

Original Research Article

Isosorbide mononitrate inhibits myocardial fibrosis in diabetic rats by up-regulating exosomal MiR-378

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Abstract

Purpose: To investigate the effect of isosorbide mononitrate on diabetic cardiomyopathy (DCM), and the potential mechanism of action.

Methods: The effects of isosorbide mononitrate and isosorbide mononitrate + GW4869 on cardiac function and myocardial fibrosis in DCM rats were determined via hemodynamics, hematoxylin-eosin (H&E) staining and Masson staining. Exosomes were extracted from the serum, and the differential expressions of microribonucleic acids (miRNAs) related to myocardial fibrosis were determined by reverse transcription-polymerase chain reaction (qRT-PCR). Western blotting was performed to determine the effects of isosorbide mononitrate and isosorbide mononitrate + GW4869 on IGF1R/STAT3 signaling pathway.

Results: Isosorbide mononitrate exerted a protective effect against DCM-induced cardiac dysfunction and myocardial fibrosis, while such a protective effect was suppressed by the exosome inhibitor GW4869 ($p < 0.05$). The expression of miR-378 in exosomes significantly rose in isosorbide mononitrate group. The increased expression of miR-378 in vitro inhibited the proliferation of primary myocardial fibroblasts, and reduced the expression of myocardial fibrosis markers ($p < 0.05$). Luciferase reporter assay data showed that miR-378 negatively regulated the expression of IGF1R by direct binding to IGF1R mRNA 3'-untranslated region (3'UTR). In primary myocardial fibroblasts, miR-378 mimic significantly reduced the protein expressions of IGF1R, p-STAT3/STAT3 and c-Myc ($p < 0.05$). Isosorbide mononitrate lowered the protein expressions of IGF1R, p-STAT3/STAT3 and c-Myc, but the inhibitory effect was weakened by the exosome inhibitor, GW4869 ($p < 0.05$).

Conclusion: Isosorbide mononitrate inhibits myocardial fibrosis in diabetic rats by up-regulating exosomal miR-378, and targeting the axis of STAT3/IGF1R. The results of this study may provide a new insight into the treatment of DCM.

Keywords: Isosorbide mononitrate, Diabetic cardiomyopathy, Myocardial fibrosis, Exosomal miR-378, STAT3 signaling pathway

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INTRODUCTION

Diabetic cardiomyopathy (DCM), an independent complication of diabetes, refers to cardiac insufficiency without other heart diseases [1].

Diabetic cardiomyopathy patients have a poorer prognosis, and they are more prone to heart failure than those with hypertension or ischemic heart disease [2]. There is considerable evidence that early-onset cardiac diastolic dysfunction and

late-onset cardiac systolic dysfunction independent of hypertension and coronary artery disease, are the main causes of heart failure in DCM patients [3]. Despite a large number of studies on DCM, its pathogenesis has not been thoroughly understood yet, so there is a lack of effective means of treatment.

The pathological mechanism of DCM is complicated and still remains unclear. However, several studies on humans and animals have demonstrated that DCM is mainly characterized by myocardial interstitial fibrosis and myocardial perivascular fibrosis [4]. In the myocardial interstitium, myocardial stiffness may be increased due to the deposition of extracellular matrix proteins and the cross-linking of matrixes, thus leading to cardiac diastolic and systolic dysfunction. There is increasing evidence that myocardial fibrosis remodeling may play a key role in the pathological process of DCM [5].

Isosorbide mononitrate is a new-generation long-acting nitrate anti-anginal drug, which mechanism of action is to stimulate guanylate cyclase by releasing nitric oxide (NO), and increase cyclic guanosine monophosphate, ultimately causing vascular dilation. Isosorbide mononitrate is an effective therapeutic drug for coronary heart disease and DCM [6]. However, the molecular mechanism of isosorbide mononitrate involved in inhibiting myocardial fibrosis still remains to be explored.

