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Original Article

Effects of Tongxinluo on myocardial fibrosis in diabetic rats

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Abstract

Background: The aim of this study was to explore the effect of Tongxinluo on myocardial fibrosis in diabetic rats and its possible mechanism of action.
Methods: Diabetic rat models were established and then divided into three groups: control, diabetes, and Tongxinluo groups. Heart function and myocardial interstitial collagen volume fraction were investigated, and the protein and mRNA expression levels of transforming growth factor beta 1 (TGF-β_1), $Smad_3$, and $Smad_7$ were measured.
Results: Heart function was clearly abnormal in the diabetes group compared with that in the control group, and the collagen volume fraction and mRNA expression levels of TGF-β_1 and $Smad_3$ were higher. However, the protein and mRNA expression levels of $Smad_7$ were lower. In the Tongxinluo group, it was observed that these indicators were improved.
Conclusion: Tongxinluo was effective for the prevention and treatment of myocardial fibrosis in diabetic rats. It probably mediates the expressions of TGF-β_1, $Smad_3$, and $Smad_7$ in rat cardiomyocytes to reduce the occurrence of myocardial fibrosis in diabetic rats.

Keywords: diabetic myocardial fibrosis; $Smad_3$; $Smad_7$; Tongxinluo; transforming growth factor beta 1

1. Introduction

In 1972, Rubler[1] first proposed diabetic cardiomyopathy (DCM) as one of the microangiopathies in diabetes with a very complicated pathogenesis, independent of the occurrence of hypertension and coronary artery disease. The prevailing opinion now is that DCM is highly associated with the renin−angiotensin−aldosterone system, oxidative stress, inflammatory factors, growth factors, and extracellular matrix (ECM), which play an essential role in the development and occurrence of DCM.[2−6] Furthermore, the major pathological changes of DCM are an increase in the size of myocardial microvascular walls, a narrow lumen, ECM accumulation, and myocardial fibrosis,[7] which is a characteristic pathological change in DCM.[8] Recent studies showed that a change in the expression of transforming growth factor beta 1 (TGF-β_1) and an abnormal ECM might be involved in the development and occurrence of DCM.[9,10] TGF-β_1 is the most powerful cytokine that has been recognized to induce fibrosis. It performs many cellular functions, including the control of fibroblast proliferation, differentiation, migration, and production of ECM. TGF-β_1 also increases protein expression in the ECM of different cell types; however, overexpression of TGF-β_1 promotes myocardial fibrosis and hypertrophy leading to ventricular remodeling.[11] The Smad protein is found in the cytoplasm; it is the downstream signal transduction molecule

Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

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of the TGF-β1 family of receptors as well as the key regulatory factor of the TGF-β_1/Smad signal transduction pathway. Moreover, the TGF-β_1/Smad signaling pathway in myocardial tissue is closely associated with DCM.

Tongxinluo, in capsule form, is a traditional Chinese medicine. It is a compound preparation consisting of many kinds of herbs and insects, developed using the traditional Chinese collateral disease theory. Tongxinluo is crushed and squeezed, which helps release the active ingredients into solution and improves bioavailability and efficacy. Some reports have indicated that Tongxinluo has a good clinical efficacy when used to treat patients with angina. This study explored the effect of Tongxinluo on the prevention and treatment of myocardial fibrosis in rats with diabetes induced by streptozotocin (STZ) and the possible mechanism of action of Tongxinluo, and examined the expression levels of TGF-β_1, $Smad_3$, and $Smad_7$ in myocardial tissue.

2. Methods

2.1. Animals

Healthy Sprague-Dawley male rats ranging in age from 7 weeks to 8 weeks and weighing 180−220 g were provided by the Experimental Animal Center of Liaoning Medical College. The certification number was SYXK (Liao) 2005-0013. This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Shandong University.

