

**Evaluating the effect of low-intensity
eccentric resistance training combined with blood
flow restriction on the systematic and genetic indices
affecting the activation and proliferation of satellite cells
in young non-athlete men**

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Abstract

Introduction: The current study was conducted to evaluate the impact of low-intensity eccentric resistance training combined with and without blood flow restriction (ECCRT with BFR *vs.* ECCRT without BFR) on some of the systematic and genetic indices affecting the activation and proliferation of satellite cells in young non-athlete men.

Materials and Methods: Twenty men with an age range of 25 ± 5 years were randomly divided into two groups of ECCRT with BFR and ECCRT without BFR (each group containing 10 subjects). ECCRT was performed using isokinetic device. It involves about 70 rpt in knee extensor muscles. Restriction was created using an air pressure meter. After local anesthesia through injecting lidocaine 1% and norepinephrine, sampling was performed from lateral broad muscle 48 hours before and 24 hours after the training, and the systematic and genetic indices affecting the activation and proliferation of satellite cells (HGF and Myf5) were examined and compared in two stages of pre-test and post-test in two situations of ECCRT with BFR *vs.* ECCRT without BFR.

Results: The results of this study showed that HGF and Myf5 increased after ECCRT with BFR and ECCRT without BFR ($P<0.05$) and the increase of HGF and Myf5 was higher after ECCRT with BFR than after ECCRT without BFR ($P<0.05$).

Conclusions: It is recommended for authorities and officials in the sports field to use low-intensity ECCRT with BFR to increase HGF and Myof5.

Key words: Low-intensity eccentric resistance exercise training, Blood flow restriction, Systematic indices, Genetic indices, Satellite cells

1. Introduction

Satellite cells are responsible for restoration of muscles against physiological stressors caused by growth, exercise, injury, and disease(1). Accordingly, satellite cells are essential to reproduce muscles. For example, the removal of satellite cells led to lack of re-production of injured muscle (2). Satellite cells that are quiescent in terms of indirect division (mitotically) are released from injured muscle fibers in response to various stimuli such as nerve disconnection, stretching, exercises with tolerating the weight, vibrations, and even burns, and after completion

of a specific program, they are activated, proliferated, and differentiated, and replaced by injured fibers. The activation of the satellite cells causes hypertrophy of the cell organelles, enlarged cytoplasm, and cell transformation (3,4). Specific tissue behaviors of the stem cells are regulated by the biochemical structure of its surrounding environment called as Niche (1,3). Fibrils, base membranes, capillaries, neurological factors, immune system cells, and systematic agents as niches of satellite cells influence the activity of these cells (1,4). The differentiation of myoblasts into multi-core myofibrils depends to expression of myogenic regulator factors (MRF) called myogenic factor 5 (Myf5), myogenin, myoblast determination protein (MyoD) and myogenic regulator factors, which the main factor is helix-loop-helix transcription (1,3,4). Myof5's initiating activity (promoter) has been observed in quiescent satellite cells. Although the expression of Myof5 gene has not been reported in quiescent satellite cells, Myof5 is proliferator of satellite cells and its expression decreases in the stage of differentiation of satellite cells. In addition, studies have been revealed that HGF is an activator of satellite cells and plays vital role in the initial phase of muscle restoration. The activation of satellite cells by HGF is performed due to the activation of HGF transcription in satellite cells and the release of HGF from the extracellular matrix by matrix metalloproteinases (MMPs). It causes that HGF to act coordinately in an autocrine and paracrine method for proliferation of satellite cells (1,4). Nowadays, exercise trainings are considered as one of the best and cost-effective ways in prevention and treatment of many muscle disorders, such as age-related muscular dysfunction, sarcopenia, and cachexia. In this regard, studies have revealed that resistance exercise training in elderly people can improve physiological conditions, increase protein biosynthesis, increase strength, increase the size and function of muscle mass and hypertrophy of FTa and FTb, and increase the ratio of FT to ST in a given cross-sectional area of the skeletal muscle (5). Resistance exercise trainings are known as the most effective intervention to increase muscle strength and size, and they are often prescribed to prevent injury, rehabilitation of skeletal muscle, reduce the risk of falling, and increase the functional ability. In order to achieve these goals, the American College of Sports Medicine (ACSM) recommends exercise intensity 70-80% of one repetition

