Original Article

# Evaluating the Efficacy of Irisin Injection in Mimicking the Molecular Responses Induced by Endurance Exercise in Mouse Liver Tissue



## Abstract

**Background:** Physical activity has been found to improve liver health by reducing oxidative stress (OS), possibly through the protein irisin. Heat shock proteins (HSPs) and microRNAs (miRNAs) help regulate the body's response to stress and maintain cellular health. This study aimed to investigate the expression of the HSP70 gene and protein, miR-223a, and serum irisin levels in the liver after 8 weeks of endurance exercise or irisin injection. **Methods:** Twenty-one mice were randomly assigned to a control group, an endurance training group, and an irisin injection group. The expression of the HSP70 gene and miR-223a was analyzed using real-time polymerase chain reaction (PCR), while HSP70 protein levels were measured using immunohistochemistry (IHC) and Western blot analysis. The concentration of irisin in the mouse serum was evaluated using the enzyme-linked immunosorbent assay (ELISA) method. **Results:** The endurance training and irisin injection groups exhibited a significant increase in the HSP70 gene (405.30% and 816.03%, respectively) and protein expression (173.89% in IHC, 36.76% in Western blot for endurance training; 206.73% in IHC, 59.80% in Western blot for irisin injection) as well as elevated serum irisin levels (49.75% for endurance training and 60.65% for irisin injection) compared with the control group. In contrast, miR-223a expression decreased in both the endurance training (21.37%) and irisin injection (52.80%) groups ($P < 0.05$ in all cases). Mice in the irisin injection group demonstrated higher levels of the HSP70 gene (81.28%) and protein expression (11.99% in IHC and 16.84% in Western blot) and lower miR-223a levels (39.97%) than those in the endurance training group ($P < 0.05$). **Conclusions:** The study concludes that irisin administration can replicate the effects of long-term endurance exercise on HSP70 and miR-223a and may have a more significant impact on their production than exercise training alone.

**Keywords:** *Endurance training, heat shock proteins, Irisin, liver, microRNAs*

**Shirin Shahabi, Fahimeh Esfarjani, Saeed Zamani[1], Fahimeh Zamani Rarani[1], Bahman Rashidi[1]**

*Department of Exercise Physiology, Faculty of Sport Science, University of Isfahan, Isfahan, Iran, [1]Department of Anatomical Sciences, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran*

## Introduction

Liver-related health issues are a prevalent and substantial concern that affects both the quality of life and the economy in numerous countries. These problems account for 3.5% of all global deaths.[1] Liver damage is believed to be heavily influenced by oxidative stress (OS), marked by the production of reactive oxygen species (ROS) and a decrease in antioxidant defenses. Although hepatocytes are the main cells affected by excessive ROS in the liver, other cell types such as Kupffer and stellate cells are also vulnerable to harm, resulting in the production of tumor necrosis factor-α (TNF-α) and substantial collagen production, respectively.[2-4]

Studies have shown that OS inflicts damage on hepatocytes by affecting their lipids, proteins, and deoxyribonucleic acid (DNA) components. One significant outcome of this process on hepatocytes is the accumulation of misfolded proteins, which can lead to liver inflammation, fat accumulation, fibrosis, cirrhosis, and even potential cancer development.[5,6]

Molecular chaperones, also known as chaperone proteins, play a pivotal role in preventing protein aggregation. They assist in the accurate folding or refolding of newly synthesized proteins and aid in the degradation of misfolded proteins. A diverse group of molecular chaperones, called heat shock proteins (HSPs), significantly contribute to maintaining protein balance and supporting cell survival under various cellular stressors such as heat shock stress, mitochondrial stress, OS, and hypoxia. Notably, HSPs exert a critical influence on liver tissue.[7,8]

HSPs have been categorized into six distinct groups or families in two different



**How to cite this article:** Shahabi S, Esfarjani F, Zamani S, Zamani Rarani F, Rashidi B. Evaluating the efficacy of irisin injection in mimicking the molecular responses induced by endurance exercise in mouse liver tissue. Int J Prev Med 2024;15:66.

