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Research Paper

Evaluation of 8 Weeks of Different Muscle Tensions on Some MicroRNAs Related to Angiogenesis of Muscular Hypertrophy of Bodybuilders

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ABSTRACT

Background and Aim: Angiogenesis is the most important foundation of exercise-induced hypertrophy. This study aimed to investigate the effect of 8 weeks of different muscle tension training on some microRNAs related to angiogenesis and muscle hypertrophy in bodybuilders.

Materials and Methods: The present study is a quasi-experimental study with a pre-test-post-test design. The study groups included resistance training group 1 (10 people), resistance training group 2 (10 people), and the control group (10 people). The study subjects were selected from male bodybuilders who have done regular training for at least three years and are aged between 25 and 30 years. The study was explained to them, and 48 hours before and after the test, 5 mL of blood was taken from each sample. Descriptive statistics, including Mean±SD, charts and tables, and inferential statistics, including analysis of variance (ANOVA) and post hoc test, were performed using SPSS software. First, the Kolmogorov-Smirnov test was used to determine the normality of the data. Then, to compare the differences between the groups, the ANOVA was used, and if it was significant, the Bonferroni post hoc test was used.

Results: This study showed that resistance groups 1 and 2 significantly increased miR-1 in bodybuilders. Also, group 1 had a greater effect than resistance group 2. The results showed that resistance training in different time points and sets had different effects on molecular pathways of hypertrophy. So, two hours after resistance training, mir-1 levels did not increase significantly, but 4 hours after resistance training, the levels of these microRNAs increased. This study showed that group 1 had a more significant effect on miR-206 levels than resistance group 2.

Conclusion: The results of this study indicated that resistance training significantly increases the vascular endothelial growth factor (VEGF) levels of bodybuilders. Also, the results showed that in group 2, an increase in VEGF levels led to a greater effect on angiogenesis in these samples.

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1. Introduction

Research on the benefits of various types of resistance training is ongoing and is of interest to clinicians, educators, and scientists [1]. Most researchers agree that it is important to customize the exercises to get the desired results or adaptations. One of the important physiological adaptations resulting from sports activities is angiogenesis. The response of angiogenesis to physical activity depends on the intensity, duration, type of exercise program, and level of physical fitness of the subjects [2]. Adaptation to exercise shifts the balance between angiogenic and angiostatic factors toward angiogenic ones. These adaptations may provide new insights into the molecular processes that increase capillary density in response to resistance activity. Nitric oxide (NO) and fibroblast growth factor (FGF) are the most important regulators of angiogenesis. These factors perform their biological actions on target cells through interaction with tyrosine kinase receptors in the cell's plasma membrane [3]. After binding to the ligand, these receptors themselves become dimers and auto-phosphorylated, which ultimately leads to a myriad of intracellular reactions [4].

In recent years, cellular and molecular fields have progressed, and there is a need for more specialized resistance training for better adaptation. Thus, the effects of time and intensity of training have been examined simultaneously [5]. It is generally believed that high-intensity resistance training (traditional strength training, or TST) with an intensity of 70%-80% of one maximum repetition (1RM) is required for angiogenesis and increased muscle strength, and low-intensity training will not produce enough stress to increase strength, muscle volume, and angiogenesis [2]. Muscle hypertrophy means an increase in the size of muscle tissue. In fact, muscle growth occurs through an increase in sarcomeres, contractile elements, and sarcoplasmic fluid, as well as the improved activity of satellite cells. Contractile muscle hypertrophy is caused by adding sarcomeres in parallel or sequentially [6]. Research has shown that certain types of exercise protocols can affect the fascicles.

Today, microRNAs are used to modify muscle function. MicroRNAs (miRNAs) are small, non-coding RNAs highly conserved in eukaryotes [1]. Currently, the important role of miRNAs in diverse biological processes, including development, differentiation, homeostasis, and diseases in vertebrate species, is evident [7]. The function of miRNAs is to fine-tune gene expression by accelerating mRNA degradation and or by inhibiting translation [8]. Identifying specific muscle miRNAs called miR-1, miR-133a/b, miR-206, miR-208b, miR-499, and miR-486 has expanded the molecular network in skeletal muscle by angiogenesis [9]. Research results have shown that miR-206 [10] modulates angiogenesis by suppressing the transcriptional vascular endothelial growth factors (VEGF) gene [11]. Angiogenesis and muscle hypertrophy can be achieved through various strength training programs [12]. The principle of training features states that some training methods promote more muscle angiogenesis than other training methods [13]. According to research, the best way to achieve angiogenesis and muscle hypertrophy is to use bodybuilding training patterns [14]. Modern methods of bodybuilding exercise use the concept of an exercise schedule,

