Research Article

The effect of aerobic exercise and ethanolic extract of bitter orange peel on cardioprotective genes expression in female rats fed a high-fat diet

Shahrzad Haji Ali Akbar[1], Mohammad Ali Azarbayjani[1*], Shahin Riyahi Malayeri[2], Hoseyn Fatolahi[3]

Abstract

The present study evaluates the effects of aerobic exercise and ethanolic bitter orange peel extract on the expression of cardioprotective genes in female rats fed a high-fat diet (HFD). From the Islamic Azad University's Central Tehran Branch animal facility, 30 adult female rats of the Wistar strain were randomly assigned to five groups (six rats per group): 1) normal diet control (ND-C), 2) HFD control (HFD-C), 3) HFD aerobic exercise (HFD-AE), 4) HFD ethanolic bitter orange peel extract (HFD-BP), and 5) HFD aerobic exercise and ethanolic bitter orange peel extract (HFD-AE-BP). A normal diet was supplemented with 20% palm oil, 1.5% cholesterol, and 0.25 cholic acid to induce obesity. Before the intervention, the subjects received a HFD for four weeks, then continued it for another four weeks during the intervention. During the four-week aerobic exercise protocol, treadmill running was performed at a moderate intensity. An ethanol extract of bitter orange peel was administered orally to rats at a dose of 100 milligrams per kilogram of body weight for four weeks. After euthanasia, left ventricle myocardium was collected for real-time PCR analysis of CTRP9, LKB1, and AMPK gene expression. In the HFD-C, CTRP9 (P=0.001), LKB1 (P=0.001), and AMPK (P=0.001) genes were significantly lower than in the ND-C. Aerobic exercise significantly increased their expression compared with the HFD-C (P=0.001). Comparing HFD-C with ethanolic bitter orange peel extract, ethanolic bitter orange peel extract increased gene expression significantly (P=0.001). This indicates that the simultaneous use of these two interventions was able to add up the effects of each and did not have a synergistic effect. However, since the magnitude of change when these two interventions were combined was greater than the effect of each alone, the combination of AE and BP was greater than the effect of each alone, suggesting that these two interventions may be used to mitigate cardiac complications under HFD conditions.

Key Words: CTRP9, LKB1, AMPK, Aerobic exercise, Bitter orange peel

1. Department of Physical Education and Sport Sciences, CT.C., Islamic Azad University, Tehran, Iran. 2. Department of Physical Education and Sport Sciences, ET.C., Islamic Azad University, Tehran, Iran. 3. Department of Physical Education and Sport Sciences, P.C., Islamic Azad University, Pardis, Iran.

*Author for correspondence: m_azarbayjani@iauctb.ac.ir

Sh HAA: 0000-0003-3074-8653; MA A: 0000-0002-3502-7487; Sh RM: 0000-0001-6989-4821; H F: 0000-0001-8438-6534

Received 30 April 2025; Accepted 5 June 2025

Introduction

The presence of obesity is a major risk factor for structural and functional changes in the myocardium, which may lead to cardiovascular complications and increased mortality (Costantino et al., 2019). Several mechanisms have been proposed for obesity-associated cardiomyopathy, including cytokines produced by hypertrophied adipose tissue (e.g.,leptin and resistin) and triglyceride accumulation (Sharma et al., 2004). It is also possible that saturated fatty acids (FAs), such as palmitic acid, are oversupplied. As a result of unoxidized FAs in cardiac myocytes, oxidative stress and endoplasmic reticulum stress are induced, which contribute to lipotoxicity (Law et al., 2018). Oxidative stress and the endoplasmic reticulum's stress-induced apoptosis signaling pathway play a critical role in the pathogenesis of obesity-induced cardiac abnormalities (Sletten, Peterson, & Schaffer, 2018). AMP-activated protein kinase (AMPK), a key cellular energy sensor in mammalian cells, regulates energy homeostasis. The pathogenesis of obesity-induced cardiac abnormalities is linked to oxidative stress and endoplasmic reticulum stress-mediated apoptotic signaling. AMPK is phosphorylated and activated by liver kinase B1 (LKB1), one of its primary upstream kinases (Hardie, 2011). The activation of AMPK is triggered by phosphorylated LKB1 translocating to the cytosol in certain conditions. Diet-induced obese mice have reduced AMPK phosphorylation, but the mechanisms behind this reduction remain unclear (Xie, Dong, Scholz, Neumann, & Zou, 2008). An adipokine that modulates metabolic and cardiovascular functions was initially identified as C1q/TNF-related protein-9 (CTRP9), the closest paralog of adiponectin. CTRP9 is abundantly produced in the heart, indicating that it may be more important to the heart than adiponectin. There is evidence that CTRP9 reduces myocardial ischemia/reperfusion injury, reverses post-infarction remodeling, and induces vasodilation (Wong et al., 2009). Additionally, CTRP9 has been implicated in the promotion of maladaptive cardiac hypertrophy under pressure overload. Moreover, CTRP9 enhances mitochondrial biogenesis and fatty acid oxidation in muscle tissue, suggesting