maximum (1RM). However, studies indicate that metabolic mechanical pressures caused by high-intensity resistance exercise trainings leave undesirable changes in indirect indices of cell injury and increase the concentration of the indices such as myoglobin (Mb) and creatine kinase (CK) and lactate dehydrogenase (LDH) in plasma. Thus, it is necessary to develop safe and effective methods for the elderly people, patients, and other groups, requiring increased muscle strength, but have no willingness and tolerance of such exercise trainings. Accordingly, resistance exercise training with intensity of 20 to 50% of 1RM combined with blood flow restriction (BFR), called as Kaatsu, has been introduced as a new training to replace traditional training. In these trainings, the inflow of the arteries and the outflow of vein in active muscles are reduced. With low intensity and under BFR conditions, these trainings can cause significant muscle strength and hypertrophy even in the short term (6,7). In this regard, Takarada et al. (2000) evaluated the performance of the brachial muscle of 24 healthy untrained women under the effect of low intensity resistance training (30-50% of 1RM) with and without BFR and high intensity resistance training (50-80% of 1RM) with and without BFR. They observed severe muscle hypertrophy as a result of low intensity resistance training combined with BFR (3). In another study lasted for 8 weeks, Takarada et al. (2002) examined knee extensor muscles of 17 male rugby players in three groups of low-intensity periodic exercises (about 50% of 1RM) combined with BFR, low-intensity periodic exercises without BFR and control group. The results showed that low intensity periodic exercise combined with BFR not only causes muscle hypertrophy and improves muscle strength of athletes, but also improves muscular resistance (4). Cramer et al. (2004) reported an increase in the number of satellite cells after high-intensity eccentric resistance training (ECCRT) of leg extensor muscles in eight healthy men, by using isokinetic dynamometer (8). Nielsen et al. (2012) also reported an increase in the number of satellite cells along with increase in muscle strength and muscle hypertrophy as a result of three weeks of low-intensity resistance training (20% of 1RM) on the knee extensor muscles of 20 healthy men (using isokinetic dynamometer) combined with BFR (9). Review of studies indicates that activation, proliferation, and differentiation of satellite cells as the main stem cells

involved in the muscle restoration, regeneration, and growth processes are affected by various sports interventions. However, cellular mechanisms responsible for creating these muscle adaptations as a result of different sports trainings have not been fully understood so far and further research is needed in this regard (8-11). Thus the present study was conducted to evaluate the impact of low-intensity eccentric resistance training combined with and without blood flow restriction (ECCRT with BFR *vs.* ECCRT without BFR) on some of the systematic and genetic indices affecting the activation and proliferation of satellite cells in young non-athlete men

2. Material & Methods

Subjects

Due to using human subjects, the current study is developmental type of study, conducted in a semi-experimental design with pre-test and post-test. The study population included young non-athlete men aged between 25 and 30 years. After different stages of screening among participants of study, 20 subjects who met the inclusion criteria of study were selected and they randomly divided into two groups ($n = 10$) of low-intensity ECCRT with BFR and ECCRT without BFR. General health status and life style parameters (smoking, using alcohol, nutritional habits, etc.), and history of any type of exercise training (number of sessions per week, duration of exercise per session), mean duration of activity, and severity of exercise were estimated using questionnaire. Subjects were ensured that the information of the questionnaires will remain confidential.

Creating blood flow restriction

In this test, blood flow restriction was created by using air pressure gauge with 5 cm band and pressure of 100 mm Hg in the upper extremity of the leg of subjects.

Biopsy on the inactive leg

In order to control and examine the interventional effect of biopsy in research findings, non-exercise leg of subjects underwent biopsy 48 hours before the intervention. For this purpose, biopsy was performed by a

specialist physician after local anesthesia with injecting lidocaine 1% using 5 mm needle from external broad muscle (15 cm higher than the patella). Then, samples were frozen at 80 ° C and quickly transferred to laboratory for perform biohistochemical tests.

