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Effect of alprostadil on myocardial fibrosis in rats with diabetes mellitus *via* TGF-β1/Smad signaling pathway

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Abstract. – OBJECTIVE: To observe the influence of alprostadil on myocardial fibrosis in rats with diabetes mellitus (DM) through the transforming growth factor beta-1 (TGF-β1)/Smad signaling pathway.

MATERIALS AND METHODS: Wistar rats were employed to induce models of DM (DM group), and alprostadil treatment group (ALPR group) and control group (NC group) were set up. After successful modeling, blood and myocardial tissues were collected from rats. Next, blood glucose level, liver function, and myocardial function were detected. In addition, hematoxylin-eosin (HE) assay was performed to determine pathological changes. The enzyme-linked immunosorbent assay (ELISA) was carried out to measure serum interleukin-6 (IL-6) and cardiac function indexes such as ejection fraction (EF), Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Western blotting, which were applied to measure the gene and protein expression levels of important molecules in the proliferation and differentiation of myocardial fibroblasts [including checkpoint kinase 1 (Chek1) and alpha-smooth muscle actin (α-SMA)] and the relevant pathway TGF-β1/Smad2.

RESULTS: The blood glucose level was increased in DM group ($p<0.01$), suggesting that modeling is successful. The tumor necrosis factor-alpha (TNF-α), IL-6, and IL-1 levels were higher in DM group than in NC group. DM group had significantly elevated serum content of alkaline phosphatase (ALP), alanine aminotransferase (ALT), and creatine kinase (CK), as well as left ventricular end-diastolic dimension (LVEDd) and left ventricular end-systolic dimension (LVESd), but it clearly decreased fractional shortening (FS) and EF in comparison with NC group. Besides, myocardial cells were orderly arranged in NC group, while myocardial fibrosis was observed in DM group. The results of RT-PCR showed that the levels of Collagen, Chek1, α-SMA, TGF-β1, and Smad2 in myocardial fibroblasts were notably lowered in ALPR group, but evidently increased in DM group ($p<0.05$). According to Western blotting, there were evident decreases in the levels of TGF-β1 and Smad2 in myocardial fibroblasts in ALPR group ($p<0.05$). The above results suggest that alprostadil represses the expression of the TGF-β1/Smad2 signaling pathway and its relevant molecules, thus further suppressing the fibrosis of myocardial cells.

CONCLUSIONS: Alprostadil treats myocardial fibrosis in DM rats by inhibiting the TGF-β1/Smad2 signaling pathway.

Key Words:
Alprostadil, TGF-β1/Smad2 signaling pathway, Rat, Diabetes mellitus, Myocardial fibrosis.

Introduction

Diabetes mellitus (DM) is a lifelong progressive disease that is common in the middle-aged and elderly populations, which is characterized by a high blood glucose level, seriously affecting the quality of life of patients and bringing a heavy burden on the family[1]. Besides, it is one of the most common chronic metabolic diseases of multiple etiologies around the world[2]. DM, caused by the disorder of insulin metabolism in the body, has diverse complications including macrovascular and microvascular diseases, which can lead to kidney diseases and cardiovascular diseases,

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such as myocardial fibrosis. Diabetic nephropathy (DN) is a complication of DM[3]. Moreover, DM has a negative impact on vision and results in non-invasive amputation, blindness, and visual impairment[4]. The prevalence rate of DM is increased with the prolonged survival of patients, and cardiomyopathy is one of its complications[5,6]. Its pathogenesis is very complex and is related to the development of hypertension and coronary artery disease. The normal heart structure is composed of several different types of cells like cardiomyocytes, fibroblasts, and extracellular matrix (ECM) including collagen[7,8]. Excessive accumulation of ECM is an important pathological change in diabetic heart diseases. Collagen, the main component of ECM, plays an important role in supporting and protecting cardiomyocytes, which keeps the normal structure and functional components of myocardial tissues[9]. Oxidative stress, inflammatory and growth factors are important contributors to the development and progression of cardiomyopathy. Interstitial fibrosis is a result of the excessive accumulation of ECM in tissues[10]. Normal cardiac ECM maintains the homeostasis between matrix protein synthesis and degradation[11]. In this lifelong disease, the sustainably high level of circulating glucose is able to impair heart structure and function through many acute and cumulative long-term changes. These pathways lead to oxidative stress and inflammation, weakening the integrity of the cardiovascular wall[12].

