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Increased Expression of *miR-1* in Fast-Twitch Skeletal Muscle in Response to Resistance Exercise

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Abstract

Background: The response of microRNA (*miR-1*), upstream regulators (Myogenic Differentiation 1 (*MyoD1*) and Myocyte Enhancer Factor 2C (*Mef2C*)), and a downstream target (Histone Deacetylase 4 (*Hdac4*)) to an acute bout of Resistance Exercise (RE) in Extensor Digitorum Longus (EDL) and soleus muscles has remained elusive.
Objectives: In this experimental study, we investigated the effect of an acute bout of RE on the expression of *miR-1*, *MyoD1*, *Mef2C*, and *Hdac4* genes in the slow and fast-twitch muscle of rats.
Methods: The current study was conducted at Tarbiat Modares University, Tehran, Iran, in 2017. Fifteen male Wistar rats were randomly divided into two groups, control (n = 5) and RE (n = 10). The RE protocol consisted of four sets of five repetitions of climbing a ladder with weights attached to the tails of rats. The soleus and EDL muscles of rats were collected at 3 h (n = 5) and 6 h (n = 5) post-RE. The real-time polymerase chain reaction was used to measure the levels of *miR-1*, *MyoD1*, *Mef2C*, and *Hdac4* mRNA expression.
Results: The *miR-1* expression in EDL muscle was significantly lower at 3 h and 6 h post-exercise in the RE group than in the control group ($P < 0.01$). The *miR-1* expression in soleus muscle was significantly lower at 3 h post-exercise in the RE group than in the control group ($P < 0.007$) but it was significantly higher at 6 h post-exercise ($P < 0.036$). The expression of *MyoD1*, *Mef2C*, and *Hdac4* genes in the EDL muscle was higher in the RE group than in the control group ($P < 0.01$). The expression of these genes in response to RE had more fluctuations in soleus muscle.
Conclusions: It can be concluded that *miR-1* expression in extensor digitorum longus and soleus muscles of rats responds to resistance exercise in different manners and this coincides with a change in upstream regulators and downstream target.

Keywords: Expression, Gene, *Hdac4*, *Mef2C*, *miR-1*, Polymerase Chain Reaction, Rat, Regulation, Resistance Exercise, Skeletal Muscle

1. Background

Endurance and resistance activities stimulate the response and adaptation of fast and slow-twitch muscle fibers (1). Different transcriptional factors among microRNAs (miRs) may contribute to this process (2, 3). Recent findings have shown that miRs have an important role in various cellular processes, such as skeletal muscle proliferation and differentiation (4).

The miRs are small, non-coding RNAs that regulate more than one-third of the mammalian genome. Several miRs are specifically expressed in striated muscle (*miR-1*, -133, -206, -208a/b, -486, and -499) (5). Among them, *miR-1* plays an important role in skeletal muscle growth (6) and is involved in myoblast differentiation by regulating the expression of *Hdac4* mRNA. The *miR-1* expression is regulated by a muscle transcriptional network involving *MyoD1* and *Mef2C* factors (7, 8), which are also important in muscle formation and plasticity (9). Skeletal muscle response to exercise is controlled by several levels of regulation, including transcriptional, post-transcriptional, and translational events. It has been suggested that the transient changes in transcription during recovery from acute bouts of exercise may accumulate and translate into cellular training adaptations if the exercise is performed for a prolonged time (10) while resistance exercise can increase muscle hypertrophy and protein synthesis in a long time (1). The changes in *miR-1* and its transcriptional network in response to resistance exercise in slow and fast twitch fibers are unclear. Concerning the role of *miR-1* and its targets in the plasticity of slow and fast-twitch fibers, we hypothesized that RE could change the expression of *miR-1* and its down and upstream targets in fast and slow-twitch muscles.

2. Objectives

The current study aimed at evaluating the potential effect of RE on *miR-1* expression and its up and downstream genes in slow and fast-twitch muscle fibers.

