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Exercise ameliorates muscular excessive mitochondrial fission, insulin resistance and inflammation in diabetic rats via irisin/AMPK activation

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This study aimed to investigate the effects of exercise on excessive mitochondrial fission, insulin resistance, and inflammation in the muscles of diabetic rats. The role of the irisin/AMPK pathway in regulating exercise effects was also determined. Thirty-two 8-week-old male Wistar rats were randomly divided into four groups (n = 8 per group): one control group (Con) and three experimental groups. Type 2 diabetes mellitus (T2DM) was induced in the experimental groups via a high-fat diet followed by a single intraperitoneal injection of streptozotocin (STZ) at a dosage of 30 mg/kg body weight. After T2DM induction, groups were assigned as sedentary (DM), subjected to 8 weeks of treadmill exercise training (Ex), or exercise training combined with 8-week cycloRGDyK treatment (ExRg). Upon completion of the last training session, all rats were euthanized and samples of fasting blood and soleus muscle were collected for analysis using ELISA, immunofluorescence, RT-qPCR, and Western blotting. Statistical differences between groups were analyzed using one-way ANOVA, and differences between two groups were assessed using t-tests. Our findings demonstrate that exercise training markedly ameliorated hyperglycaemia, hyperlipidaemia, and insulin resistance in diabetic rats ($p < 0.05$). It also mitigated the disarranged morphology and inflammation of skeletal muscle associated with T2DM ($p < 0.05$). Crucially, exercise training suppressed muscular excessive mitochondrial fission in the soleus muscle of diabetic rats ($p < 0.05$), and enhanced irisin and p-AMPK levels significantly ($p < 0.05$). However, exercise-induced irisin and p-AMPK expression were inhibited by cycloRGDyK treatment ($p < 0.05$). Furthermore, the administration of CycloRGDyK blocked the effects of exercise training in reducing excessive mitochondrial fission and inflammation in the soleus muscle of diabetic rats, as well as the positive effects of exercise training on improving hyperlipidemia and insulin sensitivity in diabetic rats ($p < 0.05$). These results indicate that regular exercise training effectively ameliorates insulin resistance and glucolipid metabolic dysfunction, and reduces inflammation in skeletal muscle. These benefits are partially mediated by reductions in mitochondrial fission through the irisin/AMPK signalling pathway.

Keywords Exercise training, Irisin, Mitochondrial fission, Inflammation, Diabetes

Type 2 diabetes mellitus (T2DM) is a prevalent chronic condition that significantly impacts adult health globally^{1–3}. The World Health Organization reports that in 2019, diabetes was responsible for 1.5 million deaths and there was a 3% increase in age-standardized death rates from diabetes between 2000 and 2019⁴. Insulin resistance (IR), commonly associated with T2DM, is often caused by mitochondrial dysfunction and chronic inflammation^{5,6}. The skeletal muscle is the largest insulin-sensitive organ, which can absorb up to 80% of post-prandial glucose and is crucial for regulating glucose and lipid metabolism. The function of mitochondria is central to the energy metabolism regulated by skeletal muscle. However, under the condition of T2DM, the homeostasis of mitochondria in skeletal muscle will be broken, and the biological properties of skeletal muscle

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will be impaired, which will affect the metabolism of the whole body^{7,8}. Thus, understanding the mechanisms of mitochondrial homeostasis in skeletal muscle and developing effective interventions are imperative.

Mitochondria play a crucial role in energy metabolism⁹. Their biogenesis and quality control hinge on a dynamic balance between fusion and fission, which governs their morphology and distribution¹⁰. In nutrient-rich environments, mitochondria may fragment into numerous smaller vesicles with reduced functionality¹¹. An increase in these vesicles beyond a certain threshold can trigger the release of reactive oxygen species and the activation of inflammatory pathways, contributing to insulin resistance^{12,13}. A variety of proteins regulates the mitochondrial fission process. One such factor is Dynamin-related protein 1 (Drp1), which is recruited to the separation site of the mitochondrial outer membrane, where it combines with the fission protein 1 (Fis1) and the mitochondrial fission factor (MFF) to form a ring structure to contract the mitochondrial outer membrane resulting in the separation of mitochondria¹⁴. Research by Liu et al. indicated that in obese mice, elevated protein expression levels of Drp1 and Fis1 correlate with disrupted mitochondrial respiratory function¹⁵. Due to the essential role of mitochondrial homeostasis in maintaining cellular energy metabolism and preventing oxidative stress, which is related to the development of IR¹⁶, strategies that target boosting mitochondrial stability could be a viable strategy to decrease IR.

Regular exercise training is an effective strategy for reducing IR and preventing T2DM¹⁷. It enhances glucolipid metabolism and decreases inflammation, both of which contribute to mitigating IR^{18,19}. Additionally, exercise reduces the expression of mitochondrial fission proteins, thereby curbing excessive mitochondrial fission. A recent study noted a marked reduction in Fis1 protein expression in the muscles of sedentary adults following aerobic exercise training, resulting in enhanced insulin sensitivity and lower fasting plasma glucose and fat mass²⁰. However, the specific mechanisms through which exercise impacts excessive mitochondrial fission are not yet fully understood.

Increasing evidence indicates that exercise benefits health by stimulating the production of myokines, proteins released from muscles during exercise that target specific organs via the bloodstream^{21–23}. One such myokine is irisin, derived from the cleavage of fibronectin type III domain-containing protein 5 (FNDC5) during exercise²⁴. Bi et al. found that exogenous irisin treatment (250 µg/kg body weight) significantly diminished the expression of Drp1 and Fis1 in the liver of mice with hepatic ischemia–reperfusion injury, thereby inhibiting excessive mitochondrial fission²⁵. Irisin also promotes energy metabolism, decreases inflammation, and alleviates IR, suggesting that it could modulate glucolipid metabolism and reduce inflammation by influencing mitochondrial fission^{24,26,27}. Nevertheless, the specific mechanisms through which irisin affects mitochondrial fission factors require further investigation.

Irisin interacts with various myokines and factors to regulate energy metabolism during exercise. AMP-activated protein kinase (AMPK), a key regulator of exercise, maintains mitochondrial stability and manages metabolic processes in skeletal muscle²⁸. Fan et al. have demonstrated that irisin can enhance AMPK phosphorylation and reduce the expression of Drp1 and MFF proteins in cardiomyocytes under high glucose conditions, this indicates that irisin collaborates with AMPK to maintain mitochondrial homeostasis²⁹. Given the integral roles of irisin and AMPK in exercise physiology, we hypothesize that Irisin/AMPK could mitigate excessive mitochondrial fission in skeletal muscle during exercise, thus improving glucose and lipid metabolism, and reducing inflammation and IR.

Materials and methods

Experimental animal model and groups

The reporting of animal experiments follows the recommendations in the ARRIVE guidelines. Male Wistar rats (8-week-old) were purchased from the Comparative Medical Centre of Yangzhou University (Yangzhou, China). The rats were housed individually in separate cages subject to a 12-h light/dark cycle at a controlled temperature of 22–24 °C and relative humidity maintained at 50% ± 5%. They had ad libitum access to food and water. All experimental protocols followed the guidelines for using experimental animals and were approved by the Ethics Committee of Yangzhou University (No. 202303139).