Exosomes are small vesicles secreted by cells, about 30-200 nm in diameter, which naturally exist in such biological fluids such as blood, urine, saliva, breast milk and cell medium. Almost all types of cells produced and released exosomes. Exosomes directly activate the recipient cells, also transport proteins, messenger ribonucleic acids (mRNAs), micro RNAs (miRNAs), lncRNAs, circRNAs and even organelles into recipient cells, and participate in inter-cellular communication [7]. According to mounting evidence, exosomes play an important role in DCM, so they are considered as an effective treatment method for DCM. In the present study, the effect of isosorbide mononitrate on myocardial fibrosis in diabetic rats was explored from the perspective of exosomes.

EXPERIMENTAL

Laboratory animals

A total of 40 male Sprague-Dawley rats weighing 180 - 200 g were fed in specific pathogen-free animal house under the

temperature of 25 °C, humidity of 45 % and 12/12 h dark/light cycle, with they had free access to feed and water. The rats were randomly divided into control group, model group, isosorbide mononitrate (15 mg/kg) group and isosorbide mononitrate + GW4869 group. This study was approved by the Animal Ethics Committee of Qingdao Municipal Hospital Animal Center (approval no. 19-QDM-AEC-023). All procedures were conducted in accordance with 'Animal Research: Reporting *in vivo* Experiments guidelines 2.0' [8].

Establishment of rat model of DCM

Streptozocin (STZ) solution was prepared in sodium citrate buffer and citric acid. In the model group, isosorbide mononitrate group and isosorbide mononitrate + GW4869 group, STZ solution was intraperitoneally injected (50 mg/kg) every other day for 30 days. In the control group, an equal volume of normal saline was intraperitoneally injected. All rats were fed normally in the same house. The fasting blood was drawn from the caudal vein to determine blood glucose, and rats with blood glucose \geq 16.7 mmol/L were selected for further studies. After 8 weeks, isosorbide mononitrate and GW4869 were intraperitoneally injected. At 12 weeks after injection of STZ, the rats were sacrificed via cervical dislocation (after being anesthetized using peritoneal administration of pentobarbital sodium at a dose of 40 mg/kg).

Extraction of primary myocardial fibroblasts

After the rats were sacrificed via cervical dislocation (after being anesthetized using peritoneal administration of pentobarbital sodium at a dose of 40 mg/kg), the heart was taken, and the blood vessels and auricles were removed. After draining the blood, the heart was collected into a 1.5 mL eppendorf (EP) tube containing 4 °C medium and cut into pieces. The liquid was discarded, and the heart tissues were transferred into a 15 mL centrifuge tube with trypsin, digested at 37 °C for 3 min and shaken evenly from time to time. Then the supernatant was recycled into a neutralization tube, digested with 5 mL of collagenase at 37 °C for 5 min, and shaken evenly from time to time on ice. The supernatant was recycled, and digested with 4 mL of collagenase at 37 °C for 5 min on ice. The supernatant was recycled again, digested with 3 mL of collagenase at 37 °C for 5 min, and fully recycled into the neutralization tube. Then some undigested tissues were filtered out through a 200-mesh sieve, and the remaining tissues were ground with the syringe core and rinsed with liquid,

followed by centrifugation at 1,000 rpm for 8 min. After the supernatant was discarded, 6 mL of culture solution was added into each tube, the bottom of the tube was tapped to resuspend the precipitate, and the cells were inoculated into the culture flask. After removing the non-adherent myocardial cells, the remaining adherent cells were obtained, which were identified to be myocardial fibroblasts. The above experiment was repeated three times to purify the myocardial fibroblasts. Then the myocardial fibroblasts were identified by inverted phase contrast microscope.

Hemodynamic test

The left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP) and maximal rate of the increase/decrease of left ventricular pressure ($\pm dp/dt_{max}$) were measured and recorded using the BL-420F hemodynamic system (Chengdu Techman Technology Co. Ltd., Chengdu, China).

Hematoxylin-eosin (HE) staining (n = 3)

The rats were sacrificed via dislocation at one time, and the heart was isolated and treated with 4 % paraformaldehyde/PBS (pH 7.4) at 4 °C for 48 h. The tissues were dehydrated with 70 %, 80 %, 95 % ethanol and 100 % ethanol sequentially. After the ethanol was removed with xylene, the tissues were embedded into paraffin (4 μ m) and stained using the HE staining kit (Beyotime, Shanghai, China) in strict accordance with the manufacturer's instructions.