Some authors[3,14] have manifested that the changed expression of transforming growth factor β1 (TGF-β1) and abnormal ECM may be involved in the development and progression of myocardial fibrosis. TGF-β1, a factor inducing fibrosis, has various cellular functions, such as controlling proliferation, differentiation, and migration of fibroblasts and the production of ECM. Besides, TGF-β1 increases protein expression in ECM in different types of cells, and overexpressed TGF-β1 promotes myocardial fibrosis and hypertrophy, thus resulting in ventricular remodeling[15]. Smad is a downstream of TGF-β1. Furthermore, the TGF-β1/Smad signaling pathway in myocardial tissue is closely related to DCM, and inhibiting the TGF-β1/Smad signaling pathway significantly attenuates myocardial hypertrophy and fibrosis, in which TGF-β1 is a crucial player[16,17]. Research has proved that alprostadil, namely prostaglandin E1 (PGE1), has many biological functions and plays good preventive and therapeutic roles in the fibrosis of the liver, lungs and kidneys. However, the study of its inhibitory effect on myocardial fibrosis in DM state is rare, and its mechanism in activating myocardial fibroblasts remains unclear.

This work aims to investigate the effects of alprostadil on myocardial fibrosis and the TGF-β1/Smad2 signaling pathway and to observe its preventive and therapeutic effects on myocardial fibrosis establishing DM models, observing various serum indicators and changes in enzyme activity and in gene and protein expressions. These will enrich and improve the theoretical basis of diabetic myocardial fibrosis and the influence on the TGF-β1/Smad2 signaling pathway.

Materials and Methods

Animal Grouping and Modeling

Male Wistar rats were divided into control group (NC group, n=30), DM model group (DM group, n=30), and alprostadil treatment group (ALPR group, 1 μg/kg, n=30). The DM models were established by intraperitoneal injection of streptozotocin (STZ). The clinical manifestations of the rats were observed every day, and detailed changes were recorded timely. After the experiment, blood and tissue samples were collected and stored for subsequent experiments. One part of heart tissues was used for hematoxylin-eosin (HE) detection and the remaining was stored at –80°C for measurement of gene and protein expression levels. This investigation was approved by the Animal Ethics Committee of Nanchang University Animal Center.

Detection of Serum Blood Glucose Level

It is well known that the successful modeling of experimental animals is a basic prerequisite for investigations. To observe whether DM models were successfully established, blood was collected from caudal vein of rats after modeling and centrifuged to collect the serum. Then, the blood glucose level was measured, recorded, and subsequently analyzed.

Determination of Liver Function and Myocardial Function

Serum biochemical indicators such as liver function indexes are affected by many diseases that certainly include DM. To predict myocardial fibrosis in DM rats in advance in clinic and provide an important reference for early diagnosis, liver function indexes alanine aminotransferase

(ALT), alkaline phosphatase (ALP), and myocardial function indicator creatine kinase (CK) were examined in this study. Blood was routinely collected from two groups of rats and centrifuged to separate the serum. Then, an automatic biochemical analyzer was used for detection.

Detection of Cardiac Physiological Function Indexes of Rats in Each Group

To observe whether DM induces myocardial dysfunction, magnetic resonance imaging (MRI), and echocardiography (ECG) systems were used to detect left ventricular function, including left ventricular end-diastolic dimension (LVEDd), left ventricular end-systolic dimension (LVESd), ejection fraction (EF), and fractional shortening (FS) according to the requirements in the instrument manuals, and the ECG was performed on each rat with a probe frequency of 10 MHz. Data were recorded and analyzed.

Observation of Changes in Heart Tissues in the Two Groups Via HE Staining

The isolated heart tissues previously obtained and immersed in formalin for 7 days were taken out, washed with running water for 24 h, dehydrated with gradient alcohol, and prepared into conventional sections (about 5 μm in thickness). Next, the sections were deparaffinized, hydrated with 95%, 90%, 80%, 75%, and 50% ethanol, respectively, permeabilized, dipped and embedded in paraffin. Then, paraffin-embedded blocks were made into pathological sections and dried. Lastly, the thin sections dried were stained with HE, mounted and observed using a light microscope. Pathological changes were recorded and photographed.

Detection of Inflammatory Factor Content Via Enzyme-Linked Immunosorbent Assay (ELISA)

Serum inflammatory factors, important indicators in heart injury, can indicate the degree of injury repair. Therefore, the content of inflammatory factors was detected in this work. The serum samples previously collected and cryopreserved at –80°C were slowly thawed at 4°C and centrifuged at low speed, and the supernatant was collected. Next, the change in each index was determined using a kit in accordance with the specific operations in the instructions. Lastly, the absorbance of inflammatory factors in each group was read using a microplate reader.

