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The Effects of High Intensity Interval Training on Mir-23a Expression and Related Factors Involved in Muscular Atrophy of Aged Rats

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ABSTRACT: Among the epigenetical factors involved in muscular atrophy, Mir-23a is subjected to change due to an alternation in intercellular calcium levels. The aim of this study is to investigate the chronic effect of high intensity interval training (HIIT) on Mir-23a expression and some other related factors, interpreting in Plantaris muscle atrophy of aged rats. Twenty-eight aged and young rats were divided into 4 different groups including exercise and control. The training protocol included 6 weeks of HIIT. Animals were sacrificed 48 hours after the last training session, and Plantaris muscle was removed. In order to measure Mir-23a, Rcan-1mRNA, Murf-1mRNA and Atrogin-1mRNA we used Real-time PCR technique. The results showed that aging significantly decreased Mri-23a (p=0.0001) as well as Rcan-1mRNA (p=0.0001) expression as upstream factors in atrophy signaling cascade, and exercise lead to a significant increase (interaction effect) of those two factors (both p=0.003). However, exercise had no significant effect on Mri-23a (p=0.238) and Rcan-1mRNA (p=0.428) in young group. Murf-1mRNA (p=0.009) and Atrogin-1mRNA (p=0.001) were significantly increased due to aging, and exercise resulted in a significant decrease in those genes in young groups (p=0.04 and p=0.003; respectively), but not a significant interaction effect in aged groups (p=0.439 and p=0.069; respectively). It seems that HIIT could improve muscular atrophy- as a result of aging- and this happens through Calcineurin signaling factors & ROS modification.

KEY WORDS High Intensity Interval Training, Muscular Atrophy, Mir-23a

INTRODUCTION

Aging, characterized as a theory of progressive degeneration in cells and tissues, increases the chance of molecular die aspects. Different theories; oxidative theory, mitochondrial theory, and cellular inflammation theory have been suggested for aging as a concept [1]. Studies shows that an elevated level of mitochondrial ROS is recognized as a key factor in muscular pathologies, such as atrophic conditions during aging [2]. There are channels to release calcium from sarcoplasmic retericium called Ryanodine receptors (RyR). RyRs are assumed as target oxidation-sensitive proteins in skeletal muscles. In fact, under oxidative stress conditions, the changes (oxidation) happened in the RyR as a result of ROS lead to RyR instabilities [3]. All of these incidences result in a defect in calcium transmission and ultimately inertial contraction.

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Failure to release calcium may be interfered with Calcineurin. In particular, it has been shown that Ca 2+ Regulated Phosphatase (Calcineurin) is an important moderator of skeletal muscle hypertrophy in all type of muscle fibers, including slow and fast twitch in-vivo environments [4]. Also, Cn functionality adjust muscle fibers' genes [4, 5]. Among these processes, miR-23a plays a key role in muscle hypertrophy as one of these factors. In fact, Cn is considered as the upstream regulator of miR-23a [6]. MiR-23a is a miRNA, associated with atrophic and hypertrophic processes. In skeletal muscle, it inhibits ubiquitin proteases expression (MuRF-1 and Atrogin-1 mRNAs) in order to prevent atrophy [7]. Suppression of Atrogin-1 and MuRF-1 in skeletal muscle reduces the muscular atrophy, which also indicates the main role of the ubiquitin-protease system in muscle atrophy [8, 9].

Wada et al reported that miR-23a suppresses Murf-1 E3 translation to protein [7]. On the other hand, Hudson et al. (2014) showed miR-23a is regulated in muscle by calcineurin / NFAT signaling. Although it has been reported that exercise leads to an acute oxidative stress response [10], but many studies show long term training significantly decrease general oxidative stress. It also has previously been shown that high intensity interval training has been proposed as a time-efficient exercise intervention that may bring about similar benefits to moderate-intensity aerobic exercise [11] and it is a practical approach to regenerate neuromuscular signaling in animal subjects [12].

