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ORIGINAL ARTICLE

Irisin/FNDC5 influences myogenic markers on skeletal muscle following high and moderate-intensity exercise training in STZ-diabetic rats

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Abstract

In the present study, we investigated the effects of high-intensity interval training (HIIT) versus moderate-intensity continuous training (MICT) on irisin and expression of myogenic markers (paired box 7 (Pax7), myogenic differentiation 1 (MyoD), myogenin) in skeletal muscle of diabetic rats. Eighty-four male Wistar rats (6 weeks of age) were randomly divided into seven groups ($n=12$): basic control (Co Basic), 8 weeks control (Co 8w), diabetes mellitus (DM), HIIT, DM + HIIT, MICT, and DM + MICT groups. Diabetes was induced by intraperitoneal injection of streptozotocin (STZ). The $\dot{V}o_{2\,max}$protocol was characterized by running on a rodent treadmill with moderate intensity (60–70% $\dot{V}o_{2\,max}$), 60 min per session, 5 days/week, for 6 weeks. HIIT consisted of six 3-min runs at a high intensity (80–90% $\dot{V}o_{2\,max}$) alternated with 2-min running at low intensity (50% $\dot{V}o_{2\,max}$), 30 min per session, 5 days/week, for 6 weeks. Results showed that DM decreased myoblast markers compared to Co Basic and Co 8w groups. Fibronectin type III domain-containing protein 5 (FNDC5) mRNA decrease was correlated with myoblast markers (Pax7 $r=0.632$, $p=0.027$; MyoD $r=0.999$, $p=0.001$; myogenin $r=1.000$, $p=0.001$) in DM group. DM + MICT significantly increased gene expression of MyoD, myogenin, and FNDC5 compared to DM and DM + HIIT. The results also showed that the intensity and duration of exercise on the treadmill were effective in stimulating irisin and myogenic markers after DM.

Keywords Satellite cells · Diabetes · Exercise training · Muscle cell · Irisin · Pax7

Abbreviations

DM	Diabetes mellitus
FNDC5	Fibronectin type III domain-containing protein 5
HIIT	High intensity interval training
MICT	Moderate intensity continuous training
Pax7	Paired box 7
MyoD	Myogenic differentiation 1
FNDC5	Fibronectin type III domain-containing protein 5
SCs	Satellite cells

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Introduction

Diabetes mellitus (DM) is a metabolic degradation disease that can cause many complications and disorders such as diabetic myopathy, cardiomyopathy, retinopathy, and neuropathy (Papatheodorou et al. 2016; D'Souza et al. 2013). It has been shown that DM can affect muscle cell and bone tissue (Bianchi and Volpato 2016; Napoli et al. 2017). Increased blood glucose caused by diabetes leads to impaired muscle function, especially at the cellular level. In other words, high blood glucose increases reactive nitrogen species (RNS) and reactive oxygen species (ROS) in most

cells (Bonnefont-Rousselot 2002). It seems that increased muscle ROS in diabetic conditions inhibits satellite cells (SCs) function and degrades myoblast marker. Aragno et al. (2004) found that the expression of vital myogenic factors (myogenic differentiation 1 (MyoD), myogenin) was reduced in the diabetic animal model (induced with STZ) compared to non-diabetic rodents. Also in this study, impaired differentiation in diabetes-derived myoblasts was observed (Aragno et al. 2004). Furuichi et al. (2021) showed that high glucose levels inhibited cell proliferation and self-renewal, by satellite cell dysfunction due to hyperglycemia (Furuichi et al. 2021). Based on the limited number of studies to date, it is obvious that different stages of the myogenic process are affected by DM. It is therefore important to consider different therapeutic modalities to reduce muscle cell damage during diabetes. The soleus muscle is one of the skeletal muscles in the animal specimen (rats and mice) that is affected by exercise training and detraining due to diseases or sedentary behavior.

Skeletal muscle cells have a high capacity for growth and regeneration in response to injury and disease (Usas and Huard 2007; Almada and Wagers 2016). This skeletal muscle adaptation largely depends on the population of resident stem cells, named SCs (Bentzinger et al. 2010). It has been reported that paired box 7 (Pax7) be one of the factors that control the establishment of the SCs lineage (Seale et al. 2000). Pax3 and Pax7 regulate stem cell function in myogenesis (Lagha et al. 2008). In addition to Pax7, MyoD and myogenin respond differently to diabetes or physical stress such as exercise training. However, few studies have investigated these factors. Akagawa et al. (2018) examined the effect of some antioxidant and vitamin D supplements with exercise training on osteopenia and muscle atrophy induced by type 2 diabetes in animal models. In this study, Pax7, MyoD, and myogenin were utilized as anabolic markers. The results of this study showed that combination therapy with exercise improves the muscle cells at an early stage by stimulating skeletal muscle differentiation and inhibiting catabolic muscle genes (Akagawa et al. 2018). Hong et al. (2018) examined changes in muscle mass in type 2 diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) rats and used alpha-lipoic acid supplementation as a treatment. In this study, rats were divided into two control and treat groups and the gastrocnemius muscle was selected for consideration and analysis. Their results showed that with the induction of diabetes, MyoD, myogenin, and myostatin were significantly inhibited in gastrocnemius muscle and the alpha-lipoic acid treatment could not control this inhibition (Hong et al. 2018). However, studies on the changes of pro-myogenic factors with DM in response to different exercise training modalities by considering the key role of irisin are limited.

Exercise is one of the most important preventive and therapeutic methods for DM (Francesconi et al. 2019). However, the improvement of pro-myogenic factors through exercise can minimize diabetes effects on muscle tissues. Furthermore, it has been reported that exercise training increases and upregulates muscle and serum irisin in healthy and diabetic subjects (Tavassoli et al. 2019). Irisin as a product of fibronectin type III domain-containing protein 5 (FNDC5), is secreted from muscle tissue in response to exercise training (Ma et al. 2021). This molecule regulates energy metabolism and acts in adipose tissue, bone tissue, and the nervous system (Jiang et al. 2021). As stated, FNDC5/irisin is expressed in muscles in response to exercise training. When people perform exercise training and irisin levels rise, the body is better able to convert white adipose tissue to brown adipose tissue and glucose tolerance improves (Lourenco et al. 2019). In addition, irisin can affect myogenic factors, as well (Reza et al. 2017a). In this regard, it has been shown that irisin increases myogenic differentiation by activating interleukin 6 (IL-6) signaling pathway (Lakshmi and Suganthi 2021). It has been claimed that the injection of irisin in mice induces hypertrophy and increases grip strength in healthy muscles (Reza et al. 2017a). Meanwhile, in injured muscles, the injection of irisin improved the repair and recovery of muscle tissue and increased hypertrophy (Reza et al. 2017b; Momenzadeh et al. 2021). Farrash et al. (2020) studied FNDC5/ irisin overexpression on muscle and adipose tissue metabolism in Wistar rats. In this study, irisin was injected exogenously and it was found that increasing irisin in the muscle environment could increase the rate of synthesis and mitochondrial biogenesis (Farrash et al. 2020). Different types of exercise training can increase endogenous irisin in skeletal muscles. It has been suggested that high-intensity interval training (HIIT) be one of the stimulation in the upregulation of irisin in muscle tissue (Jafari et al. 2019). However, moderate-intensity continuous training (MICT) also plays an important role in enhancing adipose tissue metabolism and metabolic cross-talk between muscle and adipose tissue by increasing irisin (Shirvani and Arabzadeh 2020). However, there are limited studies on the effects of different exercise modalities on irisin and myogenic markers with DM. We therefore in this study considered the effects of HIIT and MICT on irisin, Pax7, MyoD, and myogenin in skeletal muscle of diabetic rats.