Detection of Gene Expressions of Checkpoint Kinase 1 (Chek1), Alpha-Smooth Muscle Actin (α-SMA) and TGF-β1/Smad2 Through ReverseTranscription-Polymerase Chain Reaction (RT-PCR)

Myocardial tissues (100 mg) were weighed, ground in liquid nitrogen, pulverized using a homogenizer and centrifuged, and the supernatant was collected and used to extract RNAs. Next, RNAs extracted were synthesized into complementary deoxyribose nucleic acids (cDNAs) using a kit (TaKaRa, Otsu, Shiga, Japan) following the specific operating procedures in the instructions. Thereafter, the cDNAs were amplified into single-stranded cDNAs using a conventional reaction system, and stored at –20°C for PCR amplification. The samples were amplified with the gene to be detected and the internal reference gene primer, and each reaction was repeated 3 times. A 20 μL amplification system (2 μL cDNA, 10 μL qPCR mix, 2 μL primer, and 6 μL ddH_2O) was prepared for PCR amplification. The primer sequences of target genes and the internal reference glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were designed based on the sequences on GenBank (Table I), and the expression levels of target genes were measured through quantitative RT-PCR. The relative expression levels of related genes in rat myocardial tissues in each group were calculated by the $2^{-\Delta\Delta CT}$ method.

Detection of TGF-β1/Smad2 Protein Expression by Western Blotting

(1) Sterile heart tissues (about 150 mg) were accurately weighed and put in a 10 mL Eppendorf (EP) tube. Next, the tissues were ground at low temperature, rapidly pulverized at low temperature using the homogenizer, incubated in a

Table I. Primer sequences.

Gene	Primer sequence (5'-3', F-R)
Chek1	ATCAGCCCAAACCCCAAGGAGA CGCAGGAAGGTCAGCTGGATAG
α-SMA	GTCCCAGACATCAGGGAGTAA TCGGATACTTCAGCGTCAGGA
Collagen I	GGCAGTGCCTTTTGTGGAAG TCTATGGCCCGCTTCATGTC
TGF-β1	TGTGGCTCCTAG TGTTGACG GCAGTTTGGACA GGATCTGG
Smad2	GCTTCTTGACGAGAGAGTCTACGG TACTAACACTGGTGGCAGCACTGG
GAPDH	GACATGCCGCCTGGAGAAAC AGCCCAGGATGCCCTTTAGT

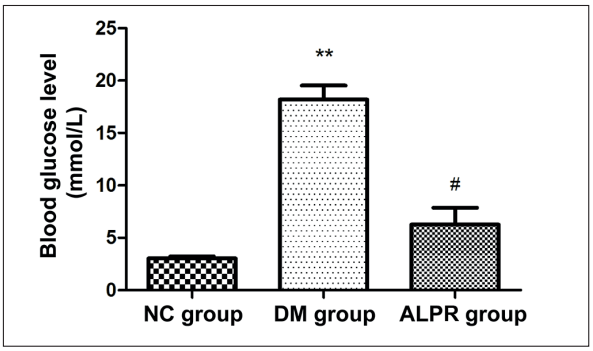

Figure 1. Blood glucose level. The blood glucose level in DM group is significantly higher than that in the other two groups (p<0.01). **p<0.01, #p<0.05.

refrigerator at 4°C for 30 min, shaken slowly at room temperature and centrifuged to collect the supernatant. (2) The supernatant was taken and dispensed into EP tubes, and a bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA) was employed to detect the protein concentration. After the protein concentration met the test requirements, the concentration of each protein was calculated. (3) Western blotting was carried out as follows: sample preparation, water bath for 8 min, centrifugation at 1000 g for 5 min, separation gel, and spacer gel preparation, sample loading, electrophoresis at room temperature, transfer of proteins to a membrane, and incubation with primary and secondary antibodies. Lastly, a membrane scanner (Bio-Rad, Hercules, CA, USA) was utilized to scan and quantify protein bands, GAPDH was used to correct the levels of proteins to be tested, and ImageLab was employed to analyze the gray value of protein bands.

Statistical Analysis

The raw experimental data recorded were processed by Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) and subjected to multiple comparisons. The experimental results obtained were expressed as mean ± standard deviation (χ±SD), and p<0.05 suggested that the difference was statistically significant. GraphPad Prism 8.0 (GraphPad Software Inc, La Jolla, CA, USA) was used for histograms.

Results

Serum Blood Glucose Level Detected

The blood glucose level was measured to determine whether DM models were successfully established. The results (Figure 1) showed that the blood glucose level was significantly higher in DM group than in the other two groups (p<0.01), suggesting that DM models are successfully established and can be used for subsequent experimental studies in this research.

