The increases in EF and FS values in the moderate-intensity exercise group were significantly higher than those in the high-, low-, and no-exercise groups ($P<0.05$). However, the improvement in EF and FS values between the high- and low-intensity exercise groups was not statistically significant ($P>0.05$) compared to that in the no-exercise group (Figures 2 and 3).

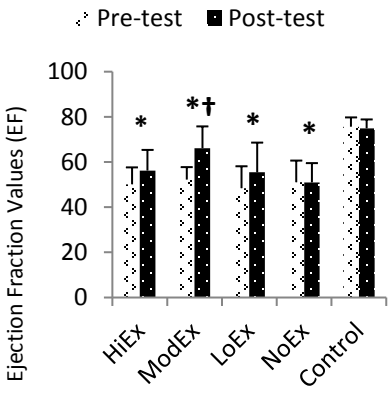

Figure 2- Ejection fraction amounts of study groups.

* Significance compared to healthy control group.

† Significance compared to no-exercise group.

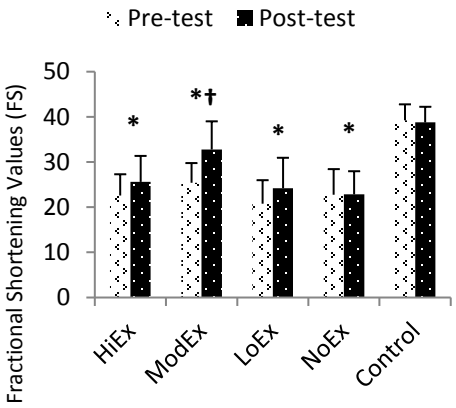

Figure 3- Fractional shortening amounts of study groups. * Significance compared to healthy control group.

†Significance compared to no-exercise group.

The results of data analysis for Parkin protein levels demonstrated that there were significant differences among the animal groups (P=0.01, P = .13 / 4) (P<0.05). Based on Bonferroni test, the Parkin protein level in the heart muscle was significantly lower in the no-exercise group compared to that in the control group (P <0.05). However, despite the increases in Parkin protein levels for all exercise groups, especially in the moderate-intensity exercise group, these changes were not statistically significant (P> 0.05) compared to the no-exercise group (Figure 4).

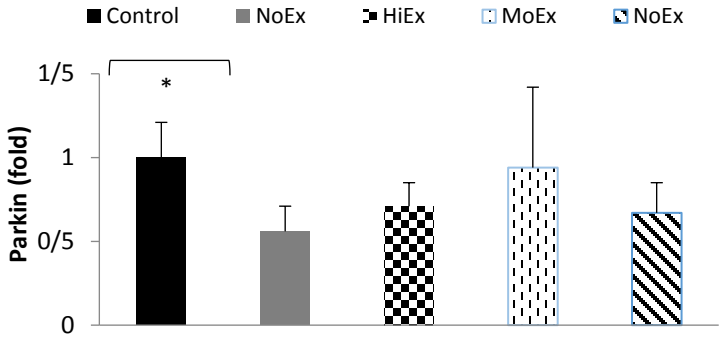

Figure 4- Parkin Changes in the Study Groups. * Significance Compared to the Control Group (P<0.05).

In addition, the P62 protein levels in the heart muscles (Figure 5) were significantly different among the experimental groups (P=0.001; F=35.9) (P<0.05).

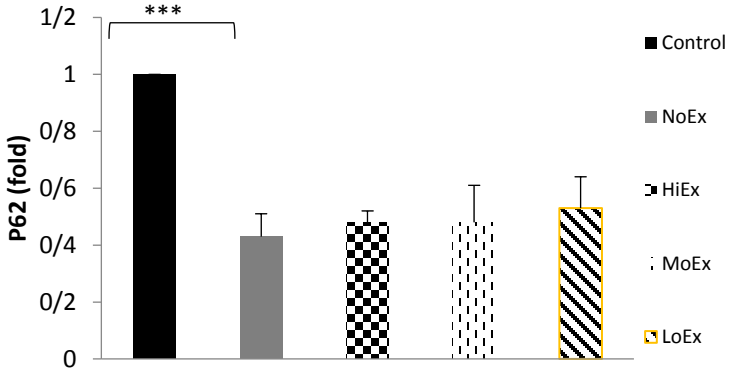

Figure5- P62 Changes in the Study Groups; * Significance Compared to the Control Group (P<0.001).**

The ROS levels among the LAD ligated groups were significantly different (P=0.001; F=7.0) (P<0.05). The results of Bonferroni post hoc test illustrated that induction of MI resulted in a significant increase in ROS compared to that in the control group (P <0.05). In contrast, all of the exercises resulted in a decrease in the ROS levels; however, these changes were statistically significant only for the moderate-intensity exercise group (P<0.05).

Based on Bonferroni test, the P62 in the no-exercise group decreased significantly compared to that in the control group (P<0.05). Moreover, P62 changes in the exercise groups were not significantly different from those in the no-exercise group (P>0.05).

Discussion

One of the most important findings of the present study was that induction of the MI led to a reduction in mitochondrial functions, secondary to reductions in the main proteins. Metabolic changes are the major mechanisms that influence the development of cardiac muscle remodeling post-MI. The myocardium is a tissue with high oxidative capacity, and the mitochondria play a key role in maintaining optimal heart function. Mitochondria function is affected by fusion, fission, and mitophagy. Therefore, changes in mitochondrial morphology serve as a mechanism for the biological adaptations and repair after MI [32]. Autophagy can be regulated by combination in bloodstream circulating or placing within the subendothelial layer of atherosclerotic plaque, it has been reported that the

macrophage-localized autophagy is associated with vascular diseases. During lesion formation, autophagy markers were observed in atherosclerotic plaques (p62/SQSTM1and LC3). Elimination of damaged mitochondria via mitophagy induces preconditioning cardiomyocytes and reduces cell death in ischemic/reperfusion. It has been shown that impaired mitophagy leads to inflammatory responses in cardiomyocytes and induces myocarditis and dilated cardiomyopathy [33].

