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Original Article

Preventive effects of intense continuous endurance training on isoproterenol-induced cardiac apoptosis and protein synthesis gene expression in wistar rats

***Majid Gholipour* [1,*], *Mehran Ghahremani* [2], *Mohammad Reza Asad* [2], *Arezoo Tabrizi* [1]**

[1] Department of Physical Education, Sharif University of Technology, Tehran, Iran
[2] Department of Physical Education and Sports Sciences, Payam-e Noor University, Alborz, Iran

*** Corresponding Author:**
Address: Department of Physical Education, Sharif University of Technology, Azadi St, Tehran, Iran. **Postal code:** 1455889694; **Tel:** 982166165152; **Email:** gholipour@sharif.edu

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Abstract

Objectives: Heart failure contributes to cellular lesions and left ventricular dysfunction. The present study aimed to determine the effects of intense continuous endurance training on protein synthesis gene expression and prevention of isoproterenol-induced cardiac apoptosis.

Methods: In this experimental study, 16 male Wistar rats were assigned to exercise and control groups. After eight-week treadmill running, with 15° inclination, at 65-75% of VO_{2max} for 30-60 minutes, Isoproterenol (3 mg/kg) was injected into the rats subcutaneously for 7 days. And 24 hours after the last injection, the left ventricular tissue was stored at -80°C for qRT-PCR and TUNEL assay. Between-group differences were determined by parametric and non-parametric tests, using SPSS software version 24.

Results: There was a significant increase in mTORC1 gene expression in the training group (P = 0.026) but AMPK alterations failed to be significant. eEF2K gene expression was suppressed in the training group (P = 0.001) which resulted in a significant increase in eEF2 expression (P = 0.005). Left ventricular weight and heart weight/body weight increased compared to the control group (P = 0.028, P = 0.010, respectively). And the training protocol effectively prevented the formation of isoproterenol-induced apoptotic cells (P <0.001).

Conclusions: The exercise training protocol increased protein synthesis gene expression, and improved cardiac protection by reducing apoptosis. This protocol can be considered a promising modality in preventing and reducing apoptosis induced by heart disease such as myocardial infarction.

Keywords: Treadmill endurance training, Cardio protection, mRNA translation, Apoptosis, Wistar rat

Introduction

The main function of the heart as a vital organ is to pump blood to the peripheral organs, including itself, to meet its demands like supplying nutrients and oxygen (1). Necrosis, apoptosis, and autophagy are the pathological complications of acute myocardial infarction (2).

Cardiomyocytes are terminally differentiated soon after birth. Recent studies have shown a potential to reactivate the cardiomyocytes' capacity for proliferation. Hence, the size of the adult heart is determined by an increase in the size of each cardiomyocyte, rather than an increase in cardiomyocyte number. Cardiac hypertrophy is a response to an increased workload (e.g. exercise training) to reduce stress on the ventricular wall to maintain function and efficiency. Both physiological and pathological hypertrophy is