a role in lipid metabolism. It has been shown that regular physical activity, particularly aerobic exercise, reduces the risk of cardiovascular disease (Y. Yang et al., 2016). An eight-week program of low-volume treadmill running (15 minutes/session) in rats with myocardial infarction significantly increased CTRP9 gene expression, which correlated with a reduction in fibrosis and mortality. It has also been reported that serum CTRP9 levels are elevated following aerobic exercise. Aerobic exercise has been shown to affect LKB1 expression in skeletal muscle, but no direct evidence exists for myocardium. Indirect evidence suggests that LKB1 may exert cardioprotective effects through kinase regulation. Conversely, aerobic exercise activates AMPK in the myocardium. Exercise increases myocardial AMPK activity through phosphorylation (Mohammadi, Rostamkhani, Riyahi Malayeri, & Shirvani, 2022), metabolic adaptation (glucose/fatty acid utilization), and cardioprotective mechanisms (anti-apoptotic signaling, mitochondrial biogenesis) (Leal, Lopes, & Batista, 2018). These responses are most beneficial for pathological cardiac conditions. A number of other pathways may contribute to the modulation of AMPK expression in medicinal plants, including those that are independent of or dependent on LKB1 (Shaito et al., 2020). CTRP9 may be directly impacted by phytochemicals, although their effects on LKB1 and AMPK are unclear. In addition to its cardioprotective potential, bitter orange peel (Citrus aurantium) also exhibits anti-oxidant activity (Benjamim et al., 2022). However, their effects on AMPK, LKB1, and CTRP9 expression in myocardial tissue are not fully understood. In spite of the fact that citrus peel compounds may influence AMPK signaling, there is no direct evidence linking them to LKB1 or CTRP9 modulation. Given the individual cardioprotective roles of aerobic exercise and bitter orange peel extract, and the lack of studies examining their combined effects, this study investigated the simultaneous impact of aerobic training and ethanolic bitter orange peel extract on AMPK, LKB1, and CTRP9 gene expression in the myocardium of female rats fed HFD.

Materials and methods

Animals

A total of 30 adult female Wistar rats were obtained from the Central Tehran Branch of Islamic Azad University for this study. There were five groups of rats (six rats each): 1) normal diet control (ND-C), 2) HFD control (HFD-C), 3) HFD aerobic exercise (HFD-AE), 4) HFD ethanolic bitter orange peel extract (HFD-BP), and 5) HFD aerobic exercise and ethanolic bitter orange peel extract (HFD-AE-BP).The animals were housed under standard conditions in transparent, autoclavable polycarbonate cages (dimensions: 15×42×26.5 cm) at a temperature of 20–22°C, 55% humidity, and a 12-hour light/dark cycle, with free access to water. The study received ethical approval from Sport Sciences Research Institute of Iran (Code: IR.SSRC.REC.1402.230). All data collection procedures adhered to the Helsinki Declaration guidelines.

High-fat diet

Standard pelleted rat chow (Behparvar Co.) was fed to rats in the normal diet control group. A normal diet was supplemented with 20% palm oil, 1.5% cholesterol, and 0.25% cholic acid to induce obesity in previous studies (Shaito et al., 2020). Before the intervention, the subjects of the HFD-C, HFD-AE, HFD-BP), and HFD-AE-BP received a HFD for four weeks, then continued it for another four weeks during the intervention. The normal diet control group received standard chow throughout the study (eight weeks).

Aerobic exercise protocol

Running on treadmills designed specifically for rats was the aerobic exercise regimen. The rats were first exposed to treadmill running for two weeks (20 minutes per day, five days per week, at 9 m/min, 0° inclined). Following familiarization, the main training protocol was implemented for four weeks. The treadmill speed increased from 16 m/min (0° incline) in the first week to 27 m/min (0° incline) by the fourth week (3.5 m/min per week). Five minutes of warm-up and five minutes of cool-down were included in each session (Nikbin et al., 2020). The total session exercise duration including warm-up, training and cool-down was 20 minutes.

Ethanolic bitter orange peel extract preparation and administration

Citrus aurantium, the scientific name for bitter orange, was obtained from reliable sources and approved by a botanist. After drying in the shade, it was powdered in a mill and prepared for extraction. Two hundred grams of the dried plant were poured into a percolator after it had been dried and ground. The extraction was performed three times with 50% ethanol in 1000 ml. For further experiments, extracts were pooled and stored in a refrigerator. Further experiments were conducted with the pooled extracts stored in a refrigerator. By drying a certain amount of liquid extract in an oven, the dry matter content could be determined. As a result, this extract had an 18% dry matter content. Rats were gavaged with 100 milligrams of bitter orange peel extract per kilogram of body weight five days a week (Han et al., 2019).

Sacrifice and heart tissue collection

After 24 hours of the final intervention (aerobic exercise or extract administration) and a 12-hour fast, the rats were anesthetized with 2.7 mL of xylazine and 10 mL of ketamine (Imalgène® 1000, Merial, Lyon, France) at a dose of 100 l/100 g body weight. After

confirming deep anesthesia and the lack of response to external stimuli, the chest cavity was opened, and blood was collected from the left ventricle. The heart was then excised, and the left ventricle was separated. Samples were homogenized in cold phosphate-buffered saline (10% w/v) for gene expression analysis.

Gene expression analysis

In order to determine the expression of CTRP9, LKB1, and AMPK genes in myocardial tissue, Real-Time PCR was employed. A set of primers was designed for each target gene. RNA was extracted from myocardial tissue and reverse transcribed into cDNA. Amplification of DNA by PCR was performed, and gene expression levels were measured. Gene expression was calculated using the $2^{-\Delta Ct}$ formula. Primer sequences used in real-time PCR presented in Table1.

Statistical analysis

Data on gene expression are expressed as mean + standard deviation. By using an independent T-test, we compared outcomes between the ND-C and the HFD-C group in order to assess whether the HFD induced obesity. The interaction effects of aerobic exercise and ethanolic bitter orange peel extract were evaluated using two-way analysis of variance (ANOVA). In this model, HFD-receiving groups were analyzed. Accordingly, the main effect of exercise, the main effect of bitter orange peel extract, and the interaction between the two were analyzed. The Bonferroni post-hoc test was used to analyze differences between groups (HFD-C, HFD-AE, HFD-BP, HFD-AE-BP). All analyses were performed at a significance level of p 0.05. The SPSS 27 software was used for all statistical calculations.

Results

CTRP9 (-64.37%, t10=6.98, P=0.001), LKB1 (-65.08, t10=4.93, P=0.001), and AMPK (-54.48, t10=3.24, P=0.001) expression levels were significantly lower in the HFD-C than in the ND-C (Fig1.A, B, C).

