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*Original Communication*

# Hesperetin Ameliorates High-Fat Diet-Induced Skeletal Muscle Dysfunction by Regulating Mitochondrial Cristae Morphology Through the Sestrin2/AMPK/OPA1 Signalling Pathway

Xiaoyang Shi[1], Xinyuan Zhang[1], Xin Ma[2], Jinjie Li[1], Amin Ullah[3], Xiaodong Zuo[1], Guangning Kou[1,3,*]

[1]Centre of Sport Nutrition and Health, School of Physical Education, Zhengzhou University, 450001 Zhengzhou, Henan, China
[2]The National Regional Medical Center, Fuwai Central China Cardiovascular Hospital, Zhengzhou University, 450001 Zhengzhou, Henan, China
[3]Department of Nutrition and Food Hygiene, School of Public Health, Zhengzhou University, 450001 Zhengzhou, Henan, China
*Correspondence: kouguangning@zzu.edu.cn (Guangning Kou)
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**Abstract**

**Background:** Long-term consumption of a high-fat diet (HFD) may cause insulin resistance (IR), leading to physiological dysfunction in skeletal muscle. IR is closely associated with mitochondrial dysfunction, and naturally occurring flavonoids may help improve mitochondrial function. **Methods:** Eight-week-old male C57BL/6J mice (n = 10/group) were fed either a 10 kcal% fat chow diet (CON) or a 60 kcal% fat HFD for 12 weeks. HFD-fed mice received daily gavage with hesperetin (50 or 100 mg/kg body weight (BW), ≥98% purity) or vehicle. BW and food intake were recorded weekly. After 10 weeks, strength and exercise performance, intraperitoneal glucose tolerance test/intraperitoneal insulin tolerance test (IPGTT/IPITT), serum lipids, mitochondrial ultrastructure (transmission electron microscopy (TEM)), adenosine triphosphate (ATP) content, molecular docking and dynamics, and Western blotting were assessed. **Results:** The findings suggested that hesperetin improved exercise capacity ($p < 0.05$ and $p < 0.01$) and reduced body fat content and distribution (all $p < 0.01$). Hesperetin also improved mitochondrial function, as primarily evidenced by increased ATP content in the skeletal muscle of HFD-fed mice (HFD vs high-fat diet with a low dose of hesperetin (HFD-LH), $p = 0.004$; HFD vs high-fat diet with a high dose of hesperetin (HFD-HH), $p < 0.01$), reduced mitochondrial cristae damage in skeletal muscle ($p < 0.05$ and $p < 0.01$), and upregulated the expression of mitochondrial function-related proteins in complexes I to V, with effects varying by muscle type and dosage ($p < 0.05$ and $p < 0.01$). Additionally, hesperetin upregulated the expression of Sestrin2 (gastrocnemius: HFD vs HFD-LH, $p = 0.047$; HFD vs HFD-HH, $p = 0.01$; soleus: HFD vs HFD-LH, $p = 0.016$; HFD vs HFD-HH, $p < 0.01$) and Optic atrophy 1 (OPA1) (gastrocnemius: all $p < 0.01$; soleus: HFD vs HFD-LH, $p = 0.017$; HFD vs HFD-HH, $p < 0.01$), and incresaed Adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) phosphorylation (gastrocnemius: HFD vs HFD-LH, $p = 0.031$; HFD vs HFD-HH, $p = 0.001$; soleus: HFD vs HFD-LH, $p = 0.01$; HFD vs HFD-HH, $p = 0.002$). **Conclusions:** We speculate that hesperetin may help preserve mitochondrial structure and function. This effect, which could contribute to reduced skeletal muscle dysfunction, may be linked to the Sestrin2/AMPK/OPA1 signalling pathway.

**Keywords:** high-fat diet; skeletal muscle; hesperetin; mitochondrial function; Sestrin2

## 1. Introduction

High-fat diets have become an important source of obtaining daily nutrients, as an unhealthy lifestyle and one of the underlying triggers for obesity that occurs [1,2]. As obesity is known to increase the risk of hyperglycemia, insulin resistance, and vascular diseases in the body, it has been a worldwide health problem [3,4].

Ectopic fat deposition associated with obesity leads to biological dysfunction in skeletal muscle, involving insulin resistance (IR), mitochondrial dysfunction, and inflammation [5]. It has been proposed that excess nutrient intake is associated with mitochondrial oxidative stress, leading to mitochondrial dysfunction [6]. Barriers to glucose uptake, impaired fatty acid oxidation, and mitochondrial dysfunction in skeletal muscle are the primary causes of the development of insulin resistance [7]. Mitochondrial dysfunction in skeletal muscle cells is mainly characterized by reduced electron transport chain activity and decreased mitochondrial fatty acid oxidation and oxidative phosphorylation, leading to the accumulation of excessive lipids in skeletal muscle, and ultimately inducing insulin resistance [8]. Although numerous studies have explored improving mitochondrial function as a therapeutic target for metabolic diseases, the potential of specifically targeting mitochondrial cristae remains unclear [9].

Mitochondria is an organelle with a double membrane structure, which can be divided into outer and inner membranes. The inner membrane consists of a boundary membrane, cristae junctions, and mitochondrial cristae that fold from the inner membrane into the mitochondrial matrix [10,11]. Mitochondrial cristae contain the respiratory chain

electron transport chain complex proteins and adenosine triphosphate (ATP) synthase and are fundamental to ATP production in mitochondria [12]. Studies demonstrated that deletion of the correlation complex proteins could affect mitochondrial cristae morphology, thereby generating mitochondrial dysfunction [13]. Transmission electron microscopy (TEM) results in a related study showed that mitochondrial cristae were absent or ruptured, and mitochondria were vacuolated in mice served a high-fat diet [14]. Numerous studies have shown that chronic metabolic diseases can be alleviated by improving mitochondrial function [15]. Therefore, it would be a promising approach to explore whether metabolic diseases can be treated by improving the morphology of damaged cristae.

Over the years, flavonoids, which are plentiful in plants, have shown promising qualities in improving mitochondrial function [16]. It has been demonstrated that Isoliquiritigenin exerts neuroprotective effects on mice with Cerebral ischemia reperfusion injury by improving mitochondrial dysfunction [17]. Mulberry leaf flavonoids could attenuate IR in skeletal muscle and improve mitochondrial function by activating Adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) [18]. Hesperetin (HES), a flavonoid, is abundantly found in various fruits and vegetables. It is widely studied that hesperetin exerts important biological activities in improving glycolipid metabolism and cardiovascular diseases, including antidiabetic, antitumor, and neuroprotective properties, hesperetin has a higher bioavailability and possesses antioxidant and anti-inflammatory effects [19,20,21]. Meanwhile, studies have shown that hesperetin activates PINK1/Parkin-mediated mitophagy and concurrently enhances mitochondrial performance [22].

Collectively, these findings indicate that while flavonoids can improve mitochondrial function, it remains unclear whether hesperetin enhances mitochondrial function by specifically modulating mitochondrial cristae. As such, this study aims to investigate the efficacy of hesperetin in mitigating mitochondrial dysfunction and to elucidate its underlying mechanisms of action in a high-fat diet mouse model.

## 2. Materials and Methods

### *2.1 Animals and Experimental Protocol*

Eight-week-old C57BL/6J male mice were purchased from Beijing HFK Biotechnology Co., Ltd. (No.110322220102378913, Beijing, China). The mice were kept in a particular animal room with a controlled temperature (22 ± 2 °C) and an automatic 12-12h light-dark circulation system (8 a.m.–8 p.m.). The mice were acclimatized to the new environment for one week before the experiment. To minimize bias, a randomized and blinded study design was employed. After one week of acclimatization period the mice were randomly divided into four groups (n = 10 per group): chow group (CON; diet with 10 kcal% fat); HFD group (high-fat diet, diet with 60 kcal% fat); HFD-LH group (high-fat diet with a low dose of 50 mg/kg/d body weight (BW) hesperetin, which was received from Huike Botanical Development Co., Ltd. Shanxi, China; HPLC, purity ≥98%.); HFD-HH group (high-fat diet with a high dose of 100 mg/kg/d BW hesperetin) [23]. Mice in the HFD-LH and HFD-HH groups were given the corresponding doses of hesperetin by gastric infusion once a day for 12 consecutive weeks (dissolved in 0.5% sodium carboxymethyl-cellulose solution); the other two groups were treated with a similar volume of solvent. Body weight (BW) and food intake were tracked weekly. The animals were given ad libitum access to food and water throughout the experimental period. This study was granted by the Zhengzhou University Life Sciences Institutional Review Board (Approval No. ZZUIRB 2021-12-02).

