

## Endurance training induces fiber type-specific revascularization in hindlimb skeletal muscles of rats with chronic heart failure

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### ARTICLE INFO

**Article type:**  
Original article

### Article history:

Received: Jul 23, 2015

Accepted: Oct 20, 2016

### Keywords:

Chronic heart failure  
Endurance training  
Fast and slow twitch muscles  
Revascularization

### ABSTRACT

**Objective(s):** Previous studies showed that skeletal muscle microcirculation was reduced in chronic heart failure. The aim of this study was to investigate the effects of endurance training on capillary and arteriolar density of fast and slow twitch muscles in rats with chronic heart failure.

**Materials and Methods:** Four weeks after surgeries (left anterior descending (LAD) artery occlusion), chronic heart failure rats were divided into 3 groups: Sham (Sham, n=10); Sedentary (Sed, n=10); Exercise training (Ex, n=10). Ex group rats were subjected to endurance training in the form of treadmill running with moderate intensity for 10 weeks.

**Results:** Exercise training significantly increased capillary density and capillary to fiber ratio ( $P<0.05$ ) in slow twitch muscle, but didn't change fast twitch muscle capillary density and capillary to fiber ratio. Furthermore, arteriolar density in fast twitch muscle increased remarkably ( $P<0.05$ ) in response to training, but slow twitch muscle arteriolar density did not change in response to exercise in chronic heart failure rats. HIF-1 increased ( $P<0.01$ ) but VEGF and FGF-2 mRNA did not change in slow twitch muscle after training. In fast twitch muscle, HIF-1 mRNA increased ( $P<0.05$ ), and VEGF and angiotatin decreased ( $P<0.01$ ) significantly after training.

**Conclusion:** Endurance training ameliorates fast and slow twitch muscle revascularization non-uniformly in chronic heart failure rats by increasing capillary density in slow twitch muscle and arteriolar density in fast twitch muscle. The difference in revascularization at slow and fast twitch muscles may be induced by the difference in angiogenic and angiostatic gene expression response to endurance training.

### ► Please cite this article as:

Ranjbar K, Ardakanizade M, F Nazem F. Endurance training induces fiber type-specific revascularization in hindlimb skeletal muscles of rats with chronic heart failure. Iran J Basic Med Sci 2017; 20:90-98; <http://dx.doi.org/10.22038/ijbms.2017.8101>

### Introduction

It has been recognized that chronic heart failure (CHF) leads to decrements in skeletal muscle blood flow and muscle performance. Studies have shown that the structural and functional properties of skeletal muscle vary greatly in response to myocardial infarction (1). The exertional fatigue is the hallmark in advanced CHF patients, caused by perfusion deficits (2). Several mechanisms have been proposed to explain the impaired skeletal muscle perfusion in heart failure. Abnormalities of the microvasculature are some of the mechanisms that contribute to the perfusion deficits (3). In this regard, research showed that soleus (SOL) and extensor digitorum longus (EDL) muscle capillary density four weeks after myocardial infarction was reduced by 18.5% and 18.2%, respectively (4).

Angiogenesis, formation of new capillaries from pre-existing capillaries, and arteriolar genesis, pre-capillary transformation into arterioles by invasion

of the smooth muscle cells into the capillaries, are recruited for revascularization of poorly vascularized muscle tissues (5). Thus, stimulation of angiogenesis and arteriolar genesis in the skeletal muscle might be expected to benefit patients with heart failure.

Previous studies confirmed that exercise training ameliorates vascularization by angiogenesis and arteriolar genesis (6, 7). On the other hand, muscle fiber type composition appears to impart fundamental differences in the biological strategy for vascular adaptation in skeletal muscle (3). Although the molecular mechanisms of these processes (angiogenesis and arteriolar genesis) within different types of muscle fibers after exercise training in heart failure is unknown, but recent studies have shown that capillary density differs among different types of muscle fibers and exercise training results in fiber type-dependent vascularization responses (8, 9).

Vascularization in skeletal muscle is a complex process mediated by the balance between angiogenic

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**Table 1.** General characteristics of rats in different experimental groups

Group	Sham	Sedentary	Exercise Training
N (At the beginning and end of experiment)	10/10	10/8	10/10
Age (week)	6-8	6-8	6-8
Heart rate	375±48	370±35	364±41
Body weight (g)	23±336	41±356	19±321
Sol weight (mg)	18±117	23±121	32±125
Gw weight (mg)	230±1232	176±1200	241±1364
Heart weight/Body weight(g/g) × 10 <sup>-3</sup>	2.74±0.21	2.97±0.12	3.72±0.7

Values are expressed as mean±SEM, heart rate, body weight, and heart weight at killing, bpm beats per min

and angiostatic factors. The main angiogenic factors are vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2), which promote angiogenesis by enhancing endothelial cell proliferation and migration. Furthermore, arteriolar growth is dependent on FGF-2. The process of skeletal muscle angiogenesis is stimulated by hypoxia. VEGF and FGF-2 are regulated by hypoxia inducible factor-1 (HIF-1) transcription factor that is expressed in hypoxic conditions. HIF-1 upregulates many of the genes that are augmented in hypoxia, and it is thought that the overall purpose of these genes and their products is to increase O<sub>2</sub> delivery to the cells (10, 11). Also, shear stress induced by exercise has profound effects on the structure and function of blood vessels. In this regard, it has been shown that shear stress stimulates the expression of factors involved in arteriolar genesis. Shear stress increases transforming growth factor-β (TGF-β) expression in endothelial cells. TGF-β is one of the most important factors involved in arteriolar genesis which stimulates vascular smooth muscle cell proliferation and migration (12) and pericyte recruitment (13). In contrast with VEGF, FGF-2, and TGF-β vascularization can be prevented by angiostatin, which inhibits endothelial and smooth muscle cells proliferation and migration (14, 15).