The main effect of AE (F1,20=11.58, P=0.003, η=0.367) and BO on CTRP9 gene expression in heart tissue was significant (F1,20=6.35, P=0.020, η=0.241), while the interaction of these two interventions on the expression of this gene was not statistically significant (F1,20=0.253, P=0.621, η=0.012). Compared to the HFD-C group, CTRP9 gene expression was significantly higher in the HFD-AE-BP (+122.9%, P=0.003), HFD-AE (+59.56%, P=0.003), and HFD-BO groups (+41.67%, P=0.020). There was no significant difference between the HFD-AE-BP group and the HFD-AE group (-28.08%, P=0.270) or the HFD-BO group (-36.26%, P=0.072). Furthermore, CTRP9 expression was not significantly different between the HFD-AE and HFD-BO groups (-11.38%P=1.000) (Figure 2, A).

The main effect of AE (F1,20=7.88, P=0.011, η=0.283) and BO on LKB1 gene expression in heart tissue was significant (F1,20=16.26, P=0.001, η=0.449), while the interaction of these two interventions on the expression this gene was not statistically

Table 1. The genes primer sequences.

Gene name	Forward	Reverse
CTRP9	ACAAAGGAGACACAGGAGAAC	TTCAACTCTTCCATCTGCTCC
LKB1	AGGTTTCAAGGTGGACATCTG	CCTCTCCCGATGTTCTCAAAG
AMPK	ACCGTTCTATTGCCACTCTG	AAGAGGTAACTGGGCAAATCC
GAPDH	AAGTTCAACGGCACAGTCAAGG	CATACTCAGCACCAGCATCACC

Note: CTRP9: C1q/TNF-related protein-9, LKB1: liver kinase B1, AMPK: AMP-activated protein kinase, GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase.

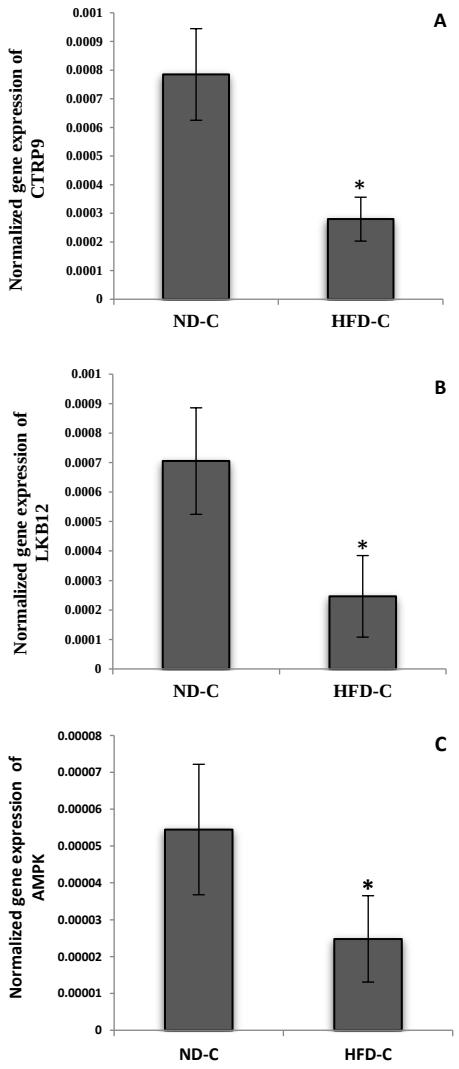

Figure 3. Comparison of CTRP9 (A), LKB1 (B), and AMPK (C) genes expression between the ND-C and HFD-C groups. Data represent Mean ± SD (n = 6). Independent t test. An asterisk symbol indicates a significant difference from the ND-C. Data are presented as mean ± standard deviation.

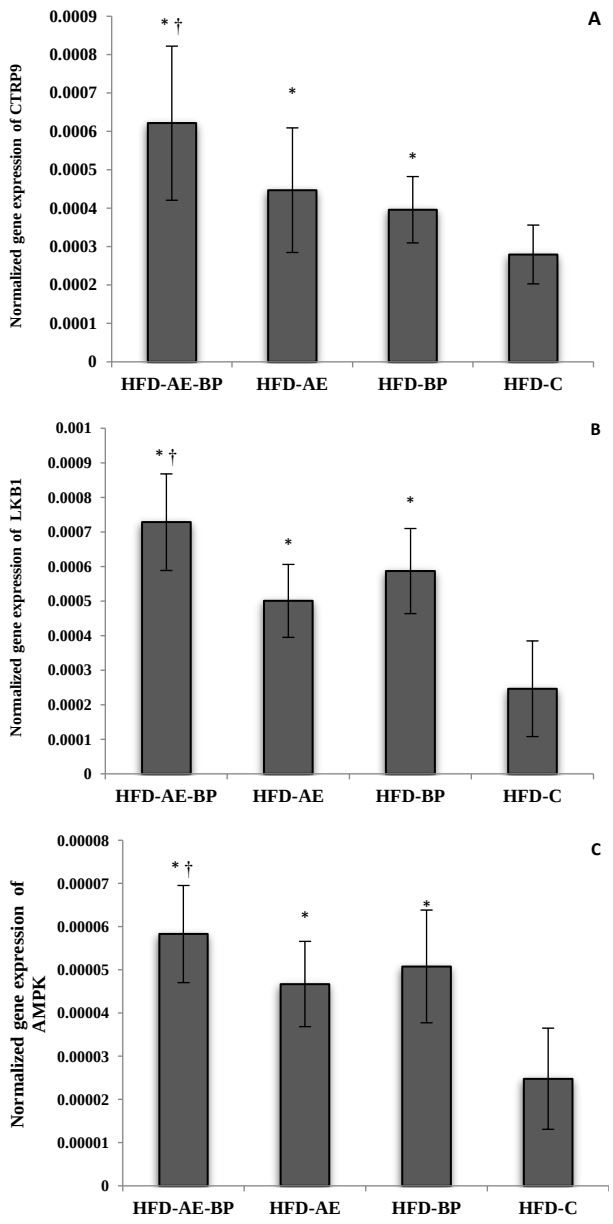

Figure 2. Compares CTRP9 (A), LKB1 (B) and AMPK (C) expression in cardiac tissue between the study groups. Data represent Mean ± SD (n = 6). Tow-way ANOVA, followed by the post hoc test of Bonferroni. An asterisk symbol indicates a significant difference from the HFD-C group. Dagger symbol indicates additive effect of these two interventions on gene expression.

