

Original Article



The effect of metformin along with high-intensity interval training on gene expression of FoxO1 and Atrogin-1 in type 2 diabetic mice

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Abstract

Background and aims: Muscle atrophy is a complication of type 2 diabetes, in which the expression of atrophy-related genes is increased. The present study aimed to investigate the effect of high-intensity interval training (HIIT) and metformin on gene expression of two atrophy-related genes (i.e., FoxO1 and Atrogin-1) in the skeletal muscle of diabetic mice.

Methods: A total of 30 mice (C57BL/6) were assigned to two groups: control (n=6), and high-fat diet (HFD) (n=24). The mice in the HFD group were fed a HFD for 16 weeks. Then, diabetes was induced in mice by HFD. Then, they were divided into 4 groups as follows: (i) diabetic control, (ii) diabetes + metformin (DM), (iii) diabetes + HIIT (DH), and (iv) diabetes + metformin + HIIT (DMH). The DM group took metformin, the HIIT group performed a HIIT program, and the DMH group had both HIIT and metformin. The real-time PCR methods were used to measure the mRNA expression of FoxO1 and Atrogin-1.

Results: The findings showed that HFD-induced diabetes caused increases in the expression of FoxO1 ($P=0.0006$) and Atrogin-1 ($P=0.0008$), and HIIT could restrain these increments ($P=0.086$, $P=0.041$). However, the decreasing effect of metformin on the expression of these genes was not significant ($P=0.15$) and the combined effect of HIIT and metformin on the expression of these genes was not greater than the individual effect of HIIT ($P=0.64$).

Conclusion: These results indicated that HIIT (but not metformin) may prevent type 2 diabetes-induced downregulation of FoxO1 and Atrogin-1 in skeletal muscle, and metformin could not affect the impact of the training on these atrophy-related genes.

Keywords: Metformin, HIIT, FoxO1, Atrogin-1, Diabetes

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Introduction

Type 2 diabetes mellitus is a chronic metabolic disease that is characterized by insulin insufficiency and insulin resistance. Globally, the incidence of diabetes increased from 11.3 million (95% UI 10.6–12.1) in 1990 to 22.9 million (21.1–25.4) in 2017, with a 102.9% increase (1).

The disease is related to many complications including retinopathy, nephropathy, neuropathy, peripheral arterial disease, and coronary disease (2). Muscular atrophy also is a common complication of type 2 diabetes characterized by decreased protein synthesis and increased protein degradation (3). The ubiquitin-proteasome is the main cellular mechanism for muscle atrophy (4). Forkhead box O family transcription factor 1 (FoxO1) is an atrophy-related transcription factor that is involved in the expression of Atrogin-1 (an E3 ubiquitin ligase) (5).

The most effective medical interventions to ameliorate type 2 diabetes include the following: (1) having a low-calorie diet, (b) administering metformin, and (c) performing exercise training (6).

Metformin is an essential drug for the treatment of insulin resistance and type 2 diabetes that has a controversial effect

on muscle atrophy and the expression of atrophy-related genes (7-10). High-intensity interval training (HIIT) is an effective exercise training that can induce muscle growth and inhibit the expression of atrophy-related genes (11).

Based on the evidence, a question was raised about whether HIIT and metformin alone and in combination could affect the atrophy-related genes such as FoxO1 and Atrogin-1 in skeletal muscle of diabetic patients. Therefore, the aim of the present study was to investigate the individual and combined effect of metformin and HIIT on gene expression of FoxO1 and Atrogin-1 in skeletal muscle of type 2 diabetic mice.

Materials and Methods

Animals

A total of 30 one-week-old C57BL/6 male mice with a mean body weight of 20 ± 2 g were obtained from Pasteur Institute of Iran and kept under standard conditions (at $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$; a humidity of $55\% \pm 10\%$; a 12-hour light/dark cycle).

The mice were randomly assigned (by restricted randomization) to two groups as follows: (a) control (C)

(n=6) and (b) HFD (n=24).

The mice of the control group were fed a standard rodent chow and water ad libitum. The mice of the HFD group were fed a HFD (55% fat) for 16 weeks. After 16 weeks, the fasting blood sugar of the mice in the HFD group was measured, and mice with a blood glucose level higher than 200 mg/dL were selected as diabetic mice.

