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The Effects of Morning Versus Evening Aerobic Exercise Training on Proteins Associated with Browning of Visceral Adipose Tissue in a Diabetic Mouse Model

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Abstract

Introduction: Converting white adipose tissue to brown-like fat (browning) has shown promise in reducing diabetes-associated complications. This study aimed to determine the effect of aerobic exercise training time on CPT1, CLOCK, and irisin protein levels in diabetic mice.

Methods: In this experimental study, 18 male NMRI mice (8-10 weeks old, mean weight 26±2.23 g) were randomized into six groups (five animals/group): Healthy controls (light and dark phases), diabetic controls (light and dark phases), and diabetic exercise intervention groups (light and dark phases). Moderate-intensity aerobic exercise (50-60% of maximum running speed) was performed for eight weeks. Differences among experimental groups were statistically analyzed using two-way ANOVA, followed by Tukey's HSD test.

Results: Significantly elevated levels of CPT1, CLOCK, and irisin were observed in the healthy control group compared to the diabetic control group ($P=0.0001$). In the dark-phase diabetic exercise training group, CPT1 ($P=0.001$), CLOCK ($P=0.001$), and irisin ($P=0.001$) protein levels were significantly elevated compared to the diabetic control group. However, morning exercise training did not show a significant difference compared to the diabetic control group ($P>0.05$).

Conclusion: In this animal model, our results indicate that aerobic exercise undertaken during the dark phase could positively influence adipose tissue metabolism in diabetic conditions.

Keywords: Diabetes mellitus, Aerobic exercise, Circadian rhythm, Adipose tissue, Brown, FNDC5 protein, Carnitine O-Palmitoyl transferase, Clock proteins

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Introduction

The Global Burden of Disease 2021 Diabetes Collaborators forecast that approximately 1.31 billion individuals globally will be affected by diabetes by 2050. It is estimated that over 90% of affected individuals will develop type 2 diabetes mellitus (T2DM) (1). Both genetic predisposition and environmental factors contribute to the risk of developing T2DM. Although genetics contributes to diabetes, excessive body fat is recognized as a significant, independent risk factor for developing diabetes (2). Over 80% of individuals with T2DM are overweight or obese (2). Excess fat, particularly in the abdominal region, disrupts the structure and function of fat cells, leading to a condition known as "adipose tissue dysfunction." This dysfunction contributes to the development of diabetes through mechanisms such as insulin resistance, chronic inflammation, and intracellular lipid accumulation (2, 3).

Adipose tissue is categorized into three main types: white (WAT), brown (BAT), and beige adipose tissue. WAT serves as an energy storage reservoir, whereas BAT expends energy to produce heat. Beige adipose tissue exhibits characteristics of both, possessing the capacity to store and expend energy (4). Browning, the conversion of WAT to brown adipose tissue, is an adaptive response to stimuli such as cold exposure and hormonal signals. This process enhances energy expenditure and mitigates the risk of metabolic disorders, including diabetes and cardiovascular diseases (5, 6).

The enzyme carnitine palmitoyltransferase 1A (CPT1A) plays a crucial role in fatty acid oxidation within the mitochondria. Increased CPT1 activity in adipose tissue is associated with enhanced glucose metabolism and reduced inflammation (4). Irisin, a product of the FNDC5 gene, promotes browning, increasing energy expenditure and

improving insulin sensitivity (6, 7).

Adipose tissue possesses an intrinsic circadian clock driven by BMAL1 and CLOCK. This molecular machinery regulates key adipose tissue functions, including lipolysis, adipogenesis, and inflammation. Circadian rhythm disruptions can significantly affect these processes, affecting whole-body energy homeostasis (4). About 10% of genes in mouse adipose tissue show circadian rhythms, highlighting the circadian clock's importance in regulating fat tissue function (4).