The Calcineurin / NFAT signaling is not the only factor in increasing miR-23a, it is essential to maintain its expression [13]. With regard to this, Mir-23a plays an important role in muscle atrophic cascades as a suppressing factor, and is subjected to changes by calcium levels. Therefore, the aim of the present study is to investigate the effect of high intensity interval training on Mir-23a expression and other dependent factors of its signaling cascade in Plantaris muscle of aged rats.

METHODS

Animals. Experiments protocols for rats were planned according to the policies of the Iranian Convention for the Protection of Vertebrate Animals, and the Ethics Committee of the School of Medicine Sciences, Tarbiat Modares University (TMU), authorized the protocol. Twenty-four male wistar rats (aged 4-5 months), obtained from Iran Pasteur Institute were collected in the current study. They were dwelled in a standard temperature room and 12-h light and dark periods with entirely access to water and food. Animals, maintained in the Animal House in School of Medical Sciences of TMU. Animals (5 in each group) were randomly divided to the groups including: Aged Control (AC) Aged Training (AT), Young Control (YC) and Young Training (YT).

Training Protocol. The training groups performed 5 sessions each week of HIIT for 6 weeks and 48 hours after the last training session, the training groups did not perform any exercise until they were sacrificed. After sacrificing animals, left foot Plantaris muscle was frozen immediately in the liquid nitrogen for cell and molecular testing, and then stored in a refrigerator for 80 days. The right leg muscle was used for conducting 10-25% formalin test for histochemical experiments. In the current study Plantaris muscle was chosen because it is predominantly composed of (II-60%) muscle fibers of type II [14].

At the beginning, in order to reduce stress as well as familiarity with running on treadmill, the subjects ran on a training program for a week at speeds of 10 to 18 meters per minute for ten minutes. Then, 48 hours after the last familiarization session, an exhausting test was performed, starting at a speed of 10 m / min and increased by 3 m for every three minutes [15]. The time to fatigue was determined by the inability of the rats to run on treadmill. After determining the maximum speed, the training groups participated in a six-week HIIT program (Table 1).

Table1. Maximum Speed & Training Session Intervals

Days in a week	Time of Trainings	Average Speed in Week	Rest Intensity	Exercise Intensity	Rest & Exercise Ratio	Sets	Week
5	90	70%	60%	80%	2;2	5	1
5	110	70%	60%	80%	2;2	6	2
5	130	70%	50%	90%	2;2	7	3
5	150	75%	50%	100%	2;2	8	4
5	150	75%	50%	100%	2;2	8	5
5	150	75%	50%	100%	2;2	8	6

Weeks 6th & 7th	Week 4th	Week 3rd	Week 1st & 2nd	Speed level	Groups
47	44	44	40	Max Speed	
47	44	40	32	Sprint Interval	YT
24	22	22	24	Rest Interval	
40	37	37	34	Max Speed	
40	37	33	27	Sprint Interval	AT
40	19	19	20	Rest Interval	

Real time PCR. Total RNA was extracted from Plantaris muscle samples by exerting QIAzol®Lysis Reagent (Qiagen) according to manufacturer's recommendations. RNA concentrations were defined by the rate of absorbance at 260 nm. RNA purity also was determined by absorbance ratio at 260 and 280 nm, and by Nano-Drop Machine. Acceptable Purification in 260/280 nm absorbance ratio above 1.8. RNA was reverse transcribed into complementary DNA (cDNA) using a Revert Aid first standard cDNA synthesis Kit (Thermo scientific, Fermentas K1622, United states) using an accepted protocol including: reverse transcription at 25 °C for 5 minutes, then inactivated reverse transcriptase at 42 °C for 60 minutes, and finally refrigeration at 70 °C for 5 minutes, with storage at -20 °C.

miRNA-cDNA synthesis. Four µl of 5x miScript HiSpec Buffer, 2 µl of 10x miScript Nucleic Mix, 2 µL of miScript Reverse Transcriptase Mix, 2 µl of RNA and 10 µl of non-nucleic acid water were mixed in pipetting so that the final volume was 20 µL . Then it was incubated at 37 ° C, then incubated for 5 minutes at 95 ° C to remove the miScript Reverse Transcriptase Mix, finally it was transferred to a 80 ° Freezer.

