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Investigating the effect of exercise on the expression of genes related to cardiac physiological hypertrophy

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ABSTRACT

Physiological hypertrophy of the heart is associated with an increase in the normal function of the heart, and it directly relates to regular exercise, especially among elite athletes. Researches about special signaling pathways that create physiological hypertrophy have recently received more attention. As a result, the present study was conducted to investigate the effect of aerobic exercise intensity on the expression of genes involved in heart physiological hypertrophy. For this purpose, 30 male Wistar rats were prepared and randomly divided into three groups: control, intense intermittent training, and submaximal continuous training. The intensive intermittent training protocol included 30 minutes of intermittent running, each interval including 4 minutes of running with an intensity of 85-90% VO2max and 2 minutes of active recovery with an intensity of 50-60% VO2max three days a week for 8 weeks. Also, the submaximal continuous exercise group had activity intensity equal to 50-55% of the maximum oxygen consumption. The expression of genes related to cardiac hypertrophy such as MMP-I, TGF-ß1, and TIMP was evaluated through real-time PCR technique. The results showed that the expression of studied genes in the three groups had significant differences ($p<0.05$). Both training methods led to a significant increase in TGF-ß1 and TIMP gene expression in the heart of rats. But the changes in MMP-I in the intermittent group were not significant compared to the control group. In general, it seems that exercise leads to the improvement of the factors involved in the physiological hypertrophy of the heart. Therefore, the findings of the current research are expressed with caution and more research is needed in the future.

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Introduction

Changes in the mass of the heart following physical activity manifest physiological hypertrophy of the heart. These activities cause changes in the expression of genes involved in this hypertrophy. Physiological hypertrophy occurs in response to regular physical activity and the increase in blood pressure and Frank Starling's mechanism leads to the initiation of hypertrophy signals (1). Physiological hypertrophy leads to changes in heart structure, expression of heart hypertrophy genes, increase in metabolism, and improvement in myocardial function. However, the reconstruction of the heart requires the expression of many genes involved in the heart muscle's structure. Regardless of damage mechanisms such as heart attack, hypertension disease, valvular problems, and myocardial infection (destruction of primary myocytes), any reduction in ventricular function activates many compensatory pathways to maintain tissue perfusion. Among these cascades, the sympathetic nervous system, renin-angiotensin-aldosterone system (RAAS), and transforming growth factor beta (TGF-β) cause myocyte hypertrophy and cardiac fibrosis (2). The fibrosis period has various stages, including the proliferation of fibroblasts, the creation and destruction of collagen, and the transformation of fibroblasts into myofibroblasts. In response to stress, myocardial ECM synthesis increases while ECM degradation decreases (3). In one hundred days of regeneration, mature collagen is gradually reduced by matrix metalloproteinase (MMP), which is controlled by tissue inhibitor of metalloproteinase (TIMP). The effect of cardiac fibrosis worsens mechanical stiffness, contractile irregularity caused by myocyte detachment, myocyte electrical connection, and tissue hypoxia. For this reason, cardiac fibrosis and ECM biology are considered crucial therapeutic targets. Previous researchers have shown the detailed profile of MMP-1/TIMP-1 expression stimulated by TGF-β1 in cardiac fibroblasts in laboratory conditions (4).

In multicellular organisms, the accumulation of cells and the integrity of tissues are rooted in ECM. ECM comprises insoluble collagen fibers and provides mechanical stability, strength, and elasticity of tissues such as skin, tendon, bone, and cartilage. But the primary role of ECM is that it has an active area that affects gene expression, cell cycle progression, cell shape, stability against migration, and cell apoptosis (1, 3). The type and amount of mechanical stress imposed on the cells that make up the extracellular matrix affects the quantity and quality of the extracellular matrix molecules. On the other hand, extracellular matrix molecules significantly affect the gene expression and behavior of neighboring cells (4).