Liver Function and Myocardial Function Determined

Liver function indicators AST and ALP, and myocardial function index CK were examined to provide important references for early diagnosis in clinical practice. The results (Table II) revealed that the levels of serum ALP, AST, and CK were clearly higher in DM group than in NC group (p<0.05). ALPR group exhibited similar or even lower levels in comparison with NC group, indicating that liver function and myocardial function indicators are notably elevated in the development and progression of myocardial fibrosis in DM rats, which are remarkably improved after treatment with alprostadil.

Arterial Blood Pressure and Cardiac Function Indexes in Two Groups of Rats

The detection results of cardiac function indexes in each group showed in Table III that DM group had significantly lowered FS and EF but notably increased LVEDd and LVESd in comparison with NC group (p<0.05). ALPR group exhibited opposite trends to DM group, indicating that rat models of DM cause changes in cardiac function, and alprostadil relieves DM-induced cardiac dysfunction.

Table II. Serum biochemical test results (U/L).

Group	AST	ALP	CK
NC group	120.5±2.4	108.5±1.2	82.5±1.8
DM group	389.8±3.8[a]	208.7±2.6[a]	188.6±1.4[a]
ALPR group	196.4±4.3[b]	152.8±3.5[b]	108.7±2.3[b]

Note: The content of serum ALP, AST, and CK is significantly increased in DM group compared with that in NC group ([a]p<0.05), implying that liver function and myocardial function indexes are abnormal ([a b]p<0.05).

Table III. Cardiac function indexes in rats.

Group	LVEDd (mm)	LVESd (mm)	FS (%)	EF (%)
NC group	4.58±0.86	3.59±0.31	54.2±2.4	60.8±2.9
DM group	7.61±1.2[a]	8.26±0.96[a]	32.4±3.5[a]	43.9±2.1[a]
ALPR group	5.26±0.64[b]	4.8±0.64[b]	48.8±1.6[b]	55.5±2.7[b]

Note: The FS and EF are significantly lower in DM group than those in NC group, while the LVEDd and LVESd are overtly higher in DM group than those in NC group ($p<0.05$), which are improved after treatment with alprostadil ([a b]$p<0.05$).

Pathological Changes in Rat Myocardial Tissues Detected Via HE Staining

HE staining was used to determine the morphology and damage of myocardial tissues in each group of rats, and the results (Figure 2) revealed that in ALPR group, the cardiomyocytes were arranged orderly, with intact structure (Figure 2A), while in DM group, thickened muscle fibers and THE relatively evident fibrosis of cardiomyocytes was observed (Figure 2B).

Serum Tumor Necrosis Factor-Alpha (TNF-α), Interleukin-1 (IL-1) and IL-6 Content

According to Table IV, the levels of IL-1, IL-6, and TNF-α were increased in DM group ($p<0.05$), but reduced in ALPR group ($p<0.05$).

Gene Expressions of Collagen, Chek1, α-SMA and TGF-β1/Smads Detected by RT-PCR

The results of RT-PCR assay (Figure 3) showed that the levels of Collagen, Chek1, α-SMA, TGF-β1, and Smad2 in myocardial fibroblasts were significantly decreased in ALPR group ($p<0.05$). They were significantly increased in DM group and were evidently higher than NC group ($p<0.05$), indicating that alprostadil inhibits the expression of TGF-β1/Smad2 signaling pathway and its related molecules, thus further suppressing cardiomyocyte fibrosis.

Protein Expression of Important Molecules of Myocardial Fibrosis and TGF-β1/Smads Determined via Western Blotting

To further determine the influence of alprostadil on the TGF-β1/Smad2 signaling pathway during myocardial fibrosis, Western blotting was employed and the results (Figure 4) revealed that the levels of TGF-β1 and Smad2 in rat cells were clearly decreased in ALPR group ($p<0.05$). Those in DM group were notably raised and higher than in NC group ($p<0.05$), implying that alprostadil represses cardiomyocyte fibrosis.