3. Methods

3.1. Animal Care

This experimental study was handled at Tarbiat Modares University, Tehran, Iran, in November 2017 (Code 60/79650 2017). In total, 15 healthy male adult Wistar rats aged 10 weeks and weighing between 175 and 200 g were purchased from the Pasteur Institute of Iran. The rats were housed under 12-hour dark (7 p.m. to 7 a.m.)/12-hour light (7 a.m. to 7 p.m.) cycles at 23 $\pm$ 2°C temperature and 30% - 70% humidity to acclimatize to the laboratory environment with free access to water and food (Behparvar Animal Chow Company, Iran). The rats were weighed before the experiments. The experimental design and procedure of the study were in complete compliance with the international guidelines (World Medical Association Declaration of Helsinki) and approved by the Ethics Committee of the School of Medicine, Tarbiat Modares University (Code 60/79650, May 2017). Any attempt was made to decrease the number of animals used and their suffering. All of the observations were performed by a single observer. Simple randomization was used to randomly assign the rats to control (n = 5) and RE (n = 10) groups. The RE group was subjected to an acute bout of RE as described below. To compare the mean values of the two groups with a type 1 error of 0.05 and a power of 80%, the sample size was determined by the following formula:

$$Sample\ size = \frac{2SD^2\left(Z^{\frac{\alpha}{2}} + Z^{\beta}\right)^2}{d^2} \quad (1)$$

Considering Z^{α} = 1.96, Z^{β} = 0.84, SD = 20, and d = 35, the maximum sample size in each group was determined to be 5.12 rats.

3.2. Resistance Exercise Protocol

The RE protocol consisted of climbing a 1-m ladder with 26 rungs inclined at 85 degrees with weights attached to the tails of rats. All rats were familiarized with the exercise in three sessions. After familiarization, the RE was administered using small bags containing weights attached to the base of the tail with an adhesive plaster tape. The bags were fastened to the upper portion of the tail; then, the proper weights were embedded into the bags. The rats were placed at the bottom of the ladder and motivated to climbing (11). The initial weight attached was equal to 50% of their body weight that was added gradually by 10% of their body weight for each set to reach the final load of 80% in the fourth set (Using the scales A&D Japan). Before and after each weighing, the digital scale was calibrated to zero. The RE consisted of four sets of five repetitions with a 30-s rest interval between each repetition and 2 min rest between each set. When the rats reached the top of the ladder, they were allowed to recover in the resting area. This procedure was repeated until all rats finished the four sets of exercise. The exercise was performed at 9:00 a.m.

3.3. Tissue Preparation

After RE, the rats were weighed and anesthetized by intraperitoneal injection of ketamine (90 mg kg^{-1}) and xylazine (10 mg kg^{-1}) mixture. Then, the experimental group was randomly divided into two groups. The first group sacrificed at 3 h (n = 5) and the second one at 6 h (n = 5) post-exercise. The EDL and soleus muscles were quickly excised, weighed, immediately frozen in liquid nitrogen, and stored at -80°C until use.

3.3.1. RNA Isolation and cDNA Synthesis

Total RNA was isolated from frozen skeletal muscles by TRIzol (Invitrogen, Inc.) and chloroform (Merck, Germany) as described in the manufacturer's manual. Briefly, ~ 50 mg of EDL or soleus muscles were pulverized in liquid nitrogen using a mortar and pestle. The pulverized sample was then transferred to TRIzol in 1 to 10 portions for removing protein components. The final product was centrifuged at 12000 $\times$ g for 10 min at 4°C. Then, it was mixed with chloroform in 1 to 5 portions and shaken vigorously for 15 s. The supernatant was centrifuged at 12000 $\times$ g for 10 min at 4°C and its water and mineral parts were removed. Finally, its RNA-containing portion was removed and mixed with isopropanol in 1 to 5 portions. It was left for 10 min at room temperature and then centrifuged at 12000 $\times$ g for 10 min at 4°C. The RNA-containing pellet was washed and dissolved in 20 μL of RNsa-free water. RNA purity was determined using the UV spectrophotometry method (Eppendorf, Germany), and 260 nm to 280 nm ratios of $\geq$ 1.8 were considered acceptable for generating cDNA. GelRed staining was used to confirm RNA purity. Total RNA was converted to cDNA using the Revert Aid First Strand cDNA Synthesis Kit (cat#K1621) according to the manufacturer's manual (Thermo Scientific, Waltham, MA, USA).