Changes in load patterns are necessary to adapt collagen-rich tissues with load transfer. Evidence in young men and animal studies shows that any change in collagen

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expression rooted in response to sports activities is related to various factors such as the amount and duration of load. Cytokine TGF-β1 causes fibrosis of different types of cells, including the heart and fibroblasts (5). TGF-β is the most potent activator of collagen expression known to date and is activated by mechanical forces. Therefore, it is most likely that the stimulation of collagen transcription is rooted in the mechanical load that takes place through the autocrine stimulation of TGF-β (6, 7).

In recent years, special sports activities for heart health and wellness have been introduced as an intervention to restore health and prevent the progression of diseases. These multiple interventions include various types of treatment, such as risk factor education, psychology, and drug therapy (8). International medical journals of rehabilitation have introduced sports activity as a necessary factor of treatment and prevention. There is no universal agreement on a specific sports prescription, so a separate exact way, including behavioral characteristics, personal goals, and priorities, is recommended (9).

Sports activities have different forms and are divided into categories based on the intensity and duration of exercise and activity (10). The most famous training model is endurance or aerobic endurance training. But nowadays, in sports environments (gyms), they rarely do this type of training. Society has turned to new exercises under the name of intense interval exercises. Therefore, there is a need to examine new training methods (9).

One of these methods is high-intensity interval training, which can be easily performed and available. Very intense sports activity is usually avoided because of the danger it has for heart patients. Ragnmo *et a*l. (11) showed that the range of adverse events related to this type of exercise is low. Kemi *et al*. (12) have shown that very intense exercise on the treadmill has caused a significant increase in the size of cardiomyocytes, and cardiac and contractile function has also been strengthened.

Many studies show that the effective increase of physical capacity, quality of life, and control of risk factors have an effect when people accept very intense exercise. This issue depicts the importance and value of training intensity (11). Gibala *et al*. (13) have stated about cardiac remodeling (left ventricular structure and resting blood pressure), the short bursts of low-volume HIIT - despite the cardiac pressure - have been able to increase the wide range of the cellular and peripheral range of the heart, in While the short time of the sports sessions effectively protects the heart against these pressures. This protective feature allows individuals to perform at higher intensities than others (who otherwise do). Several evidences show that the cardiovascular and aerobic adaptations in cardiovascular patients, patients with chronic heart failure or left ventricular dysfunction, and healthy people after participating in very intense sports activity are more compared to moderate and low-intensity levels.

Sports training with an intensity of approximately 90% VO2peak has been higher than what the guidelines for humans have ordered. This amount of aerobic exercise can be performed in the form of periodic exercise in human and animal models (12). It has been shown that aerobic interval training - periods up to -VO2peak 90% eliminates the receptive contraction of defective cardiomyocytes, reduces myocardial hypertrophy and decreases the expression of atrial sodium peptide of the myocardium after heart failure in rats (13). The beneficial effects of aerobic interval training on cardiac remodeling and myocyte function are similar to the effects of Losartan (Angiotensin II receptor antagonist). This shows that AIT can be a strong modulator of the myocardium (14).

The effect of sports activity on TGF-β secretion and stimulation of collagen production in different articles is conflicting. Perhaps the intensity of the training load challenges this contradiction. This question has been one of the regular questions of researchers that what kind of sports activity can reduce the production of TGF-β1 as the strongest driver of collagen production, which is itself dependent on mechanical load. On the other hand, it reduces the expression of TIMP and improves the balance between TIMP and MMP, because the proper balance of these two indicators contributes greatly to the health of the heart. These conditions can have good functional effects on the modulation of collagen as a therapeutic agent and stop the harmful stages of left ventricular development (15). Physiological hypertrophy is one of the most important cardiac adaptations related to health, which is created after regular physical activity. Of course, the effect of different physical activities on this kind of hypertrophy may be different. Because of the energy supply from heterogeneous aerobic and anaerobic pathways and the creation of different metabolic signals, physiological stimulations in different exercises are different from each other and these differences may lead to heterogeneous cardiac hypertrophy (14). There are very few studies that examine the effects of various physical activities (with regard to intensity, duration, activity, volume, and duration) on changes in cardiac hypertrophy (8).