Circadian rhythms affect FNDC5 gene expression and irisin levels (8). CLOCK is essential for regulating metabolism and energy use in WAT. CPT1 activity also exhibits diurnal fluctuations. Disruptions in circadian rhythms, such as irregular sleep or high-fat diets, can contribute to metabolic disorders. These disruptions alter CPT1 expression and fat metabolism in adipose tissue (9). Diurnal variations in hormone levels are associated with dietary patterns and physical activity. Perturbations in the circadian clock, including alterations in CLOCK expression, can elevate the risk of diabetes and other metabolic disorders (8, 9).

Physical activity serves as an efficacious method for enhancing insulin sensitivity and mitigating the risk of T2DM (10). Exercise stimulates the expression of FNDC5, which undergoes cleavage to form irisin. Irisin promotes the browning of white adipose tissue, converting it into beige adipocytes. These beige adipocytes exhibit increased energy expenditure and thermogenesis. This is due to the upregulation of genes such as UCP1 and CPT1, which ultimately leads to improved glucose metabolism and insulin sensitivity (10, 11).

While the precise mechanisms remain under investigation, emerging evidence suggests that exercise-induced signaling pathways, potentially involving irisin, stimulate mitochondrial biogenesis and fatty acid oxidation within adipose tissue, driving a phenotypic shift towards a more brown fat-like state (12). Exercise is known to promote white adipose tissue browning, a process with potential benefits in diabetes. However, the effect of exercise timing on this process remains largely unknown. Circadian rhythms significantly influence metabolism, suggesting that the time of day at which exercise is performed may differentially affect metabolic responses. While morning exercise training might confer benefits in terms of weight loss and insulin sensitivity, evening exercise training may better align with the body's natural rhythms, potentially improving inflammation and sleep (9).

In general, circadian rhythms regulate a wide range of physiological processes, including metabolism and energy expenditure (5). Circadian rhythms regulate adipose tissue functions, including lipolysis, adipogenesis, and inflammation. Emerging evidence suggests that the timing of exercise can significantly impact its metabolic effects. Research indicates that engaging in physical activity at different times of the day exerts distinct effects on glucose metabolism, insulin sensitivity, and gene expression in skeletal muscle (5).

Emerging evidence underscores the critical role of exercise training timing in overall health and metabolic outcomes. Exercise and nutrition are fundamental metabolic regulators that significantly influence diseases such as obesity and diabetes. Although the impact of exercise timing on systemic metabolism is gaining recognition, research on its effects on specific tissues, particularly adipose tissue, remains limited. To develop effective treatments for metabolic diseases, researchers must understand how different tissues respond to exercise at various times. This study investigated the effects of exercise training timing on white adipose tissue browning in a diabetic animal model. Researchers have examined the expression of key genes, including irisin, CPT1, and CLOCK. The hypothesis posited that aerobic exercise during the dark phase would elicit greater changes in the expression of genes associated with brown adipose tissue activation (such as irisin and CPT1) than exercise during the light phase.

Methods

Eighteen male NMRI mice aged 8–10 weeks and weighing 26 ± 3 g were procured from the Animal Breeding Center at Jundishapur University of Medical Sciences in Ahvaz, Iran. To induce diabetes, the animals were fed a high-fat diet (HFD) for five weeks, formulated to provide 60% of the total caloric intake from fat (Royan Company, Iran). This dietary intervention was followed by a single intraperitoneal injection of streptozotocin (STZ) at a dose of 20 mg/kg body weight.STZ primarily induces diabetes by selectively targeting and destroying pancreatic beta cells. This occurs through a multi-step process: STZ, which contains a glucose-like moiety, is readily taken up by beta cells via the GLUT2 transporter. Once inside, STZ exerts cytotoxic effects, including DNA damage, oxidative stress, and energy depletion, ultimately leading to β-cell death. This results in insulin deficiency and subsequent hyperglycemia, characteristic of diabetes (13).