3.3.2. Real-Time Polymerase Chain Reaction

Real-Time PCR was performed using Takara Master Mix (RR820A SYBR® Premix Ex Taq™ II. Tli RNase H Plus) for determining *MyoD1*, *Mef2C*, and *Hdac4* mRNA

expression levels (Step One Plus™ Real-Time PCR Systems, Applied Biosystem America). The reaction was performed in a final volume of 20 μL, containing 10 μL of Syber Green, 1 μL of forward primer, 1 μL of reverse primer, 1 μL of cDNA, and 7 μL of DEPC water. Each reaction was done in triplicate. To design and blast the *MyoD1*, *Mef2C*, *Hdac4*, and *gapdh* primers according to the NCBI gene bank, GeneRunner software and NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) were used, respectively. The used primer sequences are listed in Table 1. Moreover, the *gapdh* gene was used as the housekeeping gene. The thermal program used in Real-Time PCR included 95°C for 30 seconds, 95°C for 5 s, and 60°C for 30 s (40-cycle repetitions). The standard and melt curves were drawn and analyzed for optimization experiments and assessing data accuracy conditions, respectively, and *MyoD1*, *Mef2C*, and *Hdac4* expression data were normalized using housekeeping gene *gapdh*.

3.4. miR-1 Detection

Reagents for cDNA synthesis (catalog # 203300), SYBR Green master mix (catalog # 203450), and primers (*miR-1* (rno-*miR-1*-3p LNA™ PCR primer set, UniRT. MIMAT0003125) and U6 (U6 snRNA PCR primer set, UniRT, 203907) were purchased from Exiqon (Vedbaek, Denmark) to measure the *miR-1* expression (with UGGAAUGUAAAGAAGUAUGUAU sequence). The cDNA synthesis and real-time PCR for *miR-1* were done according to the manufacturer's protocols as described in Ahmadi Khatir et al. study (12). It should be noted that all the equipment used was regularly calibrated.

3.5. Statistical Analysis

All data are presented as Means ± Standard Deviation (SD). Considering the sample size, all analyses were performed based on bootstrap. Independent *t*-tests were used to assess the main effects of exercise (changes in mRNAs expression and *miR-1* muscles at three and six hours after RE) based on the bootstrap method. All analyses were performed using the IBM SPSS Statistics for Windows, version 20.0 (IBM Corp., Armonk, N.Y., USA), at the significance level of $P < 0.05$. Graphs were drawn based on means ± SD.

4. Results

4.1. Changes in miR-1 Expression in Response to RE

As shown in Figure 1, the *miR-1* expression in EDL muscle was significantly lower at 3 h ($P < 0.001$) and 6 h ($P < 0.008$) post-exercise in the RE group than in the control group. The *miR-1* expression in soleus muscle was significantly lower at 3 h post-exercise in the RE group than in the control group ($P < 0.007$) but it was significantly higher at 6 h post-exercise ($P < 0.036$; Table 2).

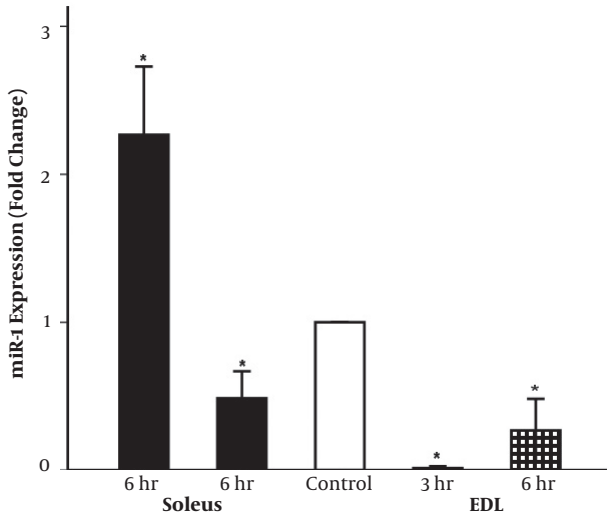

Figure 1. The expression of *miR-1* in EDL and soleus muscles in response to an acute bout of RE at 3 h and 6 h post-exercise. The expression of *miR-1* was normalized to U6 expression. The values are represented as means ± SD which indicate significant differences with the control group ($P < 0.05$)

4.2. Increased MyoD1 Expression in Response to RE

The *MyoD1* expression in EDL muscle was significantly higher at 3 h ($P < 0.01$) and 6 h ($P < 0.001$) post-exercise in the RE group than in the control group, but the *MyoD1* expression in soleus muscle was similar in the RE group (both at 3 h and 6 h post-exercise) and the control group (Figure 2).