induced by heart adaptation and response to stress, but their molecular mechanisms are quite different. Interestingly cardiac function is well-preserved over time in physiological hypertrophy, whereas pathological hypertrophy may lead to heart failure and death (1). Recent studies have demonstrated adverse cardiac remodeling and heart failure (3). Apoptosis is involved in the loss of the myocardium after myocardial infarction, left ventricle remodeling, and the development of heart failure symptoms (4). A protein synthesis pathway is essential for cardiomyocyte hypertrophy. It is regulated by phosphorylation and dephosphorylation of multiple translational factors and ribosomal proteins. Eukaryotic Elongation Factor 2 (eEF2) plays a key role in the elongation stage of protein synthesis, which is phosphorylated and inhibited by Eukaryotic Elongation Factor 2 kinase (eEF2K).Phosphorylation of eEF2 leads to inhibition of translation elongation and protein synthesis. Mammalian Target of Rapamycin Complex (mTOR) phosphorylates and inhibits eEF2K, resulting in increased eEF2 gene expression and activation (5, 6). It has been shown that pharmacological activation of AMP-activated Protein Kinase (AMPK), as a cellular energy sensor, inhibits protein synthesis of cardiac myocytes through the eEF2K/eEF2 axis (7, 8). Generally, it has been accepted that moderate to heavy load resistance training, as a modality to increase hypertrophy and muscle strength, optimizes muscle hypertrophy and strength, respectively, while low-load training optimizes local muscular endurance. In other words, the intensity and duration of exercise affect hypertrophy and muscle size (9). Note that aerobic exercise training was once believed to have a small effect on changing muscle size. Over the last two decades, however, it has been shown that aerobic exercise training improves muscle function and performance by activating protein synthesis and increasing skeletal muscle size (hypertrophy). However, further investigations are needed to make this elucidated (10). Recently, the effectiveness of moderate-intensity endurance training in preventing apoptosis-induced myocardial infarction has been reported,but the factors involved in cardiomyocyte protein synthesis have not been investigated (11). In addition, it has been reported that endurance exercise training causes a more reduction of the symptoms in old mice compared to young ones (12). The results of a study revealed that moderate-intensity endurance training is more effective in reducing and preventing aging-induced cardiac apoptosis. Whereas high-intensity interval training does not protect against cardiac damage (13). Despite the contradictory data, there are two important but unclear points: 1) More intense and prolonged exercise training is more effective on muscle hypertrophy than moderate and low intensity with short-term exercise training, 2) More intense and prolonged exercise, as a stress, can induce apoptosis (14). In other words, an effective training protocol should be performed with such intensity and duration, leading to a significant increase in protein synthesis pathway and cardiac hypertrophy while protecting the heart by reducing cardiomyocyte lesions such as apoptosis. A study reported that high-intensity interval training is more effective in reducing oxidative stress, and improving heart function in infarcted rats than moderate aerobic exercise (15). To the best of our knowledge, the effect of intense endurance exercise training on alterations of the gene expression of the protein synthesis pathway and its protective effect on the incidence of isoproterenol-induced cardiac apoptosis has not been investigated. The present study aimed to investigate the effects of intense continuous endurance exercise training, running on a treadmill with 15° slop, on the left ventricular gene expression involved in protein synthesis pathway, AMPK, mTORC1, eEF2, and eEF2k, in Wistar rats and to determine the prevention of apoptosis formation induced by isoproterenol injection at the end of the training period.

Materials and Methods

This study, which was conducted in 2019 at the animal laboratory of Tehran University, was in accordance with the Declaration of Helsinki, and ethical approval was provided by the Research Ethics Committee of the Payame Noor University, IR.PNU.REC.1398.035. A total of 16 healthy male Wistar rats, aged 8 -weeks and weighing 190 ±10 g, were obtained from Pasteur Institute and randomly divided into exercise and control groups. Three to four rats were kept in standard cages under controlled conditions, 22-24°C, relative humidity of 45% - 55%, the light-dark cycle of 12:12 h, and free access to standard water and food to adapt to the new environment. After a two-week familiarization with treadmill running, 5 sessions per week, the maximum oxygen consumption test (VO2max) was conducted before the main exercise training protocol and continued throughout the study biweekly. The treadmill speed was increased by 0.03 m/s every 2 min until the rat was no longer able to keep running, to determine the

maximum oxygen consumption following warming up at 40% -50% VO2max for 10 min(16).

Training intervention

The intensity of running on the treadmill with a slope of 15° was 65-75% of VO2max for 30 minutes in the first week. The running time included 10 minutes warm-up and cool-down. The duration of each exercise session was progressively extended up to 60 min and continued throughout an eight-week training protocol (17). Given that running at a speed of 9 m/min for 15 minutes on a treadmill at a slop of 0 causes no physiological response to exercise, the control group ran on the treadmill twice a week during the training period (16). The body weight was measured at the end of each week.

Administration of isoproterenol

To induce cardiac apoptosis, at the end of the eight-week exercise training period, 3 mg/kg isoproterenol (Sigma‐Aldrich) was injected subcutaneously for 7 days (18). And 24 h after the last injection, body weight was measured, all rats were anesthetized by intraperitoneal injection of ketamine and xylazine (50 and 10 mg/kg respectively), and the blood was drawn directly from the heart. Following complete blood drainage, the removed hearts and dissected left ventricles were weighed and stored at -80°C for later analysis.

