ORIGINAL RESEARCH

Jiedu Tongluo Decoction Attenuates Myocardial Fibrosis through Inhibition of the TGF-β1/Smad2/3 Pathway

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ABSTRACT

Jiedu Tongluo (JDTL) Decoction is a traditional Chinese medicine formula containing three herbal ingredients. It is widely used to treat myocardial fibrosis (MF). This study aimed to investigate the molecular mechanism of JDTL Decoction's effect on MF.

In this study, 6 compounds of JDTL Decoction were identified by HPLC. HE and Masson staining showed that in the isoproterenol hydrochloride-induced MF rat model, JDTL treatment can protect the myocardial structure and inhibit the expression of collagen III. The immunohistochemistry results also showed that JDTL treatment can significantly reduce vimentin and α-SMA expression, TGF-β1 expression, and phosphorylation of Smad2/3 in the rat MF model. RCF, a rat cardiac fibroblast cell line, was used as a tool for in vitro study. Using the methods of hydroxyproline detection, MTT, wound healing test, western blot, and double immunofluorescence staining, our in vitro study confirmed the inhibitory effects of JDTL Decoction on proliferation, migration, and trans-difference ability of RCF cells, as well as the molecular mechanisms underlying the inhibitory effects of JDTL Decoction, including the inhibition of TGF-β1/Smad2/3 pathway through down-regulation of TGF-β1 expression and phosphorylation of Smad2/3 as well as the inhibition of the expression of vimentin and α-SMA. In conclusion, JDTL Decoction can prolong the process of myocardial fibrosis through the inhibition of the TGFβ1/Smad2/3 signaling pathway. (*Altern Ther Health Med.* [E-pub ahead of print.])

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INTRODUCTION

Myocardial fibrosis (MF) is a common pathological phenomenon associated with the development of hypertensive heart disease, coronary heart disease, myocarditis, arrhythmia, cardiomyopathy, chronic pulmonary heart disease, and other cardiovascular diseases.[1] MF can cause microcirculation disorders and inflammatory reactions as well as cardiac systolic and diastolic function disorder, which can cause heart failure,[2] malignant arrhythmia, and sudden cardiac death in the worst cases. The pathogenesis of MF is complex, and there are many side effects of current Western medicine-based anti-MF therapies.

Studies have revealed that extracellular matrix (ECM) deposition is considered a cause of MF occurrence and that cardiac interstitial cells are the main source of the ECM, accounting for about 75% of the total number of cardiac cells, mainly cardiac fibroblasts.[3] Cardiac fibroblasts play important roles in the regulation of ECM homeostasis and the maintenance of structural, mechanical, biochemical, and electrical properties of the heart. In addition, cardiac fibroblasts also influence the function of cardiomyocytes in a variety of ways, such as direct cell-cell interactions or the secretion of various growth factors. In the case of heart injury, alterations of the cardiac environment, such as changes in the ECM, the increased amounts of growth factors and cytokines, and higher levels of mechanical stress, can induce the transdifferentiation of myocardial fibroblasts to myofibroblasts, leading to the destruction of the normal physiological structure of the myocardial interstitium and promoting MF.[4] Therefore, the suppression of proliferation and transdifferentiation of myocardial fibroblasts caused by injury or stress may be a potential and effective therapeutic

approach for preventing MF. Recently, fibroblast-based therapies have become a research hotspot in cardiac disease. Progress has been made in this field, including cell therapies and microRNA-, peptide- and drug-based interventions. Furthermore, traditional Chinese medicine (TCM) also has been found to be effective in fibroblast-targeted therapy.[5]

