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Effect of Exercise Training and Atorvastatin Supplementation on Beclin1, *LC3-I* and *LC3-II* Expression in Old Diabetic Rats

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ABSTRACT

Background and objectives: Programmed autophagy is a genetically and evolutionarily conserved process that destroys long-lived cellular proteins and organelles. This study aimed to investigate effects of continuous and interval exercise training with or without atorvastatin supplementation on Beclin1, *LC3-I* and *LC3-II* expression in old rats with type 2 diabetes.

Methods: Sixty three male Wistar rats were divided into eight groups. Continuous exercise was performed at a speed of 15-29 m/min for 5-22 minutes. Interval exercise program consisted of six 2.5-minute sets that included a four-minute rest period between each set. The rats in the supplementation groups also received 20 mg/kg body weight atorvastatin daily via intraperitoneal injection. At the end of the training period, the expression of Beclin1, *LC3-I* and *LC3-II* in soleus muscle was measured by RT-PCR. One-way ANOVA was used for data analysis at statistical significance of 0.05.

Results: The results showed that both exercise trainings with or without atorvastatin significantly reduced *LC3I*, *LC3-II* and Beclin1 compared with the diabetic control group ($P<0.05$). In addition, the effects of the trainings and atorvastatin supplement did not differ significantly ($P>0.05$).

Conclusion: The results indicate that continuous and interval exercise program alone and combined with atorvastatin supplementation could significantly reduce *LC3-1*, *LC3-II* and Beclin1 level in soleus muscle of old diabetic rats.

Keywords: Autophagy, Diabetes Mellitus, Type 2, Atorvastatin.

INTRODUCTION

Aging is associated with biological changes in an organism, leading to a decrease in vital and adaptive energy (1). From the age of 30 to 60 years, muscle mass decreases by about 1% per year, and this procedure is accelerated after 60 years. Decreased muscle mass can increase the risk of metabolic diseases, such as type 2 diabetes, metabolic syndrome and cardiovascular disease as well as chronic diseases of the musculoskeletal structure and cancer (2). It is estimated that the world's older population will be doubled to 1.2 billion by 2025. In addition, the prevalence of type 2 diabetes increases with age and reaches its maximum by the age of 60-74 years (3). Diabetes mellitus is the most common endocrine disorder among the elderly (4). It is estimated that the worldwide prevalence of diabetes will increase to 366 million by 2030 (5). Type 2 diabetes mellitus is the biggest health challenge of the 21st century (6). Skeletal muscle mass changes with the decrease in the total cross-sectional area. The proteolysis of skeletal muscle is mostly regulated via intracellular proteolytic complex systems, including the lysosomal system, activated Ca^{2+} system, cytosol and ATP-ubiquitin-dependent proteolysis (7). Diabetes reduces muscle volume and skeletal muscle mass, which consequently decrease basal metabolic rate during aging. In addition, changes in the endocrine glands, such as elevated insulin level, may lead to insulin resistance in the long run. In fact, the loss of skeletal muscle mass or muscle atrophy is due to the complex interaction of cell apoptosis, the increased production of free radicals and the activity of proteolytic systems (8).

There are several systems for the destruction of muscle proteins, the most important of which are the ubiquitin-proteasome system and the autophagy-lysosome system (9). Beclin-1 is involved in most biological processes, including stress adaptation, growth, endocytosis, immunity and aging (10). As an essential initiator of autophagy, this protein can be a key stimulator of autophagy proteins, which leads to formation of the central complex of Bcl-1, VPS34 and VPS15. Beclin1 is also a key determinant of cells exposed to autophagy or apoptosis (11).

Of three LC3 isoforms expressed in mammalian tissues (LC3-I, LC3-II, LC3-III), LC3-ll is associated with autophagy (12). Various stressors can strongly regulate LC3 expression and turns it into a cytosolic form. Moreover, LC3-II has been considered as the most reliable autophagy index so far (13).

Atorvastatin is a statin that stops the conversion of mevalonate to hydroxy β-methylglutaryl-CoA by inhibiting 3-hydroxymethylglutaryl coenzyme reductase, which will consequently reduce cholesterol production. Despite lowering blood cholesterol, this medicine has some antioxidant, anti-inflammatory, anti-apoptotic and tissue-protective effects in some pathological conditions (14, 15). Statins stimulate Langerhans cells to release more insulin in order to lower blood glucose. In general, this drug can inhibit diabetes-related pancreatic tissue damage and necrosis by limiting oxidative stress and pancreatic inflammation (16). However, statins have dose-dependent side effects on skeletal muscles such as congestion, muscle pain, weakness and acceleration of skeletal muscle decomposition, leading to cell death (17).

Since aging is associated with physiological changes, such as structural skeletal muscle change, it can enhance insulin resistance and diabetes. Given the limited number of studies in this regard, we aimed to investigate effects of continuous and interval aerobic exercise along with statin administration on Beclin1 and LC3 expression in old rats with type 2 diabetes.

