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High-intensity interval training, but not Spirulina supplementation, changes muscle regeneration signaling proteins in aged rats with obesity and diabetes

Roya Askari,[1] Marzieh Sadat Azarniveh,[1,2] Amir Hossein Haghighi,[1] Hadi Shahrabadi,[1] Paulo Gentil[3,4]

[1]Department of Exercise Physiology, Faculty of Sport Sciences, Hakim Sabzevari University, Sabzevar, Iran; [2]Department of Sports Sciences, Faculty of Literature and Humani-ties, Zabol University, Zabol, Iran; [3]College of Physical Education and Dance, Federal University of Goias, Goiânia, Brazil; [4]Hypertension League Federal University of Goias, Goiânia, Brazil.

Abstract

This study aimed to investigate changes in protein signaling associated with muscle regeneration in aged rats with obesity and diabetes following high-intensity interval training (HIIT) and SP supplementation. Forty male Wistar rats weighting 280-325 g were used in this study. Obesity was induced by eight weeks of a high-fat diet, and diabetes was induced by intraperitoneal injection of 40 mg/kg streptozocin. Rats were randomly divided into control (CON), sham, SP, HIIT, and HIIT+SP groups. HIIT was performed five times per week during the 8-week period. SP dose was 50 mg/kg. Real-time PCR was used to evaluate the expression of myogenin, MyoD1, and Pax7. The decreases in body mass in the HIIT, HIIT+SP and SP groups were significantly higher than those in the sham and CON groups ($p=0.0001$). The soleus muscle mass increased significantly only in the HIIT and HIIT+SP groups ($p<0.01$). HIIT+SP improved fasting blood glucose and insulin levels more than HIIT alone and SP ($p<0.05$), while HIIT increased the expression levels of myogenic factors more than other groups ($p=0.0001$). In conclusion HIIT alone had a significant impact on myogenic factors, whereas Spirulina had an effect only when combined with HIIT.

Key Words: high-intensity interval training; Spirulina; muscle regeneration; rats; resistance training.

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Aging is associated with a decrease in physical activity and functional capacity concomitant with increases in fat mass[1]. Increased secretion of pro-inflammatory cytokines associated with aging can disrupt insulin signaling and increase systemic insulin resistance, predisposing individuals to diabetes.[2] Moreover, inflammation and oxidative stress caused by obesity and diabetes act as regulators of cell signaling, leading to increased proteolysis and muscle atrophy.[3]

Stem cells (satellite cells) are closely associated with muscle fiber regeneration and their potential decreases with increasing age, leading to poor muscle regeneration after trauma or muscle injury.[4] A popular method for estimating satellite cells activity is through myogenic regulatory factors (MRFs), including MyoD, Myf5, myogenin, and myogenic regulatory factor4 (MRF4), which can provide comparisons of the number of cells between different situations.[5]

Exercise training and nutritional support are effective modulators of skeletal muscle proteins that work synergistically to increase skeletal muscle mass.[6] In this regard, high-intensity interval training (HIIT) has recently become popular for its benefits in clinical populations. As for diabetes, a review by Arrieta-Leandro *et al.* (2023) concluded that HIIT improves glycemic control, aerobic resistance, and % fat and waist circumference in individuals with diabetes.[7]

HIIT has also been shown to improve transcriptional and translational responses of muscle cells.[8] Considering that a single bout of HIIT increased the activity of satellite cells (*i.e.*, MyoD+/Pax7+ cells) 24 and 48 h after exercise in older men,[9] investigating satellite cell activity in response to HIIT can provide mechanistic insights into the potential factors regulating skeletal muscle regeneration.