Regarding the cause of myocardial fibrosis, Dr. Huang Yongsheng, a well-known doctor of traditional Chinese medicine (TCM), thought that the cardiac function disorder caused by injury or stress might be due to the internal injuries of "qiging," based on the theory of TCM. The internal injuries of "qiqing" could be explained by the abnormality in homeostasis. In detail, the stagnation of qi in the body will induce the conversion of the stagnated qi into the fire. At the same time, the stagnation of qi will also block blood circulation, leading to blood stasis, which can induce heat. The heat can be turned into fire, so the fire and the blood stasis work together to form a toxin that is obstructed in the veins, thus blocking blood circulation. Suppose the fire poison and the blood stasis cannot be terminated in the body rapidly and completely. In that case, the blood will be damaged, followed by putrefaction, resulting in a coronary blood supply disorder, heart injury, and eventually MF. Based on the overall concept mentioned above, Dr. Huang has developed a novel TCM-based therapy for MF, using several Chinese medicinal herbs that can promote the qi, improve blood circulation, disperse the fire, and detoxify, leading to smooth venation, harmony of the qi and blood, and removal of toxins and pathogens. The mixture of these herbs (including Prunella Spica, Achyranthis Bidentatae Radix,and Leonuri Fructus) is called the Jiedu Tongluo (JDTL) decoction, which has been widely used in the treatment of MF and in animal models.[6] It is notable that the main components of Prunella vulgaris include caffeic acid, ursolic acid, rutin, and other active ingredients. The main components of Achyranthes bidentata are β moltsterone and other active ingredients. Related studies have shown that ursolic acid, caffeic acid, and rutin have a pronounced anti-fibrosis effect.[7-10]

To better understand the mechanisms underlying the effectiveness of the JDTL decoction in fibroblast-targeted therapy, this study aimed to investigate its effects on MF using a rat MF model established by subcutaneous injection of isoproterenol hydrochloride (ISO). The action mechanism of JDTL was found to be related to the regulation of the myocardial transforming growth factor-beta 1 (TGF-β1)/Smad2/3 signaling pathway, which provides new ideas and methods for treating patients with MF.

MATERIALS AND METHODS

Reagents

ISO and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were products of Sigma Aldrich. TGF-β1 and SB431542 were purchased from MedChemExpress, Inc. Caffeic acid, oleanolic acid, ursolic acid, rosmarinic acid, rutin, hyperin, β-ecdysterone, chlorogenic acid, and ginsenoside Ro were purchased from HongYang, Biotech Co. (JiLin, China). Antibodies against TGF-β1, p-Smad2/3, type III collagen (COL3), alpha-smooth muscle actin (α-SMA), and vimentin as well as FITC-labeled rabbit IgG and Cy3-labeled mouse IgG were provided by Affinity Biosciences Ltd. (Jiangsu, China). The following items were purchased from the indicated vendors: the rat cardiac fibroblast (RCF) cell line, Beina-Chuanglian Biotechnology Co. (BNCC, Beijing, China) fetal bovine serum (FBS), YuSheng, Biotech Co. (Shanghai, China); high-glucose Dulbecco's modified Eagle medium (DMEM), Gibco, YiXiang, Biotech Co. (Jilin, China); hydroxyproline content determination kit A030 (alkaline hydrolysis method), Jiancheng, Biotechnology Co. (Nanjing, China); bicinchoninic acid (BCA) protein determination kit and enhanced chemiluminescence (ECL) color development kit, Beyotime Biotechnology Co. (Shanghai, China); and 3,3'-diaminobenzidine (DAB) immunohistochemistry (IHC) kit, Zhongshan Jinqiao Biotech Co. (Beijing, China).

Experimental animals

The specific pathogen free (SPF) male 40 Wistar rats, weighing 180–200 g, were purchased from Liaoning Changsheng Biotechnology Co. Ltd. (Certificate No. SCXK (Liao) 2015–0001, Liaoning, China). The rats were housed in the Laboratory Animal Center of the Changchun University of Chinese Medicine under standard laboratory conditions.

Animal model establishment and drug administration

After seven days of adaptive breeding, the rats were randomly divided into the following four groups: normal control group (Ctrl), model group (ISO), low-dose JDTL group (Jie L), and high-dose JDTL group (Jie H), with 10 rats in each group. Except for the Ctrl group, the rats in the other three groups were injected subcutaneously with ISO at 5 mg/kg body weight, while the Ctrl control group was injected subcutaneously with an equal volume of saline. From the second day of model establishment, the experimental rats in the Jie L and Jie H groups were given JDTL decoction at 5 g/kg and 50 g/kg, respectively, by gavage daily for 7 consecutive days; meanwhile, the Ctrl and model groups were given (by gavage) an equal volume of normal saline daily. To evaluate the effect of JDTL (alone) on normal rats, another batch of 30 Wistar rats was randomly divided into the following three groups: a normal control group (Ctrl), a low-dose JDTL (alone) group (Jie aL), and a high-dose JDTL (alone) group (Jie aH), with 10 rats in each group. Rats in the Jie aL and Jie aH groups were given JDTL at 5 g/kg and 50 g/kg daily by intragastric administration, respectively, while the rats in the Ctrl group were intragastrically administered with an equal volume of normal saline daily for 7 consecutive days.