Discussion

DM models were established to further observe whether myocardial fibrosis injury can be induced, and the pathogenesis of diabetic myocar-

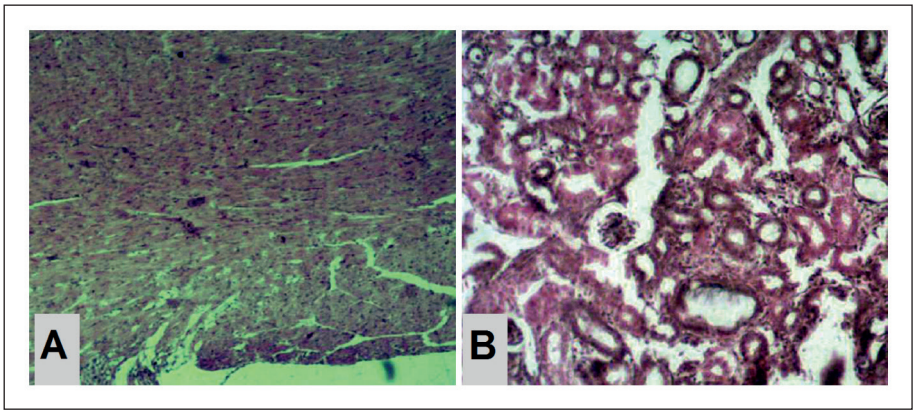

Figure 2. HE staining results. Cardiomyocytes are arranged orderly and have intact structure in ALPR group, showing no differences from those in NC group (**A**, magnification 200×), while in DM group, cardiomyocytes have disordered arrangement and are fibrotic, with thickened muscle fibers (**B**, magnification 200×).

Table IV. Serum TNF-α, IL-1, and IL-6 content.

Group	IL-1 (mg/L)	TNF-α (fmol/mL)	IL-6 (mg/L)
NC group	25.35±5.67	15.34±3.25	14.29±5.58
DM group	89.64±7.25[a]	35.52±5.24[a]	80.35±7.45[a]
ALPR group	29.78±3.86[b]	18.29±5.54[b]	30.12±6.29[b]

Note: The levels of inflammatory factors IL-6, TNF-α, and IL-1 are raised in DM group ($p<0.05$) but lowered in ALPR group ($p<0.05$), [a]$p<0.05$, [a]$p<0.05$.

dial fibrosis was explored to find potential treatment methods. DM models had the most similar signs to those of DM patients, so they could be commendably used as research subjects. It was observed that the blood glucose level was significantly increased in DM rats. Furthermore, HE staining results showed that cardiac muscle fibers were thickened and the fibrosis of cardiomyocytes was relatively evident in DM group. These results suggest that the DM models used in this study are successful.

Diabetic cardiomyopathy (DCM), whose main pathological feature is cardiomyocyte hypertrophy or hyperplasia, is characterized by an excessive accumulation of myocardial interstitial collagens and myocardial necrosis, often resulting in cardiac hypertrophy and cardiac dysfunction[18]. Moreover, the non-enzymatic advanced glycation end products alter the structure and function of the matrix proteins and the expression of collagens, which is capable of leading to myocardial fibrosis and stiffness[19]. Excessive collagen I and collagen III may result in deposition and disorder of myocardial interstitial collagens, or even heart injury. Furthermore, myocardial contractile function is impaired with aggravating myocardial ischemia[20]. In this study, the results of cardiac function index examination showed that DM group had significantly lowered FS and EF but evidently increased LVEDd and LVESd in comparison with NC group ($p<0.05$). ALP group exhibited opposite tendencies, suggesting that the rat models of DM have changes in cardiac function, and alprostadil attenuates cardiac dysfunction due to DM. Besides, liver function indicators AST and ALP and myocardial function index CK were detected and it was discovered that the serum ALP, AST, and CK levels were significantly increased in DM group. Such indexes in ALPR group were similar to or even lower than those in NC group, suggesting that the liver function and myocardial function indexes are notably raised in the development and progression of myocardial fibrosis in DM rats, which is overtly improved after treatment with alprostadil. In addition, inflammatory responses, characterized by chronic low-level inflammation and immune response disorders, further aggravate cardiomyopathy. Here, the levels of IL-6, IL-1, and TNF-α were elevated in DM group, suggesting that elevated levels of IL-6 and TNF-α further promote the progression of diabetic myocardial fibrosis, thereby aggravat-