***Address for correspondence:***
*Dr. Fahimeh Esfarjani, Department of Exercise Physiology, Faculty of Sport Science, University of Isfahan, Isfahan, Iran.*
*Email: f.esfarjani@spr.ui.ac.ir*

**Access this article online**

**Website:** www.ijpvmjournal.net/www.ijpm.ir

**DOI:** 10.4103/ijpvm.ijpvm_124_23

**Quick Response Code:**

ways: based on their molecular weight (including small HSPs (HSP27), HSP40, HSP60, HSP70, HSP90, and HSP100 families) or according to the nomenclature designated by the Human Genome Organization (HUGO) Gene Nomenclature Committee (HGNC) (such as HSPA (HSP70), HSPB (small HSPs, including HSP27), HSPC (HSP90), HSPD/HSPE (HSP60/HSP10), HSPH (HSP110), and DnaJ (HSP40)). HSPs may be constitutively expressed or induced by stress-dependent pathways.[9]

HSP70 is considered the primary cellular sensor that detects issues stemming from OS. Studies have suggested that the expression of HSP70 may function as a biomarker of OS, with low concentrations of this protein indicating a favorable physiological state within cells. Nevertheless, under stress conditions, there is a notable increase in HSP70 expression.[8]

Recent research has shown that microRNAs (miRNAs)—small, single-stranded, noncoding ribonucleic acid (RNA) molecules about 19–25 nucleotides long—have the ability to modulate the expression of HSPs. These miRNAs usually attach to mRNA to regulate gene expression posttranscriptionally. They commonly trigger mRNA silencing activity by binding to the 3′ untranslated region (UTR) of target mRNAs, subsequently decreasing the expression of associated proteins. This process can occur through actions such as cleaving the mRNA strand into two pieces or destabilizing mRNA by shortening its poly (A) tail.[10-12] In a study by Duan *et al*., it was shown that miR-34a and miR-122 are involved in reducing liver damage caused by OS.[13,14] Other research indicates that individuals with liver cancer and cirrhosis had increased levels of miR-34a in their bloodstream.[15] Tranter *et al*.[16] have proposed that during stress conditions such as heat and ischemia, the binding site of miR-378, an inhibitor of HSP70, becomes unavailable, resulting in an upsurge of HSP70 gene expression in cardiomyocytes. Moreover, miR-223 expression has been found to be dysregulated in various liver diseases, including hepatitis, cirrhosis, fibrosis, and alcohol-induced liver injury. Online tools and studies suggest that miR-223 targets HSP70. Both *in vitro* and *in vivo* studies have shown that miR-223 diminishes HSP70 expression, leading to retinal ganglion cell apoptosis and inflammation.[17,18]

It seems that modulating the expression of HSP70 and miR-223 could potentially serve as a mechanism to enhance the liver's resilience against OS, thereby potentially reducing the occurrence of liver-related diseases and disorders. Research has indicated that both acute and long-term endurance exercise, known as aerobic or cardiorespiratory exercise and recommended by the World Health Organization for promoting health and well-being in children, adolescents, and adults, can impact the levels of HSP70 in various tissues. Studies have demonstrated that the increase in HSP70 levels following acute exercise is linked to the OS induced by this form of exercise and generally returns to a physiologically low level shortly after the exercise stops. However, the elevation in HSP70 levels after long-term exercise happens independently of OS and persists for a longer duration compared with acute exercise.[19-21] Hence, it seems that continual endurance exercise can enhance the body's ability to handle subsequent stress. However, the impact of prolonged aerobic exercise on miR-223 expression and its specific targets, particularly HSP70 in the liver, has not been extensively investigated thus far.

Due to the global lack of physical activity, numerous studies have sought to identify exercise mimetics. These studies propose that exercise mediators could offer an effective means to replicate some of the effects of exercise in tissues.[22,23] Research indicates that irisin, a hormone newly identified in mouse skeletal muscle in 2012, is a crucial mediator of exercise benefits. Irisin is a small peptide composed of 112 amino acids and has a molecular weight of 12 kDa. It is mainly released after exercise through the cleavage of fibronectin type III domain-containing protein 5 (FNDC5).[24,25] *In vitro* studies have shown that irisin can regulate OS and HSP70 expression in cultured hepatocytes. These studies suggest that irisin can improve OS conditions in these cells and potentially protect the liver from hepatic steatosis.[26] Similar results have been reported in cardiomyocytes exposed to OS induced by doxorubicin, a chemotherapy drug. Zhang *et al*.[27] showed that adenovirus-mediated irisin mRNA transfer into cardiac cells under OS can increase HSP70 and HSP20 concentrations and decrease OS complications.

The objective of this investigation was to evaluate the levels of expression of miR-223a and HSP70 in the liver, in addition to the concentration of irisin, following prolonged endurance exercise and irisin administration. In particular, it aimed to analyze the impact of prolonged endurance exercise on miR-223a and HSP70 expression in the liver, as well as the serum concentration of irisin. The study aimed to determine whether the external administration of irisin could replicate the effects induced by exercise.