significant (F1,20=0.642, P=0.432, η=0.031). Compared to the HFD-C group, LKB1 gene expression was significantly higher in the HFD-AE-BP (+195.72%, P=0.001), the HFD-AE group (+103.8%, P=0.011), and the HFD-BO group (+138.33%, P=0.001). There was no significant difference between the HFD-AE-BP group (-31.26%, P=0.200) and the HFD-BO group (-19.41%, P=1.000). Moreover, there was no significant difference in LKB1 expression between the HFD-AE and HFD-BO groups (+17.24, P=1.000) (Figure 2, B).

Myocardial tissue expresses AMPK in a similar manner to CTRP9 and LKB1. The main effect of AE (F1,20=6.06, P=0.023, η=0.233) and BO on AMPK gene expression in heart tissue was significant (F1,20=9.88, P=0.005, η=0.331), while the interaction of these two interventions on the expression of this gene was not statistically significant (F1,20=1.46, P=0.241, η=0.068). In comparison to the HFD-C group, HFD-AE-BP (+135.04%, P=0.023), HFD-AE (+88.42%, P=0.005), and HFD-BO (+104.85%, P=0.005) groups showed significantly higher expression of this gene. There were no significant differences between HFD-AE-BP, HFD-AE (-19.83%, P=1.000), and HFD-BO (-12.84%, P=1.000) groups. Likewise, there was no significant difference in AMPK gene expression between HFD-AE and HFD-BO (+8.71%, P=1.000) (Figure 2, C).

There was no statistically significant interaction effect between aerobic exercise and an extract on the expression of CTRP9, LKB1, and AMPK genes in the HFD-AE-BP group.

Discussion

It was demonstrated in this study that obesity caused by a HFD reduces the expression of the genes AMPK, LKB1, and CTRP9 in myocardial tissue. Previously, hasten et al. (2023) and Zhou et al. (2020) documented decreased CTRP9 levels in myocardial tissue following HFD feeding (Zuo et al., 2020). As a paracrine hormone, CTRP9 is mainly secreted by endothelial cells in the heart, where it is expressed approximately 100 times greater than adiponectin. It is interesting to note that endogenous CTRP9 expression declines during myocardial ischemia, while exogenous CTRP9 supplementation promotes cell survival and improves ventricular remodeling (Sun et al., 2013). Due to the significant decrease in cardiac and plasma CTRP9 levels during HFD, the question of whether this reduction contributes to myocardial dysfunction under these conditions is clinically important. Interestingly, CTRP9 knockout mice subjected to long-term (26 weeks) HFD exhibited worsening systolic performance, increased oxidative damage, and increased lipid accumulation (Zuo et al., 2020), supporting CTRP9's cardioprotective role. Diet-induced obesity and metabolic disorders can be prevented by CTRP9 expression in mice by reducing food intake, increasing oxygen consumption partly through increased mitochondrial content, upregulating fatty acid oxidation enzymes, and chronically activating AMPK (Peterson et al., 2013).

As a result of reduced CTRP9 expression during HFD feeding in knockout mice, Hasten et al. (2023) found that insulin signaling and glucose uptake were impaired in the myocardium. This indicates that decreased myocardial CTRP9 leads to insulin resistance in diabetic hearts, causing decreased glycolysis and glucose oxidation, as well as a greater dependence on fatty acids (Haustein et al., 2023). During contraction, this substrate shift inc-

-reases oxygen consumption costs and decreases myocardial efficiency. As a result of CTRP9 activation, mitochondrial biogenesis is promoted in target cells (Rohrbach et al., 2021). Thus, decreased CTRP9 expression results in reduced glucose uptake and mitochondrial numbers, resulting in diminished mitochondrial respiration. As a result of these changes and mitochondrial dysfunction, fatty acid metabolism increases in myocardial tissue, producing reactive oxygen species (ROS) and lipotoxicity (Wende, Symons, & Abel, 2012). During HFD-induced myocardial lipotoxicity, oxidative stress plays a critical role. The accumulation of excessive lipids from HFD triggers pathological apoptosis in cardiomyocytes due to elevated oxidative stress. In neonatal mice stimulated with palmitate, ROS levels were increased in cardiomyocytes. The dose-dependent inhibition of ROS production by exogenous CTRP9 was observed under these conditions. Furthermore, CTRP9-deficient mice fed HFD had significantly higher levels of 4-hydroxynonenal (4-HNE), a highly reactive aldehyde produced during lipid peroxidation. A CTRP9-induced reduction in myocardial lipotoxicity may be due to its antioxidant properties (Zuo et al., 2020). Myocardial tissue is disrupted by HFD-induced obesity through downregulation of CTRP. In a previous study, Millar et al (2015) reported that myocardial LKB1 expression decreased. According to this study, decreased LKB1 expression in HFD-fed mice was associated with extensive myocardial dysfunction, particularly impaired systolic function (Miller et al., 2015). A mutation in LKB1 causes Peutz-Jegher's syndrome and cancers that affect cell growth and polarity. LKB1-mediated phosphorylation at Thr172 on the AMPK-α catalytic subunit is a key regulatory step to activate cardioprotective AMPK during myocardial ischemia (Sakamoto et al., 2005). A 50% increase in mortality is observed in mice with cardiac homozygous LKB1 deficiency at four months of age, confirming LKB1's cardioprotective role.