Quantitative real time-PCR (qRT-PCR)

Real-time PCR was used to determine the expression of the genes. According to the manufacturer's instructions, total RNA was isolated using Trizol reagent (Qiazol, cat. no. 79306, USA). Purity and concentration of RNA were determined spectrophotometrically by NanoDrop 2000 (Thermo Scientific, Rockford, USA), and 1% agarose gel stained with Nancy-520 was used to determine RNA integrity electrophoretically (Sigma-Aldrich, Sao Paulo, SP, Brazil). After designing the primer, RNA was extracted from the left ventricle tissues and converted to cDNA using reverse transcriptase using cDNA synthesis kit (Fermentas, Glen Burnie, MD, USA). The quantitative real-time polymerase chain reaction (qRT-PCR) was run to assess the messenger RNA (mRNA) levels of the target genes and reference gene (GAPDH) separately. The primer sequences of the genes used in this study are shown in Table 1.

Table 1. Primer sequence utilized for qRT-PCR

Gene	Forward primer	Reverse primer	Gene ID
AMPK	5′-TGTGTTCAAAGTCTGCTGCC-3′	5′-ACGCTGTAAGGTCTGGTCAA-3′	78975
mTORC1	5′-TGATTTTGGGAGAACAGAAGATGA-3′	5′- GAGGTAACAGGATGGTGGAGTG -3′	56718
eEF2	5′-AGTGAGGACAAGGACAAGGAGGG-3′	5′-GGGACGGCAAGTGGATGGTGA-3′	29565
eEF2K	5′-GGGAGAGAGGAGAAGTGTGGGAG-3′	5′-TGGATGAAGACGGGGAGGAAGG-3′	25435
GADPH	5′-AAGTTCAACGGCACAGTCAAGG-3′	5′-CATACTCAGCACCAGCATCACC-3′	24383

Staining using the TUNEL method

TUNEL kit (Roche Company, Germany) was used to identify and determine apoptosis, and the procedure was performed according to the manufacturer's protocol. Dissected left ventricles were stained after being paraffinized and incubated with proteinase K using a TUNEL apoptosis cell detection kit. Following staining, samples of left ventricular tissue were observed by fluorescence microscope (Zeiss LSM 5). To determine the number of apoptotic cells, 5 fields in each group were counted. The green dots specified during TUNEL staining indicate the apoptotic cells.

Statistical method

Shapiro-Wilk test was used to evaluate the normality of data distribution. Independent *t*-test and Mann-Whitney U test were used to determine between-group differences using SPSS 24 at a significance level of $\alpha<0.05$. Results were reported as mean ± standard error of the mean (SEM).

Results

Shapiro-Wilk test revealed a normal distribution of data except the AMPK gene, heart weight/body weight, and left ventricular weight/body weight ($P = 0.002$, $P = 0.027$, and $P = 0.014$,

respectively), which were analyzed by the Mann-Whitney U test.

Effect of exercise training on the morphological characteristics

The characteristics of 16 rats in 2 groups are presented in Table 2. At the end of the eighth weeks of training and after isoproterenol injection, a significant difference was observed between left ventricular weight and body weight/heart weight (P = 0.028, P = 0.010, respectively). Although heart weight/body weight and left ventricular weight/body weight considerably increased in the exercise group, the difference was not significant compared to the control group (P = 0.080, P = 0.130, respectively). Given the isoproterenol injection for both groups at the end of the exercise training, these differences indicate the effectiveness of the exercise training protocol in enhancing physiological hypertrophy in the left ventricle.

Table 2. Effects of exercise and isoproterenol on physiological properties in rats

Variables	Control	Exercise
Body weight (g)	326.00 ± 8.53	331.50 ± 10.55
Heart weight (mg)	912.50 ± 25.41	984.25 ± 28.37
Left ventricular weight (mg)	642.13 ± 14.57	694.25 ± 15.41 *
Heart weight/Body weight (mg/g)	2.80 ± 0.05	2.97 ± 0.05 *
Left ventricular weight/Body weight (mg/g)	1.97 ± 0.03	2.10 ± 0.06
Left ventricular weight/Heart weight (mg/mg)	0.70 ± 0.01	0.71 ± 0.01

Data are presented as mean ± SEM. *Significant difference with the control group (α <0.05)

Expression of genes involved in protein synthesis signaling

According to Figure 1A, increased gene expression of AMPK in the exercise group failed to be significant compared to the control group (P = 0.065), and eight-week strenuous endurance exercise training did not influence AMPK gene expression. In contrast, mTORC1 gene expression in the exercise group significantly increased compared to the control group (P = 0.026), which indicates the effectiveness of the exercise training protocol on mTORC1 gene expression (Figure 1B).