Then, the diabetic mice were randomly divided (by restricted randomization) into four groups as follows: (i) diabetic control (DC) (n=6), (ii) diabetes + metformin (DM) (n=6), (iii) diabetes + HIIT (DH) (n=6), and (iv) diabetes + metformin + HIIT (DMH) (n=6).

The mice in the DM group received metformin and continued HFD feeding for eight weeks. The mice in the HIIT group performed HIIT and continued HFD feeding for eight weeks. The mice in the DMH group performed HIIT received metformin, and continued HFD feeding for eight weeks. The mice in the DC group were fed only HFD for eight weeks. After the experiment, mice were sacrificed, and blood and tissue samples were collected.

HFD-induced diabetes

The mice in the HFD group were fed HFD for 16 weeks (from 6 to 28 weeks old). The HFD contained 45% fat, 35% carbohydrate, and 20% protein (total calorie of 4.73 kcal/g) (12). The mice in the control group were fed a normal diet (15% fat, 60% carbohydrates, and 20% protein; a total calorie of 3.80 kcal/g) (13).

The fasting blood sugar of the mice was measured using a glucometer (01-Mini, Japan), and the mice with a blood glucose level higher than 200 mg/dL were considered diabetic mice.

Metformin

Metformin [1,1-dimethylbiguanide hydrochloride] (Abidipharma, Iran) was added to HFD before pellet production (2.5 g/kg diet) (14). Indeed, the mice in the DM and DMH groups received metformin when they feed the pellets.

HIIT protocol

Before the commencement of the experiment, the mice were acclimatized to treadmill running for one week by making them run on the treadmill at 0% inclination.

The HIIT was performed for eight weeks (5 sessions per week). The training protocol consisted of 4-minute high-intensity interval running at 90% VO_2 peak, interspersed with 2-minute recovery periods by moderate-intensity running at 70% VO_2 peak (15). The number of intervals was four in the first weeks and it progressively increased to seven in the last weeks.

Each HIIT session included warm-up (5 minutes), the main training (20-36 minutes), and cool-down (5 minutes), lasting 30 to 50 minutes (16).

VO_2 peak

At the beginning of the training and the end of each

two-week training period, the mice were subjected to a progressive exercise test on the treadmill (at 0% inclination) to determine the VO_2 peak (6). The test consisted of two-minute stages with alternating increases in speed (increments of two m/min). The VO_2 peak was reached when the animal could not keep running at a new speed for 10 seconds (6). The obtained VO_2 peak values were utilized to define the intensity of future training programs.

Tissue and plasma collection

Finally, 48 hours after the last training session, the mice were anesthetized by intraperitoneal injection of a mixture of xylazine (10 mg/kg) and ketamine (90 mg/kg) (17). The quadriceps muscle was dissected out, weighed, and snap-frozen in liquid nitrogen before storage at $-80^{\circ}C$ for future analysis.

The blood samples (5-10 mL) were obtained from a cardiac puncture using a heparinized syringe and put immediately into siliconized disposable glass tubes with EDTA as an anticoagulant. Then, plasma was separated by centrifugation (10 minutes, 3000 rpm, $4^{\circ}C$) and frozen ($-78^{\circ}C$) for future measurement.

Evaluation of biochemical parameters

Fasting plasma glucose was measured by a glucose oxidase method using a Biochemistry Auto Analyzer (Hitachi 902, Boehringer Mannheim, Mannheim Germany). Plasma insulin concentrations were measured by ELISA technique via enzyme immunoassay (EIA) kit (10-1247-01; Mercodia AB, Uppsala, Sweden) and ELISA microplate reader (Stat Fax 2000).

The homeostasis model assessment of insulin resistance (HOMA-IR) was computed using the

following formula: fasting insulin in $\mu U/mL \times$ fasting glucose in mmol/L/22.5.

Gene expression (mRNA) evaluation

RNA extraction

Approximately 50 mg of rectus femoris muscle was homogenized in 1 mL of TRIzol reagent (Thermo Fisher Scientific, USA). The total RNA was isolated using TRIzol following the manufacturer's instructions (Cat. No. 15596026). RNA concentration and purity were assessed (OD: 260/280) using spectrophotometers (Ultrospec 3000, Pharmacia Biotech, Sweden).

cDNA synthesis

First-strand complementary DNA (cDNA) was synthesized from 1 μg of RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche, Switzerland), according to the manufacturer's instructions (Cat. No. 04897030001).

