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The Effect of Reduced Neuromuscular Activity on Class IIa Histone Deacetylase and Selected Downstream Pathways

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Abstract

Introduction: Decreased neuromuscular activity, in terms of different models including hind-limb suspension (HS), can result in muscular atrophy by changing different genes expression. Hdac 4&5 and their downstream pathways are 2 important pathways involved in the preservation of muscle mass.

Methods: In the current study, in order to survey the changes of Hdac4&5 and their downstream cascade mRNA expression, Wistar rats were assigned into 2 groups (1) weight bearing (WB) and (2) HS (n=5 in each group). Hindlimb's rats were suspended for 14 days. After 2 weeks, the rats were sacrificed and the soleus and plantaris muscles were gathered. Thereafter, gene expression was determined using eal-time polymerase chain reaction (PCR) technique.

Results: The results showed that 14 days HS decreased muscle mass in both the plantaris and soleus muscles and the latter changes definitely outweighed the former. In addition, the cross-sectional area of the muscle fibers of the plantaris and soleus muscles decreased and, with changes in muscle mass, the observed decrease in soleus was higher. Also, the increase in mRNA expression of Hdac4&5, myogenin, and Gadd45a, was observed in both muscles. But the expression of the Dach2 gene was significantly reduced in the plantaris and soleus muscles of the HS group as compared to the WB group.

Conclusion: Hdac/Myogenin and Hdac/Dach2/Gadd45a participate in muscular atrophy progression.

Keywords: Muscular atrophy, Hindlimb suspension, Histone Deacetylase

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Introduction

It is clear that under some specific conditions or chronic diseases such as bed rest, aging, cancer, diabetes, and even space flight, a muscle would be subjected to lose its mass and this could lead to muscular weakness and even death.[1-4] It is proposed that preservation of muscle mass depends on optimal innervation and neuronal activity, in a way that, dropping off the neuronal support from the muscle, results in atrophy.[5,6]

Also, decreased neuromuscular activity in the form of immobilization, hind-limb suspension (HS), space flight, and denervation is strongly linked to the modification of some genes such as muscle ring finger 1 (Murf-1) and atrogen-1 in skeletal muscle, which are considered as the main factors for proteolysis of muscle proteins and atrophy.[7-10] It is suggested that some pathways such as histone deacetylase/dachshund family transcription factor2/myogenin (Hdac/Dach2/myogenin) are involved in muscular atrophy by decreased neuromuscular activity and this happens whenever neurogenic atrophy is induced by Murf-1 and atrogin-1.[3,11,12] Generally, histone deacetylases, through deacetylation, can suppress or activate transcriptional process and this mediates the proliferation and growth of skeletal muscle. Some studies have shown that among the histone deacetylases family, Hdac4&5 play a major

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role in the maintenance and degradation of muscular proteins.[3,13]

In this regard, it has been shown that increase of Hdac4&5 suppresses Dach2 expression, which is an inhibitor of myogenin[11]; thus, the myogenin rising regulates Murf-1 and atrogin-1 expression.[3]

In addition to the Dach2/myogenin pathway, the effects of Hdacs on skeletal muscle mass could be exerted by another element, named growth arrest and DNA damage-inducible alpha (Gadd45a).[5] In other words, Gadd45a mediates some downstream effect of Hdac4 such as myogenin induction.[5] The elevation of Gadd45a would raise up genes, which are related to protein degradation and anabolic signaling inhibitor, and finally, result in muscular atrophy.[14] In fact, activation of some transcriptional regulators such as Hdac and activating transcription factor 4 (ATF4) induces Gadd45a gene expression in stress conditions.[14]

In order to study the cellular and molecular changes happening during the process of muscle atrophy, different models have been used. Most atrophy processes in mice have the same features as in man.[14] However, these models run different molecular pathways because of their different traits apiece.[14] For example, unlike the denervation model, myogenin does not play a substantial role in muscle atrophy due to starvation, which means that the Murf-1 and atrogin-1 mRNA expression after 48 hours fasting had been raised in both healthy and myogenin suppressed mice.[3] It is believed that after fasting, upregulation of Gadd45a is independent of Hdac4 and ATF4 is responsible for Gadd45a expression.[5]

To date, despite the studies which have been done on muscle atrophy, the cellular and molecular mechanisms involved in muscle atrophy have not been completely understood. It can, therefore, be argued that having more knowledge about these pathways, would lead to the development of new treatment methods to control muscle mass changes in some clear-cut conditions such as hindlimb casting, bed rest, and even space flight.