Masson staining

The heart tissues were fixed in 4% paraformaldehyde/phosphate buffered saline (PBS) (pH 7.4) at 4 °C for 48 h and then were dehydrated with 70 %, 80 %, 95 % ethanol and 100 % ethanol sequentially. After the ethanol was removed with xylene, the tissues were embedded in paraffin (2 μ m), deparaffinized, washed with water and air-dried. The Masson staining was according to the protocols of the manufacture. Finally, the sections were observed under a microscope.

Extraction of exosomes (n = 3)

The plasma of rats in each group was thawed on ice and centrifuged at 3,000 \times g and 4°C for 10 min to remove particles. Then the plasma was diluted in an equal volume of ice-cold PBS, and centrifuged at 200,000 \times g and 4 °C for 60 min. The supernatant was taken, and the exosome particles were resuspended in ice-cold

PBS and centrifuged at 200,000 \times g and 4 °C for 60 min. Finally, the supernatant was taken to verify exosome markers CD63 and CD9 via Western blotting [9].

Western blotting

An appropriate amount of radioimmuno-precipitation assay (RIPA) lysis buffer was prepared, and the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (RIPA:PMSF = 100:1) was added and mixed evenly. After the cells were trypsinized, they were collected and lysed with lysis buffer, and the lysate was collected and transferred into an EP tube, followed by centrifugation at 14,000 rpm and 4 °C for 30 min using a refrigerated high-speed centrifuge. Then the protein supernatant was collected and subjected to heating in a bath at 95 °C for 10 min for protein denaturation. The protein samples prepared were placed in a refrigerator at -80 °C prior to use, and the protein was quantified using the bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA). Afterwards, the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel was prepared, and the protein samples were loaded into the loading well for electrophoresis under a constant pressure of 80 V for 2.5 h. Then the protein was transferred onto a polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) using a semi-dry transfer method. The PVDF membranes was immersed in tris-buffered saline with Tween-20 (TBST) containing 5 % skim milk powder, and shaken slowly for 1 h on a shaking table to be sealed. Subsequently, the protein was incubated with primary antibodies, diluted with 5% skim milk powder, rinsed with TBST for 3 times (10 min/time), incubated again with the secondary antibodies at room temperature for 2 h, and rinsed again with TBST twice and with TBS once (10 min/time). Finally, the protein was determined using the ECL reagent, followed by exposure in a darkroom. The relative expression of protein was analyzed using Image-Pro Plus v6 (Media Cybernetics, Silver Spring, MD, USA). The following primary antibodies were used in this study: Collagen I (ab260043, Abcam, Cambridge, MA, USA), Collagen III (ab6301, Abcam, Cambridge, MA, USA), Phosphorylated-signal transducer and activator of transcription 3 (p-STAT3) (ab76315, Abcam, Cambridge, MA, USA), STAT3 (ab119352, Abcam, Cambridge, MA, USA), insulin-like growth factor-1 receptor (IGF1R) (ab119843, Abcam, Cambridge, MA, USA) and c-Myc (ab32072, Abcam, Cambridge, MA, USA).

Luciferase reporter assay

In this experiment, the wild-type and mutant-type IGF1R 3'UTR were amplified and cloned into psiCHECK-2 luciferase plasmids (Promega, Madison, WI, USA) to generate the wild-type and mutant-type reporter plasmids respectively. The HEK293 cells were cultured in a 24-well plate, and co-transfected with miR-378 or miR-con and wild-type or mutant-type plasmids. Luciferase activity was determined at 48 h after transfection using dual-luciferase reporter reagent (Promega, Madison, WI, USA).