MATERIALS AND METHODS

In this experimental study, 63 old male Wistar rats (weighting 300-350 g) were selected. The animals were randomly divided into eight groups of healthy control (CN), diabetic control (CD), diabetic + continuous exercise (CED), diabetic + interval exercise (IED), diabetic + atorvastatin (AD), diabetic + continuous exercise + atorvastatin (ACED), diabetic + interval exercise + atorvastatin (AIED) and saline (SD). Diabetes was induced in rats by intraperitoneal injection of 50 mg/kg streptozotocin. Blood samples were taken from the corners of the eye and glucose level of above 250 mg/dl confirm the induction of diabetes (18). All study procedures were carried out according to the standard guidelines of working with laboratory animals. Before starting the main protocol, the rats became familiar with the exercise by running

on a treadmill for five minutes, five sessions a week, at speed of 8-10 m/min. The exercise program consisted of two continuous and interval exercise protocols (19). Daily atorvastatin supplement (20 mg/kg) was intraperitoneally injected to the supplementation groups (18).
Forty eight hours after the last exercise session and after 10-12 hours of fasting, the rats were anesthetized by intraperitoneal injection of ketamine-xylazine, and soleus muscle tissues were separated and stored at -80 °C.
Then, the samples were sent to the laboratory for measuring LC3I, LC3-II and Beclin1 expression via RT-PCR using specific primers (Table 1).

Table 1- The primers sequence for studied genes

Gene	Type	Sequences
LC3 I	Forward	5'TGGGTGCTGGCTGGGTTGGGAG3'
	Reverse	5'AAAGCCTCAGGTGGATGAGGG3'
LC3 II	Forward	5'GAAACAGGTCAGGTGTATAGGA3'
	Reverse	5'TCTGAGCAGTGGTGCATGTGGT3'
Beclin1	Forward	5'AGGCTGAGGCGGAGAGATTG3'
	Reverse	5'TGTGGAAGGTGGCATTGAAGAC3'
GAPDH	Forward	5'CAT ACT CAG CAC CAG CAT CAC C3'
	Reverse	5'AAG TTC AAC GGC ACA GTC AAG G3'

Quantification of the values of target gene expression was performed using the $2^{-CT\Delta\Delta}$ formula.
After confirming the normality of data distribution using the Shapiro–Wilk test, descriptive statistics including mean and standard deviation were used to describe the data. Inferential statistics, one-way ANOVA and Tukey post hoc test were used for data analysis. All statistical analyses were performed in SPSS 20 and at significance of 0.05.

RESULTS

Based on the results of ANOVA, LC3-I expression was significantly lower in AD, CED, IED, ACED and AIED groups than in the CD group (p=0.001) (Figure 1).

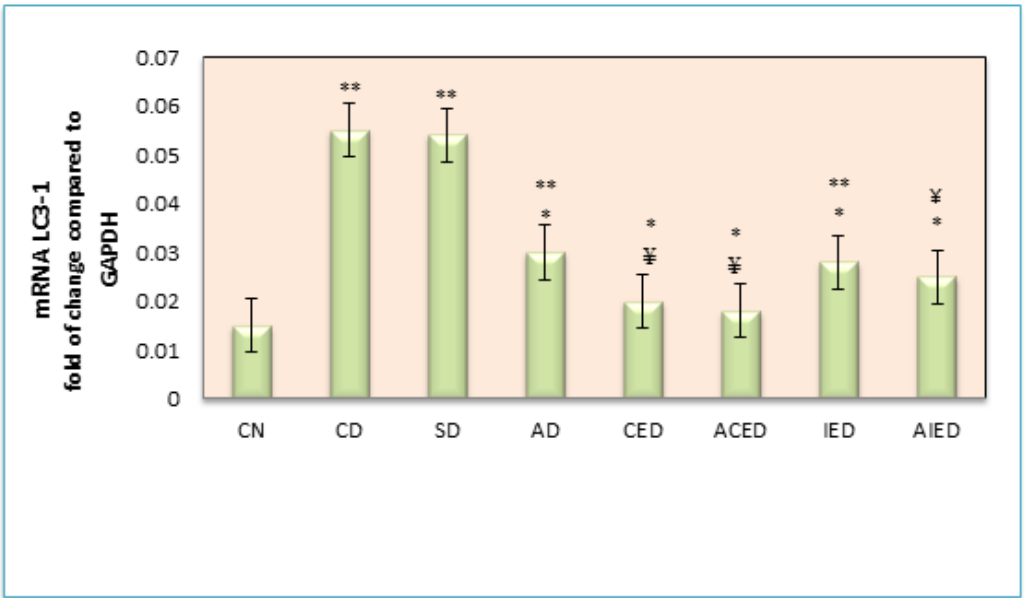

Figure 1- Comparison of mean LC3-I mRNA expression between study groups.
****: Significant difference compared to healthy control group (CN)**
***: Significant difference between diabetic (CD) and diabetic + saline (SD) groups**
¥: Significant difference between supplement (AD) and interval (IED) groups

As shown in figure 2, LC3-II expression differed significantly between the study groups (p<0.001). The LC3-II expression level in groups AD, CED, IED, ACED and AIED was significantly lower than in the CD group (p=0.001).

