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Türk Hijyen ve Deneysel Biyoloji Dergisi

Comparable effects of continuous exercise training and intermittent hypoxia on angiogenic upregulations in the heart tissue of male Wistar rats

Erkek wistar sıçanlarının kalp dokusundaki anjiyojenik yükselmelerde sürekli egzersiz eğitimi ve aralıklı hipoksinin etkilerinin karşılaştırılması

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ABSTRACT

Objective: Angiogenesis can be influenced by many factors. The main purpose of the present study was to explore the effect of aerobic training and intermittent hypoxia on angiogenic factors in the cardiac tissue of male Wistar rats.

Methods: Thirty male Wistar rats were randomly divided into one of three groups; normal control (NC), hypoxia (H), and training (T). The Hypoxia group was exposed to chronic periodic and isobaric hypoxia (total pressure PiO_2≈760 mmHg, 14% oxygen) for 8 weeks. Animals in the training group ran on a motorized treadmill (22-26 meters per min) for 8 weeks, 5 sessions per week. At the end of the procedure, protein expression of Tie-1, HIF-1a, VEGF, and P-AKT were calculated by an immunoblotting technique in the cardiac tissue.

Results: The findings indicated that periodic hypoxia and exercise training substantially raised the expression of the HIF-1a, VEGF, and P-AKT proteins as compared to the NC (p = 0.001). Moreover, chronic treadmill training significantly increased the phosphorylation of AKT as

ÖZET

Amaç: Anjiyogenez birçok faktörden etkilenebilir. Bu çalışmanın temel amacı, erkek Wistar sıçanlarının kalp dokusunda aerobik antrenman ve aralıklı hipoksinin anjiyojenik faktörler üzerindeki etkisini araştırmaktı.

Yöntem: Otuz erkek Wistar sıçanı rastgele üç gruba ayrıldı; normal kontrol (NC), hipoksi (H) ve eğitim (T). Hipoksi grubu, 8 hafta boyunca kronik periyodik ve izobarik hipoksiye (toplam basınç PiO_2~760 mmHg, %14 oksijen) maruz bırakıldı. Eğitim grubundaki hayvanlar, 8 hafta boyunca haftada 5 seans olmak üzere motorlu koşu bandında (dakikada 22-26 metre) koştu. Prosedürün sonunda, kalp dokusunda bir immünoblotlama tekniği ile Tie-1, HIF-1a, VEGF ve P-AKT'nin protein ekspresyonu hesaplandı.

Bulgular: Bulgular, periyodik hipoksi ve egzersiz eğitiminin, NC'ye kıyasla HIF-1a, VEGF ve P-AKT proteinlerinin ekspresyonunu önemli ölçüde artırdığını gösterdi (p = 0.001). Ayrıca, kronik koşu bandı eğitimi, hipoksi grubuna kıyasla AKT'nin fosforilasyonunu

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compared to the hypoxia group (p = 0.001), indicating that PI3K/Akt signalling pathway is influenced by exercise training rather than hypoxia.

Conclusion: It seems that both hypoxia and exercise training are potent stimulators for the induction of angiogenesis signalling pathway.

Key Words: Intermittent hypoxia, angiogenesis, training

önemli ölçüde arttırdı (p = 0.001), bu da PI3K/Akt sinyal yolunun hipoksiden ziyade egzersiz eğitiminden etkilendiğini gösterir.

Sonuç: Hem hipoksi hem de egzersiz eğitiminin, anjiyogenez sinyal yolunun indüksiyonu için güçlü uyarıcılar olduğu görülmektedir.

Anahtar Kelimeler: Aralıklı hipoksi, anjiyogenez, eğitim

INTRODUCTION

Angiogenesis is defined as a process of generating new blood capillaries from pre-existing blood vessels through endothelial sprouting or non-sprouting microvascular growth. This process may be up-regulated by physical and chemical stimuli, including increased shear stress, tumor, muscle contraction, stretching, and hypoxia. Hypoxia is one of the key regulators of angiogenesis, and is identified as a clinical factor broadly related to a number of pathological, physiological, or environmental conditions. Maintenance of oxygen homeostasis is requisite for the survival of all multi-cellular organisms (1). Upon an excess or deficiency of O2 levels in the circulatory blood, a variety of acute and chronic responses are triggered quickly in the cardiovascular and respiratory systems to maintain the normal metabolism (2).