Materials and methods

Ethical approval

The experimental protocol was approved by the local ethics committee of Baqiyatallah University of Medical Science (ethical code: IR.BMSU.REC.1396.632) and was in

accordance with the current legislation on animal experimentation (Guide for the Care and Use of Laboratory Animals, Eighth Edition 2011). In the meantime, the study was conducted in adherence to the NIH Guide for the Care and Use of Laboratory Animals.

Animal model and experimental groups

Eighty-four adult (6-week-old) male Wistar rats were purchased from Pastor Institute of Tehran and were housed individually in an air-conditioned room with a controlled temperature (21 °C) and a reverse 12/12 h light/dark cycle. Water and food were available ad libitum. After acclimatization to laboratory environment and running on a rodent treadmill (including 5 days of treadmill exercise), rats were selected for the study. Rats were pre-tested to specify their treadmill running eagerness and those refusing to run were excluded prior to the beginning of experiments. The runners rats were then randomly divided into the basic control (Co Basic), 8 weeks' control (Co 8w), DM, HIIT, DM + HIIT, MICT, and DM + MICT groups ($n = 12$ for each group). Calculation of sample size was carried out based on the resource equation method in which: E = Total number of animals − Total number of groups, and any sample size which keeps E between 10 and 20 was considered adequate (Charan and Kantharia 2013).

Diabetes induced by streptozotocin (STZ)

The experimental diabetes was induced by STZ. To this end, 55 mg/kg of STZ (Sigma Chemical, Saint Louis, MO, USA) in 0.4 ml citrate buffer (0.1 M, pH 4.5) at pH 4.5 was injected intraperitoneally (ip). Two days after STZ injection, blood glucose was measured employing a glucometer (Accu-chek Advantage; Boehringer Mannheim, Mannheim, Germany). Accordingly, the animals with a fasting glucose > 200 mg/dL were considered as diabetic (Yoon et al. 2015; Grant et al. 2012). Since it is possible to recover from diabetes after STZ injection, we weekly checked the blood glucose levels of animals.

Maximal oxygen consumption ($\dot{V}o_{2\,max}$) tests and exercise training protocols

To determine the $\dot{V}o_{2\,max}$ in Wistar rats, the standard incremental test of Bedford et al. was used (Leandro et al. 2007; Bedford et al. 1979). The test consisted of ten stages (each stage consists of 3 min). The speed in the first stage was 0.3 km/h and at the later stages, 0.3 km/h was added to the speed of the treadmill. The slope was zero in all stages. During the test, when each rat was unable to run in a stage, the velocity at that stage was regarded as the speed of the animal at the $\dot{V}o_{2\,max}$ (Leandro et al. 2007). $\dot{V}o_{2\,max}$ test was taken at the end of weeks 2 and 4 to determine the principle of exercise overload.

The MICT protocol was characterized by running on a rodent treadmill with moderate intensity (60–70% $\dot{V}o_{2\,max}$), 60 min per session, 5 days/week, for 6 weeks.

HIIT protocol consisted of six 3-min periods running at a high intensity (80–90% $\dot{V}o_{2\,max}$) alternated with 2-min (active rest) running at low intensity (50% $\dot{V}o_{2\,max}$) (30 min per session), 5 day/week, for 6 weeks (de Lade et al. 2018). Each exercise training protocol consisted of 10 min as a warm-up (50% $\dot{V}o_{2\,max}$) and 5–10 min as a cool-down (20–30% $\dot{V}o_{2\,max}$).

Tissue preparation for staining

All animals were anesthetized with ketamine (90 mg/kg, intraperitoneally (IP)) and xylazine (10 mg/kg, IP) and killed 48 h after the last training session. The blood samples were collected from right ventricle. The soleus muscles of the rats were removed (cut into two pieces), frozen (for gene expression and western blot analysis), and fixed (for immunohistochemical staining) by 10% formaldehyde for three days. Then, fixed muscle were dehydrated in the ascending alcohol series, rinsed by xylene, and embedded in paraffin. Afterwards, all of the blocks were divided into 6 µm transverse sections.

To prepare tissue homogeneity from soleus muscle, a cold solution of 0.015 potassium chloride (KCl) with a ratio of (1:10) was added to each sample. Tissue homogenization was prepared using a mechanical homogenizer. After centrifugation at 3000 revolutions per minute (RPM) for 10 min at 4 °C, the clear supernatant was separated from the substrate to be used for biochemical analysis. All of the assays were performed blind to the treatment group.

Immunostaining assessment

For immunohistochemical staining, the fixed sections of soleus muscle were deparaffinized and rehydrated, then washed in phosphate-buffered saline (PBS) solution for 5 min. The sections were placed in sodium citrate buffer at 70 °C for 30 min. After that, they were washed with PBS three times for 5 min. Toblock non-specific binding sites, after incubation with 0.3% Triton-X100, slides were also treated with a blocking solution containing 10% goat serum in PBS for 45 min at 37 °C. To detect Pax7, MyoD, and myogenin markers, the sections were treated with mouse monoclonal antibody against Pax7 (1:100, Santa Cruz Biotechnology, USA), MyoD (1:100, Santa Cruz Biotechnology, USA), and myogenin (1:100, Santa Cruz Biotechnology, USA) left at 4 °C overnight. After it was washed by PBS three times, the second antibody FITC (1:200, Santa Cruz

Biotechnology, USA) was applied at 37 °C for 2 h in darkness. Then, the section was rinsed with PBS three times, the nuclei were counterstained by 4′,6-diamidino-2-phenylindole (DAPI) at 0.1 µg/ml for 5 min, and the slides were washed in PBS. The proteins were detected on the same tissue sections using the fluorescent microscope (Olympus, type CH2). The quantification of the immunohistochemical assay was based on the fluorescence intensity obtained by image J software.