Some studies have shown that both continuous and intense intermittent exercise activate different signaling pathways that increase the content of heart muscle mitochondria, the activity of transfer proteins (such as citrate synthase and cytochrome c oxidase), and the expression of transfer proteins of the plasma membrane, glycogen content and energy consumption after exercise. But some researchers have stated that intense periodic activity provides stronger stimulations to initiate cell signals than continuous activity because it causes hypoxia and more intense mechanical pressure to the heart (15).

Most of the researches have investigated the indirect variables that are involved in hypertrophy. On the other hand, the studies to investigate physiological hypertrophy were based on endurance or continuous exercises, and no research directly examined the role of training intensity on the characteristics of physiological hypertrophy changes(4, 15). In addition, intense interval training is more economical and attractive in terms of time, and one can expect a suitable mechanical load from it, but its effects are still unclear. Perhaps HIIT as an exercise protocol can be a positive and appropriate treatment factor in preventing heart diseases and on the other hand changes in the structure and physiological hypertrophy of the heart. According to this topic, the aim of this study is to compare the effect of eight weeks of HIIT and continuous aerobic exercise on the effective indices in the remodeling and functioning of the heart muscle of rats.

Materials and Methods

The current research method is experimental in terms

of implementation method and terms of goal, it is an applied study. In this study, 30 Wistar male desert rats (200-250 grams) were used. The animals were kept in 3 groups of 10 including control, intense intermittent exercise and continuous submaximal exercise in a standard rodent laboratory (12-hour light-dark cycle and average temperature of 22 ± 2 degrees Celsius) with free access to food and water. All animals used the same food in the form of pellets. In addition to being the same in terms of age, the animals were also homogenized in terms of weight at the beginning of the protocol, the age of the rats was eight weeks, and they were kept in the same conditions and under the temperature, humidity, ventilation and light-dark cycle desirable for keeping laboratory animals.

Familiarization of rats with intense periodic exercise protocol and submaximal continuity was done with 5 training sessions in one week. In this way, on the first day of the training, the rats were placed on the treadmill with the utmost care and calmness, and they started to train at a very low and uniform speed, and in the following sessions, the rats came well and in sync with the program for familiarization. With the desired intermittent and continuous protocol, intermittent and continuous training was used at low speeds so that the rats get used to the type of training and become familiar with the protocol. This work was done until the end of the 5 familiarization sessions and all the rats were familiar with these protocols and without any problems in the protocol and familiarization of rats after that, the main training started and ended for eight weeks.

Sports training and the performing method of the sports test

The exercise program on the specially designed animal treadmill was three days a week for 40 minutes, which included 5 minutes of warming up and cooling down with an intensity of 40-50% of the maximum oxygen consumption (VO2max) and 30 minutes of intermittent running. Each interval consisted of 4 minutes of very high-intensity running (approximately 85-90% VO2max) and 2 minutes of active recovery (approximately 50-60% VO2max intensity).

The training intensity during the weeks was adjusted based on previous studies (15) and the relationship between running speed and VO2max. Therefore, training intensity was increased by 0.02 m/sec every week (16). In the submaximal continuous training group, they trained three times a week for eight weeks (activity intensity equivalent to 50-55% of the maximum oxygen consumption) based on the percentage of the maximum oxygen consumption (which was converted to meters per minute). In addition to the treadmill speed, which was increased by 0.02 m/sec every week, the training time was 30 minutes in the first weeks and 60 minutes in the final weeks. All the training sessions were done from 8:00 to 13:00. The rats of the two groups practiced regularly during different times between 8:00 and 13:00.