Reverse transcription-polymerase chain reaction (RT-PCR)

The expressions of miR-378 and other mRNAs in myocardial tissues and exosomes were determined via RT and quantitative PCR (qPCR). MiRNAs in exosomes were reversely transcribed using the PrimeScript RT reagent kit and then quantified using TaqMan quantitative kit (Invitrogen, Carlsbad, CA, USA). A 500 ng of RNA samples in RT were divided into three portions, the total RNA was diluted (10-fold) and 3 μ L of total RNA was taken for PCR amplification. The amplification level of the target gene was verified via 5% agarose gel electrophoresis. The data were quantified and processed using LabWorks 4.0 image acquisition and analysis software. In this study, U6 was selected as an internal reference. The primers of miR-378 were purchased from ABM (Peterborough, Camb, Canada). To obtain reliable data, the experiment was repeated for 3 times in each group. The relative expression levels of target genes were analyzed using the $2^{-\Delta\Delta Ct}$ method. The primer sequences used are shown in Table 1.

Table 1: Primer sequences used in PCR

Primer	Sequence (5'-3')
MiR-378	5'-TACCACTTCACAAGTCGGAGGC-3' 5'-CTGCAAGTGCATCATCGTTGTTTC-3'
MiR-30c	5'-GAGAGTGATTGAGAGTGGACCAC-3' 5'-CACAACCCTCTGCACCCAGTTT-3'
MiR-208	5'-TGGACCTCCAGGATGAGGACA-3' 5'-GTTTCATCTCGGAGCCTGTAGTG-3'
MiR-21	5'-GGTGCCATATGTCTCAGCCTCTT-3' 5'-GCCATAGAAGTATGAGAGGGAG-3'
IGF1R	5'-CGGGATCTCATCAGCTTCACAG-3' 5'-TCCTTGTTCCGAGGCAGGTCTA-3'
U6	5'-CGCTTCGGCAGCACATATACTA-3' 5'-CGCTTCACGAATTTGCGTGTCA-3'

Cell counting kit-8 (CCK-8) assay

Primary myocardial fibroblasts transfected with miR-378 mimic or miR-con were uniformly

inoculated into a 96-well plate (1×10^4 cells/well). CCK-8 assay was performed according to the manufacturer's protocols. Finally, the absorbance was measured at a 450 nm using a microplate reader.

5-ethynyl-2'-deoxyuridine (EdU) staining

Primary cells in logarithmic growth phase were inoculated into the 96-well plate (1×10^5 cells/well). After normal growth, the cells were treated with miR-378 mimic or miR-con for 24 h. EdU solution was diluted (1 : 1000) with culture medium, and 100 μ L of 50 μ M EdU solution (Sigma-Aldrich, St. Louis, MO, USA) was added into each well for incubation for 2 h.

After discarding the medium, the cells were washed twice with PBS, incubated with 100 μ L of fixative (PBS containing 4 % paraformaldehyde) in each well at room temperature for 30 min, and stained with 100 μ L of 1 \times Apollo[®] dye at room temperature in the dark for 30 min. After the dye was discarded, the cells were treated with 100 μ L of penetrant (PBS containing 0.5% TritonX-100) and washed 2 - 3 times (10 min/time), and the penetrant was discarded. Finally, the cells were observed under the microscope.

Statistical analysis

SPSS statistical analysis software (version 26.0) was used for statistical analysis. The data were analyzed using *t*-test or one-way ANOVA test followed by Post hoc test (least significant difference) among groups. The data are expressed as mean \pm standard deviation (SD) and percentage.

RESULTS

Isosorbide mononitrate significantly improved cardiac function in DCM rats

As shown in Table 2, LVSP, +dp/dt_{max} and -dp/dt_{max} were significantly reduced, while LVEDP significantly rose in the model group when compared with those in control group, ($p < 0.01$). LVSP, +dp/dt_{max} and -dp/dt_{max} considerably rose, while LVEDP remarkably declined in isosorbide mononitrate group compared with those in model group, also showing statistically significant differences ($p < 0.01$). It can be seen that isosorbide mononitrate improved DCM-induced cardiac dysfunction. In GW4869 + isosorbide mononitrate group, the protective effect of isosorbide mononitrate on the cardiac function in DCM rats was reversed ($p < 0.01$).