As a result of HFD, ROS production and lipid peroxidation are increased at the molecular level. LKB1 expression is suppressed by elevated 4-hydroxy-2-nonenal, a marker of lipid peroxidation. MiR-451 upregulation also contributes to reduced LKB1 expression in HFD-induced obesity. By suppressing LKB1 mRNA translation or stability, and decreasing LKB1 protein levels, HFD positively regulates miR-451, which directly targets the LKB1/AMPK pathway. Lipotoxicity, mitochondrial dysfunction, and cardiac hypertrophy are exacerbated by this suppression. A significant increase in miR-451 expression is observed in HFD-fed mice, which correlates with reduced LKB1 protein, impaired AMPK signaling, and worsened cardiac dysfunction (H. Yang et al., 2020). Due to decreased AMPK activation, reduced LKB1 expression impairs mitochondrial biogenesis and oxidative metabolism. In contrast, LKB1 deficiency enhances caspase-3 and p53/PUMA signaling, resulting in pathological cardiomyocyte death. HFD-induced obesity exacerbated diastolic/systolic dysfunction, fibrosis, and hypertrophy (Miller et al., 2015).

There was a significant decrease in AMPK gene expression in the HFD group compared to the ND-C group in this study. Through interconnected mechanisms, HFD reduces myocardial AMPK expression and activity. By elevating oxidative stress, HFD induces lipid peroxidation and the accumulation of reactive aldehydes like 4-hydroxy-2-nonenal, which inhibit AMPK phosphorylation and downstream metabolic regulation. In addition, mitochondrial dysfunction and lipotoxicity are exasperated in HFD-fed mice (Lindholm et al., 2013). Inflammation of the system and tissues is another common consequence of HFD. The HFD promotes macrophage infiltration into adipose tissue and the heart, causing inflammation. M1 macrophages release cytokines (e.g., IL-1β, NOS2) that suppress AMPK activity. O-GlycNAcylation, a post-translational modification induced by inflammation, further inhibits AMPK activity and phosphorylation (Almabrouk et al., 2018). Furthermore, HFD-induced metabolic disturbances (e.g., hyperglycemia, hypercholesterolemia) indirectly inhibit AMPK. Adiponectin, an AMPK activator, is also reduced in hypertrophic adipose tissue, impairing AMPK activity further. The activity of AMPK in myocardium decreased by 35–44% in mice fed a HFD (Lindholm et al., 2013). In the present study, CTRP9 gene expression significantly increased in myocardium compared with the HFD-C group. It has been shown that endocrine signaling and transcriptional regulation work together to upregulate CTRP9 expression in the myocardium following aerobic exercise. In cardiomyocytes, aerobic exercise elevates insulin-like growth factor-1 (IGF-1), which activates the nuclear receptor subfamily 2 group F member 2 (NR2F2). CTRP9's expression is regulated by NR2F2, which binds directly to the gene's promoter region. Exercise (15 minutes/day) increased cardiac CTRP9 expression by more than 1.5-fold in mice with myocardial infarction (Tan et al., 2023). The increase in serum CTRP9 levels after acute aerobic exercise (e.g., 38 minutes of moderate cycling) correlates with improved endothelial function (flow-mediated dilation) in obese and normal-weight people. Apoptosis and oxidative stress in cardiomyocytes are suppressed by CTRP9 upregulation induced by aerobic exercise. Following ischemia-reperfusion injury, this molecular pathway is critical for reducing infarct size and enhancing survival. LKB1 gene expression was also significantly increased following aerobic exercise in this study. Exercise increases myocardium energy demand, which may activate LKB1 as a key regulator of AMPK (Sadat-Ebrahimi et al., 2022). In the context of maintaining energy homeostasis, this activation could potentially result in increased LKB1 expression. There is evidence that muscle contraction alters LKB1 gene expression in skeletal muscle. Due to the similarity between skeletal muscle and the myocardium, aerobic exercise may also affect LKB1 expression in the myocardium (Tanner et al., 2013). Metabolite signaling may also explain the increased AMPK expression observed in the current study.

Exercise elevates glycolysis and pentose phosphate pathways intermediates such as glucose-6-phosphate (G6P) and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR). Exercise-induced metabolic changes are linked to the activation of AMPK by these metabolites. ROS can also increase AMPK expression following aerobic exercise. By reducing mitochondrial ROS production, aerobic exercise prevents AMPK and its downstream regulators from being damaged by oxidative stress. The inhibition of ROS improves the stability and activity of AMPK, resulting in improved myocardial metabolism. As a result of aerobic exercise, AMPK expression and function in myocardial tissue are maintained (Fulghum & Hill, 2018). In addition, aerobic exercise stimulates AMPK expression and activity in cardiomyocytes by activating catecholamine signaling cascades (e.g., epinephrine). According to several molecular studies on catecholamines, they promote mitochondrial fission and regulate PGC-1α, indirectly enhancing AMPK activity. CTRP9 gene expression in myocardium was also significantly increased by ethanolic bitter orange peel extract in this study. According to a literature review, bitter orange peel does not appear to have a significant effect on myocardial CTRP9 expression. Although bitter orange peel contains numerous bioactive compounds, such as flavonoids, phenolic acids, and other phytochemicals, its molecular influence on CTRP9 expression seems plausible. CTRP9 expression is suppressed by chronic inflammation induced by HFD through NF-κB activation. Therefore, reducing inflammation can increase CTRP9 expression. As a result, bitter orange peel extract modulates NF-κB activation by blocking phosphorylation and nuclear translocation of NF-κB p65 subunits (Kang et al., 2011), and thus mitigates the negative effects of cellular inflammation on CTRP9. Furthermore, bitter orange peel contains flavonoids (hesperidin, naringenin) and phenolic acids, which reduce oxidative stress by scavenging ROS and enhancing antioxidant defenses. Antioxidant compounds in bitter orange peel may counteract the effects of oxidative stress on myocardial CTRP9 expression (Shen, Wan, Wang, & Jiang, 2019). By reducing fat accumulation and insulin resistance, bitter orange peel also improves metabolic status. Enhanced metabolic function may enhance adipokine signaling and increase CTRP9 expression in adipose tissue or the heart (Shen et al., 2019).