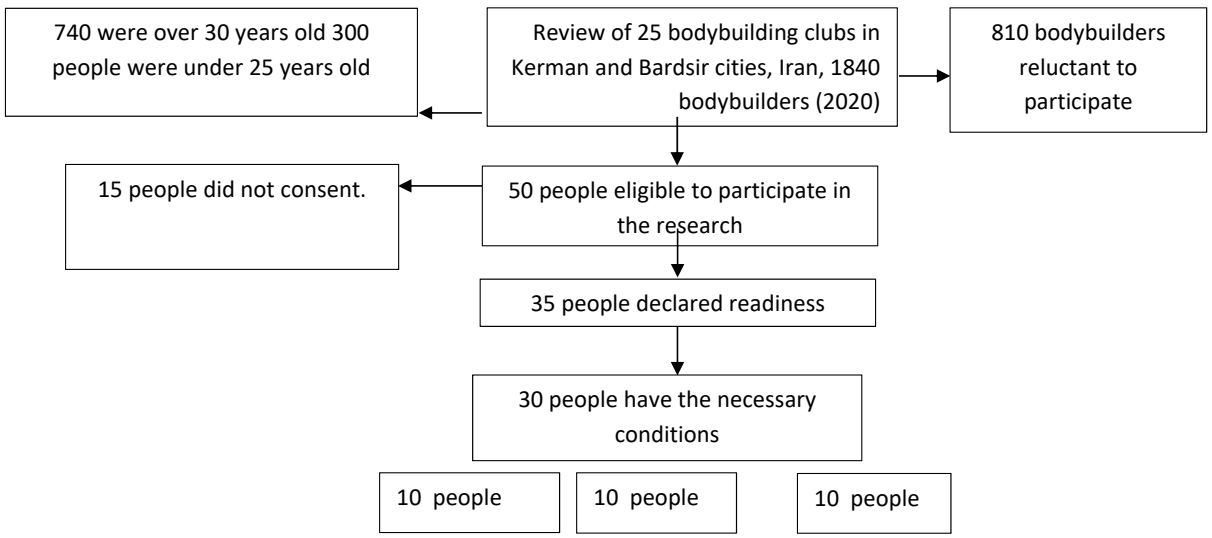

Figure 1. Steps of implementing the research plan in selecting samples

which includes 6 stages (structural adaptation, bulking, integration, maximum strength, separation, and transfer) [15], meaning that at each stage, athletes use methods to develop muscles [16] better. They use different exercises, but it is unclear which method is more suitable for maximizing the development of angiogenesis and hypertrophy or which training method is superior to other methods. So far, no study has been done on the effect of different muscle tensions on some microRNAs related to angiogenesis and muscle hypertrophy, and no scientific study has been found in this field. Therefore, this study aimed to investigate the effect of 8 weeks of different muscle tensions on some microRNAs related to angiogenesis and muscle hypertrophy in bodybuilders.

2. Materials and Methods

Considering that the subjects of the present study are male bodybuilders and were not completely under control in some variables, the present study is a quasi-experimental study with a pre-test-post-test design. The study groups were resistance training group 1 (10 people), resistance training group 2 (10 people), and a control group (10 people). Figure 1 shows the beginning to the end of the research process.

The study population comprised male bodybuilders who had regularly trained for at least three years. Thirty of them were selected as a research sample. The selected people of their age range should be between 25 and 30 years old. The selected samples should not have any heart disease or mental health problems, then 30 people are randomly divided into three groups: resistance training 1 (10 people), resistance training 2 (10 people), and control (10 people) groups. Then, after selecting the samples, the study was explained to them, and 48 hours before and after the test, 5 mL of blood was taken from each sample. The experimental group performed three sessions of exercises with different muscle tensions each week for 8 weeks. Each session started with 15 minutes warm-up and finished with 15 minutes of cool down.