Western blot analysis

The frozen soleus muscles of the rats were powdered in liquid nitrogen. Soleus muscle tissues (50 mg) were homogenized in 400 µl lysis buffer (RIPA, Beyotime Institute of Biotechnology) supplemented with protease inhibitor cocktails (PMSF, Sigma-Aldrich Co). After this stage, homogenates were centrifuged (12,000*g* for 15 min at 4 °C), and the supernatant was stored at − 80 °C for further western blot analysis. The equal amounts of protein (20 µg) were mixed with an equal volume of 5 × sample buffer. The mixture and pre-stained molecular weight markers were boiled for 10 min and separated by 10% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to standard western blot analysis in a Bio-Rad electrophoresis system (Hercules, CA, USA). Proteins were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham TM Hybond, Merk, Germany). The membranes were blocked with 5% dried skim milk in TBST (20 mM Tris, 150 mM NaCl, and 0.05% Tween-20). After 1 h at room temperature, the membranes were washed and incubated with primary antibody against Pax7 (1:500, Santa Cruz Biotechnology, USA), MyoD (1:500, Santa Cruz Biotechnology, USA), myogenin (1:100, Santa Cruz Biotechnology, USA), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1: 1000; Santa Cruz Biotechnology Inc., Dallas, TX, USA). The membrane was washed with TBST buffer and then incubated with goat anti-mouse IgG-HRP (1:5000, Santa Cruz Biotechnology, USA). Enhanced chemiluminescence was performed according to the manufacturer's instructions (Amersham Life Sciences Inc., Arlington Heights, IL). The results were subjected to densitometry analysis using the image J software. To ensure that equal amounts of protein were loaded; the GAPDH protein was employed as an internal control.

RNA extraction and cDNA synthesis

A 35 ± 3 mg piece of soleus muscle was homogenized in 800 µl of TRIzol (Invitrogen, Carlsbad CA) using an electric homogenizer (Power Gen 125, Fisher Scientific, Pittsburgh PA). Samples were then incubated at room temperature for 5 min, after which 180 µl of chloroform was added and then tubes were shaken vigorously. After an additional incubation for 2–3 min, the samples were centrifuged at 12,000*g* (gravities) for 15 min and the aqueous phase was transferred to a fresh tube. Next, mRNA was precipitated by adding 400 µl of isopropyl alcohol and incubated overnight at − 20 °C. The next morning, samples were centrifuged at 12,000*g* for 10 min at 4 °C and the mRNA was washed by removing the supernatant and adding 800 µl of 75% ethanol. Samples were vortexed and centrifuged at 7500*g* for 5 min at 4 °C. The supernatant was removed, and the dried mRNA pellet was re-dissolved in 100 µl of RNase-free water. The RNA was further purified using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol using the additional DNase digestion step (RNase-free DNase set, Qiagen). Next, RNA was quantified utilizing a nano-spectrophotometer (nano-drop 2000 C, Wilmington, DE). The RNA integrity was assessed using an Agilent RNA 6000 kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions, and read on a 2100 Bioanalyzer (Agilent Technologies). These quality control measures confirmed pure, intact RNA (260:280 ratios 1.98 ± 0.14; 260:230 ratios 1.38 ± 0.55; RIN 8.4 ± 0.3) (Heesch et al. 2016; Vosadi et al. 2016). cDNA was synthesized using Thermo fisher Reverse Transcription (Qiagen, Germany) according to the manufacturer's instructions.

Real time-PCR

The gene expression of MyoD, Pax7, myogenin, and FNDC5 in soleus muscle tissue was measured by quantitative real-time PCR using SYBR Green (Applied Biosystems Step One, USA). The reaction mixture had a final volume of 20 µl (including 1 µL of cDNA, 1 µl of forward primer, 1 µl of reverse primer, 7 µl of Diethylpyrocarbonate (Depc) water, and 10 µl of SYBR Green) and each reaction was duplicated. The sequence of primers used is shown in Table 1. GAPDH was analyzed as potential stable reference genes. The real-time PCR temperature program included one cycle at 95° C for 10 min, followed by 40 cycles of 95° C for 15 s and 60 °C for 1 min. Melt diagrams were drawn to check the accuracy of the data and standard diagrams were drawn to optimize the test conditions. Gene expression of the data was calculated by the ratio of the expression of MyoD, Pax7, myogenin, and FNDC5 genes to the reference gene. The gene expressions of MyoD, Pax7, myogenin, and FNDC5 were also measured by $2^{\Delta\Delta}$CT method (Schmittgen and Livak 2008).

Enzyme-linked immunosorbent assay (ELISA)

Irisin levels of soleus muscle were measured using ELISA kits (BioVendor, Brno, Czech Republic). 25 µl of standard solution and serum from each sample was added to

Table 1 A pattern of primers used for the desired genes

Genes	Primer sequence	Product size (bp)	T_m	Gene bank
MyoD	F: AAGGAAGAAGGAAGGGGCGA R: GGACATGGTCTGGGCTGGAT	124	60.84	XM_006244475.3
Pax7	F: GTGCCCTCAGTGAGTTCGATT R: AGGATGCCATCGATGCTGTG	128	60.34	NM_001191984.1
Irisin	F: CAGCTAGCCACAGGTTCTCC R: CTCTCTCCCAGGGCTTTGTG	228	20	NM_001270981.1
Myogenin	F: ACAGGCCTTGCTCAGCTC R: CGCTGTGGGAGTTGCATT	102	32	NM_001320842.1
GAPDH	F: ATCACTGCCACTCAGAAGAC R: ACATTGGGGGTAGGAACAC	179	57.24	XM_017593963.1

the wells coated with antibody. After adding 100 µl of the reagent conjugated buffer, the solution was incubated for 2 h at 37 °C. The contents of each well were removed and the wells were washed three times with a wash buffer. Then, 100 µl of TMB reagent was added to each well and incubated for 30 min at 37 °C. After adding 50 µl of stop solution, ELISA reader (Biorad laboratories, USA) was used to measure the absorption of 450 nm in 10 min and was also measured in standard curve.

Statistical analysis

To determine the normality of the distribution of the data, the Shapiro–Wilk test was employed. Histology alterations as well as gene and protein expression analysis were performed using two-way ANOVA using Graph Pad Prism 5.0 (Graph Pad Software, San Diego, CA). The p-values < 0.05 were considered statistically significant. All data were shown as mean ± SD from at least three independent experiments.