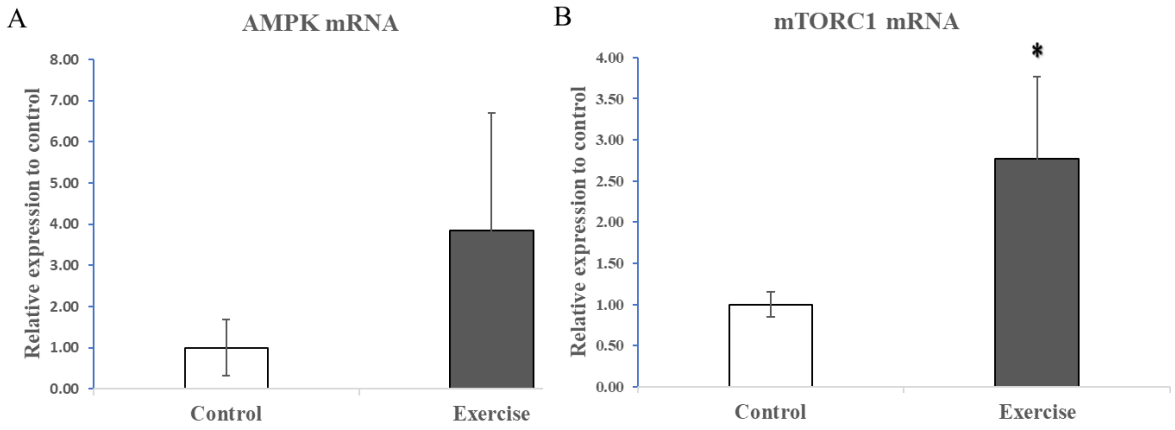

Figure 1. The effect of eight-week continuous endurance running on a treadmill with a slope of 15° on gene expression of AMPK (A) and mTORC1 (B) compared to the control group. □ Significant difference with the control group (α <0.05).

The exercise training protocol significantly inhibited the expression of the eEF2K gene compared to the control group (P = 0.001). Due to inhibition of eEF2K, a significant increase in eEF2 gene expression was observed compared to the control group (P = 0.005). In other words, the eight-week intense continuous endurance training inhibited eEF2K (Figure 2A) while eEF2 gene expression increased (Figure 2B).

Exercise protocol attenuated isoproterenol-induced apoptosis

Based on the results of TUNEL, an eight-week of intense endurance exercise training protected the heart against apoptosis due to the isoproterenol injection at the end of the study period, compared with the control group (Figure 3).

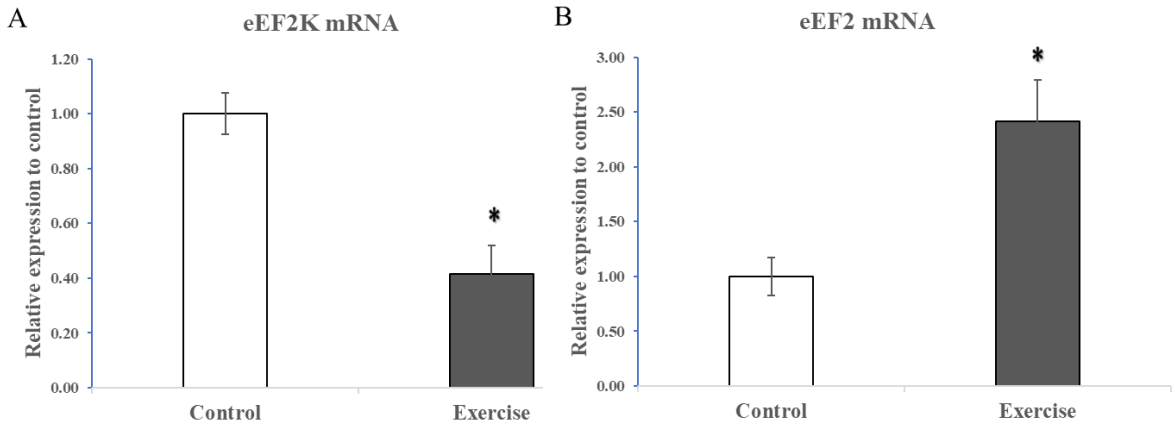

Figure 2. The effect of eight-week continuous endurance running on a treadmill with a slope of 15° on gene expression of eEF2K (A) and eEF2 (B) compared to the control group. □ Significant difference with the control group (α <0.05).