Table 2: Effect of isosorbide mononitrate on cardiac function in rats

Group	LVSP (mm Hg/s)	LVEDP (mm Hg/s)	+dp/dt _{max} (mm Hg/s)	-dp/dt _{max} (mm Hg/s)
Control	93±9	4.2±0.97	6841±376	5821±573
Model	46±7**	8.7±1.9**	5457±497**	4678±531**
Isosorbide mononitrate	83±7.21&&	5.1±1.3&&	6641±610&&	5269±454&&
GW4869 + isosorbide mononitrate	39±4.9###	11.2±2.1###	5458±546###	4632±379###

** $P < 0.01$ model group vs. control group, && $p < 0.01$ isosorbide mononitrate group vs. model group, ### $p < 0.01$ GW4869 + isosorbide mononitrate group vs. isosorbide mononitrate group

Isosorbide mononitrate improved myocardial morphology and fibrosis in DCM rats

In control group, the myocardial cells had normal size and morphology, and they were arranged orderly, without infarction lesions. In model group, the myocardial cells were arranged disorderly and varied in size, some muscle fibers had degeneration and swelling, and there was massive inflammatory cell infiltration. In isosorbide mononitrate group, isosorbide mononitrate had a good protective effect on the myocardium, the myocardial cells were arranged regularly and stained uniformly, there were clear intercellular boundaries and no interstitial edema, and the inflammatory cell infiltration was relieved when with that in model group. In GW4869 + isosorbide mononitrate group, the morphology of myocardial tissues became worse compared with isosorbide mononitrate group (Figure 1).

Myocardial fibrosis is one of the main causes of ventricular remodeling and cardiac dysfunction. Due to the imbalance of collagen synthesis and degradation in myocardial fibers, the myocardium becomes stiff, and the cardiac compliance declines, affecting cardiac systolic and diastolic function, and causing diminished cardiac function and even heart failure. According to the results of Masson staining (Figure 1), normal myocardial tissues were replaced with a large area of fibrotic tissues, myocardial interstitial collagen had obvious proliferation, and a large number of blue-stained fibrous connective tissues separated and wrapped myocardial muscle bundles in model group. In isosorbide mononitrate group, the myocardial fibrosis was markedly alleviated in rats than that in model group, thereby protecting the myocardium. In GW4869 + isosorbide mononitrate group, the morphology of myocardial tissues was markedly poorer than that in isosorbide mononitrate group. Western blotting showed that the expressions of Collagen I and Collagen III were statistically increased in model group compared with control group. In isosorbide mononitrate group, the levels of Collagen I and Collagen III were decreased significantly when compared with model group. However, the improvement was neutralized in GW4869 + isosorbide mononitrate group.

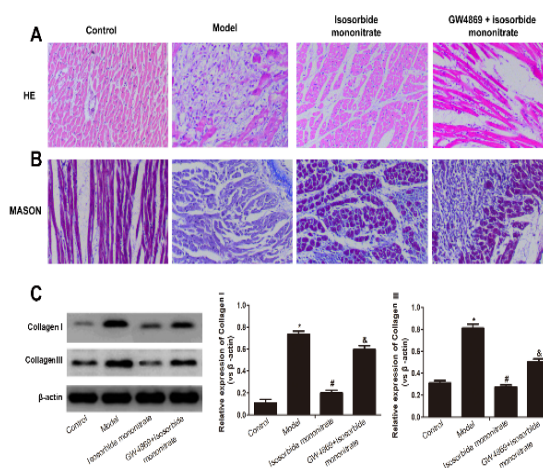


Figure 1: Isosorbide mononitrate improves myocardial morphology and fibrosis in DCM rats. (A) Isosorbide mononitrate improves myocardial morphology by H & E staining (magnification: 400 \times). (B) Isosorbide mononitrate ameliorates myocardial fibrosis (Masson staining (magnification: 400 \times)). (C) Isosorbide mononitrate decreases the expression of Collagen I and Collagen III by Western blotting. * $p < 0.01$ vs control group; # $p < 0.01$ vs model group; & $p < 0.01$ vs isosorbide mononitrate group

Isosorbide mononitrate raised content of exosomal miR-378 in serum

As can be seen from Table 2 and Figure 1, isosorbide mononitrate had a protective effect against DCM-induced cardiac dysfunction and myocardial fibrosis, while this protective effect was suppressed by the exosome inhibitor GW4869. The above findings indicate that isosorbide mononitrate may protect the myocardial tissues in DCM rats through the exosome pathway. To further explore the protective effect of isosorbide mononitrate on myocardial tissues in DCM rats, changes in miRNAs related to myocardial fibrosis in exosomes were determined via qRT-PCR, and it was found that the expressions of miR-378, miR-30c, miR-208 and miR-21 in exosomes rose in isosorbide mononitrate group (Figure 2). Among them, miR-378 was selected as the following research.

