

Review Article

Aerobic exercise promotes MiR-29c-3p to regulate apoptosis and autophagy of rat skeletal muscle cells through Sirt1 pathway

Peng Zhang¹, Qing Zhao², Peng Yang², Yong Wang³, Shaoyong Wan⁴

¹Department of Sports Training and Rehabilitation, Sports College, China University of Mining and Technology, Xuzhou 221000, Jiangsu, China; ²Department of Sports Human Medicine, Sports Department, Harbin Institute of Technology, Harbin 150000, Heilongjiang, China; ³Department of Sports Training and Research, Sports College, Jiangxi Normal University, Nanchang 330000, Jiangxi, China; ⁴Department of Sports Training and Research, Physical Education College, Jinggangshan University, Ji'an 343009, Jiangxi, China

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Abstract: Background: To verify that aerobic exercise (EX) promotes miR-29c-3p to regulate apoptosis and autophagy of skeletal muscle cells in rats through Sirt1 pathway. Methods: Seventy male rats were selected and stochastically divided into healthy control group (ND group; normally fed), a type 2 diabetes mellitus group (T2DM group; injected with streptozocin (STZ) at 35 mg/kg), an aerobic exercise intervention group (T2DM + EX group), a T2DM + miR-29c-3p-mimics group, a T2DM + EX + miR-29c-3p-mimics group, a T2DM + Sirt1 siRNA group and a T2DM + EX + Sirt1 siRNA group. The expression changes of miR-29c-3p and Sirt1 were detected dynamically by real-time fluorescence quantitative PCR. Flow cytometry was adopted to measure cell apoptosis in each group, and Western blotting (WB) was utilized to detect the expression of apoptosis-related proteins (caspase-9, caspase-3, Bax, Bcl-2) and autophagy proteins (LC3-II, LC3-II/I ratio, Beclin1) in the cells. Results: EX inhibited the apoptosis of myotube cells. T2DM can be alleviated by down-regulating pro-apoptotic proteins Bax, caspase-3 and caspase-9 and up-regulating anti-apoptotic proteins Bcl-2, as well as by reducing autophagy proteins LC3-II, LC3-II/I ratio and Beclin1. MiR-29c-3p was remarkably up-regulated in T2DM, while Sirt1 was notably down-regulated. MiR-29c-3p overexpression or Sirt1 inhibition aggravated T2DM, increased apoptosis and decreased autophagy activity of myotube cells, while the above trend was reversed after EX intervention. Both miR-29c-3p up-regulation and Sirt1 down-regulation could eliminate the protective effect of EX on T2DM. Conclusion: EX can improve apoptosis and autophagy of skeletal muscle cells by down-regulating miR-29c-3p-mediated Sirt1 pathway.

Keywords: Aerobic exercise, miR-29c-3p, Sirt1, apoptosis

Introduction

Skeletal muscle, which accounts for approximately 40% of human body weight, is mainly responsible for insulin-stimulated glucose uptake in healthy subjects and is also considered to be the main site of peripheral insulin resistance [1]. Studies have shown that skeletal muscle is not only a mechanism for generating strength, but an important organ for glucose storage and metabolism, so improving and maintaining skeletal muscle mass is one of the effective methods to control blood glucose [2]. In addition, it is reported that many adverse health risks, including T2DM, coronary heart

disease, and the risk of shortening life, can be induced by lack of exercise [3]. Physical exercise can increase the level of basal metabolism and facilitate blood circulation in various parts of the human body, and doing it in a regular manner contributed to lowering the possibility of getting T2DM and improving blood glucose control in patients with diabetes [4]. Currently, it has been reported that the protective mechanism of EX on T2DM can be reflected in the following several pathways: one is to regulate the signaling pathway to improve the mechanism of apoptosis and necrosis, and the other is to reduce the expression of autophagy protein by reversing the decline of skeletal muscle therapy

caused by diabetes [5, 6]. In this study, the signaling pathway, apoptosis mechanism and autophagy are integrated to investigate the potential regulatory mechanism of EX in skeletal muscle of T2DM rats and to find a new therapeutic target.

MiRNA is a type of short non-coding RNA, which is essential in the regulation of cellular processes in response to environmental changes, and can also change the biosynthesis of miRNA under stress conditions [7]. At present, a flood of miRNAs have been identified to be differentially expressed in skeletal muscle of T2DM patients, indicating their roles as key metabolic regulators in this disease [8]. In the study of Esteves et al. [9], miR-29c-3p affected glucose metabolism, insulin response and lipid metabolism in skeletal muscle, and was also an important regulator of glucose metabolism and lipid oxidation. In addition, Peng et al. showed that the imbalance of miR-29c-3p was related to the pathogenesis of diabetic nephropathy [10]. The preceding research suggests that miR-29c-3p participates in the pathological mechanism of T2DM. While Sirt1 has now become a key metabolic sensor for various metabolic tissues as it can regulate glucose and lipid metabolism through its deacetylase activity as proposed in numerous studies [11]. Also, Sirt1 expression is abnormally low in the blood of T2DM patients, suggesting that it may be involved in the disease process of T2DM [12].

At present, there are few studies on the regulation mechanism of EX in T2DM, and whether EX can play a protective role by mediating miR-29c-3p and Sirt1 remains to be studied.

