

Fasudil attenuates myocardial fibrosis in rats with diabetes mellitus *via* TGF- β 1/Smad signaling pathway

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ABSTRACT

This research aimed to study the influence of fasudil (FAS) on myocardial fibrosis in rats with diabetes mellitus (DM) *via* the transforming growth factor-beta 1 (TGF- β 1)/small mothers against decapentaplegic (Smad) signaling pathway. A total of 30 Sprague-Dawley rats were randomly divided into a blank control group (Control group, n=10), DM model group (DM group, n=10) and FAS treatment group (FAS group, n=10), and their blood and myocardial tissues were collected. Then blood glucose (GLU) content, hepatic and myocardial function indexes, ejection fraction (EF) and other cardiac function parameters were detected, and ELISA was employed to determine the content of serum IL-6, IL-1 and TNF- α . The pathological changes and cell apoptosis in myocardial tissues were observed through hematoxylin-eosin (HE) staining combined with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. The gene expression levels of Collagen I, α -smooth muscle actin (α -SMA), and the TGF β 1/Smad3 pathway were determined *via* qRT-PCR, and the expression levels of the pathway-associated proteins were measured *via* Western blotting. DM group exhibited an increase in GLU level ($P < 0.05$), suggesting successful modeling. The content of serum Fas, aspartate aminotransferase (AST), creatine kinase-MB (CK-MB), IL-6, IL-1 and TNF- α in DM group were higher than those in Control group ($P < 0.05$). The fractional shortening (FS) and EF in DM group were lower than those in Control group ($P < 0.05$), and the left ventricular end-diastolic diameter (LVEDD) and left ventricular end-systolic diameter (LVESD) were distinctly greater than those in Control group ($P < 0.05$). Myocardial cells in Control group were regularly arranged, and DM group exhibited fibrosis in myocardial cells, with a substantial increase in apoptotic myocardial cells ($P < 0.05$). Besides, FAS group showed notable declines in myocardial fibrosis and apoptotic myocardial cells ($P < 0.05$). Gene assay results revealed that the rats in FAS group had markedly lowered levels of Collagen I, α -SMA, TGF- β 1 and Smad3 in myocardial tissues ($P < 0.05$), but their expression levels were remarkably raised in DM group ($P < 0.05$). According to the results of Western blotting, the levels of TGF- β 1 and Smad3 in rat myocardial tissues were decreased in FAS group ($P < 0.05$), implying that FAS inhibits the expressions of the TGF- β 1/Smad signaling pathway and the relevant molecules. FAS can attenuate myocardial fibrosis in DM rats through suppressing the TGF- β 1/Smad signaling pathway.

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Introduction

Diabetes mellitus (DM) is a group of chronic and metabolic diseases. The leading cause of the death of DM patients is cardiovascular complications, including atherosclerosis, myocardial infarction and cardiomyopathy (1). Diabetic cardiomyopathy (DCM) refers to the impairment of cardiac structure and function in DM patients, which serves as the main contributor to heart failure (2). However, it is not associated with hypertension, coronary arteriosclerosis and other complications (3). DCM has multi-factorial pathophysiological mechanisms, and massive animal models and clinical data have proven that this disease is induced by the combination of increases in apoptosis and myocardial inflammation and changes in extracellular matrix (ECM) components with aggravated myocardial fibrosis (4,5). In DCM, cardiac inflammation and fibrosis are inseparable. Currently, there are few research reports on myocardial fibrosis that is regulated by various cell growth factors. Myocardial fibroblasts maintain the

contraction and relaxation in a healthy heart, and once activated, they aberrantly proliferate and differentiate, causing myocardial fibrosis and affecting the connection and contraction of myocardial cells, as well as further inducing the development and progression of cardiovascular diseases, such as coronary atherosclerosis, arrhythmia and cardiomyopathy (6,7). The normal structure of the heart comprises myocardial cells, fibroblast, ECM and collagen (8), and it is the excessive deposition of ECM that is a major pathological change in diabetic heart disease. Collagen is the main component of ECM, which maintains the normal structure and function of myocardial tissues (9). Interstitial fibrosis refers to the excessive deposition of ECM in tissues (10), and in such a lifelong disease, the circulating glucose at persistently high concentration can impair cardiac structure and function through multiple acute and accumulated long-term changes. These pathways lead to apoptosis and inflammation, weakening the integrity of cardiovascular wall (11,12). Ultimately, the diastolic and systolic dysfunctions of the heart result in contractile heart

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failure and cardiac interstitial fibrosis. Therefore, the anti-inflammation and anti-fibrosis treatment strategies may provide novel pathways for the treatment of DCM.