## Materials and Methods

The present experimental study utilized twenty-one mature male mice (Naval Medical Research Institute (NMRI), mean weight 18g and 5 weeks old). These mice were housed under standard conditions in the animal facility, with *ad libitum* access to standardized food and water, and were maintained at a stable temperature of 21°C, with a 12:12-h light/dark cycle, and humidity levels maintained at 50 ± 3%. Following a 2-week adaptation period, the mice were assigned randomly to one of three groups: a control group (no intervention was performed), an endurance exercise group, and an irisin group.[28-30]

## Endurance exercise

The endurance exercise was conducted thrice a week for eight consecutive weeks on a running treadmill (Exer6M, Columbus Instruments). During the initial 2 weeks of the study, the treadmill's incline was set to 0°, and the subjects ran for 10 minutes at a speed of 5 m.min-1. In the subsequent 2 weeks (i.e., weeks 3 and 4), the exercise duration was increased to 12 minutes, and the incline was set to 2°, with the running speed raised to 7 m.min-1. Over the following 2 weeks, the mice ran for 13 minutes at a speed of 8 m.min-1, with an incline of 4°. Finally, during the last 2 weeks of the study, the exercise intensity was raised to a speed of 10 m.min-1, an incline of 5°, and an exercise duration of 15 minutes.[30]

## Irisin administration

Irisin injections (Canada Phoenix Pharmaceuticals) were administered intraperitoneally at a dose of 100 µg/Kg/week, three times a week for eight consecutive weeks. Irisin powder was dissolved in 1% dimethyl sulfoxide (DMSO). It is worth noting that Colaianni *et al*.[31] have reported this irisin dosage to have a significant impact on cortical bone mass.

## Enzyme-linked immunosorbent assay (ELISA) test

Blood samples were collected via cardiac puncture, in an ethylenediaminetetraacetic acid (EDTA) tube, 72 hours after the final training bout or irisin injection. The collected samples were then centrifuged at 4000 rpm for 15 minutes to isolate the serum. The concentration of serum irisin levels was determined using commercially available ELISA kits (Aviscera Biosciences, Santa Clara, CA).[29]

## Real-time polymerase chain reaction (PCR) for HSP70 expression

The level of HSP70 expression was determined by real-time PCR**.** The mouse liver tissue was ground using a mortar and pestle in liquid nitrogen to produce homogeneous samples. The total RNAs were isolated from the mouse liver samples by the Total RNA Prep Kit (BIOFACT, Korea). After the isolated RNA purity assessment, DNase I (Sinaclon) was employed to prevent potential contamination with genomic DNA. The first-strand cDNA was synthesized by the BioFACT™ 5 × RT Pre-Mix Kit (BIOFACT, Korea). Designation and synthesis of HSP70 forward and reverse primers (CCCGCCTACTTCAACGACT and CGTCGTCGATCGTCAGGA, respectively) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as the internal reference, forward and reverse primers (CAGAACATCATCCCAGCCTCC and TTGGCAGGTTTCTCAAGACGG, respectively) were performed by AlleleID 7.6 software (PREMIER Biosoft) and metabion (Germany). Finally, the real-time PCR was performed using the BioFACT™ 2 × Real-Time PCR Master Mix Kit (BIOFACT, South Korea) and StepOnePlus™ Real-Time PCR detection System (Applied Biosystems), and relative fold change was calculated using the $2^{-\Delta\Delta Ct}$ method.[32]

## Real-time PCR for miR-223a expression

The level of HSP70 expression was determined using real-time PCR. Total RNA was extracted from the samples using the Total RNA Prep Kit (BIOFACT, Korea). After assessing RNA purity, DNase I (Sinaclon) was utilized to prevent potential genomic DNA contamination. First-strand cDNA synthesis was performed using the BioFact™ 5 × RT Pre-Mix Kit (BIOFACT, Korea). The HSP70 forward and reverse primers (CCCGCCTACTTCAACGACT and CGTCGTCGATCGTCAGGA, respectively) and GAPDH forward and reverse primers (CAGAACATCATCCCAGCCTCC and TTGGCAGGTTTCTCAAGACGG, respectively) were designed and synthesized using the AlleleID 7.6 software (Premier Biosoft) and metabion (Germany). Finally, real-time PCR was performed using the BioFACT™ 2 × Real-Time PCR Master Mix Kit (BIOFACT, South Korea) and the StepOnePlus™ Real-Time PCR Detection System (Applied Biosystems). The relative fold change was calculated using the $2^{-\Delta\Delta Ct}$ method.[33]