Materials and methods

Laboratory animals, materials and reagents

Seventy male C57BL/6 rats (Junke Biological Engineering Co., Ltd., Nanjing, China, J006) with an average body weight of 27 ± 3 g and an age of 16 weeks were purchased, and raised at temperature and humidity of $22 \pm 2^\circ\text{C}$ and 50-65% under normal 12 h circadian rhythm. According to the standard regulations of animal care, water and food were available for the rats, and the adaptation time was one week. L6 myotube cells (Xuanke Biotechnology Co., Ltd., Shanghai, China, XK-XB-1478), microplate reader (Yanhui Biotechnology Co., Ltd., Shanghai,

China, HBS-1096A), flow cytometer (FC) (Ranger Apparatus Co., Ltd., Shanghai, China, NovoCyte), ultraviolet spectrophotometer (PKUCare Industrial Park Technology Co., Ltd., Beijing, China, UV-1100), PCR instrument (Image Trading Co., Ltd., Beijing, China, 100073), Double luciferase reporting system (Top Biotech Co., Ltd., Shenzhen, China). Annexin V-FITC/PI double staining apoptosis kit, trypsin, PVDF membrane (Create Biotechnology Co., Ltd., Beijing, China, 120248, 14260, ISEQ00011), phosphatic buffer solution (PBS), BCA kit, ECL luminescent reagent (Junrui Biotechnology Co., Ltd., Shanghai, China, JR01521, LCB004, UFML05294), DMEM medium containing 10% fetal bovine serum (FBS) (Xinyu Biotechnology Co., Ltd., Shanghai, China, 19-0040-100). Lysate (Zhenyu Biotechnology Co., Ltd., Shanghai, China, PS0033), horseradish peroxidase-labeled goat anti-rabbit secondary antibody (Bersee Science and Technology Co., Ltd., Beijing, China, BHR201). Trizol Kit (Mingjing Biotechnology Co., Ltd., Shanghai, China, 5003050), Reverse Transcription Kit (Qiming Biotechnology Co., Ltd., Shanghai, China, OX02700), SYBR Premix Ex Taq TM Kit (Yihui Biotechnology Limited Company, Shanghai, China, HRR420A), Lipofectamine™ 2000 (Mito Biotechnology Co., Ltd., Shanghai, China, 11668019), TUNEL Apoptosis Detection Kit (Create Biotechnology Co., Ltd, Beijing, China, 10462). Gas analyzer, modular incubator (Yuyan Instrument Co., Ltd., Shanghai, 53222, customized).

Modeling

Seventy male rats were stochastically divided into healthy control group (ND group; normally fed), type 2 diabetes mellitus group (T2DM group; injected with streptozocin at 35 mg/kg), aerobic exercise intervention group (T2DM + EX group), T2DM + miR-29c-3p-mimics group, T2DM + EX + miR-29c-3p-mimics group, T2DM + Sirt1 siRNA group and T2DM + EX + Sirt1 siRNA group. Model establishment method [13]: The ND group was raised normally and grew naturally, while the other groups were fed with self-made high-sugar and high-fat diet, and were given a one-time injection of STZ (35 mg/kg). In addition, T2DM group, T2DM + EX group, T2DM + miR-29c-3p-mimics group, T2DM + EX + miR-29c-3p-mimics group, T2DM + Sirt1 siRNA group and T2DM + EX + Sirt1

Table 1. Primer sequences

	Forward	Reverse
miR-29c-3p-mimics	5'-UAGCACCAUCUGAAAUCGGUUA-3'	5'-ACCGAUUUCAGAUGGUGCUAUU-3'
Sirt1 siRNA	5'-GGUCAAGGGAUGGUUUUA-3'	5'-UAAAUACCAUCCCUUGACC-3'
NC-mimics	5'-UUCUCCCAACGUGUCACGUTT-3'	5'-ACGUGACACGUUCGAGAATT-3'

Table 2. Primer sequences

	Forward	Reverse
miR-29c-3p	5'-CGATTTCTCCTGGTGTTC-3'	5'-ACCGATTTCAAATGGTGC-3'
U6	5'-CTCGCTTCGGCAGCACA-3'	5'-AACGCTTCACGAATTGCGT-3'
Sirt1	5'-GCAACAGCATCTTGCTGAT-3'	5'-GTGCTACTGGTCTCACTT-3'
GAPDH	5'-CCGTATTCAGCATTCTATGCTCTC-3'	5'-TGGATACACACTCTGGGGCT-3'

siRNA group were given normal saline, EX, miR-29c-3p-mimics, EX + miR-29c-3p-mimics, Sirt1 siRNA + EX + Sirt1 siRNA, respectively. After 12 hours of fasting, blood samples were taken from the caudal vein of all rats, and the fasting blood glucose (FBG) was measured. A FBG value of ≥ 11.1 mmol/L indicated that the modeling was successful.