All the rats were randomly divided into a control group (Con) and three experimental groups. The Con group was fed a standard diet (1010002, Jiangsu Xietong Medicine Bioengineering Co., China), while the experimental groups were fed a high-fat diet (XTHF45, Jiangsu Xietong Medicine Bioengineering Co., China). Four weeks later, rats in the experimental groups were given intraperitoneal injections of a single dose of streptozotocin (30 mg/kg, 18883-66-4, Sigma-Aldrich Co., USA)³⁰. In contrast, the Con group received an equivalent dose of sodium citrate buffer via intraperitoneal injection. Seven days post-injection, rats exhibiting a fasting glucose level above 16.7 mmol/L were diagnosed with T2DM and subsequently divided into three subgroups: (1) a sedentary T2DM model group (DM); (2) an exercise training group (Ex), where DM rats underwent an 8-week exercise training intervention; (3) an exercise training and cycloRGDyK treatment group (ExRg), where DM rats received both eight weeks of exercise training and cycloRGDyK (irisin inhibitor) injection, with 8 rats in each group (Fig. 1)³¹.

Exercise training and drug intervention protocols

Two weeks after streptozotocin injection, the rats in the Ex and ExRg groups commenced a treadmill exercise training regimen, conducted 5 days a week for 8 weeks. In the initial week, the rats underwent adaptive training; the treadmill speed was progressively increased from 10 to 18 m/min, the incline was set at a 5° uphill gradient, and the duration of exercise gradually extended from 10 to 45 min. From the second through the eighth week, each training session began with a 5-min warm-up at 10 m/min, after which the speed was raised to 18 m/min and maintained for 35 min before being reduced to 0 m/min. The exercise training protocol was designed as moderate-intensity treadmill training and was based on the internationally recognized Bedford exercise load

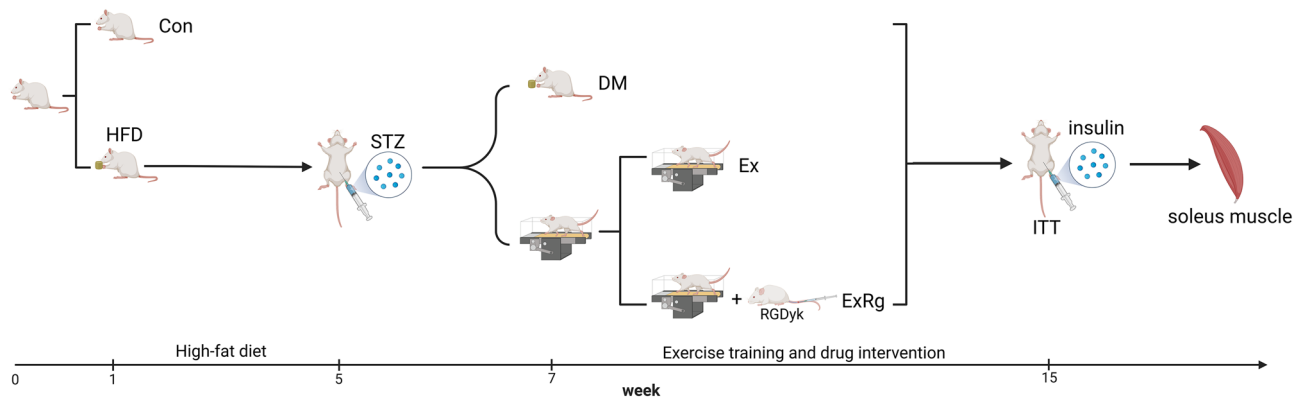


Figure 1. The schematic diagram of the experimental process. *Con* control, *DM* Type 2 diabetes, *Ex* Type 2 diabetes combined with exercise, *ExRg* Type 2 diabetes combined with exercise plus cycloRGDy, *ITT* Insulin tolerance test, *HFD* High fat diet feeding.

standard. This regimen, including the duration and speed of exercise and the principle of progressive overload, was informed by the study conducted by Bedford et al.³².

During treadmill training, rats in the ExRg group were injected with cycloRGDy (GC13923, GLPBIO Biological Company, USA) through the tail vein twice a week. CycloRGDy was prepared in 10% DMSO (GK12002, GLPBIO Biological Company, USA) at a concentration of 1 mg/ml and administered at a dose of 2.5 mg/kg body weight^{31,33}. Rats in the other groups received equivalent doses of 10% DMSO. The weight of the rats was measured prior to each injection to adjust the dosage accordingly.

Insulin tolerance test (ITT)

Upon completion of the exercise training and pharmacological interventions, rats from each group were subjected to a 6-h fast. They then received an intraperitoneal injection of 0.5 IU/kg of regular human insulin. Blood glucose levels were measured prior to and at 30, 60, 90, and 120 min post-injection. Additionally, the area under the insulin tolerance curve was calculated to assess insulin sensitivity.

Detection of glycolipid metabolism parameters

The fasting blood glucose (FBG) values of blood samples collected from the abdominal aorta of rats in each group were determined using a blood glucose detector. Subsequently, the remaining blood samples were centrifuged at 5000 rpm for 10 min to separate the serum for further biochemical analysis. Serum levels of total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and glycated hemoglobin (HbA1c) were measured by an automatic biochemical analyser (BK-400, Biobase, China). Additionally, Serum irisin and fasting insulin (FIN) concentrations were assessed using enzyme-linked immunosorbent assay (ELISA) kits (JL21442-96T & JL10692-96T, Jianglai Biological Technology, China). The Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) was calculated using the formula: $HOMA-IR = FBG \text{ (mmol/L)} \times FIN \text{ (mU/L)} / 22.5$ ³⁴.

H&E staining

Fresh soleus muscle from the left side of the rat was excised and fixed in a 4% paraformaldehyde solution to preserve its structural integrity. After fixation, the soleus muscle samples were dehydrated through a graded series of ethanol concentrations. The dehydrated samples were subsequently processed with xylene and embedded in paraffin for sectioning. Following embedding and sectioning, 5 μm thick paraffin sections of soleus muscle tissue were prepared³⁵.

The paraffin was then removed from the sections using xylene to prepare them for staining. Initially, nuclei were selectively stained blue-purple with hematoxylin. Following rinsing, cytoplasmic components and extracellular matrix elements were stained pink with eosin, creating a stark contrast with the hematoxylin-stained nuclei³⁵. The stained sections were then dehydrated, cleared, and sealed with resin before being examined under an optical microscope (Nikon, Japan).

Immunofluorescence analysis

The sections cut from soleus muscle tissue were deparaffinized and subjected to antigen retrieval under high temperature and pressure. The sections were then blocked with 5% skim milk powder for two hours and subsequently incubated with primary antibodies against IL-1 β (1:250, SC-12742, Santa Cruz), TNF- α (1:250, SC-52746, Santa Cruz), Drp1 (1:200, A17069, ABclonal), and Fis1 (1:200, A19666, ABclonal) overnight at 4 $^{\circ}\text{C}$. After rinsing off the primary antibodies, the sections were incubated with the corresponding secondary antibodies for 2 h. The target proteins in the soleus muscle sections were then visualized using fluorescently labeled tyramine (G1222, Servicebio, China), washed with TBST, and counterstained with DAPI (G1012, Servicebio, China). Images were acquired with a confocal microscope (Carl Zeiss AG, Germany) and analyzed quantitatively using Image-J software.

Real-time quantitative PCR analysis

Total RNA extracted from the right soleus muscle of each group of rats by using Trizol reagent and an equal amount of RNA was reverse transcribed into cDNA using HiScript II Q RT SuperMix for qPCR (R223-01, Vazyme, China). For quantitative PCR, the reagents were added sequentially to a 96-well optical reaction plate and then centrifuged briefly at 2000 rpm to ensure that the mixture was fully settled at the bottom of the wells. The reflector plate was placed in the Real Time PCR System, and amplification was conducted following the protocol of the qPCR SYBR Green Master Mix (Q121-02, Vazyme, China). The primers, synthesized by GENERAL BIOL, are listed in Table 1. To determine primer efficiency, a series of tenfold dilutions of cDNA were prepared, and repeated qPCR analyses were conducted to establish a standard curve. Primer efficiency (E) is calculated using the slope value with the following formula: $E = 10^{-1/\text{slope}} - 1$. Subsequently, the expression of the target gene in the rat soleus muscle was quantified using the comparative method ($2^{-\Delta\Delta C_t}$), with GAPDH serving as the reference gene.