Primers

Primers were designed based on previous studies (18), verified using primer-blast/NCBI and Oligo Analyzer

(1.02), and synthesized by Pishgam Company (Iran). The sequences of primers were as follows:
 FOXO1: F- CAAAGTACACATACGGCCAATCC;
 R-CGTAACCTTGATTTGCTGTCCTGAA;
 Atrogin-1(MAFbx): F- CGTCTCACTTCCCCTCAAG;
 R-GACTCCCAGCCATCCAATTAG
 Myostatin: F-TGGCCATGATCTTGCTGTAA;
 R - C C T T G A C T T C T A A A A G G G A T T C A ;
 GAPDH: F-AGCTTGTTCATCAACGGGAAG;
 R-TTTGATGTTAGTGGGGTCTCG

Real-time PCR

The mRNA contents were determined by real-time PCR performed using a Corbett Research Thermocycler (Australia) with Sahara RT-qPCR Master Mix (2X) (Santa Clara, US) and specific primers, according to the manufacturer’s instructions (Cat. No. R02310). The thermal cycling profile was 95°C for 10 minutes, followed by 40 cycles at 95°C for 10 seconds and 60°C for 60 seconds.

Quantification of mRNA

The efficiency of PCR products and primers was determined by LinRegPCR software (version 2020.0), and the melting curve confirmed the specificity of the PCR products. The relative gene expression was quantified by the Pfaffl method using Genex software (7.0).

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Two-way analysis of variance (ANOVA) was used to assess the effect of HFD, ET, and HFD*ET on dependent variables. The intergroup comparisons were analyzed by one-way ANOVA followed by the post hoc Tukey test. A P value of less than 0.05 (P≤0.05) was considered statistically significant. GraphPad Prism 8 (GraphPad, USA) was used to analyze data and design graphs.

Results

General characteristics

Table 1 shows the general characteristics of the study groups at the end of the experiment. Fasting glucose, insulin, and insulin resistance estimated by HOMA-IR were greatly increased in diabetic mice, and metformin,

HIIT, and a combination of metformin and HIIT mitigated the increase of them.

Body weight

As is shown in Figure 1, the mice that were fed the HDF gained more body weight as compared to the control groups. However, in the second phase of the experiment (16 to 24 weeks), the mice that had metformin (DM group), HIIT (DH group), and the combination of metformin and HIIT (DMH group) gained less weight as compared to the diabetic control group. Based on the above-mentioned results, the final body weight (at end of the experiment) was higher in the DM group than in the control group (P=0.0001) and the body weight was lower in the experimental group that had metformin (P=0.03), HIIT (P=0.001), and the combination of them (P=0.001) as compared to diabetic control (Figure 2).

Muscle mass

The diabetic control group had lower quadriceps muscle mass compared to the control group (P=0.001). Diabetic mice that had HIIT (DH group) and also the combination of metformin and HIIT (DMH group) had greater quadriceps muscle mass as compared to diabetic control (P=0.01, P=0.04, respectively). Moreover, the muscle mass did not differ between experimental diabetic groups (P=0.89) (Figure 3).

Gene expression of atrophy-related factors

A two-way ANOVA test showed that HIIT had a

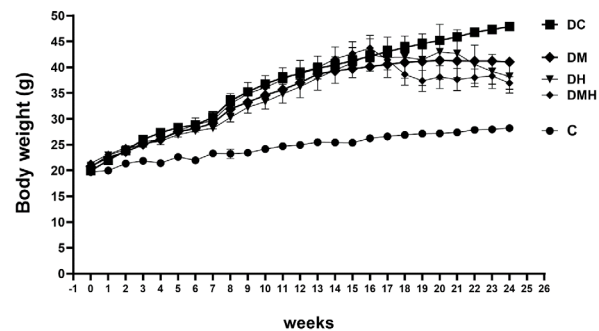


Figure 1. The weight gain of different study groups during the experiment. C: control group; DC: diabetic control group; DM: diabetes + metformin; DH: diabetes + high-intensity interval training; DMH: diabetes + metformin + high-intensity interval training.