VO2max measurement method in male Wistar rats

According to the study of Hoydal *et al*. (16), each rat first spent 10 minutes at a speed of 10 m/min in the warm-up phase, then the incremental exercise test began, and every two minutes the speed of the treadmill was automatically increased by 0.03 m/sec until the rats Not able to continue sports activities. According to the final speed obtained at the end of the incremental exercise test and based on the study of Hoydal *et al*., (16) the desired speed was obtained in different intensities of the training program and was used for the desired training protocol. 48 hours after the last training session of the rats, heart tissue sampling was provided, and to collect the tissues, the animal was first anesthetized with a combination of xylazine (10 mg/kg) and ketamine (75 mg/kg) by intraperitoneal injection. Then, the hearts of the rats were separated from their bodies and washed in physiological serum until the blood in it was completely emptied with a little pressure, and then it was placed on filter paper to absorb moisture and be ready for weighing. The hearts of the rats were weighed on a digital scale with an accuracy of 0.001 g and then immediately frozen using liquid nitrogen and transferred to a freezer at -80 centigrade for RNA purification.

Genetic evaluations

In this study, the qRT-PCR technique was used to investigate the changes in the gene expression of the dependent variable of the research. For this purpose, first, the RNA of the cells was extracted and then it was treated with DNaseI. In this method, if there is excess DNA in the sample, the DNA is deleted. Finally, cDNA was made and qRT-PCR reactions were performed. RNA was extracted from the sample tissue using the Qiazol kit from Qiagen, Germany, according to the manufacturer's recommendation. In order to eliminate the possibility of contamination of RNA with DNA, an RNase-free DNase enzyme was used. The necessary amounts were determined according to the concentration of the extracted RNA. Thus, for one microgram of extracted RNA, one microliter of DNase (Fermentase, 1 µl) and one microliter of 10X buffers were added, and the volume of the solution with DEPC-treated water was brought to 10 microliters. The resulting solution was incubated for 15 minutes at 37 degrees Celsius, and then placed at 65 degrees Celsius for 15 minutes to inactivate the enzyme. RNA concentration was determined by UV spectrophotometry (Eppendorf, Germany). To make cDNA, 1 microliter of Oligo dta was added to 1-0.2 micrograms of extracted RNA. The final volume of this step should be 12 microliters. In this order, if the RNA was more concentrated, a smaller amount was removed and it was brought to a final volume of 12 microliters with water treated with DEPC. The reaction was placed at -70°C for 5 minutes and then immediately placed in ice. 4 microliters of buffer 2, 5 microliters of dNTP and 1 microliter of RNase were added to the microfuge until the final volume reached 19 microliters. The reaction solution was incubated for 5 minutes at 37°C. One microliter of RT enzyme was added to the reaction and incubated for 1 hour at 42°C. To stop the reaction, the microtube was placed at 70°C for 10 minutes. The resulting cDNA was placed on ice and kept in a freezer at -20°C until the PCR reaction was performed. To design the primers, first, the mRNA sequence related to TIMP, TGF-ß1 and MMP-I genes was extracted using the NCBI site. The primers were designed by Allele ID software and then each primer was evaluated by BLAST software to ensure the uniqueness of the primer pairing site. In this research, the beta-actin gene was used as an internal control (Table 1).

Each PCR reaction was performed using SYBR Green (Applied Biosystems) according to the manufacturer's protocol. 40 cycles were considered for each Real-time PCR

Table 1. Primer Sequences, product length and annealing temperature of studied genes.

Gene	Primer Sequence (5'-3')		Product Length	Temp.
ß-actin	Forward	TGGAATCCTGTGGCATCCATGAAAC	104bp	60°C
	Reverse	TAAACGCAGCTCGTAACAGTCCA		
TGF-ß1	Forward	CAACAACGCAATCTATGACAA	240bp	64°C
	Reverse	CAAGGTAACGCCAGGAAT		
TIMP	Forward	ACAGAGGAGACCATAGTGA	89bp	58°C
	Reverse	ATAACCAGGTCCGAGTT		
MMP-I	Forward	ACAGAGGAGACCATAGTGA	190bp	58°C
	Reverse	TGAGCCGTAACATAGAACAA		

cycle, and the temperatures of each cycle were set at 94°C for 15 seconds, and 60°C for 30 seconds. For the studied genes, the reference gene, beta-actin, was used to obtain the appropriate temperature. Also, to check the efficiency of the primers, the specific standard curve of each gene was drawn. The melting chart was also evaluated to check the accuracy of PCR reactions performed specifically for each gene and in each series of reactions along with the negative control chart to check the presence of contamination in each reaction. By putting the data in the ΔΔCt and $2^{-\Delta\Delta Ct}$ formulas, the expression level of the target gene was normalized with the reference gene.