Western blot analysis

Protein concentrations in 30 mg of soleus muscle tissue extracted from the right limb of rats were measured using a BCA kit. Subsequently, 45 μg of protein, dissolved in loading buffer, was subjected to electrophoresis and then transferred onto polyvinylidene difluoride (PVDF) membranes. After blocking with 5% skim milk powder, the PVDF membranes were incubated with primary antibodies against IL-1 β (1:1000, SC-12742, Santa Cruz), TNF- α (1:1000, SC-52746, Santa Cruz), Drp1 (1:2000, A17069, ABclonal), Fis1 (1:2000, A19666, ABclonal), MFF (1:2000, GB114102, Servicebio), MFN2 (1:5000, 12186-1-AP, Proteintech), FNDC5 (1:2000, A18107, ABclonal), AMPK (1:500, A12718, ABclonal), and p-AMPK (1:1000, AP0432, ABclonal) for 12 h at 4 $^{\circ}\text{C}$. This was followed by incubation with the appropriate secondary antibodies. The expression of the target proteins was analyzed using ImageJ software, with β -actin (1:50,000, AC048, ABclonal) serving as a reference.

Statistical analysis

For statistical analysis of the experimental data, we utilized GraphPad Prism software (version 9.5.0, CA, USA). Results are presented as mean \pm standard deviation (SD). To determine statistical differences among multiple groups, one-way analysis of variance (ANOVA) was employed, with post-hoc comparisons conducted using Tukey's or Bonferroni correction method to control for multiple testing errors. Data normality was assessed using the Shapiro–Wilk test prior to applying ANOVA. For comparing two independent groups, an unpaired two-tailed t-test was used. All tests were two-sided, and a p-value of less than 0.05 was considered statistically significant.

Results

Exercise ameliorates hyperglycaemia, hyperlipidaemia, and insulin resistance in diabetic rats

We established a diabetic rat model using a combined protocol of high-fat diet feeding and STZ injection, as previously described³⁰. Following T2DM induction, the rats consistently exhibited hyperglycemia (> 16.7 mmol/L) over three consecutive days, confirming the successful establishment of the T2DM model³⁰. At the end of the 8-week intervention protocol, DM rats demonstrated a significant reduction in body weight, whereas control group rats gained weight relative to their initial measurements at the study's onset. Notably, the body weight of DM rats receiving exercise training slightly decreased after the 8-week intervention, but it was better than sedentary DM rats ($p < 0.05$). These results underscore the detrimental effects of T2DM on body weight and suggest the potential for exercise training to mitigate these effects (Fig. 2).

In the absence of any intervention, the diabetic rats experienced elevated levels of HbA1c, FBG, FIN, and significant insulin resistance (measured by HOMA-IR and ITT) compared to normal rats ($p < 0.05$). However, these adverse effects, including hyperglycemia and insulin resistance, were ameliorated following 8 weeks of treadmill exercise training ($p < 0.05$). The same period of exercise training also significantly mitigated hyperlipidemia. These findings highlight the efficacy of exercise training as a therapeutic strategy to manage conditions induced by T2DM, such as hyperglycemia, hyperlipidemia, and insulin resistance (Fig. 2).

Exercise improves morphology and inflammation of skeletal muscle in diabetic rats

Skeletal muscle plays a vital role in glucose transport and is a primary site of IR in T2DM⁸. Previous research has shown that skeletal muscle dysfunction and chronic inflammation are pivotal in T2DM pathology³⁶. To assess the effects of T2DM on soleus muscle morphology and inflammation, we measured the weight and histological features of the soleus muscle in the left hind limb of DM rats. The soleus muscle weight in the DM group was

Gene	Sense (5'–3')	Antisense (5'–3')	E
Drp1	CAGCGAGATTGTGAGGTTATTGA	CGGATTCAGTCAGAAGGTCATC	94.6%
Fis1	ATCCGTAGAGGCATCGTG	CCTTGAGCCGGTAGTTG	96.3%
MFF	CTGCCGCCACTTCTAATCCTCATC	TCAATGAAGCCGCATCTACCACAG	95.9%
MFN2	CCATGATGCCAACCTGTGA	TCCTGTGGGTGTGTCTCAAGGA	94.7%
FNDC5	AAGTGGTCATTGGCTTGC	GTTGTTATTGGGCTCGTTGT	92.8%
GAPDH	TGCTTACCACCTTCTTGA	TCACCATCTCCAGGAGC	99.2%

Table 1. Primer sequence for real-time qPCR.