On the other hand, physical exercise, even during normoxic conditions, potentially leads to a drop in level of oxygen availability and induces hypoxia (3). Earlier studies have demonstrated that endurance exercise training such as (running, cycling, rowing, swimming, skiing, etc.) and high altitudes stimulate the process of angiogenesis and increase the capillary frequency in the active muscle fibers (4). Recently, studies which revealed the effect of exercise on angiogenesis, have focused on cell signaling pathways and the expression of angiogenesis-related genes (5), although, in this regard, the results are not consistent. Li et al. reported that following eight weeks of intermittent exposure to 12% O_2, aerobic training increased serum level of VEGF in sedentary men (4). Conversely, Lundby et al. showed no notable changes in capillary frequency and expression of angiogenesis regulatory factors in skeletal muscle at 4100 meters altitude (6).

However, several studies have shown that under hypoxia conditions, angiogenesis is partly determined by over-expression of hypoxia-inducible factor-1α (HIF-1α) (7). HIF-1α is the main regulator of the genes such as VEGF, which are connected to the hypoxia adaptation feedback in most mammalian cells (8). Evidence shows that HIF-1α is a potent trigger of the VEGF transcription (7). VEGF is a strong endothelial specific-mitogen, which extends endothelial proliferation through the recruitment of endothelial cells into the hypoxic centers (9). In fact, VEGF acts as an effective mobilizer of blood circulating stem cells and initiates angiogenesis, while the regulation of VEGF in endothelial cells is mediated by the PI3K/AKT pathway (8) .

In addition to VEGF, the orphan receptor tyrosine kinase Tie-1 is essential for normal vascular development and plays an important role in angiogenic

processes. Tie-1 is expressed entirely in endothelial cells and plays a crucial role during sprouting angiogenesis (10). Previous studies indicated that tie-1 expression is enhanced following endothelial activation by hypoxia, VEGF, and shear stress (11). According to earlier studies, hypoxia could act to enhance tie-1 by increasing production of VEGF (12).

Moreover, exposure to hypoxia activates PI3K/AKT signaling pathway, which protects cells from apoptosis. The interplay between the AKT pathway and HIF-1 signaling is essential for hypoxic cell survival. The AKT pathway enhances HIF-1 signaling by increasing HIF-1α protein levels (13). Kontos et al. revealed that Tie-1 suppressed UV irradiation-induced apoptosis by activation of PI3K/AKT signaling channel and inhibition of caspase-3 cleavages (14).

To the best of our knowledge, hypoxia and exercise training are now used as a therapeutic strategy for angiogenesis in cardiovascular patients. Therefore, the present study was performed to elucidate which of the hypoxia and exercise training stimuli plays an important role in activating the angiogenesis signaling pathway.

MATERIAL and METHOD

Animals

Thirty male Wistar rats, weighing 220±20 g, were obtained from the laboratory animal colony of Tabriz University of Medical Sciences. Animals were housed (2-3 rats per cage) at 22 ± 1 °C, 50% humidity, and low noise in a controlled room with a 12:12 light/dark cycle. The animals were allowed free access to standard chow and water. After one week of adaptation, animals were randomly divided into 3 groups (10 rats in each group) as follows; normal control (NC), intermittent hypoxia (H), and training groups (T).

Aerobic training and intermittent hypoxia induction protocols

The rats in the T group were exercised according to the exercise training protocol which was previously described to adequate systemic and cellular adaptation with this level of aerobic exercise (15). Briefly, a motorized rodent treadmill with 5 separate lanes was used. In the first week, animals ran at a speed of 10 -20 m/min, with a slope of 10%. Then, the speed and duration of exercise gradually increased over the next weeks. The training group ran on a treadmill (22-26 m/min) for 8 weeks, 5 session/ week.