Researchers diagnosed diabetes in mice five days after STZ injection if their fasting blood glucose levels exceeded 6.5 mmol/L. Blood glucose levels were measured using a GALA glucometer (Taiwan) using samples collected from the tail vein. Animals were randomly assigned to six groups: three groups exercised in the early light phase (ZT3, 9:00 AM) and three groups exercised in the early dark phase (ZT15, 9:00 PM). Each time point included a Healthy Control (HC), a Diabetic Control (DC), and a Diabetic + Exercise Training (DE) group (n = 5/group). The DE groups underwent 60-80 minutes of aerobic exercise, while the HC and DC groups were placed on a stationary treadmill at both time points as a sham-exercise control. The code of ethics for this research is EE/1401.2.24.173079/scu.ac.ir.

Exercise Performance Test

The exercise performance was evaluated through a treadmill running protocol, adapted from previously published studies (14). The progression of aerobic

capacity in diabetic mice was evaluated through a maximal running speed (Vmax) test during the fourth and eighth weeks of the eight-week exercise training period. The experimental protocol initiated with a 5-minute warm-up phase conducted at a speed of 6 m/min. Subsequently, the running speed was incrementally advanced by 2 m/min at 2-minute intervals. This progressive increase continued until the animal exhibited signs of volitional exhaustion. We recorded the final speed as Vmax (14, 15). In the following weeks, we adjusted the exercise intensity based on Vmax measured in the fourth week.

Protocol for Moderate-Intensity Aerobic Exercise Training

After confirmation of diabetes, two Exercise Training groups were formed: One group performed aerobic exercise training in the morning (9:00 am), and the other group performed exercise training in the evening (9:00 pm). The exercise training protocol was implemented over 8 weeks, consisting of five sessions per week, each lasting 80 minutes at a moderate intensity corresponding to 50–60% of the maximum running velocity (Vmax) (Table 1) (14, 15). The body weight and blood glucose levels of the mice were measured weekly.

Tissue Collection and Preparation

At the end of the eight-week exercise training intervention, all mice were brought to the laboratory for sample collection. Following the induction of general anesthesia through intraperitoneal administration of ketamine (100 mg/kg) and xylazine (10 mg/kg), surgical procedures were carried out to collect tissue samples. Blood samples were collected by cardiac perfusion. After centrifugation, blood serum was separated and stored at -20°C for biochemical analysis, including the measurement of glucose and other relevant biomarkers. Visceral adipose tissue, the target tissue in this study, was carefully removed from the abdominal cavity. For molecular analyses, samples were rapidly frozen in liquid nitrogen and stored at -80°C, and protein content was subsequently analyzed using Western blot and ELISA (16). Given the crucial role of circadian rhythms in the regulation of metabolic processes, the timing of sample collection from animals in both Exercise Training groups (morning and evening) was precisely matched to the Exercise Training period during the intervention phase. This approach allows detailed investigation of the biological changes associated with the time of day and their interaction with exercise training (16).

Table 1. Weekly Progression of Speed and Duration

Week	Speed (m/min)	Duration (min)
Week 1	8	60
Week 2	9	60
Week 3	10	65
Week 4	10	65
Week 5	11	70
Week 6	11	75
Week 7	12	80
Week 8	12	80

m/min = Meters per minute; min = Minutes.

Western Blot Analysis of CPT1 and CLOCK

Adipose tissue samples containing CPT1 and CLOCK proteins were lysed in lysis buffer and homogenized to release the proteins. The resulting protein mixture was separated by size on a polyacrylamide gel (SDS-PAGE) under an electric field. Owing to their specific molecular weights, the CPT1 and CLOCK proteins migrated to different positions in the gel. Following separation, the proteins were immobilized onto nitrocellulose or PVDF membranes to enable downstream analysis. Membranes were blocked to prevent non-specific antibody binding. The membrane was then incubated with a primary antibody specific for CPT1, which specifically binds to the CPT1 protein. The membrane was washed and incubated with a primary antibody specific for CLOCK. After washing, the membrane was incubated with a secondary antibody bound to the primary antibody. The secondary antibody is usually conjugated to an enzyme or fluorophore. Using a chemiluminescent substrate or a fluorescence detection system, the positions of CPT1 (Santa Cruz Biotechnology, sc-393070) and CLOCK (Santa Cruz Biotechnology, D45B10) proteins on the membrane were visualized and recorded with an imaging system. The intensity of the bands corresponding to CPT1 and CLOCK indicates the amount of protein in the sample (17, 18).