In the current study, HS was implemented in order to survey the changes in the expression of Hdac/Dach2/myogenin and Hdac/Gadd45a pathways followed by a reduction in neuromuscular activity, which was accompanied by muscle and nerve connection.

Methods

Animals

Experiments protocols about rats were managed 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. Ten male Wistar rats (aged 4-5 months), obtained from Iran Pasteur Institute were collected in the current study. They dwelled in a standard temperature room and 12-hour light and dark periods with entire 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 into the groups including weight bearing (WB) and HS.

Hindlimb Suspension Procedure

Rats were hanged by tail harnessing and placed in individual and different cages. The mice of both test and control group had been fed with the same food during the study. The tail harness consisted of a triangular shaped wire (18 gauge), which was put between layers of vinyl cloth glued with Dural dispatch cement to the dorsal proximal four-fifths of the tail. Beforehand, the tail was cleaned and covered with cement before linking the cloth strips. The harness then strengthened by loosely wrapping the tail by vinyl strips and an elastic tape (Medipore-3M). With this method we distributed the load alongside the length of the tail, so excessive tension on a small area was avoided. Animals were suspended similar to the non-invasive procedure.[15] Daily their health were checked and confirmed that the exposed tail remained in natural color, indicating normal blood flow. Moreover, a flsh swivel was attached to the subjected apex of the wire triangle, and fishing line tied to the swivel to boost the rat's hindquarters, suspending the hindlimbs. The height was adjusted in a way that the hind feed just cleared the grid floor. Forelimbs held contact with the floor, therefore, allowed the animal access to food and water. At the end of the first week, we checked the food of both groups. They were 2 rats in HS group, which were very weak, so they were overlooked.

Tissue Preparation

After 14 days HS, animals were comatose by intraperitoneal (IP) injection of 200 mL/100 g body weight of 40 mg/mL ketamine and 5 mg/mL xylazine solution. After that, they were sacrificed immediately. The muscles of both left and right legs were quickly extracted and weighted. The right leg was stored in liquid N_2 for real-time polymerase chain reaction (PCR) analysis, and the other leg was kept in paraformaldehyde for histopathological measurements. The soleus and plantaris muscles were assumed slow-twitch and fast-twitch muscles, respectively.

Histopathological Examination

For histopathological examination, the muscles were fixed in 10% neutral formalin. The fixed organs tissues were then embedded in paraffin. The paraffinized tissue specimens were cut into 5 um thick slices and were stained with hematoxylin and eosin (H&E) for histopathological observation using a previously established procedure.[16] Photomicrographs were captured using a normal spectra fluorescent microscope (Olympus DP 72) at ×6100 magnification with an attached digital camera (Olympus, Tokyo, Japan).

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Real-Time Polymerase Chain Reaction

Total RNA was extracted from soleus and 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 was also determined by absorbance ratio at 260 and 280 nm, and by ethidium bromide staining. Acceptable purification in 260/280 nm absorbance ratio above 1.8. RNA was reverse transcribed into complementary DNA (cDNA) using a RevertAid First Standard cDNA Synthesis Kit (Thermo scientific, Fermentas K1622, USA) using an accepted protocol including reverse transcription at 25°C for 5 minutes, then incubated reverse transcriptase at 42°C for 60 minutes, and finally refrigeration at 70°C for 5 minutes, with storage at -20°C.

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 1. Gene expression measurement was 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 minutes, followed by 40 cycles at 95°C for 15 seconds, and 60°C for 30 seconds. 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 expressions 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. All data are represented as fold change from the weight-bearing group.[17]

Statistical Analysis

Statistical methods were performed in SPSS software (version 20, SPSS Inc., Chicago, IL, USA). Normal distribution was examined using one-sample Kolmogorov-Smirnov test. Independent sample student *t* tests were used to compare groups regarding under study variables and significance level was determined at $P<0.05$.