Intermittent hypoxia (total pressure PiO_2 ≈ 760 mmHg, under 14% O_2) was applied 8 to 12 hours per day, for 8 weeks in an air-conditioned isobaric hypoxia chamber (Australia Pty. Ltd, Melbourne GO2 Altitude, Biomedtech). The amount of this hypoxia in terms of PiO_2 was similar to the 3400-meter altitude (16). At the end of the hypoxia, rats were moved to the animal house along with the other groups.

Sampling

Forty-eight hours after the last training session, rats were deeply anesthetized by intraperitoneal injection of ketamine (90 mg/kg), and xylazine (10 mg/kg) and sacrificed. The heart tissue was immediately removed and washed with normal saline and then frozen in liquid nitrogen and stored at −80 °C for further analysis.

Histological examination

For evaluation of vascular density, myocardial tissues in the left ventricle were collected and fixed in 4% formalin then embedded in paraffin for histological examination. Paraffin-embedded tissues were serially cut into 5 µm sections. Hematoxylin-Eosin (H&E) was used to stain the sections. Images were visualized under a light microscope (Olympus, Japan) at ×100 and ×400 magnifications.

Western blotting

The isolated heart tissues were homogenized in ice-cold RIPA buffer (50 mM Tris buffer, pH 8.0; 150 mM NaCl; 1% NP-40; 0.5% deoxycholate; and 0.1% SDS) and centrifuged at 12000 RPM for 15 min at 4 °C. Equal amounts (40 µg) of total protein were subjected to SDS-PAGE using 8-12% denatured

ready gel (Bio-Rad, Hercules, CA, USA). Protein bands were transferred from the gel to Hybond-LFP polyvinylidene fluoride (PVDF) membranes (GE Healthcare Bioscience, Arlington Heights, IL, USA). Then, the membranes were incubated in skimmed milk 5% in phosphate-buffered 0.1% Tween (PBST) 20 to block nonspecific bindings. Subsequently, blots were exposed overnight to different rabbit polyclonal primary antibodies of HIF-1a (1:500, #sc-13515, Santa Cruz Biotechnology, Santa Cruz, CA, USA), VEGF (1:500#sc -507; Santa Cruz, USA), P-AKT(1:500#sc -7985-R; Santa Cruz, USA) and ß-action (1:500#sc -47778; Santa Cruz, USA). After four times washing the membrane, each time for 5 minutes with PBS, blots were incubated with Horseradish peroxidase (HRP) conjugated secondary anti-rabbit antibody. The enhanced chemiluminescence (ECL) kit (Bio-Rad) was used for detection of protein band development and quantified by Image J software. ß-Actin was used as an internal loading control.

Immunohistochemical (IHC) staining

The total number of positively stained cells for five sections per rat was counted by a blinded person to the experiment under a light microscope (x10).

Statistical analysis

Data were expressed as mean ± SD. Data were analyzed using IBM SPSS Statistics software (version 22; SPSS Inc., USA). One-way ANOVA followed Bonfeeroni's multiple comparisons post-hoc test was used to determine significant differences between the groups. The level of statistical significance was set at $p < 0.05$.

All procedures were in compliance with Helsinki declaration guidelines for the use and care of animals and the study was approved by the Research Ethics Committee (REC) of Tabriz University of Medical Sciences (TUOMS) (Date: 06.02.2020 and Number: IR.TBZMED.REC.1395.723).

RESULTS

HIF-1a and VEGF

As shown in Fig. 1 the expression of HIF-1a and VEGF proteins significantly increased in the heart tissue of T and H groups as compared to the NC group ($p < 0.01$). However, no significant difference was seen between the hypoxia and training groups ($p = 0.99$).