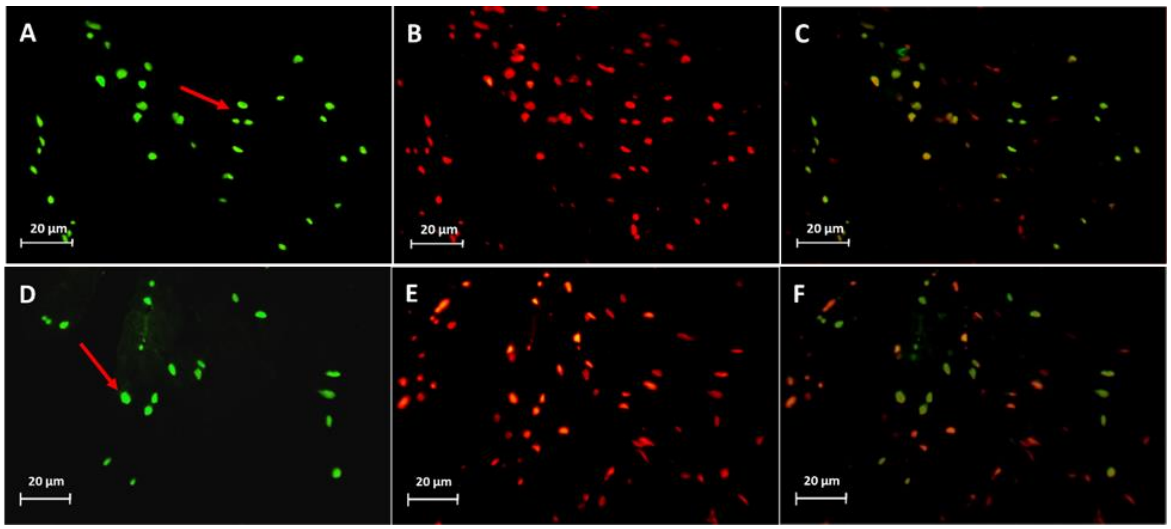

Figure 3. TUNEL staining with 400 magnifications, representative images from the control group (A, B, and C) and exercise group (D, E, and F). Green color indicates detected apoptosis (left images), red indicates stained nuclei (middle images), and right images indicate the merging. The percentages of the detected apoptosis cells are 59% and 35% for the control (C) and exercise (F) group samples respectively.

As shown in Figure 4A, isoproterenol augmented myocardial apoptosis in the control group (52.00% ± 6.37%). Eight weeks of intense exercise training protocol attenuated the apoptosis in the exercise group (31.25% ± 4.10%), so the difference was significant compared to the control group (P < 0.001). This protective effect was observed while the positive effects of exercise training protocol on protein synthesis components increased left ventricular mass, which indicates the physiological

hypertrophy of cardiomyocytes. As illustrated in Table 2, left ventricular weight (Figure 4B) and the heart weight/body weight (Figure 4C) of the exercise group were significantly higher than the control group. In other words, the eight-week exercise training activated the protein synthesis signaling components, as a result, increased left ventricular mass and protected the heart by preventing cardiomyocyte apoptosis.

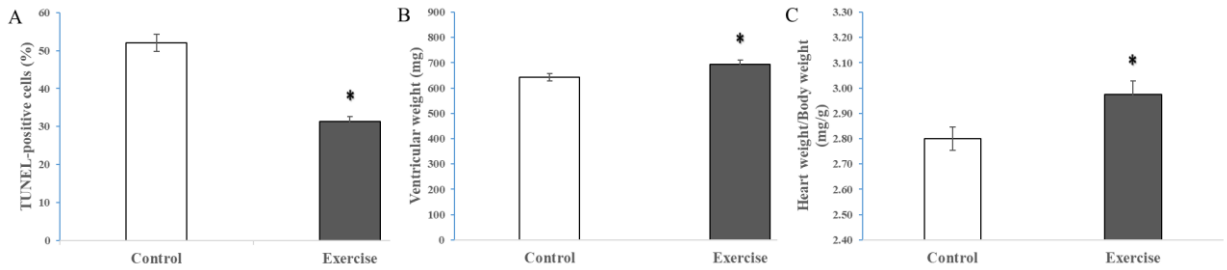

Figure 4. Data are presented as mean ± SEM.
A: Percentage of the positive apoptosis cells in left ventricular, B: Left ventricular weight, C: Heart weight/body weight in the exercise and control groups. □ Significant difference with the control group ($\alpha < 0.05$).