The rats were anesthetized and euthanized at 1–2 h after the last intragastric administration. The heart was immediately removed after euthanizing the animal. Meanwhile, the residual roots of the large blood vessels, the auditory ear, and the left and right atrium were also removed. The heart tissue was immediately immersed in liquid nitrogen for

preservation, which was used for western blot detection. The remaining ventricular tissue was fixed with 4% paraformaldehyde for preparation of the paraffin-embedded tissue slices. Blood samples were collected, and the serum was inactivated at 56°C for 30 min for future use.

High-performance liquid chromatography (HPLC) fingerprint analysis

HPLC analyzed the composition of the JDTL decoction (water extract) according to a standard procedure. First, the water extract was separated via a ZORBAX SB-C18 column (4.6 mm × 250 mm, 5-μm pore size, Agilent, Santa Clara, CA, USA), with the following conditions: mobile phase, acetonitrile (A) and 0.4% phosphoric acid/water (B); column temperature, 30°C; flow rate, 1 mL/min; UV detection wavelength, 203 nm; and injection volume, 5 μL. The authorized software Agilent 1200 was used for chromatographic data collection and analysis of specific components. Several chemicals, such as caffeic acid, oleanolic acid, ursolic acid, rosmarinic acid, rutin, hyperoside, β-ecdysterone, chlorogenic acid, and ginsenoside RO, were used as standards to determine if they were components of the JDTL decoction.

Hematoxylin and eosin (HE) staining and Masson staining.

The ventricular muscle tissues were collected from the experimental rats in each group and were used to make 5-μm-thick paraffin-embedded sections. After dewaxing with xylene, HE staining and Masson trichrome staining was performed on the ventricular muscle tissue sections. The images of the stained sections were recorded, and the degree of MF was analyzed using ImageJ software (U. S. National Institutes of Health, Bethesda, MD, USA).

Immunohistochemical staining

The collected myocardial tissue of the experimental rats was fixed with 4% paraformaldehyde for IHC staining. The expression of TGF-β1, p-Smad2/3, and COL3 in the different groups was analyzed using a standard IHC staining procedure (primary antibody dilution, 1:300). The secondary antibody was then added, according to the instructions of the IHC kit (PV-6000; Zhongshan Jinqiao Biotech Co., Beijing, China). The staining results were visualized using the DAB chromogenic method.

Cell culture and experimental groups

RCFs were cultured with high-glucose DMEM containing 10% FBS. Cells in the logarithmic growth phase were divided into five groups: normal control group, TGF-β1 stimulation group, low-dose JDTL group, high-dose JDTL group, and SB431542 group. The cells in the normal control group were cultured in a medium containing 2% normal rat serum. In the TGF-β1 stimulation group, TGF-β1 was added to the medium containing 2% normal rat serum at a final concentration of 10-8 μg/mL. In the low-dose and high-dose JDTL groups, the cells were cultured in a medium containing 2% normal rat serum, low-dose or high-dose JDTL, and TGF-β1 (10-8 μg/mL). In the SB431542 group, both TGF-β1 (10-8 μg/mL) and SB431542 (10-5 μg/mL) were added into the medium containing 2% normal rat serum.

Cell viability assay

RCFs were plated into 96-well plates at a density of 2 × 103 cells/well. The corresponding medium of each group, as mentioned above, was added to the wells of the different groups, and the cells were incubated with 5% CO2 at 37°C for 24 h. The proliferation activity of the cells was determined using an MTT assay, which is a colorimetric assay that involves the reduction of the tetrazolium dye MTT into its insoluble formazan). The optical density (OD) of each well was measured with a microplate reader at a wavelength of 490 nm to determine the viability of the RCFs for evaluating their proliferation potentials. Meanwhile, the blank control wells (with culture medium only but without cells) were used for zeroing. The cell proliferation activity was calculated as follows: cell proliferation activity (%) = 100 × (OD of the experimental group – blank control group / (OD of control – blank control group).