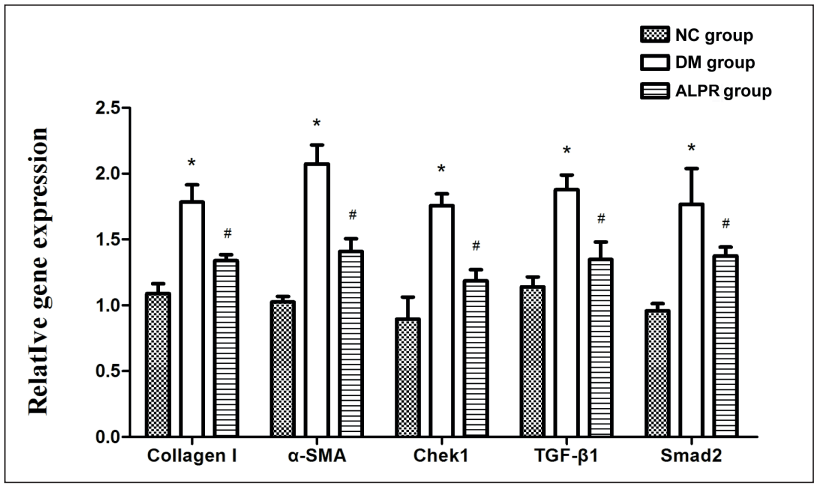

Figure 3. Results of RT-PCR assay. ALPR group has overtly lowered levels of Collagen, Chek1, α-SMA, TGF-β1, and Smad2 in myocardial fibroblasts ($p<0.05$), while such levels in DM group are markedly elevated and evidently higher than those in NC group ($p<0.05$). *$p<0.05$, #$p<0.05$.

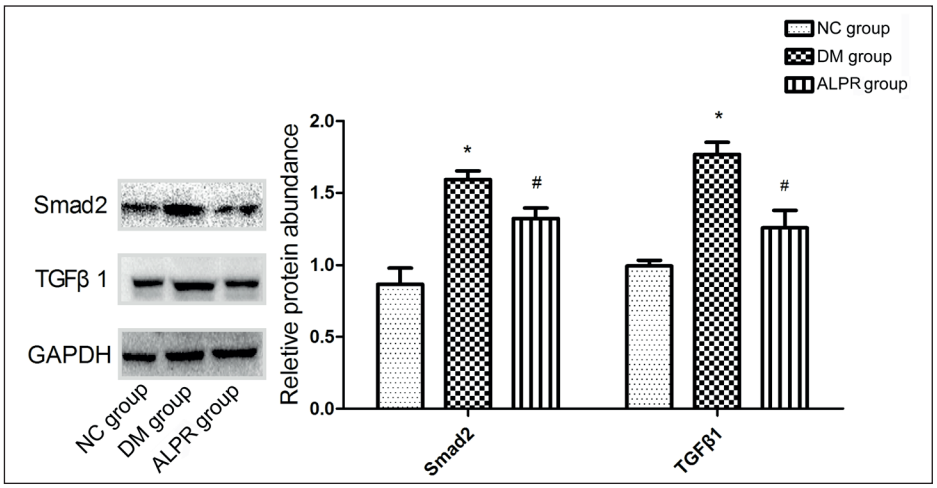

Figure 4. Protein expression level. The level of TGF-β1 and Smad2 is significantly increased in ALPR group (p<0.05), but overtly lowered in DM group (p<0.05). *p<0.05, #p<0.05.

ing inflammatory responses. Such levels were decreased after treatment with alprostadil, implying that the condition of the disease is improved after treatment with alprostadil, which indicates that alprostadil is very effective in treating diabetic myocardial fibrosis. TNF-α plays an indispensable role in the development of inflammation in DM rats and IL-6 can also trigger excessive production of other inflammatory mediators[21,22]. The results of this study are in line with the findings of previous studies. It shows that alprostadil can suppress excessive inflammatory cytokines and prevent irreversible damage due to excessive cell production.

Scholars[23,24] have observed that myocardial fibrosis leads to heart failure, increased left ventricular stiffness, and reduced ventricular wall compliance, thus resulting in systolic function, especially diastolic dysfunction. Excessive ECM, caused by an imbalance between synthesis and degradation, plays an important role in heart failure. Collagens have good toughness, so that they maintain heart elasticity[25]. Therefore, excessive collagen deposition destroys the structure of the heart, thus cardiac dysfunction occurs. Furthermore, Chek1 and α-SMA are important in myocardial fibrosis. Chek1 is a key molecule for mitotic proliferation regulation of cardiomyocytes, and α-SMA is an important component of cardiac fibroblast expression. Their expressions will be remarkably increased in case of cardiomyocyte fibrosis[26,27]. It was found in this study that the expressions of Collagen, Chek1, and α-SMA were low in ALP group and high in DM group. Such expressions in DM group were clearly higher than those in NC group (p<0.05), implying that alprostadil inhibits the expression of molecules related to myocardial fibrosis, which is consistent with the above studies. In the TGF-β1/Smad2 pathway, TGF-β1 binds to its receptor TGF-β1-RI to phosphorylate the c-terminal serine residue of the Smad2 protein, thus producing different downstream signals[28,29]. TGF-β1 inhibits the degradation of ECM and induces tissue inhibitors of metalloproteinases by repressing matrix metalloproteinases, which may lead to the deterioration of myocardial damage and the development and progression of myocardial fibrosis[30]. The results of pathway gene detection showed that the levels of TGF-β1 and Smad2 were markedly decreased in ALPR group (p<0.05), but evidently lowered in DM group, and the levels of TGF-β1 and Smad2 were higher in DM group than in NC group. Besides, the results of protein detection revealed that ALPR group had markedly reduced levels of TGF-β1 and Smad2 (p<0.05). DM group exhibited clearly raised levels of TGF-β1 and Smad2, which were higher than those in NC group (p<0.05). The above results indicate that alprostadil can treat cardiomyocyte fibrosis by inhibiting TGF-β1 and Smad2, which is similar to the findings of the previous studies. This research proved the effect of alprostadil on diabetic myocardial fibrosis, but there were some shortcomings: only some *in vivo* investigations in experimental animals were performed for verification and no typical cell lines were used for mutual confirmation of this effect with the *in vivo* experiments. Therefore, more molecular investigations including immunofluorescence assay, flow cytometry, and electrophoretic mobility shift as-