Intracellular signaling pathways can be activated by numerous substances produced in myocardial fibrosis-induced myocardial injury, such as transforming growth factor-beta 1 (TGF- β 1) that has many functions in cells, including modulating the proliferation, differentiation and migration of fibroblasts and production of ECM (13). Such substances may trigger molecular cascades, thereby activating the downstream molecules. Small mothers against decapentaplegic (Smad) are the downstream of the TGF- β 1 molecular cascade. In myocardial tissues, the TGF- β 1/Smad signaling pathway is closely associated with DCM, and it can be repressed to significantly alleviate myocardial hypertrophy and fibrosis. This pathway exerts vital effects (14), such as regulation of myocardial infarction and ventricular remodeling, and as an important signaling transduction pathway in myocardial regulation, it is implicated in the development and progression of myocardial fibrosis (15). TGF- β 1 also increases the protein expression in ECM in various types of cells, and its over-expression promotes myocardial fibrosis and hypertrophy, inducing ventricular remodeling (16). Fasudil (FAS) is a widely used drug for the treatment of myocardial diseases. Whether it represses myocardial fibrosis in DM through the TGF- β 1/Smad signaling pathway is rarely investigated, and the specific mechanism of action of FAS on myocardial fibroblasts remains less clear now.

Therefore, the present study explored the role of FAS in the treatment of myocardial fibrosis and its relationship with the TGF- β 1/Smad3 signaling pathway through observing the changes in tissue inflammation and cell apoptosis as well as gene and protein expressions.

Materials and Methods

Instruments and reagents

Interleukin-6 (IL-6), IL-1 and tumor necrosis factor- α (TNF- α) enzyme-linked immunosorbent assay (ELISA) kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), TRIzol (Invitrogen, Carlsbad, CA, USA) reagent, diethyl pyrocarbonate (DEPC)-treated water, SuperScript III reverse transcriptase kit and SYBR quantitative polymerase chain reaction (qPCR) mix (ABI), radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, Shanghai, China), loading buffer, protease inhibitor and bicinchoninic acid (BCA) protein concentration assay kit (Biosharp, Heifei, China), β -actin and secondary antibodies (Boster Biological Technology Co., Ltd., Cambridge, MA, USA), primary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), tissue homogenizer and electrophoresis apparatus (Bio-Rad, Hercules, CA, USA), microplate reader (Thermo Fisher Scientific, Waltham, MA, USA), 2500 gel imaging system (Bio-Rad, Hercules, CA, USA), and 7900HT Fast qPCR instrument (Applied Biosystems, Waltham, MA, USA).

Establishment of animal model

Male Sprague-Dawley rats were assigned into the Control group (n=10), DM model group (DM group, n=10) and 1.5 μ g/kg FAS treatment group (FAS group, n=10). After 35 mg/kg streptozotocin (STZ) was intraperitoneally injected once to establish the DM model, the rats in each

group were fed for another 4 weeks, during which their clinical manifestations were observed at regular times daily. Finally, blood and myocardial tissues were sampled from each group of rats for subsequent experiments. This study was approved by the Animal Ethics Committee of No. 940 Hospital, Joint Logistics Support Force of the Chinese People's Liberation Army Animal Center.

Measurement of cardiac physiological function parameters in all groups of rats

In the present study, left ventricular function parameters [fractional shortening (FS), ejection fraction (EF), left ventricular end-diastolic diameter (LVEDD) and left ventricular end-systolic diameter (LVESD)] were measured using magnetic resonance imaging and echocardiogram (ECG) systems to observe whether DM induced myocardial dysfunction. ECG examination was performed in each rat at a probe frequency of 10 MHz as required in the instructions.