For real-time PCR, primers were designed using NCBI and gene runner software and synthesized by Cinnagen Company (Iran). The primer sequences have been represented in table 2. Gene expression measurement done with Master Mix and SYBR Green in an Applied Biosystems, StepOne™ thermal cycler. The thermal cycle protocol was divided into such protocols including: 1 cycle at 95° C in 10 min, followed by 40 cycles at 95° C for 15 s, and 60° C for 30 s. PCR amplification also was performed with duplication in a total reaction volume in 20 µl. The reaction mixture had 3 µl diluted template, 10 µl SYBR Premix Ex Taq™Kit (Perfect Real Time, Takara Code RR041A, Japan), and 2 µl primers. Amplification specificity was monitored by analysis of melting curve. Genes Relative expression were normalized by subtracting the housekeeping levels of the mean of glyceraldehyde 3-phosphate dehydrogenase (Gapdh) $2^{-\Delta\Delta CT}$, which was amplified as housekeeping gene (Table 2). All data are represented as fold change from the weight-bearing group [16].

Statistical Analysis. All statistical analyses were done by using SPSS software (version 20, SPSS Inc., Chicago, IL, USA). Normal assumption was examined using 1-sample Kolmogorov-Smirnov test. Two-way Manova tests was used to compare groups regarding under study variables and significant level was determined at $p<0.05$.

Table 2 sequences of the primer designed in this study

primer	sequences
Atrogin-1	forward 5'CCAGTACATTGAGCACCTAC3' reverse 5'GCAAATGATCTACTGGGTTG3'
Murf-1	forward 5'TAACTGTCGGCGTGTACGAG3' reverse 5'GCAACAGAAAGCACGAATGA3'
Rcan-1	forward 5'GACATGCCGCCTGGAGAAA3' reverse 5'AGCCCAGGATGCCCTTTAGT3'

STATISTICAL RESULTS

Body mass. Changes in body mass before and after exercise are shown in Table 1. The results of t-test for intra-group comparison of body mass changes showed that at the end of six weeks, the weight of both young groups increased significantly compared to the baseline measurements (P=0.001). However, the aged control & training group showed a non-significant increase compared to the pre-test data (P=0.655, P=0.83, respectively) (Table 3).

Table 3. Weight Changes in the experimental groups

Variable	Group	Pre Test	Post test
Body Mass	YC	314.15±17.63	370.23±18.52*
	YT	299.82±10.54	347.03±17.15*
	AC	386.02±9.55	390.37±55.09
	AT	407.23±11.51	400.35±1.76

YC: Young Control, **YT:** Young Training, **AC:** Aged Control, **AT:** Aged Training, ***:** Significant Difference to pre-test (p = 0.001).

The results for Atrogin-1 mRNA expression showed that HIIT and aging did not have a significant interactive effect (p=0.493) on Atrogin mRNA expression in the Plantaris muscle of the aged rats. However, a significant effect of HIIT (p=0.003) and aging (p=0.009) was observed separately. Exercise training reduced Atrogin mRNA expression in both training groups, but aging solely was significantly associated with Atrogin mRNA expression (Figure 1-A).

It was observed that HIIT & aging did not have a significant interactive effect on Murf-1 mRNA expression in Plantaris muscle of Aged rats (p=0.069). However, either of the training factor (p=0.04) and aging (p=0.001) showed a significant effect on Murf-1 mRNA expression. Exercise training reduced Murf-1 mRNA in both training groups, otherwise aging raised it (Figure 1-B).

Rcan-1 mRNA expression was also affected interactively by HIIT and aging (p=0.003). There was a change in Rcan-1 expression in AT group, but the effect of HIIT on Rcan-1 mRNA expression was not solely significant (p=0.228). Aging reduced Rcan-1 mRNA expression, while exercise increased it in Young control group (Figure 1-C).