ELISA Assay for the Measurement of Irisin

Irisin levels in adipose tissue samples were measured using a commercially available ELISA kit (Cat Number: SL0837Mo, Sunlong Bioteck China). Adipose tissue specimens were homogenized using a Heidolph homogenizer (Germany) in 250 µL of RIPA lysis buffer. This buffer comprised 150 mM NaCl, 0.1% SDS, 25 mM Tris (pH 7.4), 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 50 mM sodium fluoride, and a protease inhibitor cocktail, with all reagents obtained from Sigma-Aldrich. The homogenized samples were centrifuged at 10,000 RPM and at a temperature of 4°C for 15 minutes. The resulting clear supernatants were precisely collected, aliquoted, and the samples were promptly stored at −70°C until further analysis. Protein levels were quantified using the Bradford assay. Samples and irisin standards of known concentrations were then added to the wells of a microplate coated with a primary antibody specific for irisin. After incubation at the optimal temperature and rigorous washing, a horseradish peroxidase (HRP)-conjugated secondary antibody was applied to the wells. This antibody binds to the primary antibody, irisin. After washing again, the TMB substrate was added to the wells. This substrate reacts with HRP and produces a blue color. An acidic solution was added to stop the reaction, causing the color to turn yellow. The optical density was measured at a wavelength of 450 nm using a microplate reader. Using a standard curve generated from different irisin concentrations, the irisin concentration in the unknown samples was determined. For this purpose,

the optical density of the samples was compared with the standard curve, and the irisin concentration was calculated by taking into account the sample dilution factor.

Statistical Analysis

GraphPad Prism and SPSS statistical software were used to analyze the data. After assessing the normality of the data distribution using the Shapiro-Wilk test and testing the homogeneity of variances with Levene's test, the group means were compared using a two-way ANOVA, followed by Tukey's Honestly Significant Difference (HSD) post-hoc test. All data are expressed as mean ± standard deviation, and the significance level was set at 0.05.

Results

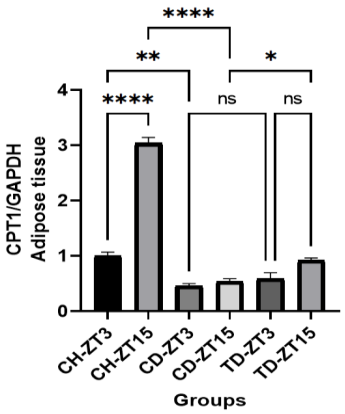
Analysis of variance revealed significant differences across groups in maximum running speed ($F = 40.950$, $P < 0.0001$), glucose levels ($F = 22.217$, $P < 0.0001$), and weight ($F = 5.480$, $P = 0.008$). Post-hoc analysis (Table 2) revealed significantly higher glucose ($P = 0.0064$) and weight ($P = 0.0075$) values in the diabetic mice than in the healthy control group. Aerobic exercise training significantly reduced glucose ($P = 0.0031$) and weight ($P = 0.007$) levels in the exercise training groups compared to those in the diabetic group. However, there was no significant difference in glucose levels between the light and dark phases in any of the groups ($P = 0.4140$). The evaluation of maximum running speed showed a significant increase in the exercise training groups compared to the other groups ($P = 0.0087$). There was no significant difference in the maximum running speed between the light and dark phases ($P = 0.9996$).