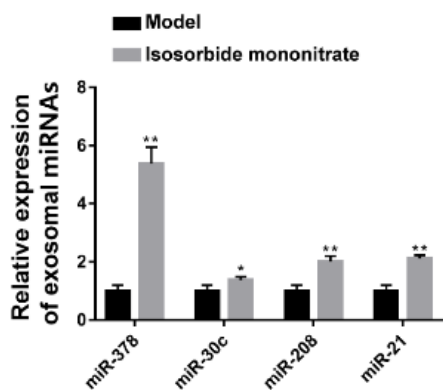


Figure 2: Isosorbide mononitrate increases the content of exosomal miR-378 in serum. ** $P < 0.01$ model group vs. isosorbide mononitrate group

MiR-378 mimic suppressed fibroblast proliferation

To determine the effect of miR-378 on the proliferation of primary myocardial fibroblasts at the *in vitro* level, primary myocardial fibroblasts were transfected with miR-378 mimic and miR-con. First, CCK-8 assay and EdU staining were used to determine the effect of miR-378 expression on the proliferation of primary myocardial fibroblasts. The results manifested that miR-378 mimic distinctly inhibited the proliferation of primary myocardial fibroblasts (Figure 3 A and B). Then the effects of miR-378 expression on the protein expressions of fibrosis markers collagen I, collagen III and matrix metalloproteinase 9 (MMP9) were determined using Western blotting. The results showed that the increased expression of miR-378 distinctly reduced the protein expressions of collagen I, collagen III and MMP9 (Figure 3 C and D).

MiR-378 negatively regulated IGF1R expression via direct binding to IGF1R mRNA 3'UTR

Based on bioinformatics prediction, IGF1R might be a potential target of miR-378 (Figure 4A). To further confirm the interaction between miR-378 and IGF1R, the responses of wild-type and mutant-type IGF1R in HEK293 cells to miR-378 and miR-con was determined by luciferase reporter assay. The results revealed that the fluorescence response intensity declined in miR-378-transfected HEK293 cells with wild-type IGF1R, while it produced no obvious changes in HEK293 cells with mutant-type IGF1R (Figure 4B). Then RT-qPCR was performed for further evidence, and it was found that transfection of miR-378 greatly reduced the mRNA level of IGF1R (Figure 4C). To sum up, miR-378

negatively regulate the IGF1R expression via binding to the IGF1R mRNA 3'UTR.

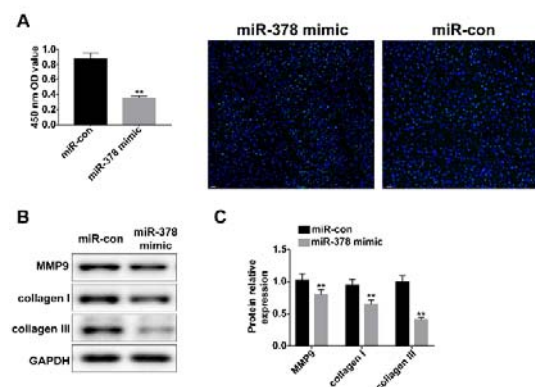


Figure 3: MiR-378 mimic distinctly suppresses fibroblast proliferation. (A) Effect of miR-378 expression on the proliferation of primary myocardial fibroblasts determined via CCK-8 assay. (B) Effect of miR-378 expression on the proliferation of primary myocardial fibroblasts detected via EdU staining. (magnification: 400 ×). (C) & (D) Effects of miR-378 expression on the protein expressions of collagen I, collagen III and MMP9 determined using Western blotting. ** $p < 0.01$ miR-378 mimic group vs. miR-con group