Determination of blood glucose (GLU) content and hepatic and myocardial function indexes

To observe whether the DM model was successfully established, venous blood was drawn from rat tails after modeling and centrifuged for serum, and then GLU content was determined. Besides, to provide an important reference for the prediction of the development of myocardial fibrosis in DM rats in clinical practices, blood was sampled from the two groups of rats and centrifuged, and then the serum was isolated to determine the hepatic function indexes Fas and aspartate aminotransferase (AST) and myocardial function index creatine kinase-MB (CK-MB) using an automatic biochemistry analyzer.

Determination of inflammatory factor content using ELISA

The serum samples collected earlier and cryopreserved at -80°C were thawed slowly at 4°C and subjected to low-speed centrifugation. Then the supernatant was collected, and each indicator was determined using the kits according to the instructions. Finally, the absorbance of inflammatory factors in each group was read using a microplate reader.

Detection of myocardial apoptosis via terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

Myocardial apoptosis assay was performed in paraffin-embedded sections using the in situ cell death detection kit (Roche, Basel, Switzerland) according to the following steps. The paraffin-embedded sections were first deparaffinized, washed with phosphate buffer saline (PBS), and then they were added with protease K working solution, fixed in blocking solution, rinsed and penetrated using 0.1% Triton X-100, followed by fluorescein isothiocyanate (FITC) end labeling of apoptotic DNA fragments using the TUNEL assay kit, and the images of the FITC-labeled TUNEL-positive cells were observed under a fluorescence microscope. Finally, the number of TUNEL-positive cells was calculated in 5 fields.

Observation of changes in myocardial tissues via hematoxylin-eosin (HE) staining

The isolated myocardial tissues soaked in formalin

were swashed using running water overnight, dehydrated in gradient alcohol, embedded in paraffin and routinely sectioned. The sections were immersed in xylene, hydrated using a series of ethanol, and baked dry. The resulting thin sections were stained with hematoxylin for 20 min and separated using hydrochloric acid-ethanol mixture for 30 s, stained again with eosin for 15 min, disassociated using 90% ethanol for 60 s and blocked. Finally, all the stained sections were placed in aqueous solution, and the pathological changes were observed under a light microscope.

Detection of gene expressions of Collagen I, α -SMA, TGF- β 1 and Smad3 via quantitative real-time polymerase chain reaction (qRT-PCR)

A total of 100 mg of myocardial tissues were taken carefully and accurately weighed at low temperature, then smashed with a homogenizer and centrifuged for the supernatant. Subsequently, ribonucleic acids (RNAs) were extracted from the supernatant collected and synthesized into complementary deoxyribonucleic acids (cDNAs) using the kit in accordance with the specific steps in the instructions. Afterward, the cDNAs were amplified into single-stranded ones in the amplification system (20 μ L) comprising 2 μ L of cDNAs, 10 μ L of qPCR mix, 2 μ L of primers and 6 μ L of ddH₂O, and stored at -20°C for later use. Then PCR amplification was conducted as follows: pre-denaturalization at 95°C for 2 min and PCR at 94°C for 20 s, 60°C for 20 s and 72°C for 30 s, for 40 cycles in total. The samples were amplified using the primers of the genes to be detected and the internal reference gene in triplicate. The sequences of the target genes and the internal reference glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed based on those from the GenBank (Table 1), and the expression levels of the target genes were measured via qRT-PCR. The relative expression levels of related genes in rat myocardial tissues in each group were calculated using $2^{-\Delta\Delta Ct}$.