EX

After successful modeling, animal experimental treadmills were used, and all groups except the ND group were subjected to adaptive training for two weeks, followed by receptive training. The training regimen was as follows: the rats were given EX on a treadmill five days a week for eight weeks. At the last 4 weeks, the rats were required to gradually run for 30 min/day at a speed of 15 m/min, until they gradually run for 40 min/day at a 0% slope of 18 m/min. The rats were fast for 12 hours before the exercise, and 24 hours after exercise, they were sacrificed to remove the soleus muscle for subsequent experiments. The Ethics Committee at the Affiliated Hospital of Harbin University of Technology approved the animal experimental protocol and the study was conducted in accordance with the Declaration of Helsinki (7th amendment). All the operation was performed in strict accordance with and National Institutes of Health (NIH) guidelines on laboratory animal care and use.

Cell culture and transfection

The purchased L6 myotube cells were placed in DMEM containing 10% FBS and cultured in an incubator of 5% CO₂ at 37°C. Transfection with miR-29c-3p-mimics (overexpression of miR-

29c-3p), Sirt1 siRNA (inhibition of Sirt1 expression) and NC-mimics (negative control) was then performed after cells finished passage. L6 myotube cells were randomly divided into control group, T2DM + NC group, T2DM + EX group, T2DM + miR-29c-3p-mimics group, T2DM + EX + miR-29c-3p-mimics group, T2DM + Sirt1 siRNA group, and T2DM + EX + Sirt1 siRNA group. The T2DM model was established in all other groups except the control group. After the cells in each group adhered to the wall and grew to a certain density, the culture medium was abandoned and replaced with a DMEM containing 25 mmol/L glucose (**Table 1**).

RT-PCR detection for miR-29c-3p and Sirt1 expression

The total RNA was extracted using Trizol kit, and its purity, concentration and integrity were determined by ultraviolet spectrophotometer and agarose gel electrophoresis. RNA was reverse-transcribed to cDNA following the reverse transcription kit instructions. With GAPDH or U6 as internal parameters, the reaction was carried out on the PCR apparatus with SYBR Premix Ex Taq TM kit. PCR amplification cycle conditions: first to pre-denature at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, then annealing and extension at 60°C for 60 s. Data were obtained after three repeated experiments, and the relative expression was calculated using 2- $\Delta\Delta CT$ (**Table 2**).

WB detection

Soleus muscle (50 mg) was lysed with 500 μ L lysate. After homogenization in ice bath, it was centrifuged at 12000 \times g and 4°C for 20 min. The supernatant was taken and the protein

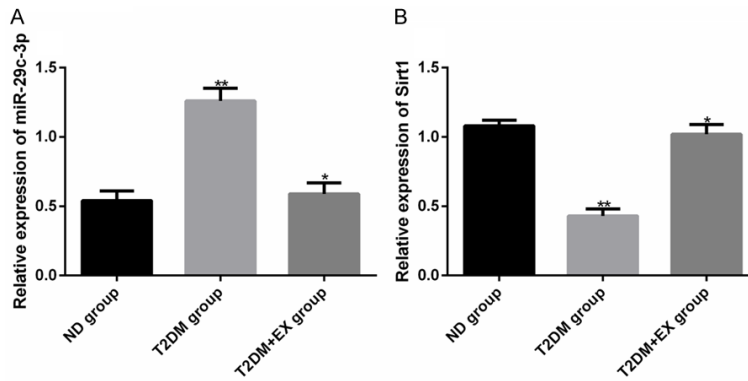


Figure 1. Effects of EX on miR-29c-3p and Sirt1. A: MiR-29c-3p level in each group. B: Sirt1 level in each group. Note: * indicates $P < 0.05$.

Sirt1 3'-UTR containing the 3'-untranslated region (3'-UTR) of the predicted binding site wild-type (Sirt1 wt) or mutation (Sirt1 mut) was subsequently cloned in the vector. After validation by DNA sequencing, the plasmids (miR-29c-3p-mimics, NC mimic) were transfected into cardiomyocytes according to the instructions of Lipofectamine™ 2000, and the cells were collected 48 h after transfection for analysis with the dual luciferase reporter (DLR) system.

concentration was determined by BCA. After separation by 12% SDS-PAGE electrophoresis, the protein was transferred to a PVDF membrane before it was sealed in 5% skim milk. Followed by an immune response, the membrane was incubated with a primary antibody (1:1000) at 4°C all night long. Next, the membrane was rinsed to remove the primary antibody, incubated with horseradish peroxidase-labeled goat anti-rabbit secondary antibody (1:1000) for 1 h at 37°C, and rinse 3 times with TBST for 5 min each. Upon completion, ECL luminescence reagent was used to develop and fix, and Quantity One infrared imaging system was applied to take pictures. The relative expression level of the protein to be tested = the gray value of the band to be tested/the gray value of the internal reference protein band.

Apoptosis detection (flow cytometry, FC)

FC was utilized to detect the apoptosis rate of cardiomyocytes in each group as instructed by the annexin V-FITC/PI double staining apoptosis kit. The cells were digested fully with trypsin, washed twice with PBS before collecting them in the centrifuge tube. Then, annexin-V-FITC labeling solution (20 µL) was added to buffer solution (1 ml), followed by the addition of 20 µL PI reagent, and cultivated at room temperature and in darkness for 5 min. The results were detected by FC and the average value was obtained by repeating the experiment for 3 times.