Results

Blood glucose

Two-way ANOVA demonstrated significant effects of exercise ($p = 0.001$), diabetes (p = 0.0001), interaction exercise and diabetes ($p = 0.003$) on blood glucose levels (Fig. 1). In addition, an exercise-dependent effect of HIIT on glucose levels was observed, since this modality decreased blood glucose levels in MICT and control rats, while higher levels were detected in diabetic ones. The interaction of exercise and diabetes shows that all exercise modalities attenuated diabetes-induced hyperglycemia in circulating since MICT and especially HIIT modalities reduced the level of blood glucose.

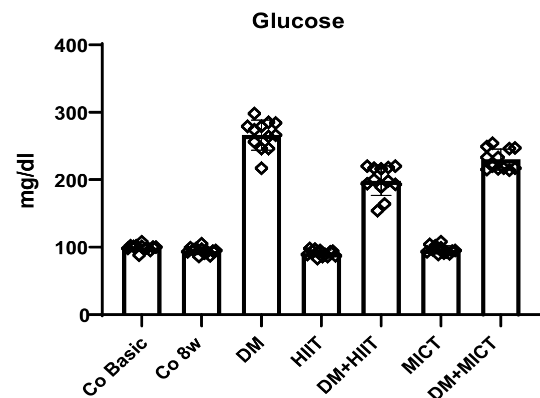

Fig. 1 Blood glucose levels of rats in different groups. DM, DM + HIIT, and DM + MICT groups have higher blood glucose than healthy groups (with and without exercise). However, exercise and diabetes groups (DM + HIIT and DM + MICT) show a lower increase in blood glucose than DM. All values were expressed as a mean ± SD ($n = 12$/group). *Co Basic* Basic Control, *Co 8w* 8 weeks Control, *DM* diabetes mellitus, *HIIT* high-intensity interval training, *DM + HIIT* diabetes mellitus + high-intensity interval training, *MICT* moderate intensity continues training, *DM + MICT* diabetes mellitus + moderate intensity continues training

Expression and correlation of Pax7, MyoD, myogenin, and FNDC5 genes

Interestingly, two-way ANOVA demonstrated a significant interaction between exercise and diabetes in Pax7 gene expression ($p = 0.0001$). The MICT exercise modalities increased the Pax7 gene expression in soleus muscle in adult rats without diabetes, while Pax7 gene expression increased for animals experiencing HIIT exercise after diabetes induction (Fig. 2a).

The analysis of MyoD gene expression revealed a significant interaction ($F = 24.435$, $p = 0.0001$). The results indicated DM + MICT had significantly increased gene expression of MyoD compared with DM and DM + HIIT (Fig. 2b). Also, the analysis of myogenin gene expression demonstrated a significant interaction ($F = 13.985$, $p = 0.0001$).

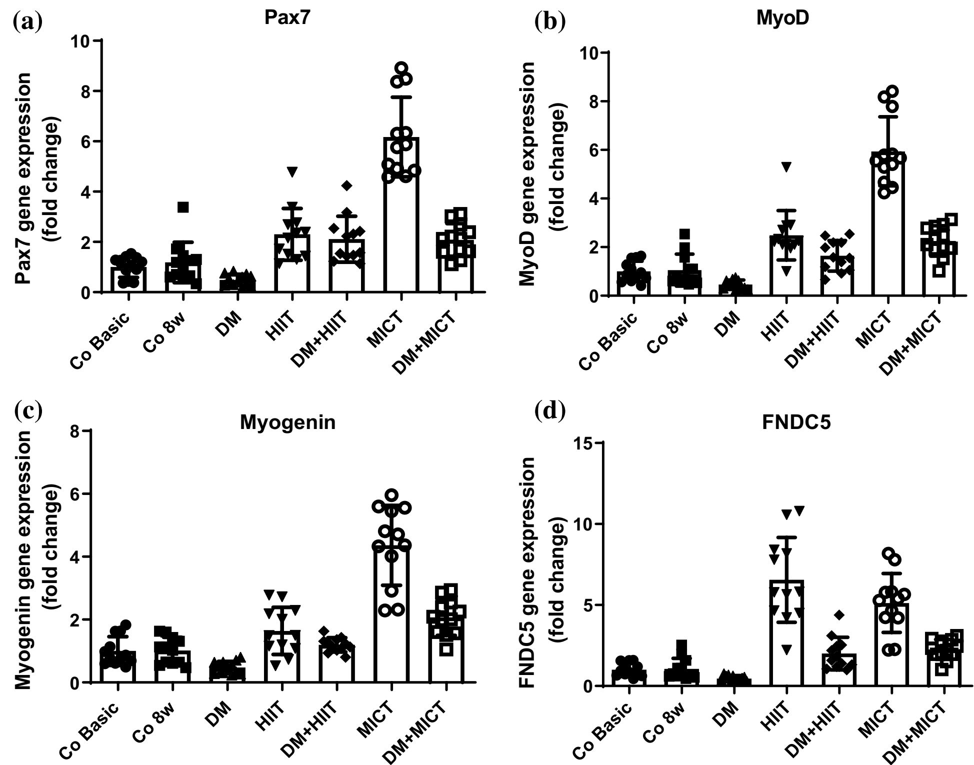

Fig. 2 The expression of **a** Pax7, **b** MyoD, **c** myogenin and **d** FNDC5 genes in soleus muscle. Exercise groups (with and without diabetes) showed higher mRNA levels of Pax7, MyoD, myogenin, and FNDC5 than DM group. HIIT exercise significantly increased FNDC5. All values were expressed as a mean ± SD ($n = 12$/group). *Co Basic* Basic Control, *Co 8w* 8 weeks Control, *DM* diabetes mellitus, *HIIT* high-intensity interval training, *DM + HIIT* diabetes mellitus + high-intensity interval training, *MICT* moderate intensity continues training, *DM + MICT* diabetes mellitus + moderate intensity continues training

The results revealed DM + MICT had significantly increased gene expression of myogenin compared with DM and DM + HIIT (Fig. 2c). In addition, the analysis of FNDC5 gene expression displayed a significant interaction ($F = 11.558$, $p = 0.0001$). The findings revealed DM + MICT had significantly increased gene expression of FNDC5 compared with DM and DM + HIIT (Fig. 2d).

With the induction of diabetes, a significant correlation between the FNDC5 mRNA and simultaneously the reductions in Pax7, MyoD and myogenin mRNA ($r = 0.63$, $p < 0.05$; $r = 0.99$, $p < 0.05$; $r = 1.00$, $p < 0.05$ respectively) were observed. There was also a correlation between Pax7 and the FNDC5 mRNA ($r = 0.84$, $p < 0.05$) during the MICT protocol (Table 2). Furthermore, MyoD mRNA levels were positively correlated with FNDC5 mRNA in the DM + MICT group ($r = 0.99$, $p < 0.01$) (Table 2).