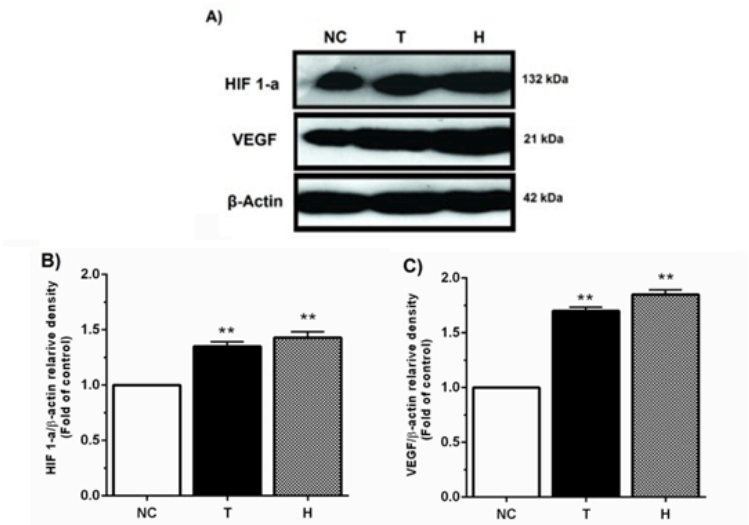

Figure 1. Up-regulation of HIF-1a and VEGF proteins in the cardiac tissue of hypoxia (H) and exercise training (T) groups. A) Immunoblotting images of HIF1-α. And VEGF in the cardiac tissue. Semi-quantitative analyses of HIF-1a (B) and VEGF (C) proteins. **p < 0.01 vs. Normal control (NC) group

Phospho-AKT

Our results also showed that chronic exercise training ($p < 0.001$, Fig. 2) as well as intermittent hypoxia ($p < 0.05$) significantly increased the phosphorylation of AKT protein (p-AKT) as compared to the NC group. In addition, there was a significant difference between the training and hypoxia group in p-AKT levels ($p < 0.01$).

Tie 1

The results of immunohistochemistry staining revealed that exercise training and intermittent hypoxia increased Tie-1 positive cells (those with brown granules within their cytoplasm) in the heart tissue (Fig. 3A). One-way ANOVA analysis also showed a significant increase in the tissue expression of Tie 1 in the T ($p < 0.001$) and H ($p < 0.01$) groups as compared to the NC group (Fig. 3B).

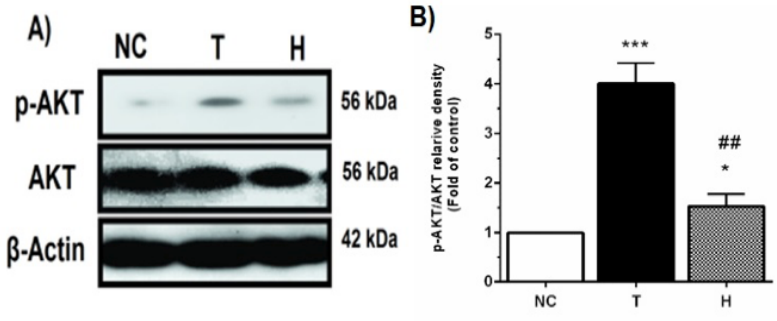

Figure 2. Exercise training (T) and hypoxia (H) increased Phosphorylation levels of AKT (p-AKT) in the heart tissue. A) Immunoblotting images of P-AKT and total AKT protein expression. B) Semi-quantitative analyses of P-Akt in the hypoxia and training groups. *p<0.05 and ***p<0.001 vs. normal control (NC), ##p<0.01 vs. training group