Table 1. Sequences of the Primer Designed in This Study

Primer	Sequences
Hdac4	Forward 5′GCAGAGGTTGAATGTGAGCA3′ Reverse 5′GGAAGAAGTTCCCATCGTCA3′
Hdac5	Forward 5′TGTCACCGCCAGATGTTTTG3′ Reverse 5′TGAGCAGAGCCGAGACACAG3′
Dach2	Forward 5′CAGCAAGAAAAGAAGGAACTAC3′ Reverse 5′ACCGCTGTCACCAGATGT3′
Myogenin	Forward 5′CCAGTACATTGAGCACCTAC3′ Reverse 5′GCAAATGATCTACTGGGTTG3′
Gadd45a	Forward 5′TAACTGTCGGCGTGTACGAG3′ Reverse 5′GCAACAGAAAGCACGAATGA3′
Gapdh	Forward 5′GACATGCCGCCTGGAGAAA3′ Reverse 5′AGCCCAGGATGCCCTTTAGT3′

Results

The Effects of Hind-Limb Suspension on Muscle Mass

The changes in relation to weight indexes are shown in Table 2, representing a decrease in total body weight in the HS group, but 2 other groups had no significant differences in total body weight at the beginning of the study.

Two weeks unloading resulted in atrophy of the slow soleus muscle, indicated by a significant lower ratio of soleus (muscle mass-to-body) weight in the HS group as compared to the WB group and there was no significant difference in the plantaris muscle-to-body weight ratio. Although, the weight of the plantaris muscle diminished significantly in the HS group as compared to the WB group, the ratio of this muscle to body weight did not differ significantly among the groups.

Effect of Hind-Limb Unloading on Genes Expression Changes

In the soleus muscle, 14 days after hind-limb suspension, Hdac4 expression was significantly upregulated in the HS group as compared to the WB group ($P=0.01$) (Figure 1A). Also, the HS group showed an increase in Hdac4 mRNA expression in plantaris (Figure 2A). Similar differences were also observed in Hdac5 expression in the HS and WB groups in the same muscle ($P=0.002$) (2.1-fold change), but the plantaris muscle of the HS group showed a slight increase in Hdac5 as compared to the WB group ($P=0.865$) (Figures 1 and 2B). Dach2 expression was significantly downregulated in both the plantaris and soleus muscles after unloading in the HS group (Figures 1 and 2C). Regarding the influence of Dach2 on myogenin expression; in the HS group, myogenin was upregulated after hind-limb suspension in the soleus muscle (Figure 1D). However, it was significantly increased in the plantaris muscle of that same group ($P=0.05$) (Figure 2D). The soleus and plantaris muscles showed an increase in Gadd45α in HS and WB groups, with 27.6 and 2.2 fold,

Table 2. Characteristic Features of Rats in Different Groups

	WB	HS
Initial body weight (g)	335.87±6.67	323.4±14.76
Final Body weight (g)	383.4±4.53[b]	297.4±2.39[a]
Soleus muscle mass (mg)	131.37±8.36	62.7±4.37[a]
Soleus-to-body mass (mg g^{-1}×100)	34.3±2.43	21.08±1.43[a]
Plantaris muscle mass (mg)	404.17±12.87	329.9±11.15[a]
Plantaris-to-body mass (mg g^{-1}×100)	105.43±3.24	110.91±3.24

Abbreviations: HS; hindlimb suspension, WB; weight bearing.
[a] $P<0.05$ versus WB group; [b] $P<0.05$ versus pre-test.
Data were presented as mean ± SE and analyzed by independent and paired sample *t* test, n=3-4 for each group.