2.2. Establishment of diabetic model and groups

Thirty healthy Sprague-Dawley male rats that were deprived of food and water for 12 hours were injected with 1% STZ solution (Sigma, 22 chaowai street, fanli mansion, 4 floor, room 407, Chaoyang, Beijing) through the caudal vein. An STZ (1%) solution was prepared with 0.5 mol/L of sodium citrate buffer (pH 4.5). As controls, 10 rats were injected with the same dose (0.5 mol/L) of the sodium citrate buffer. After 72 hours, blood samples were collected to measure blood glucose level using a blood glucose monitor (SureStep Plus; Johnson Company). If the blood glucose concentration exceeded 16.7 mmol/L for 3 consecutive days, a diabetic model was assumed to have been successfully established. Thereafter, 28 diabetic rats were successfully established, which were then randomly divided into a diabetic group and a Tongxinluo group; each group had 14 rats. In the Tongxinluo group, 1.0 g Tongxinluo ultrafine powder (equivalent to 1.43 g of crude drug; lot number: 070406; HebeiYiling Pharmaceutical Institute, Shijiazhuang, China), consisting of ginseng, leech, scorpion, chilopod, woodlouse, red paeonia, cicada, and borneol, was dissolved in physiological saline and intragastrically administered to rats at a dose of 0.5 g/kg once a day. The same volume of water was administered to the control and diabetic groups for 12 weeks. The rats were weighed and blood samples were collected to determine blood glucose level once a week. In addition, drug dose was regulated according to weight.

2.3. Measurement of heart function

At the end of 12 weeks, rats from the three groups were anesthetized by injecting them with a 20% urethane solution (5 mL/kg). Tracheal cannulation was performed on the rats; one end of the tube was inserted into the left ventricular chamber and the other end was connected to a pressure transducer to record left ventricular pressure (LVP) curves. Then, LVP signals were inputted into a BL-420F biological signal collection system (Taimeng Tech. Ltd., Chengdu, China) and the heat rate, LVP maximum rise or fall speed ($\pm dp/dt_{max}$), left ventricular systolic pressure (LVSP), and left ventricular end diastolic pressure (LVEDP) were recorded.

2.3.1. Determination of heart weight index and left ventricular weight index

After functional measurement was obtained, the heart was removed and washed at 4°C and weighed after being dried. The left ventricle was weighed and rat heart weight index (whole heart weight/body weight) and left ventricular weight index (left ventricular weight/body weight) were calculated. Next, the myocardial specimen of the left ventricle was cut into two parts; one part was stored at −80°C for reverse transcriptase polymerase chain reaction (RT-PCR) assay, and the other part was fixed with 10% formalin and then embedded in paraffin for spectroscopic analysis and detection of the protein expressions of TGF-β_1, $Smad_3$, and $Smad_7$ through immunohistochemistry.

2.4. Measurement of myocardial interstitial collagen volume fraction

After the paraffin sections were dewaxed, they were stained with Masson's dye and observed under a light microscope. Myocardial cells and collagen fibers were dyed in red and blue, respectively. Next, the sections were photographed and scanned to calculate the interstitial collagen volume fraction (CVF) using an image analyzer (CVF = collagen area/total area). In each section, the measurements were performed by choosing five high-power fields and then obtaining mean area values.

2.5. Immunohistochemistry assay

Myocardial tissue sections were dewaxed with xylene and ethanol, and antigen repair and serum blocking were performed. The primary antibody (at 4°C overnight) and secondary antibodies (rabbit antirat TGF-β_1, $Smad_3$, and $Smad_7$ antibodies; Beijing Bioss Ltd., Beijing, China) were added, and 3,3′-diaminobenzidine (DAB) coloration, hematoxylin redyeing, dehydration, and mounting were performed in order. A brown-yellow coloration represented a positive expression under the microscope. Ten high-power fields were chosen in sections from each group to be observed randomly and

analyzed using a CIAS-1000 cell image analyzer (Beijing Daheng Image Vision, Ltd., Beijing, China).