For CPT1 expression (Figure 1, Table 3), a significant interaction between exercise training status and circadian time was observed ($F = 192.8$, $P < 0.0001$). Consequently, the results are presented based on subgroup comparisons. In the healthy control group, CPT1 levels were significantly elevated during the dark phase (CH-ZT15) compared to the light phase (CH-ZT3) ($P < 0.0001$). In the diabetic control groups, CPT1 expression was significantly reduced relative to the healthy controls at both time points ($P < 0.01$), with no significant difference between ZT3 and ZT15 (ns). In diabetic mice subjected to exercise training, CPT1 levels were increased compared to the diabetic controls; however, this increase achieved statistical significance only in the

Table 2. Comparison of mean values of assessed variables post-intervention across different groups

Groups	Maximum speed m/min	Glucose mg/dL	Weight g
CH-ZT3	15.16 ± 1.04^{b}	101.18 ± 4.10^{e}	33.00 ± 3.60^{b}
CH-ZT15	15.50 ± 1.32^{b}	116.40 ± 4.22^{d}	34.00 ± 2.64^{b}
CD-ZT3	14.83 ± 1.60^{b}	143.58 ± 5.09^{b}	33.41 ± 1.52^{a}
CD-ZT15	15.60 ± 0.91^{b}	157.33 ± 5.47^{a}	40.66 ± 3.05^{a}
TD-ZT3	24.66 ± 1.15^{a}	143.20 ± 5.33^{b}	34.33 ± 1.52^{b}
TD-ZT15	24.80 ± 1.70^{a}	127.40 ± 3.36^{c}	36.33 ± 1.52^{b}

Significant differences were found between the groups in Tukey's HSD post-hoc test. Statistical significance was set at $P < 0.05$. Different superscript letters above each column indicate that the mean of that column differs significantly from those of the other columns. For instance, in the Weight column in Table 2, if values are marked with different letters such as 'a' and 'b', it signifies that the means for those groups are statistically significantly different from each other. Conversely, if values share the same letter (e.g., 'a' and 'a', or 'b' and 'b'), it indicates no statistically significant difference between the means of those groups. CH: Control Healthy group, CD: Control Diabetic group, TD: Exercise Training Diabetic group, ZT3: Light phase, ZT15: Dark phase, m/min = Meters per minute, mg/dl = Milligrams per deciliter, g = Grams.



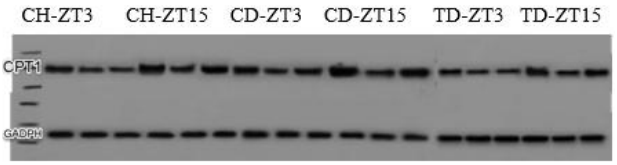


Figure 1. CPT1 protein levels in adipose tissue of different groups. Data are presented as mean ± standard deviation. Pairwise comparisons between bars indicate significant differences at the $P < 0.05$ level. CH-ZT3: Healthy mice in the light phase; CH-ZT15: Healthy mice in the dark phase; CD-ZT3: Diabetic mice in the light phase; CD-ZT15: Diabetic mice in the dark phase; TD-ZT3: Diabetic mice exercised in the light phase; TD-ZT15: Diabetic mice exercised in the dark phase. Stars above each bar indicate a significant difference between that group and the other groups. The number of stars indicates the level of significance (more stars indicate more significant differences).

Table 3. Two-way analysis of variance (ANOVA) related to the measured parameters in adipose tissue

	Training	F	ss	F	sig
CPT1	Time	2	7.969	108.6	$P < 0.0001$
	Training*time	1	2.995	256.5	$P < 0.0001$
	Residual	2	3.373	192.8	$P < 0.0001$
	Training	12	0.01553	-	-
Clock	Time	2	1.185	54.58	$P < 0.0001$
	Training*time	1	0.02004	1.846	$P = 0.1992$
	Residual	2	0.01877	0.8642	$P = 0.4460$
	Training	12	0.1303	-	-
Irisin	Time	2	7646	162.0	$P < 0.0001$
	Training*time	1	1930	81.83	$P < 0.0001$
	Residual	2	603.3	12.79	$P = 0.0011$
	Training	12	283.1		