### *2.2 Sample Collection*

After 12 weeks of hesperetin intervention, mice from all four groups were dissected for sample collection. The mice were deeply anesthetized using sodium pentobarbital (100 mg/kg, intraperitoneal injection). Following the achievement of a surgical-plane depth of anesthesia (confirmed by loss of pedal and corneal reflexes), mice were euthanized by cervical dislocation to ensure death prior to tissue harvesting. Blood was collected via eyeball extraction and transferred into pre-prepared EP tubes for subsequent analysis of four blood lipid parameters. The gastrocnemius muscle and soleus muscle were dissected and stored at –80 °C for subsequent Western blot analysis.

### *2.3 Strength and Exercise Performance Tests*

The behavioral and motor examinations were conducted at Week 10 of the intervention. A wire mesh hanging apparatus was used for the hanging test, as described elsewhere. The mice were subjected to a wire mesh. In order to make the mice grasp the wire mesh, the wire mesh was then gently inverted. The time from grasping to falling was recorded for analysis [24]. The muscle strength of mice was measured by using a grip strength meter SA417 (Jiangsu Science Biotechnology Co., Jiangsu, China). Each of the mice was placed onto the grid with whole limbs attached to the grid and gently pulled backward from the tail until the grid was released. All the mice underwent three trials for grip strength, and the highest value for each mouse was recorded for data analysis [25]. Mice were subjected to a treadmill-running test using a treadmill (XR-PT-10B, Shanghai, China). The animals were adapted to treadmill running with uniform velocity exercise for two days, followed by low-speed running (those mice were run at an increase of 1 m every 3 min until 40 m/min) and high-speed running (those mice were run at a rise of 5 m every 2 min until 40 m/min) tests on the third day. The duration of the run was noted for analysis. Following the completion of a specific testing procedure, mice were subsequently used

for other assessments, provided that their health and welfare were not compromised.

### *2.4 Body Composition, Fat Distribution, and Metabolic Characterizations Study*

The metabolic assessments were then performed at the end of the treatment period in Week 12. The body composition of mice was screened to detect fat mass and lean mass by using a QMR06-060H small animal body composition analysis system (Niumag Analytical Instrument Corporation, Shanghai, China). When measuring fat distribution in mice, the animals were first anesthetized before being placed in a probe coil, which permitted coronal and horizontal scanning. Oxygen consumption ($VO_2$), carbon dioxide production ($VCO_2$), and levels of energy expenditure were monitored by the Oxymax lab animal monitoring system (Columbus Instruments, OH, USA, CLAMS), in which the mice were placed in individual cages to acclimate for 24 h, followed by 24 h of experimental runtime, both of 12 h of the light cycle and 12 h of the dark cycle.

### *2.5 IPGTT and IPITT*

For the intraperitoneal glucose tolerance test (IPGTT), the mice were fasted for 12 h, followed by an intraperitoneal injection of 2 g/kg body weight 20% glucose solution. Two days after IPGTT, the mice were fasted for 6 h, and then human insulin at a dose of 0.75 U/kg body weight was administered intraperitoneally (IPITT) [26]. At 0, 15, 30, 60, 90, and 120 min after injection, serum glucose concentrations were quantified immediately by collecting the blood samples from the tail vein of the mice tested by a glucometer (YUWELL, 590, Jiangsu, China). The data on glucose levels were graphed, and the area under the curve (AUC) in each group was calculated.

### *2.6 Serum Lipid Analysis*

The mice were fasted overnight before being sacrificed. Blood was collected and centrifuged for 5 min at 3000 ×g to obtain serum. Serum total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were determined by the appropriate commercial kits (Nanjing Jiancheng, Nanjing, China) according to the manufacturer's instructions.

### *2.7 Transmission Electron Microscopy (TEM)*

In this experiment, the microstructure of mitochondria was studied using TEM. Pieces of fresh gastrocnemius and soleus muscles in 3 mice (1 mm × 1 mm) were fixed with a 2.5% glutaraldehyde buffer solution for 24 h at 4 °C and then immersed in 1% osmium tetroxide for 2 h. After being subjected to dehydration in gradient ethanol concentrations and embedded with Epon 812 resin, the samples were cut into ultrathin sections (70 nm) and stained with uranyl acetate/lead citrate. The pictures were taken under a transmission electron microscope (Hitachi, HT-7800 Tokyo, Japan), and quantitated using Image J software, Version 1.54i, manufactured by National Institutes of Health (NIH), located in Bethesda, Maryland, USA.

### *2.8 Detection of ATP Content*

ATP levels in skeletal muscle were measured by luciferin/luciferase method using an Enhanced ATP Assay Kit (Beyotime Biotechnology, Shanghai, China) following the manufacturer's instructions.

### *2.9 Molecular Docking and Molecular Dynamics (MD) Simulations*

To perform molecular docking, the 3D structure of hesperetin and the crystal structure of the target protein SESN2 were downloaded from the PubChem database and the PDB database, respectively. Before docking started, water molecules, salt ions, etc., were removed using PyMol 2.5 (Schrödinger, Inc., New York, NY, USA) software to clean the crystal structure. The protein structure was saved in pdbqt format, and molecular docking was carried out using AutoDock Vina 1.1.2 (The Scripps Research Institute, La Jolla, CA, USA)software [27]. When docking was completed, the output of the highest-scoring structural conformation (considered the best binding conformation) was saved for subsequent Molecular Dynamics (MD) simulations [28]. The temperature of the system was increased for 200 ps to further distribute the solvent molecules uniformly in the solvent box when the system temperature was increased and maintained at 298.15 K, the simulation was carried out successively under NVT (isothermal-isovolumetric) and NPT (isothermal-isobaric) conditions for 500 ps, and the last two composite systems were subjected to the simulation of the NPT (isothermal isobaric) system for a duration of 100 ns, respectively.

### *2.10 Western Blot Assay*

Extraction of total protein within gastrocnemius and soleus muscles was achieved by homogenizing with the addition of radioimmunoprecipitation assay (RIPA) buffer (Beyotime Biotechnology, Shanghai, China) contenting Phenylmethanesulfonyl fluoride, a serine protease inhibitor (PMSF) (Beyotime Biotech, Shanghai, China) solution, and subsequent protein supernatants were obtained by centrifugation of 12,000 ×g for 15 min at 4 °C. Equivalent protein samples were denatured after the quantification of protein concentrations using a Bicinchoninic acid (BCA) protein assay kit (Beyotime Biotech, Shanghai, China). The prepared samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and later electroblotted to polyvinylidene difluoride (PVDF) membranes (Millipore, MA). The membranes with target proteins were blocked for 2 h with 5% nonfat milk or bovine serum albumin at room temperature, incubated in the company of the corresponding primary antibodies in-

cluding complexI (Proteintech, China), complexII (Proteintech, China), complexIII (Proteintech, China), complexIV (Proteintech, China), complexV (Proteintech, China), Sestrin2 (Proteintech, China), AMP-activated protein kinase (AMPK, Proteintech, China), phospho-AMPK (Thr172, Proteintech, China), OPA1 (Proteintech, China), GAPDH (Proteintech, China) overnight at 4 °C, and finally incubated for 1 h at room temperature with the relevant secondary antibody followed by three washes with TBST. The protein bands were visualized via a SmartChemiTM 610 Plus Imaging Analysis System (SinSage Technology, Beijing, China) by adding enhanced chemiluminescence (ECL) reagent (Beyotime, China) and then quantitated intensities using Image J software.

### *2.11 Statistical Analysis*

Statistical analyses were performed using GraphPad Prism 8.0.2 (GraphPad Software Inc., San Diego, CA, USA) and SPSS 25.0 (Statistical Package for Social Sciences, Inc., Chicago, IL, USA) software. Results are shown as mean ± standard deviation (SD). Significant differences among the data were identified by applying one-way ANOVA with the Dunnett test for assessing differences among more than two groups. The biological sample size n is as follows: ATP assay, n = 10 per group; Western blot analyses for both gastrocnemius and soleus muscles, n = 4 per group. Differences were considered statistically significant if $p < 0.05$.

## 3. Results

### *3.1 Hesperetin Alleviates Body Weight and Boosts Muscle Function in HFD-fed Mice*

By analyzing the data recorded on weekly body weight, it was found that the initial body weight of the four groups of mice did not change significantly; however, at the end of the experiment, the body weight of the mice in the HFD group significantly increased when compared to the mice in CON group. In contrast, the body weight of the mice in the HFD-LH and HFD-HH groups significantly decreased compared to HFD mice (all $p < 0.05$) (Fig. 1A). The fat distribution was observed by magnetic resonance imaging system, as shown in Fig. 1B that in HFD mice, the fat distribution was the largest, focusing on the abdominal and subcutaneous areas; compared with the HFD group, hesperetin treatment dramatically decreased the fat distribution in obese mice (Fig. 1B). Body composition results showed that the fat content of mice in the HFD group was significantly greater than that of the CON group. There was no significant difference in the amount of food consumed by mice on a high-fat diet (all $p > 0.05$) (Fig. 1C), so we believe that the weight loss was not due to food consumption. After treatment with hesperetin, the fat content of the other two groups of obese mice also significantly declined (all $p < 0.01$) (Fig. 1D). To investigate the role of hesperetin on muscle function in a high-fat diet-induced obese model, we performed a grip strength test, suspension test, and exercise tolerance test. We first examined data related to grip strength, and compared with the chow group, it was dramatically diminished in the HFD mice; compared with mice in the HFD group, it was elevated in the HFD-LH group ($p = 0.016$) and significantly elevated in the HFD-HH group ($p = 0.002$) (Fig. 1E). For the low-speed running test, the running duration of the HFD group was significantly decreased compared to the CON group. In contrast, the running duration of the mice in both HFD with hesperetin groups were significantly increased compared to the HFD group (all $p < 0.01$) (Fig. 1F,G). The suspension time test obtained the same results as grip strength test (HFD vs HFD-LH $p = 0.361$, HFD vs HFD-HH $p < 0.01$) (Fig. 1H). Concurrently, comparable results were found in the high-speed running test (all $p < 0.01$) (Fig. 1I,J). This finding indicates the importance of hesperetin in alleviating body weight and promoting muscle strength and exercise performance in the HFD model.