Target gene detection

TargetScan was adopted to predict the binding sites of miR-29c-3p and Sirt1. A fragment of

Statistical analysis

Data analysis and picture rendering were performed by GraphPad 6. All the data were described as mean \pm standard deviation (mean \pm sd). An independent sample t-test was adopted for inter-group comparisons, one-way ANOVA was used for multi-group comparisons (expressed as F), and the post-hoc pairwise comparison was verified by the LSD-t test. Multi-time expressions were analyzed by repeated measures ANOVA (expressed as F), and the post hoc test was conducted by Bonferroni. $P < 0.05$ indicated a statistically significant difference.

Results

Effects of EX on miR-29c-3p and Sirt1

QRT-PCR results showed that the miR-29c-3p level in ND and T2DM + EX groups was evidently down-regulated, while that in T2DM group was obviously boosted ($P < 0.05$). Regarding Sirt1 expression, it presented dramatically enhanced level in ND and T2DM + EX groups, while its level in T2DM was notably reduced ($P < 0.05$), and the difference was statistically significant ($P < 0.05$) (**Figure 1**).

Effects of EX on skeletal muscle cell apoptosis-related proteins

Observing cell apoptosis in each group, it was found that the apoptosis rate in T2DM group elevated remarkably compared with ND group ($P < 0.05$), while T2DM + EX group could alleviate apoptosis and lower the apoptosis rate ($P < 0.05$). The detection of apoptosis-related protein levels in each group revealed that the

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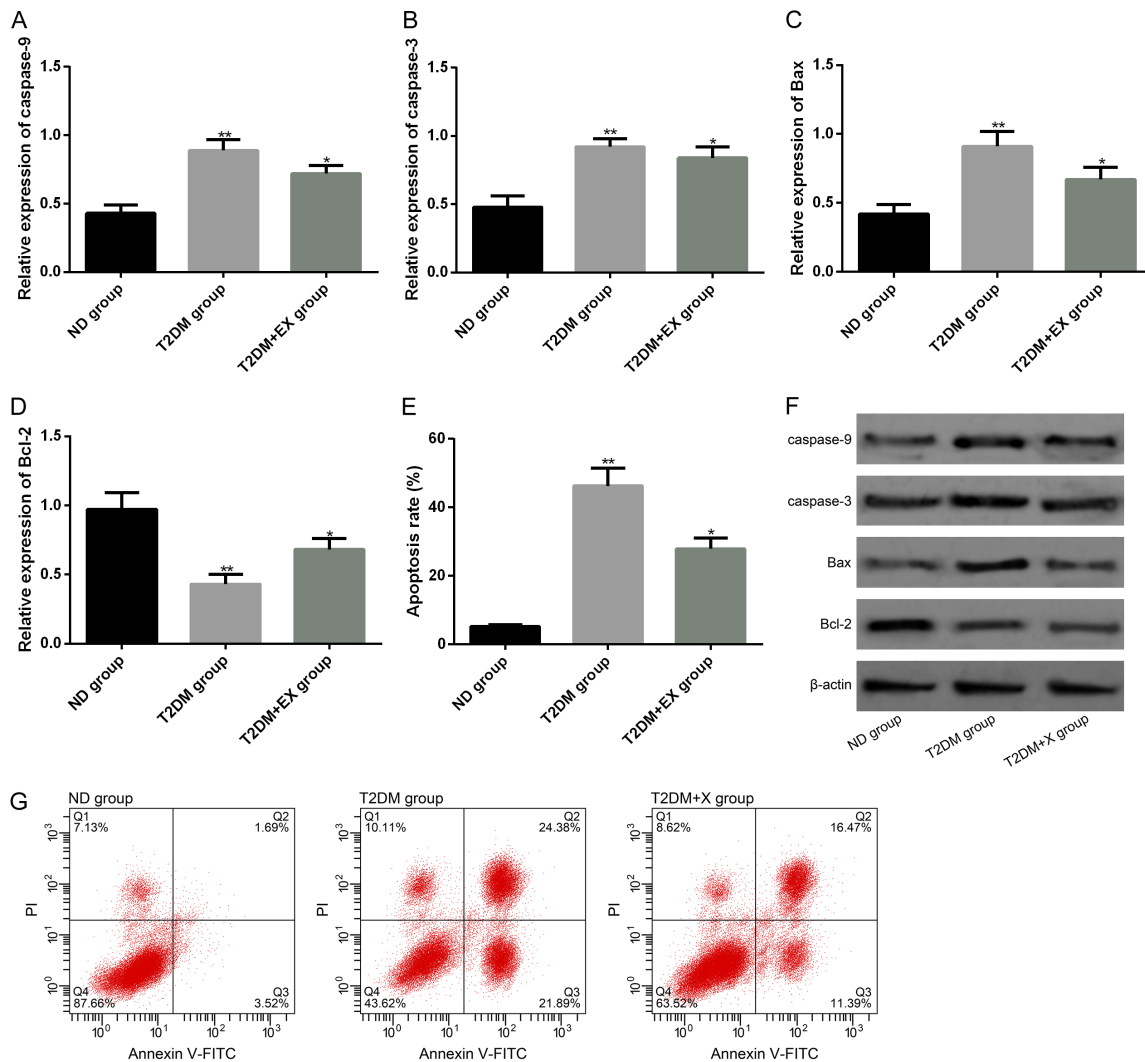


Figure 2. Effects of EX on skeletal muscle cell apoptosis-related proteins. A-D: Apoptosis-related protein expression in each group. E: Comparison of apoptosis rate among the three groups. F: Apoptosis-related protein map. G: FC diagram of apoptosis. Note: * indicates $P < 0.05$.

expression and content of caspase-9, caspase-3 and Bax were noticeably elevated in T2DM group compared with the other two groups ($P < 0.05$), whereas the expression and content of Bcl-2 were markedly decreased ($P < 0.05$). While after EX intervention, T2DM + EX group showed down-regulated caspase-9, caspase-3 and Bax, and up-regulated Bcl-2 ($P < 0.05$) (**Figure 2**).