LKB1 is also elevated in myocardial tissue by ethanolic extract of bitter orange peel. Hesperidin and naringenin in the peel scavenge ROS and lipid peroxidation byproducts such as 4-hydroxy-2-nonenal (4-HNE). Under HFD conditions, bitter orange peel likely increases myocardial LKB1 expression by inhibiting 4-HNE expression and activity (Suntar, Khan, Patel, Celano, & Rastrelli, 2018). LKB1 expression is sensitive to inflammation, and chronic inflammation suppresses it. The bitter orange peel reduces pro-inflammatory cytokines (TNF-α, IL-6) and NF-κB activity, preventing LKB1 downregulation during HFD feeding (Kang et al., 2011). Citrus peels improve insulin sensitivity and lipid metabolism in HFD models. LKB1 expression is reduced by obesity and metabolic dysfunction (Shen et al., 2019), so alleviating these conditions increases it. Additionally, LKB1 plays an important role in AMPK activation. According to studies, bitter orange peel naringenin activates AMPK, possibly reflecting an upstream modulation of LKB1 (Benjamim et al., 2022).

As a result of bitter orange peel extract administration, AMPK expression was significantly increased. Molecular evidence suggests that the extract underlying this molecular effect reduces inflammation and oxidative stress. Furthermore, naringenin activates AMPK directly by mimicking AMP effects. By activating AMPK via bitter orange peel, mitochondrial biogenesis and oxidative phosphorylation are also promoted. By improving mitochondrial efficiency, ATP production increases, which further enhances AMPK expression and activity. As myocardial lipid content increases, ceramide accumulates, an inhibitor of AMPK (Jin et al., 2009). By enhancing lipid metabolism and insulin sensitivity, bitter orange peel reduces ectopic fat deposits in the myocardium. Ceramide levels are decreased and AMPK expression is increased as a result. When aerobic exercise and ethanolic bitter orange peel extract were combined, the greatest increases in gene expression were observed because both interventions reduce inflammation, oxidative stress, and improve metabolic status through similar mechanisms. Their interaction, however, was not statistically significant. Accordingly, these interventions did not synergistically enhance gene expression, but rather had additive effects.

Conclusion

The lack of measurement of myocardial function and histological changes was a limitation of the present study. Since the genes measured in this study indicate mitochondrial biogenesis capacity, energy turnover, and myocardial ischemia/reperfusion injury, it is suggested that researchers simultaneously study changes in myocardial function and histology in future studies after induction of aerobic exercise and bitter orange peel extract mode.

What is already known on this subject?

The presence of obesity is a major risk factor for structural and functional changes in the myocardium, which may lead to cardiovascular complications and increased mortality.

What this study adds?

The present study shows that HFD negatively affects myocardial tissue by suppressing the CTRP9, LKB1, and AMPK genes. They play a critical role in energy metabolism, fat accumulation, apoptosis, and myocardial function. Both aerobic exercise and ethanolic bitter orange peel extract significantly reduced the adverse effects of HFD on the expression of these genes. In spite of the fact that these two interventions did not synergistically enh-

-ance gene expression, the highest gene expression was observed when they were combined. This indicates the additive effect of these two interventions on gene expression. As a result of reducing inflammation, oxidative stress, and improving metabolic status, aerobic exercise and bitter orange peel extract appear to increase expression of the studied genes. In obese subjects induced by HFD, aerobic exercise and ethanolic bitter orange peel extract exhibit cardioprotective effects and protect myocardial tissue. CTRP9, LKB1, and AMPK genes are upregulated in order to accomplish this. In order to reduce cardiac complications under HFD conditions, these two interventions should be used.

Organ Cross-Talk Tips:

- Aerobic exercise likely triggers muscle-derived factors that signal to the heart, increasing expression of protective genes (CTRP9, LKB1, AMPK).
- Bitter orange peel extract potentially influences gut or liver metabolism, generating signals that enhance cardioprotective gene expression in the heart under HFD stress.

Acknowledgements

This study is derived from a doctoral thesis in Exercise Physiology. It was approved by the Department of Physical Education and Sport Sciences at the Islamic Azad University, Central Tehran Branch. The authors would like to express their sincere gratitude to the staff of the exercise physiology departments.

Funding

No sources of funding were sought or awarded for this study.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval the study protocol conformed to the Declaration of Helsinki and was approved by the animal care and use committee of Islamic Azad University Central Tehran branch.

Informed consent Animal study.

Author contributions

Conceptualization: SH.H.A.A, M.A.A, SH.R.M, H.F,; Methodology: SH.H.A.A, M.A.A, SH.R.M, H.F,; Software: SH.H.A.A, M.A.A, SH.R.M, H.F,; Validation: SH.H.A.A, M.A.A, SH.R.M, H.F,; Formal analysis: SH.H.A.A, M.A.A, SH.R.M, H.F,; Investigation: SH.H.A.A, M.A.A, SH.R.M, H.F,; Resources: SH.H.A.A, M.A.A, SH.R.M, H.F,; Data curation: SH.H.A.A, M.A.A, SH.R.M, H.F,; Writing - original draft: SH.H.A.A, M.A.A, SH.R.M, H.F,; Writing - review & editing SH.H.A.A, M.A.A, SH.R.M, H.F,; Visualization: SH.H.A.A, M.A.A, SH.R.M, H.F,; Supervision: SH.H.A.A, M.A.A, SH.R.M, H.F,; Project administration: SH.H.A.A, M.A.A, SH.R.M, H.F,; Funding acquisition: SH.H.A.A, M.A.A, SH.R.M, H.F.