It is generally known that microcirculation density in slow twitch muscles is higher than fast twitch muscle (16). The contribution of each of these factors in different types of muscle fibers and their response to exercise training in skeletal muscle of rats with CHF is unknown. It is likely that the skeletal muscle microcirculation disparity in diverse types of muscle is induced by differences in angiogenic and angiostatic genes expression.

Therefore, in this paper to our knowledge for the first time, researchers sought to answer whether endurance exercise training can restore microcirculation of skeletal muscle in CHF rats.

## Materials and Methods

### Animals

Male Wistar rats (6–8 week old and 180–200 g) were housed under standard conditions (temperature (22±2 °C), humidity (40–60%) and light/dark 12:12 hr). Rat chow and water were available ad libitum. Animals used in these experiments were treated in

accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the study protocols were approved by the Institutional Animal Care and Use Committee of Hamedan University of Medical Sciences, Hamedan, Iran.

### Myocardial infarction procedure

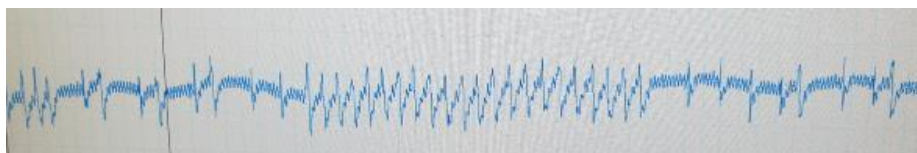
Myocardial infarction (MI) was performed in accordance with a previous method from our laboratory. Myocardial infarction was induced by permanent ligation of the left anterior descending coronary artery as described previously (17). Briefly, after intubation, left thoracotomy and pericardio-tomy, 6-0 silk suture was placed around the left anterior descending coronary artery localized 2 mm below the left atrium. The chest was closed and lung reinflated using positive end expiratory pressure. A standard limb lead-II electrocardiogram (ECG) was continuously monitored and recorded throughout the experiment, using a computerized data acquisition system (ML750 Power Lab/4sp, AD Instruments). Change of ST-segment (elevation) Premature Ventricular Contraction (PVC), Ventricular Tachycardia (V Tach) and ventricular fibrillation were indicators of a successful operation and heart failure confirmation (Figure 1). Respiratory functions were preserved through the use of a ventilator (Small Animal Ventilator, Model 683, Harvard Apparatus, 15 ml/kg stroke volume and 60–70 breaths/min) and body temperature was maintained with an incubator that was fixed to a laboratory bench. The sham group underwent the same procedures except that myocardial ischemia was not induced. The chest was closed with a silk suture.

### Experimental design

CHF rats were divided into three groups of 10 rats: Sham (Sham, n=10); Sedentary (Sed, n=10); Exercise training rats (Ex, n=10). General characteristics of experimental groups were shown in Table 1.

### Endurance training program

Endurance exercise training was performed 4 weeks after MI surgery. Endurance training was performed on a motorized treadmill (Panlab/Harvard Apparatus Treadmills, Holliston, MA, USA) at 60% of maximal oxygen uptake (VO<sub>2</sub>max) for 10 weeks (18). To determine VO<sub>2</sub>max, as described



**Figure 1.** ECG showed premature ventricular contraction (PVC) and ventricular tachycardia (V Tach)

previously (19), the treadmill was placed into a metabolic chamber. Ambient air was pumped through the metabolic chamber at a flow rate of  $4.5 \text{ l min}^{-1}$ , and samples of extracted air ( $200 \text{ ml min}^{-1}$ ) were directed to an oxygen analyzer that was based on a paramagnetic oxygen transducer (Servomex type 1155, Servomex, UK) and a carbon dioxide analyzer (LAIR 12, M&C Instruments, The Netherlands). The  $\text{VO}_2\text{max}$  protocol involved step-wise increases in the treadmill speed as follows: a 15-min period of acclimation, after which the treadmill was started at  $10 \text{ m/min}$  and then the speed was incrementally increased  $5 \text{ m/min}$  every 3 min until the rat reached exhaustion.  $\text{VO}_2\text{max}$  was measured for each animal by using three criteria: (i) no change in  $\text{VO}_2$  when speed was increased, (ii) rats could no longer keep their position on the treadmill, and (iii) respiratory quotient ( $\text{RQ} = \text{VCO}_2/\text{VO}_2$ )  $> 1$ . Then, based on the level of  $\text{VO}_2\text{max}$ , the speed corresponding to 60%  $\text{VO}_2\text{max}$  was determined and used for daily training for 50 min, five times a week for 10 weeks. The  $\text{VO}_2\text{max}$  was measured every other week, and running speed was adjusted to maintain 60%  $\text{VO}_2 \text{ max}$  (19). Sham and sed groups remained sedentary throughout the experiment period.

Forty-eight hr after the last exercise session, training rats were anesthetized with sodium pentobarbital overdose. Soleus and superficial, white portion of gastrocnemius (Gw) skeletal muscles (as slow and fast twitch muscles, respectively) were removed and freeze-clamped in RNase-free microtubes for angiogenic and angiostatic genes analysis. For immunohistochemistry analysis, muscle tissues were then fixed with 4% paraformaldehyde for at least 72 hr. After dehydrating in alcohol, xylene, baptist wax, and paraffin embedding, the muscle tissues were cut into  $5 \mu\text{m}$  sections with a microtome, HM500 OM (Walldorf, Germany).