Effects of EX on skeletal muscle autophagy-related protein expression

The expression of autophagy protein in skeletal muscle of each group was observed. Compared with ND group, LC3-II, LC3-II/I ratio and Beclin1 in the other two groups up-regulated obviously,

while EX alleviated this phenomenon and down-regulated LC3-II, LC3-II/I ratio and Beclin1 ($P < 0.05$) (**Figure 3**).

MiR-29c-3p eliminated the protective effect of EX on T2DM-induced skeletal muscle

The preceding research demonstrated that EX played a protective role in the T2DM model. While after miR-29c-3p-mimics transfection, we found that the miR-29c-3p overexpression reversed the effect of EX on apoptosis and autophagy protein, resulting in increased apoptosis rate and autophagy protein activity ($P < 0.05$), and there were little differences in the above indexes between T2DM + miR-29c-3p-mimics group and T2DM + EX + miR-29c-3p-mimics group ($P > 0.05$) (**Figure 4**).

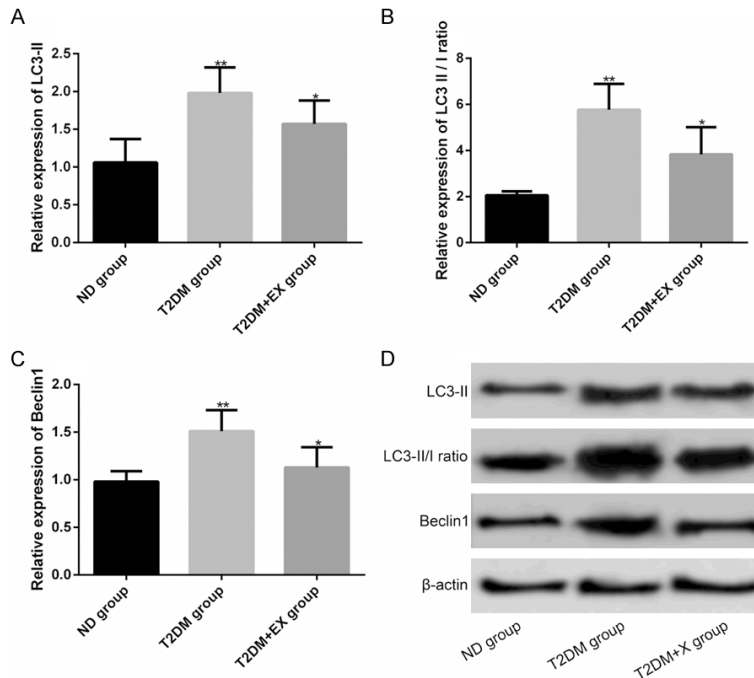


Figure 3. Effects of EX on skeletal muscle autophagy-related protein expression. A-C: Autophagy-related protein expression in skeletal muscle of rats in each group. D: Autophagy-related protein map. Note: * indicates $P < 0.05$.

Sirt1 eliminated the protective effect of EX on T2DM-induced skeletal muscle

After Sirt1 siRNA transfection, silencing Sirt1 was found to evidently inhibit the increase of Sirt1 induced by EX, resulting in decreased Sirt1 content ($P < 0.05$). In addition, silencing Sirt1 remarkably reversed the effect of EX on apoptosis and autophagy proteins, as well as the protective effect of EX on T2DM model (Figure 5).

Target gene identification

We found through Targetscan7.2 that there were targeted binding sites between Sirt1 and miR-29c-3p. After miR-29c-3p-mimics, Sirt1-Wt's luciferase activity was significantly inhibited ($P < 0.05$). Moreover, compared with NC, the expression and content of Sirt1 in miR-29c-3p-mimics group were dramatically reduced ($P < 0.05$) (Figure 6).

Discussion

EX has been used as a basic treatment to control blood glucose levels and prevent T2DM complications, and it is also an important fac-

tor in preventing muscle atrophy in T2DM patients [14]. Yet, the mechanism is not fully elucidated. In order to explore the protective effect of EX on T2DM and its molecular mechanism, this study was therefore carried out. As we find out, EX effectively improves cell autophagy and promotes apoptosis, and plays its role by regulating miR-29c-3p and Sirt1.