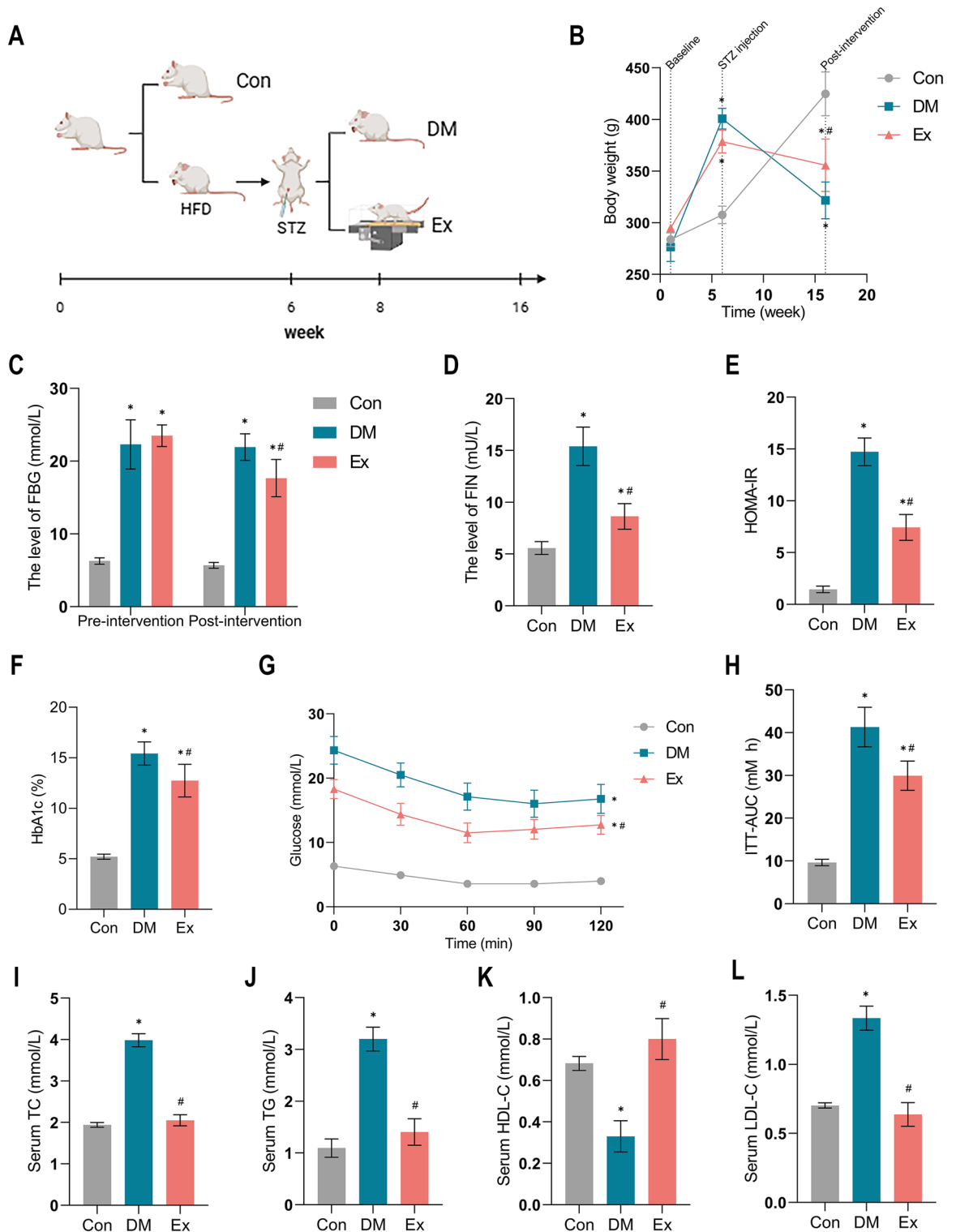


Figure 2. Physiological parameters of rats between groups. (A) Experimental schedules. (B) Trends in body weight. (C,D) Levels of FBG and FIN. (E) Insulin resistance index. (F) Levels of HbA1c. (G) Fasting blood glucose level was determined after insulin injection for 30, 60, 90 and 120 min. (H) Area under the insulin tolerance curve in different groups. (I–L) Levels of lipid parameters. *Con* control, *DM* Type 2 diabetes, *Ex* Type 2 diabetes combined with exercise, *TC* Total cholesterol, *TG* Triglyceride, *HDL-C* High-density lipoprotein cholesterol, *LDL-C* low-density lipoprotein cholesterol, *FBG* Fasting blood glucose, *FIN* Fasting insulin, *HbA1c* Glycated haemoglobin, *ITT* Insulin tolerance test. *Denotes the significant difference compared with *Con* group ($p < 0.05$); # indicates the significant difference compared with *DM* group ($p < 0.05$).

significantly reduced compared to the control group (Fig. 3). Notably, eight weeks of treadmill exercise training intervention substantially reversed the T2DM-related weight loss in these muscles ($p < 0.05$). Moreover, while muscle fibers in the control group were regularly aligned, those in the DM rats were disorganized and fragmented, with considerable gaps filled with inflammatory cells. This structural damage was notably remedied following the exercise training intervention, alongside a reduction in inflammatory cell presence. Further, we utilized immunofluorescence and Western blot analyses to quantify the inflammatory markers IL-1 β and TNF- α in the rat soleus muscle. These markers were significantly elevated in the DM group but were markedly reduced after

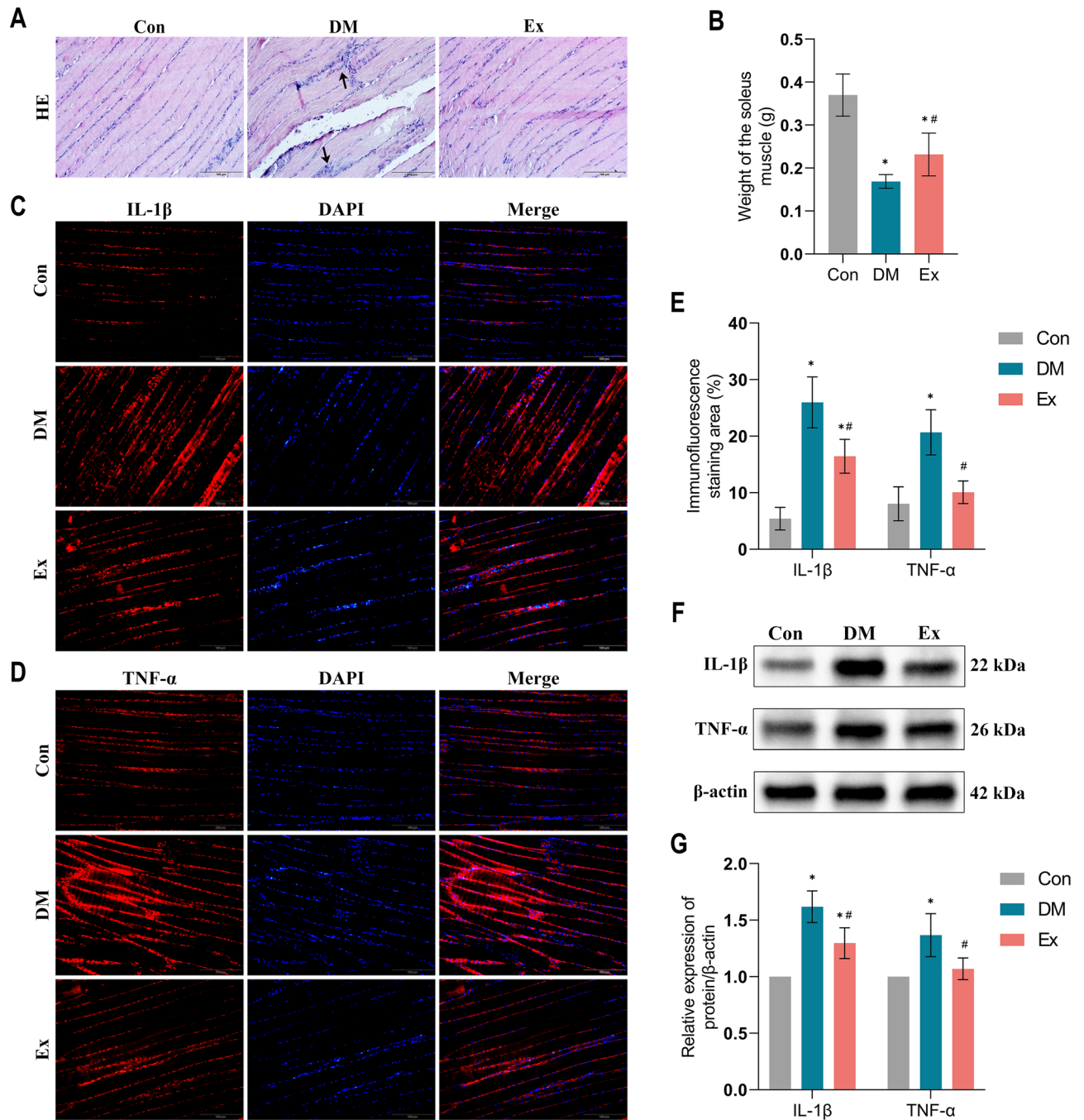


Figure 3. Exercise improves morphology and inflammation of skeletal muscle in diabetic rats. (A) H&E staining of representative Skeletal muscle sections. (B) Weight of soleus muscle. (C–E) Immunofluorescence and its semiquantitative analysis for evaluating IL-1 β and TNF- α expression in soleus muscle of rats between groups. (F–G) Western blot analysis of IL-1 β and TNF- α expression in soleus muscle of rats between groups. Con control, DM Type 2 diabetes, Ex Type 2 diabetes combined with exercise, IL-1 β interleukin-1 β , TNF- α tumor necrosis factor- α . *Denotes the significant difference compared with Con group ($p < 0.05$); #indicates the significant difference compared with DM group ($p < 0.05$).

the exercise training intervention ($p < 0.05$). Collectively, these findings suggest that treadmill exercise training can significantly restore skeletal muscle integrity and diminish inflammation in diabetic conditions.