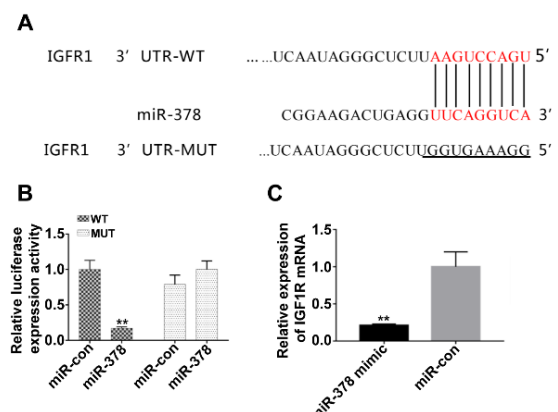


Figure 4: MiR-378 negatively regulates IGF1R expression via directly binding to IGF1R mRNA 3'UTR. (A) Based on bioinformatics prediction, miR-378 binds directly to the IGF1R mRNA 3'UTR. (B) The response of wild-type and mutant-type IGF1R in HEK293 cells to miR-378 and miR-con, determined by luciferase reporter assay. (C) The effects of transfection of miR-con and miR-378 on mRNA level of IGF1R determined using qRT-PCR. ** $p < 0.01$ miR-378 mimic group vs. miR-con group

MiR-378 regulated STAT3 signaling pathway by targeting IGF1R

The *in vitro* results manifested that the protein expressions of IGF1R, p-STAT3/STAT3 and c-Myc in primary myocardial fibroblasts were

remarkably reduced in miR-378 mimic group compared with those in miR-con group ($p < 0.01$, Figure 5).

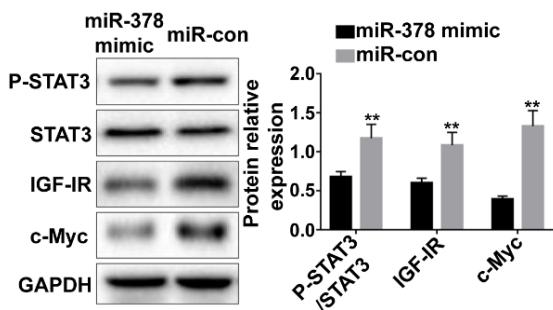


Figure 5: MiR-378 regulates STAT3 signaling pathway via targeting IGF1R. MiR-378 mimic remarkably reduces the protein expressions of IGF1R, p-STAT3/STAT3 and c-Myc; ** $p < 0.01$ miR-378 mimic group vs. miR-con group

Effect of exosome inhibitor GW4869 on IGF1R/STAT3 signaling pathway

As shown in Figure 6, isosorbide mononitrate remarkably lowered the protein expressions of IGF1R, p-STAT3/STAT3 and c-Myc at the *in vivo* level, but such an inhibitory effect was remarkably weakened by the exosome inhibitor GW4869, displaying statistically significant differences ($p < 0.01$).

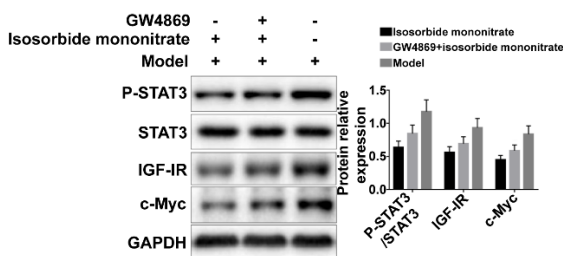


Figure 6: The inhibitory effect of isosorbide mononitrate on the protein expressions of IGF1R, p-STAT3/STAT3 and c-Myc is weakened by the exosome inhibitor GW4869; ** $p < 0.01$ miR-378 mimic group vs. miR-con group