The removal of damaged mitochondria by mitophagy is one of the most important dynamic features involved in the repair and remodeling of the heart. Mitophagy is essential in maintaining cardiac homeostasis, a concept supported by studies in mice lacking mitophagy components. Mice deficient in PINK1 protein develop left ventricular dysfunction, cardiac hypertrophy, increased oxidative stress and mitochondrial dysfunction [34]. Furthermore, Parkin-free mice are more susceptible to doxorubicin-induced cardiomyopathy [35] and are more prone to MI than normal mice [36].

In the current study, induction of MI was accompanied by a decrease in the levels of major proteins involved in the mitophagy process. Both P62 and Parkin proteins in the heart muscle in the infarcted areas decreased, which is consistent with the findings reported by previous studies [36,37]. This suggests that P62 and Parkin proteins are likely to be involved in the post-MI recovery of the damaged myocardial tissue. In contrast, ischemia results in the attachment of Parkin to mitochondria for its protective role [38]. Recently, Parkin has been suggested to be involved in the clearance of mitochondria from the ischemic regions of rat heart tissue [38]. Considering the involvement of mitophagy proteins in the repair processes of heart tissue, investigation of these proteins can be a useful approach in the prevention of heart failure after MI.

After the acute phase of the MI, a series of structural and functional changes occurs in the myocardium, collectively known as the cardiac remodeling process. The process involves fibrosis, inflammation, mitotic hypertrophy, and apoptosis, leading ultimately to advanced heart failure [39]. Although heart tissue changes are well documented after MI, cardiac remodeling is a complex dynamic process, involving such biological events as improvement in mitochondrial function. A defective remodeling after MI can lead to advanced heart failure [40]. Conversely, performing regular exercises can prevent or minimize the development of cardiovascular disease after MI [41]. Therefore, formulating treatment protocols to prevent cardiac damages secondary to MI is an important clinical significance. It has been well documented that performing regular exercises has beneficial effects on the management of post-MI pathologies [18, 42]. Moreover, the beneficial effects of exercising have been reported to improve mitophagy capacity [43]. The significant finding of the ongoing study was that performing interval exercises, independent of exercise intensity, could lead to improved regulation of

proteins involved in mitochondrial dynamics. The results of the present study demonstrated that P62 and Parkin proteins increased in all of the three exercise intensities. There are limited studies examining the effects of athletic activities on the levels of proteins in cardiac myopathy. In this regard, it has been reported an increase in proteins involved in the mitochondrial process including P62, LC3BI in response to exercise. In addition, increased expression of mitochondrial proteins such as P62, Parkin, and PINK has been reported in response to exercise in various tissues, including skeletal muscle [44-46]. The precise mechanism of mitochondrial control in myocardial remodeling, especially versus mitophagy is not quite clear.

Though the exercises reduced the ROS levels of the heart tissue, these changes were significant only in the MIIT group (Figure 6).

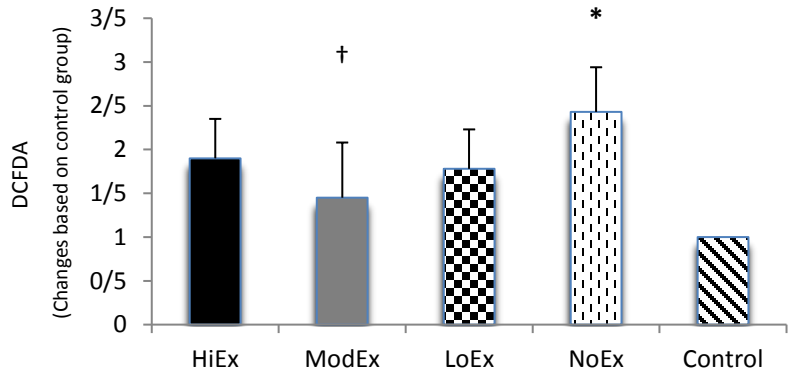

Figure 6- ROS Variations Between Study Groups, *= Significance Compared to the Healthy Control Group.

†= Significance compared to the no-exercise group.

In this relationship, exercise training with a moderate intensity leads to an appropriate balance between the production of ROS and its buffer [47], in contrast, high-intensity exercise training appears to have resulted in a transient increase in the inflammatory and oxidative state of the cell [48]. On the other hand, low-intensity interval training is not a suitable stimulant for activating cellular signaling involved in the development of PGC-1a. Therefore, moderate-intensity interval exercise seems to be a more suitable stimulus for activating the pathways involved in the mitochondrial process due to the optimal regulation of ROS production and, possibly, PGC-1a. The findings of the ongoing study supported the hypothesis in terms of improvement in ROS levels and a significant improvement in the proteins involved in the mitophagy process (P62 & Parkin) in the mean intensity of the interval exercise.

Conclusions

It was concluded that the MI caused an increase in ROS levels in the rat heart muscle. Moderate-intensity exercise significantly increased the levels of P62 and Parkin proteins and reduced ROS in the heart tissue of rats with MI compared to those noted for the no-exercise group. The findings of this study supported the hypothesis that the levels of free P62 and Parkin proteins increased while that of the ROS decreased due to performing interval exercises, especially after the moderate-intensity exercise.

Conflict of Interests

The authors declare no conflict of interest.

Acknowledgments

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