## Immunohistochemistry (IHC) staining

The level of HSP70 protein was assessed using IHC. After sacrificing the mice, liver samples were fixed in 10% natural buffered formalin for 24 hours and processed using Autotechnicon. The paraffin-embedded samples were then cut into 4-µm sections, deparaffinized, and rehydrated before being placed in Tris-buffered saline (TBS) 1× (T5912, Sigma) in a microwave. Membrane permeabilization was achieved using 3% Triton (Sigma, T8787), and 10% goat serum (Sigma, G9023) was used to block the secondary antibody reaction to endogenous mouse IgG. Diluted primary antibody (1:100, Rabbit, Biorbyte, orb556728) and secondary antibody (1:150, Goat, Biorbyte, orb688925) were added, and the slides were incubated for 24 hours at 2–8°C and for 1 hour and 30 minutes at 37°C in the dark, respectively. 4',6-Diamidino-2-phenylindole (DAPI) (D9542, Sigma) was applied for nuclei staining, and fluorescence imaging was performed using an Olympus microscope.[34]

## Western blot analysis

The HSP70 protein expression was determined by the Western blot test. The liver tissue samples were lysed using radioimmunoprecipitation assay (RIPA) lysis buffer, and the proteins were separated by electrophoresis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel based on their molecular mass. The proteins were then transferred to a polyvinylidene difluoride fluoride (PVDF) membrane. After blocking with a solution containing skim milk, Tween-20, water, and glycerol, the samples were incubated overnight at 4°C with the Santa Cruz Biotechnology HSP70 antibody (Santa Cruz, sc-32239), followed by incubation with the secondary

antibody (Santa Cruz, sc-516102) for 2 hours at room temperature (from mice). The band intensity of GAPDH was used as the internal control. Finally, the protein bands were detected using an enhanced chemiluminescence (ECL) Western blotting detection system (GE Amersham, UK), and the densitometry of the protein bands was evaluated using ImageJ software (NIH, MD, USA).[35]

### Statistical methods

In this study, statistical analyses were performed using Statistical Package for the Social Sciences (SPSS), version 22. Variance analysis was performed by the one-way analysis of variance (ANOVA) test, and the data were presented as the mean ± standard deviation (SD). *P* values of ≤0.05 were considered statistically significant.

## Result

### Serum irisin

The serum concentration of irisin was assessed using ELISA [Figure 1]. The levels of irisin were notably higher in both the endurance training and irisin injection groups compared with the control group ($P < 0.05$ in both cases), with the highest concentration observed in the irisin group. However, there was no significant difference between the exercise and irisin groups ($P > 0.05$).

### HSP70 gene expression

This study utilized real-time PCR to determine the expression of HSP70 gene in response to endurance exercise or irisin injection. The results indicated a significant upregulation of HSP70 gene expression in both the exercise and irisin groups compared with the control group ($P < 0.001$ for both cases). Notably, the relative expression of HSP70 was higher in the irisin training group compared with the endurance group, and this difference was statistically significant ($P < 0.01$, as shown in Figure 2).

### HSP70 protein production

The concentration of HSP70 protein in the liver tissue of mice was evaluated using IHC staining and Western blot analysis (Figures 3 and 4, respectively). The IHC results indicated that the level of HSP70 protein was significantly elevated in the exercise and irisin groups as compared to the control group ($P < 0.001$ in both cases). The maximum protein level was observed in the mice that received irisin, which was significant when compared to the exercise group ($P < 0.05$).

The Western blot analysis confirmed the results of the IHC test. As shown in Figure 4, the level of HSP70 protein was significantly higher in all treated groups than in the control group ($P < 0.01$ in both cases). Additionally, there was a significant difference between the exercise group and the exogenous irisin injection group ($P < 0.05$), and mice in the irisin group exhibited higher levels of HSP70 protein than those in the exercise group. Overall, the IHC [Figure 3] and Western blot [Figure 4] results demonstrated that both endurance exercise and exogenous irisin injection increased the expression of HSP70 protein, with the highest level observed in the irisin group.