Data were analyzed by two-way ANOVA to examine the effects of Training, Time, and their interaction (Training × Time) on CPT1, Clock, and Irisin protein levels in visceral adipose tissue. Statistical significance was set at $P < 0.05$. Abbreviations used include ANOVA (Analysis of Variance), SS (Sum of Squares), df (Degrees of Freedom), F (F-statistic), CPT1 (Carnitine Palmitoyltransferase 1), Clock (Circadian Locomotor Output Cycles Kaput), and Irisin (Exercise-induced myokine).

dark-phase exercise training group (TD-ZT15) ($P<0.05$). No significant difference was observed between the light- and dark-phase training groups (ns). These findings suggest that exercise training during the dark phase most effectively restores CPT1 expression in diabetic mice.

Regarding CLOCK protein levels (Figure 2, Table 3), the interaction between exercise training and time was not statistically significant ($F=0.8642$, $P=0.446$). Therefore, the main effect of training can be assessed. CLOCK expression was significantly elevated in healthy controls compared to diabetic controls ($P<0.001$). Among diabetic mice, exercise training during the dark phase (TD-ZT15), but not during the light phase (TD-ZT3), resulted in a significant increase in CLOCK expression relative to diabetic controls ($p<0.05$ vs. ns). No significant difference was observed between TD-ZT3 and TD-ZT15 ($P>0.05$).

For Irisin levels (Figure 3, Table 3), a significant interaction between exercise training and time was identified ($F=12.79$, $P=0.0011$). In healthy controls, Irisin levels were significantly elevated during the dark phase (CH-ZT15) compared to during the light phase (CH-ZT3) ($P<0.0001$). Conversely, diabetic controls exhibited substantially lower Irisin levels than healthy controls at both time points ($P<0.0001$). Among diabetic mice subjected to training, Irisin levels were significantly increased only in the dark-phase training group (TD-ZT15) compared to the diabetic controls ($P<0.001$). Furthermore, a pronounced difference was observed between the light- and dark-phase training groups, with higher values in TD-ZT15 ($P<0.0001$). These findings clearly indicate that dark-phase exercise training exerts the most potent stimulatory effect on Irisin expression in diabetic adipose tissue.

Discussion

The present study demonstrated that the concentrations of CLOCK, irisin, and CPT1 proteins in adipose tissue were lower in the diabetic cohort than in the healthy control group. This observation aligns with the findings of Ertuna et al. (2023), Al-Arideh et al. (2020), and Montazerifar et al. (2022) (19-21).

Studies have indicated that individuals with diabetes exhibit significantly lower irisin levels than healthy controls. This reduction in irisin levels is associated with multiple metabolic factors, including increased waist circumference, elevated triglycerides, oxidative stress, higher nitrate levels, and elevated fasting blood glucose (21, 22). Lower irisin levels may lead to diminished browning of visceral adipose tissue in individuals with diabetes (23). Our study showed that moderate-intensity aerobic exercise increased irisin protein expression in visceral adipose tissue. This effect is likely due to heightened irisin secretion during physical activity, which activates browning genes in WAT via the p38 MAPK and ERK signaling pathways. Higher irisin levels in visceral adipocytes boost UCP-1 expression, enhance mitochondrial energy metabolism, and promote thermogenesis, leading to browning and anti-inflammatory effects in WAT (6, 24).

The observed increase in CPT1 protein levels in the exercise training group during the dark phase, compared with the control group, can be attributed to several factors. First, the increased energy demands associated with physical activity drive the body to utilize stored energy reserves. Fatty acid oxidation is the primary pathway for energy production, and CPT1 plays a crucial role in this process. CPT1 facilitates β-oxidation and the subsequent synthesis of ATP by promoting the movement of long-chain fatty acids from the cytosol into the mitochondria.