Determination of TGF- β 1/Smad3 protein expression levels via Western blotting

Under low-temperature conditions, 100 mg of sterile myocardial tissues were accurately weighed, placed into a 10 mL Eppendorf (EP) tube, ground and quickly broken into pieces using a homogenizer. Then the resulting tissues were incubated in a refrigerator at 4°C for 30 min and centrifuged in a centrifuge. The supernatant was collected and stored in EP tubes separately, and the concentration of pro-

teins was measured using the BCA kit. Finally, the concentration of each protein was calculated. Subsequently, the protein samples were prepared, bathed in water for 8 min and centrifuged 1,000 g for 5 min, and the separation and stacking gels were prepared, followed by Western blotting, namely the proteins were loaded for electrophoresis at room temperature, transferred onto a membrane and incubated with primary and secondary antibodies. The protein bands were scanned using a scanner, and the levels of the proteins to be detected were corrected using GAPDH. Finally, the grayscale value of protein bands was analyzed using Image Lab (Media Cybernetics, Silver Springs, MD, USA).

Statistical analysis

The raw experimental data recorded were processed by Statistical Product and Service Solutions (SPSS) 22.0 (IBM, Armonk, NY, USA) analysis software, and subjected to multiple comparisons. The experimental results obtained were expressed as mean \pm standard deviation ($\bar{x} \pm SD$), and $P < 0.05$ indicated statistically significant differences. GraphPad Prism 8.0 (La Jolla, CA, USA) was employed to analyze histograms.

Results

Cardiac function parameters of rats in all groups

According to the measurement results of cardiac function parameters of rats, the FS and EF in DM group were obviously lower than those in Control group, but the LVEDD and LVESD were evidently greater than those in Control group ($P < 0.05$), while FAS group exhibited the opposite changes in the cardiac function parameters (Figure 1), suggesting that the DM rat model alters cardiac function, and that FAS can relieve the DM-induced cardiac dysfunction.

GLU content and hepatic and myocardial function indexes

Based on the detection results of GLU content and hepatic and myocardial function indexes, DM group had a substantially higher level of GLU in rats than the other two groups ($P < 0.05$) (Figure 2), implying that the rat model was constructed successfully. Moreover, compared with that in Control group, the content of serum Fas, AST and CK-MB was obviously raised in DM group ($P < 0.05$), but notably lowered in FAS group ($P < 0.05$), indicating that the hepatic and myocardial function indexes are obviously

Table 1. Primer sequences.

Gene	Primer sequence (F-R, 5'-3')
α -SMA	TCCCAGACATCAGGGAGTA CGGATACTTCAGCGTCAGG
Collagen I	GCAGTGCCTTTTGTGGAA CTATGGCCCGCTTCATGT
TGF- β 1	GTGGCTCCTAG TGTTGAC CAGTTTGGACA GGATCTG
Smad3	CTTCTTGACGAGAGAGTCTAC CTAACACTGGTGGCAGCACT
GAPDH	ACATGCCGCTGGAGAAA GCCCAGGATGCCCTTTAG

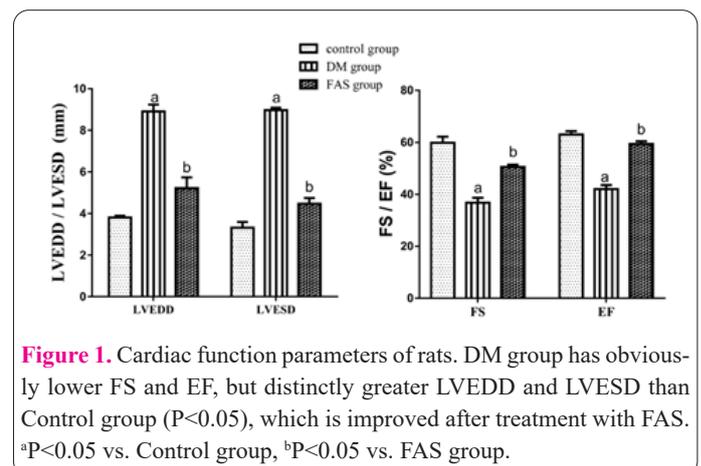


Figure 1. Cardiac function parameters of rats. DM group has obviously lower FS and EF, but distinctly greater LVEDD and LVESD than Control group ($P < 0.05$), which is improved after treatment with FAS. ^a $P < 0.05$ vs. Control group, ^b $P < 0.05$ vs. FAS group.