Table 1. General characteristics of mice in the experimental groups

	C	DC	DM	DH	DMH
Body weight (g)	28.20± 0.30	47.88±0.75***	41.04±0.51 †	38.20±2.92†††	36.92±1.10†††
Body weight gain (g)	8.52± 0.14	27.94±0.25***	20.24±0.04 †	17.52±2.29†††	15.56±0.65†††
Quadriceps (g)	152.5± 4.59	131.66±5.04***	137.5±6.55	147.16±4.50††	144.33±5.02†
Glucose (mg/dL)	119.2± 1.39	279.80±3.22***	184.60±3.65†††	169.40±4.91†††	150.40±3.05†††
Insulin (µIU/mL)	8.11±0.822	36.43±2.36***	11.45±1.07†††	12.04±1.05†††	10.38±1.036†††
HOMA-IR	2.39±0.51	25.23±2.94***	5.23±0.86 †††	5.05±0.7†††	3.86±0.7†††

C: control group; DC: diabetic control group; DM: diabetes + metformin; DH: diabetes + high-intensity interval training; DMH: diabetes + metformin + high-intensity interval training.

Data are expressed as mean ± SEM and were analyzed by one-way ANOVA followed by the Tukey test. *** P<0.001 as compared to control; † P<0.05, ††† P<0.001 as compared to DC group.

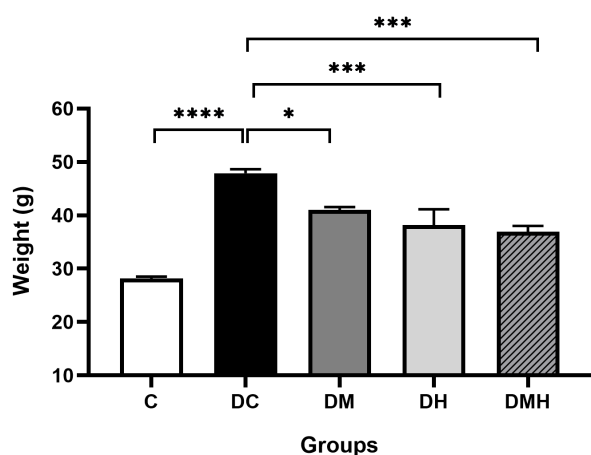


Figure 2. The final body weight (at the end of the experiment) in different study groups. Data are expressed as the mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. C: control group; DC: diabetic control group; DM: diabetes + metformin; DH: diabetes + high-intensity interval training; DMH: diabetes + metformin + high-intensity interval training.

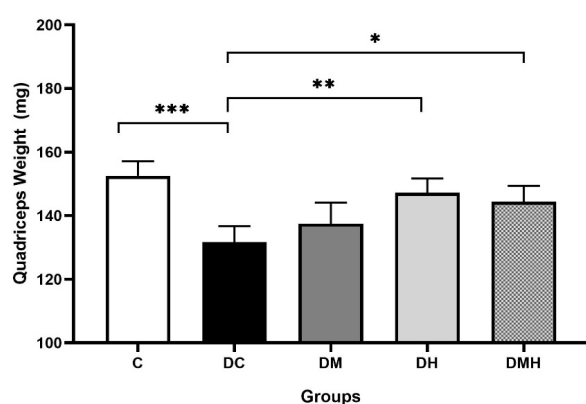


Figure 3. The weights of quadriceps muscle in different groups. Data are expressed as the mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. C: control group; DC: diabetic control group; DM: diabetes + metformin; DH: diabetes + high-intensity interval training; DMH: diabetes + metformin + high-intensity interval training.

significant effect on the gene expression of FoxO1 and Atrogin-1 ($P < 0.001$). Metformin had a significant effect on the gene expression of FoxO1 ($P = 0.04$) but not on Atrogin-1 ($P = 0.46$). However, there was not a significant interaction between metformin and HIIT regarding the gene expression of FoxO1 and Atrogin-1 in skeletal muscle ($P = 0.21$, $P = 0.92$, respectively) (Figures 4 and 5).

Comparison between groups using one-way ANOVA showed that the gene expression of FoxO1 and Atrogin-1 was significantly higher in diabetic control as compared to the control group ($P = 0.0006$, $P = 0.0008$, respectively). The diabetic group that performed HIIT showed lower FoxO1 and Atrogin-1 gene expression as compared to the diabetic control group ($P = 0.086$, $P = 0.041$). Although the gene expression of FoxO1 and Atrogin-1 was not lower in the diabetic group that took metformin as compared to diabetic control ($P = 0.15$), the gene expression of these genes was lower in the diabetic group that took metformin and performed HIIT as compared to diabetic control ($P = 0.036$). Moreover, the gene expression of FoxO1 and Atrogin-1 did not differ between experimental diabetic groups (i.e., DH, DM, and DMH) ($P = 0.64$) (Figures 4 and 5).