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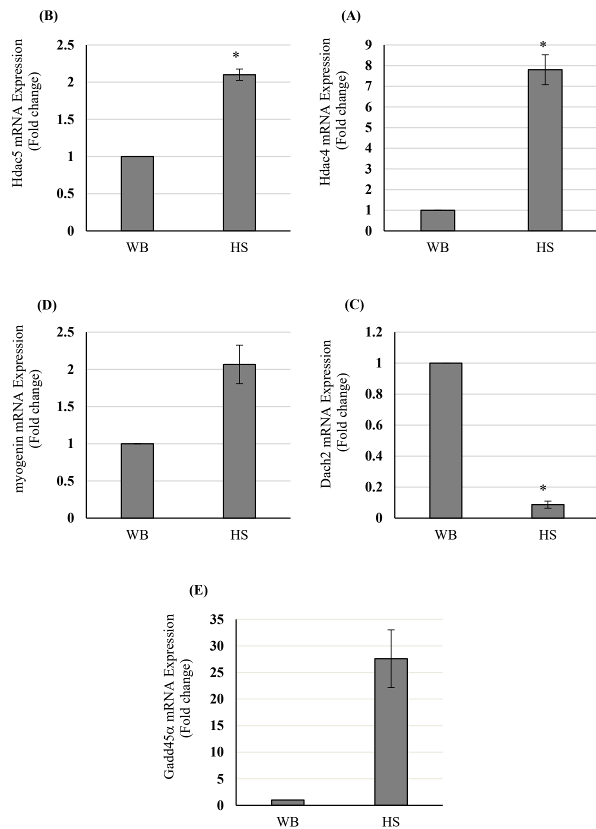

Figure 1. Effects of Hind-Limb Suspension on Gene Expression in Soleus Muscle. Results are expressed as fold changes. WB, weight bearing; HS, hind-limb suspension. mRNA values were normalized to GAPDH levels and expressed as fold changes in comparison to the WB group. n=3-4 animal per group. *$P \leq 0.05$ versus WB group.

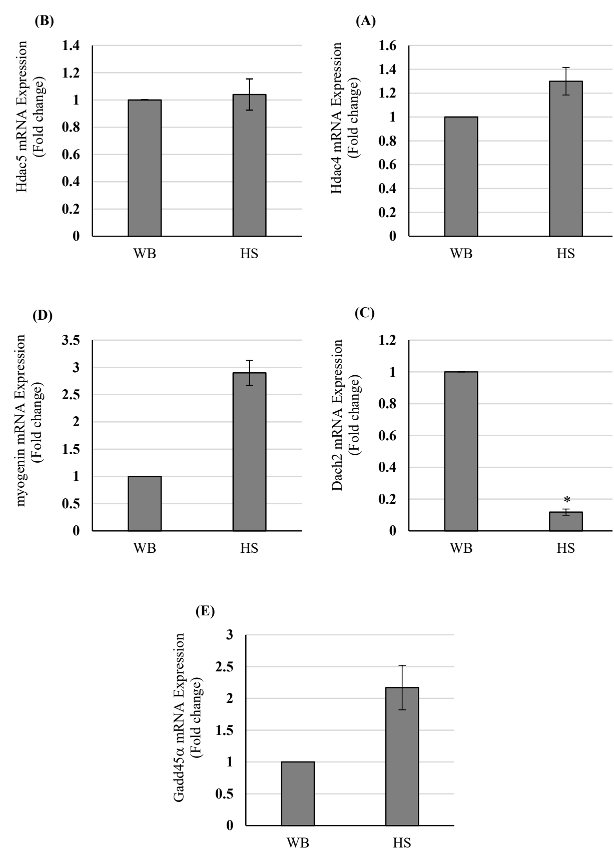

Figure 2. Effects of Hind-Limb Unloading on Gene Expression in Plantaris Muscle. Results are expressed as fold changes. WB, weight bearing; HS, hind-limb suspension. RNA values were normalized to GAPDH levels and expressed as fold changes in comparison to the WB group. n=3-4 animal per group. *$P \leq 0.05$ versus WB.

respectively (Figures 1 and 2E).

The Effects of Hindlimb Suspension on Cross-Section Area of Myofibrils

At the end of the period, a decrease was witnessed in cross-section area of myofibril in the HS group as compared to the WB group in plantaris (Figure 3F and D) and more decrease in the soleus muscle (Figure 3E and B). Also, the nuclei were peripherally marginated (green arrow) in some myofibrils of the soleus muscle in the HS group (Figure 3B).