T2DM is caused by multiple organ defects, the most important of which are insufficient insulin secretion and insulin resistance. Of them, insulin resistance in skeletal muscle is of particular concern because muscle is the main site for glucose management [15]. T2DM is related to a series of pathological and physiological events, such as apoptosis and autophagy [16, 17]. It is well-

established that EX can protect T2DM-induced skeletal muscle damage. For example, research pointed out that physical exercise such as EX exerted positive effects on maintaining blood glucose control [18]. Others revealed that EX could improve the regulation of autophagy markers and reduce apoptosis to protect skeletal muscle from T2DM damage [19]. In our study, it was found that EX effectively reduced miR-29c-3p and increased Sirt1 after T2DM induction in vivo. Further cell experiments exhibited that EX alleviated the injury of L6 myotube cells induced by T2DM and inhibited the apoptosis of myotube cells, resulting in sharp decrease in the transcriptional expression of pro-apoptotic genes caspase-9, caspase-3 and Bax, and significant increase in anti-apoptotic gene Bcl-2, thus increasing cell activity. Just as what is shown in the research by Dang et al. [20], 6 months of EX could significantly prevent the apoptosis of cardiomyocytes in mice, and it was an important way to effectively regulate Bcl-2 and Bax levels in cardiomyocytes. The pathological basis of skeletal muscle myocardium is the change of autophagy signals and pathways. Autophagy is a process of catabolism, and the increase of autoph-

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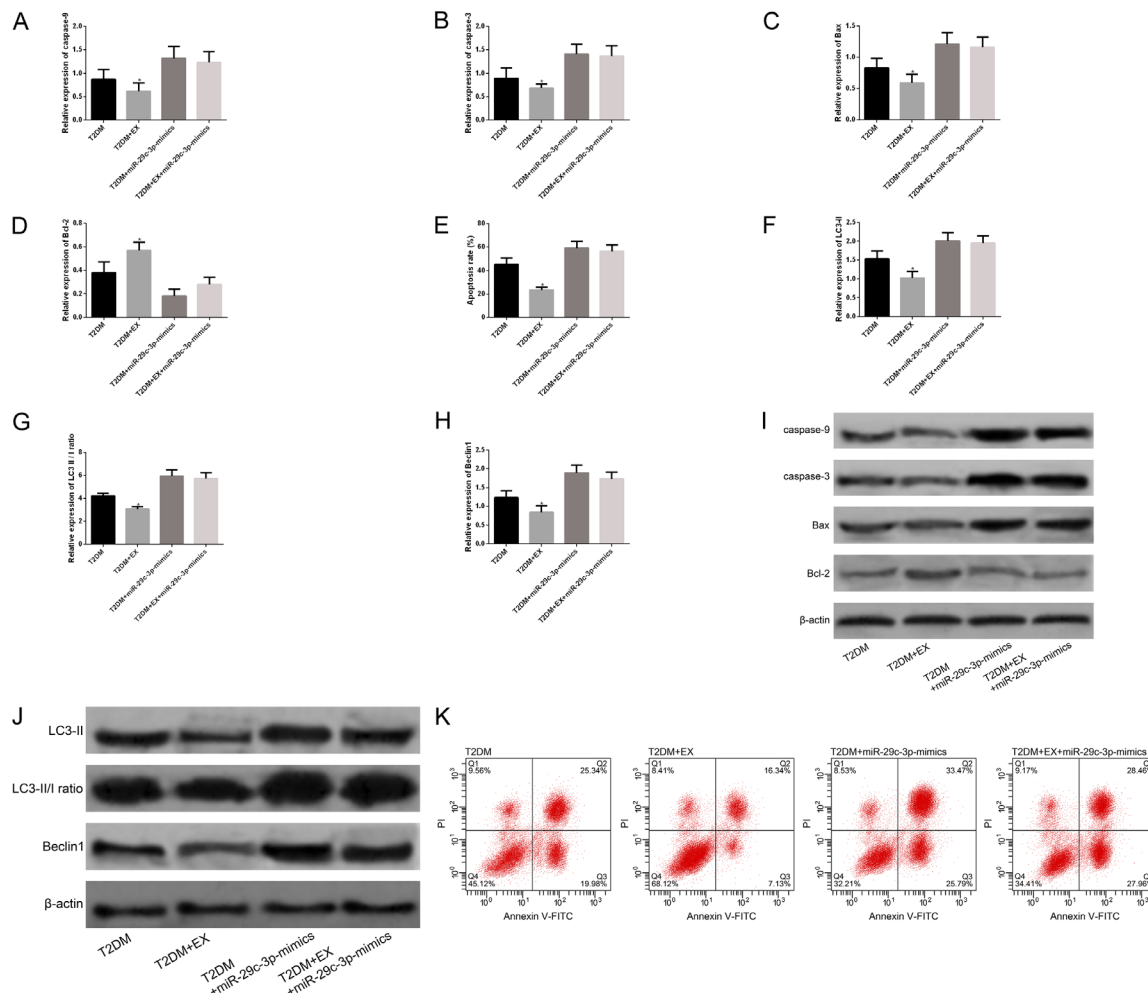


Figure 4. MiR-29c-3p eliminated the protective effect of EX on T2DM-induced skeletal muscle. A-D: Effects of overexpression of miR-29c-3p on apoptosis-related proteins. E: Apoptosis after transfection. F-H: Effects of overexpression of miR-29c-3p on cell autophagy-related proteins. I: Apoptosis-related protein map. J: Autophagy-related protein map. K: FC diagram of apoptosis. Note: * indicates $P < 0.05$.

agy will lead to the remodeling of pathological tissues [21, 22]. Evidence has shown that EX can restore the Sestrin 2 protein content in skeletal muscle of aged mice to normal and induce autophagy, thus improving insulin sensitivity in aging animal models [23]. In this study, we observed the effect of EX on autophagy protein expression in T2DM-induced mouse model, and found that autophagy protein LC3-II, LC3-II/I ratio and Beclin1 were dramatically enhanced after T2DM induction, while this phenomenon was alleviated and LC3-II, LC3-II/I ratio and Beclin1 were down-regulated after EX intervention in rats, suggesting that EX could reduce T2DM induced autophagy dysfunction and effectively activate autophagy activity. In the study of Kwon et al. [24], long-term resistance

exercise training could promote autophagy and reduce muscle degeneration caused by autophagy dysfunction induced by sporadic inclusion body myositis.