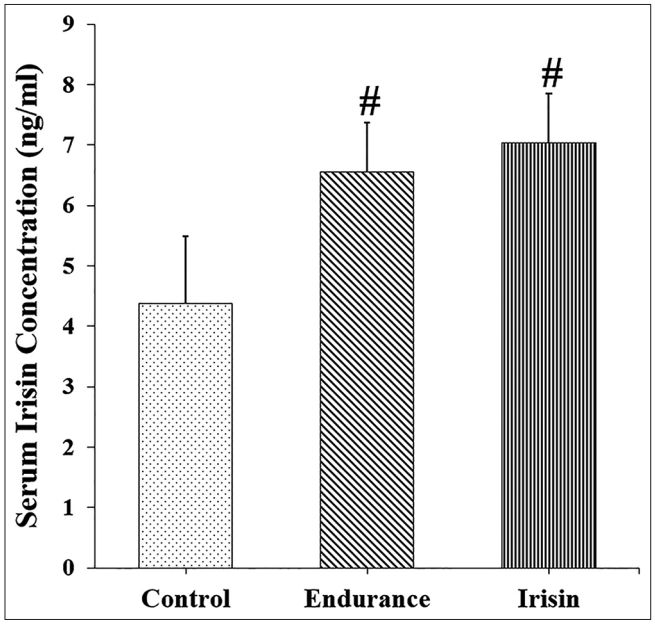


**Figure 1: Irisin level in the mouse serum. The concentration of irisin was significantly higher in the endurance and irisin groups as compared to the control group (*P* < 0.05). The mice in the irisin group showed the highest serum irisin level. There was no statistically significant difference between the endurance and irisin groups (*P* > 0.05). Data were presented as mean ± SD, and *P* < 0.05 was considered statistically significant**

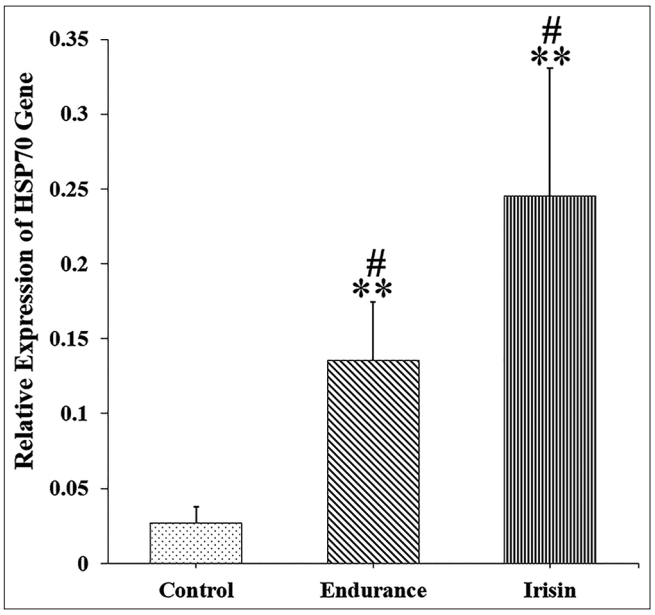


**Figure 2: Expression of the HSP70 gene in the liver of mice was evaluated using real-time PCR. The results showed a significant increase in the expression levels of the HSP70 gene in both the endurance training and irisin groups, as compared to the control group (#*P* < 0.001). Furthermore, the mice in the irisin group exhibited significantly higher levels of HSP70 gene expression when compared to the exercise group (***P* < 0.01). The data were presented as mean ± standard deviation (SD), and a *P* value of less than 0.05 was considered statistically significant**