Discussion

There are many studies, which have attempted to determine the signaling pathways involved in different types of skeletal muscle atrophy such as denervation or disuse of limb muscles and to date, there is no clear explanation about their mechanism. The results of the present study showed that inactivity in form of HS leads to shrinkage of muscle mass in both the soleus and plantaris muscles. However, it had a greater effect on the soleus as a slow-twitch skeletal muscle. Also, HS activated Hdacs/Dach2/myogenin and Hdac/Gadd45a pathways in both the plantaris and soleus skeletal muscles, the genes, which have been represented earlier with their roles in muscle atrophy resulted from denervation and starvation.[3,5] Moreover, in line with the changes in muscle mass, the changes of genes expression were also more in the soleus compared to the plantaris.

Some studies have described Hdac4 as a linking element between neuronal activity and transcriptional machine in skeletal muscles, in a way that, there is a negative correlation between neuronal activity and Hdac4 gene expression.[13] In this regard, it has been reported that after dropping off the neuronal activity due to denervation,[13] Hdac and its downstream cascades changed.[5,11] Also, recently, another study was conducted with high intensity and continuous training, to reduce Hdac4&5 mRNA expression in the slow and fast muscles of both aged and young rats (unpublished). Moreover, Drummond et al[18] showed that after an acute exercise, Hdac4 protein expression decreased in young and aged subjects.

Since HS is associated with a reduction in both neuronal activity and mechanical load, decreased neuronal activity in the HS model can regulate the expression of the genes, which are related to the occurrences, and finally, control muscular remodeling. The change in intercellular calcium content of muscles would be considered as a reason for

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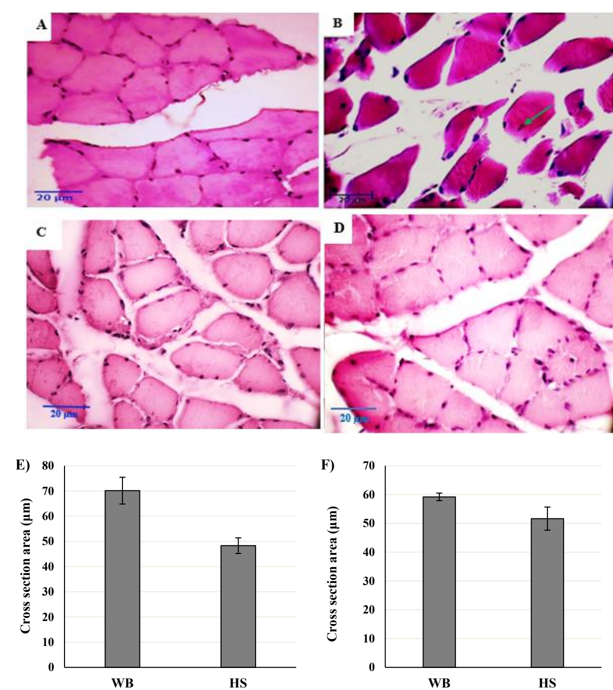

Figure 3. A Photomicrograph of a Transverse Section in the Soleus Muscle of HS (A, B) and WB (C, D). WB, weight bearing; HS, hind-limb suspension. Note: The nuclei showed peripherally marginated (green arrow) in some myofibrils. The figures E-F represent the effects of hind-limb suspension on the myofibril area changes (soleus and plantaris, respectively) (H & E ×400).

the increase in Hdac4 and this resulted from decreased activity condition. In fact, it was found that calcium leaked continuously from the sarcoplasmic reticulum, after observing the HS group in both fast and slow muscles[19] as well as their cytosol.[20] To further support this, it should be noticed that there is a binding site in the Hdac4 promoter gene for Sp3 and Sp1 transcriptional factors; therefore, Hdac4 expression is controlled by Sp3 and Sp1, and if they are suppressed, Hdac4 expression would fall off.[21] It is argued that an increase in intercellular calcium content is followed by an up-regulation in Sp1 expression.[22] It seems that an increase in intercellular calcium in the muscle fiber could lead to increased SP1 transcriptional factor, which results in the development of Hdac4 mRAN transcription by linking to its promoter.