Spirulina (SP) has been shown to be a potential supplement for controlling and reducing complications caused

by advanced age.[10] SP could have antioxidant effects, promote weight loss,[11] anti-inflammatory effects, and improve glycemic control.[12] A study on rats showed that SP supplementation, when combined with resistance training, improved antioxidant capacity and attenuated exercise-induced increases in ROS and inflammation without compromising the positive physiological adaptations to exercise training.[13] Lu *et al.* (2006) provided SP supplementation for three weeks before a muscle damaging protocol and reported a reduction on skeletal muscle damage in trained men.[14]

A literature review by Calella *et al.* (2022) concluded that SP has ergogenic potential during submaximal exercise, increasing oxygen uptake, and improving exercise tolerance. However, the authors highlighted that there is a lack of evidence supporting the benefits of SP supplementation on the immune system, and the benefits of SP supplementation in healthy people performing physical exercise are not consistent.[15] In contrast, Chauoachi *et al*. (2024) suggested positive effects of SP on body composition, especially in overweight and obese subjects, which could not be the case in some pathologies. Moreover, this review highlights the improvements in aerobic fitness and muscle performance, especially in untrained and moderately trained subjects, and highlights that most studies show improvements in antioxidant status and a reduction in muscle damage in accelerated recovery.[16]

In addition to the apparent controversy, we are not aware of any studies investigating the effects of SP on skeletal muscle at the cellular and molecular levels in clinical situations. Moreover, studies investigating the interaction between exercise training and SP supplementation have reported different results, with either improvement[17] or no change[18] in glycemic and weight control. Therefore, this study aimed to investigate the effects of HIIT and SP supplementation, alone or in combination, in the expression of myogenic factors (*i.e.*, MyoD1, Pax7, myogenin, and MyoD1/Pax7) in aged rats with obesity and diabetes.

Materials and Methods

Animals

This study was approved by the Research Ethics Committee of Hakim Sabzevari University (code IR. HSU. REC.1400.007). Forty male Wistar rats, aged 20-month and with an average weight of 280-325 g were purchased and transferred to the laboratory environment. The rats were kept at a temperature of 22±2°C, humidity of 40-50% and light and 12:12 h.

Induction of obesity and diabetes

Obesity was induced by a high-fat diet derived from soybeans and vegetable oil (40% fat, 13% protein, and 47% carbohydrates), which was prepared and used under the supervision of livestock and poultry specialists for eight weeks. Rats required 10 g of pellets and 10-15 ml of water per 100 g of body weight daily[19] and had free access to food and water. Type 1 diabetes was induced after the rats' weight exceeded 310 g by an intraperitoneal single-dose injection of streptozotocin (STZ; Sigma, Germany). STZ (40 mg/kg body weight) was dissolved in sodium citrate buffer solution (pH 4.5) and injected into the rats after a 12-h fasting period. After five days, blood glucose levels were measured using blood samples collected from the tails of the animals using a glucometer (Beurer, GL42, Germany) and the glucose oxidase enzyme method. Rats were diagnosed with diabetes if their glucose concentration was > 200 mg/ml.[8] They were then weighed using a digital scale (Rat Grimace Scale) with an accuracy of 0.0001 g. The obese and diabetic rats were then randomly divided into four groups of eight: HIIT, SP, HIIT + SP and sham (normal saline). We also selected 8 rats as the control (CON) group before induction of obesity and diabetes (Basic CON).

Spirulina and placebo supplementation

The supplement was SP algae powder (Far East Microalgae, Taiwan), prepared by Sina Riz Algae (Qeshm, Iran). SP algae powder was diluted with normal saline solution at a rate of 50 mg/kg body weight,[23] and was administered by gavage to the rats in the supplement-consuming groups, five days per week for a period of eight weeks. To establish the same conditions, the same amount of a normal saline solution was used as a placebo in the groups that did not consume SP.

Determination of VO_{2max} and training protocol

A progressive test was performed to determine the VO_{2max}.[20] The rats were familiarized with the treadmill for one week at a speed of 5 m/min for 5 min in five sessions. The test began with a warm-up for 10 min at an intensity of 10 m/min. Then, every 2 min, the treadmill speed was automatically increased by 3 m/min until the rats were unable to continue running. The VO_{2max} was calculated according to the following formula, and the training intensity was adjusted accordingly:[21]

$$Y = 162 X - 1$$

Y indicates VO2 (ml/kg/$m^{0.75}$ per min) and X indicates the treadmill speed (m/s). The maximum speed obtained in the tests was 29.41±3.12 m/min.