Exercise suppresses muscular excessive mitochondrial fission in diabetic rats

Increasing evidence suggests a correlation between IR, chronic inflammation, and mitochondrial dysfunction, including disruptions in mitochondrial fusion and fission processes^{15,37}. We investigated the impact of T2DM on the mitochondrial quality control system through immunofluorescence analyses, protein synthesis evaluations, and transcriptional studies.

In DM group, the fluorescence area of mitochondrial fission proteins Drp1 and Fis1 was significantly higher than in the control group, indicating enhanced mitochondrial fission in skeletal muscle (Fig. 4). This aberrant fission was substantially mitigated after an 8-week treadmill exercise training regimen in the Ex group, where Drp1 and Fis1 fluorescence area markedly decreased ($p < 0.05$). Additionally, mRNA and protein levels of Drp1, Fis1, and MFF were considerably elevated in the soleus muscle of DM rats relative to the Con group. Post-exercise training intervention, these levels significantly declined ($p < 0.05$). Conversely, the expression of the mitochondrial fusion protein MFN2 decreased in DM rats and partially recovered following exercise training, although these changes were not statistically significant. These results demonstrate that exercise training can effectively reduce excessive mitochondrial fission in skeletal muscle of diabetic rats.

Exercise up-regulated irisin/AMPK expression, but this is inhibited by cycloRGDyk treatment

Irisin, a myokine discovered recently, is recognized for linking exercise with metabolic disorders^{24,26}. Our prior research demonstrated that irisin secretion induced by exercise plays a crucial role in regulating glucose transport, lipid metabolism, and IR³⁸. These findings suggest that irisin may be the pivotal mediator of exercise's beneficial effects on energy metabolism. To explore the influence of treadmill exercise training on irisin levels, we analyzed its expression in the soleus muscle. We found that both FNDC5 mRNA and the FNDC5/irisin protein levels were significantly reduced in DM rats compared to controls (Fig. 5). However, these levels substantially recovered after an 8-week exercise training regimen ($p < 0.05$). Further studies have indicated that irisin often interacts with other molecular signals to enhance exercise's positive impacts on metabolic diseases, such as through the phosphorylation of AMPK, another key myokine involved in metabolic processes³⁹. Consequently, we investigated whether blocking irisin signaling would alter exercise-regulated AMPK phosphorylation. We observed that phosphorylation levels of AMPK were lower in diabetic rats than in controls, but 8 weeks of exercise training significantly reversed this reduction ($p < 0.05$). Nevertheless, the enhanced phosphorylation of AMPK due to exercise training was inhibited by blocking irisin receptor signaling. This indicates that the beneficial effects of exercise training on diabetic rats might be partly due to the upregulation of irisin, which facilitates AMPK phosphorylation.

Exercise-related beneficial effects could be partially blocked by cycloRGDyk treatment in diabetic rats

We investigated whether inhibiting irisin signaling could obstruct the positive effects of exercise training on hyperglycemia, hyperlipidemia, IR, and inflammation in diabetic conditions. H&E staining revealed that blocking the irisin receptor pathway diminished the beneficial enhancements in skeletal muscle fiber characteristics typically induced by exercise training. This led to greater disorganization of muscle fibers, and enhanced infiltration of inflammatory cells. Administration of cycloRGDyk similarly curtailed improvements in FIN levels, HOMA-IR scores, and insulin tolerance associated with exercise training (Fig. 6). Furthermore, cycloRGDyk treatment effectively obstructed positive alterations in hyperlipidemia observed in diabetic rats undergoing exercise training ($p < 0.05$). Additionally, cycloRGDyk reduced the anti-inflammatory effects of exercise training, as indicated by increased protein expression and fluorescence imaging of IL-1 β and TNF- α in the soleus muscle of exercised rats receiving this treatment ($p < 0.05$).

The effect of exercise in improving muscular excessive mitochondrial fission was blocked by cycloRGDyk injection

To explore the role of irisin signaling in mitigating excessive mitochondrial fission induced by exercise training, diabetic rats undergoing treadmill routines were treated with cycloRGDyk. Immunofluorescence analysis indicated that the fluorescence intensities of Drp1 and Fis1 proteins were significantly elevated in the ExRg group compared to the exercise training-only group (Ex). Subsequent Western blot and RT-qPCR analyses showed that the expressions of mitochondrial fission proteins Drp1 and Fis1 in the ExRg group were markedly higher than those in the Ex group ($p < 0.05$), as depicted in Fig. 7. These results underscore the importance of irisin signaling in controlling mitochondrial fission in the skeletal muscle of diabetic rats engaged in treadmill exercise training.

Discussion

Our findings indicate that exercise training activates the irisin/AMPK pathway, thereby decreasing excessive mitochondrial fission in skeletal muscle. This process is linked to the amelioration of glucolipid metabolism and the reduction of IR and inflammation associated with T2DM, aligning with our initial hypothesis. While previous research has explored the interaction between irisin and mitochondrial dynamics during exercise in various disease models, studies involving T2DM rodent models have been lacking⁴⁰. This study contributes novel experimental evidence that addresses this gap in the literature.

Extensive research has established that regular exercise offers multiple benefits for T2DM management, including enhanced glucose uptake, increased muscle mass, accelerated lipid metabolism, and improved insulin sensitivity^{41–43}. Our results are consistent with these findings. However, these positive effects were attenuated by