Second, the increased energy demands during exercise

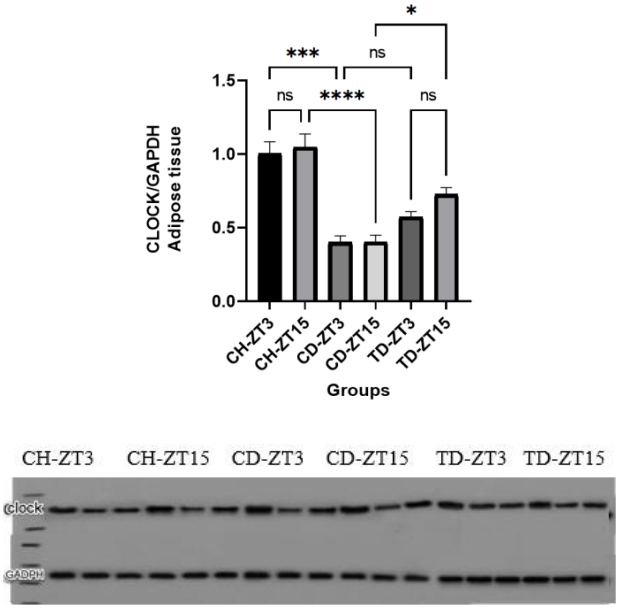


Figure 2. Comparison of CLOCK protein levels in adipose tissue among various groups. Data are reported as mean ±standard deviation. Pairwise comparisons between bars indicate significant differences at the $P<0.05$ level. CH-ZT3: Healthy mice in the light phase; CH-ZT15: Healthy mice in the dark phase; CD-ZT3: Diabetic mice in the light phase; CD-ZT15: Diabetic mice in the dark phase; TD-ZT3: Diabetic mice exercised in the light phase; TD-ZT15: Diabetic mice exercised in the dark phase. Stars above each bar indicate a significant difference between that group and the other groups. The number of stars indicates the level of significance (more stars indicate more significant differences).

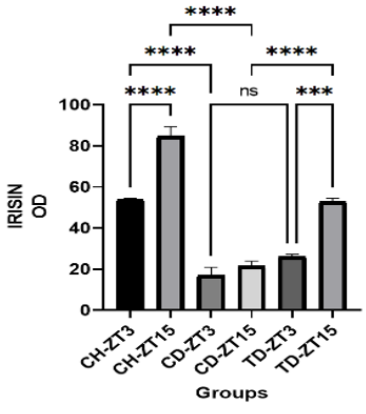


Figure 3. Irisin protein levels (pg/ml) in fat tissue for each group. Data are reported as the mean ±standard deviation. Pairwise comparisons between bars indicate significant differences at the $P<0.05$ level. CH-ZT3: Healthy mice in the light phase; CH-ZT15: Healthy mice in the dark phase; CD-ZT3: Diabetic mice in the light phase; CD-ZT15: Diabetic mice in the dark phase; TD-ZT3: Diabetic mice exercised in the light phase; TD-ZT15: Diabetic mice exercised in the dark phase. Stars above each bar indicate a significant difference between that group and the other groups. The number of stars indicates the level of significance (more stars indicate more significant differences).

stimulate CPT1 gene expression, leading to increased synthesis of this protein (25). Consequently, the cell's capacity for fatty acid oxidation increases, and fat storage is depleted to meet the energy demands of physical activity (26).

On the other hand, exercise training activates AMP-activated protein kinase (AMPK), which increases CPT1 expression and improves fatty acid oxidation while preventing lipogenesis (27, 28). Hormones affect how the body breaks down fats. Leptin, a hormone made by fat tissue, helps control weight by increasing the production of CPT1 and the breakdown of fatty acids. Irisin, a hormone released during exercise, also improves how the body uses insulin and breaks down fats, which is supported by the findings of our research (29). Additionally, physical activity promotes mitochondrial biogenesis. This process, in which PGC-1α plays a key regulatory role, increases PGC-1α expression. The upregulation of PGC-1α promotes mitochondrial biogenesis and elevates CPT1 expression, thereby enhancing the cellular capacity for fatty acid oxidation (29). In conclusion, physical activity regulates fatty acid oxidation and body weight through complex mechanisms, including AMPK activation, hormonal changes, and mitochondrial biogenesis. This study demonstrates that aerobic exercise, particularly in the evening, enhances clock gene expression in adipose tissue. This finding supports existing evidence that exercise can restore disrupted circadian rhythms, notably in individuals with diabetes. As a key component of the body's internal clock, the CLOCK gene is crucial for maintaining metabolic balance. By synchronizing peripheral clocks, especially in adipose tissue, exercise-induced Clock expression may offer significant benefits for individuals with diabetes, who frequently exhibit circadian rhythm disruption (30, 31).