### *3.2 Supplementation With Hesperetin Improves Energy Expenditure in HFD-fed Mice*

It is widely known that obesity is mainly characterized by metabolic disorders. To evaluate the influence of hesperetin on the metabolism of obese mice, we analyzed the $VO_2$ and $VCO_2$. Compared with the CON group, the HFD mice resulted in a significant decrease in $VO_2$ and $VCO_2$. Mice in the HFD+hesperetin groups showed higher $VO_2$ (Fig. 2A,B) (Light: HFD vs HFD-LH $p = 0.008$, HFD vs HFD-HH $p < 0.01$; Dark: all $p < 0.01$) and $VCO_2$ (**Supplementary Fig. 1A,B**) (Light: all $p < 0.01$; Dark: all $p < 0.01$) than the HFD-fed group. In addition, as shown in Fig. 2, obesity leads to a reduction in energy expenditure in the mouse organism. Hesperetin treatment resulted in a significant increase in energy expenditure capacity (Light: HFD vs HFD-LH $p = 0.291$, HFD vs HFD-HH $p = 0.011$; Dark: HFD vs HFD-LH $p = 0.02$, HFD vs HFD-HH $p < 0.01$) (Fig. 2C,D) in mice compared to HFD mice. These findings suggest that supplementation with hesperetin may ameliorate the reduced metabolic capacity caused by a high-fat diet and enhance energy expenditure levels.

### *3.3 Hesperetin Mitigates Lipid Metabolism Disorders and Promotes Glucose Homeostasis in HFD-fed Mice*

Serum lipid levels were detected to explore better the impact of hesperetin on lipid metabolism in obese mice. Compared with the CON group, the high-fat diet significantly increased the levels of TG (all $p < 0.01$), TC (HFD vs HFD-LH $p = 0.003$, HFD vs HFD-HH $p < 0.01$) and LDL-C (all $p < 0.01$) in obese mice, which were significantly alleviated by hesperetin replenishment (Fig. 3A–C). In HFD mice we also found a reduction in serum HDL-C levels. Hesperetin therapy potentially improves HDL-C levels compared with the HFD group (HFD vs HFD-LH $p = 0.005$, HFD vs HFD-HH $p < 0.01$) (Fig. 3D). IPGTT and

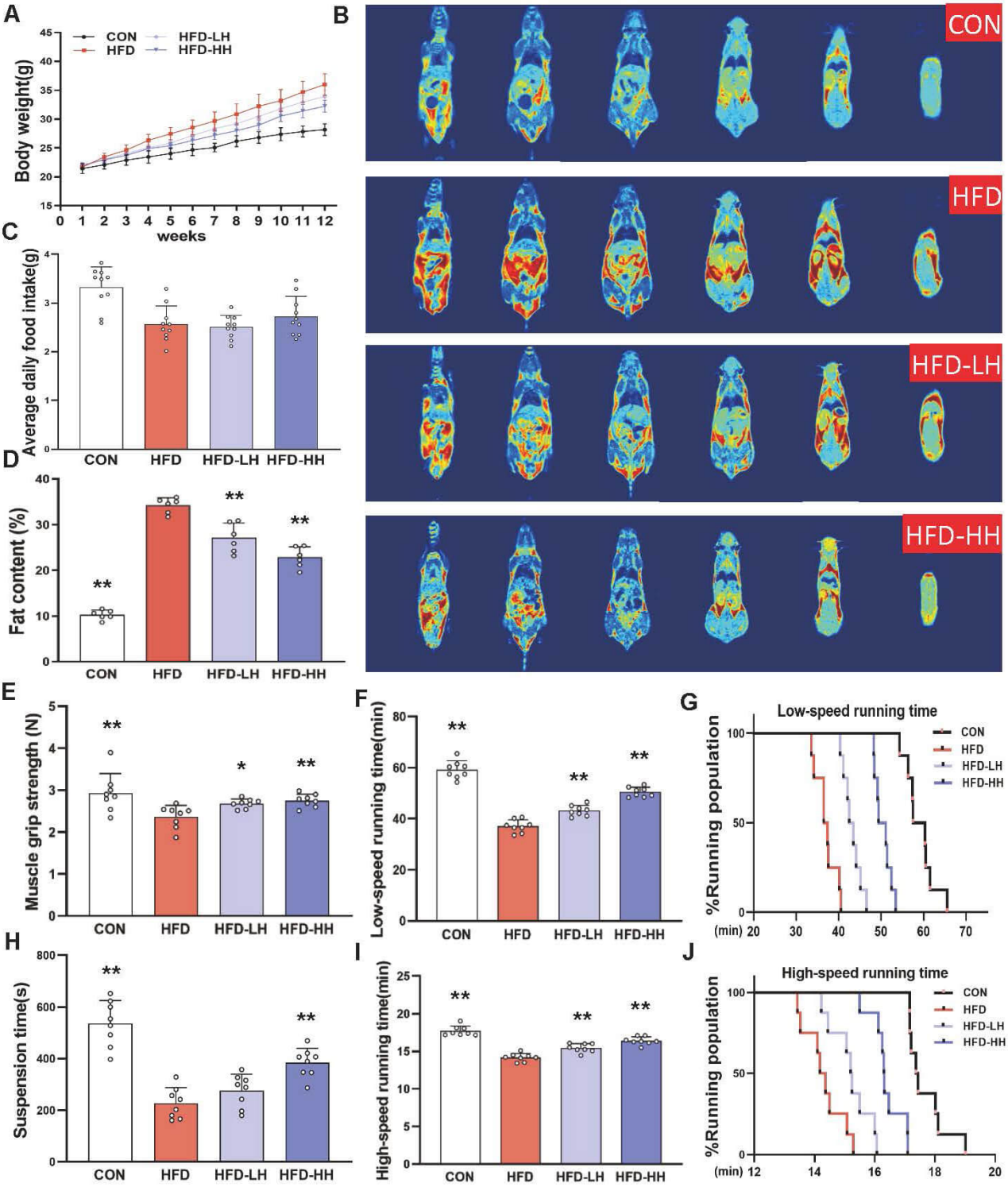


**Fig. 1. Hesperetin alleviates body weight and boosts muscle function in HFD-fed mice**. (A) Body weight changes of mice fed for 12 weeks (n = 10). (B) The fat distribution (red areas) in four groups (n = 3). (C) Food intake (n = 10). (D) Fat mass percentage (n = 6). (E) Muscle grip strength (n = 8). (F) Time in low-speed running (n = 8). (G) The percentage of mice in low-speed running at a particular moment (n = 8). (H) Duration of suspension test in mice (n = 8). (I) Time in high-speed running (n = 8). (J) The percentage of mice in high-speed at a particular moment (n = 8). For Fig. 1G/J, data are shown for descriptive purposes only; no statistical comparisons were performed. For the other panels in Fig. 1, data are the mean ± SD. Differences between groups were assessed by one-way ANOVA with Dunnett test. *$p < 0.05$, **$p < 0.01$ compared with the HFD group. CON, control group; HFD, high-fat diet; HFD-LH, high-fat diet with a low dose of hesperetin; HFD-HH, high-fat diet with a high dose of hesperetin; SD, standard deviation.