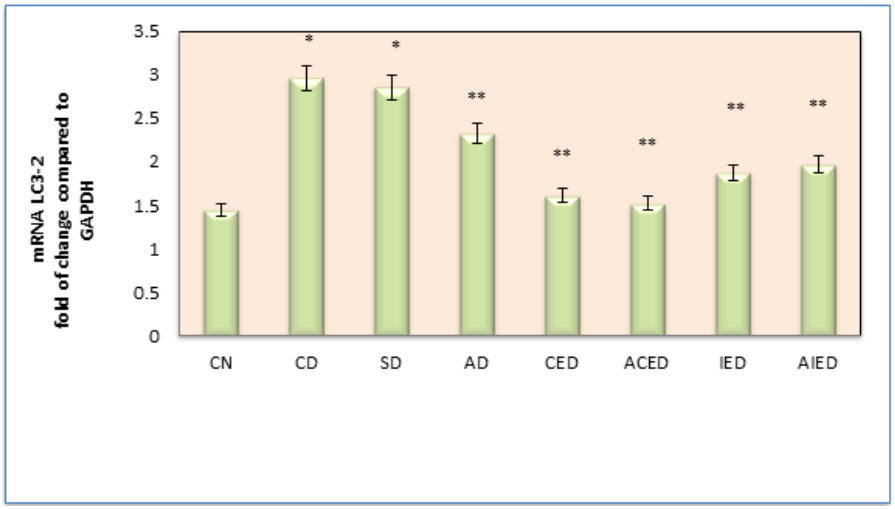

Figure 2. Comparison of mean LC3-II mRNA expression between study groups.
****: Significant difference compared to healthy control group (CN)**
****: Significant difference between diabetic (CD) and diabetic + saline (SD) groups**

As shown in figure 2, LC3-II expression differed significantly between the study groups ($p<0.001$). The LC3-II expression level in groups AD, CED, IED, ACED and AIED was significantly lower than in the CD group ($p=0.001$).

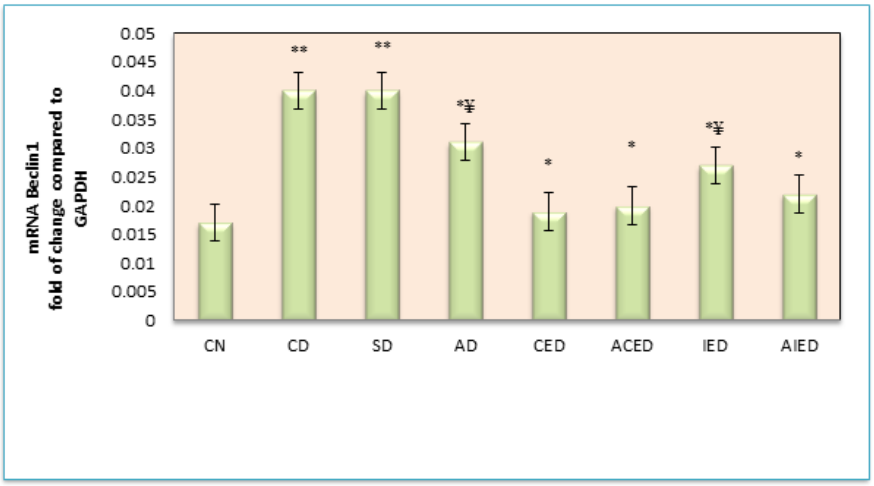

Figure 3- Comparison of mean Beclin1 mRNA expression between study groups.
****: Significant difference compared to the healthy control group (CN)**
***: Significant difference between diabetic (CD) and saline (SD) groups**
¥: Significant difference between continuous + supplement (ACED) and interval + supplement (AIED) groups

DISCUSSION

The present study was conducted to investigate the effects of interval and continuous exercise along with atorvastatin supplementation on Beclin1, LC3-I and LC3-II expression in muscle of aged rats with type 2 diabetes mellitus. The results showed that continuous and interval exercise, atorvastatin supplementation and their combination significantly reduced Beclin1, LC3I and LC3-II expression in muscle of diabetic rats compared to the control diabetic group. Choi et al. reported that treadmill running significantly decreased body weight and improved biochemical indices and insulin resistance in obese rats. However, they reported no significant change in Beclin1 and LC3 levels (19). Our results provide new information on molecular pathways involved in muscular autophagy in diabetic rats.