#### Real-time reverse transcriptase PCR

Fifty mg of frozen muscle tissue was homogenized in TRIzol reagent (Sigma, St. Louis, MO) buffer, and RNA isolation was completed using total RNA purification kits (Jana Bioscience GmbH, Germany) following manufacturer's instructions. Briefly, the tissue sample was ground, and the resulting powder was resuspended in  $200 \mu\text{l}$  of TRIzol Reagent. The suspension was then homogenized and incubated for 5 min at room temperature. The homogenate was extracted with  $500 \mu\text{l}$  of chloroform, and after centrifugation ( $10000 \text{ g}$ , 10 min,  $4^\circ\text{C}$ ) the aqueous

phase was mixed with  $300 \mu\text{l}$  of isopropanol. The resulting pellet was washed with  $700 \mu\text{l}$  of ethanol and resuspended in  $40\text{--}50 \mu\text{l}$  of RNase-free water. Total RNA samples were stored at  $-80^\circ\text{C}$  until use.

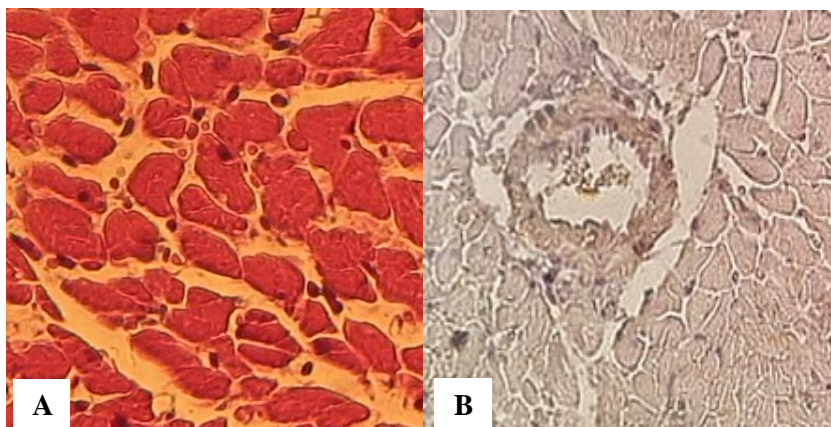
Total RNA ( $1 \mu\text{g}$ ) was reverse transcribed to complementary DNA using cDNA synthesis kit (AccuPower® RT PreMIX, BIONEER, USA) according to the manufacturer's instructions. It should be noted that RNA contamination (with DNA) was removed by adding  $2 \mu\text{l}$  of RNase A ( $10 \text{ mg/ml}$ , Fermentas) to 20 microlitres of DNA dissolved in TE buffer (Tris-EDTA,  $\text{pH} = 8.0$ ) and incubated for 3 hr at room temperature.

Quantitative real-time PCR was conducted using SYBR Green PCR Master Mix Kit (Applied Biosystems, USA) to measure the expression of VEGF, FGF-2, HIF-1, TGF- $\beta$ , and angiostatin. Real-time PCR reactions were performed using the Rotor-Gene 3000 real-time PCR system from Corbett using the following program: step1:  $95^\circ\text{C}$  for 20 sec-3 min and step 2: 40 cycle of  $95^\circ\text{C}$  for 5 sec and  $60^\circ\text{C}$  for  $>20$  sec. The last heating step in phase 2 was carried out for generation of a melting curve of the product. The mRNA expression was assessed by oligonucleotides primers for analysis of the genes VEGF-A (F: 5'- ATC TTT CAT CGG ACC AGT CG-3'; R: 5'- CCC AGA AGT TGG ACG AAA AG-3'), angiostatin (F: 5'- GAC CTC TGG TTT GCT TCG AG-3'; R: 5'-TTG GTT TGA TTG CTG TCA GG-3'), FGF-2 (F: 5'-CAG AAG CTC CTG CAA AAA GG-3'; R: 5'-AGT CTG CAG CTC CTC CAC AT-3'), TGF- $\beta$  (F: 5'-GGATACCAACTATTGCTTCAGCTCC-3'; R: 5'-AGGCTCCAAATATAGGGGCAGGGTC-3') and  $\beta$ -actin (F: 5'-AGC CAT GTA CGT AGC CAT CC-3'; R: 5'- CTC TCA GCT GTG GTGGTG AA-3'). The relative expression of VEGF, FGF-2, HIF-1, TGF- $\beta$ , and angiostatin mRNA was normalized to the control  $\beta$ -actin using the comparative threshold cycle ( $2^{-\Delta\Delta\text{Ct}}$ ) method.

#### Histological analysis

The hindlimb muscles of rats were prepared and collected for measurements of capillary density, capillary to fiber ratio, and arteriolar density. Transverse  $5 \mu\text{m}$ -thick serial sections were cut from paraffin-embedded muscle slices and mounted onto glass microscope slides. Hematoxylin and eosin (H&E) stains were used for capillary density and capillary to fiber ratio evaluation (20) (Figure 2A).