ECCRT protocol

ECCRET protocol, included about 70 repetitions of eccentric resistance exercise of knee extensors, was used with 30% intensity of 1RM in 3 sets (15-15-25) and 30 seconds of resting between each set using isokinetic device which can set the workload and speed of 120 degrees per second. Subjects with BFR experienced BFR before performing the test on the upper extremity of the thigh. Twenty and four hours after performing the training, the active muscle of the subjects underwent biopsy. Then, samples were frozen at -80⁰C and quickly transferred to laboratory to perform biohistochemical tests.

Blood sampling

Blood sample was taken at the amount of 5cc before and immediately after training from medulla of the subjects and kept in laboratory tubes. After being kept at room temperature for about 15 minutes, sample was isolated for 15 minutes by serum and blood plasma centrifugation device. Then, it was kept in a freezer at a temperature of -80⁰C until the measurement time. To measure the serum level of HGF hormone, human HGF hormone kit manufactured by the System Immunodiagnostic Company of Germany was used and samples were measured using ELISA method.

Estimating the Myf5 gene expression

Myf5 was measured in this research by using immunohistochemical method. RNA was extracted using 50 mg of external broad muscle. To extract the RNA, RNeasy Mini Kkit manufactured by Kiagen Company (Catalog No74124) and its instructions were used. RNA level and purity was determined using column method trough picodrape device and the absorbance ratio of RNA was measured at wavelengths of 260 nm to 280 nm.

Real time stage (the process of generating cDNA) for mRNA

RNeasy Mini Kit manufactured by French Diaclone Company was used in this research to measure the Myf5 index. We converted 1 μg of RNA to single-stranded cDNA using the Random Hexamer primer, according to the manufacturer's protocol. Two incubation steps were used here:

The first incubation was performed first for 5 minutes at 65°C and a second incubation was performed for 10 minutes at 25°C and then 60 minutes at 42°C . Finally, the reaction ended with 10 minutes of heat at 72°C (12). Myf5 gene was considered as the target gene and GAPDH gene was considered as a reference in this research. Then, primers were designed using the GENERUNNER software and the NCBI database (Table. 1).

Table 1. Characteristics of primers

Gene	Primer sequence (f)	Primer sequence (p)	Fragment length
Myf5	TGTACCACGA CCAACCCCAAC	ATAGTAGTTCTC CACCTGCTCTCTC	192
GAPDH	CCAGGTGGTCTCC TCTGACTTCAACAG	AGGGTCTCTCTCT TCCTCTTGCTCTCT	223

All Real Time PCR reactions were conducted on a Rotor Gene TM 6000 device. The time-temperature schedule of device was adjusted at three stages. The first stage, resulting in spell of cDNA molecules, was conducted at 95°C for 5 minutes, and the second stage was conducted at 95°C for 100 seconds for splitting, at 56°C for 10 seconds for pairing, and at 60°C for 24 seconds for elongation in 40 cycles. In the final stage, the temperature in each repetition was increased from 50°C to 99°C (1°C per 5 seconds) (12).

Statistical analysis

Results were expressed as the mean \pm SD. Kolmogorov-Smirnov test also used to examine the normal distribution of quantitative data in groups. Independent sample t-test was used to compare the mean variations of each of the considered variables in the two groups in the post-test stage. Paired sample t- test was used to compare the variations of indexes in the post-test stage compared to the pre-test in each group. The significance level of the tests was considered at the level of $P < 0.05$. All

statistical calculations were analyzed using SPSS-17 and Graph Pad software.

3. Results

Given the first row of Table 1, in post-test of ECCRT without BFR, the HGF level of young non-athlete men increased by 3.75 compared to the pre-test ($P < 0.05$). Given the second row of Table 1, in post-test of ECCRT with BFR, the HGF level of young non-athlete men increased by 13.7 compared to the pre-test ($P < 0.05$). Given the third row of Table 1, in post-test of ECCRT without BFR, the Myf5 level of young non-athlete men increased by 2.33 compared to the pre-test ($P < 0.05$). Given the fourth row of Table 2, in post-test of ECCRT with BFR, the Myf5 level of young non-athlete men increased by 3.44 compared to the pre-test ($P < 0.05$).