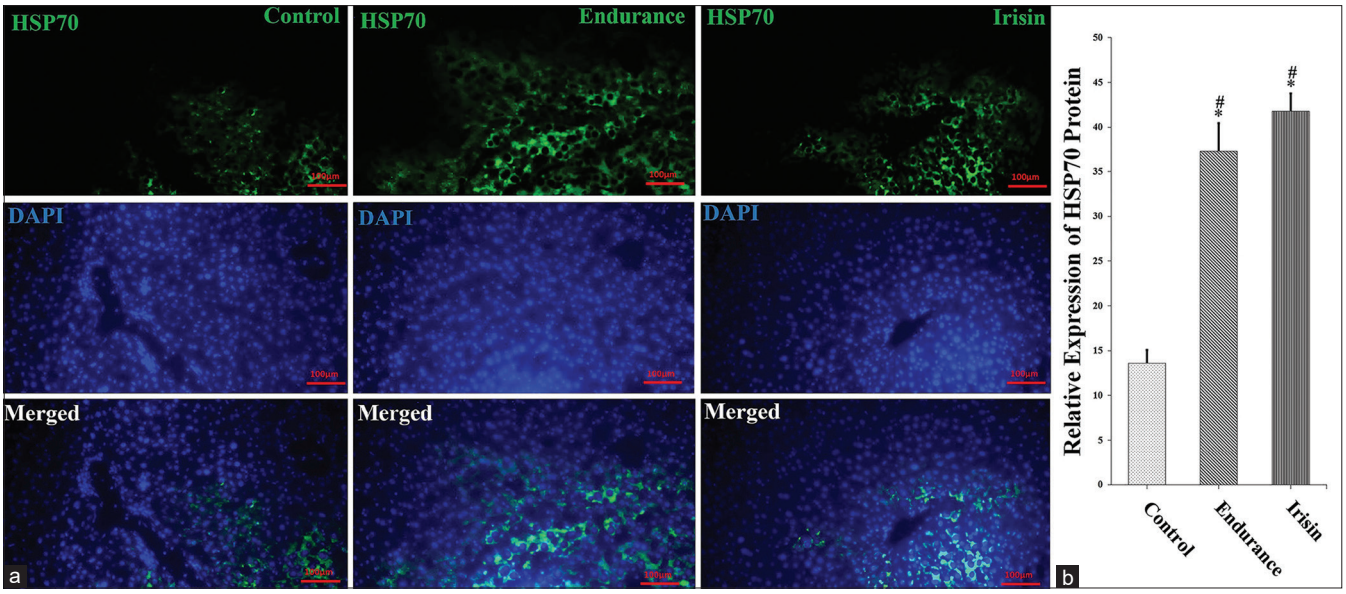


**Figure 3: HSP70 protein expression in the mouse liver was evaluated using IHC. (a) IHC images showed HSP70 protein in the liver. (b) Quantitative analysis of HSP70 protein expression revealed that it was significantly increased in all treated groups compared with the control group (#*P* < 0.001). The irisin group had significantly higher levels of HSP70 protein compared with the endurance exercise group (**P* < 0.05). Data were presented as mean ± SD, and a *P* value of less than 0.05 was considered statistically significant**

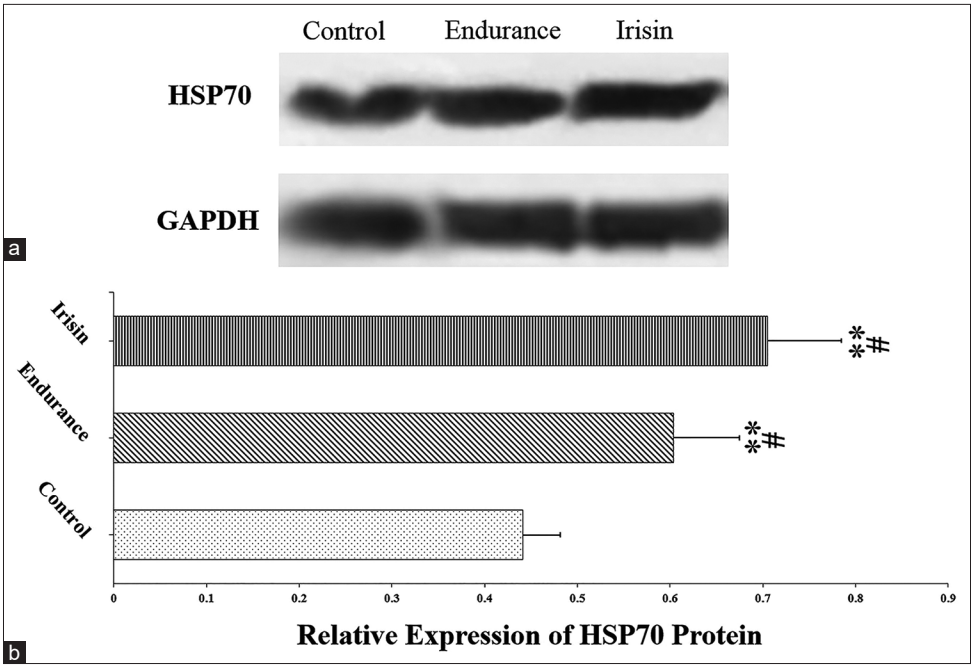


**Figure 4: Expression of HSP70 protein in the liver was evaluated by the Western blot analysis. (a) HSP70 protein bands are shown in the Western blot. (b) Quantitative analysis of HSP70 protein expression revealed a significant increase in the endurance exercise and irisin groups compared with the control group (#*P* < 0.01, in both cases). Moreover, there was a significant difference observed among the treatment groups (***P* < 0.05). The data were presented as mean ± SD, and a *P* value of less than 0.05 was considered statistically significant**