Finally, Mir-23a expression in Plantaris muscle of aged rats showed significant changes due to exercise and aging interaction (p=0.003). However, the effect of training on Mir-23a expression was not significant by itself (p=0.238). The significant effect of aging (p=0.0001) on Mir-23a expression was also observed. Aging reduced Mir-23a expression, while exercise increased its expression in Aged control group (Figure 1-D).

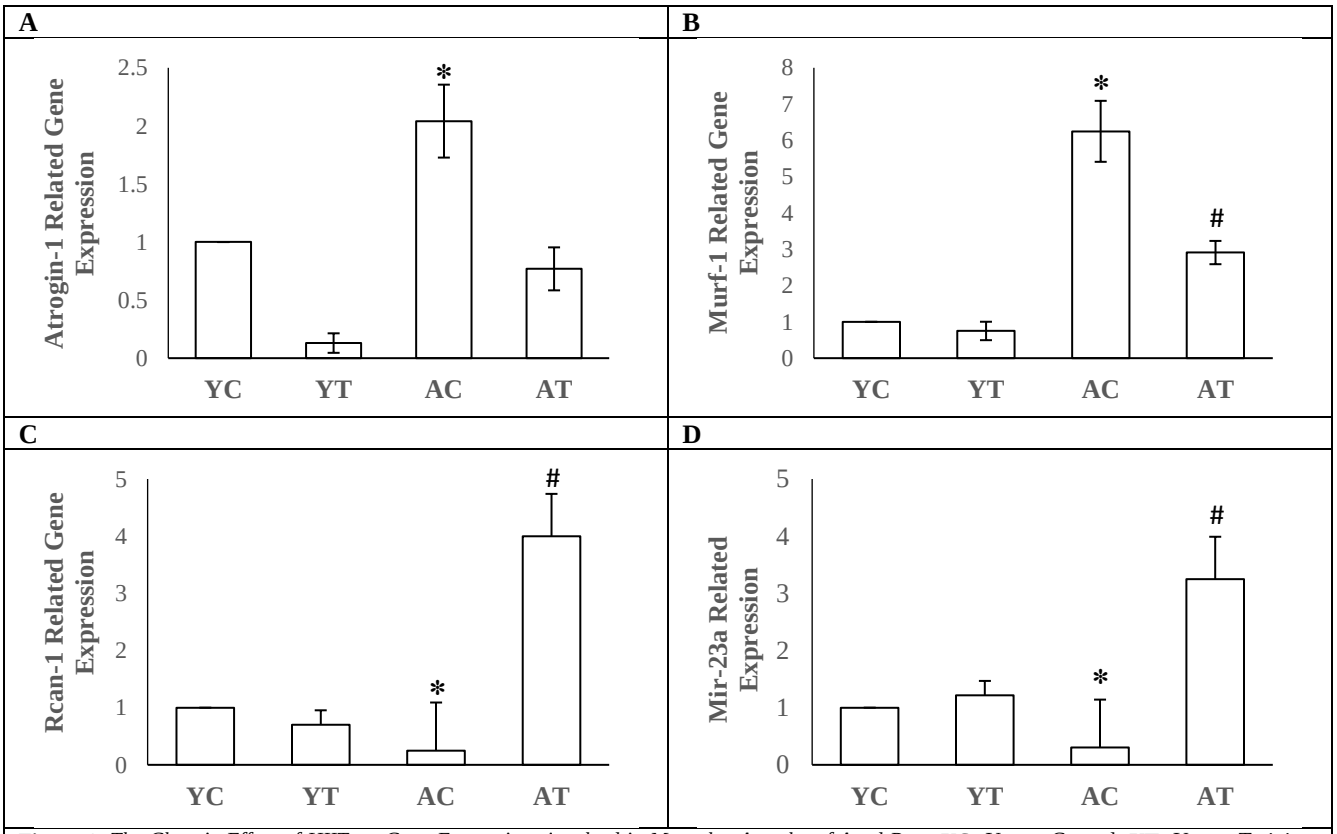

Figure 1. *The Chronic Effect of HIIT on Gene Expressions involved in Muscular Atrophy of Aged Rats*. **YC:** Young Control, **YT:** Young Training, **AC:** Aged Control, **AT:** Aged Training, ***:** Significant Difference to YC, **#:** Significant Difference to AC.