Discussion

The main finding of this study is that HFD-induced diabetes can cause upregulation in the expression of the atrophy-related genes (i.e., FoxO1 and Atrogin-1), and HIIT can decrease this upregulation in diabetic subjects. However, the decreasing effect of metformin on the genes was not significant.

A long-term HFD could lead to an abnormality in body mass named sarcopenic obesity which is characterized by an increment of body fat accompanied by muscle mass loss (19). Further, muscle loss is a complication of type 2 diabetes that is associated with an increase in atrophy-related gene expressions (3,20). Consistently, our findings showed that type 2 diabetes induced by feeding HFD resulted in a reduction in muscle mass and an increment in the expression of FoxO1 and Atrogin-1. This finding could suggest that chronic HFD feeding resulting in type 2 diabetes could induce the expression of ubiquitin-proteasome factors (i.e., FoxO1 and Atrogin-1). Previous

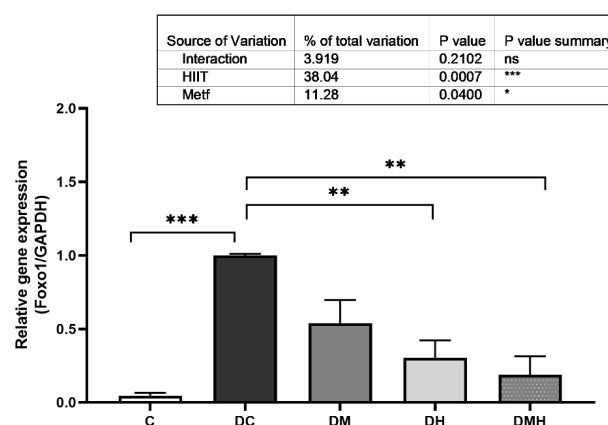


Figure 4. The gene expression of FOXO1. Data are expressed as the mean \pm SEM. The effects of variables were analyzed using two-way ANOVA and the results are shown in Figure 4. Comparisons between groups were analyzed by one-way ANOVA and post hoc Tukey test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. C: control group; DC: diabetic control group; DM: diabetes + metformin; DH: diabetes + high-intensity interval training; DMH: diabetes + metformin + high-intensity interval training.

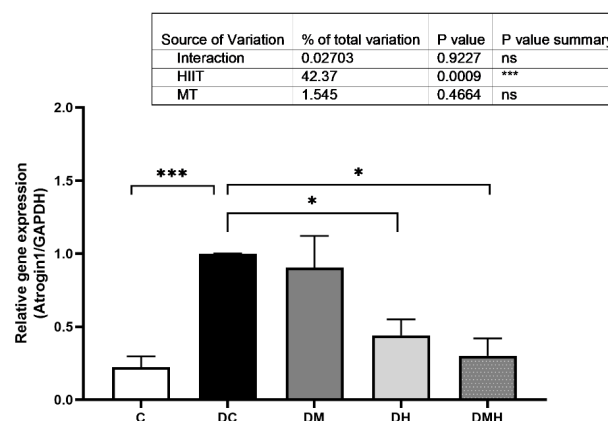


Figure 5. The gene expression of Atrogin-1. Data are expressed as the mean \pm SEM. The effects of variables were analyzed using two-way ANOVA and the results are shown in Figure 5. Comparisons between groups were analyzed by one-way ANOVA and post hoc Tukey test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. C: control group; DC: diabetic control group; DM: diabetes + metformin; DH: diabetes + high-intensity interval training; DMH: diabetes + metformin + high-intensity interval training.

studies also established that HFD could stimulate the expression of atrophy-related genes and cause muscular atrophy (21,22).

The mechanism underlying the upregulation of FoxO1 and Atrogin-1 gene expression in HFD-induced diabetic mice may be related to inflammation that is developed in obesity and diabetes. Cytokines have been established to induce atrophy-related gene expression (23). Further, Le et al showed that obesity-induced atrophy could be modulated by a decline in muscle inflammation (22).