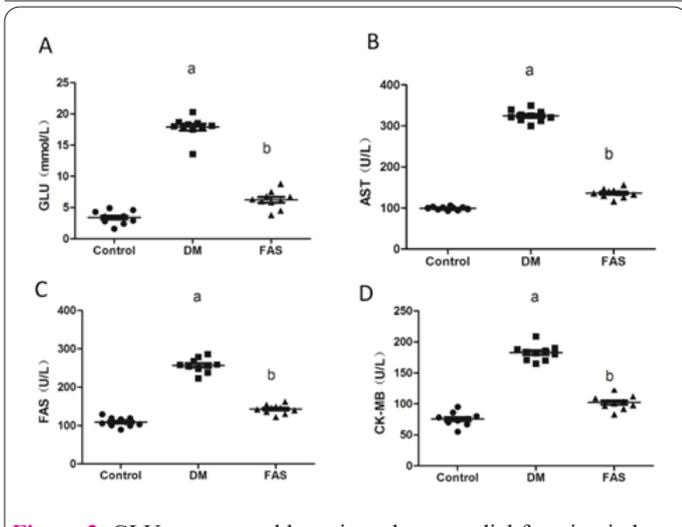


Figure 2. GLU content and hepatic and myocardial function indexes determined. The content of GLU, AST, Fas and CK-MB in the three groups is shown in Figures A, B, C and D, respectively. The content of serum GLU, AST, Fas and CK-MB in DM group is obviously higher than that in the Control group ($P < 0.05$), implying the abnormalities in the hepatic and myocardial function indexes. ^a $P < 0.05$ vs. Control group, ^b $P < 0.05$ vs. FAS group.

elevated in the development and progression of myocardial fibrosis in DM rats.

Serum TNF- α , IL-1 and IL-6 content determined

As shown in Table 2, the content of IL-1, IL-6 and TNF- α was elevated in DM group ($P < 0.05$), while their content declined in FAS group ($P < 0.05$). TUNEL staining results revealed that the TUNEL-positive cells in DM group were substantially more than those in the other two groups ($P < 0.05$), while no distinct TUNEL-positive cells were observed in both Control group and FAS group ($P < 0.05$). According to the HE staining results, DM group exhibited more obvious fibrosis in myocardial cells, thickening of muscle fibers and structurally disordered myocardial cells, while FAS group had arranged orderly myocardial cells and basically intact myocardial structure.

Expressions of related genes detected via qRT-PCR

According to the qRT-PCR results (Figure 3), the levels of Collagen I, α -SMA, TGF- β 1 and Smad3 in myocardial tissues of rats were markedly elevated in DM group ($P < 0.05$), but their expression levels declined remarkably in FAS group ($P < 0.05$), suggesting that FAS inhibits the expressions of the TGF- β 1/Smad3 signaling pathway and its relevant molecules to effectively prevent myocardial fibrosis.

Expression levels of TGF- β 1/Smad3 pathway-associated proteins determined via Western blotting

Furthermore, protein assay was performed in this study

Table 2. Serum TNF- α , IL-8 and IL-6 content.

Group	IL-1 (mg/L)	IL-6 (mg/L)	TNF- α (fmol/mL)
Control group	20.39 \pm 4.12	18.20 \pm 5.21	19.34 \pm 3.71
DM group	99.12 \pm 4.25 ^a	86.78 \pm 7.05 ^a	55.55 \pm 5.50 ^a
FAS group	32.85 \pm 3.16 ^b	28.41 \pm 6.10 ^b	28.39 \pm 5.04 ^b

Note: The content of the inflammatory factors IL-6, TNF- α and IL-1 is elevated in DM group ($P < 0.05$), but their content is lowered in FAS group ($P < 0.05$). ^a $P < 0.05$ vs. Control group, ^b $P < 0.05$ vs. FAS group.