4.3. Differential Expression of Mef2C in Response to RE

The *Mef2C* gene expression in EDL muscle was significantly higher at 3 h ($P < 0.001$) and 6 h ($P < 0.02$) post-exercise in the RE group than in the control group. In contrast, the *Mef2C* gene expression in soleus muscle was significantly lower at 3 h post-exercise in the RE group than in the control group ($P < 0.033$); however, this difference did not remain at 6 h post-exercise (Figure 3).

4.4. Hdac4 Expression in Response to RE

The *Hdac4* gene expression in EDL muscle was significantly higher at 3 h post-exercise in the RE group than in the control group ($P < 0.015$) but not at 6 h post-exercise. The *Hdac4* gene expression in soleus muscle was not different between the RE and control groups.

Table 1. Primer Sequences Used for Real-time Polymerase Chain Reaction[a, b]

Gene Name		Sequence 5 - 3	NCBI Reference Sequence	Product Size
Gapdh	F	AACCCATCACCATCTTCCAG	NM_017008	74
	R	CACGACATACTCAGCACCAG		
Mef2C	F	CCATTGGACTCACCAGACCT	XM_003749164.1	84
	R	ATGTTGCCCATCCTTCAGAG		
Hdac4	F	AACCTAACCTGAAATTACGGTC	NM_053449.1	137
	R	ACATGCGGAGTCTGTAACATC		
MyoD1	F	TCTGATGGCATGATGGATTAC	NM_176079.1	74
	R	TAGTAGGCGGCGTCGTAG		

Abbreviations: F, forward; *gapdh*, Glyceraldehyde-3-phosphate dehydrogenase; *Hdac4*, Histone Deacetylase 4; *Mef2C*, Myocyte Enhancer Factor 2c; *MyoD1*, Myogenic Differentiation 1; R, reverse.
[a] miR-1: 205104 rno-miR-1, LNA™ PCR primer set, UniRT. miRCURY LNA™ Universal RT microRNA PCR, microRNA primer set.
[b] U6: 203907, U6 snRNA (hsa, mmu, rno) PCR primer set, UniRT. miRCURY LNA™ Universal RT microRNA PCR, reference gene primer set.

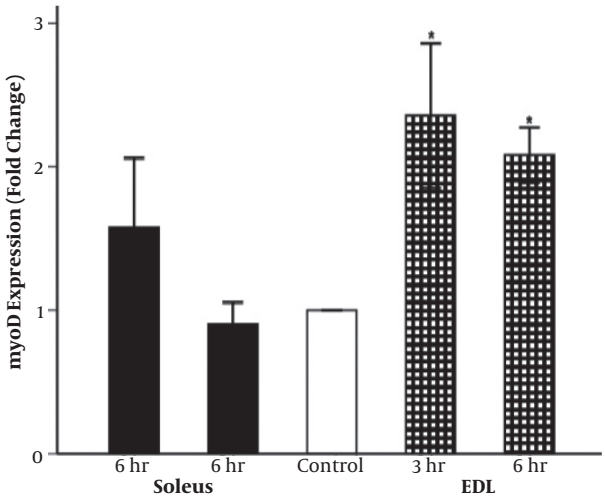

Figure 2. The expression of *MyoD1* in EDL and soleus muscles in response to an acute bout of RE at 3 h and 6 h post-exercise. The expression of *MyoD1* was normalized to *gapdh* expression. The values are represented as means ± SD which indicate significant differences with the control group ($P < 0.05$).