Additional sections were immunostained with  $\alpha$ -actin smooth muscle primary antibodies (SantaCruz Biotechnology, Santa Cruz, CA, USA) to visualize the



**Figure 2.** A) Capillary density and capillary to fiber ratio in soleus muscle by H & E staining and B) large artery in gastrocnemius muscle by alpha-Actin smooth muscle staining

arterioles. Briefly, sections were fixed in ice-cold acetone for 10 min and blocked with phosphate buffer saline (PBS) containing 5% bovine serum albumin (BSA), followed by 70 µg/ml AffiniPure anti-mouse fab fragments diluted in 5% BSA (Jackson ImmunoResearch, West Grove, PA) for 20 and 30 min, respectively. Primary antibodies were diluted to a concentration of 1:100 in PBS with 1% BSA and were independently applied to the tissue sections for 60 min at room temperature. All sections were counterstained with Hematoxylin to visualize the cell nuclei. The stained sections were examined under the Olympus BX53 microscope (Shinjuku, Tokyo, Japan). Smooth muscle  $\alpha$ -actin-positive vessels were used to calculate the arteriolar number density. Arterioles were defined as vessels with an internal diameter in the range of 10–150 µm that had at least one layer of smooth muscle cells (Figure 2B). All parameters were estimated separately for soleus and gastrocnemius muscles. Images for number of capillaries, arteriole, and myocyte evaluation were processed with the ImageJ software. To determine capillary density, capillary to myocyte ratio and arteriole density, the numbers of capillary, arteriole and myocyte were counted in a blind fashion in 10 fields per section of the muscle at  $\times 200$  magnification and normalized to the section area.

### Statistical analysis

After confirming that all continuous variables were normally distributed using the Kolmogorov-

Smirnov test, statistical comparisons among groups were performed by one-way analysis of variance (ANOVA) followed by *post-hoc* comparison via Tukey's test in SPSS. It should be noted that homogeneity of variance was established by Levene's test.  $P < 0.05$  was considered significant. The data are presented as the mean  $\pm$  SEM.

## Results

### Slow twitch muscle capillary density

As shown in Table 2, the soleus muscle capillary density was significantly different between groups ( $P=0.003$ ). In the sed group, capillary density significantly decreased in comparison with the sham group. Exercise training significantly increased capillary density compared to the sedentary infarcted group ( $P=0.02$ ) and there was no significant difference with the sham group ( $P=0.69$ ).

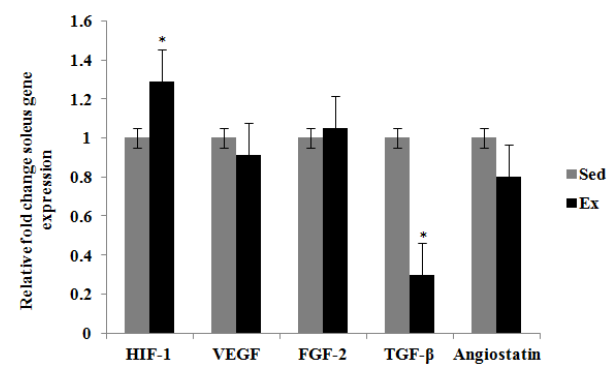
### Fast twitch muscle capillary density

On the other hand, capillary density in the superficial, white portion of gastrocnemius muscle was significantly different in the studied groups ( $P=0.04$ ). Gastrocnemius muscle capillary density in the infarcted groups was significantly reduced compared to the sham group. Exercise training increased gastrocnemius capillary density by 8%, but this change was not significant ( $P=0.056$ ). Gastrocnemius capillary density in the Ex group was significantly lower than in the sham group ( $P=0.01$ ).

**Table 2.** Revascularization of Sol and Gw muscles after exercise training in chronic heart failure rats

Group	Sol muscle			Gw muscle		
	Capillary density	Capillary to fiber ratio	Arteriolar density	Capillary density	Capillary to fiber ratio	Arteriolar density
Sham	813 $\pm$ 23	2.2 $\pm$ 0.6	65 $\pm$ 5	567 $\pm$ 29	1.2 $\pm$ 0.1	21 $\pm$ 5
Sedentary	600 $\pm$ 17*	1.6 $\pm$ 0.3*	69 $\pm$ 10	465 $\pm$ *30	1.1 $\pm$ 0.1	17 $\pm$ 4
Exercise Training	772 $\pm$ 36&	2.0 $\pm$ 0.5&	77 $\pm$ 18	504 $\pm$ 16	1.5 $\pm$ 0.3	28 $\pm$ 11&*

The data was represented as mean  $\pm$  SEM; \*  $P < 0.05$  vs. sham group;  $P < 0.05$  vs. sed group



**Figure 3.** Changes of angiogenic and angiostatic factors gene expression in trained soleus muscle in comparison to sedentary soleus muscle. \*  $P < 0.05$  vs. sed group, the data was represented as mean  $\pm$  SEM

#### Capillary to fiber ratio at slow twitch muscle

In this regard, results showed that capillary to fiber ratio at soleus muscle in response to exercise increased significantly in comparison with the sed group ( $P = 0.01$ ), but there was no significant difference compared to the sham group.

#### Capillary to fiber ratio at fast twitch muscle

Also, the capillary to fiber ratio, was not significantly different among experimental groups in gastrocnemius muscle ( $P > 0.05$ ).

#### Slow twitch muscle arteriolar density

Furthermore, immunohistochemistry results showed that arteriolar density of soleus was not significantly different among groups ( $P = 0.7$ ).

#### Fast twitch muscle arteriolar density

In this regard, gastrocnemius muscle arteriolar density in sham and sed groups was similar, but 10-week long-term aerobic exercise training increased gastrocnemius muscle arteriolar density in comparison to sham and sed groups ( $P < 0.05$ ) (Table 2).