DISCUSSION

In this study, it was first confirmed that isosorbide mononitrate had a protective effect against DCM-induced cardiac dysfunction and myocardial fibrosis. Isosorbide mononitrate is a new-generation long-acting nitrate antianginal drug, whose mechanism of action is to stimulate guanylate cyclase via releasing NO, and increase cyclic guanosine monophosphate, ultimately causing vascular dilation. Isosorbide mononitrate is an effective therapeutic drug for coronary heart disease. Studies have shown that

the bioavailability of NO significantly declines in diabetes, and a large number of studies have also pointed out that NO suppressed myocardial fibrosis. For example, Zhu *et al* [10] reported that DDAH2 can raise the expression of NO via activating the DDAH/ADMA/NOS/NO signaling pathway, thereby relieving myocardial fibrosis in DCM. Consistent with the above study, Ren *et al*. [11] pointed out that in the isoproterenol-induced myocardial hypertrophy model, inhibiting the activity of NO synthase enhanced myocardial fibrosis. In addition, Kazakov *et al* [12] showed that in the mouse model of TAC, inhibiting the eNOS expression suppressed myocardial fibrosis in mice. Thus, a large number of studies on NO have proved the reliability of results in this paper.

In this study, it was found that the protective effect of isosorbide mononitrate was abolished by exosome inhibitor GW4869, suggesting that exosomes play a key role in the protective effect of isosorbide mononitrate against DCM-induced cardiac dysfunction and myocardial fibrosis. The results of RT-qPCR revealed that the expression of miR-378 in exosomes markedly rose in isosorbide mononitrate group, and the increased expression of miR-378 *in vitro* obviously inhibited the proliferation of primary myocardial fibroblasts, and reduced the expression of myocardial fibrosis markers. The research results of Sun *et al* [13], consistent with the findings in this study, manifested that lncRNA PCFL adsorbs miR-378 as a molecular sponge, and regulates the GRB2 signaling pathway, thus promoting myocardial fibrosis. In addition, Yuan *et al*. [14] proved that in the early stage of myocardial hypertrophy, mechanical stimulation inhibited myocardial fibrosis through miR-378 via the paracrine pathway. Moreover, Nagalingam *et al* [15] pointed out that miR-378 derived from myocardial cells inhibited the stimulation of fibroblasts by TGF- β .

To further explore the effects of exosomal miR-378 on the proliferation and fibrosis of fibroblasts, the targets of miR-378 were screened using bioinformatics software and luciferase reporter assay. It was found via luciferase reporter assay that miR-378 negatively regulated the expression of IGF1R via directly binding to the IGF1R mRNA 3'UTR. Besides, the results of Western blotting confirmed that in primary myocardial fibroblasts, miR-378 mimic evidently reduced the protein expressions of IGF1R, p-STAT3/STAT3 and c-Myc. At the *in vivo* level, isosorbide mononitrate also remarkably reduced the protein expressions of IGF1R, p-STAT3/STAT3 and c-Myc, but such an inhibitory effect was remarkably weakened by the exosome inhibitor GW4869. The above findings demonstrate that exosomal miR-378

inhibits the JAK/STAT3 signaling pathway via targeting IGF1R, thereby suppressing the proliferation and fibrosis of myocardial fibroblasts.

IGF1R, a transmembrane tyrosine kinase, binds to the ligands, causing phosphorylation. Then phosphorylated IGF1R activates the PI3K, MAPK and JAK/STAT signaling pathways [16]. According to a large number of studies, the STAT signaling pathway plays an important role during myocardial fibrosis. For example, Dai *et al* [17] showed that the increased protein expressions of p-STAT1 and STAT3 enhanced the expressions of myocardial fibroblastic protein and the glial deposition under high glucose conditions. Moreover, Dai *et al* [18] argued that H₂S inhibited oxidative stress, endoplasmic reticulum stress and inflammatory response by suppressing the JAK/STAT signaling pathway, thereby restraining myocardial fibrosis in diabetic rats. However, the extract fibroblasts from the DCM rat model was adopted, rather than that from the normal rat model. This was a limitation of the present study.

CONCLUSION

Isosorbide mononitrate inhibits myocardial fibrosis in diabetic rats through the up-regulation of exosomal miR-378 and the regulation of STAT3 signaling pathway, by targeting IGF1R. However, the potential mechanism involved in up-regulating the expression of exosomal miR-378 by isosorbide mononitrate, will need to be further explored in a future study.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Kaidi Zhao and Junyi Zhang contributed equally to this work.

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