say (EMSA) can be employed to study such an effect from multiple levels and perspectives to provide important theoretical and experimental evidence for relevant subsequent research.

Conclusions

It is demonstrated by a series of *in vivo* experiments and gene and protein experiments in animals that alprostadil may regulate the progression of myocardial fibrosis in DM rats by suppressing the TGF-β1/Smad2 signaling pathway. Its therapeutic effect can be observed through the TGF-β1/Smad2 signaling pathway, providing experimental evidence and certain theoretical basis for the treatment of diabetic myocardial fibrosis and the effect on the TGF-β1/Smad2 signaling pathway.

Conflict of Interests

The Authors declared that they have no conflict of interests.

References

1) Bao SL, Pan J, Sun HX, Liu WT. Valsartan improves cardiac function in mice with diabetes mellitus by CaMKII/AngII. Eur Rev Med Pharmacol Sci 2018; 22: 5327-5334.
2) Olokoba AB, Obateru OA, Olokoba LB. Type 2 diabetes mellitus: a review of current trends. Oman Med J 2012; 27: 269-273.
3) Mestry SN, Dhodi JB, Kumbhar SB, Juvekar AR. Attenuation of diabetic nephropathy in streptozotocin-induced diabetic rats by Punica granatum Linn. leaves extract. J Tradit Complement Med 2016; 7: 273-280.
4) Sharma S, Oliver-Fernandez A, Liu W, Buchholz P, Walt J. The impact of diabetic retinopathy on health-related quality of life. Curr Opin Ophthalmol 2005; 16: 155-159.
5) Aneja A, Tang WH, Bansilal S, Garcia MJ, Farkouh ME. Diabetic cardiomyopathy: insights into pathogenesis, diagnostic challenges, and therapeutic options. Am J Med 2008; 121: 748-757.
6) Ward ML, Crossman DJ. Mechanisms underlying the impaired contractility of diabetic cardiomyopathy. World J Cardiol 2014; 6: 577-584.
7) Cunnington RH, Nazari M, Dixon IM. C-Ski, Smurf2, and Arkadia as regulators of TGF-beta signaling: new targets for managing myofibroblast function and cardiac fibrosis. Can J Physiol Pharmacol 2009; 87: 764-772.
8) Fujiu K, Wang J, Nagai R. Cardioprotective function of cardiac macrophages. Cardiovasc Res 2014; 102: 232-239.
9) Liu X, Liang E, Song X, Du Z, Zhang Y, Zhao Y. Inhibition of Pin1 alleviates myocardial fibrosis and dysfunction in STZ-induced diabetic mice. Biochem Biophys Res Commun 2016; 479: 109-115.
10) Falcão-Pires I, Leite-Moreira AF. Diabetic cardiomyopathy: understanding the molecular and cellular basis to progress in diagnosis and treatment. Heart Fail Rev 2012; 17: 325-344.
11) Talior-Volodarsky I, Connelly KA, Arora PD, Gullberg D, McCulloch CA. α11 integrin stimulates myofibroblast differentiation in diabetic cardiomyopathy. Cardiovasc Res 2012; 96: 265-275.
12) Brownlee M. The pathobiology of diabetic complications: a unifying mechanism. Diabetes 2005; 54: 1615-1625.
13) Jiang C, Huang H, Liu J, Wang Y, Lu Z, Xu Z. Fasudil, a Rho-kinase inhibitor, attenuates bleomycin-induced pulmonary fibrosis in mice. Int J Mol Sci 2012; 13: 8293-8307.
14) Harmancey R, Taegtmeyer H. The complexities of diabetic cardiomyopathy: lessons from patients and animal models. Curr Diab Rep 2008; 8: 243-248.
15) Sharma A, Tate M, Mathew G, Vince JE, Ritchie RH, de Haan JB. Oxidative stress and NLRP3-inflammasome activity as significant drivers of diabetic cardiovascular complications: therapeutic implications. Front Physiol 2018; 9: 114.
16) Tijsen AJ, van der Made I, van den Hoogenhof MM, Wijnen WJ, van Deel ED, de Groot NE, Alekseev S, Fluiter K, Schroen B, Goumans MJ, van der Velden J, Duncker DJ, Pinto YM, Creemers EE. The microRNA-15 family inhibits the TGF β-pathway in the heart. Cardiovasc Res 2014; 104: 61-71.
17) Bai YW, Ye MJ, Yang DL, Yu MP, Zhou CF, Shen T. Hydrogen sulfide attenuates paraquat-induced epithelial-mesenchymal transition of human alveolar epithelial cells through regulating transforming growth factor-beta1/Smad2/3 signaling pathway. J Appl Toxicol 2019; 39: 432-440.
18) Kayama Y, Raaz U, Jagger A, Adam M, Schellinger IN, Sakamoto M, Suzuki H, Toyama K, Spin JM, Tsao PS. Diabetic cardiovascular disease induced by oxidative stress. Int J Mol Sci 2015; 16: 25234-25263.
19) Joshi M, Kotha SR, Malireddy S, Selvaraju V, Satoskar AR, Palesty A, McFadden DW, Parinandi NL, Maulik N. Conundrum of pathogenesis of diabetic cardiomyopathy: role of vascular endothelial dysfunction, reactive oxygen species, and mitochondria. Mol Cell Biochem 2014; 386: 233-249.
20) Zhang Y, Zhang L, Zhang Y, Xu JJ, Sun LL, Li SZ. The protective role of liquiritin in high fructose-induced myocardial fibrosis via inhibiting NF-κB and MAPK signaling pathway. Biomed Pharmacother 2016; 84: 1337-1349.
21) Christman JW, Sadikot RT, Blackwell TS. The role of nuclear factor-kappa B in pulmonary diseases. Chest 2000; 117: 1482-1487.