2.5.1. RT-PCR

Myocardial tissue, which was stored at −80°C, was taken and 1 mL of Trizol solution was added to extract total RNA. RT-PCR assay was performed according to the RT-PCR kit instructions. Additionally, primers were designed and synthesized by Dalian Takara Ltd (Dalian treasure biological engineering co., LTD, economic and technological development zone of northeast 19 second street of Dalian city, Liaoning province). Primers were as follows: TGF-β_1 (329 bp)—sense: 5′ATGGTGGACCGCAAC AAC3′, antisense: 5′TGAGCACTGAAGCGAAAGC3′; $Smad_3$ (74 bp)—sense: 5′GATGTGGCTGGGAAATAC3′, antisense: 5′TTCTAGTCAGTCTGCCTGTAC3′; $Smad_7$ (345 bp)—sense: 5′CCCTTTGGATCAGCATTTC3′, antisense: 5′GGTTCTGG TTCAGCCTTCTA3′; β-actin (477 bp)—sense: 5′GAAATCGT GCGTGACATTA3′, antisense: 5′GGACTCATCGTACTCCT GCT3′. PCR conditions were as follows: initial melting at 94°C for 5 minutes followed by 33 cycles of denaturation at 94°C for 30 seconds, annealing at an appropriate temperature for 30 seconds, and extension at 72°C for 30 seconds. The PCR was then given a final extension step of 72°C for 7 minutes. The transcripts were checked subsequently using 2% agarose gel electrophoresis, and integrated optical density was presented by the ratio of TGF-β_1, $Smad_3$, $Smad_7$, and β-actin.

2.6. Statistical analysis

Statistical analysis was performed using the SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). Measured data were presented as means ± standard deviation. The Q-test and one-way analysis of variance were used to compare the data between two groups; $p < 0.05$ denoted a significant statistical difference.

SPSS 13.0 statistical software (SPSS, Inc.) was used for data processing. The data were expressed as ($x \pm s$). The multigroup comparison was performed using analysis of variance, and the intergroup comparison was performed using the standard pairwise q test (Student–Newman–Keuls method). A p value < 0.05 was considered a statistically significant difference.

3. Results

3.1. General condition

After injection of STZ, the rats presented typical symptoms of diabetes (diuresis, polydipsia, and polyphagia) and their weight increased slowly along with gradually increasing listlessness; meanwhile, their hair became thin and lackluster. At the end of the study, two rats died due to infection in the diabetic group and one rat was dead in the Tongxinluo group. Therefore, 25 rats survived.

3.2. Effect of Tongxinluo on heart function

Heart function was judged by the measurement of LVP ($\pm dp/dt_{max}$). We also measured LVSP and LVEDP, which reflected systole and abnormal diastolic function, respectively. Compared with the control group, LVSP and $\pm dp/dt_{max}$ were lower, but LVEDP was higher in the diabetic group. Furthermore, LVSP and $\pm dp/dt_{max}$ were clearly higher in the Tongxinluo group than in the diabetic group. However, LVEDP was significantly lower in the Tongxinluo group than in the diabetic group (Table 1).

3.3. Effect of Tongxinluo on blood glucose level, heart weight index, and left ventricular weight index

At the end of 12 weeks, the blood glucose level was higher in the diabetic group than in the control group. The heart weight index and left ventricular weight index were also clearly higher in the diabetic group. Furthermore, compared with the diabetic group, there was no obvious difference in blood glucose level in the Tongxinluo group. The heart weight index and left ventricular weight index were significantly reduced, but were still higher than those in the control group (Table 2).

3.4. Measurement of myocardial interstitial CVF

Under the light microscope, myocardial cells were seen surrounded by only a few collagen fibers that were blue, thin, and well distributed in the control group. However, many large collagen fibers that were poorly aligned and unevenly distributed were observed in the diabetic group. In addition, compared with the diabetic group, the number of blue collagen fibers was lower in the Tongxinluo group. CVF was determined by image analysis, and when compared with the control group, it was significantly higher in the diabetic group. Compared with the diabetic group, CVF was clearly reduced in the Tongxinluo group, but was still higher than that in the control group (Table 2).

3.5. Protein expressions of TGF-β_1, $Smad_3$, and $Smad_7$

TGF-β_1 and $Smad_3$ were brownish-yellow particles located in the myocardial cytoplasm, periplasm, and surrounding

Table 1
Comparisons of heart function in three groups ($\bar{x} \pm s$).