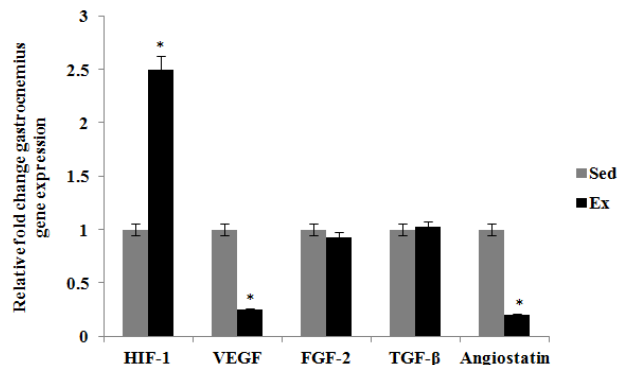
#### Slow twitch muscle gene expression

As shown in Figure 3, HIF-1 and TGF- $\beta$  gene expressions were increased ( $P < 0.01$ ) and decreased ( $P < 0.001$ ), respectively in the soleus muscle after training. But, VEGF, FGF-2, and angiostatin gene expression did not change in response to exercise.

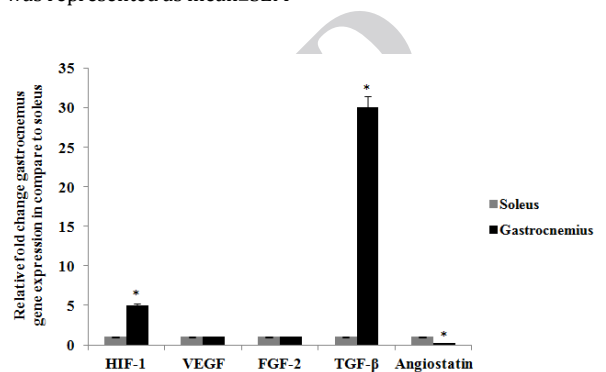
#### Fast twitch muscle gene expression

Also in the gastrocnemius muscle VEGF and angiostatin significantly decreased ( $P < 0.01$ ) in response to training, but HIF-1 significantly increased ( $P < 0.05$ ). The FGF-2 and TGF- $\beta$  gene expression levels did not change in response to training (Figure 4).

#### Comparison of gene expression between slow and fast twitch muscles at the end of training



**Figure 4.** Changes of angiogenic and angiostatic factors gene expression in trained gastrocnemius muscle in comparison to sedentary gastrocnemius muscle. \*  $P < 0.05$  vs. sed group, the data was represented as mean  $\pm$  SEM



**Figure 5.** Changes of angiogenic and angiostatic factors gene expression in trained gastrocnemius muscle in comparison to trained soleus muscle. \*  $P < 0.05$  vs. ex group soleus muscle, the data was represented as mean  $\pm$  SEM

At the end of training, angiostatin mRNA gastrocnemius muscle was less than soleus muscle ( $P < 0.01$ ). Also, HIF-1 and TGF- $\beta$  gene expression was higher in training gastrocnemius muscle than in training soleus muscle ( $P < 0.05$ ) (Figure 5).

## Discussion

Endurance training is emerging as an enormously powerful treatment strategy for patients with peripheral vascular and ischemic heart disease. Briefly, in this paper results showed that myocardial infarction reduced soleus and gastrocnemius capillary density by 26% and 17% respectively, but arteriolar density was not affected by myocardial infarction in these muscles. Endurance training for 10 weeks improved the capillary density of soleus muscle and did not change gastrocnemius capillary density. Instead, training increased gastrocnemius arteriolar density, but did not change arteriolar density in the soleus muscle.

Endurance training increased capillary density and capillary to fiber ratio in soleus muscle. In this regard, research showed that 10 week aerobic

exercise corrects capillary rarefaction in soleus muscle in hypertensive rats (21).

According to our knowledge, this is the first study to assess angiogenesis responses to exercise training after myocardial infarction in rat skeletal muscles. Furthermore, the molecular mechanism of this process in response to exercise after myocardial infarction in rat skeletal muscle is not yet clear.

In order to gain a deeper understanding about skeletal muscle revascularization and identify fiber-type differences in this rat model, we evaluated angiogenic and angiostatic factors gene expression, which have a pivotal role in angiogenesis and arteriolar genesis.

Results showed that soleus HIF-1 gene expression increased significantly in response to endurance training. However, the expression of VEGF, FGF-2, and angiostatin in response to training did not change and TGF- $\beta$  decreased significantly. These seemingly contradictory changes in the expression of angiogenic factors, happened while soleus muscle capillary density after training increased by 28%. VEGF has an initial increase during the early phase of the training followed by a progressive decline in control values over the remainder of the training program (22). Although HIF-1 increased in response to aerobic exercise and is one of the best regulators of VEGF and FGF-2 gene expression, but researchers have questioned its role in the exercise response (23). It is likely that exercise-induced nitric oxide promote HIF-1 gene expression in absence of hypoxia (24).

In this regard, previous studies showed that during exercise oxygen pressure decreases inside skeletal muscle and this change triggers angiogenesis process. With the increase in capillary supply after training, oxygen pressure reduces inside skeletal muscle. Therefore, signals from inside muscle fiber for the syntheses of VEGF, FGF-2, and TGF- $\beta$  decreased. This observation supports the concept that increased VEGF mRNA levels are necessary for the untrained state to promote angiogenesis and thus to facilitate the observed increase in tissue oxygen flux. However, in the trained state, the achievement of this capillary proliferation and its associated benefits lead to an attenuation of this process, suggesting a negative feedback mechanism (25, 26). It is presently unclear whether the tempered response of VEGF mRNA to continued exercise training is due to reduced transcription and/or an altered half-life of the message because both of these mechanisms are known to regulate VEGF mRNA levels (22).