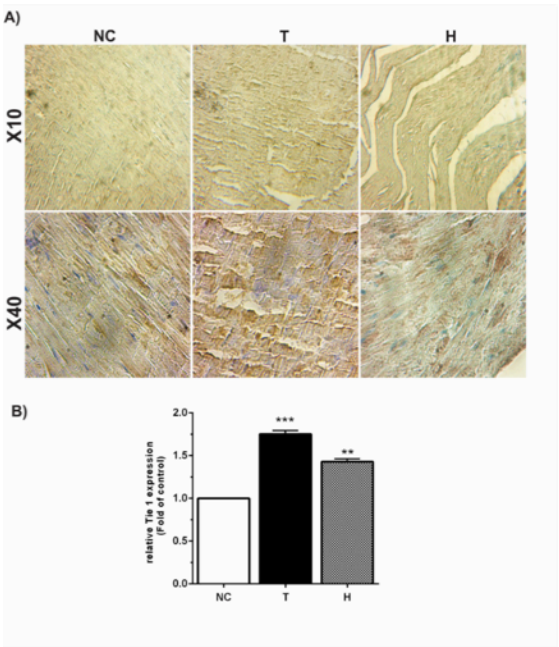

Figure 3. Exercise training (T) and hypoxia (H) increased Tie-1 expression in the heart tissue. A) Immunohistochemistry of Tie-1 expression in the heart tissue of male rats. **p < 0.01, ***p < 0.001 vs. normal control (NC)

The results of histological examination also showed that the control hearts were relatively avascular, with only occasional capillary spaces, whereas aerobic exercise training and exposure to hypoxia were associated with increased capillary density (Fig. 4).

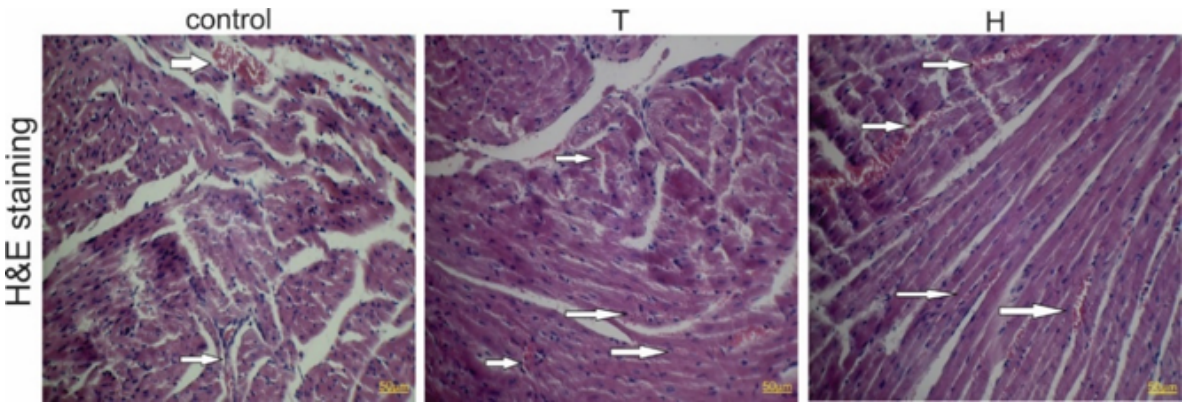

Figure 4. Capillary density in left ventricle of the different groups (H&E staining, 40X). White arrows show capillary

DISCUSSION

In the current study, our results demonstrated that Eight-week intermittent hypoxic condition and moderate intensity aerobic exercise training up-regulated HIF-1α, and VEGF proteins in the cardiac tissue. Furthermore, the level of phosphorylated Akt (p-Akt) increased in both condition (fig 2). Under the hypoxic condition, extensive stress is induced to the cell, and all body cells, sense and respond to hypoxia. Therefore, a number of adaptative responses take place in cells, including elevated oxygen-carrying capacity by increasing the number of cells reduced affinity of hemoglobin for O_2 in the vicinity of tissues, increased capillary network, reduced oxygen consumption and metabolic activity, and the shift from aerobic to anaerobic metabolism and lactate production (17). In addition, physical exercise might create hypoxia-like conditions due to rapid oxygen consumption by active muscle fibers. Intermittent hypoxia has a protective role in the CNS and cardiovascular system, and defends against oxidative stress. A Previous study showed skiing in the Alps, which simulates intermittent hypoxia, reduces blood cholesterol, and mental stress as well as preventing hypertension and diabetes (18).