Exercise protocol

The resistance training protocol was implemented for 3 sessions per week for 8 weeks. Initially, one maximum repetition of individuals was performed using the following formula: the resistance training protocol is circular and includes leg presses, back foot, front foot, chest press, front arm, and bilateral downward stretching. All movements, except the movement of the chest press and the front of the arm, were performed with special devices and under the supervision of the instructor and researcher. The chest press was performed lying on the table with a barbell and weights. The movement of the front of the arm would be done by the barbell and standing next to the wall. Because the subjects were not familiar with this movement, they were asked to lean against the wall 1st and bend their legs from the pelvis to place their legs slightly forward along the trunk. In this way, the back is supported, and the possibility of injury is reduced. To perform similar training for two groups, the following formula was used to equalize the training load of the two groups: (muscle tension duration×number of repetitions)×intensity=training load volume×intensity. The training load resistance in the 1st group was implemented in three 12-repetition sets with 75% of one maximum repetition (1MR) with 2 minutes of break between sets and 1 second of muscle tension (1 second of flexion and 1 second of extension). The training load resistance in the 2nd group was implemented in three 3-repetition sets with 50% of 1MR and with 2 minutes of breaks between sets, with a duration of 6 seconds of muscle tension (6 seconds of flexion and 6 seconds of extension). The resting time between stations was considered 30 seconds, and to observe the principle of overload and gradual improvement, 5% was added to the weight after every 6 training sessions [15].

The inclusion and exclusion criteria

The inclusion criteria were as follows: no history of any surgery; not suffering from cardiovascular diseases, chronic systemic diseases such as diabetes and hyperthyroidism, hormonal or immune system disorders, and mental and emotional diseases; not smoking and drinking alcohol; no problems or physical defects in the musculoskeletal or nervous system that prevents exercise, no history of severe hypertension (more than 160/90 mm Hg). The exclusion criteria were as follows: increase of blood pressure more than three degrees at rest due to exercise pressure, nausea, and dizziness after exercise, having repetitive and chronic pain in different areas of the body that prevents exercise, any heart and respiratory problems which occurs during the implementation of the protocol, excessive fatigue and shortness of breath before or during exercise that causes bruising or discoloration of the patient.

The study instruments to measure various indicators are a digital scale for measuring the weight of the subjects with an accuracy of 0.001 kg made in Japan, a digital altimeter for measuring the height of the subjects made in Iran, a digital Polar heart rate monitor made in Finland, a mercury barometer Picado made in Germany, ELISA STAT FAX 2100 made in Japan to measure biochemical variables, and real-time PCR to measure microRNAs [3].

Measurement of blood indicators

Subjects were told not to engage in physical activity for 48 hours before and after the blood sampling. Blood samples were taken from the study subjects in two stages (48 hours before the 1st training session and 48 hours after the last training session), followed by 12 hours of night fasting. At each stage, 10 mL of blood was taken from each sample's forearm vein. Blood samples were centrifuged at 1500 rpm for 10 minutes to separate the serum, and then the extracted sera were distributed in Eppendorf special containers for laboratory analysis and measurement of the indicators studied in this study. The samples were kept at -80°C [1].

For the microRNA expression assay, red blood cells were 1st lyzed, and after centrifugation, samples of precipitated white blood cells were collected. If necessary, the secreted microRNAs in the serum can also be isolated after centrifugation. MicroRNA extraction was performed using a kit. Specific primers were used to amplify the microRNAs. These primers are usually ribonucleotides containing a methylene bridge to which oxygen binds 2' carbon of the ribose sugar to its 5' carbon. The creation of this bridge leads to an increase in specific properties and affinity in hybridization to complementary sequences and an increase in melting point (Tm) by several degrees. Then, according to the manufacturer's protocol of microRNA primer, water was added to it, and quantitative microRNA amplification was performed using real-time PCR. Finally, the data were normalized using the control reference gene. For this purpose, the expression of microRNAs is measured and compared to control genes such as U6 snRNA and mir-99a-5p. The internal control gene must be a gene whose expression does not change under various cellular conditions. Finally, the expression of the desired microRNAs will be studied and analyzed quantitatively [2].