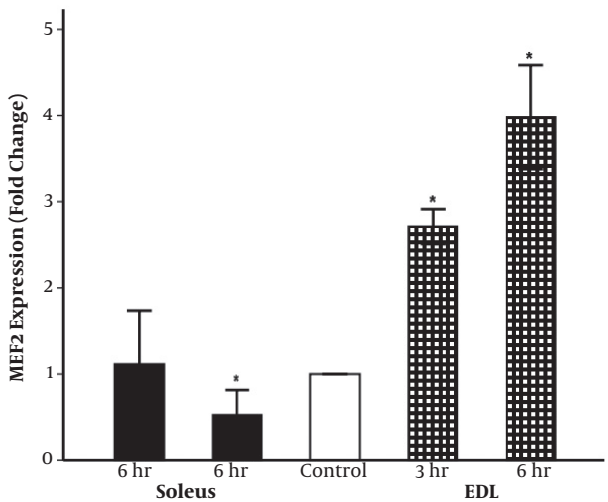

Figure 3. The expression of *Mef2C* in EDL and soleus muscles in response to an acute bout of RE at 3 h and 6 h post-exercise. The expression of *Mef2C* was normalized to *gapdh* expression. The values are represented as means ± SD which indicate significant differences with the control group ($P < 0.05$).

5. Discussion

The main findings of the present study were: (1) an acute bout of RE significantly induced the downregulation of *miR-1* expression in both EDL and EDL muscles and (2) in response to RE, *MyoD1*, *Mef2C* and *Hdac4* gene expression increased in the EDL muscle, but it had more fluctuations in the soleus muscle (see Figures 5 and 6).

It is estimated that 1% - 2% of myonuclei in a normal adult rat muscle are replaced every week (13). Minor lesions by day-to-day wear and tear can elicit only a slow turnover of their constituent multinucleated muscle fibers. Nonetheless, mammalian skeletal muscle has the ability to complete a rapid and extensive regeneration in response to severe damage whether the muscle injury is inflicted by direct trauma (i.e., extensive physical activity and especially resistance training) (14). Elevated *MyoD1* mRNA levels in soleus and EDL muscles may reflect a primary attempt to generate new muscle fibers (15). It coincides with the early phase of muscle injury that occurs 1 to 6 h after exercise-induced muscle damage (16). Our finding of *MyoD1* gene expression in response to RE is consistent with the findings of Bickel et al. (17).

Analysis of the muscle cells regulatory promoter regions of various exercise responsive genes shows that

Table 2. Changes in the Expression Level of *miR-1* and Genes After RE in Control and Experimental Groups (3 h and 6 h Post-RE)

Muscle Type	Mean ± SD	P Value[a]
EDL		
miR-1		
Control	1.06 ± 0.029	
3 h	0.01 ± 0.010	0.001
6 h	0.26 ± 0.172	0.008
MyoD1		
Control	1.05 ± 0.051	
3 h	2.35 ± 0.406	0.01
6 h	2.08 ± 0.154	0.001
Mef2C		
Control	1.05 ± 0.036	
3 h	2.71 ± 0.162	0.001
6 h	3.98 ± 0.485	0.020
Hdac4		
Control	1.05±.040	
3 h	2.42 ± 0.265	0.015
6 h	1.24 ± 0.240	0.190
Soleus		
miR-1		
Control	1.06 ± 0.040	
3 h	0.486± 0.146	0.007
6 h	2.267± 0.371	0.036
MyoD1		
Control	1.05 ± 0.039	
3 h	0.900 ± 0.123	0.096
6 h	1.57 ± 0.387	0.172
Mef2C		
Control	1.05 ± 0.040	
3 h	0.524± 0.234	0.033
6 h	1.11 ± 0.498	0.78
Hdac4		
Control	1.07 ± 0.079	
3 h	1.15 ± 0.208	0.449
6 h	0.84 ± 0.407	0.317

Abbreviations: *Hdac4*, Histone Deacetylase 4; *Mef2C*, Myocyte Enhancer Factor 2c; *miR-1*, microRNA-1; *MyoD1*, Myogenic Differentiation 1.
[a]P values are based on bootstrap (1000 bootstrap samples) compared to the control group found by the independent *t*-test.