HIF-1 is the main transcriptional mediator of the hypoxic adaptive response that react to changes in cellular oxygen tension (19). It accumulates in conditions of low oxygen and stimulates the expression of several genes that encode important angiogenic factors to compensate the decreased oxygen availability (17). HIF is composed of the two subunits of α and ß, HIF-1α being more sensitive to oxygen deficiency. Low oxygen at the intracellular level stabilizes the α-subunit of HIF leading to the nuclear translocation and dimerization with HIF-1β and transcription of numerous genes involved in angiogenesis such as VEGF (20). In addition, studies have shown that various factors, including acute exercise, acidosis, oxidative stress, and temperature can regulate HIF-1α protein stability (21). HIF-1 is an important regulator of angiogenesis leading to neovascularization and protection to the cells against hypoxic damage (22). Animal studies also showed that one hour of systemic hypoxia is sufficient to increase the protein expression of HIF-1α in the skeletal muscle (7). The adaptive mechanisms to maintain oxygen

homeostasis during exercise and in hypoxic conditions involve similar pathways. Upon the exercise training, the demand for oxygen and glucose is augmented, so a mild form of hypoxia can be possible (23).

It was also reported that the rapid consumption of oxygen in active muscles during exercise leads to up-regulation of HIF-1α. For example, Li et al. reported that even one bout of exercise under hypoxic condition has a critical role in gene expression of HIF-1α in skeletal muscle (24). Exercise training independently exerts great metabolic stress, and subsequently increases O_2 consumption by active muscles, whereas acute hypoxic exposure leads to metabolic stress by reducing SaO_2 level. One solution for this problem, may be using anaerobic metabolic pathways. Recently, studies have demonstrated that exercise training under the normobaric hypoxia condition for 8 weeks enhanced the expression of glucose-insulin-dependent transporters (GLUT4) in the heart tissue and decreased fibrosis in the heart cardiomyopathy (16). The influx of glucose in cells intensifies anaerobic metabolic pathways, which in turn, results in the adaptation of the cells to O_2 deficiency and may stimulate angiogenesis. Intermittent hypoxia also promotes the release of nitric oxide (NO) from vascular endothelial cells that is an important regulation of vascular tone, and useful protective factor against severity and prolonged hypoxia (25).

Angiogenesis, the process of sprouting of new capillaries from preexisting vessels, is a physiological response to hypoxia that is controlled by VEGF. Previous studies indicated a significant increase in the number of myocardial capillaries in physiological cardiac hypertrophy (26), whereas capillary density is reduced in pathological hypertrophy. Myocardial angiogenesis is regulated by secreted angiogenic growth factors, including VEGFs, angiopoietin-1 and -2, fibroblast growth factors, transforming growth factors, and platelet-derived growth factors (27, 28). Among them, VEGF is the most potent endothelial mitogen and the initiator of angiogenesis (29). VEGF mobilizes the blood circulating stem cells and initiates endothelial proliferation and angiogenesis by recruitment of endothelial cells into the hypoxic area (30). The results of the current study showed that protein expression of HIF-1α and VEGF were increased in both exercise training and hypoxia situations. Our results are consistent with the previous studies showing up-regulation of VEGF under hypoxia conditions and exercise training (24, 31, 32). Wang et al. also reported that 8-hour exposure to intermittent hypoxia with 15 $\%O_2$ for 8 weeks up-regulated VEGF mRNA expression (33). One of the proposed mechanisms underlying the upregulation of VEGF by hypoxia is that hypoxia stimulates expression of HIF which via activation of PI3K/AKT pathway induces the expression of VEGF protein (34). Previous studies also showed that physical exercise induces endothelial progenitor cells to proliferate leading to endothelial regeneration and angiogenesis (35). Since angiogenic factor VEGF is a target gene for HIF-1α, it is possible that just as in cases of exposure to hypoxia, exercise may also increase the expression of VEGF in the heart tissue.