Table 2. Effect of ECCRT on mean differences of HGF and Myf5 in young non-athlete men

Variables	Mean \pm SD	Paired sample t-test	P Value
Effect of ECCRT without BFR on HGF (Pg/mg)	3.7 \pm 0.9	10.89	0.01*
Effect of ECCRT with BFR on HGF (Pg/mg)	13.7 \pm 4.8	7.96	0.01*
Effect of ECCRT without BFR on Myf5	2.3 \pm 0.5	4.53	0.006*
Effect of ECCRT with BFR on Myf5	3.4 \pm 0.2	12.00	0.01*

* Significant differences ($P < 0.05$)

Comparison the effect of ECCRT with BFR and ECCRT without BFR on HGF is shown in the Table 3. Based on the Table 3, the increase of HGF was higher after ECCRT with BFR than after ECCRT without BFR ($P < 0.05$).

Table 3. Comparing the effect of ECCRT without BFR and ECCRT with BFR on HGF

	Levene test for equality of variances		Testing independent means equality		
	F	P Value	t	P Value	Mean difference
Confirmation of assumption of equality of variance	2.01	0.17	5.20	0.01*	14.67

* Significant differences ($P < 0.05$)

Comparison the effect of ECCRT with BFR and ECCRT without BFR on Myf5 is shown in the Table 4. Based on the Table 4, the increase of Myf5 was higher after ECCRT with BFR than after ECCRT without BFR ($P < 0.05$).

Table 4. Comparing the effect of ECCRT without BFR and ECCRT with BFR on Myf5

	Levene test for equality of variances		Testing independent means equality		
	F	P Value	t	P Value	Mean difference
Confirmation of assumption of equality of variance	5.13	0.04	2.54	0.026*	4.72

* Significant differences ($P < 0.05$)

4. Discussion

In the present study, ECCRT with BFR caused an increase in level of HGF and Myf5 gene expression, suggesting the effect of this training on the activation and proliferation of satellite cells. Increasing level of Myf5 gene expression, which is one of the key transcription factors in muscle building process, allows more proliferation for satellite cells (1,3,4,12). It seems that response of the satellite cells to be completed within 96-72 hours after exercise training, as the number of satellite cells in this period is maximized and then decreases (5, 9). In addition, HGF is necessary for muscle hypertrophy and growth, moderated by activated satellite cells. In the muscle that is in the resting state, there is an inactive HGF separately as a local storage to facilitate quick response to restoration of injured muscle. These growth factors can be activated quickly by proteolytic enzymes available in serum or interstitial agents such as thrombin, serine proteases, and matrix metalloproteinases (MMPs). This highly- coordinated process acts to stimulate the survival, activation, and proliferation of satellite cells after injury to the muscle (1). After ECCRT with BFR, HGF transcription increases depending on the degree of injury, and an active form of HGF is released from the extracellular matrix without any need for proteolytic cleavage of pre-HGF (10,11,13). Released HGF and newly-generated HGF act directly

on satellite cells and myoblasts through receptor c-Met, found on the surface of the quiescent satellite cells and activated satellite cells, and they activate the quiescent satellite cells (13-15). ECCRT in the present study showed an increase in gene expression level in Myf5. Previous studies have reported that one session of resistance exercise training could increase the number activation indices of satellite cells such as PaX7, MyoD, and Myf5 in subjects. This response was significant in young subjects, and the time period in which this increase was reported was 24 hours to 8 days after resistance exercise. There are few studies for shorter periods and significant change has not been reported (3,11). In most of the studies conducted in this regard, satellite cells' response has been investigated in men after a period of high-intensity ECCRT to induce muscle injury. In some of the cases, BFR has also been investigated along with exercise. One important aspect of the current study is evaluating the low-intensity ECCRT with BFR, which it was performed in single session. This issue can be investigated in another study using long-term exercise periods or in a different study using people with different gender and age groups, which might lead to different responses.

5. Conclusion

Findings of the present study revealed that low-intensity ECCRT with BFR increased the serum level of HGF and also increased MyF5 gene expression in non-athlete subjects. These two factors play key role in activation and proliferation of satellite cells. Thus, it can be useful for patients or injured people such as professional athletes, who are not able to perform training with high-intensity with the aim of hypertrophy or muscle mass retention, while complementary studies are required in this regard.

Conflict of interests: No conflict of interests amongst authors.

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