Group	N	LVSP (mmHg)	LVEDP (mmHg)	$+dp/dt_{max}$ (mmHg/s)	$-dp/dt_{max}$ (mmHg/s)
Control group	10	128.07 ± 7.51	4.91 ± 0.47	3809.17 ± 142.23	3411.55 ± 175.35
Diabetic group	12	89.38 ± 5.97	9.65 ± 0.94	2561.37 ± 320.49	1989.89 ± 248.76
Tongxinluo group	13	107.58 ± 6.09	7.68 ± 0.93	2960.58 ± 384.64	2483.39 ± 355.35
F value		97.19	88.98	45.21	72.57
p		<0.001	<0.001	<0.001	<0.001

The pairwise q test showed that there existed significant differences between two indicators among the three groups, $p < 0.05$.
LVEDP = left ventricular end diastolic pressure; LVSP = left ventricular systolic pressure; $\pm dp/dt_{max}$ = maximum rise or fall speed of left ventricular pressure.

Table 2
Comparisons of blood sugar, heart weight index, left ventricular weight index, and CVF in three groups ($\bar{x} \pm s$).

Group	n	Blood sugar (mmol/L)	Heart weight index (mg/g)	Left ventricular weight index (mg/g)	CVF (%)
Control group	10	5.12 ± 0.47	1.68 ± 0.40	1.24 ± 0.23	9.22 ± 1.91
Diabetic group	12	26.13 ± 2.85	3.41 ± 0.33	2.47 ± 0.32	20.60 ± 3.32
Tongxinluo group	13	24.11 ± 2.69	3.07 ± 0.23	2.08 ± 0.14	18.15 ± 2.27
F value		257.83	87.77	73.88	56.83
p		<0.001	<0.001	<0.001	<0.001

The pairwise q test showed that there were significant differences in blood glucose, heart weight index, left ventricular mass index, and CVF among the three groups, $p < 0.05$.
CVF = collagen volume fraction.

vessels and were weakly expressed. However, $Smad_7$ was strongly expressed in the control group. In the diabetic group, the expressions of TGF-β_1 and $Smad_3$ were strong, but the expression of $Smad_7$ was significantly lower. Compared with the diabetic group, the expressions of TGF-β_1 and $Smad_3$ were clearly lower, but the expression of $Smad_7$ was significantly higher in the Tongxinluo group (Fig. 1, Table 3).

3.5.1. Expressions of mRNA in TGF-β_1, $Smad_3$, and $Smad_7$

Compared with the control group, the mRNA expressions of TGF-β_1 and $Smad_3$ were higher, but the expression of $Smad_7$ was significantly lower in the diabetic group. Furthermore, compared with the diabetic group, the mRNA expressions of TGF-β_1 and $Smad_3$ were clearly lower, but the expression of $Smad_7$ was significantly higher in the Tongxinluo group (Table 4, Fig. 2).

4. Discussion

DCM is a chronic complication of diabetes. In diabetes mellitus, alterations in cardiac function in the absence of ischemic heart disease, hypertension, or other cardiac pathologies are termed DCM.[12] Recent studies have found that intermyocardial collagen deposition and myocardial fibrosis are important features of DCM. Fibrosis is characterized by ECM accumulation and often by a change in the quality of the ECM, such as Type I and Type III collagen.[13] Myocardial fibrosis is a major pathological characteristic in the