DISCUSSION

There is plenty of evidence that Calcineurin's capability to re-generate skeletal muscle (physiologically and morphologically) through Mir-23a signaling pathway [17]. Exercise on the other hand would be a positive intervention in this area. The most important finding of this study was the effect of exercise training on Mir-23a expression and Rcan-1 mRNA in the Plantaris muscle of both aged and young groups. As a result, six weeks of HIIT exercise significantly increased Mir-23a expression as well as Rcan-1 mRNA; as the most important activator of calcineurin [14, 15, 18]. Reducing Mir-23a & Rcan-1 mRNA expression as the two main elements of calcineurin signaling cascade could indicate a huge calcium shuttle defect in muscle cells caused by excessive ROS. Although in the present study we had faced to some limitations to measure ROS levels, but considering many studies representing intramuscular proteins oxidation induced by aging, it would be considered as a definite cause of calcium deficiency in muscle cells [16, 17, 19], so that it can be argued that ROS did its role priority and then Rcan-1 gene was reduced.

In the present study, aging resulted in a significant increase in Atrogin and Murf-1 gene expression, and HIIT did not change the those genes in the aged subjects, However, in the young group HIIT significantly decreased Atrogin and Murf-1 mRNA, identified as the two known

factors located at downstream of many muscular atrophic signaling pathways. Many studies have been done on the role of Atrogin and Murf-1 in muscular atrophy process due to various pathologic and physiological abnormalities, and the results shows the effectiveness of these two genes in different signaling pathways that ultimately lead to skeletal muscle atrophy.

According to the mentioned literature, Murf-1 over expression degenerate muscle fibers and increases mitochondrial ROS production [20, 21]. In addition, calcium deficiency disrupts nerve function through activating apoptotic pathways and impair mitochondrial function, this leads to reduce skeletal muscle mass [22]. On the other hand, exercise have a direct effect on the atrophic factors as a result of aging in a way that reduces their effectiveness. For example, TNF-α increases atherogen 1 / MAFbx and MuRF1 expression ligands in skeletal muscles and exercise can modify it through of p38 MAPK and NFκB pathways [23]

Inflammation is one of the mechanisms involved in muscle atrophy during aging induced by Atrogin and Murf-1. It is assumed exercise training can affect this cascade with its anti-inflammatory effects including enhancing antioxidant capacity; it can also prevent raising of the muscular proteasomes expression caused by ROS [23]. Thus, it can be argued that Atrogin and Murf-1 are downstream factors of many atrophic paths, such as IGF-1/Akt, TNFalpha [24], Akt / mTor, Foxo-1 [25] that would be affected by exercise. The results of this study represents an alternate increase and decrease of Atrogin & Murf-1 gene expression in aged control group and young training group respectively, which were according to published and expected literature. However, having non-significant changes of the interactive effects of aging & exercise could be attributed to the involvement of other signaling pathways except Mir-23a pathway, in which are likely to have an effective role of aging on these changes. In other words, Regarding we used high intensity exercise in this study and possibly fast twitch muscles were implemented, Mir-23a expression and Rcan-1 mRNA were significantly decreased in Plantaris muscle of both young and aged subjects. In support of this theory, we can refer to others' studies [26-28].

In conclusion, the current study showed Rcan-1 gene expression was reduced in Plantaris muscle of aged rats, and the same happened to Mir-23a expression as the upstream factor of muscle atrophy signaling; therefore, both resulted in more destruction in skeletal muscle due to aging and HIIT reversed these processes. Therefore, according to the results, elderly people may benefit high intensity exercises to increase their muscular health in order to reduce oxidative stress in muscle mass, and ultimately prevent skeletal muscles from metabolic diseases, and be able of doing daily routine during aging.

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