References

Almabrouk, T. A. M., White, A. D., Ugusman, A. B., Skiba, D. S., Katwan, O. J., Alganga, H., . . . Kennedy, S. (2018). High Fat Diet Attenuates the Anticontractile Activity of Aortic PVAT via a Mechanism Involving AMPK and Reduced Adiponectin Secretion. Front Physiol, 9, 51. doi: https://doi.org/10.3389/fphys.2018.00051

Benjamim, C. J. R., Júnior, F. W. S., Porto, A. A., Rocha É, M. B., Santana, M. D., Garner, D. M., . . . Bueno Júnior, C. R. (2022). Bitter Orange (Citrus aurantium L.) Intake Before Submaximal Aerobic Exercise Is Safe for Cardiovascular and Autonomic Systems in Healthy Males: A Randomized Trial. Front Nutr, 9, 890388. doi: https://doi.org/10.3389/fnut.2022.890388

Costantino, S., Akhmedov, A., Melina, G., Mohammed, S. A., Othman, A., Ambrosini, S., . . . Paneni, F. (2019). Obesity-induced activation of JunD promotes myocardial lipid accumulation and metabolic cardiomyopathy. Eur Heart J, 40(12), 997-1008. doi: https://doi.org/10.1093/eurheartj/ehy903

Fulghum, K., & Hill, B. G. (2018). Metabolic Mechanisms of Exercise-Induced Cardiac Remodeling. Front Cardiovasc Med, 5, 127. doi: https://doi.org/10.3389/fcvm.2018.00127

Han, H. Y., Lee, S. K., Choi, B. K., Lee, D. R., Lee, H. J., & Kim, T. W. (2019). Preventive Effect of Citrus aurantium Peel Extract on High-Fat Diet-Induced Non-alcoholic Fatty Liver in Mice. Biol Pharm Bull, 42(2), 255-260. doi: https://doi.org/10.1248/bpb.b18-00702

Hardie, D. G. (2011). AMP-activated protein kinase: an energy sensor that regulates all aspects of cell function. Genes Dev, 25(18), 1895-1908. doi: https://doi.org/10.1101/gad.17420111

Haustein, R., Trogisch, F. A., Keles, M., Hille, S., Fuhrmann, M., Weinzierl, N., . . . Heineke, J. (2023). C1q and Tumor Necrosis Factor Related Protein 9 Protects from Diabetic Cardiomyopathy by Alleviating Cardiac Insulin Resistance and Inflammation. Cells, 12(3), 443.

Jin, J., Mullen, T. D., Hou, Q., Bielawski, J., Bielawska, A., Zhang, X., . . . Hsu, Y. T. (2009). AMPK inhibitor Compound C stimulates ceramide production and promotes Bax redistribution and apoptosis in MCF7 breast carcinoma cells. J Lipid Res, 50(12), 2389-2397. doi: https://doi.org/10.1194/jlr.M900119-JLR200

Kang, S. R., Han, D. Y., Park, K. I., Park, H. S., Cho, Y. B., Lee, H. J., . . . Kim, G. S. (2011). Suppressive Effect on Lipopolysaccharide-Induced Proinflammatory Mediators by Citrus aurantium L. in Macrophage RAW 264.7 Cells via NF-κB Signal Pathway. Evidence-based complementary and alternative medicine : eCAM, 2011. doi: https://doi.org/10.1155/2011/248592

Law, B. A., Liao, X., Moore, K. S., Southard, A., Roddy, P., Ji, R., . . . Cowart, L. A. (2018). Lipotoxic very-long-chain ceramides cause mitochondrial dysfunction, oxidative stress, and cell death in cardiomyocytes. Faseb j, 32(3), 1403-1416. doi: https://doi.org/10.1096/fj.201700300R

Leal, L. G., Lopes, M. A., & Batista, M. L. (2018). Physical Exercise-Induced Myokines and Muscle-Adipose Tissue Crosstalk: A Review of Current Knowledge and the Implications for Health and Metabolic Diseases. Frontiers in Physiology, 9. doi: https://doi.org/10.3389/fphys.2018.01307

Lindholm, C. R., Ertel, R. L., Bauwens, J. D., Schmuck, E. G., Mulligan, J. D., & Saupe, K. W. (2013). A high-fat diet decreases AMPK activity in multiple tissues in the absence of hyperglycemia or systemic inflammation in rats. J Physiol Biochem, 69(2), 165-175. doi: https://doi.org/10.1007/s13105-012-0199-2

Miller, E. J., Calamaras, T., Elezaby, A., Sverdlov, A., Qin, F., Luptak, I., . . . Colucci, W. S. (2015). Partial Liver Kinase B1 (LKB1) Deficiency Promotes Diastolic Dysfunction, De Novo Systolic Dysfunction, Apoptosis, and Mitochondrial Dysfunction With Dietary Metabolic Challenge. J Am Heart Assoc, 5(1). doi: https://doi.org/10.1161/jaha.115.002277

Mohammadi, S., Rostamkhani, F., Riyahi Malayeri, S., & Shirvani, H. (2022). High-intensity interval training with probiotic supplementation decreases gene expression of NF-κβ and CXCL2 in small intestine of rats with steatosis. Sport Sciences for Health, 18(2), 491-497. doi: https://doi.org/10.1007/s11332-021-00829-5

Nikbin, S., Tajik, A., Allahyari, P., Matin, G., Hoseini Roote, S. S., Barati, E., . . . Azarbayjani, M. A. (2020). Aerobic exercise and eugenol supplementation ameliorated liver injury induced by chlorpyrifos via modulation acetylcholinesterase activation and antioxidant defense. Environ Toxicol, 35(7), 783-793. doi: https://doi.org/10.1002/tox.22913

Peterson, J. M., Wei, Z., Seldin, M. M., Byerly, M. S., Aja, S., & Wong, G. W. (2013). CTRP9 transgenic mice are protected from diet-induced obesity and metabolic dysfunction. Am J Physiol Regul Integr Comp Physiol, 305(5), R522-533. doi: https://doi.org/10.1152/ajpregu.00110.2013