The rats had a one-week exercise adaptation period with a progressive increase in treadmill speed before starting the training protocol. After the adaptation period, the animals exercised five times per week during the 8-week period with 90% VO_{2max} for 30s, with no inclination,[22] interspaced by 1 min of active recovery at 8.7 m/min. The number of intervals started at five and increased by one per week until it reached 12 in the eighth week. Five minutes of warm-up and cool-down were performed at 40–50% and 20–30% of the maximum speed, respectively.

Blood sampling, tissue extraction and biochemical monitoring

Blood sampling was performed 24 h after the last training session and after 8 h of fasting to eliminate the acute effects of exercise. Rats were anesthetized by intraperitoneal

injection of xylazine (10 mg/kg) and ketamine (90 mg/kg) to collect samples. The chest of the animal was opened after complete anesthesia and a blood sample was collected directly from the heart of the animal. The soleus muscle was then separated from the left leg under sterile conditions, washed with physiological serum, and weighed. The tissue was frozen in liquid nitrogen and transferred to a-80°C freezer for further measurements. Fasting insulin levels were measured using enzyme immunoassay. Serum glucose levels were measured using a biochemical kit and an enzymatic method (the glucose oxidase method). HOMA-IR was used to measure insulin resistance. The HOMA-IR index was calculated as [fasting serum glucose (mmol/L) × fasting serum insulin (μIU/ml)/22.5].[8]

RNA extraction, cDNA synthesis and real-time PCR

The efficiency of the reference gene (GAPDH) was evaluated based on the instructions of the real-time PCR technique. The soleus muscle was homogenized at a 1:10 ratio in QlAzol® Lysis Reagent to extract the total RNA. It was then centrifuged at a temperature of 4C for 10 min at 12000 rpm in order to separate the protein components. The mixture was then mixed with chloroform at a 2:1 ratio and shaken vigorously for 15 s. The mixture was centrifuged at a temperature of 4C for 15 min at 12000 rpm and the mineral and aqueous parts were separated. The remaining content was mixed with isopropanol at a 2:1 ratio, incubated at room temperature for 10 min, and centrifuged at 4C for 10 min at 12000 rpm. The pellet containing RNA was washed in ethanol and dissolved in 20 μL RNAS-free water. RNA concentration was measured using an Eppendorff (Germany), and a 260:280 ratio between 1.8 and 2 was defined as the optimal purity. cDNA synthesis was done using 1μg of RNA, a cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA, cat NO: K1621), and Mulv Reverse Transcriptase enzyme.

The expression levels of MyoD1, Pax7, and myogenin were measured by real-time quantitative PCR using Primix syber Green II (Applied Biosystems, Step One, USA). Primers were designed based on the information on MyoD1, Pax7, myogenin, and Gapdh genes in the NCBI gene bank and by the Macrogen Company, Seoul, Korea. Primer sequences used are listed in Table 1. The temperature program used in real-time PCR was 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min, and was repeated for 40 cycles, according to the manufacturer's instructions. The expression levels of target genes were measured using the $2^{-\Delta\Delta CT}$ method. The primers used are listed in Table 1.

Statistical analysis

The means and standard deviations were used to describe the data. Shapiro-Wilk and Levene tests were used to check the normality of the data and homogeneity of variances, respectively. One-way ANOVA was used to determine the differences between variables among groups, and Tukey's post-hoc test was used when necessary. Analyses were performed using the statistical software SPSS version 23, and the significance level was set at $p < 0.05$.

Results

Table 2 shows the total body and soleus muscle mass before and after the training period, with the soleus muscle mass reported in absolute and relative terms, respectively. The results of the statistical test for body mass showed a significant difference between the studied groups ($F=76.808$; $p=0.002$), and the results of Tukey's test indicated a significant decrease in body mass in the HIIT, HIIT+SP, and SP groups compared with the CON ($p=0.0001$) and sham ($p=0.0001$) groups. There was no significant difference in body mass among HIIT, HIIT+SP, and SP groups ($p>0.05$).