## miR-223a expression

The expression of miR-223a was determined using the real-time PCR method. The findings showed that miR-223a expression was significantly reduced in the endurance and irisin groups when compared to the control group ($P < 0.01$) and there was a significant difference between the irisin and endurance groups ($P < 0.05$). Furthermore, the lowest level of miR-223a expression was observed in mice that received exogenous irisin [Figure 5].

## Discussion

Irisin, a hormone discovered in the skeletal muscle of mice in 2012, serves as a key factor in the advantages gained from exercise. Its primary release follows exercise

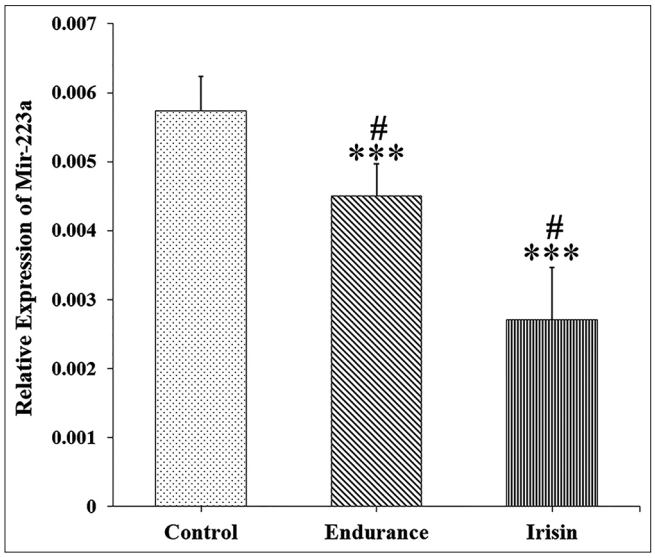


**Figure 5: miR-223a expression in the mouse liver. miR-223a expression was significantly decreased in all treated groups compared with the control group (#*P* < 0.01). Additionally, there was a significant difference between the endurance and irisin groups (****P* < 0.001). Data were presented as mean ± SD, and *P* < 0.05 was considered statistically significant**

through the cleavage of FNDC5. Studies conducted *in vitro* have indicated that irisin holds the capacity to regulate OS and influence HSP70 expression.[24-26] OS can cause the accumulation of misfolded proteins in hepatic cells, potentially contributing to the development of hepatic diseases.[1,2,6] Chaperone proteins, such as HSPs, play a crucial role in supporting cell survival against various cellular stressors by aiding in the refolding or degradation of misfolded proteins. Under stressful conditions, HSP70 serves as a primary cellular sensor for OS, significantly increasing its ability to prevent excessive cell injury caused by free radicals.[7-9,36] The expression of HSPs can be regulated by miRNAs, with miR-223 targeting HSP70, potentially affecting the liver's capacity to cope with stress. While some studies have evaluated the effect of long-term aerobic exercise on miR-223 and HSP70 expression separately, none have concurrently assessed the levels of HSP70 and miR-223 in liver tissue. This investigation compares the expression of the HSP70 gene and protein, miR-223, and serum irisin levels following 8 weeks of endurance exercise and exogenous irisin administration, 72 hours after the final exercise session or irisin injection.

As illustrated in Figure 1, this study demonstrates that irisin levels were significantly elevated in both the endurance training and irisin groups in comparison with the control group ($P < 0.05$ in both cases). Furthermore, subjects who received irisin injections had higher levels of irisin compared with those who underwent endurance training, albeit without statistical significance ($P > 0.05$). The observed rise in blood irisin levels following endurance exercise or irisin injection aligns with previous research findings.[29]

In the present study, endurance training significantly enhanced the expression of both the HSP70 gene and protein compared with the control group ($P < 0.5$). According to Campisi *et al*.,[37] active rats exposed to mobile running wheels for 8 weeks exhibited greater and faster HSP72 responses in various tissues, including the brain, adrenal gland, liver, spleen, and heart, compared with sedentary rats exposed to locked, immobile wheels for 8 weeks, following exposure to inescapable tail-shock stress (100 1.6-mA tail shocks, five-second duration, and 60-second intertrial interval) and/or exhaustive exercise stress (treadmill running until exhaustion). Similarly, Mikami *et al*.[38] reported that a training program consisting of 60 minutes of treadmill running 5 days per week for 4 weeks significantly increased liver HSP70 levels by 4.5 times compared with sedentary mice. Similar results have been reported following exposure to some stressors induced after the last exercise bout.