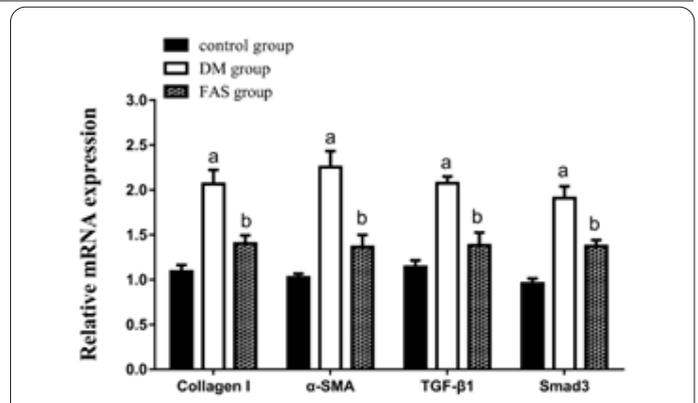


Figure 3. QRT-PCR results. DM group has markedly raised levels of Collagen I, α -SMA, TGF- β 1 and Smad3 in myocardial tissues of rats ($P < 0.05$), but their expression levels decline substantially in FAS group ($P < 0.05$). ^a $P < 0.05$ vs. Control group, ^b $P < 0.05$ vs. FAS group.

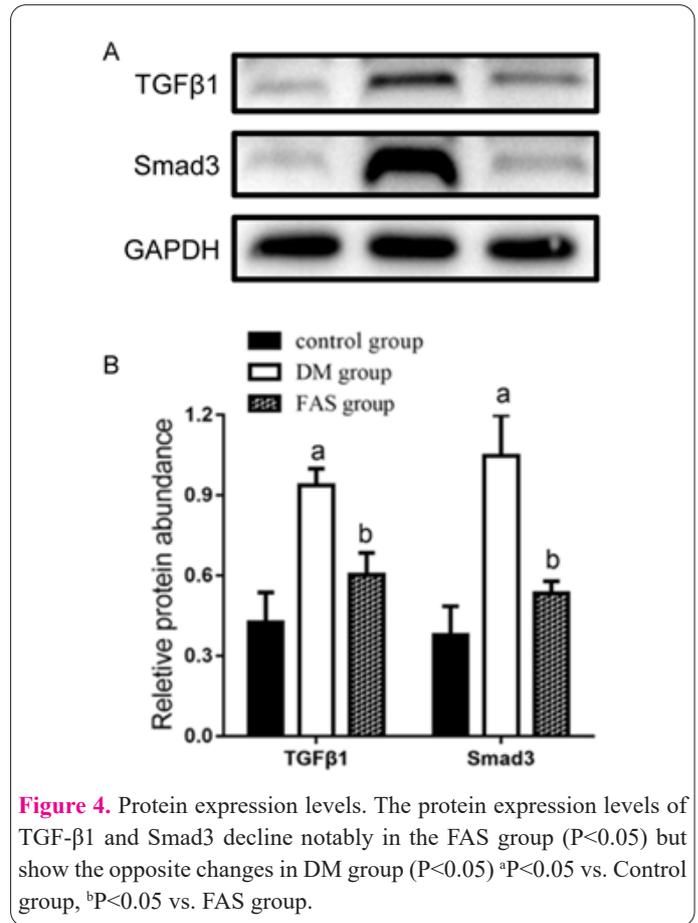


Figure 4. Protein expression levels. The protein expression levels of TGF- β 1 and Smad3 decline notably in the FAS group ($P < 0.05$) but show the opposite changes in DM group ($P < 0.05$) ^a $P < 0.05$ vs. Control group, ^b $P < 0.05$ vs. FAS group.

to determine the influence of FAS on the TGF- β 1/Smad3 signaling pathway in myocardial fibrosis, and it was found that FAS group showed substantial declines in the protein levels of TGF- β 1 and Smad3 in myocardial tissues ($P < 0.05$), while their protein expression exhibited the opposite changes in DM group ($P < 0.05$), indicating that FAS plays an inhibitory role in myocardial fibrosis (Figure 4).