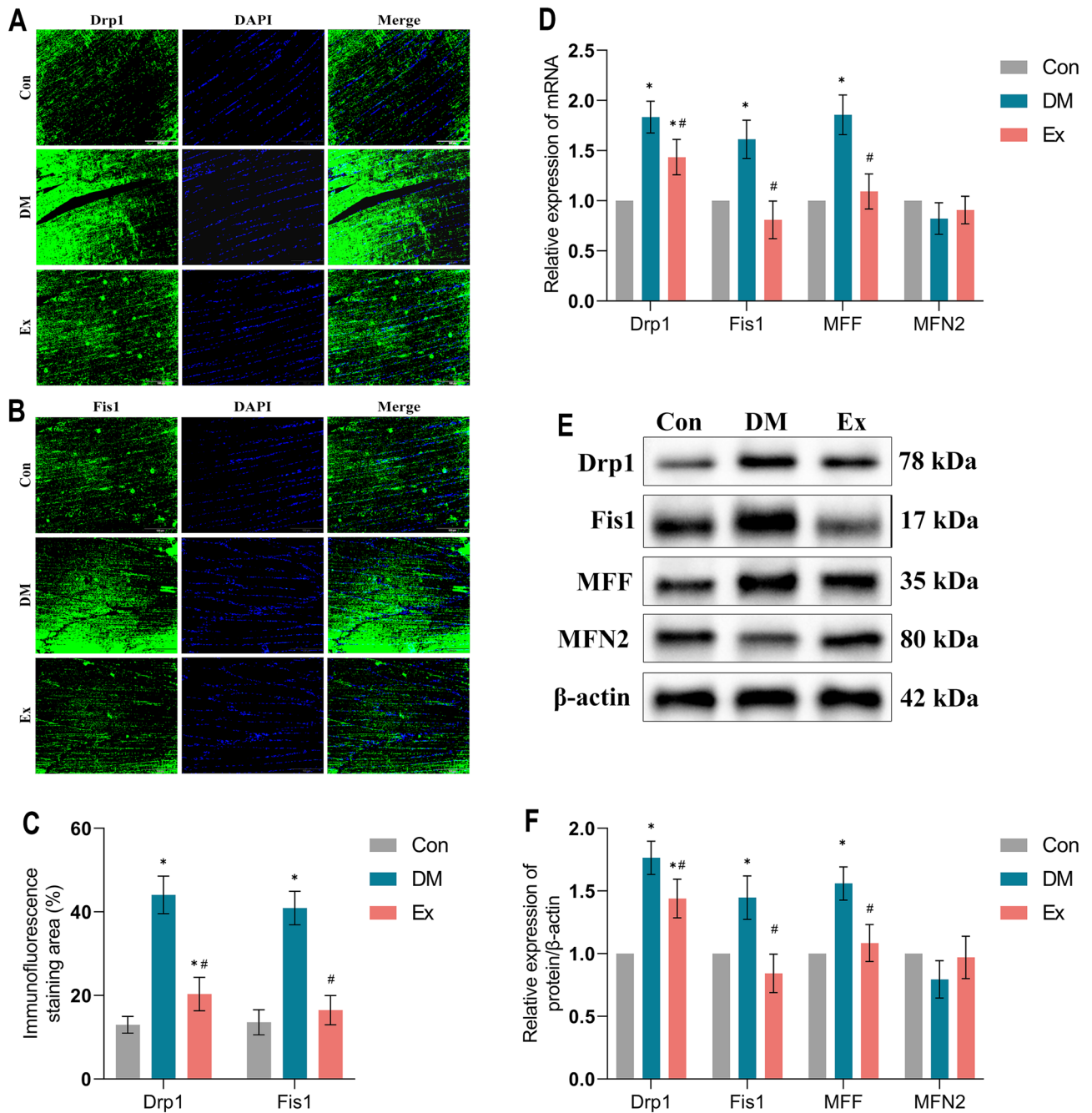


Figure 4. Exercise suppresses muscular excessive mitochondrial fission in diabetic rats. (A–C)

Immunofluorescence and its semiquantitative analysis for evaluating Drp1 and Fis1 expression in soleus muscle of rats between groups. (D) RT-qPCR analysis of the mRNA expression of Drp1, Fis1, MFF and MFN2 in soleus muscle of rats between groups. (E, F) Western blot analysis of Drp1, Fis1, MFF and MFN2 expression in soleus muscle of rats between groups. *Con* control, *DM* Type 2 diabetes, *Ex* Type 2 diabetes combined with exercise, *Drp1* Dynamin-related protein 1, *Fis1* Fission protein 1, *MFF* Mitochondrial fission factor, *MFN2* Mitofusin 2. *Denotes the significant difference compared with *Con* group ($p < 0.05$); # indicates the significant difference compared with *DM* group ($p < 0.05$).

the administration of an irisin antagonist (RGDyk), indicating the potential therapeutic role of irisin in T2DM management. Zheng et al. demonstrated that irisin significantly boosts the PI3K/AKT insulin signaling pathway both in vivo and in vitro, and markedly reduces serine phosphorylation of IRS-1 and IRS-2, which aligns with our observations that irisin enhances insulin sensitivity in diabetic rats³⁹. In this study, we monitored the dynamic insulin response in rats via insulin tolerance tests and computed the HOMA-IR index as a static measure of insulin resistance. The integration of these dynamic and static metrics confirmed that irisin contributes to improved insulin sensitivity and reduced insulin resistance during exercise training. Moreover, Luo et al. reported that mice

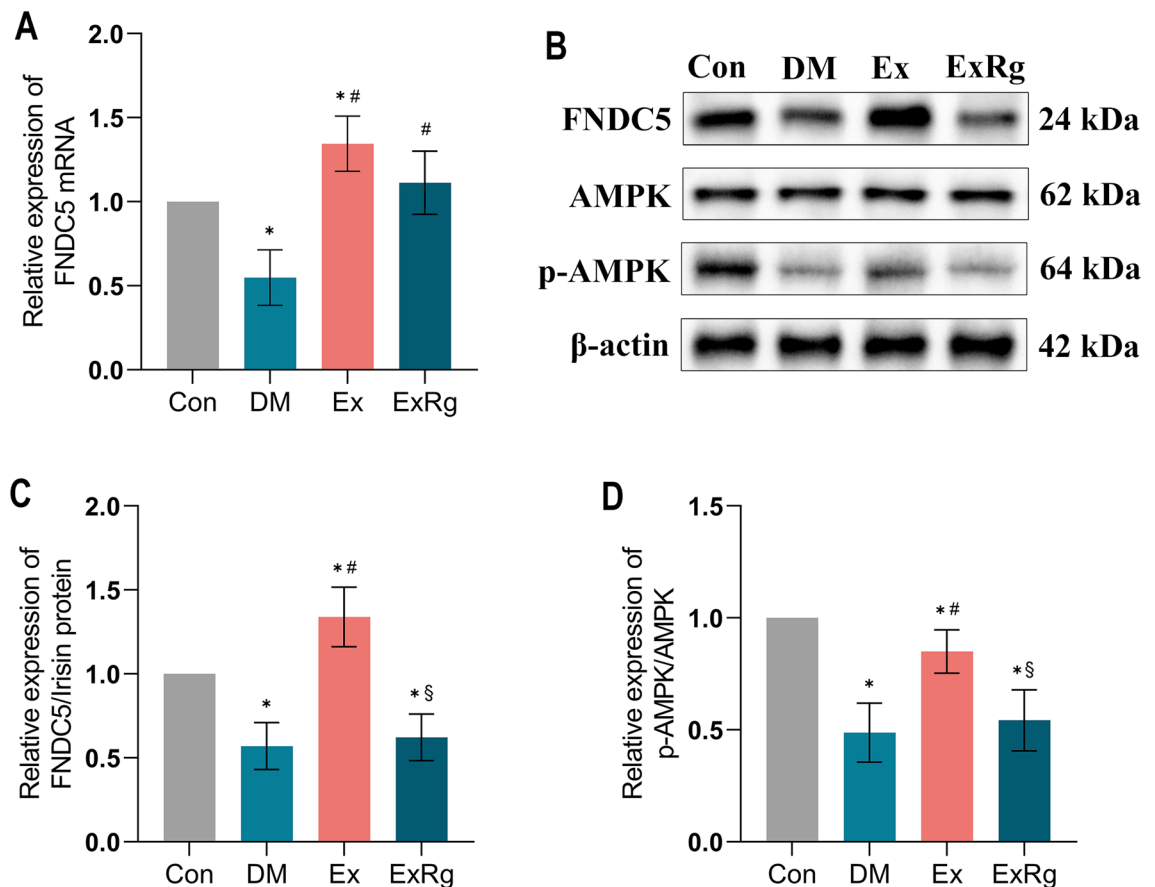


Figure 5. Exercise and cycloRGDyk treatment affect the Irisin/AMPK pathway in the skeletal muscle of diabetic rats. **(A)** RT-qPCR analysis of soleus muscle FNDC5 expressions. **(B–D)** Western blot analysis of FNDC5/irisin and p-AMPK expression in soleus muscle of rats between groups. *Con* control, *DM* Type 2 diabetes, *Ex* Type 2 diabetes combined with exercise, *ExRg* Type 2 diabetes combined with exercise plus cycloRGDyk, *FNDC5* Fibronectin type III domain-containing protein 5, *AMPK* AMP-activated protein kinase. *Denotes the significant difference compared with Con group ($p < 0.05$); #indicates the significant difference compared with DM group ($p < 0.05$); §presents the significant difference compared with Ex group ($p < 0.05$).

deficient in FNDC5/irisin displayed lower HDL-C levels, higher LDL-C levels, and significantly decreased insulin sensitivity, findings that resonate with our own⁴⁴. Additional research has elucidated the anti-inflammatory effects and mechanisms of irisin across various animal and cell models^{27,39,45}, suggesting that irisin signaling enhances the beneficial impacts of exercise on T2DM.