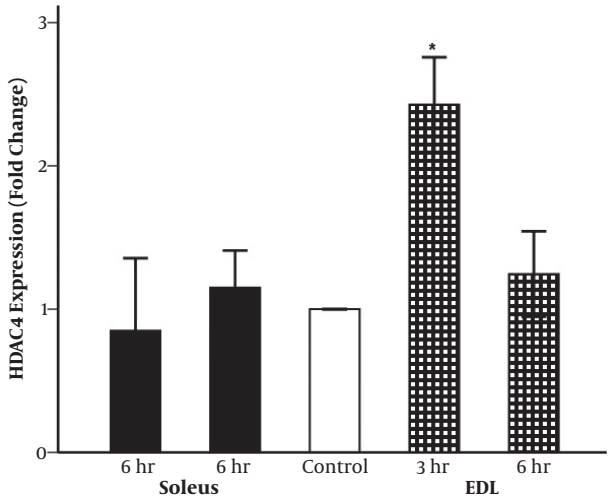

Figure 4. The expression of *Hdac4* in EDL and soleus muscles in response to an acute bout of RE at 3 h and 6 h post-exercise. The expression of *Hdac4* was normalized to *gapdh* expression. The values are represented as means ± SD which indicate significant differences with the control group ($P < 0.05$).

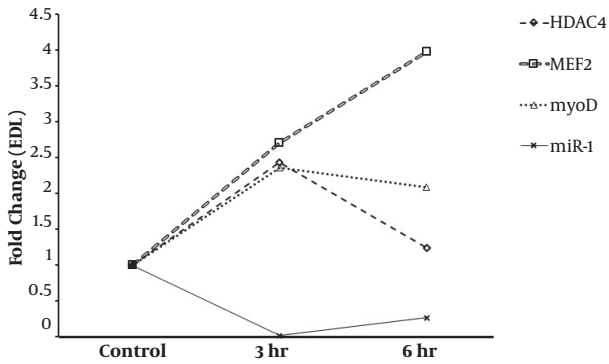

Figure 5. The changes in the expression of *miR-1* and up and downstream genes of EDL muscle following an acute bout of RE in the control and experimental groups (3 h and 6 h post-exercise). The values are represented as means.

many of them possess a binding region for the myocyte enhancer factor 2 family of transcription factors (18). Physical exercise induces mechanical stress activities at rest and 30 and 60 min after a single resistive bout of exercise (19). Skeletal muscle development (induced by exercise) is controlled by the *MyoD1* and its partner myocyte enhancer factor 2 families of transcription factors, which interact to establish a unique transcriptional code for activation of skeletal muscle-specific genes (20). Studies have shown that twist, as a bHLH transcription factor, regulates *Mef2C* gene expression by two upstream enhancers (21). It has also been shown that PKB can phosphorylate twist transcription factor that actives twist (22). It is likely that the increased expression of *Mef2C* in EDL and soleus muscles in this study was due to the activation of twist transcription factors induced by PKB and physical activity. The mRNA ex-

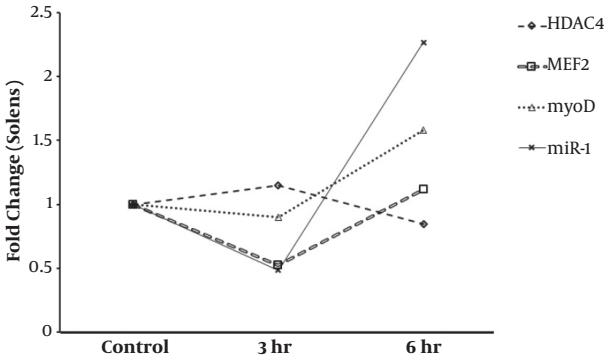

Figure 6. The changes in the expression of *miR-1* and up and downstream genes of EDL muscle following an acute bout of RE in the control and experimental groups (3 h and 6 h post-exercise). The values are represented as means.

pression of *Mef2C* in EDL muscle was about three and four times higher at 3 h and 6 h post-exercise, respectively, in the group receiving an acute bout of RE than in the control group. This finding is consistent with a study that showed the rate of *Mef2C* gene expression increased after physical activity (23). *MyoD1* and *Mef2C* are upstream transcription factors regulating *miR-1*. They control the rate of *miR-1* expression, which is encoded by bicistronic pre-miRNAs (9).

The difference in the *miR-1* expression between the two muscles may be related to muscle fiber type and the MHC transition (IIb→type IId/x→type IIa→type I (reported by many studies to occur in response to increased neuromuscular activity by strength (or resistance) training (24). Thus, increasing neuromuscular activity results in the slowing of the muscle contractile properties resulting from increased expression of the two MHC isoforms (MHC-I and MHC-IIa) (25). This coincided with increased expression of *Mef2C* transcription factor, which was sufficient to enhance skeletal muscle oxidative capacity and mitochondrial content (26).