Discussion

DM is a lifelong progressive disease with the feature of a significant increase in the concentration of blood glucose, and as one of the most common multi-cause chronic metabolic diseases worldwide (17,18), it has a wide variety of complications, including macrovascular and microvascular diseases, and can induce cardiovascular diseases such as nephropathy and myocardial fibrosis. In addition, DM endangers vision and leads to non-invasive amputation, blindness and visual impairment (19,20). The morbidity rates of diabetic diseases are raised with the extending of the survival time of patients, and cardiomyopathy is a DM complication (21). In the present study, the DM rat model was established, and notably raised blood GLU content was detected therein, implying that the model was successfully established. Furthermore, HE staining was performed to observe whether the model induced myocardial fibrosis, and it was discovered that DM group exhibited thickening of myocardial fibers and more obvious fibrosis in myocardial cells. Additionally, according to the measurement results of rat cardiac function parameters, the FS and EF in DM group were obviously lower than those in Control group, but the LVEDD and LVESD were evidently greater than those in Control group, and FAS group showed the opposite conditions. These results suggest that the DM model induces changes in cardiac function, and FAS can improve the DM-induced cardiac function abnormality. The hepatic function indexes AST and Fas and myocardial function index CK-MB were also measured in the present study, and the results showed that DM group had obviously elevated content of serum Fas, AST and CK-MB, while their content declined notably in FAS group, indicating obvious improvement in FAS group. Inflammation is a widely recognized crucial factor for DM and its vascular complications. The levels of such proinflammatory cytokines as IL-6 and TNF- α are elevated in DCM, and inflammatory responses, which are characterized by chronic low-grade inflammation and immune response disorder, further promote the aggravation of cardiomyopathy (22,23). The elevation of the levels of IL-6, IL-1 and TNF- α was detected in DM group in this study, suggesting that the increases in their levels accelerate the progression of myocardial fibrosis in DM to aggravate inflammatory responses. After the application of FAS, the levels of these three factors declined, illustrating that FAS treatment alleviates myocardial fibrosis in DM with favorable efficacy. Consistent with previous studies, the present study corroborated that FAS can repress the over-production of inflammatory factors to prevent myocardial fibrosis.

According to a study, the generation of apoptosis factors can be inhibited to suppress the development of myocardial fibrosis, and the wastes produced when cells sustain life activities can be timely eliminated through apoptosis, thereby keeping cells stable (24). The present study found that TUNEL-positive cells in DM group were notably more than those in the other two groups, while no distinct TUNEL-positive cells were observed in the Control group and FAS group, illustrating that FAS can prevent myocardial apoptosis. Studies have established that cardiac dysfunction occurs when the cardiac structure is destroyed due to the excessive deposition of collagen. Once myocardial cells have fibrosis, the expression level of α -SMA, an important component expressed by myocardial fibroblasts,

will be substantially raised (25-30). According to the findings in the present study, Collagen I and α -SMA were lowly expressed in FAS group, while their expression levels were considerably raised in DM group, implying that FAS represses the expressions of myocardial fibrosis-related molecules, which accords with the results of the above studies. Besides, it was found through the pathway-related gene assay that the expression levels of TGF- β 1 and Smad3 in rats were remarkably lowered in FAS group, but substantially raised in DM group, which were also detected through the protein assay. These results manifest that FAS exerts the therapeutical effect on myocardial fibrosis through inhibiting the expressions of TGF- β 1 and Smad3, which is similar to the conclusion of previous research. The present study confirmed the influence of FAS on myocardial fibrosis in DM, and subsequent multi-level and multi-angle studies can be performed through more molecular experiments such as immunofluorescence assay and flow cytometry, so as to provide vital theoretical and experimental bases for relevant research.

In conclusion, through the detection of indicators in animals *in vivo* as well as gene and protein expressions, this study found that FAS may suppress the TGF- β 1/Smad3 signaling pathway to regulate the degree of myocardial fibrosis in DM rats, and it can be clinically applied to control the progression of myocardial fibrosis in DM patients, which provide important experimental support for the treatment of myocardial fibrosis in DM and serve as the theoretical and experimental references for subsequent research of the TGF- β 1/Smad3 signaling pathway.

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Conflict of interest

The authors declared no conflict of interest.

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