To remove RNA from microtubes and sampler heads, all samplers and microtubes used to extract the microRNAs required in this study were placed overnight in DEPC (Diethyl pyrocarbonate) solution at a concentration of 0.1%. After drying at 45°C in an oven, the samplers and microtubes were doubled. The next time they were autoclaved regularly. To extract the microRNA, we used the TRIzol RNA extraction kit, produced by Rena Biotechnology Company with access code (RB1001), according to the manufacturer's instructions. Also, to check the levels of microRNA, the RT steam-loop housekeeping method was used. 1st, 500 µL of the blood sample was mixed with 1 mL of the irisol buffer to obtain a homogeneous solution. The mixed solution was then transferred to a 2 ml microtube and kept at room temperature for 5 minutes. Next, 200 µL of chloroform was added to the solution and shaken vigorously for 10 to 15 seconds to be mixed. The tube containing this mixture was again exposed to ambient temperature for 5 minutes and then centrifuged at 8000 g for 5 minutes. At the end of these stages, two phases were visible. The clear supernatant containing the RNA was added to 1000 µL of 100% cold ethanol and then placed in a freezer at -20°C for 8 minutes after separate transfer to another tube. Finally, the tube was taken out of the freezer and centrifuged according to the previous step. After centrifugation, the liquid inside the tube was separated and discarded. At this stage, colorless or whitish spots were observed on the body and floor of the tube, as usual. The centrifugation operation was repeated by adding 80% ethanol for washing. After removing the ethanol and drying the contents of the tube by air-dry method, 20 to 50 µL of double distilled water was added. The pipette dissolved the RNAs inside the tube. Then, to confirm the quality, 5 µL of this solution was analyzed by electrophoresis on 1% agarose gel so that if the quality is confirmed, this solution can be used in cDNA synthesis. For DNA replication of this product, the extracted RNAs were removed from the DNA strands with the help of DNase I. For this purpose, after adding the DNase enzyme, the reaction mixture was incubated at 37°C for 30 minutes. To confirm RNA quality, the DNA samples taken from the total RNA were analyzed on 1% agarose gel [1].

Table 1. Sequence of primers of microRNAs examined

Pmir	Primer Design From miRBase Database
M miR-1	GAGGUUUUCUGGGUUUCUGUUUC
M miR-206	UAGCAGCACAUAAUGGUUUGUG
miR-139-5p	UCUACAGUGCACGUGUCUCCAGU

MiRNA synthesis

According to Tables 1 to 5, cDNA synthesis was performed using a cDNA synthesis kit produced by Rena Biotechnology. After completing the process and obtaining the threshold cycles (Ct), the expression of the desired variables was measured using mathematical calculations. ΔCt of each sample was calculated using reference control (miR-139-5p) and miRNA (subtracting Cts from reference control). The corresponding ΔΔCt for each sample was then calculated by subtracting the ΔCt of that sample from the mean ΔCt of the control group. Finally, the mathematical relation was used to report changes in the quantitative expression of variables.

Statistical analyses

Descriptive statistics, including Mean±SD, plotting charts and tables, and inferential statistics, including analysis of variance and post hoc test, were performed using SPSS software. The significant level of the measured items will be considered $P \leq 0.05$. 1st, the Kolmogorov–Smirnov test was used to determine the normality of the data. Then, to compare the differences between the groups, the analysis of variance (ANOVA) test was used, and if it were significant, the Bonferroni post hoc test would be used.

3. Results

The study aimed to investigate the effect of 8 weeks of different muscle tensions on some microRNAs associated with angiogenesis of muscle hypertrophy in bodybuilders. This study showed that 8 weeks of resistance training significantly increased miR-1 in bodybuilders in resistance groups 1 and 2. Also, resistance training in group 1 had a greater effect than resistance in group 2.

4. Discussion

The results of this study are consistent with the results of Shi et al. [16]. They reported that resistance training in different periods and sets had different effects on molecular pathways of hypertrophy so that 2 hours after resistance training, miR-1 levels did not increase significantly but 4 hours after resistance training the levels of these microRNA increased [16]. The study's results are inconsistent with Torma et al. They examined the effect of blood restriction on bodybuilding exercises with the intensity of 70% and 90% [17]. The results of the Torma research showed that blood limitation at intensities of 70% and 90% of IMR did not cause any change in miR-1 levels in the research samples [17]. Measurement of study samples also showed that 8 weeks of resistance training increased miR-206 levels in bodybuilders. This study showed that 8 weeks of resistance training in group 1 had a more significant effect on miR-206 levels than in resistance group 2. The results of this study are consistent with the results of Fallah et al. [18]. They reported that the expression of miR-206 in the flexor hallucis longus muscle increased in both resistance training groups compared to the control group but decreased in soleus muscle in both groups [18]. In a study of C57 mice, McCarthy et al. found an 18.3-fold increase in miR-206 levels and a 7-fold increase in hypertrophy [19]. The results of this study were consistent with those