On the other hand, the results of this study demonstrated that gastrocnemius capillary density and capillary to fiber ratio did not change in response to endurance training. Gastrocnemius capillary density in response to exercise increased by

8%, but this change was not significant. This result was not in agreement with the findings of Medeiro *et al* (2008) who reported a significant increase in gastrocnemius capillary density in mice with sympathetic hyperactivity-induced heart failure after exercise training (27). A probable explanation for the difference between the results of this study and the findings of Medeiro is related to the type of exercise that Medeiro used swimming training for 8 weeks.

Gastrocnemius angiogenic and angiostatic gene expression changes in response to exercise showed that HIF-1 mRNA increased and VEGF and angiostatin mRNA decreased significantly in response to exercise training. Furthermore, FGF-2 and TGF- $\beta$  mRNA did not change in comparison to Sed in response to training.

Olfert *et al* (2001) in line with the results of this study showed that rats trained in hypoxia exhibited an attenuated gastrocnemius VEGF mRNA response to exercise (28) and this researcher in another study showed that prolonged exposure to hypoxia attenuated gene expression of VEGF and its receptors in rat gastrocnemius muscle (29). These observations are in line with the deficit of skeletal muscle angiogenesis after hypoxia.

Although, gastrocnemius HIF-1mRNA in this study increased, this factor cannot explain the launching of the angiogenesis process alone. In fact, angiogenesis effects of HIF-1 apply by VEGF, FGF-2, and TGF- $\beta$ . But, in this study, FGF-2 and TGF- $\beta$  did not change and VEGF decreased significantly.

These results showed that gastrocnemius muscle felt oxygen pressure reduction, but couldn't create new capillaries or did not need to create new capillaries. Why gastrocnemius muscle felt oxygen pressure reduction, but couldn't create new capillaries is a question that needs more studies to answer.

The reason is that VEGF mRNA is necessary for the angiogenic response to exercise training. In this regard, study showed that training-induced angiogenesis was inhibited in skeletal muscle VEGF gene-deleted mice (skmVEGF<sup>-/-</sup>) gastrocnemius muscle, but arteriogenesis (smooth muscle actin+, artery number, and diameter) and remodeling [vimentin+, 5'-bromodeoxycytidine (BrdU)+, and F4/80+ cells] occurred in skmVEGF<sup>-/-</sup> mice, even in the absence of training (30). VEGF gastrocnemius decreased significantly in response to exercise. Probably, one of the most important obstacles that prevents training-induced increased capillary density is VEGF mRNA reduction.

Microcirculation in oxidative and glycolytic muscle differs not only according to their shape but also according to their density. In this study, results showed that 10-week aerobic exercise training ameliorated soleus muscle angiogenesis but had no effect on gastrocnemius muscle in myocardial

infarction rats. However, it is not known whether the fiber type-specific changes in the mRNAs of angiogenic and angiostatic factors lead to a fiber type-specific increase in capillary density.

The changes in angiogenic and angiostatic expression in trained gastrocnemius muscle strikingly differ compared with the trained soleus muscle. Results showed that HIF-1 expression was higher in trained gastrocnemius muscle than in trained soleus muscle. This finding showed gastrocnemius muscle lack oxygen in comparison to soleus muscle. This change is in line with unchanged gastrocnemius capillary density after exercise training. The unforeseen finding was that VEGF and FGF-2 genes expression between trained soleus and gastrocnemius were similar, despite obvious differential in capillary density in both types of muscle. Trained gastrocnemius angiostatin mRNA was 5 times more than in soleus muscle, but this factor can't promote angiogenesis after training.

The observed discrepancies in the Fiber type-specific differential expression of angiogenic and angiostatic factors in response to aerobic exercise training between oxidative and glycolytic skeletal muscle in myocardial infarction rats may result from both the intrinsic differences between the muscle types and from functional differences between these fiber types; the different stimuli to these muscles may also contribute to altered gene expression (31).

It is proposed that the signals for stimulation of angiogenesis and arteriolar genesis originate from within the contracting muscle fibers (32). In this regard, it is worth noting that Angiopoietin-2/Angiopoietin-1 (Ang-2/Ang-1) ratio is a more decisive factor than VEGF mRNA in the later stages of angiogenesis (22). Study showed that the Ang-2/Ang-1 ratio was more in soleus muscle in comparison to gastrocnemius muscle during exercise training (23). Also, Peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1 $\alpha$ ) regulates critical processes in muscle physiology, including mitochondrial biogenesis, lipid metabolism, and angiogenesis. In this direction, related studies have shown that PGC-1 $\alpha$  is the master expression regulator of angiogenic factors through coactivation of estrogen-related receptor- $\alpha$  (ERR- $\alpha$ ) during exercise and in response to ischemia in skeletal muscle. However, whether a muscle fiber type-specific PGC-1 $\alpha$  content exists, or PGC-1 $\alpha$  content relates to capillarity, are questions that have been poorly addressed (33).

In addition, recent reports suggest that reactive oxygen species (ROS) play an important role in angiogenesis; however, its underlying molecular mechanisms remain unknown (34). So according to the fiber recruitment pattern, it is likely that fiber-type difference in angiogenesis may be due to fiber

type-specific differential Ang-2/Ang-1, PGC-1 $\alpha$ , and ROS production during exercise training.

Arteriolar density after 10 weeks of aerobic training in gastrocnemius muscle rose by 64% but the increase in soleus muscle was 11%. A similar result was reported by a study that revealed that 10-week aerobic training increased arteriolar density in white gastrocnemius muscles, but not in soleus muscle rats (35).