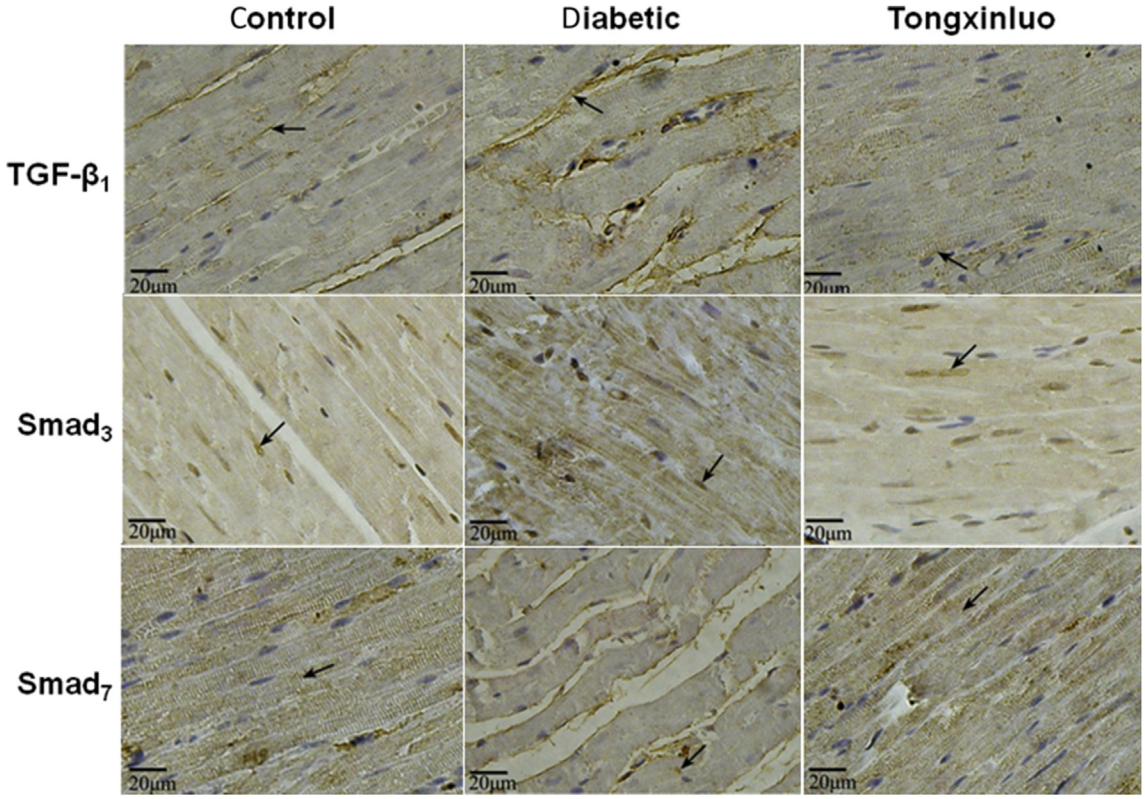

Fig. 1. In the control group, the protein expressions of TGF-β1 and $Smad_3$ were presented as weakly positive, and the protein expression of $Smad_7$ was strongly positive. In the diabetic group, the protein expressions of TGF-β1 and $Smad_3$ were strongly positive, but the expression of $Smad_7$ was reduced significantly. In the Tongxinluo group, protein expressions of TGF-β1 and $Smad_3$ obviously reduced, but the expression of $Smad_7$ increased significantly compared with that in the diabetic group. TGF-β_1 = transforming growth factor beta 1.

Table 3
Comparisons of the protein expressions of TGF-β_1, $Smad_3$, and $Smad_7$ ($\bar{x} \pm s$).

Group	*n*	TGF-β_1 (%)	$Smad_3$ (%)	$Smad_7$ (%)
Control group	10	19.75 ± 1.78	19.29 ± 0.94	48.77 ± 1.54
Diabetic group	12	52.27 ± 1.24	50.03 ± 2.33	24.15 ± 0.91
Tongxinluo group	13	32.10 ± 2.79	39.59 ± 2.37	40.49 ± 5.03
F value		692.11	622.62	168.73
p		<0.001	<0.001	<0.001

The pairwise *q* test showed that there existed significant differences between two indicators among the three groups, $p < 0.05$.
TGF-β_1 = transforming growth factor beta 1.

Table 4
Comparisons of the mRNA expressions of TGF-β_1, $Smad_3$, and $Smad_7$ in three groups ($\bar{x} \pm s$).