Table 2. Results of the Two-way analysis of variance to investigate the effect of exercise and nutrition interventions on variables

Variables	Source	Sum of Squares	df	Mean of Square	F	Sig.	Eta	Observed Power
	Control	1.274	1	1.274	2.041	0. 120	0.085	0.329
miR-1	Training 1	19.512	1	19.512	107.799	0.000***	0.777	1.000
	Training 2	0.685	1	0.685	3.785	0.061	0.109	0.470
	Control	0.540	1	0.540	1.091	0.304	0.034	0.173
miR-206	Training 1	67.728	1	67.728	136.962	0.000***	0.815	1.000
	Training 2	2.163	1	2.163	4.374	0.045*	0.124	0.527
	Control	4.171	1	4.171	8.159	0.008**	0.208	0.790
VEGF	Training 1	31.693	1	31.693	61.994	0.000***	0.667	1.000
	Training 2	0.705	1	0.705	1.379	0.249	0.043	0.207

Table 3. Bonferroni test results for miR-1 variables

Group(I)	Group(j)	Mean±SE	Sig.
Resistance 1	Resistance 2	3.25±1.421	0.0002
	Control	5.45±1.414	0.0002
Resistance 2	Control	5.11±1.422	0.0001

SE: Standard error

Table 4. Bonferroni test results for the miR-206 variables

Group(I)	Group(j)	Mean±SE	Sig.
Resistance 1	Resistance 2	2.370±0.615	0.002
	Control	0.563±0.599	0.1
Resistance 2	Control	2.934±0.433	0.001

E: Standard error

Table 5. Bonferroni test results for the VEGF variables

Group(I)	Group(j)	Mean±SE	Sig.
Resistance 1	Resistance 2	2.125±0.657	0.001
	Control	0.475±0.526	0.0001
Resistance 2	Control	2.474±0.524	0.0001

SE: Standard error

of Drummond et al., who examined the expression of progressive and adult microRNA in young and older men after an open-leg session with essential amino acids as anabolic stimulants [20]. They found that miR-206 pri-in old and young men increased in lateral extensor muscle after 3 and 6 hours of training, while in the case of miR-206, the changes were not significant. He also disagreed with the results of Drummondnd et al. [20]. In the study of skeletal muscle microRNA expression after 1 session of resistance training [21]. Necessary biopsies were taken from the lateral extensor muscle at 0, 6, and 24 hours after resistance training. They observed that miR-206 decreased expression 6 and 24 hours after exercise, but at the time 0, there was no change in miR-206 [22]. Regarding the conflicting results of the above study and other results, factors such as type of exercise, muscle type, type of species, studied time points, and stimuli may be the reasons for the differences. Circulating miRNAs, which are present in the bloodstream and are highly stable, have been suggested as potential new biomarkers of sports responses, including weight training [23]. These microRNAs have many proteins and targets and act through these pathways. Regarding the molecular pathway of hypertrophy, all eukaryotic cells have an innate ability to adapt to various extracellular stimuli, which are caused by physiological situations [24]. This activity is mainly mediated through multiple ways of intracellular messaging and genomic modification and the activity of copying the factors and cellular proteins involved in the existing synthesizing proteins [25]. Also, the results showed that resistance training significantly increases the vascular endothelial growth factor (VEGF) levels of bodybuilders [26].

5. Conclusion

The results of this study indicated that resistance training significantly increases the VEGF levels of bodybuilders. Also, it showed that in group 2, the increase in VEGF levels leads to a greater effect on angiogenesis in these people.

Ethical Considerations

Compliance with ethical guidelines

The Ethics Committee of the University of Qom approved this study (Code: IR.QOM.REC.1399.002).

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Authors' contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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Journal of Vessels and Circulation
Qom University of Medical Sciences

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