More and more researchers have studied the regulation mechanism of miR-29c-3p and Sirt1 in human diseases. For example, according to Massart et al. [25], miR-29c-3p was an important regulator of insulin-stimulated lipid oxidation and glucose metabolism, and that miR-29c-3p was associated with human physiology and T2DM. As pointed out by Luan et al. [26], Sirt1 decreased in T2DM patients and was an important therapeutic target for T2DM. The results of this study showed that miR-29c-3p elevated remarkably in T2DM patients, while

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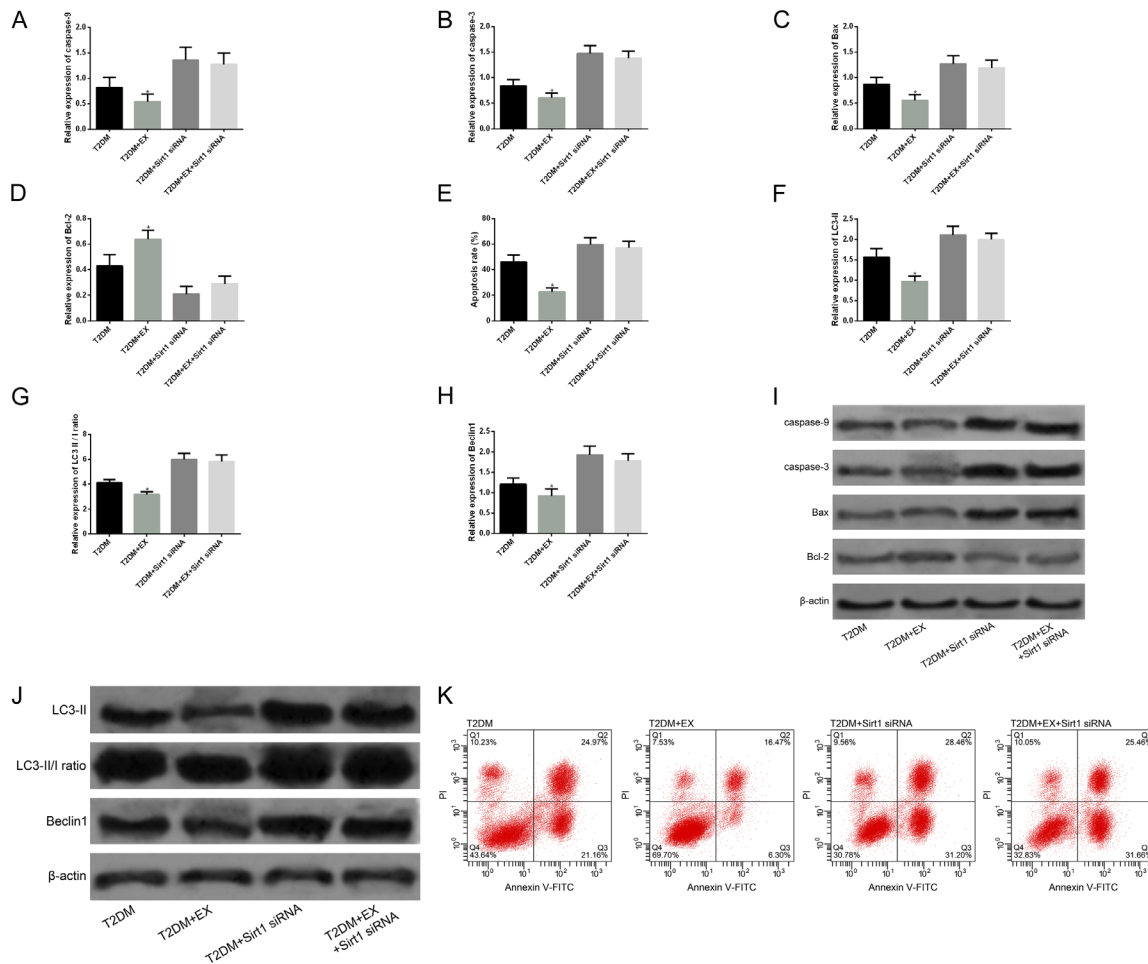


Figure 5. Sirt1 eliminated the protective effect of EX on T2DM-induced skeletal muscle. A-D: Effects of inhibition of Sirt1 on apoptosis-related proteins. E: Apoptosis after transfection. F-H: Effects of inhibition of Sirt1 on autophagy-related proteins. I: Apoptosis-related protein map. J: Autophagy-related protein map. K: FC diagram of apoptosis. Note: * indicates $P < 0.05$.