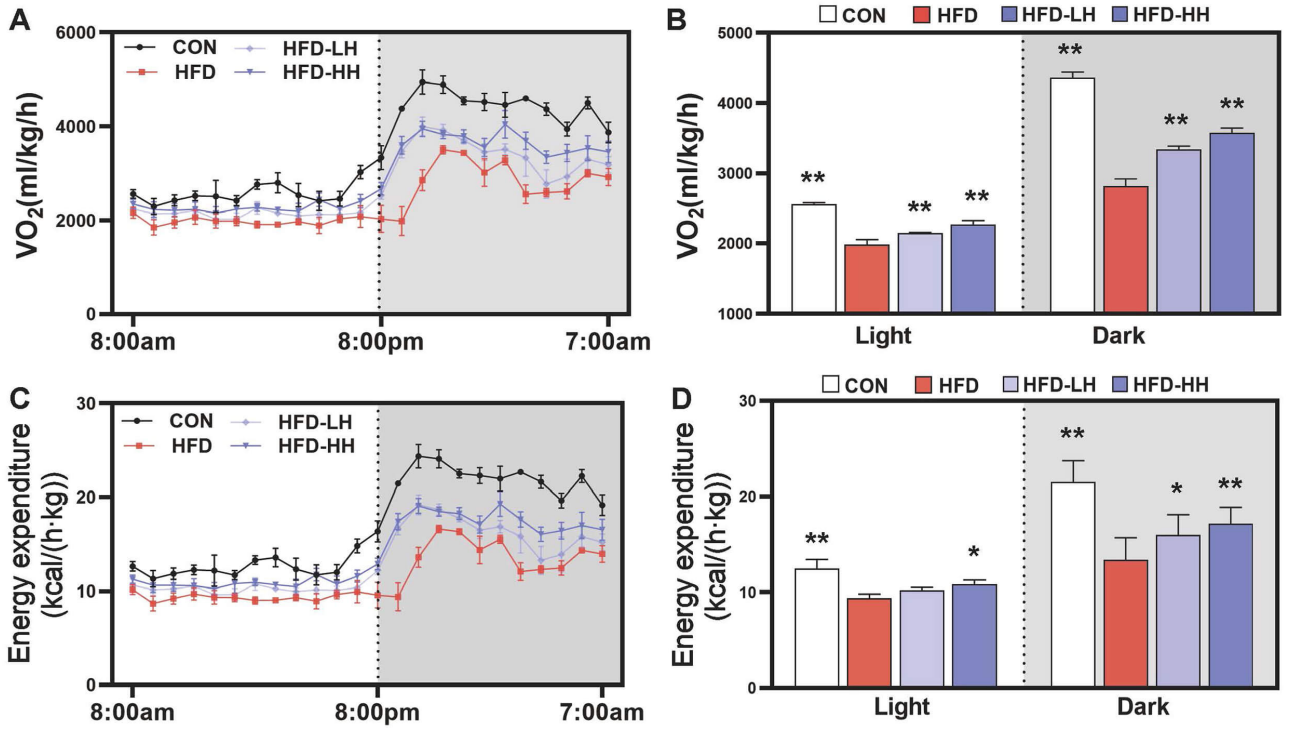


**Fig. 2. Supplementation with hesperetin improves energy expenditure in HFD-fed mice**. (A,B) Oxygen consumption ($VO_2$) of mice throughout light and dark cycle. (C,D) Energy expenditure. All data are the mean ± SD for n = 3 per group. Differences between groups were assessed by one-way ANOVA with Dunnett test. *$p < 0.05$, **$p < 0.01$ compared with the HFD group. CON, control group; HFD, high-fat diet; HFD-LH, high-fat diet with a low dose of hesperetin; HFD-HH, high-fat diet with a high dose of hesperetin.

IPITT experiments were carried out to determine the beneficial effects of hesperetin on glucose metabolism. We observed that HFD mice had higher blood glucose levels than the CON group (assessed by AUC, $p < 0.01$), which were perceived to have decreased glucose tolerance (assessed by AUC, HFD vs HFD-LH $p < 0.01$, HFD vs HFD-HH $p < 0.01$) (Fig. 3E,F) and insulin resistance (assessed by AUC, HFD vs HFD-LH $p < 0.01$, HFD vs HFD-HH $p < 0.01$) (Fig. 3G,H). After treatment with hesperetin, the AUC was markedly reduced in both treatment groups, suggesting a noticeable improvement in glucose tolerance and insulin sensitivity in HFD mice. In light of these results, hesperetin may have beneficial effects on alleviating lipid metabolism disorders and maintaining glucose homeostasis.

### *3.4 Hesperetin Ameliorates Skeletal Muscle Ultrastructure and Cristae Structure Attributed to High-Fat Diets*

In the development of insulin resistance, mitochondrial dysfunction is known to play a significant role. The observed enhancements in both energy expenditure and glycemic control are consistent with an underlying improvement in mitochondrial function. Therefore, this study aims to investigate whether hesperetin can ameliorate the impaired mitochondrial structure in the skeletal muscle of high-fat diet-fed mice. In the case of the gastrocnemius muscle, we quantified the TEM images (Fig. 4A) and observed that a high-fat diet led to an increase in mitochondrial area (all $p < 0.01$), perimeter (all $p < 0.01$), and diameter (HFD vs HFD-LH $p < 0.01$, HFD vs HFD-HH $p < 0.01$) (Fig. 4B–D), possibly caused by mitochondrial dysfunction and swelling resulting from accumulation of lipids. Further examination of the mitochondrial ultrastructure showed that the width between the mitochondrial cristae increased (HFD vs HFD-LH $p = 0.021$, HFD vs HFD-HH $p < 0.01$), cristae density (HFD vs HFD-LH $p = 0.001$, HFD vs HFD-HH $p < 0.01$), and the length ratio between the mitochondrial cristae membranes and outer membranes (HFD vs HFD-LH $p = 0.022$, HFD vs HFD-HH $p = 0.001$) decreased in the HFD group compared with the control group (Fig. 4E–G). The above indicators showed an improvement with treatment with hesperetin (all $p < 0.05$). Similar results were found in the study of the soleus muscle (area: all $p < 0.01$; perimeter: all $p < 0.01$; diameter: HFD vs HFD-LH $p = 0.017$, HFD vs HFD-HH $p < 0.01$; the width between the mitochondrial cristae: all $p < 0.01$; cristae density: HFD vs HFD-LH $p = 0.001$, HFD vs HFD-HH $p < 0.01$; the length ratio between the mitochondrial cristae membranes and outer membranes: HFD vs HFD-LH $p = 0.035$, HFD vs HFD-HH $p < 0.01$) (Fig. 5A–G). Altogether, these TEM images and quantitative data suggest that hesperetin could improve mitochondrial morphology and cristae structure appreciably.

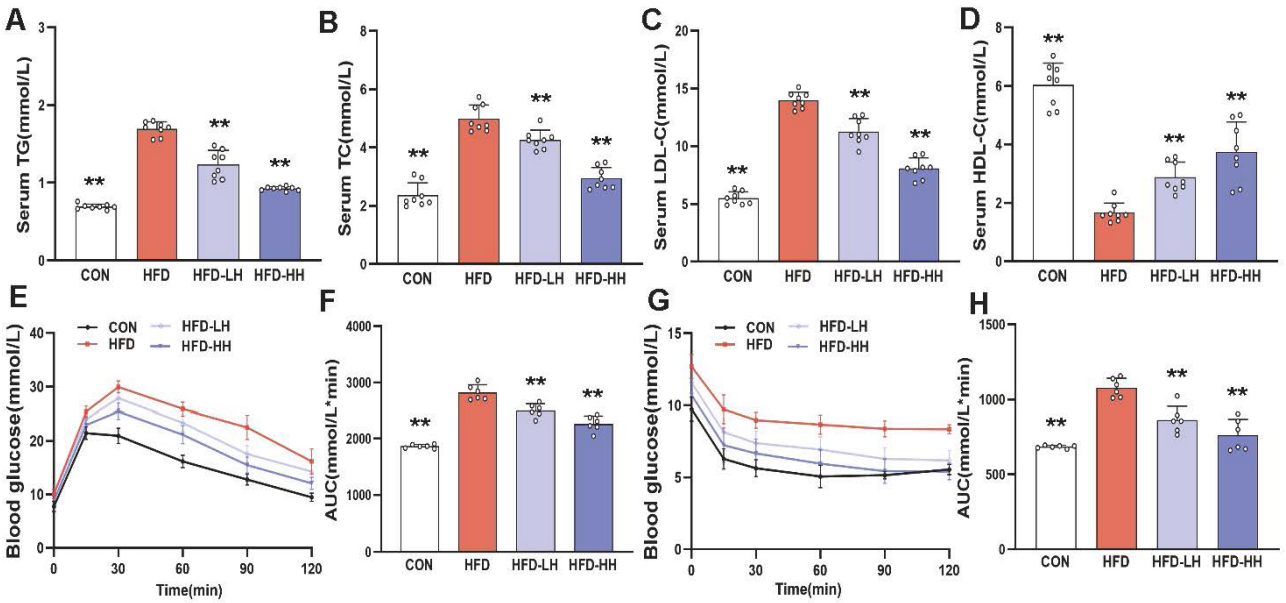


**Fig. 3. Hesperetin mitigates lipid metabolism disorders and promotes glucose homeostasis in HFD-fed mice**. (A) Serum TG levels. (B) Serum TC levels. (C) Serum LDL-C levels. (D) Serum HDL-C levels (n = 8). (E) Blood glucose changes were recorded during IPGTT (n = 6). (F) The area under the curve (AUC) during IPGTT. (G) Blood glucose changes were recorded during IPITT (n = 6). (H) The area under the curve (AUC) during IPITT. All data are the mean ± SD. Differences between groups were assessed by one-way ANOVA with Dunnett test. $^{**}p < 0.01$ compared with the HFD group. CON, control group; HFD, high-fat diet; HFD-LH, high-fat diet with a low dose of hesperetin; HFD-HH, high-fat diet with a high dose of hesperetin; TG, triglyceride; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; IPGTT, intraperitoneal glucose tolerance test; IPITT, intraperitoneal insulin tolerance test.