In support of above finding, results showed, TGF- $\beta$  and angiostatin have a great role in arteriolar genesis. TGF- $\beta$  signaling stimulates smooth muscle cell (SMC) differentiation (36) and proliferation (12) and pericyte recruitment during vascular development and neointimal formation. In contrast, angiostatin has been observed to inhibit endothelial and SMC proliferation and pericyte recruitment during blood vessel formation (15, 37). Trained gastrocnemius TGF- $\beta$  mRNA was 30 times more than trained soleus muscle, and gastrocnemius angiostatin mRNA was 4 times less than soleus after exercise training. So it is likely that one of the reasons for the gastrocnemius arteriolar density promotion after exercise training was increase and decrease in TGF- $\beta$  and angiostatin in gene expression, respectively.

In this regard Francis showed that capillary density increased at 3 weeks and then returned to control levels by continuing training; concomitantly, the number of arterioles (20–30  $\mu$ m) increased at 16 weeks. They speculated that the "extra" capillaries observed at 3 weeks were the source of the new arterioles (6).

Thus, as the gastrocnemius muscle capillary density did not change and TGF- $\beta$  gene expression increased significantly in response to exercise, it is likely that the gastrocnemius capillaries develop into small arterioles during later weeks of aerobic exercise training. Therefore, probably arteriolar genesis process is an important factor in fixed capillary density in response to exercise in myocardial infarction rat.

## Conclusion

Taken together, the data presented here demonstrated that 10-week long-term endurance exercise training ameliorates soleus and gastrocnemius muscle revascularization non-uniformly in CHF rats by increasing capillary density in soleus muscle and arteriolar density in gastrocnemius muscle. The difference in revascularization at slow and fast twitch muscles may be induced by difference in angiogenic and angiostatic gene expression response to exercise training. This study demonstrates that the magnitude and direction of change in angiogenic and angiostatic factors gene expression depend on muscle fiber type. A challenge for the future will be to determine the

other pivotal factors that are involved in this complex process.

### Acknowledgment

This study is part of a PhD dissertation in exercise physiology and has been funded by a research grant from Bu-Ali Sina University, Hamedan, Iran.

### Conflict of interest

The authors declare that there are no conflicts of interest.

### References

- Castillero E, Akashi H, Wang C, Najjar M, Ji R, Kennel P, *et al.* Cardiac myostatin upregulation occurs immediately after myocardial ischemia and is involved in skeletal muscle activation of atrophy. *Biochem Biophys Res Commun* 2014; 457:106-111.
- Thomas DP, Hudlická O. Arteriolar reactivity and capillarization in chronically stimulated rat limb skeletal muscle post-MI. *J Appl Physiol* 1999; 87:2259-2265.
- Laughlin M, Roseguini B. Mechanisms for exercise training-induced increases in skeletal muscle blood flow capacity: differences with interval sprint training versus aerobic endurance training. *J Physiol Pharmacol* 2008; 59:71-88.
- Ogoh S, Hirai T, Nohara R, Taguchi S. Adaptation in properties of skeletal muscle to coronary artery occlusion/reperfusion in rats. *Nihon Seirigaku Zasshi* 2002; 64:225-236.
- Benest AV, Stone OA, Miller WH, Glover CP, Uney JB, Baker AH, *et al.* Arteriolar genesis and angiogenesis induced by endothelial nitric oxide synthase overexpression results in a mature vasculature. *Arterioscler Thromb Vasc Biol* 2008; 28:1462-1468.
- White FC, Bloor CM, McKirnan MD, Carroll SM. Exercise training in swine promotes growth of arteriolar bed and capillary angiogenesis in heart. *J Appl Physiol* 1998; 85:1160-1168.
- Roudier E, Forn P, Perry ME, Birot O. Murine double minute-2 expression is required for capillary maintenance and exercise-induced angiogenesis in skeletal muscle. *FASEB J* 2012; 26:4530-4539.
- Waters RE, Rotevatn S, Li P, Annex BH, Yan Z. Voluntary running induces fiber type-specific angiogenesis in mouse skeletal muscle. *Am J Physiol Cell Physiol* 2004; 287:1342-1348.
- Gute D, Laughlin MH, Amann JF. Regional changes in capillary supply in skeletal muscle of interval-sprint and low-intensity, endurance-trained rats. *Microcirculation* 1994; 1:183-193.
- Lundby C, Pilegaard H, Andersen JL, van Hall G, Sander M, Calbet JA. Acclimatization to 4100 m does not change capillary density or mRNA expression of potential angiogenesis regulatory factors in human skeletal muscle. *J Exp Biol* 2004; 207:3865-3871.
- Jin K, Mao X, Nagayama T, Goldsmith P, Greenberg D. Induction of vascular endothelial growth factor and hypoxia-inducible factor-1 $\alpha$  by global ischemia in rat brain. *Neuroscience* 2000; 99:577-585.
- Tsai S, Hollenbeck ST, Ryer EJ, Edlin R, Yamanouchi D, Kundi R, *et al.* TGF- $\beta$  through Smad3 signaling stimulates vascular smooth muscle cell proliferation and neointimal formation. *Am J Physiol Heart Circ Physiol* 2009; 297:540-549.
- Chantrain CF, Henriot P, Jodele S, Emonard H, Feron O, Courtoy PJ, *et al.* Mechanisms of pericyte recruitment in tumour angiogenesis: a new role for metalloproteinases. *Eur J Cancer* 2006; 42:310-318.
- Nyberg P, Xie L, Kalluri R. Endogenous inhibitors of angiogenesis. *Cancer Res* 2005; 65:3967-3979.
- Walter JJ, Sane DC. Angiostatin binds to smooth muscle cells in the coronary artery and inhibits smooth muscle cell proliferation and migration *in vitro*. *Arterioscler Thromb Vasc Biol* 1999; 19:2041-2048.
- Murakami S. Comparison of capillary architecture between slow and fast muscles in rats using a confocal laser scanning microscope. *Acta Med Okayama* 2010; 64:11-18.
- Sun Y, Zhang J, Zhang JQ, Weber KT. Renin expression at sites of repair in the infarcted rat heart. *J Mol Cell Cardiol* 2001; 33:995-1003.
- Bansal A, Dai Q, Chiao YA, Hakala KW, Zhang JQ, Weintraub ST, *et al.* Proteomic analysis reveals late exercise effects on cardiac remodeling following myocardial infarction. *J Proteomics* 2010; 73:2041-2049.
- Wisløff U, Brubakk AO. Aerobic endurance training reduces bubble formation and increases survival in rats exposed to hyperbaric pressure. *J Physiol* 2001; 537:607-611.
- Wagatsuma A, Tamaki H, Ogita F. Capillary supply and gene expression of angiogenesis-related factors in murine skeletal muscle following denervation. *Exp Physiol* 2005; 90:403-409.
- Fernandes T, Roque FR, Magalhães FdC, Carmo ECd, Oliveira EMd???. Aerobic exercise training corrects capillary rarefaction and alterations in proportions of the muscle fibers types in spontaneously hypertensive rats. *Rev Bras Med Esporte* 2012; 18:267-272.
- Lloyd PG, Prior BM, Yang HT, Terjung RL. Angiogenic growth factor expression in rat skeletal muscle in response to exercise training.