Table 2 Correlation between FNDC5 mRNA expression and other genes

Groups	Pax7 mRNA	MyoD mRNA	Myogenin mRNA
Co Basic	$r = 0.291$ $p = 0.360$	$r = 0.996$ $p = 0.001$*	$r = 0.901$ $p = 0.001$*
Co 8w	$r = 0.247$ $p = 0.440$	$r = 1.000$ $p = 0.001$*	$r = 0.502$ $p = 0.097$
DM	$r = 0.632$ $p = 0.027$*	$r = 0.999$ $p = 0.001$*	$r = 1.000$ $p = 0.001$*
HIIT	$r = 0.255$ $p = 0.432$	$r = 0.316$ $p = 0.318$	$r = 0.258$ $p = 0.419$
DM + HIIT	$r = -0.031$ $p = 0.925$	$r = 0.541$ $p = 0.069$	$r = 0.259$ $p = 0.416$
MICT	$r = 0.843$ $p = 0.001$*	$r = -0.500$ $p = 0.098$	$r = 0.532$ $p = 0.075$
DM + MICT	$r = 0.448$ $p = 0.144$	$r = 0.999$ $p = 0.001$*	$r = 0.501$ $p = 0.097$

*Significant difference ($p < 0.05$) indicates a variable correlation between each variable

Co Basic Basic Control, *Co 8w* 8 weeks Control, *DM* diabetes mellitus, *HIIT* high-intensity interval training, *DM + HIIT* diabetes mellitus + high-intensity interval training, *MICT* moderate intensity continues training, *DM + MICT* diabetes mellitus + moderate intensity continues training

Levels of irisin by ELISA

As expected, irisin was markedly affected after treatment with the exercise training. The effects of exercise and diabetes on the irisin levels are shown in Fig. 3. Like MICT, HIIT exercise had a significant effect on irisin levels. The interaction between exercise and diabetes for irisin was significant ($F = 53.996$, $p = 0.0001$). Following diabetes induction, alterations in the irisin levels were significantly higher in DM + MICT and DM + HIIT rats than in DM rats ($p < 0.05$).

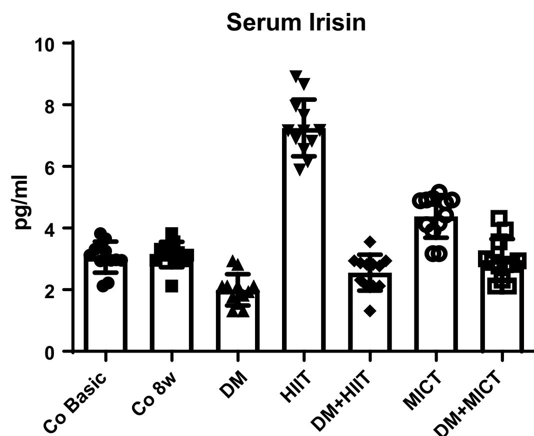

Fig. 3 Change of irisin by ELISA methods. Exercise groups, especially HIIT, had a significant increase in irisin in the soleus muscle. Two exercise training methods in diabetic groups also increased irisin levels in the soleus muscle. All values were expressed as a mean ± SD ($n = 12$/group). *Co Basic* Basic Control, *Co 8w* 8 weeks Control, *DM* diabetes mellitus, *HIIT* high-intensity interval training, *DM + HIIT* diabetes mellitus + high-intensity interval training, *MICT* moderate intensity continues training, *DM + MICT* diabetes mellitus + moderate intensity continues training

Protein levels of MyoD, Pax7, and myogenin by immunohistochemistry (IHC)

Figure 4a–f show the effects of exercise and diabetes on protein levels of MyoD, Pax7, and myogenin by IHC. Diabetes effects were observed for MyoD ($p < 0.0001$), Pax7 ($p < 0.0001$), and myogenin ($p < 0.0001$). Meanwhile, exercise effects were observed for MyoD ($p < 0.0001$), Pax7 ($p < 0.0001$), and myogenin ($p < 0.0001$). Interactions between exercise and diabetes were also observed for Pax7 ($p < 0.05$). Diabetic rats presented MyoD and Pax7 activities, as well as myogenin activities, that were significantly lower ($p < 0.001$) than non-diabetic rats. MICT and HIIT respectively have a higher MyoD, Pax7, and myogening activity compared to non-exercised rats (Fig. 4).

Western blot of MyoD, Pax7, and myogenin proteins

Figure 5a–d show the effects of exercise and diabetes on protein levels of MyoD, Pax7, and myogenin by western blotting. Diabetes effects were observed for MyoD ($p < 0.05$), Pax7 ($p < 0.001$), and myogenin ($p < 0.001$). Furthermore, exercise effects were observed for MyoD ($p < 0.001$), Pax7 ($p < 0.0001$), and myogenin ($p < 0.0001$). Interactions between exercise and diabetes were not observed for MyoD, Pax7, and myogenin ($p > 0.05$). Diabetic rats presented less activity of MyoD, Pax7, and myogenin which was significantly lower ($p < 0.001$) than that of non-diabetic rats. MICT and HIIT respectively have a higher MyoD, Pax7 and myogenin activity compared to rats without exercise (Fig. 5).

We compared myogenic function by measuring the expressions of MyoD, Pax7, and myogenin proteins from soleus muscle that were harvested from Co Basic, Co 8w, DM, HIIT, DM + HIIT, MICT and DM + MICT group rats by western blotting (Fig. 5a–d). Both exercise modalities, including MICT (2.022% ± 0.33), ($p < 0.0023$) and HIIT (1.61% ± 0.15), ($p < 0.05$) groups significantly elevated the expression level of MyoD in the soleus muscle compared with DM group (0.78% ± 0.35) (Fig. 5a, b). Additionally, the expression of Pax7 increased significantly in MICT (2.48% ± 0.52), ($p < 0.0001$) and HIIT (1.81% ± 0.17), ($p < 0.001$) groups compared with DM group rats (Fig. 5a, c). Also, myogenin expression was upregulated significantly in MICT (2.46% ± 0.44) and HIIT (2.05% ± 0.18) group rats compared with DM group ($p < 0.001$), (Fig. 5a, d). Meanwhile, MyoD, Pax7 and myogenin in DM + HIIT and DM + MICT groups show a significant increase compared to DM groups ($p < 0.05$ for all).

Discussion

Diabetes is associated with various health problems especially in muscle tissue. It can disrupt the hemostasis of muscle cell signaling with effect on myogenic marker. In the present study, we investigated the changes of FNDC5, irisin and myogenic markers with different modalities of exercise training in diabetic rats.