### *3.5 Hesperetin Improved Skeletal Muscle Mitochondrial Dysfunction Caused by a High Fat Diet*

Since mitochondria are essential for the production of ATP, we tested the ATP content of gastrocnemius to detect whether the mitochondrial function was impaired and noticed that the ATP content of the mice in the HFD group was remarkably lower than in the CON group (all $p < 0.01$), after treatment with hesperetin, there was an elevation of ATP content in skeletal muscle compared with HFD mice (HFD vs HFD-LH $p = 0.004$, HFD vs HFD-HH $p < 0.01$) (Fig. 6A). As an essential portion of the mitochondrial cristae, oxidative phosphorylation (OXPHOS) subunits could directly reflect the levels of mitochondrial damage. Hence, we performed Western blot (WB) assays on the protein subunit complexes to better characterize the effect of hesperetin on ameliorating the mitochondrial damage induced by a high-fat diet. Take the gastrocnemius as an example, the results showed that protein expression of complexes I (HFD vs HFD-LH $p = 0.452$, HFD vs HFD-HH $p = 0.013$), II (HFD vs HFD-LH $p = 0.007$, HFD vs HFD-HH $p < 0.01$), III (HFD vs HFD-LH $p = 0.289$, HFD vs HFD-HH $p = 0.049$), IV (all $p > 0.05$), V (HFD vs HFD-LH $p = 0.024$, HFD vs HFD-HH $p = 0.011$) were reduced to varying degrees in the HFD group (HFD vs CON $p < 0.01$), whereas the complex protein levels were slightly higher in the hesperetin-treated groups than in the high-fat diet without intervention group (Fig. 6B,D). Similar results were obtained for the soleus muscle complexes I (HFD vs HFD-LH $p = 0.203$, HFD vs HFD-HH $p = 0.001$), II (HFD vs HFD-LH $p = 0.006$, HFD vs HFD-HH $p < 0.01$), III (HFD vs HFD-LH $p = 0.048$, HFD vs HFD-HH $p = 0.003$), IV (HFD vs HFD-LH $p = 0.284$, HFD vs HFD-HH $p = 0.008$), V (HFD vs HFD-LH $p = 0.022$, HFD vs HFD-HH $p < 0.01$) (Fig. 6C,E). Based on the above results, hesperetin is essential in ameliorating high-fat diet-induced mitochondrial damage.

### *3.6 Molecular Docking and MD Simulations of Hesperetin-Sestrin2*

Molecular docking techniques have recently been widely used to predict small molecule-protein binding modes quickly. In this study, the interaction between hesperetin and Sestrin2 was predicted by using AutoDock Vina 1.1.2 software. It can be seen from the three-dimensional plot that hesperetin binds to the active site of the Sestrin2 protein (Fig. 7A), and from the 2D interaction diagram (Fig. 7B), it can be found that it was bound to the protein primarily through hydrophobic interactions as well as hydrogen bonding interactions, such as hydrophobic contacts with TRP-444, ILE-378, TYR-375, LEU-389, PHE-447, LEU-85, etc., and also hydrogen bonding interactions with ARG-448 and THR-374. As shown in the above figure, the protein without ligand has a large root mean square deviation

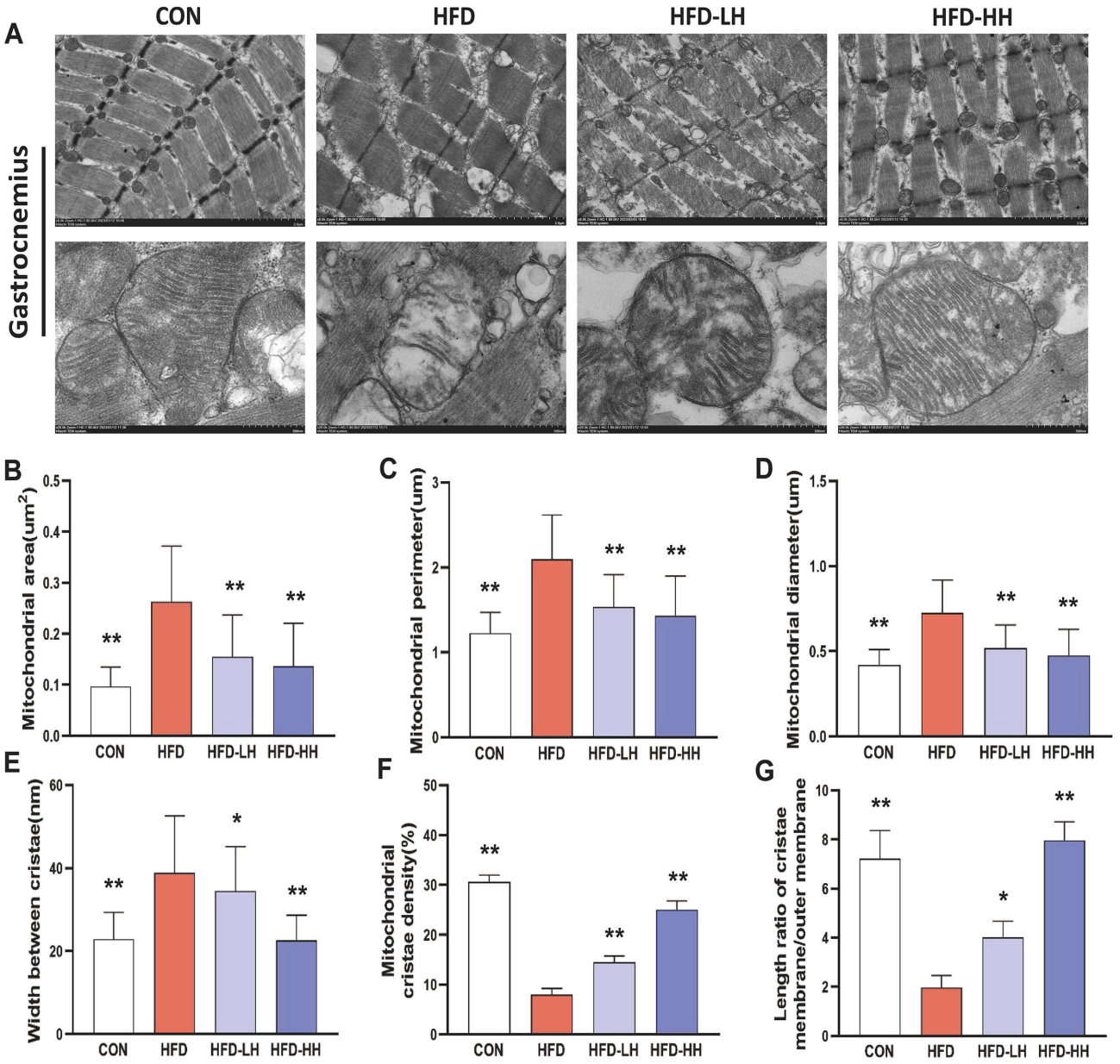


**Fig. 4. Hesperetin ameliorates skeletal muscle ultrastructure and cristae structure attributed to high-fat diets**. (A) Mitochondrial images of gastrocnemius muscle obtained by transmission electron microscopy (TEM). The scales are 2 μm and 500 nm, respectively. (B–G) Quantified TEM images of gastrocnemius muscle from 3 mice in each group, including (B) mitochondrial area, (C) mitochondrial perimeter, (D) mitochondrial diameter, (E) width between cristae, (F) mitochondrial cristae density, and (G) length ratio of cristae perimeter/mitochondrial perimeter. All data are the mean ± SD. Differences between groups were assessed by one-way ANOVA with Dunnett test. *$p < 0.05$, **$p < 0.01$ compared with the HFD group, n refers to mice (n = 3 per group). CON, control group; HFD, high-fat diet; HFD-LH, high-fat diet with a low dose of hesperetin; HFD-HH, high-fat diet with a high dose of hesperetin.