Sirt1 dropped notably. MiR-29c-3p overexpression or Sirt1 inhibition brought aggravated myotube cell injury and increased apoptosis rate in T2DM patients. Further, we used prediction software to predict possible targets to explore the possible biological functions of miR-29c-3p. There was a complementary pairing region between miR-29c-3p and Sirt1 sequences, indicating that Sirt1 was the target of miR-29c-3p. After transfection, miR-29c-3p overexpression was found to significantly reduce the expression of Sirt1, suggesting that miR-29c-3p could decrease Sirt1 and aggravate the development of T2DM. While EX down-regulated miR-29c-3p and up-regulated Sirt1, indicating that EX intervention could bolster the body's protective mechanisms such as increasing anti-apoptosis and enhancing the autophagy

activity through the above mechanisms. We also found by transfection of miR-29c-3p and Sirt1 that overexpression of miR-29c-3p or inhibition of Sirt1 could eliminate the protective effect of EX on T2DM treatment.

This study finds the regulatory mechanism of EX in skeletal muscle injury, and miR-29c-3p and Sirt1 may be potential therapeutic targets for this disease. However, there is still room for improvement, for instance, we can supplement whether the regulation mechanism of EX is related to exercise time and amount of exercise in our follow-up research.

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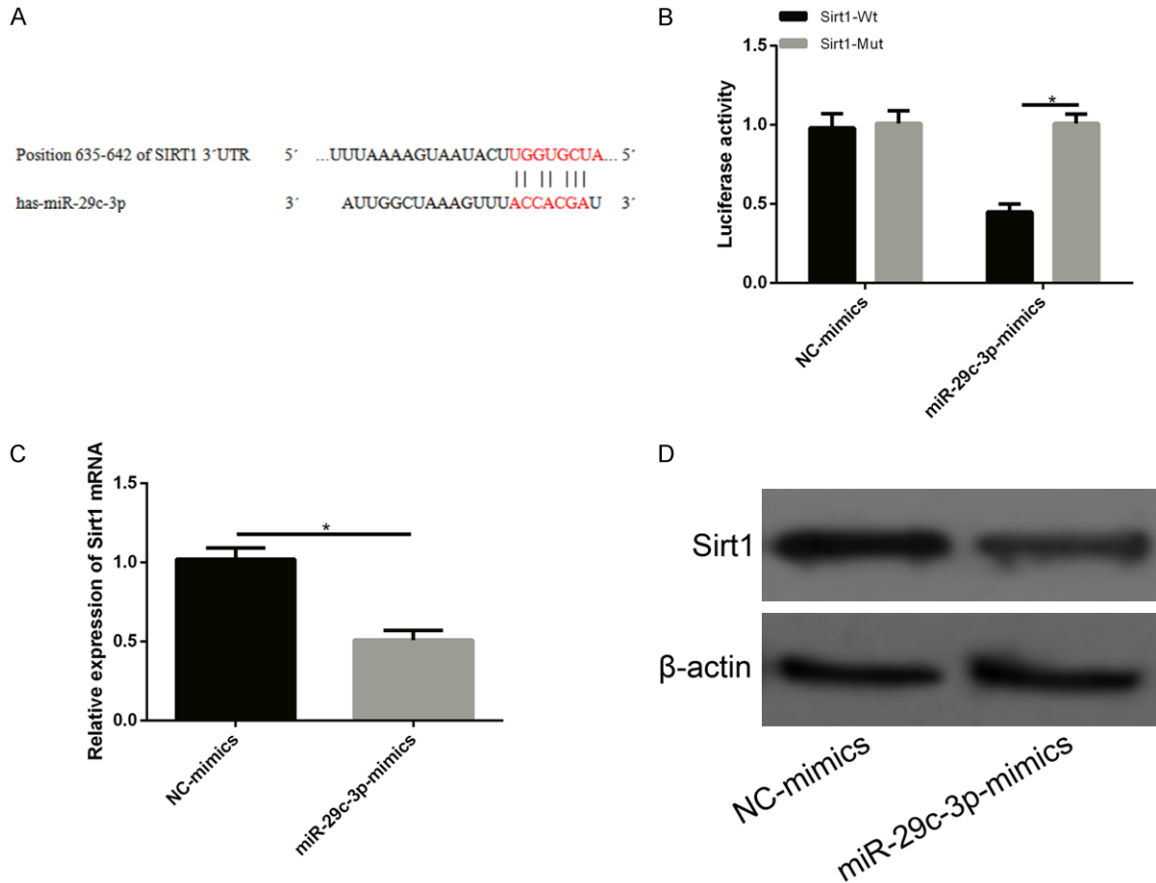


Figure 6. Target gene identification. A: Binding site between miR-29c-3p and Sirt1. B: DLR results. C: Effects of miR-29c-3p-mimics on Sirt1 protein. D: Protein map of the effects of miR-29c-3p-mimics on Sirt1 protein. Note: * indicates $P < 0.05$ compared between two groups.

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Disclosure of conflict of interest

None.

Address correspondence to: Shaoyong Wan, Department of Sports Training and Research, Physical Education College, Jinggangshan University, No. 28 Xueyuan Road, Qingyuan District, Ji'an 343009, Jiangxi, China. E-mail: wanshaoyong@outlook.com

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