The results of the present study showed that exercise groups (especially HIIT) have a significant increase in the FNDC5 gene expression. Also, we showed that DM + MICT significantly increased the expression levels of FNDC5 compared to the diabetic group. But DM + HIIT group did not display significant differences in FNDC5. The gene expression of Pax7, MyoD, and myogenin were similar to FNDC5 gene expression changes. In consideration of the correlation between FNDC5 mRNA and other mRNAs, our result showed that the FNDC5 in diabetes group had a significant correlation with Pax7, MyoD, and myogenin. Based on these results, it seems that in pathological conditions such as diabetes, FNDC5 and irisin downregulation in muscle cells can have effects on myoblast marker. However, examining the effect of exercise training revealed that only MyoD mRNA was significantly correlated with FNDC5 gene expression in the DM + MICT group. Moreover, by evaluating protein changes in this study (irisin with ELISA and Pax7, MyoD and myogenin by IHC and Western blotting), we showed similar changes compared to their gene expression. In consideration of all variables, it was observed that the changes of myogenic markers were higher in the healthy and diabetic MICT groups. It has been reported that during skeletal

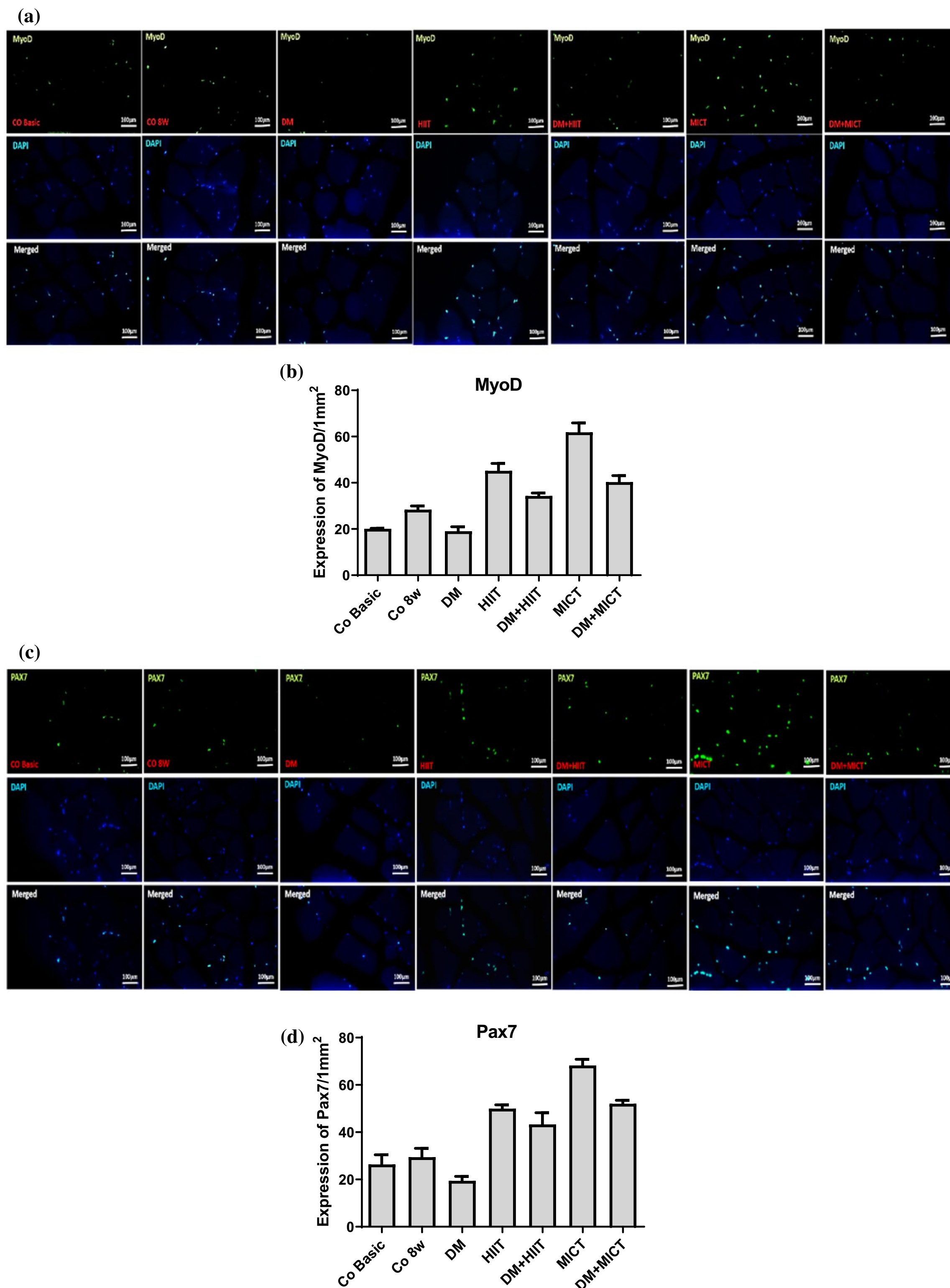

Fig. 4 IHC of MyoD (**a**, **b**), Pax7 (**c**, **d**) and myogenin (**e**, **f**) proteins in soleus muscles of rats. Nuclei are stained with 49, 6-diamidino-2-phenylindole (DAPI, *blue*). Percentages of MyoD, Pax7 and myogenin positive nuclei are displayed in *green*. Protein levels of MyoD, Pax7 and myogenin in soleus muscle decreased with diabetes. However, exercise training, especially MICT, increased these myogenic proteins. All values were expressed as a mean ± SD (n = 5–7/group). *Co Basic* Basic Control, *Co 8w* 8 weeks Control, *DM* diabetes mellitus, *HIIT* high-intensity interval training, *DM + HIIT* diabetes mellitus + high-intensity interval training, *MICT* moderate intensity continues training, *DM + MICT* diabetes mellitus + moderate intensity continues training