It has been shown that Sp1 and Sp3 can modulate the expression of *Hdac4*; they control and affect *Hdac4* promoter activity, and increase the expression of *Hdac4* mRNA (27). Studies showed that contractile activity (28) and increased intercellular calcium (29) can increase the Sp1 expression. It is likely that physical activity increases Sp1 transcription activity by contractile activity and increased intracellular calcium, inducing the increased expression of *Hdac4* gene.

Chen et al. reported that *miR-1* was able to modulate skeletal muscle cell differentiation by repressing the activity of *Hdac4* (30), which has been shown to repress *Mef2C* activity (31). It is reasonable to propose that the decrease of *miR-1* expression in EDL muscle may be indicative of starting exercise-induced muscle growth. This idea is supported by the finding that the decrease in *miR-1* expression coincided with increased *MyoD1* expression in EDL muscle as a marker of satellite cell activation (32).

Exercise-induced muscle damage is known to stimulate satellite cell activation (33). The workload placed on skeletal muscle during RE is at or near maximum capacity, which produces significant perturbations to the skeletal muscle fibers and the associated extracellular matrix (34). These perturbations can lead to significant muscle damage, especially with lengthening contractions (eccentric exercise) using supra-maximal loads (35). Although there is not a complete consensus within the field, there is evidence demonstrating that eccentric exercise is more effective in inducing skeletal muscle hypertrophy, presumably by satellite cell activation as the result of greater muscle damage. Furthermore, the distinct transcriptional response to RE between the EDL and soleus muscles is likely the result of differences in the integration of metabolic, mechanical, neuronal, and immune signals. A previous study showed that *miR-1* expression in the plantaris muscle decreased due to functional overload (6), which is in agreement with our results at 3 h post-RE. However, a study by Davidsen et al. reported that *miR-1* expression was unaffected by 12 weeks of RT in human skeletal muscle (36).

It is reported that class II HDAC proteins block the formation of slow-twitch oxidative myofibers through the repression of MEF2 activity. Conversely, the expression of a hyperactive form of *Mef2C* in skeletal muscle of transgenic mice promoted the formation of slow fibers and enhanced running endurance, enabling mice to run almost twice the distance of wild-type littermates. Thus, the selective degradation of class II HDACs in slow-twitch skeletal muscle provides a mechanism for enhancing physical performance and resistance to fatigue by augmenting the transcriptional activity of MEF2 (26). We observed that the decrease of *miR-1* expression in the EDL at 3 h post-exercise coincided with an increase in *Hdac4* expression, suggesting a probable sign for the fiber-type transition known to occur with training. The finding that *Hdac4* expression was unchanged in both soleus and EDL muscles 6 h post-exercise in response to RE was unexpected. Despite observing no change in *Hdac4* expression, *Hdac4* may still be involved in regulating gene expression in response to RE given the importance of post-translational modifications (nuclear export of the *Hdac4* following phosphorylation) to *Hdac4* function (37).

However, as these upstream (regulatory factors) and downstream (*miR-1* targets) factors were only measured at gene levels, the findings cannot be generalized to protein changes. These responses and adaptations to exercise require further study to determine protein changes during exercise and training.

In conclusion, the results of this study provided the

first evidence that the expression of *miR-1* differs between slow-twitch and fast-twitch muscles in response to an acute bout of RE, and that the response of *miR-1*-related genes to RE is more remarkable in fast-twitch muscles than in slow-twitch muscles.

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Footnotes

Authors' Contribution: Study concept and design, statistical analysis and interpretation of data: Mohammad Fathi; drafting of the manuscript: Mohammad Fathi, Reza Gharakhanlou, and Hamid Rajabi; critical revision of the manuscript for important intellectual content: Mohammad Fathi and Masoud Soleimani.

Conflict of Interests: The authors do not have any conflict of interest to declare.

Ethical Approval: The study were in complete compliance with the international guidelines (World Medical Association Declaration of Helsinki) and approved by the Ethics Committee of the School of Medicine of the Tarbiat Modares University, Iran (Code 60/79650, May 2017).

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