Am J Physiol Heart Circ Physiol 2003; 284:1668-1678.

23. Gustafsson T, Puntchart A, Kaijser L, Jansson E, Sundberg CJ. Exercise-induced expression of angiogenesis-related transcription and growth factors in human skeletal muscle. Am J Physiol Heart Circ Physiol 1999; 276:679-685.

24. Kimura H, Weisz A, Kurashima Y, Hashimoto K, Ogura T, D'Acquisto F, et al. Hypoxia response element of the human vascular endothelial growth factor gene mediates transcriptional regulation by nitric oxide: control of hypoxia-inducible factor-1 activity by nitric oxide. Blood 2000; 95:189-197.

25. Richardson R, Wagner H, Mudaliar S, Saucedo E, Henry R, Wagner P. Exercise adaptation attenuates VEGF gene expression in human skeletal muscle. Am J Physiol Heart Circ Physiol 2000; 279:772-778.

26. Wood R, Sanderson B, Askew C, Walker P, Green S, Stewart I. Effect of training on the response of plasma vascular endothelial growth factor to exercise in patients with peripheral arterial disease. Clin Sci 2006; 111:401-409.

27. Medeiro A, Vanzelli A, Rosa K, Irigoyen M, Brum P. Effect of exercise training and carvedilol treatment on cardiac function and structure in mice with sympathetic hyperactivity-induced heart failure. Braz J Med Biol Res 2008; 41:812-817.

28. Olfert IM, Breen EC, Mathieu-Costello O, Wagner PD. Skeletal muscle capillarity and angiogenic mRNA levels after exercise training in normoxia and chronic hypoxia. J Appl Physiol 2001; 91:1176-1184.

29. Olfert IM, Breen EC, Mathieu-Costello O, Wagner PD. Chronic hypoxia attenuates resting and exercise-induced VEGF, flt-1, and flk-1 mRNA levels in skeletal muscle. J Appl Physiol

2001; 90:1532-1538.

30. Delavar H, Nogueira L, Wagner PD, Hogan MC, Metzger D, Breen EC. Skeletal myofiber VEGF is essential for the exercise training response in adult mice. Am J Physiol Regul Integr Comp Physiol 2014; 306:586-595.

31. Cherwek DH, Hopkins MB, Thompson MJ, Annex BH, Taylor DA. Fiber type-specific differential expression of angiogenic factors in response to chronic hindlimb ischemia. Am J Physiol Heart Circ Physiol 2000; 279:932-938.

32. Yan Z, Okutsu M, Akhtar YN, Lira VA. Regulation of exercise-induced fiber type transformation, mitochondrial biogenesis, and angiogenesis in skeletal muscle. J Appl Physiol 2011; 110:264-274.

33. Gouspillou G, Sgarioto N, Norris B, Barbat-Artigas S, Aubertin-Leheudre M, Morais JA, et al. The Relationship between Muscle Fiber Type-Specific PGC-1 $\alpha$  Content and Mitochondrial Content Varies between Rodent Models and Humans. PLoS One 2014; 9:1-14.

34. Moriyama M, Metzger S, van der Vlies AJ, Uyama H, Ehrbar M, Hasegawa U. Antioxidants: Inhibition of Angiogenesis by Antioxidant Micelles. Adv Healthcare Mater 2015; 4:480-488.

35. Laughlin MH, Cook JD, Tremble R, Ingram D, Colleran PN, Turk JR. Exercise training produces nonuniform increases in arteriolar density of rat soleus and gastrocnemius muscle. Microcirculation 2006; 13:175-186.

36. Guo X, Chen SY. Transforming growth factor- $\beta$  and smooth muscle differentiation. World J Biol Chem 2012; 3:41-52.

37. Wajih N, Sane DC. Angiostatin selectively inhibits signaling by hepatocyte growth factor in endothelial and smooth muscle cells. Blood 2003; 101:1857-1863.