There was a significant difference in the soleus muscle mass between the different groups ($F=4.242$; $p=0.004$). Tukey's test showed a significant increase in the HIIT ($p=0.002$) and HIIT+ SP ($p=0.010$) groups compared with the basic CON group, and no significant difference was observed between the other groups ($p>0.05$).

The glycemic indices are presented in Table 3. One-way ANOVA results showed significant differences in fasting glucose concentration ($F=140.51$; $p=0.0001$), insulin ($F=136.52$; $p=0.0001$), and HOMA-IR ($F=14.04$; $p=0.0001$) between the studied groups. Tukey's post-hoc test showed that fasting glucose levels in the SP, HIIT+SP, and HIIT groups were significantly lower than those in the sham group ($p=0.0001$). Moreover, the values for the HIIT+SP group were lower than those for the sham, SP, and HIIT groups ($p<0.05$).

Table 1. *Sequences of specific primers used for real-time PCR.*

Gene	F-Primer	R-Primer	Product length
MyoD1	AAGTGAACGAGGCCTTCGAG	CCGCTGTAATCCATCATGCC	271 bp
Pax7	TAAGAGGGAGAACCCCGGAA	GGCTAATCGAACTCACTGAGGG	104 bp
Myogenin	GAAGCGCAGGCTCAAGAAAG	GCTGCGAGCAAATGATCTCC	300 bp
Gapdh	GCATCTTCTTGTGCAGTGCC	GATGGTGATGGGTTTCCCGT	262 bp

Insulin levels were higher in the HIIT and SP groups than those in the sham group (p=0.0001), with higher values in the HIIT+SP group than those in the HIIT, supplement, and sham groups (p=0.0001).
HOMA-IR decreased significantly in the HIIT, SP, and HIIT+SP groups compared with that in the sham group (p<0.05). There was no significant difference in HOMA-IR among HIIT, HIIT+SP, and SP groups (p>0.05). In addition, a significant difference was observed in all glycemic indices between the CON and sham groups (p=0.0001).
There were significant differences in MyoD1 (F=50.57; p=0.0001), myogenin (F=24.62; p=0.0001), Pax7 (F=79.95; p=0.0001), and MyoD1/Pax7 (F=13.93; p=0.0001) gene expression levels in the soleus muscle of rats in the different groups (Figure 1).
The post-hoc test showed that MyoD1 gene expression levels in the soleus muscle of the HIIT and HIIT+SP groups were significantly higher than those in all other groups (p=0.0001) and were higher in HIIT than in HIIT+SP (p=0.0001). MyoD1 gene expression levels were higher in the SP group than in the CON group (p=0.033). Pax7 expression was higher in the HIIT group than in all investigated groups (p=0.0001), whereas the HIIT+SP group showed a significant increase compared with the SP (p=0.030), CON (p=0.020), and sham (p=0.0001) groups. No other significant differences were observed between the groups (p>0.05).
According to post-hoc tests, myogenin gene expression levels in the HIIT group were significantly higher than those in all the investigated groups (p=0.0001). The HIIT+SP values were higher than those in the CON group (p=0.004).
The results of the post-hoc test of the MyoD1/Pax7 ratio

Table 2. *Changes in body mass and soleus muscle mass before and after the training period in the studied groups.*

Groups	HIIT+SP	HIIT	SP	CON	Sham
Baseline body mass(g)	508.18±6.83	504.55±9.57	507.48±7.33	508.54±7.08	507.65±7.33
Post body mass (g)	484.83±7.63^{B}	486.65±8.08^{B}	501.38±7.70^{B}	569.23±15.36	568.63±26.59
Soleus muscle mass (g)	0.66±0.05^{A}	0.67±0.02^{A}	0.64±0.04	0.62±0.04	0.62±0.05
Sole muscle mass ratio to body mass (%)	0.14	0.14	0.13	0.11	0.11

CON, control; SP, Spirulina; HIIT, high-intensity interval training; HIIT + SP, high-intensity interval training combined with Spirulina. ^{A}Significant differences compared with the basic CON group. ^{B}Significant difference compared with the CON and sham groups.