Group	*n*	TGF-β_1 mRNA	$Smad_3$ mRNA	$Smad_7$ mRNA
Control group	10	0.81 ± 0.04	0.63 ± 0.06	2.29 ± 0.16
Diabetic group	12	1.59 ± 0.14	1.77 ± 0.13	0.90 ± 0.05
Tongxinluo group	13	1.18 ± 0.05	1.37 ± 0.06	1.41 ± 0.09
F value		107.00	440.71	480.31
p		<0.001	<0.001	<0.001

The pairwise *q* test showed that there existed significant differences in the expressions of TGF-β_1, $Smad_3$, and $Smad_7$ mRNA among the three groups, $p < 0.05$.
TGF-β_1 = transforming growth factor beta 1.

development and occurrence of DCM. It causes a significant increase in ECM, and diastolic dysfunction is induced by diffused myocardial fibrosis.[14] Diastolic dysfunction represents the earliest preclinical manifestation of DCM.[15] This is followed by the development of systolic dysfunction and a clear reduction of the left ventricular ejection fraction,[16,17] which can easily cause arrhythmia and heart failure, even sudden death.[18] TGF-β_1 is a polypeptide member of the TGF-β superfamily of cytokines. A high expression of TGF-β_1 promotes the occurrence of fibrosis in myocardial tissue, by increasing the synthesis and secretion of ECM and the production of collagen and matrix proteins. TGF-β_1 reduces the degradation of ECM, which leads to myocardial ECM accumulation, ultimately leading to myocardial fibrosis.[19] A previous study showed that TGF-β_1 was overexpressed and the synthesis of Type I and Type III collagen increased in an animal model of ventricular remodeling.[20] TGF-β1 binds to specific receptors on the cell surface to exert its biological effects; Type I (TGF-β1 receptor I, TGF-β1RI) and Type II (TGF-β1RII) receptors have high affinities and signal transduction functions.

Smad protein is a frequently studied substrate. It is the only substrate transducing the signal downstream of the TGF-β1RI activating enzyme. Smads are intracellular signal transduction proteins of the TGF-β1 pathway and are involved in the