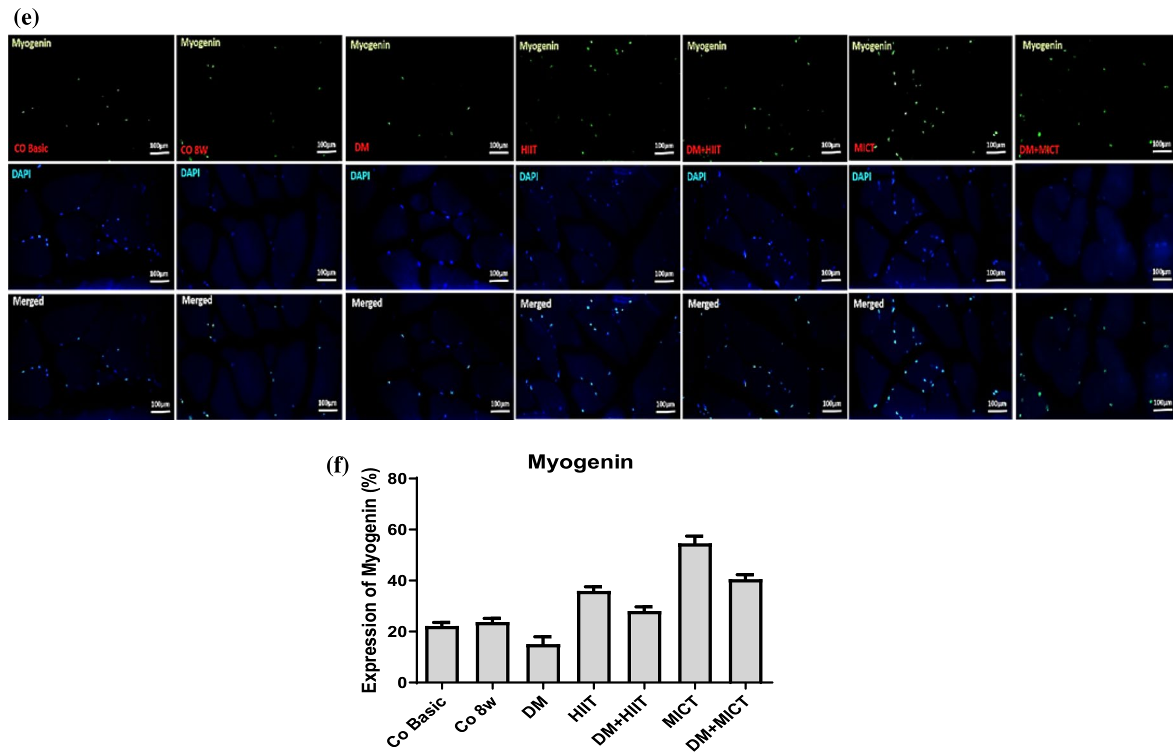

Fig. 4 (continued)

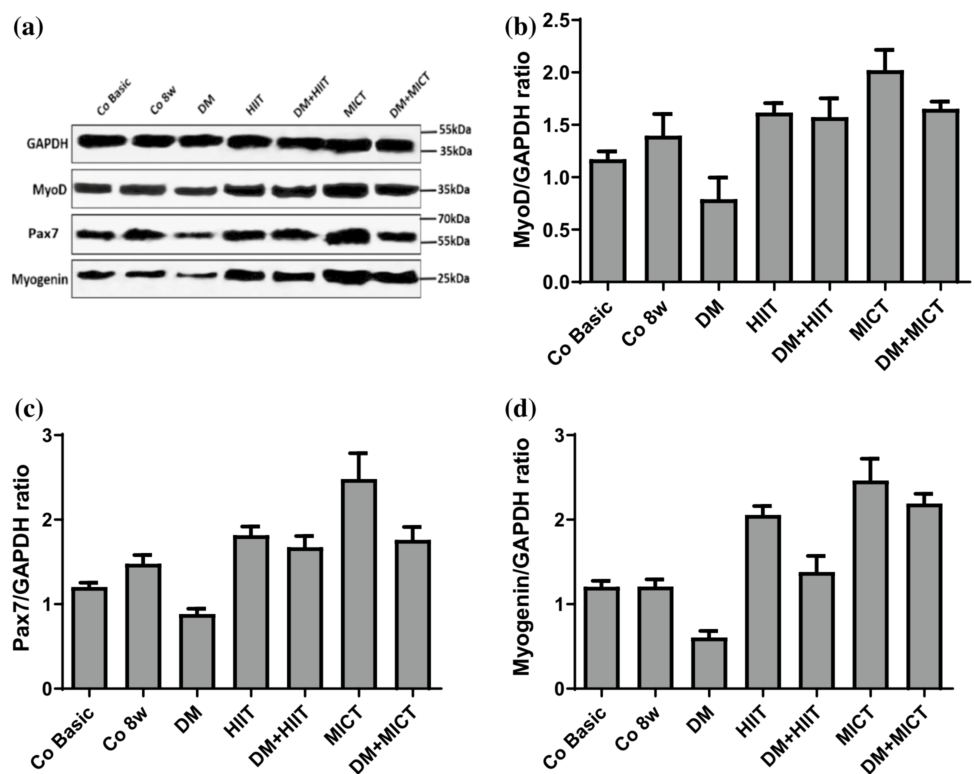

Fig. 5 Western blot of MyoD, Pax7, and myogenin proteins (**a**–**d**). The protein expression of MyoD, Pax7 and myogenin by western blotting method also decreased in the diabetes group. However, both exercise training modalities (with and without diabetes) increased the expression of these proteins in the soleus muscles of Wistar rat. All values were expressed as a mean ± SD (n = 5–7/group). *Co Basic* Basic Control, *Co 8w* 8 weeks Control, *DM* diabetes mellitus, *HIIT* high-intensity interval training, *DM + HIIT* diabetes mellitus + high-intensity interval training, *MICT* moderate intensity continues training, *DM + MICT* diabetes mellitus + moderate intensity continues training

muscle differentiation, Pax7 is essential for the activation of satellite cells (Seale et al. 2000). Furthermore, MyoD plays a critical role in the differentiation of satellite cells into myoblasts (Smith et al. 1994), and also controls the differentiation of myoblasts into myotubes (Nabeshima et al. 1993). The effects of exercise alone on increasing these markers have been shown in numerous studies (Bazgir et al. 2017). In the present study, the changes of these markers were investigated both in HIIT and MICT training modalities. It was found that MICT in the diabetic model was effective on these anabolic markers. According to the measurement of FNDC5 gene expression and protein levels of irisin, it seems that irisin changes in pathological conditions are effective on myoblast anabolic markers. Concerning irisin, it is stated that upregulation of this factor is enhanced by endurance training that plays an important role in the production of brown adipose tissue (Shirvani and Arabzadeh 2020). However, in the present study, the modality of MICT (DM + MICT) was more effective in modifying this protein, and myoblast markers such as Pax7, MyoD, and myogenin increased further in the MICT groups. Moreover, in this study, we evaluated slow-twitch muscle (soleus), thus an increase in exercise duration and endurance nature could be more effective in these changes in MICT groups. In contrast to these anabolic factors, there are catabolic factors such as myostatin, whose incremental changes inhibit myoblast marker (Cohen et al. 2015). Among the mechanisms that influence the effects of exercise training is the control and inhibition of these markers (myostatin) because exercise training with myostatin downregulation can increase MyoD, myogenin. Cohen et al. (2015) showed that factors associated with muscle atrophy in T2DM include increased myostatin, endogenous glucocorticoids, and insulin resistance (Cohen et al. 2015). However, one of the positive effects of exercise training, especially endurance exercise, in addition to inhibiting the myostatin signaling pathway, is to counteract glucocorticoids, and insulin resistance (Passos et al. 2015). Increasing glucocorticoids itself is a factor in the enhancement of catabolic pathways and inhibition of anabolic pathways in muscle tissue such as myoblastic factors (Wang et al. 2017). Thus, controlling and reducing these factors may be effective in increasing Pax7, MyoD, and myogenin. However, in the present study, changes in glucocorticoids were not evaluated.