Rohrbach, S., Li, L., Novoyatleva, T., Niemann, B., Knapp, F., Molenda, N., & Schulz, R. (2021). Impact of PCSK9 on CTRP9-Induced Metabolic Effects in Adult Rat Cardiomyocytes. Front Physiol, 12, 593862. doi: https://doi.org/10.3389/fphys.2021.593862

Sadat-Ebrahimi, S. R., Amini, H., Rahbarghazi, R., Habibollahi, P., Ghaderi, S., Rajabi, H., & Rezabakhsh, A. (2022). Putative therapeutic impacts of cardiac CTRP9 in ischaemia/reperfusion injury. J Cell Mol Med, 26(11), 3120-3132. doi: https://doi.org/10.1111/jcmm.17355

Sakamoto, K., McCarthy, A., Smith, D., Green, K. A., Grahame Hardie, D., Ashworth, A., & Alessi, D. R. (2005). Deficiency of LKB1 in skeletal muscle prevents AMPK activation and glucose uptake during contraction. Embo j, 24(10), 1810-1820. doi: https://doi.org/10.1038/sj.emboj.7600667

Shaito, A., Thuan, D. T. B., Phu, H. T., Nguyen, T. H. D., Hasan, H., Halabi, S., . . . Pintus, G. (2020). Herbal Medicine for Cardiovascular Diseases: Efficacy, Mechanisms, and Safety. Front Pharmacol, 11, 422. doi: https://doi.org/10.3389/fphar.2020.00422

Sharma, S., Adrogue, J. V., Golfman, L., Uray, I., Lemm, J., Youker, K., . . . Taegtmeyer, H. (2004). Intramyocardial lipid accumulation in the failing human heart resembles the lipotoxic rat heart. Faseb j, 18(14), 1692-1700. doi: https://doi.org/10.1096/fj.04-2263com

Shen, C. Y., Wan, L., Wang, T. X., & Jiang, J. G. (2019). Citrus aurantium L. var. amara Engl. inhibited lipid accumulation in 3T3-L1 cells and Caenorhabditis elegans and prevented obesity in high-fat diet-fed mice. Pharmacol Res, 147, 104347. doi: https://doi.org/10.1016/j.phrs.2019.104347

Sletten, A. C., Peterson, L. R., & Schaffer, J. E. (2018). Manifestations and mechanisms of myocardial lipotoxicity in obesity. J Intern Med, 284(5), 478-491. doi: https://doi.org/10.1111/joim.12728

Sun, Y., Yi, W., Yuan, Y., Lau, W. B., Yi, D., Wang, X., . . . Ma, X. L. (2013). C1q/tumor necrosis factor-related protein-9, a novel adipocyte-derived cytokine, attenuates adverse remodeling in the ischemic mouse heart via protein kinase A activation. Circulation, 128(11 Suppl 1), S113-120. doi: https://doi.org/10.1161/circulationaha.112.000010

Suntar, I., Khan, H., Patel, S., Celano, R., & Rastrelli, L. (2018). An Overview on Citrus aurantium L.: Its Functions as Food Ingredient and Therapeutic Agent. Oxid Med Cell Longev, 2018, 7864269. doi: https://doi.org/10.1155/2018/7864269

Tan, Y., Feng, P., Feng, L., Shi, L., Song, Y., Yang, J., . . . Yi, W. (2023). Low-dose exercise protects the heart against established myocardial infarction via IGF-1-upregulated CTRP9 in male mice. MedComm (2020), 4(6), e411. doi: https://doi.org/10.1002/mco2.411

Tanner, C. B., Madsen, S. R., Hallowell, D. M., Goring, D. M., Moore, T. M., Hardman, S. E., . . . Thomson, D. M. (2013). Mitochondrial and performance adaptations to exercise training in mice lacking skeletal muscle LKB1. Am J Physiol Endocrinol Metab, 305(8), E1018-1029. doi: https://doi.org/10.1152/ajpendo.00227.2013

Wende, A. R., Symons, J. D., & Abel, E. D. (2012). Mechanisms of lipotoxicity in the cardiovascular system. Curr Hypertens Rep, 14(6), 517-531. doi: https://doi.org/10.1007/s11906-012-0307-2

Wong, G. W., Krawczyk, S. A., Kitidis-Mitrokostas, C., Ge, G., Spooner, E., Hug, C., . . . Lodish, H. F. (2009). Identification and characterization of CTRP9, a novel secreted glycoprotein, from adipose tissue that reduces serum glucose in mice and forms heterotrimers with adiponectin. Faseb j, 23(1), 241-258. doi: https://doi.org/10.1096/fj.08-114991

Xie, Z., Dong, Y., Scholz, R., Neumann, D., & Zou, M. H. (2008). Phosphorylation of LKB1 at serine 428 by protein kinase C-zeta is required for metformin-enhanced activation of the AMP-activated protein kinase in endothelial cells. Circulation, 117(7), 952-962. doi: https://doi.org/10.1161/circulationaha.107.744490

Yang, H., Xin, X., Yu, H., Bao, Y., Jia, P., Wu, N., & Jia, D. (2020). microRNA Expression Profiles in Myocardium of High-Fat Diet-Induced Obesity Rat. Diabetes Metab Syndr Obes, 13, 1147-1159. doi: https://doi.org/10.2147/dmso.s248948

Yang, Y., Li, Y., Ma, Z., Jiang, S., Fan, C., Hu, W., . . . Yi, W. (2016). A brief glimpse at CTRP3 and CTRP9 in lipid metabolism and cardiovascular protection. Prog Lipid Res, 64, 170-177. doi: https://doi.org/10.1016/j.plipres.2016.10.001

Zuo, A., Zhao, X., Li, T., Li, J., Lei, S., Chen, J., . . . Guo, Y. (2020). CTRP9 knockout exaggerates lipotoxicity in cardiac myocytes and high-fat diet-induced cardiac hypertrophy through inhibiting the LKB1/AMPK pathway. J Cell Mol Med, 24(4), 2635-2647. doi: https://doi.org/10.1111/jcmm.14982