Although, in this regard, the scientific findings in literature are inconsistent. Contrary to our results, Mounier et al. demonstrated a significant decrease in the serum levels of VEGF in 3 weeks of intermittent hypoxic training (36). Oltmanns et al. also reported a significant reduction in serum VEGF after 150 minutes of acute hypoxia (37). In addition, Lundby et al. found that 2-8 weeks exposure to a hypoxic condition (altitude 4100 meters) has no effect on the mRNA expression of VEGF and HIF-1, and capillary density (6). It seems that the possible reason for these inconsistencies is due to the type, intensity and duration of exercise, time spent in height, severity and duration of hypoxia, chronic or intermittent hypoxia exposure, and type of sample for measurements (serum against heart tissue) (38).

The PI3K/Akt pathway has a fundamental role in the proliferation, adhesion, migration, invasion, metabolism, and survival of the cells (34). Exposure to hypoxia also activates PI3K/Akt signaling pathway,

which protects the hypoxic cells from apoptosis (22). Activation of the PI3K/AKT pathway leads to stabilization of HIF-1α, promotes the transcription of VEGF (34), and regulates the expression of other angiogenic factors such as nitric oxide and angiopoietins (22). This study also showed that the PI3K/AKT is activated in both situations of exercise training and hypoxia. However, the increase of p-AKT in the training group was more rigorous than in the hypoxia group. Exercise has been shown to decrease apoptosis and improve cardiac function by increasing the expression of AKT and AKT phosphorylation. Moreover, the PI3K inhibitor eliminates the beneficial effects of exercise on cardiac function (35).

Tie-1 is a transmembrane glycoprotein that consists of an extracellular domain and an intracellular portion containing a tyrosine kinase domain. Previous studies showed that hypoxia, VEGF and shear stress increase Tie-1 expression (39). Similar to VEGF, Tie-1 is required for normal vascular development and angiogenesis. It has been demonstrated that the expression of Tie-1 is enhanced in endothelial cells involved in angiogenesis during wound healing and tumor vascularization (40). According to earlier studies, hypoxia increases Tie-1 protein in the vascular endothelial cells through VEGF production (41). Kontos et al. showed that Tie1 inhibited UV irradiation-induced apoptosis through activation of PI3K/Akt signaling pathway and inhibition of caspase 3 cleavage (14). Tie-1 regulates biological functions of endothelial cells and recently has been targeted for anti-angiogenesis therapy of tumor. Although Tie-1 deletion from the endothelium of adult mice inhibits tumor angiogenesis and growth, Tie-1 suppression did not affect the normal vasculature (42). In the present study, we found that both hypoxia and exercise training increased the number of Tie-1 positive cells in the heart tissue. Similar to our finding, Mcarthly et al. reported that both hypoxia (2% O_2) and VEGF increased Tie-1 expression time-dependently (12). It seems that hypoxia increased Tie-1 expression directly or indirectly through the up-regulation of VEGF.

All in all, the results of the present study demonstrated that 8 weeks of exercise training (running on a treadmill 22-26 m/min for 8 weeks, 5 session/ week) and intermittent hypoxia (~14% PiO_2; 12h daily for 8 weeks) triggered cardiac angiogenic factors in male rats. It seems that both moderate intensity exercise and intermittent systemic hypoxia increase the protein expression of HIF-1 and NO release from vascular endothelial cells, which can upregulate VEGF-1 and Tie-1 expression, necessary for angiogenesis and positive changes in heart tissue. By the way, continuous exercise training and hypoxia also activate PI3K/Akt signaling pathway, which can protect the hypoxic cells from apoptosis. Activation of the PI3K/AKT pathway leads to stabilization of HIF-1α, promotes the transcription of VEGF, and regulates the expression of other angiogenic factors such as nitric oxide and angiopoietins. So, both of hypoxia and exercise training (separately or in combination) can be considered as a therapeutic strategy for angiogenesis in studies with cardiovascular patients. But more research is needed to improve clinical practice for clients with cardiovascular problems.

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ETHICS COMMITTEE APPROVAL

* The study was approved by the Research Ethics Committee (REC) of Tabriz University of Medical Sciences (TUOMS) (Date: 06.02.2020 and Number: IR.TBZMED.REC.1395.723).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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