The beneficial impact of exercise on T2DM management is closely associated with improved mitochondrial homeostasis. Studies have established that mitochondrial fission is vital for maintaining mitochondrial integrity in skeletal muscle cells, which is crucial for optimal energy metabolism^{15,37,46}. Our findings indicate that diabetic rats exhibited elevated levels of mitochondrial fission regulatory proteins such as Drp1, Fis1, and MFF, correlated with increased mitochondrial fission, an abundance of damaged mitochondria, and heightened inflammatory pathways. Regular physical activity has been demonstrated to facilitate the removal of damaged or dysfunctional mitochondria and to stimulate mitochondrial biogenesis—the formation of new mitochondria⁴⁷. This process leads to an increase in the number of functional mitochondria in skeletal muscle cells, thereby boosting energy production and enhancing metabolic health. Research by Fealy et al. showed that 12 weeks of aerobic exercise decreased Drp1 activity in the skeletal muscles of obese individuals, improving their insulin sensitivity and fat oxidation⁴⁸. Likewise, our results imply that exercise training may enhance glycolipid metabolism by reducing excessive mitochondrial fission in skeletal muscle.

Irisin, a myokine produced through the activation of PGC-1 α (Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-Alpha) during exercise²⁴, plays a crucial role in cellular energy metabolism. PGC-1 α activates transcription factors that enhance gene expression for mitochondrial complexes, thus improving mitochondrial function. This enhancement is potentially linked to irisin⁴⁹. Zhang et al. discovered that exogenous irisin can enhance the activity of mitochondrial complex I in the brains of mice with Parkinson's disease, thereby improving mitochondrial function⁵⁰. Similarly, Bi et al. demonstrated that irisin administration significantly alleviated the expression of Drp1 and Fis1 in the liver of mice with hepatic injury, inhibiting excessive mitochondrial fission²⁵. This finding parallels those of the current study. In this study, we chose the soleus muscle for analysis to evaluate the role of irisin in skeletal muscle. Soleus muscle is one of the major skeletal muscles stimulated by mechanical forces during treadmill training, and can secrete large amounts of irisin. In addition, Chen et al. reported that

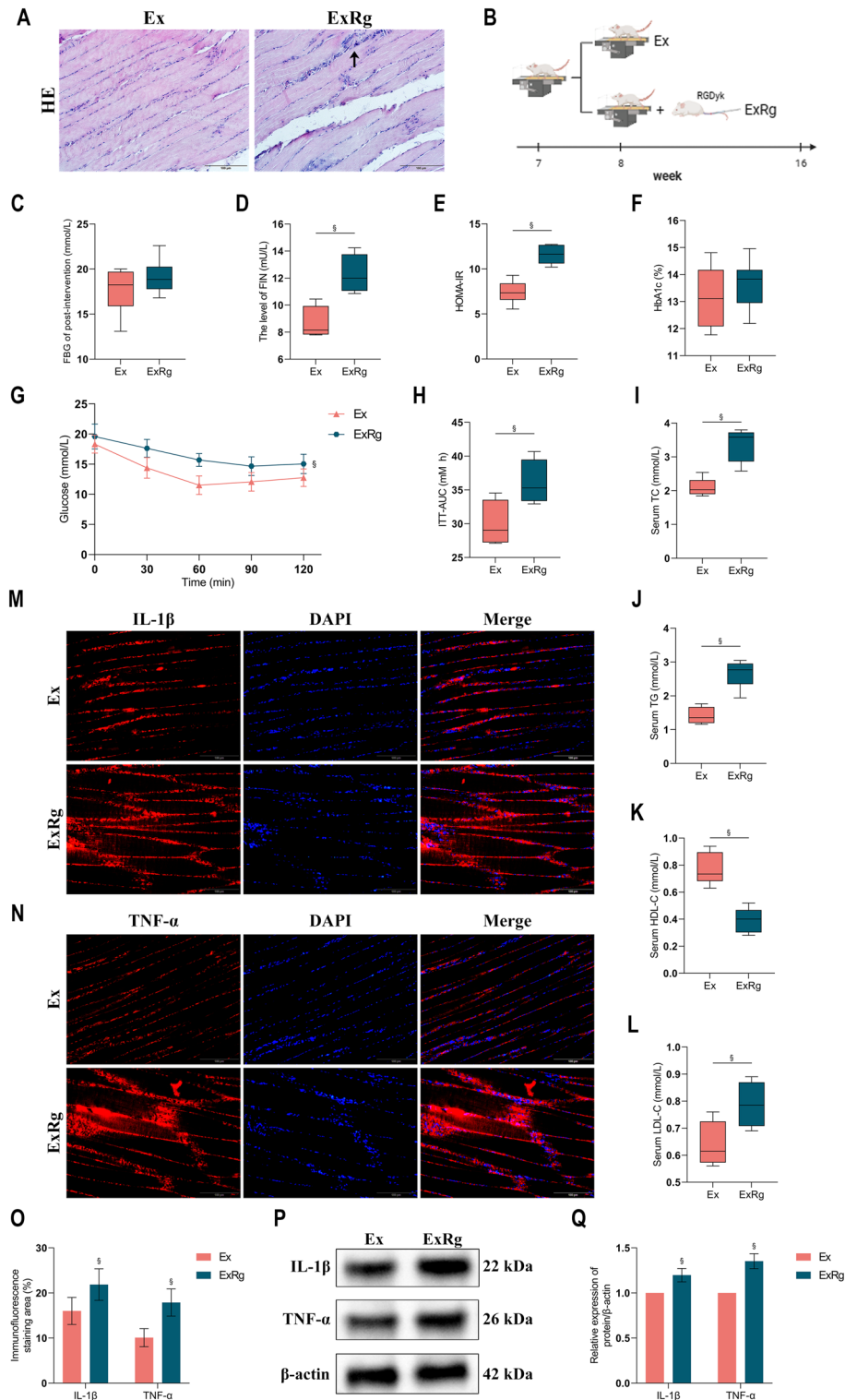


Figure 6. Exercise-related beneficial effects could be partially blocked by cycloRGDyK treatment in diabetic rats. **(A)** H&E staining of representative Skeletal muscle sections. **(B)** Experimental schedules. **(C,D)** Levels of FBG and FIN. **(E)** Insulin resistance index. **(F)** Levels of HbA1c. **(G)** Fasting blood glucose level was determined after insulin injection for 30, 60, 90 and 120 min. **(H)** Area under the insulin tolerance curve in different groups. **(I–L)** Levels of lipid parameters. **(M–O)** Immunofluorescence and its semiquantitative analysis for evaluating IL-1β and TNF-α expression in soleus muscle of rats between groups. **(P–Q)** Western blot analysis of IL-1β and TNF-α expression in soleus muscle of rats between groups. *Ex* Type 2 diabetes combined with exercise, *ExRg* Type 2 diabetes combined with exercise plus cycloRGDyK, *FBG* Fasting blood glucose, *FIN* Fasting insulin, *TC* Total cholesterol, *TG* Triglyceride, *HDL-C* High-density lipoprotein cholesterol, *LDL-C* Low-Density Lipoprotein Cholesterol, *IL-1β* interleukin-1β, *TNF-α* tumor necrosis factor-α, *HbA1c* Glycated haemoglobin, *ITT* Insulin tolerance test. §Presents the significant difference compared with the Ex group ($p < 0.05$).