Discussion

To the best of our knowledge, this is the first study that investigates the effects of continuous endurance training on the treadmill with 15° inclination on the expression of genes involved in left ventricular protein synthesis in Wistar rats, concomitant with the evaluation of preventing isoproterenol-induced cardiac apoptosis. The results revealed that the left ventricular weight, as well as the heart weight to body weight ratio in the exercise group, were significantly higher than the control group, which indicates the protein synthesis signaling and physiological hypertrophy. In addition, the exercise training protocol protected the heart by reducing cardiomyocytes apoptosis. Contrary to the finding of the present study, AMPK gene expression temporarily increased immediately after resistance exercise and remained elevated for 1 h post-exercise. In addition, phosphorylation of 4E-BP1, as the protein synthesis suppressor, reduced immediately after the cessation of exercise, while phosphorylation of mTORC1, as a 4E-BP1 inhibitor, increased. The reduction in phosphorylated and activated form of 4E-BP1 can be interpreted by increasing AMPK expression and inhibiting the mTORC1 activity, which lasted 2 h following exercise (19). In nutrient availability and energy balance conditions, mTORC1 regulates protein synthesis by phosphorylating and inhibiting 4EBP1 and activating p70S6K. In addition, mTORC1 is inhibited by AMPK during nutrient starvation and low-energy status, for example, during exercise (20). In other words, increasing or decreasing the amount of AMPK and its activity depends on the level of intracellular energy. Under low energy states, AMPK, as a cellular energy sensor, is activated and inhibits the protein synthesis pathway by phosphorylating and activating eEF2K. In contrast, in nutrient availability and cellular energy balance conditions, e.g., after cessation of exercise, AMPK is inactivated, and the activation of mTORC1 and its downstream targets leads to increased protein synthesis, which is consistent with our findings (21). In the present study, AMPK in the exercise group did not significantly differ from the control group (Figure 1A). mTORC1 activates S6K1 to translation initiation and inhibits eEF2K by phosphorylation on Ser^{366}. eEF2K, as an upstream suppressor, phosphorylated and inactivated eEF2 on Thr^{56}, otherwise, eEF2 could interact with ribosomes to move along mRNAs during the elongation stage of protein synthesis (22). Moreover, low energy levels during each exercise session to AMPK and eEF2K activity, which in turn, mTORC1 and eEF2 activities suppressed, and protein synthesis attenuated eventually (23).

Similar to our results, when AMPK expression and its activity are reduced, the increased mTORC1 gene expression induced by exercise training (Figure 1B) not only activates S6K1 for initiation of the translation but also inhibits eEF2K to increase the elongation stage of protein synthesis. These results emphasize that alterations