Some previous studies have shown that metformin protects against skeletal muscle loss induced by aging and a sedentary lifestyle (6,10,24). Accordingly, there was some evidence demonstrating that metformin plays a restraining role in the upregulation of the atrophy-related gene expression induced by obesity and diabetes (25). However, in this study, no significant effect of metformin on muscle mass and the expression of Atrogin-1 and FoxO1 in HFD-induced diabetic mice was observed. On the contrary, Kang et al in 2022 showed an upregulation of Myostatin gene expression following metformin administration in skeletal muscle of wild-type and db/db mice (8). The findings raise the possibility that the expression pattern of myostatin is different from other atrophy genes such as Atrogin-1 and FoxO1. It seems that metformin may not stimulate ubiquitin-proteasome factors such as FoxO1 and Atrogin-1.

The results of the present study showed that HIIT could inhibit the diabetes-induced upregulation of FoxO1 and Atrogin1 expressions in skeletal muscle, and accordingly, it could prevent diabetes-induced muscle mass loss. This finding is in line with a previous study establishing that exercise training can stimulate hypertrophy-related gene expression and suppress atrophy-related gene expression (26). These effects also have been reported for HIIT (11). Kordi et al in 2019 found that 5 weeks of HIIT could inhibit the gene expression of Atrogin-1 and Myostatin in skeletal muscle of STZ-induced diabetic rats (27). Overall, these findings suggest that HIIT can inhibit the upregulation of atrophy-related gene expression in diabetic subjects.

Although the exact molecular mechanism of the HIIT for inhibiting the expression of atrogenes has not been elucidated, it seems that HIIT via activation of the signaling pathway of AKT/mTOR may inhibit the transcription of atrogenes (FOXO1, Atrogin1, and Myost) (26). However, this needs further research.

The main aim of this study was to survey the combined effect of HIIT and metformin on the atrophy-related gene expressions (Atrogin1 and FoxO1) in HFD-induced diabetic subjects. Our findings showed that the combination of HIIT and metformin had a restricting effect on the diabetes-induced upregulation of Atrogin1 and FoxO1 expression and the diabetes-induced muscle mass loss. However, this effect was not greater than the single effect of HIIT on the expression of these genes and muscle mass. This was predictable since metformin was not found to affect the expression of these genes, and

two-way ANOVA did not show an interaction between Metformin and HIIT in relation to the expressions of these genes. In conflict with our findings, Walton et al in 2019 showed that metformin could restrain the resistance training-induced hypertrophy in older adults likely by activating AMPK and inhibiting mTOR signaling (28). However, similar to our results, Hernández-Álvarez et al in 2019 found that metformin could not affect the muscle growth induced by endurance training (9). Altogether, these findings suggested that metformin may not negate the decreasing effect of HIIT on Atrogin1 and FoxO1 expression in HFD-induced diabetic subjects.

Conclusion

The results of the present study showed that HFD-induced diabetes caused muscle mass loss and increment of gene expression of some atrophy-related genes (i.e., FoxO1 and Atrogin-1). Interestingly, HIIT (but not metformin) could prevent muscle mass loss and upregulation of the atrophy-related gene expression induced by diabetes. Further, metformin could not affect the inhibiting effect of HIIT on muscle mass loss and atrophy-related gene expression.

These results would suggest that exercise training such as HIIT but not metformin may prevent atrophy induced by HFDs and by type 2 diabetes likely by downregulation of the expression of atrophy-related genes such as FoxO1 and Atrogin-1.

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Authors' Contribution

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Data curation: Maryam Delfan.

Formal analysis: Maryam Delfan, Saeed Daneshyar.

Funding acquisition: Alzahra University.

Investigation: Atefeh Rahimi, Maryam Delfan, Saeed Daneshyar.

Methodology: Maryam Delfan, Saeed Daneshyar.

Project administration: Maryam Delfan.

Resources: Atefeh Rahimi, Maryam Delfan, Saeed Daneshyar.

Supervision: Maryam Delfan.

Validation: Maryam Delfan, Saeed Daneshyar.

Visualization: Maryam Delfan, Saeed Daneshyar.

Writing—original draft: Atefeh Rahimi, Maryam Delfan, Saeed Daneshyar.

Writing—review & editing: Maryam Delfan, Saeed Daneshyar.

Competing Interests

The authors declare no competing interests.

Ethical Approval

All experimental procedures were conducted based on international guidelines for the care and use of laboratory animals and were approved by the Ethics Committee of Sport Sciences (IR.SSRI.REC.1400.1352).

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