Statistical analysis

Descriptive statistics were used to classify the raw data and describe the data. The Shapiro-Wilk test was used to check the normality of the data in the studied groups, and the one-way analysis of variance test and the LSD post hoc test were used to check the changes between groups and pairs of groups. The significance level for all statistical tests was considered as $\alpha \leq 0.05$. Statistical analysis was done using SPSS21 software and graphs were drawn using Excel 2007 software.

Results

The results of the one-way analysis of variance for TGF-β gene expression are given in Table 2. Considering the calculated F value (3.8) and its significance at the p=0.038 level, the significant difference between TGF-β gene expression in different research groups was confirmed with 95% confidence.

Also, the results of the one-way analysis of variance for TIMP gene expression are given in Table 3. According to the calculated F value (5.9) and its significance at the level of p=0.009, a significant difference between TIMP gene expression in different research groups was confirmed.

Also, the results of the one-way analysis of variance for MMP-I gene expression are given in Table 4. According to the calculated F value (4.2) and its significance at the p=0.02 level, the significant difference between MMP-I gene expression in different research groups was confirmed.

LSD test was used to check the desired difference. The results showed that TGF-β gene expression was significantly increased in intense intermittent exercises (p=0.01) and submaximal endurance (p=0.04) compared to the control group. Also, the results showed that there is no difference between the submaximal continuous group and the severe intermittent group (p=0.6). Figure 1 shows the difference in TGF-β gene expression in the intermittent and continuous training group compared to the control group after 8 weeks.

The results showed that TIMP gene expression increased significantly in intense intermittent (p=0.003) and submaximal continuous (p=0.03) exercises compared to the control group. Also, the results showed that there is no difference between the submaximal continuous group and the severe intermittent group (p=0.29). Figure 2 shows the difference in TIMP gene expression in the intermittent trai-

Table 2. TGF-β gene expression in intense interval training, continuous submaximal and control groups at the end of the protocol.

Groups	Gene Expression	Mean Square	Degree of Freedom	F-value	P-value
Intense interval training	2.7±2.2	20.07	2	3.8	0.038*
Continuous submaximal	2.3±1.6				
Control	0.6±0.1				

Table 3. TIMP receptor gene expression in intense interval training, continuous submaximal and control groups at the end of the protocol.

Groups	Gene Expression	Mean Square	Degree of Freedom	F-value	P-value
Intense interval training	2.08±0.6	5.6	2	5.9	0.009*
Continuous submaximal	1.7±0.8				
Control	0.9±0.5				

Table 4. MMP-I receptor gene expression in intense interval training, continuous submaximal and control groups at the end of the protocol.

Groups	Gene Expression	Mean Square	Degree of Freedom	F-value	P-value
Intense interval training	1.4±0.2	0.6	2	4.2	0.02*
Continuous submaximal	1.9±0.6				
Control	1.3±0.1				

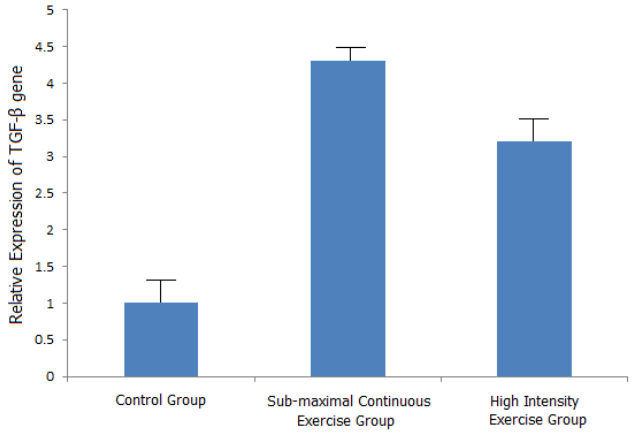

Figure 1. The difference in TGF-β gene expression among the three studied groups after 8 weeks of training.