(RMSD) fluctuation in the first 40 ns of the simulation and arrives at a converged state after 40 ns, and the hesperetin-Sestrin2 complex gets to a converged state at 10 ns (Fig. 7C). By comparison, the RMSD of the hesperetin-Sestrin2 complex showed a more stable trend. From the perspective of potential energy during the simulation, we noticed that the potential energy of both the hesperetin-Sestrin2 complex and the protein without ligand remained stable during the simulation period, and the hesperetin-Sestrin2 complex had a smaller potential energy, implying a more stable binding of the complex (Fig. 7D). At 100 ns, our analysis of the binding conformation of the hesperetin-Sestrin2 complex suggests that the protein pocket had further wrapped around the small molecule hesperetin, which indicates that small molecule binding may become more stable in the later stage of the simulation (Fig. 7E). In the end, it was cal-

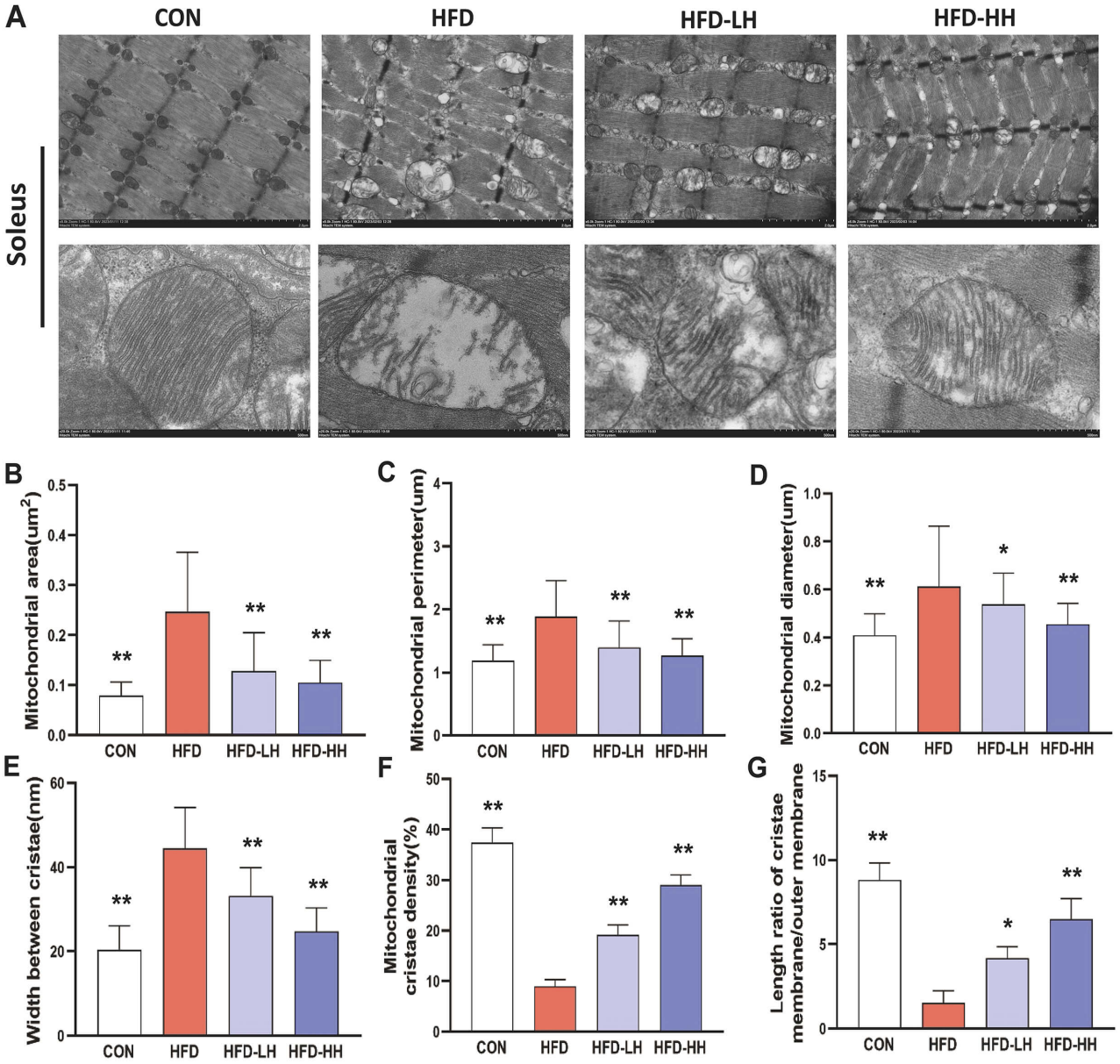


**Fig. 5. Hesperetin ameliorates skeletal muscle ultrastructure and cristae structure attributed to high-fat diets**. (A) Mitochondrial images of soleus muscle obtained by transmission electron microscopy (TEM). The scales are 2 μm and 500 nm, respectively. (B–G) Quantified TEM images of soleus muscle from 3 mice in each group, including (B) mitochondrial area, (C) mitochondrial perimeter, (D) mitochondrial diameter, (E) width between cristae, (F) mitochondrial cristae density, and (G) length ratio of cristae perimeter/mitochondrial perimeter. All data are the mean ± SD. Differences between groups were assessed by one-way ANOVA with Dunnett test. *$p < 0.05$, **$p < 0.01$ compared with the HFD group, n refers to mice (n = 3 per group). CON, control group; HFD, high-fat diet; HFD-LH, high-fat diet with a low dose of hesperetin; HFD-HH, high-fat diet with a high dose of hesperetin.

culated that the binding energy of hesperetin-Sestrin2 was –29.84 ± 2.18 kcal/mol by using the Molecular Mechanics–Generalized Born Surface Area (MM-GBSA) method (Fig. 7F), which showed that the binding affinity of hesperetin-Sestrin2 was potent.

### *3.7 Role of Hesperetin in Alleviating Mitochondrial Impairment in HFD Mice That May Be Mediated by the Sestrin2/AMPK/OPA1 Signalling Pathway*

The molecular docking showed that the hesperetin monomer has better binding energy with the protein Sestrin2. We next investigated the possible underlying mechanisms by which hesperetin exerts beneficial action on high-fat-induced impairment of mitochondrial function. Our

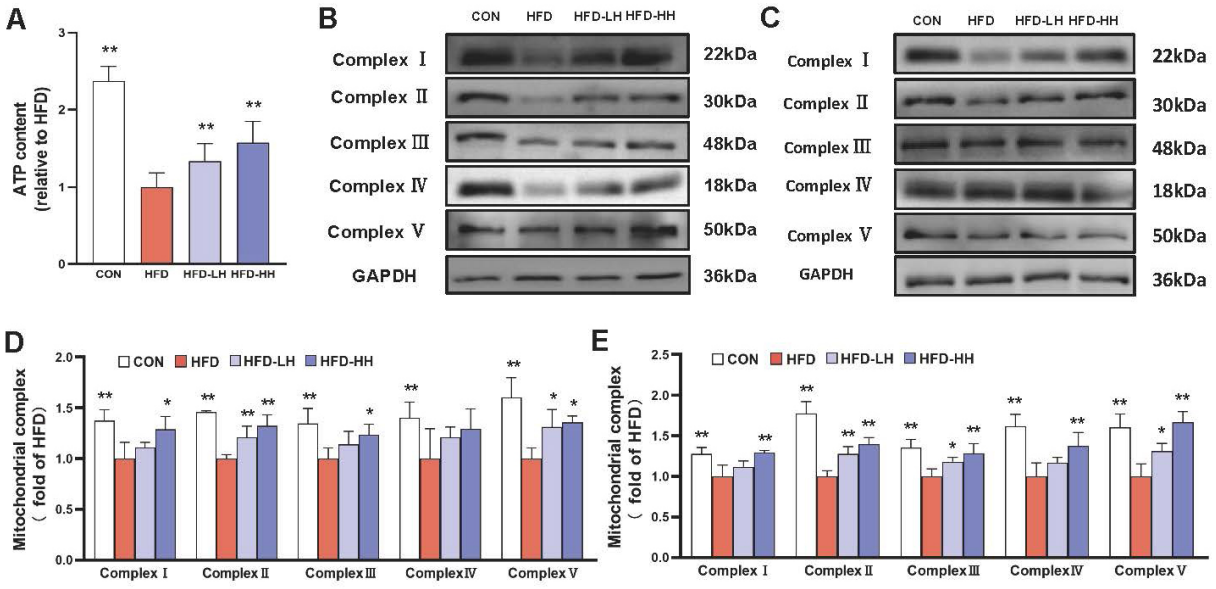


**Fig. 6. Hesperetin improved skeletal muscle mitochondrial dysfunction caused by a high fat diet**. (A) ATP content in gastrocnemius muscle relative to HFD group. (B,D) The expression levels of mitochondrial complex subunits in gastrocnemius were detected by Western blot and quantified by Image J. (C,E) The expression levels of mitochondrial complex subunits in soleus were detected by Western blot and quantified by Image J. All data are the mean ± SD. Differences between groups were assessed by one-way ANOVA with Dunnett test. *$p < 0.05$, **$p < 0.01$ compared with the HFD group. The biological sample size n is as follows: ATP assay, n = 10 per group; Western blot analyses for both gastrocnemius and soleus muscles, n = 4 per group. CON, control group; HFD, high-fat diet; HFD-LH, high-fat diet with a low dose of hesperetin; HFD-HH, high-fat diet with a high dose of hesperetin; ATP, adenosine triphosphate.

gastrocnemius findings showed that the expression of Sestrin2, p-AMPK/AMPK, and OPA1 was significantly lower in HFD mice than in the normal diet group. In turn, hesperetin augmented the phosphorylation of AMPK (HFD vs HFD-LH $p = 0.031$, HFD vs HFD-HH $p = 0.001$)and upregulated the expression of Sestrin2 (HFD vs HFD-LH $p = 0.047$, HFD vs HFD-HH $p = 0.01$) and OPA1 (all $p < 0.01$)(Fig. 8A–D). Similar results were found in the study of the soleus Sestrin2 (HFD vs HFD-LH $p = 0.016$, HFD vs HFD-HH $p < 0.01$), the phosphorylation of AMPK (HFD vs HFD-LH $p = 0.01$, HFD vs HFD-HH $p = 0.002$) and OPA1 (HFD vs HFD-LH $p = 0.017$, HFD vs HFD-HH $p < 0.01$)(Fig. 8E–H). These results indicated that hesperetin may enhance mitochondrial function by activating the Sestrin2/AMPK/OPA1 signalling pathway.