Several studies have reported the role of irisin in anabolic pathways of muscle cells and shown that irisin is a pro-myogenic factor (Reza et al. 2017a, b). As stated in the present study, correlation of Pax7, MyoD, and myogenin with irisin expression, most were approved in the diabetic and DM + exercise groups. Consistent with the results of this study, it has been shown that other pro-myogenic factors, such as semaphorin 3F (SEMA3F), IL-6, androgen receptor 1 (AR1), interleukin 7 (IL-7), bone morphogenetic protein 4 (BMP4), tumor necrosis factor receptor superfamily member 11B (TNFRSF11B), and alanine-glyoxylate aminotransferase (2AGXT2), are upregulated with irisin treatment (Reza et al. 2017a). Exercise itself is one of the contributing factors to irisin upregulation, hence it can have positive effects on muscle tissue. It is stated that exogenous injection of irisin improved myogenic differentiation in myoblasts. It has been demonstrated that irisin increases myogenesis in C2C12 cultures. Studies have shown that irisin induces myomaker and caveolin-3 upregulation (Millay et al. 2013). The expression of these two genes is essential in the phase of myoblast fusion, thus the findings of the present study confirmed those of the previous studies, except that the present study highlighted the role of irisin in inhibiting diabetes-disrupted hemostasis of myogenic marker. Other mechanisms of the effect of irisin are its indirect role. Moreover, it has been reported that irisin is a potent regulator of IL-6. Previous studies have shown that IL-6 levels are induced during myogenic differentiation and that the absence of IL-6 mRNA reduced myogenic differentiation while overexpression of IL-6 mRNA enhanced myogenic differentiation (Baeza-Raja and Muñoz-Cánoves 2004). In the present study, it seems that irisin has participated in muscle hypertrophy and satellite cell activation by increasing IL-6 (Serrano et al. 2008). However, it is suggested that future studies examine IL-6 changes with these markers after HIIT and MICT.

According to the results of the present study, it also seems that the type of exercise training (HIIT Vs. MICT) was effective in different expressions of these factors in soleus muscle, because the expression level of FNDC5 and irisin was higher in HIIT groups, while most myogenic factors (especially Pax7 and myogenin) showed a greater increase in MICT groups. Several studies have shown that there is a difference in the effect of HIIT and MICT on several factors in different organs (O'Donovan et al. 2005; Stein et al. 1990; Rahmati-Ahmadabad et al. 2021; Mirdar et al. 2019; Ghardashi-Afousi et al. 2018). In a recent study, Rahmati-Ahmadabad et al. (2021) showed that HIIT has a greater effect compared to MICT in modulating the components of the reverse cholesterol transport process, which can have greater cardioprotective effects (Rahmati-Ahmadabad et al. 2021). Consistent with the results of the present study, it has been shown that HIIT induces higher levels of fat tissue irisin compared to MICT in metabolic disease (Tine Kartinah and Rosalyn Sianipar 2018). By contrast, it has been revealed that MICT (50–60% aerobic power) has a greater effect on plasma irisin levels and expression of intramuscular FNDC5 gene compared to HIIT (Teimourian et al. 2020). This was while we examined irisin in muscle tissue and use MICT with 60–70% $\dot{V}o_{2\,max}$. Hazrati Molaee et al. also reported that ten weeks of intense interval training resulted in a significant increase in serum irisin in rats (HAZRATI et al. 2015). It seems that higher intensity exercise trainings

due to more effect on α Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) are more effective in irisin secretion. Therefore, the reason for further increase in irisin due to intense interval training may be attributed to PGC-1α activating signals (Reisi 2016). HIIT also has an anaerobic stage, and since adenosine triphosphate (ATP) levels decrease immediately after anaerobic exercise, it is hypothesized that irisin may have short-term effects on ATP balance restoration (28). Therefore, the amount of irisin in this type of anaerobic exercise increases more to restore more ATP (Bell et al. 2016; Timmons et al. 2012). Although in this study, the amount of muscle ATP was not measured, higher levels of muscle irisin in the HIIT group were confirmed. However, HIIT had fewer effects on myogenic factors (MyoD, Pax7 and myogenin). Consistent with these results, Pugh et al. (2017) showed that HIIT training did not interfere with myogenic factors and increased the total number of satellite cells compared to resistance training in type I muscle fibers (Pugh et al. 2018). It seems that the aerobic future of MICT, to be one of the reasons for the increase in myogenic factors in the present study because it is stated that in diabetic conditions, aerobic exercise suppresses the catabolic muscle pathway in several directions, which may increase MyoD and other myogenic factors (Akagawa et al. 2018). It has already been stated that since the muscle used to analyze was slow twitch (soleus), further changes in myogenic factors with this training model (aerobic) seem reasonable.

In conclusion, our results have shown that MICT was a stronger stimulus for the myogenic markers (such as Pax7, MyoD, and myogenin) in diabetic skeletal muscle. However, the expression of myokine irisin as a pre-myogenic factor was greater in response to HIIT. The finding of this study may lead to introducing the treatments that restore myogenic markers to maintain muscle health and reduce diabetic complications, especially in muscle tissue. A potential limitation of our study is that we only investigated the myogenic markers in diabetic rats. It is better in a future study to measure SCs activity (stain satellite stem cells), and muscle atrophy marker with a myogenic markers in diabetic mice after exercise training.

Author contributions HS, EA, and MEZ designed and performed the research, analyzed data, performed histological analysis, and performed western blotting analysis. SRM, GM, and FR designed and performed the research, analyzed data, and wrote the manuscript. All authors revised the manuscript and approved the final version. The authors declare that all data were generated in-house and that no paper mill was used.

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Data availability Experimental data can be available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

Research involving animals The experimental protocol was approved by the local ethics committee of Baqiyatallah University of Medical Science (ethical code: IR.BMSU.REC.1396.632), and was in accordance with the current legislation on animal experimentation (Guide for the Care and Use of Laboratory Animals, Eighth Edition 2011).

Informed consent Not applicable.

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