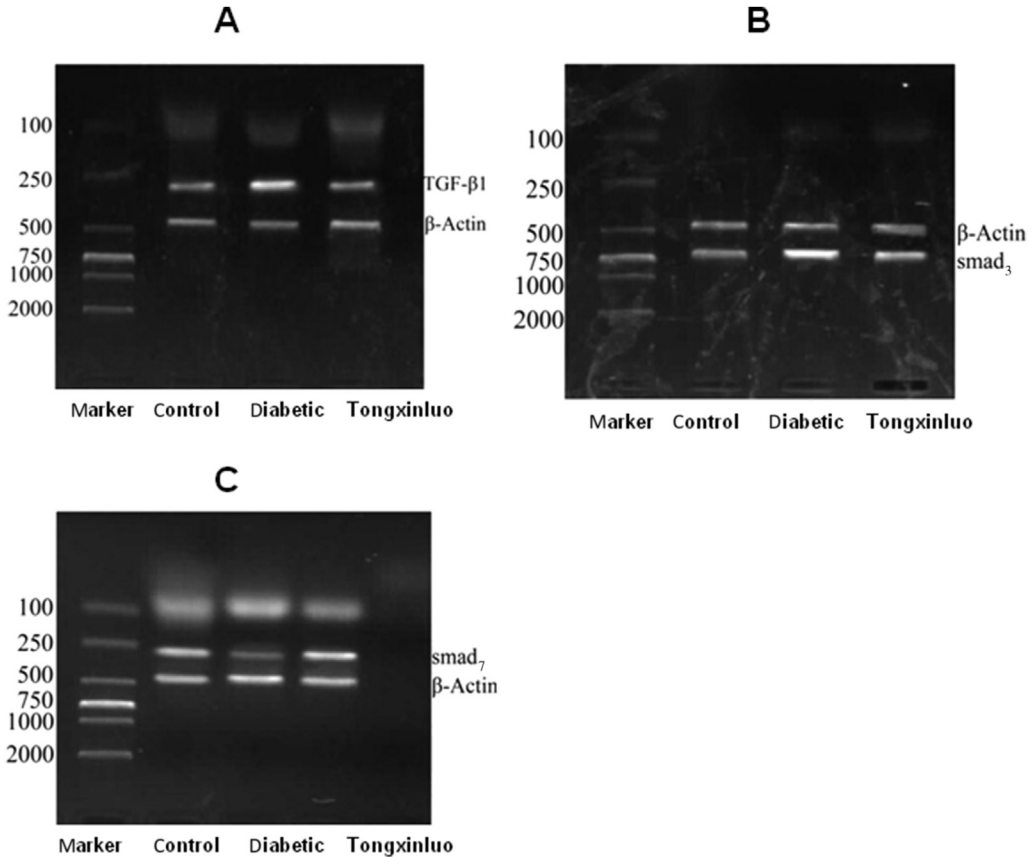

Fig. 2. Contents of (A) TGF-β1 mRNA; (B) $Smad_3$ mRNA; and (C) $Smad_7$ mRNA in rat myocardial tissue of the three groups. TGF-β_1 = transforming growth factor beta 1.

pathological changes of DCM. Many studies have shown that Smads could play an important role in neovascularization, myocardial hypertrophy, and heart failure. Eight types of Smad proteins are expressed in the mammalian cytoplasm, which are divided into three categories based on their functions: (1) receptor-modulating Smad proteins (R-Smads) include Smads1, Smads2, Smads3, Smads5, and Smads8, among which Smads2 and Smads3 mediate TGF-β1 signal transduction; (2) comediator Smad proteins (Co-Smads) include the only mammalian Smad, Smads4, which is involved in TGF-β1 signal transduction; and (3) inhibitory Smad proteins (I-Smads) include $Smad_6$ and $Smad_7$. In the TGF-β1/Smad pathway, first, TGF-β1 binds to its receptor TGF-β1RI, which phosphorylates the C-terminal serine residue of the R-Smads protein, leading to the generation of different downstream signals. $Smad_7$, an inhibitory Smad protein, is involved in the modulation of TGF-β1 by negatively regulating signal transduction. $Smad_7$ binds competitively with TGF-β1RI to stop $Smad_{2,3}$ phosphorylation and TGF-β1 signaling.[21,22]

Some studies have shown that Tongxinluo could regulate the TGF-β1/Smad signaling pathway, thereby improving the functions of the target organs. Wang et al[23] applied Tongxinluo while treating diabetic nephropathy, and used technologies such as RT-PCR, cell transfection, *in situ* hybridization, and laser confocal microscopy. It was found that Tongxinluo could block the miR-21-regulated TGF- β_1/$Smad_3$ pathway and increase the expression of $Smad_7$, thus improving renal structures and functions, and playing a protective role in diabetic nephropathy. Our study showed that the protein and mRNA expression levels of TGF-β_1 were clearly higher in the diabetic and Tongxinluo groups than in the control group. However, compared with the diabetic group, the protein and mRNA expression levels of TGF-β_1 were lower in the Tongxinluo group, which suggested that Tongxinluo could reduce the expression of TGF-β_1 in rats with DCM and alleviate the occurrence and development of DCM.

This study also found that the protein and mRNA expressions of $Smad_3$ were higher in the myocardial tissue of diabetic rats. However, the expression of $Smad_7$ decreased. In the Tongxinluo group, the expression of $Smad_7$ was significantly higher, but $Smad_3$ expression was lower. This implies that $Smad_3$ might be involved in the occurrence and development of DCM and that $Smad_7$ plays a protective role in DCM through the $Smad_7$/TGF-β_1 signaling pathway. The results of this study further showed that Tongxinluo could prevent and treat myocardial fibrosis in diabetic rats. The mechanism of action of Tongxinluo could be through its effect on the intracellular signal transduction of TGF-β_1, wherein it downregulates the expressions of TGF-β_1 and $Smad_3$, and upregulates the expression of $Smad_7$ to relieve myocardial fibrosis and improve heart function in diabetic rats. These results indicate that Tongxinluo has a protective effect on the myocardial tissue of diabetic rats. However, Tongxinluo is composed of many herbs and insects, including *Panax ginseng, leech, scorpion, Radix paeoniaerubra, periostracum cicadae, ground beetle, centipede, sandalwood, rosewood, heartwood, frankincense, semen Ziziphi Spinosae, and borneol*; whether the protective effect on myocardial tissue observed in this study is imparted by a single ingredient or multiple compounds is still not clear. Therefore, further studies are needed to determine the exact mechanism.

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