It is worth noting that some studies have reported contradictory findings. Sharifi *et al*.[39] found that 12 weeks of moderate- and high-intensity combined training (resistance training + aerobic interval) significantly decreased serum levels of HSP70 in women with type 2 diabetes. In contrast, Min *et al*.[40] observed that nasal expression levels of HSP70 remained unchanged after exercise in nonathlete subjects who ran for 30 minutes at 70–80% of their maximal heart rate, while HSP70 expression declined markedly in professional volleyball players. These inconsistencies may be attributed to factors such as the use of healthy or unhealthy subjects (e.g., diabetics), different species (e.g., mice or rats), different tissues, or varying exercise protocols.

In the present study, it was observed that the group receiving irisin injections exhibited a significant increase in the expression of both the HSP70 gene and protein compared with the control group ($P < 0.5$). Notably, the expression of the HSP70 gene and protein was found to be statistically higher in the irisin injection group than in the endurance exercise group ($P < 0.5$). These findings suggest that the administration of irisin, as an exercise mediator, not only mimics the molecular effects of long-term endurance exercise but also engages in these molecular production processes to a greater extent than exercise training. As previously noted, the injection of exogenous irisin leads to a higher increase in serum irisin concentration compared with long-term endurance exercises, which may account for the higher expression of the HSP70 gene and protein in the irisin group compared with the endurance group.

It has been demonstrated that miRNAs can regulate the expression of HSPs, and miR-223 has been identified as a potential target for HSP70 regulation (https://mirtarbase.cuhk.edu.cn/~miRTarBase/miRTarBase_2022/php/detail.php?mirtid=MIRT100353 and https://www.mirbase.org/cgi-bin/mirna_entry.pl?acc=hsa-mir-223). Therefore, it can be

expected that miR-223a would lead to a reduction in HSP70 expression.[13] In a study conducted by Nielsen *et al*.,[41] it was demonstrated that acute aerobic exercise significantly increased circulating miR-223 expression. This finding is consistent with the results of a study by Qu H *et al*., which showed that long-term endurance exercise significantly upregulated miR-223 expression in the hippocampus of a chronic unpredictable mild stress (CUMS) depression model.[42]

In contrast, this study revealed a significant reduction in miR-223a expression in the endurance training and irisin injection groups, compared with the control group ($P < 0.05$ in both cases). miRNAs function as posttranscriptional regulators of gene expression by silencing RNA.[13] There have not been any studies looking into how miR-223 changes in liver tissue after long-term endurance exercise or receiving irisin injections. Changes in miRNA profiles have been noticed in different liver-related conditions. These changes in miRNA expression seem to be linked with OS and how HSPs are expressed in liver tissue.[13-16] The ELISA results showed an increased serum irisin concentration following endurance exercise or irisin injection, and previous studies have demonstrated that irisin interacts with several miRNAs, including miR-135a-5P, microRNA-19b, miR-199a, and microRNA-18a, and their targets.[16,43-45] Therefore, the findings of this study may provide insight into how miR-223a modulates the protein expression and gene regulation of HSP70 in response to irisin, although further detailed research is required. Furthermore, this investigation indicated that miRNA expression was significantly lower in the irisin injection group compared with the endurance exercise group ($P < 0.5$). As previously mentioned, the elevated serum irisin concentration in the irisin group may account for this difference.

## Conclusions

Based on our study and what other research studies have found, it can be inferred that irisin has the capacity to replicate several advantageous molecular effects of prolonged endurance exercise, exhibiting even greater efficacy in liver tissue. Thus, utilizing irisin as a surrogate for physical activity in situations where exercise is not practical may represent a promising strategy for enhancing liver health.

### Acknowledgments

The authors would like to thank all the participants in the study.

### Ethics approval

The current study was performed in accordance with the Research Ethics Committees of the University of Isfahan guidelines and principles set out for the Care and Use of Laboratory Animals (Isfahan, Iran, IR.UI.REC.1401.042).

### Financial support and sponsorship

Nil.

### Conflicts of interest

There are no conflicts of interest.

**Received:** 06 May 23 **Accepted:** 20 Feb 24
**Published:** 28 Nov 24

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