Aerobic exercise can enhance CLOCK gene expression through multiple mechanisms. Improved insulin sensitivity, positively influencing CLOCK transcription in diabetic models (31, 32), and PGC-1α-mediated mitochondrial biogenesis, known to regulate circadian rhythms (32, 33), contribute significantly. Furthermore, exercise reduces inflammation (34), creating a more favorable environment for CLOCK gene expression in adipose tissue (35). Notably, exercise-induced increases in intracellular calcium levels activate signaling pathways that enhance CLOCK expression by stimulating transcription factors involved in circadian CLOCK gene regulation (36).

This study demonstrates that the time of day significantly affects the expression of proteins, particularly irisin, with increased expression observed during evening exercise. This is influenced by the body's internal clock, which regulates 24-hour rhythms in various biological processes. The central clock, located in the hypothalamus, along with peripheral clocks in tissues such as muscle and adipose, responds to both internal and external cues, including light and exercise. Exercise, especially during the dark phase, can synchronize these tissue clocks, potentially enhancing the expression of circadian-regulated proteins involved in glucose metabolism and offering a promising approach for managing metabolic diseases such as T2DM (37). Furthermore, hormones such as cortisol and testosterone fluctuate throughout the day and can influence protein synthesis and muscle adaptation. Exercise during the dark phase, due to its alignment with natural body rhythms, optimal physiological conditions, and hormonal responses, is often recommended for optimal exercise training outcomes, including muscle protein synthesis and recovery (38).

The efficacy of aerobic exercise in modulating metabolic functions is significantly dependent on the time of day it is performed. This is due to the interplay between exercise and circadian rhythms, which regulate cellular pathways and gene expression. Exercise timing can affect the response of various metabolic markers, such as irisin, which exhibits a distinct circadian rhythm (8). Understanding these mechanisms can lead to the development of personalized exercise programs for the management of metabolic diseases, although further research is still needed.

This study has several limitations. First, the assessment of protein expression at only two time points limits our understanding of the temporal dynamics of the exercise response. More frequent sampling throughout the day is needed. Second, despite controlling for time of day, factors such as meal timing, sleep-wake cycles, and physical activity may have influenced the results. Future studies should comprehensively assess these factors. Third, the focus on adipose tissue limits our understanding of the systemic effects of time-dependent exercise. Investigations in other relevant tissues, such as liver and skeletal muscle, are warranted. Finally, future studies should explore the potential benefits of high-intensity interval training (HIIT) in modulating mitochondrial dynamics and circadian rhythms.

Conclusion

In summary, these findings have implications for future research and clinical practice. Firstly, our results suggest that exercising during the dark phase may be more beneficial for diabetes management. Further human studies are needed to determine the optimal exercise timing for individuals with diabetes. Secondly, considering individual circadian rhythms in exercise recommendations may personalize treatment and improve outcomes. Third, additional studies are required to elucidate the mechanisms through which exercise timing exerts beneficial effects on adipose tissue function and glucose metabolism, potentially paving the way for novel therapeutic strategies for diabetes management.

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Competing Interests
The authors declare no conflict of interest.

Data Availability Statement
The data underlying this article will be shared upon reasonable request to the corresponding author.

Ethical Approval
The study was approved by the Ethics Committee of Shahid Chamran University of Ahvaz (Ethical code: EE/1401.2.24.173079/scu.ac.ir).

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