of these factors during and after exercise are temporary and are affected by changes in metabolism and cellular energy levels. It has also been shown that extrinsic and intrinsic stresses such as mechanical, metabolic, or oxidative stresses, cause muscle hypertrophy or atrophy by altering the expression or activity of components of the protein synthesis and breakdown pathways (24). In the present study, increased mTORC1 and eEF2 expression induced by exercise training and decreased eEF2K levels (Figures 2A and 2B) could lead to cardiac adaptation and physiological hypertrophy. Accordingly, as reported in Table 2, left ventricular weight and heart weight/body weight of the exercise group increased significantly compared to the control group, indicating the effectiveness of intense continuous endurance training in increasing the expression of genes involved in protein synthesis and consequently left ventricular hypertrophy On the other hand, many studies have shown that injection of isoproterenol even at lower doses, e.g., 0.3 mg/kg, in addition to activating the components involved in the autophagy pathway, leads to cellular lesions such as necrosis and apoptosis (25). Moreover, some diseases such as myocardial infarction can trigger apoptosis and autophagy. Although there are functional differences between autophagy and apoptosis, in some cases, they work together to turnover proteins and organelles within the cell (26). Macroautophagy is one of the primary types of autophagy in which LC3, as an indicator of autophagy binds to the autophagosome to deliver cytosolic organelles and proteins to the lysosome to be degraded for cell survival and maintenance. Autophagy is regulated by AMPK and mTORC1, and their expression and activity mainly depend on the availability of nutrients and the cellular energy balance, although other stresses also play a role. Stress due to starvation and decreased cellular energy levels - the ratio between AMP and ADP to ATP- significantly affect AMPK and AKT and their downstream targets in the protein synthesis and degradation pathways. However, these effects are temporary. In other words, a decrease in blood insulin concentration during starvation or exercise increases AMPK activity and inhibits AKT. Conversely, an increase in blood insulin concentration, for example under nutrient-rich conditions or similar to the present study in post-exercise recovery, leads to inhibition of AMPK and activation of AKT and downstream targets in the protein synthesis signaling and elongation by increasing the expression and activity of eEF2; this is consistent with the findings of the present study (27, 28). Given the fact that the left ventricular tissues were dissected 24 hours after the last injection and animals had free access to water and food, the issue of nutritional deficiency stress eliminated. The results of TUNEL staining in the present study confirmed the isoproterenol-induced apoptosis in both groups. The intense endurance exercise training protected the heart by reducing apoptosis, so the percentage of apoptotic cells in the exercise group was significantly lower than in the control group (Figures 3 and 4A). In the present study, higher intensity of the exercise training protocol and consequently the significant expression of mTORC1 can contribute to the prevention of apoptosis and left ventricular hypertrophy. Accordingly, apart from playing a major role in cell proliferation, regeneration, and differentiation, the Hippo pathway as a controller of tissue growth and organ size is effective in cardiomyocyte apoptosis. MST1/2 (Mammalian STE20-like protein kinase 1/2), the upstream and essential components of the Hippo signaling pathway, are involved in cardiomyocyte apoptosis. In the absence of disease and stress conditions, in which MST1/2 and LATS1/2, and consequently the Hippo pathway are inactive, YAP/TAZ (Yes) Associated Protein/Coactivator with PDZ-binding motif) can translocate to the nucleus and bind with the TEAD (TEA domain transcription factor) to initiate several gene expressions involved in cell proliferation, differentiation, growth, and death, including cardiomyocytes. Conversely, when the Hippo pathway is active during stress and heart disease, MST1/2 phosphorylate activates LATS1/2 (Large Tumor Suppressor homolog 1/2). Phosphorylation and activation of these factors promote YAP/TAZ cytoplasmic retention and proteasomal degradation. Therefore, MST1/2 and LATS/2, and consequently the Hippo pathway are activated during heart disease and cardiomyocytes damage (29). As mentioned above, activation of mTORC1 and its downstream targets leads to protein synthesis and hypertrophy,so the Hippo pathway is activated under conditions such as cell proliferation and apoptosis to control tissue and organ growth.Conversely, when the Hippo pathway is active during stress and heart disease, MST1/2 phosphorylate activates LATS1/2 (Large

Tumor Suppressor homolog 1/2). Phosphorylation and activation of these factors promote YAP/TAZ cytoplasmic retention and proteasomal degradation. The crosstalk and interaction between mTORC1 and Hippo pathways have been documented. Activation of LATS1/2 under apoptotic conditions reduces mTORC1 activity to inhibit protein synthesis and cell growth. YAP, on the other hand, increases protein synthesis by activating the PI3K-mTOR pathway (30)The results of the present study in healthy rats showed that endurance exercise training increased left ventricular hypertrophy by inhibiting the expression of MST1 and LATS1 followed by increased YAP1 gene expression (31). On the one hand, increasing mTORC1 expression in the present study suppressed AMPK expression and its effects on activating the protein degradation signaling. On the other hand, increasing the expression of genes involved in the elongation stage of the protein synthesis pathway led to left ventricular hypertrophy concomitant with the prevention of apoptosis in rats. Considering the vital role of the Hippo pathway in heart disease-induced apoptosis and controlling cardiac hypertrophy, the Hippo pathway may have contributed to our findings, which require further investigation.

The present study revealed that intense endurance exercise training could lead to an increase in left ventricular mass. However, one limitation of the present study was the absence of a histological analysis such as Hematoxylin and Eosin (H&E).

Conclusion

To sum up, the uphill continuous endurance exercise training and cardiac hypertrophy can protect the heart by preventing, and reducing apoptosis. According to the results, the exercise training protocol can be employed to reduce the lesions induced by heart disease such as myocardial infraction, health promotion, and rehabilitation of heart patients. The impact of Hippo pathway components on these changes needs further study.

Conflicts of Interest

There are no conflicts of interest to be declared.

Acknowledgments

Not applicable

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