Table 3. *Comparison of glycemic indices between the different groups.*

Groups	Fasting blood glucose (mg/dl)	Insulin (µIU/ml)	HOMA-IR
HIIT	201.12±16.84^{E}	7.17±0.14^{A}	3.55±0.23^{C}
HIIT+SP	165.25±18.51^{D}	9.24±0.39^{D}	3.73±0.34^{A}
SP	230.25±39.23^{A}	6.99±0.29^{A}	3.98±0.74^{A}
CON	90.37±8.07	13.12±1.56	2.94±0.53
Sham	372.00±28.83^{B}	5.13±0.12^{B}	4.71±0.39^{B}

CON, control; SP, Spirulina; HIIT, high-intensity interval training; HIIT + SP, high-intensity interval training combined with Spirulina. ^{A}Significant differences compared to the CON and sham groups. ^{B}Significant difference compared to the CON group; ^{C}Significant difference compared to the sham group; ^{D}Significant difference compared to all groups ^{E}Significant difference compared to HIIT+SP, sham and CON groups.

were higher in the CON group than in all other groups ($p<0.05$); however, no other significant differences were observed between the groups ($p>0.05$).

Discussion

This study aimed to investigate the changes in myogenic signaling proteins in aged rats with obesity and diabetes following HIIT or SP supplementation. The results of the present study showed that HIIT alone (14%) and in combination with SP (9%) caused a significant decrease in body mass. There was also an increase in soleus muscle mass in the HIIT+SP (17.8%) and HIIT (19.6%) groups compared to that in the basic CON group. Many studies have been conducted on the effect of HIIT on body mass, which is mostly consistent with the present findings, indicating the effects of weight loss following HIIT.[24,25] Moreover, previous studies have shown that HIIT is likely associated with promote both anabolic and anticatabolic stimuli and stimulate muscle hypertrophy.[26]

The results showed that SP alone and in combination with HIIT caused weight loss, reduced fasting blood glucose levels, increased insulin levels, and improved insulin resistance. Notably, these changes were significantly higher in the HIIT+SP group than in the SP group, indicating a synergy between HIIT and SP supplementation. However, some studies have reported results that are contrary to those of the current study. For example, Lee *et al.* (2008) indicated that there was no significant decrease in serum glucose and insulin levels in diabetic patients owing to supplementation.[27] The positive effects of HIIT on diabetes management have been reported in several studies[12,28,29] and is be associated with improvements in glucose metabolism,[29] changes in insulin receptor signaling, increased expression of glucose transporter proteins, reduced release of free fatty acids, and increased release of blood glucose into the muscle.[28] SP has also been found to improve diabetes control by reducing the weight and production of proinflammatory cytokines.[12] The effectiveness of SP is mainly attributed to the water-soluble part of this alga, which consists of a protein called phycocyanin, and is considered a blood glucose-lowering agent. Furthermore, other factors that have been associated with the effects of SP on glycemic control are the high fiber content, which reduces the absorption of glucose in the digestive system,[30] and vitamin B6, which helps in insulin production.[31]

An important goal of the present study was to investigate the effects of HIIT and SP supplementation on the expression of myogenic proteins in the soleus muscle. The results showed that the expression of myogenin, MyoD1 and Pax7 increased more in the HIIT group than in other groups and the MyoD1/Pax7 ratio in all groups was lower than the CON. This increase in myogenin expression is consistent with the results of some studies.[8,32] however, there are others that do not show significant changes.[33,34] The increase in Pax7 expression and decrease in the MyoD1/Pax7 ratio in the intervention