22) Naka T, Nishimoto N, Kishimoto T. The paradigm of IL-6: from basic science to medicine. Arthritis Res 2002; 4 Suppl 3: S233-S242.

23) Li CJ, Lv L, Li H, Yu DM. Cardiac fibrosis and dysfunction in experimental diabetic cardiomyopathy are ameliorated by alpha-lipoic acid. Cardiovasc Diabetol 2012; 11: 73.

24) Kain V, Kumar S, Sitasawad SL. Azelnidipine prevents cardiac dysfunction in streptozotocin-diabetic rats by reducing intracellular calcium accumulation, oxidative stress and apoptosis. Cardiovasc Diabetol 2011; 10: 97.

25) Asbun J, Villarreal FJ. The pathogenesis of myocardial fibrosis in the setting of diabetic cardiomyopathy. J Am Coll Cardiol 2006; 47: 693-700.

26) Castro-Chaves P, Cerqueira R, Pintalhao M, Leite-Moreira AF. New pathways of the renin-angiotensin system: the role of ACE2 in cardiovascular pathophysiology and therapy. Expert Opin Ther Targets 2010; 14: 485-496.

27) Porrello ER, Johnson BA, Aurora AB, Simpson E, Nam YJ, Matkovich SJ, Dorn GW 2nd, van Rooij E, Olson EN. MiR-15 family regulates postnatal mitotic arrest of cardiomyocytes. Circ Res 2011; 109: 670-679.

28) Schneiders D, Heger J, Best P, Michael PH, Taimor G. SMAD proteins are involved in apoptosis induction in ventricular cardiomyocytes. Cardiovasc Res 2005; 67: 87-96.

29) Pan Z, Zhao W, Zhang X, Wang B, Wang J, Sun X, Liu X, Feng S, Yang B, Lu Y. Scutellarin alleviates interstitial fibrosis and cardiac dysfunction of infarct rats by inhibiting TGFβ1 expression and activation of p38-MAPK and ERK1/2. Br J Pharmacol 201 1; 162: 688-700.

30) Cutroneo KR. TGF-beta-induced fibrosis and SMAD signaling: oligo decoys as natural therapeutics for inhibition of tissue fibrosis and scarring. Wound Repair Regen 2007; 15 Suppl 1: S54-S60.