## 4. Discussion

Obesity, a metabolic disease characterized by insulin resistance and inflammation, is also a topical issue of ongoing concern worldwide, and commonly leads to frustrating results [29]. Mitochondrial oxidative damage contributes to an excessive accumulation of fatty acids in the body, which is closely related to the occurrence of insulin resistance [30]. Skeletal muscle is a crucial tissue for glucose metabolism, and alterations in mitochondrial properties are frequently observed in skeletal muscle that develops insulin resistance [31]. Improving mitochondrial function to alleviate insulin resistance in skeletal muscle has long been a subject of considerable interest [32].

Hesperetin is one of those flavonoids that has attracted attention for its anti-inflammatory and antioxidant properties [33]. Flavonoids, such as quercetin, chrysin, and hesperetin methyl chalcone, are known for their anti-obesity and insulin-resistance-reducing properties [34,35,36,37]. In a previous study, sechium edule shoots slowed down weight gain and fatty liver in HFD-fed rats, while caffeic acid and hesperetin were the main components in *Sechium edule* shoots (SWE), with caffeic acid and hesperetin acting mainly in the reduction of lipid accumulation in the cells [38]. In line with the results of the present work, mice fed a high-fat diet showed an apparent reduction of weight gain and a decrease in fat content after 12 consecutive weeks given hesperetin. A growing body of evidence indicates that flavonoids can manage metabolic syndrome by targeting mitochondrial function [39]. We also examined the effects of hesperetin on motor performance and metabolic capacity in HFD mice, increasing both performance and metabolic capacity, including $VO_2$, $VCO_2$, and energy expenditure. This may be in relationship to the enhancement of mitochondrial function. A study suggests that hesperetin could promote muscle function in senescent mice by improving mitochondrial function [40], which is in accordance with our findings.

A large number of flavonoids play important roles in improving glucose and insulin tolerance [41,42], simi-

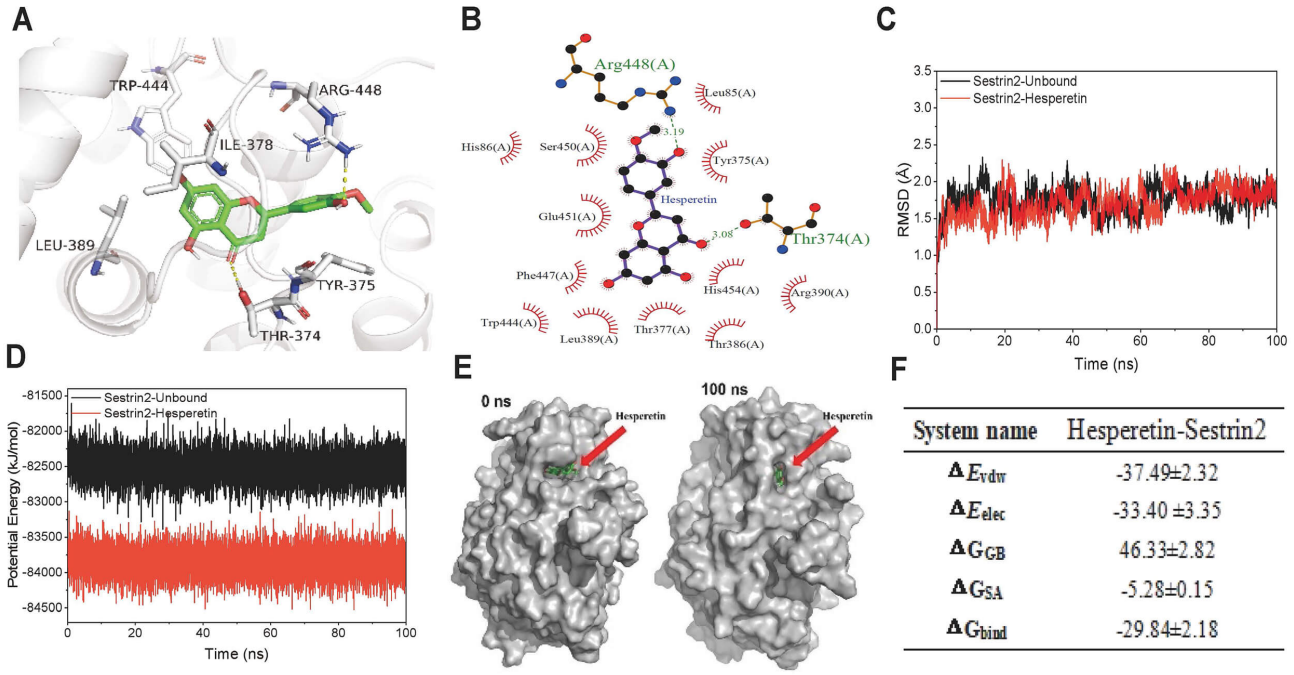


**Fig. 7. Molecular docking and Molecular Dynamics (MD) simulations of hesperetin-Sestrin2**. (A) Three-dimensional binding mode of the small molecule hesperetin (green) and the protein Sestrin2. (B) The two-dimensional plot shows that hesperetin predominantly binds to Sestrin2 protein through hydrophobic interaction as well as hydrogen bonding. (C) Changes in the root mean square deviation (RMSD) difference of complexes with time during molecular dynamics simulations. (D) Potential energy changes in the system during molecular dynamics simulations. (E) Changes in the binding conformation of the hesperetin-Sestrin2 complex at 0 ns as well as at the last moment (100 ns). (F) Hesperetin-Sestrin2 binding energy predicted using the Molecular Mechanics–Generalized Born Surface Area (MM-GBSA) (kcal/mol).

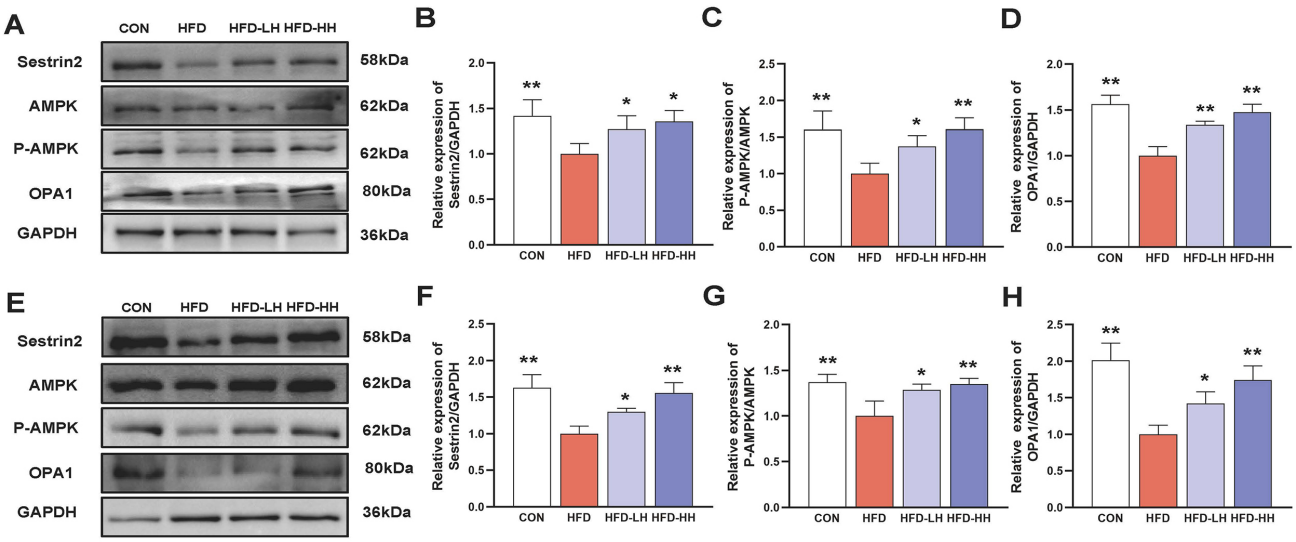


**Fig. 8. Role of hesperetin in alleviating mitochondrial impairment in HFD mice through the Sestrin2/AMPK/OPA1 signalling pathway**. (A–D) Western blot analysis of the proteins Sesn2, AMPK and OPA1 in gastrocnemius muscle (n = 4). (E–H) Western blot analysis of the proteins Sesn2, AMPK and OPA1 in soleus muscle (n = 4). All data are the mean ± SD. Differences between groups were assessed by one-way ANOVA with Dunnett test. *$p < 0.05$, **$p < 0.01$ compared with the HFD group. CON, control group; HFD, high-fat diet; HFD-LH, high-fat diet with a low dose of hesperetin; HFD-HH, high-fat diet with a high dose of hesperetin; SESN2, Sestrin2; AMPK, Adenosine 5'-monophosphate (AMP)-activated protein kinase; OPA1, Optic atrophy 1.