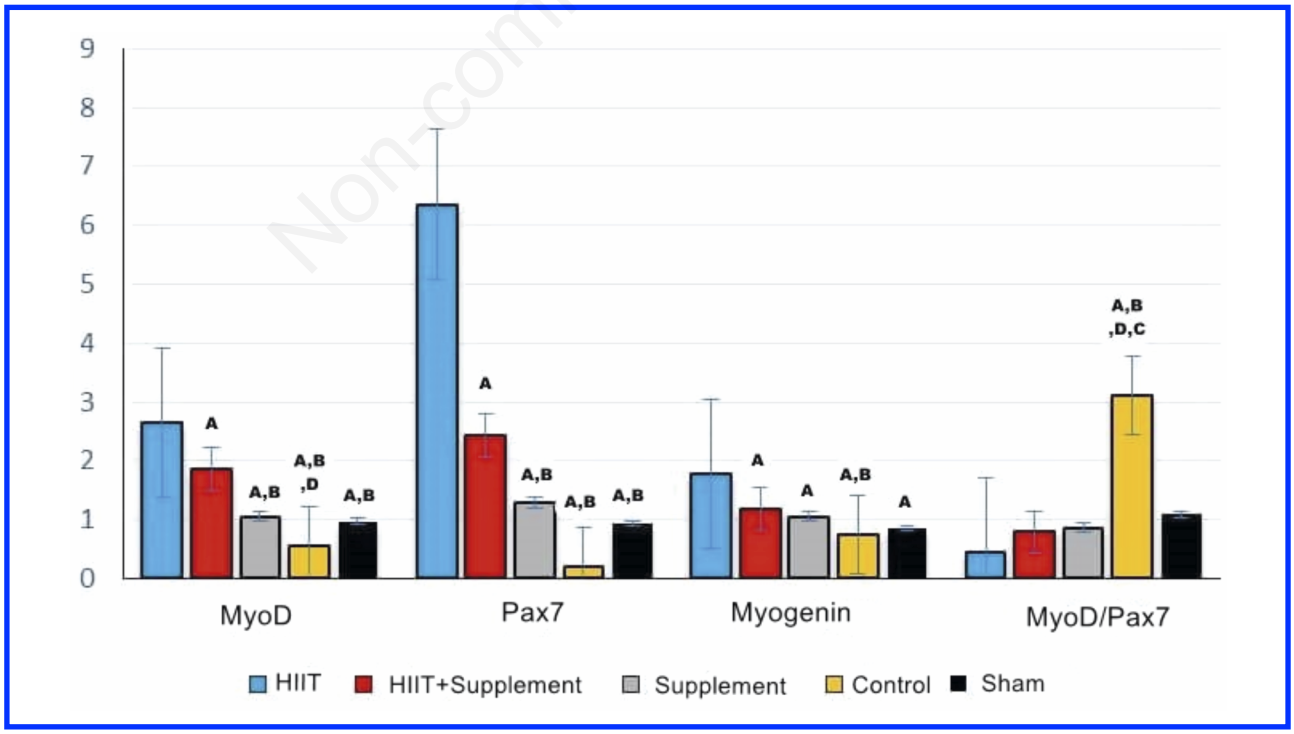

Figure 1. *Changes in muscle regeneration signaling proteins in the studied groups. A, Significant difference compared to the HIIT group. B, Significant difference compared to the HIIT+SP group; C, Significant difference compared to the SP group; D, Significant difference compared to the sham group.*

groups in the present study are in agreement with the results of previous study,[35] whereas the increase in MyoD1 expression is in line with studies using different exercise modes (e.g., HIIT, resistance, and endurance training).[33,36]

Although the present study did not directly test for some mechanisms associated with changes in myogenic factors, there are some potential changes that could be presented, such as muscle damage, stimulation of growth factors, and inhibition of myostatin following HIIT, which help activate satellite cells.[37,38] Moreover, exercise training has been shown to increase the expression of MRFs, especially myogenin, via the activation of the IGF-1/PI3K/Akt pathway.[38] Another possible mechanism for changing satellite cells is calcium stimulation, which activates calcineurin and MEF2 signals, ultimately leading to the stimulation of myogenin transcription.[39]