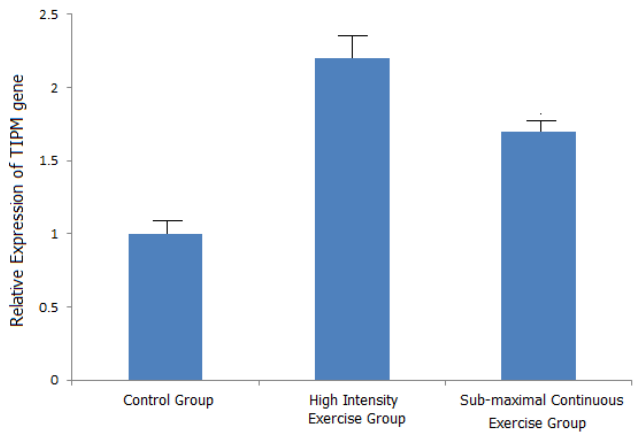

Figure 2. The difference in TIMP gene expression among the three studied groups after 8 weeks of training.

ning group compared to the control group after 8 weeks.

Also, the results showed that MMP-I gene expression was significantly increased in submaximal continuous exercise compared to the control (p=0.01) and intermittent (p=0.04) groups. Also, the results showed that there is no difference between the intense interval group and the control group (p=0.4). Figure 3 shows the difference in MMP-I gene expression in the intermittent exercise group compared to the control group after 8 weeks.

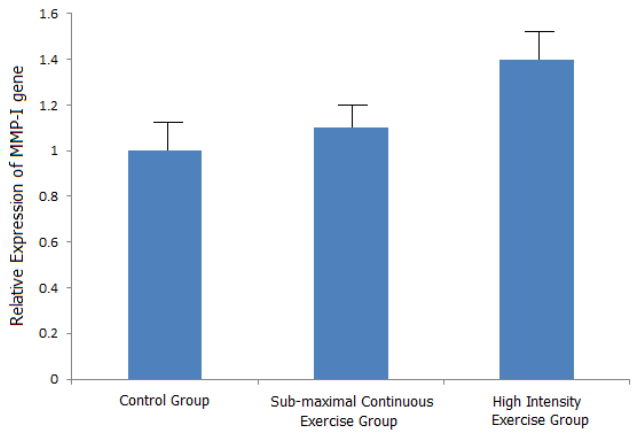

Figure 3. The difference in MMP-I gene expression among the three studied groups after 8 weeks of training.

Discussion

The present research showed a significant difference in gene expression changes in all three variables between eight weeks of intense intermittent exercise and submaximal continuous exercise groups compared to the control group. Intermittent and continuous exercise training (compared to the control) leads to a significant increase in TGF-ß1 and TIMP gene expression in the hearts of male Wistar rats. However, in the other variable involved in the physiological hypertrophy process, the results of the present study showed that the amount of MMP-I in the continuous group significantly increased compared to the intermittent group. Still, in the intermittent group, this increase was not significant compared to the control group.

The results of the present study regarding TGF-ß1 changes are consistent with the results of Li *et al*.'s research (17). They investigated the role of sports exercises on serum levels of factors involved in ventricular hypertrophy. Their study showed that sports activity could increase the factors involved in cardiac hypertrophy.