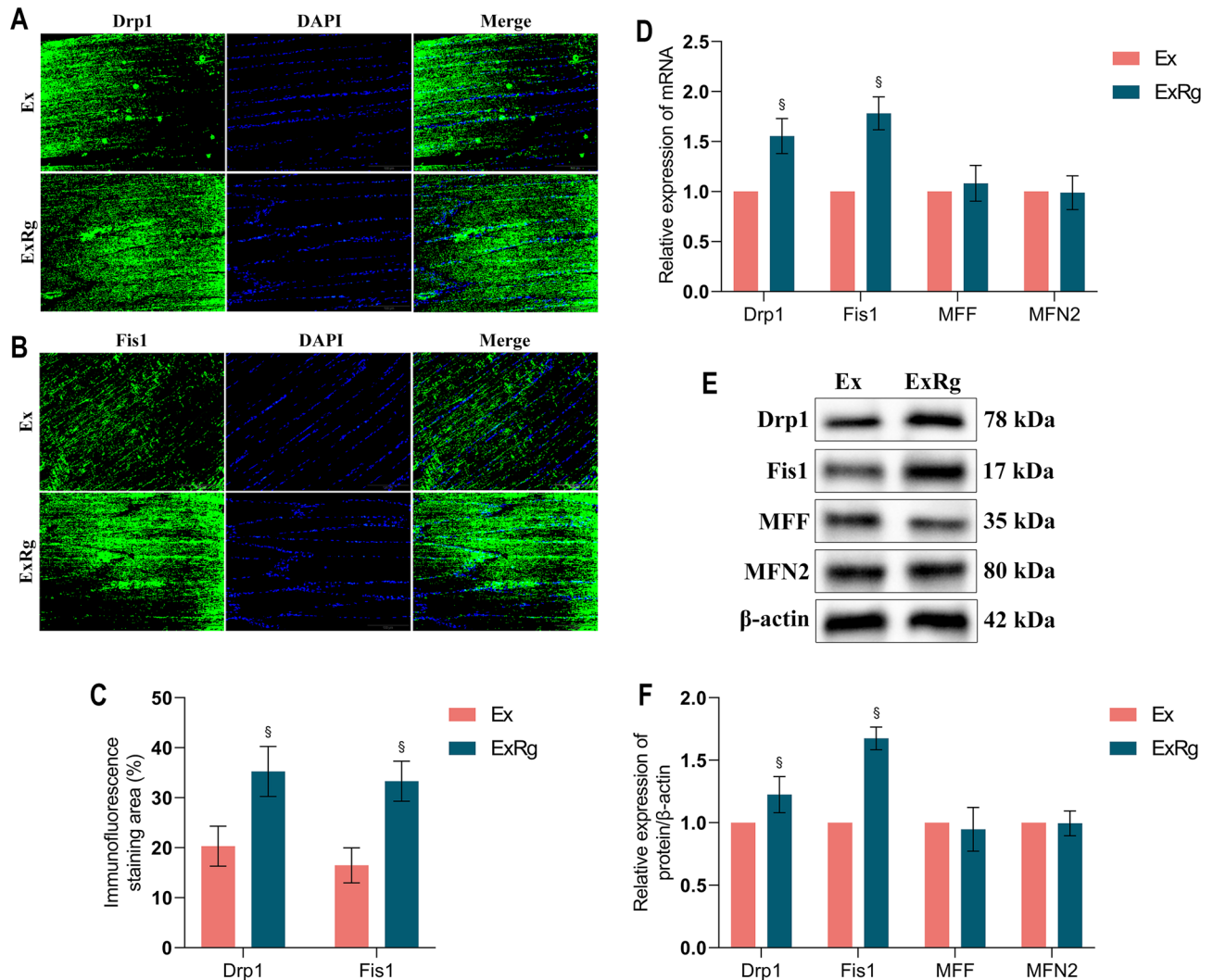


Figure 7. The effect of exercise in regulating muscular excessive mitochondrial fission was blocked by cycloRGDyK injection. (A–C) Immunofluorescence and its semi-quantitative analysis for evaluating Drp1 and Fis1 expression in soleus muscle of rats between groups. (D) RT-qPCR analysis of the mRNA expression of Drp1, Fis1, MFF and MFN2 in soleus muscle of rats between groups. (E,F) Western blot analysis of Drp1, Fis1, MFF and MFN2 expression in soleus muscle of rats in Ex and ExRg group. *Ex* Type 2 diabetes combined with exercise, *ExRg* Type 2 diabetes combined with exercise plus cycloRGDyK, *Drp1* Dynamin-related protein 1, *Fis1* Fission protein 1, *MFF* Mitochondrial fission factor, *MFN2* Mitofusin 2. [§]Presents the significant difference compared with the Ex group ($p < 0.05$).

oxidative muscles such as soleus muscle can secrete more irisin than glycolytic muscles such as gastrocnemius or quadriceps muscle⁵¹. The results we obtained suggest that irisin, which binds to the $\alpha V\beta 5$ integrin, may regulate excessive mitochondrial fission in the skeletal muscle of diabetic rats during exercise.

Extensive research has shown that irisin collaborates with various myokines and metabolic factors to regulate energy metabolism during exercise^{39,52}. Specifically, AMPK serves as a crucial downstream signaling molecule for irisin, essential for maintaining mitochondrial homeostasis and managing glucose and lipid metabolism²⁸. Our research indicates that AMPK activation is closely linked to exercise training, as evidenced by enhanced phosphorylation at Thr172 in T2DM rats during treadmill running, an effect that is inhibited by an irisin antagonist (RGDyK). Studies by Tang et al. and Lee et al. have further established that irisin treatment augments AMPK phosphorylation at Thr172 in a time- and dose-dependent manner^{53,54}. These findings highlight the role of irisin in promoting AMPK phosphorylation during exercise. Moreover, it has been demonstrated that AMPK activation facilitates AKAP1 phosphorylation at Ser103 and its interaction with protein kinase A (PKA), which in turn reduces the activity and expression of Drp1^{48,55,56}. Consequently, the irisin/AMPK pathway may mitigate excessive mitochondrial fission in the skeletal muscle of T2DM rats during exercise.

In summary, our research supports the hypothesis that exercise training can enhance glycolipid metabolism, insulin resistance (IR), and inflammation by reducing excessive mitochondrial fission in the skeletal muscle of diabetic rats. Our findings also suggest that the irisin/AMPK pathway plays a role in this process. However, our study has several limitations that future research should address. Primarily, while this study provides in vivo evidence of the effects of exercise on mitochondrial dysfunction and IR in diabetic rats, including the role of the

irisin/AMPK pathway, further in vitro studies are necessary to clarify how irisin influences the molecular mechanisms involved, potentially through cell culture and transgenic experiments in diabetic models. Moreover, while we have shown that the irisin/AMPK pathway can decrease insulin resistance and improve insulin sensitivity during exercise, its specific impacts on insulin resistance pathways remain unclear. Additionally, our study does not include measurements of mitochondrial function markers such as mitochondrial complexes. Furthermore, the lack of an Oral Glucose Tolerance Test (OGTT) also constitutes a limitation. Consequently, more studies are essential to explore these aspects thoroughly.

Data availability

The authors of this article will make the raw data supporting their conclusions available, without any hesitation or reservation. Renqing Zhao (renqing.zhao@yzu.edu.cn) should be contacted if someone wants to request the data from this study.

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Author contributions

J.L. and R.Z. designed the study. J.L. and X.Z. wrote the first draft of the manuscript. J.L., B.Z., C.Z., B.W., Y.W., X.T., Y.S., H.X., X.Z. and N.L. performed the data curation and analysis. All authors have read and agreed to the published version of the manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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