Diabetes affects protein homeostasis by causing insulin resistance, leading to disruption of protein synthesis through the PI3K/Akt pathway.[40] As a result, there is an interference in insulin signaling and reduction in the transport of amino acids into muscle cells and decreases in protein synthesis.[41] The results of some studies confirm the current findings that HIIT improves glycemic status and protein synthesis in patients with diabetes via the activation of the AKT/mTOR/4EBP1 pathway.[42]

From a practical standpoint, it is important to note that HIIT may be adapted for different populations, including older people with obesity and diabetes, due to the diverse possibilities and variables involved in interval training.[43] For example, in light of musculoskeletal limitations, cycling and even water activities can be used. Intensity should also be individualized; for example, for frail people, high intensity can be achieved with slow walking.[44] When observing this, previous studies have shown that HIIT brought higher improvements, when compared to milder activities, in health markers, glycemic control and weight management in different populations.[44,45] Specifically in older people, a systematic review and meta-analysis concluded that HIIT is more effective than moderate-intensity exercise in improving glucose metabolism.[46]

This study had some important limitations that should be addressed. The soleus muscle is predominantly (~75%) composed of type I fibers,[47] and the responses could be associated with be different in muscles with different compositions. However, we opted for a muscle predominantly composed of type I fibers to better reflect aging, as aging in humans is associated with an increase in type 1 and a decrease in the proportion of type 2 fibers.[48] Another important limitation is the lack of a more robust analysis of molecular mechanisms, PCR, and histological data, which are not possible due to logistical and financial constraints. Based on the present results, we conclude that the combination of HIIT and SP supplementation and/or HIIT alone could be used to manage obesity and diabetes in older people. Nevertheless, future studies can be of great help in expanding our understanding of this issue, with more robust molecular analysis involving other muscles, with different fiber type distribution.

List of acronyms

HIIT, high-intensity interval training
CON, control
SP, Spirulina
MRFs, myogenic regulatory factors
ROS, reactive oxygen species
AKT, protein kinase B
PI3K, phosphatidylinositol 3-kinase
mTOR, mammalian target of rapamycin
4EBP1, eukaryotic translation initiation factor 4E-binding protein 1
MyoD1, myoblast determination protein 1
MEF2, myocyte enhancer factor 2
HOMA-IR, homeostatic model assessment for insulin resistance
GAPDH, glyceraldehyde-3-phosphate dehydrogenase
VO_{2max}, maximal oxygen consumption

Contributions of Authors

Conceptualization and Supervision, RA; Writing (original draft, and formal analyses), MSA and HS; Data curation and Investigation, MSA; Methodology, Project and Administration, AHH; Resources, MSA and HS; Review & editing and Validation, PG; Visualization, PG and AHH. All the authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare no financial, personal, or other conflicts of interest.

Ethics approval

This study was approved by the Research Ethics Committee of Hakim Sabzevari University (code IR. HSU. REC.1400.007). The study is conformed with the Helsinki Declaration of 1964, as revised in 2013, concerning human and animal rights.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Corresponding author

Paulo Gentil, PhD. College of Physical Education and Dance, Federal University of Goias, Goiânia, 74690-900, Brazil.
Tel./Fax: +55 62 3521-1141.
ORCID ID: 0000-0003-2459-497
E-mail: paulogentil@hotmail.com

Roya Askari:
ORCID ID: 0000-0003-4331-2293
E-mail: r.askari@hsu.ac.ir

Marzie Sadat Azarnive
ORCID ID: 0000-0003-2655-5947
E-mail: m.azarnive@uoz.ac.ir

Amir Hossein Haghighi
ORCID ID: 0000-0002-7258-9737
E-mail: ah.haghighi@hsu.ac.ir

Hadi Shahrabadi
ORCID ID: https://orcid.org/0000-0001-8404-6927
E-mail: h.shahrabadi@gmail.com

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