Deng *et al*. (6) investigated the effect of 8 weeks of swimming training on cardiac levels of matrix metalloproteinase-2 (MMP-2) and transforming growth factor-beta one (TGF-β1) in diabetic rats. The results of their research showed that swimming can lead to a significant decrease in TGF-β1 in diabetic rats. Among the possible reasons for the difference in the results of the present study, we can probably refer to the type of subjects. In the aforementioned study, the subjects were sick rats, while healthy rats were used as samples in the present study. They used diabetic samples and Chen *et al*. (18) also used ischemia rats. No research investigated the direct effect of sports activity on the factors involved in physiological hypertrophy.

The results regarding the beta growth factor showed that this increase is significant in both sports intervention groups. Transforming growth factor beta is a multi-functional cytokine that plays an essential role in migration, proliferation, differentiation, cell death, and production of ECM proteins and is a strong driver of collagen synthesis. Also, it mediates collagen synthesis by increasing its translation and decreasing collagen degradation by decreasing MMPs and increasing TIMPs; therefore, it increases the density of ECM, especially collagen. Sports activities with high metabolic and mechanical intensity (such as intense intermittent activities) can stimulate the synthesis of TGF-β in smooth muscles, skeletal muscles, and blood flow as a physiological response in the heart (19).

The activity of TGF-β1 is done by the secretion of a group of proteins connected to TGF-β by proteases. The activity starts with the connection of TGF-β1 with its type II receptor (TβRII) and the TβRI-TβRII complex is formed. By phosphorylating Smad2/3 and adding Smad4 to the complex, it enters the nucleus and the site of gene transcription regulation. Smad7 is an inhibitory protein of this pathway. In an independent pathway, and Ras-Raf-MEK-ERK by MAPK kinase-1 pathway activated by TGF-β by TAK1-TAK1-related protein forms a group, TAK1 activity with MKK3-p38 and MKK4-JNK-AP-1 ATF2 pathways and NF-Kβ mediates the profibrotic response (6).

The present study showed that intense interval training and resistance training increased the level of TGF-ß1 and TIMPs, and on the other hand, the level of MMP-I did not change in the interval training group. As it was said, these types of exercises can lead to more physiological hyper-

trophy than endurance exercises due to the increase in mechanical and metabolic pressure they put on the heart. The lack of significant change in MMP-I gene expression in normal people can be the beginning of cardiovascular problems and disorders, but these changes seem to be different in athletes (20). Also, the increase in TIMP increases the cooperative activities between this gene with Gata4 and Nkx2.5, which prevents pathogenesis. Following these findings, this research emphasizes the role of MMP-I in regulating the structure of the septum during the formation of the heart, and in fact, the reduction of this factor in intense intermittent exercises is considered a risk factor in normal people and possibly a physiological adaptation for athletes (16).

The balance of ECM remodeling by collagen breakdown and formation is necessary for the normal structure and function of the heart. Collagen rearrangement is modulated by regulatory proteins, hormonal factors, cytokines and growth factors. Cardiac remodeling depends on the balance between MMP/TIMP (16). This balance is observed in the endurance training group, and in fact, the amount of rearrangement has increased with sports activity and indicates cardiac hypertrophy. But in the severe intermittent group, this balance between MMP/TIMP was not established, which requires further investigations in future research.

Collagen is the most important ECM protein that forms the necessary mechanical structures and provides its tensile strength and stability (21). The structural changes in the left ventricle in athletes show that an adaptation to hemodynamic overload is induced by exercise, and this type of adaptation is proportional to the type of exercise and physical activities, while in endurance athletes, the work capacity during exercise increases Preload is positively affected, while in athletes of speed disciplines and intense intervals, the increase in afterload caused by training causes higher systolic resistance and plays a decisive role (20-24). It is normal that along with the structural changes, tissue changes also occur, and several signaling pathways in the heart cause the restructuring of its tissue. Sports activities create different signaling pathways for muscle hypertrophy and fibrosis. In fact, the results show that in a normal state, the increase of TGF-ß1 leads to fibrosis (5). It seems that when another interfering factor, such as sports activity, leads to an increase of TGF-β1, it activates the physiological hypertrophy signaling pathway, which the research results show (8). Our study has also confirmed this issue.

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