During the aging process, autophagic dysfunction occurs in many organs and tissues. In this condition, cells are unable to maintain proteins and healthy microorganisms, which leads to cellular dysfunction and often cell

death. This may make cells more vulnerable to stressors and cellular pathogens (20). In this regard, many studies have reported the positive effects of intense continuous and interval aerobic exercise on improving the autophagy process in healthy and sick rats (21,22).

Patients with type 2 diabetes have significant muscle atrophy, which is accelerated during aging. These changes may be due to the decreased activity of the Akt/mTOR pathway, which reduces the glucose uptake and protein synthesis (23). On the other hand, this pathway interacts with proteasome-ubiquitin and autophagy-lysosomal pathways, thereby increasing autophagy enzymes (24). Guan et al. showed that Beclin1 plays an important role in hippocampal cell autophagy in old diabetic rats. They also showed that Beclin1 expression is reduced in the hippocampus of old diabetic rats (25). Sun et al. showed that Beclin1 expression in diabetic rats was significantly higher than in healthy counterparts, indicating the initiation of cellular autophagy process (26). The autophagy process plays an important role in maintaining tissue and cellular homeostasis. Beclin1 is a key protein in autophagic, apoptotic and inflammatory processes (27). However, autophagy is mainly modulated via the interaction between the Beclin1 protein and members of the Bcl-2 family (28).

A limited number of studies have investigated the effect of exercise on Beclin1 modifications. In this regard, a study reported that endurance exercise did significantly affect Beclin1 level in the soleus muscle of obese mice (29). Accordingly, He et al. stated that the JNK1-Bcl-2 signaling activity and subsequent Beclin1-Bcl-2 complex degradation are critical for AMPK to regulate the change between autophagy and cellular apoptosis pathways in diabetic patients (30). Our results showed that atorvastatin supplementation along with interval and continuous exercise significantly reduced LC3-I and LC3-II expression. It is known that LC3 can form a covalent bond to phospholipids, and the membrane vesicles components are autophagic (31). Talaei et al. reported that 40 mg/day statin could reduce inflammatory markers, such as leptin, C-reactive protein and tumor necrosis factor-α in diabetic patients (32). Mohammadi et al. showed that atorvastatin could inhibit the production of free radicals in diabetes (33). It has been reported that exercise could help reduce the side effects of statins. Azamian Jazi et al. showed that combination of endurance training and atorvastatin has a favorable effect on vascular endothelial growth factor expression in angiogenesis process following myocardial infarction (34). Mejías-Peña et al. reported that eight weeks of aerobic training could alter autophagy indices (35). In another study, the expression of Beclin1, Autophagy Related 12 and Lysosomal Associated Membrane Protein 2 increased following exercise (36). Another study demonstrated that eight weeks of swimming exercise five sessions a week increases the amount of Beclin1 protein (37).

In a study by Agha-Alinejad et al., six weeks of interval exercise reduced Bcl-2 and increased LC3 expression in tumor tissue of female rats with breast tumor (38). In our study, atorvastatin along with continuous and interval aerobic exercise significantly decreased LC3-1, Beclin1 and LC3-II expression. The anti-inflammatory and anti-oxidative role of atorvastatin in pathological conditions has been well-demonstrated (15, 39). The atorvastatin antioxidant property increased the active oxygen species resistance by increasing the antioxidant enzyme induced by exercise activity, thereby reducing the release rate of cytochrome and initiating the autophagic process in the skeletal muscle of diabetic rats. The level of active oxygen species is high in animals and humans with type 2 diabetes (40).

Kim et al. reported that a 50-min running session at the speed of 12 m/min could significantly decrease LC3 and Beclin1 levels in the skeletal muscle (41). Brent et al. stated that exercise intensity is an important and effective variable in LC3-II and LC3-I protein content. In our study, there was a significant difference in the LC3-II/LC-I ratio between the continuous and interval exercise groups. Consistent with this result, a study showed that LC3-II decreased significantly 3, 6 and 12 hours after one treadmill running session but did not change significantly immediately after the exercise (39).

CONCLUSION

Diabetes in older people is associated with autophagy-mediated skeletal muscle atrophy. The results of our study showed that the

continuous and interval exercise program alone and combined with atorvastatin could significantly reduce the LC3-I, LC3-II and Beclin1 levels in the skeletal muscles of old diabetic rats.

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DECLARATIONS

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This research was conducted at the personal expense of researcher Dr. Farnaz Zanganeh.

Ethics approvals and consent to participate

Ethics approval was obtained from the local ethics committee. All experiments involving animals were carried out according to the Guide for the Care and Use of Laboratory Animals.

Conflicts of interest

The authors declare that there is no conflict of interest.

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