larly, in our results, we found that the flavonoid hesperetin-intervened HFD mice had better glucose tolerance and insulin sensitivity. Flavonoids are known to ameliorate lipid metabolism disorders caused by lipid overaccumulation [43]. Simultaneously, the serum indexes in this study also showed improvement trend. So far, our results indicate that hesperetin has a vast potential to decrease obesity and ameliorate dysfunction of skeletal muscle physiology attributed to high-fat diets.

In the mitochondrial cristae, mitochondrial functions such as metabolism and production of ATP are carried out; therefore, it is crucial to maintain the shape of the mitochondrial cristae [11]. Mitochondria are the location of ATP production, and mitochondrial damage is always associated with decreased ATP production. It has been shown that a high-fat diet results in mitochondrial dysfunction, mitochondrial swelling, and cristae disruption in skeletal muscle in mice [44]. We got similar findings in HFD mice with TEM images; the HFD group displayed broken myofilaments and disorganized arrangement of mitochondria, accompanied by a swollen and reduced number of mitochondria, and cristae were shorter or shallower. It should be noted that while the small sample size (n = 3) is common in TEM studies, it may limit the generalizability of the findings, and potential fixation artifacts were also considered in our interpretation.

A previous study has shown that flavonoids exhibit promising prospects for ameliorating structural damage in mitochondria [45]. An oral administration of (–)-epicatechin to rats significantly increased cristae densities on the surface of each unit of mitochondria [46]. Curcumin enhanced mitochondrial function in mildly hypoxic Bone marrow mesenchymal stem cells and improved cristae structure as one of its manifestations [47]. Hesperetin is a highly prospective natural compound; it has been shown that hesperetin promotes mitochondrial biogenesis and ameliorates age-related mitochondrial function [40], and hesperetin nanocrystals provided a significant increase in mitochondrial respiratory chain complex activity [48]. In our study, hesperetin treatment mitigated the absence of high-fat diet-induced mitochondrial cristae and disordered alignment; TEM image visual quantification also exhibited positive results. We also detected an increase in the protein expression levels of mitochondrial respiratory chain complexes located on the cristae, along with elevated ATP levels in skeletal muscle after 12 weeks of hesperetin treatment, suggesting that the mitochondrial cristae structure was restored to some extent and mitochondrial function was moderately enhanced. Hence, we presumed that hesperetin might enhance mitochondrial function via cristae remodeling and ameliorate HFD-induced skeletal muscle dysfunction.

We evaluated the Sestrin2/AMPK/OPA1 signal pathway to investigate how hesperetin affects mitochondrial cristae remodeling. Optic atrophy 1 (OPA1) is on the inner membrane of mitochondria, facilitates cristae formation and modification, and plays a vital role in the regulation of mitochondrial fusion and the production of ATP [49]. OPA1 overexpression was found to protect the stability of mitochondrial cristae and greatly improved mitochondrial damage [50]. Studies show that Aconitine promoted OPA1-mediated remodeling of mitochondrial cristae, thus boosting mitochondrial function [51]. Recent studies have shown that OPA1 mediates mitochondrial cristae remodeling to ameliorate diabetes [52]. We speculated that the rupture or disappearance of mitochondrial cristae and the diminished expression of mitochondrial respiratory chain complexes exhibited in skeletal muscle tissues of HFD mice in the current study might be relevant to OPA1. Adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) is essential in keeping mitochondrial function at bay, and OPA1-associated mitochondrial function and kinetics are also under the regulation of AMPK in aging and obesity [53]. Melatonin has been reported to promote mitochondrial fusion with the AMPK-OPA1 pathway [54]. Aconitine improved mitochondrial function through the induction of AMPK phosphorylation and OPA1 expression [51]. In agreement with previous studies, our present study observed increased AMPK phosphorylation and OPA1 expression after hesperetin treatment. Sestrin2 (SESN2) is an antioxidant protein belonging to the Sestrin gene family that protects cells during oxidative stress [55]. Excitingly, it has been proposed that Sestrin deficiency may contribute to insulin resistance and that SESN2 is a promising insulin sensitizer [56]. It has also been demonstrated that HFD reduced the expression level of SESN2 protein in cardiac tissues of C57BL/6J mice and improved mitochondrial function by empagliflozin to promote SESN2 expression to upregulate AMPK phosphorylation [57]. Resveratrol could induce the expression of a novel antioxidant protein, SESN2, and suppress hepatic lipogenesis [58]. Quercetin has been reported to reduce mitochondrial dysfunction and improve amyotrophic lateral sclerosis by activating the SESN2 and AMPK [59]. To verify whether hesperetin worked by upregulating SESN2 expression, we performed molecular docking and molecular dynamics simulations exhibiting a powerful hesperetin-Sestrin2 binding affinity with a binding energy of –29.84 ± 2.18 kcal/mol. Consequently, we have hypothesized in this study that the flavonoid hesperetin could play a role in treating metabolism-related diseases through the Sestrin2/AMPK/OPA1 signalling pathway, as evident from our findings.

*Limitations*

This study provides preliminary evidence that hesperetin may enhances mitochondrial function through the restoration of cristae structure; however, several limitations should be acknowledged. The small sample size and the exclusive use of male mice limit the generalizability of our findings. Although we observed elevated ATP levels and

increased expression of respiratory chain complexes, direct functional assessments of mitochondrial respiration are lacking. Despite improved glucose tolerance, insulin sensitivity was not evaluated using the HOMA-IR index. Potential systemic metabolic interactions among key organs such as liver, adipose tissue, and skeletal muscle remain unaddressed. And the absence of mechanistic inhibition experiments prevents us from definitively establishing a causal link between the observed improvements and the specific molecular pathways targeted by hesperetin.

## 5. Conclusions

In conclusion, our research suggests that hesperetin is linked with improved mitochondrial structure and function, possibly by activating the Sestrin2/AMPK/OPA1 pathway. The modulation of mitochondrial cristae presents a prospective avenue for addressing metabolic disorders associated with obesity.

Future research will focus on incorporating female mice to assess sex-specific responses; investigating systemic metabolic interactions between liver, adipose tissue, and skeletal muscle using metabolic tracers; and conducting both *in vivo* and *in vitro* inhibition experiments to elucidate the precise molecular mechanisms underlying hesperetin's effects on mitochondrial cristae structure through tissue-specific knockout models or pharmacological inhibitors.

## Abbreviations

AMPK, Adenosine 5'-monophosphate (AMP)-activated protein kinase; ATP, adenosine triphosphate; HE, Hematoxylin-Eosin; HDL-C, high-density lipoprotein cholesterol; IPGTT, intraperitoneal glucose tolerance test; IPITT, intraperitoneal insulin tolerance test; IR, insulin resistance; LDL-C, low-density lipoprotein cholesterol; OPA1, Optic atrophy 1; OXPHOS, oxidative phosphorylation; SESN2, Sestrin2; TC, total cholesterol; TEM, transmission electron microscopy; TG, triglyceride.

## Availability of Data and Materials

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

## Author Contributions

XS contributed to investigation, data curation, formal analysis, visualization, and writing (both original draft and review/editing). XZ, XM, and JL performed investigation and formal analysis. AU participated in methodology and writing-review. XZ provided methodology. GK was responsible for conceptualization, methodology, funding acquisition, and supervision. All authors contributed to editorial changes in the manuscript. All authors have read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

## Ethics Approval and Consent to Participate

All procedures were conducted in accordance with the Guidelines for the Care and Use of Animals established by the Chinese Animal Management Committee, as granted by the Zhengzhou University Life Sciences Institutional Review Board (Approval No. ZZUIRB 2021-12-02). This animal study is reported in accordance with the ARRIVE guidelines (**Supplementary Table 1**).


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## Conflicts of Interest

The authors declare no conflicts of interest.

## Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/IJVNR46645.

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