In the present study, after 14 days HS, Hdac4 and Hdac5 mRNA expression in the soleus muscle were by far higher compared to the plantaris muscle, with ratios of 8 to 1.5 and 2 to 0.4, respectively. As mentioned earlier, these genes are completely relevant to neuronal activity and the changes in muscular activity during HS would be considered as a reason for the difference between the 2 types of muscles. For example, it has been shown that the electromyography (EMG) activity of the soleus muscle, which came after HS, was substantially less than that of the plantaris muscle, 473 and 90 mv/h, respectively.[23,24]

Like previous studies by Cohen et al[13] and Moresi et al,[3] who have done mirror experiments with a denervation model, it was observed that the increase in Hdac4&5 was accompanied with an increase in myogenin mRNA expression in unloaded muscles. Although up-regulated myogenin was introduced as an adaptive mechanism to prevent muscle atrophy,[25,26] evidence revealed that Myogenin activates some important genes involved in protein degradation such as atrogin-1 and Murf-1.[27] Moresi et al stated that after denervation, ubiquitin ligases (E3s) will not be increased in mice with suppressed myogenin and they resisted neurogenic atrophy.[3] Despite the fact that muscle-specific E3 including atrogin-1 and Murf-1 were not measured in the present study, other studies such as Polge et al[28] have shown the upregulation of these genes after HS. Generally, these genes play a major role in the process of skeletal muscle atrophy in all degenerative conditions.[9] On the contrary, Hughes et al reported that the overexpression of myogenin is not enough for running the atrophy process alone.[29] Hence, it has been stated that the effects of myogenin could be different and it totally depends on growth or pathological condition.[3]

It is approved that Dach2 mediates myogenin expression in innervated muscle, in a way that, suppression of Dach2 leads to myogenin transcription and its downstream pathways.[12] In the current study, just like the denervation model,[12] Dach2 mRNA expression was observed to diminish in both the soleus and plantaris muscles. The accumulation of Hdac4 through the decrease of neuronal activity reduces Dach2 expression and that is necessary for the suppression and expression of Dach2 and myogenin. Therefore, regarding the increased Hdac4 mRNA expression in the present study, HS could be considered as a mechanism for increasing myogenin.

It is argued that myogenin is mostly expressed in slow twitch fiber.[29,30] In this study, if the 2 types of muscles were combined, myogenin will be expressed in soleus more than in the plantaris (Figures 1 and 2). Alway et al[26] reported the same results for myogenin after HS. They believed that high myogenin levels in slow muscle, will not allow more increase. In addition, this could be a mechanism to prevent more atrophy.[31]

Researchers have stated that the termination of neurogenic atrophy by removing Hdac4&5 is more efficient than Myogenin suppression in animal models.[3] Clearly, Bongres et al noted the upregulation of Gadd45a, after Hdac4 increment. They also demonstrsted that Hdac4 is one of the regulators for the induction of Gadd45 during denervation.[5]

Gadd45a can exert some effects of Hdac4 on skeletal muscle by myogenin mRNA induction.[5] In fact, whenever an atrophic process is simulated for example by starvation, Gadd45a is controlled through other pathways apart from Hdac4, such as ATF4. Although, ATF4 expression was not measured in the present study, but the compatibility of increasing Hdac4 and Gadd45a could explain the mediatory role of Hdac4 in Gadd45a expression after

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a period of HS. Here, it has been shown that Gadd45a expression in the soleus muscle after HS, is significantly much higher than in the plantaris muscle (about 9 fold). Therefore, it appears that in the slow muscles, Gadd45a is the main factor involved in the atrophy process instead of myogenin and this tells us why the optimized Gadd45a in the soleus muscle was in good agreement with stronger Hdac4&5 activity and atrophy.

Conclusion

This study showed that muscular atrophy as a result of HS is due to decreased mechanical load and neuromuscular activity. This has been associated with increased Hdac4&5 genes expression specifically in slow muscles. In fact, they are linked with modification of downstream pathways of class II Hdacs including Hdac/Dach2/Myogenin and Hdac/Gadd45a. It can be stated that through the pharmacological elements, which could regulate these pathways, it may be possible to prevent muscular atrophy in some specific conditions such as bed rest and space flight.

Ethical Approval

Experiments protocols about rats were managed 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.

Competing Interests

None.

Acknowledgment

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