Wound-healing assay

RCFs were placed into a six-well plate at a cell density of 4 × 105 cells/well and cultured to near confluence. In the wells of the different groups, the old culture medium was replaced with the corresponding medium of each group, as mentioned above. At 24 h after treatment, the cells were scratched and washed with phosphate-buffered saline three times to remove the suspended cells, followed by an additional 24-h culture in the corresponding medium of each group. The image of each well was recorded with an optical microscope. A comparison of the width of the scratch between the different groups was performed using ImageJ software to evaluate the wound-healing ability of the treated cells.

Hydroxyproline content determination

To assess the degree of cell fibrogenesis, hydroxyproline released in the cell culture supernatant of the RCFs was measured by a spectrophotometric method (alkaline water method).

Immunofluorescence staining

Both cultured cells and collected tissue samples on the glass slide were fixed with 4% formaldehyde and then incubated with mouse anti-α-SMA or vimentin antibodies (1:50) at 4°C overnight. The next day, the slides were incubated with a secondary antibody coupled with FITC or Cy3 (1:1000) at room temperature in the dark for 1 h, followed by DAPI nuclei staining for 10 min. Finally, the stained slides were examined using a fluorescence microscope. The images of the stained slides were recorded and analyzed using ImageJ software.

Western blot analysis

The cultured cells were lysed with radioimmunoprecipitation assay buffer and centrifuged at 12 000 rpm for 15 min before the supernatant was collected. The protein concentration was measured with a BCA protein assay kit. After denaturation at 95°C for 5 min, the protein samples (100 μg each) were separated by electrophoresis on 10% or 12% sodium dodecyl sulfate–polyacrylamide gels and transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% skimmed milk and then incubated with primary antibodies (1:1500 dilution) against total Smad2/3 and p-Smad2/3, respectively, at 4°C overnight. The next day, after incubation with a secondary antibody (1:1500 dilution) at room temperature for 1 h, the bands on the membrane were visualized by ECL detection. The western blot results were analyzed using ImageJ software.

Statistical analysis

In this study, all data were expressed as the mean ± standard deviation ($\bar{x} \pm s$). SPSS19.0 software (IBM Corp., Armonk, N.Y., USA) was used for statistical analysis. Group comparison was performed by one-way analysis of variance. The least significant difference method was used for pairwise comparison between groups. $P < .05$ was considered as a significant difference.

RESULTS

Identification of the composition of JieDuTongLuo decoction

The composition of the water extract of the JDTL decoction was determined by HPLC analysis to include six compounds, which were identified by the comparison of the retention times of the standard products, as shown in Figure 1.

Comparison of the cardiac histological changes in the different groups

The HE staining results (Figure 2A) showed that in the normal control group (Group 1), the myocardial tissue was arranged in order, the nucleus size was uniform, and no obvious inflammatory infiltration or necrosis was found. However, in the other groups treated with ISO (Groups 2–4), the rat myocardial tissue was irregularly arranged, and myocardial necrosis of different sizes and with clear boundaries was found at 7 days after ISO injection (Figure 2A). The presence of a necrotic area suggests that there were obvious changes in MF due to increases of the myocardial collagen composition and myocardial fibroblasts. Masson trichrome staining also demonstrated that the necrotic area in the rat myocardial tissue was filled by blue-stained collagen components and fibroblasts (Figure 2B). The results of image analysis revealed that compared with the normal control group, the collagen components in the myocardial tissue collected from either the model group or the JDTL treatment groups were significantly increased ($P < .001$). Moreover, compared to the model group, the collagen composition in either the low-dose or high-dose JDTL treatment group was significantly reduced ($P < .001$); however, as mentioned above, there was no statistically significant difference in the collagen composition of the myocardial tissue between the two groups.

As shown in Figure 2C, E (IHC staining), the expression intensity of COL3 of the myocardial tissue in the model group was significantly greater than that in the normal control group ($P < .001$). However, compared with the model group, the expression intensity of COL3 in the Jie H group was significantly decreased ($P < .01$).

Figure 1. Identification of the components of JDTL decoction(water extract) (A) β-Ecdysterone (B) Ursolic acid (C) Caffeic Acid; (D) Zidanolic acid (E) Rutin (F) Ginsenoside RO

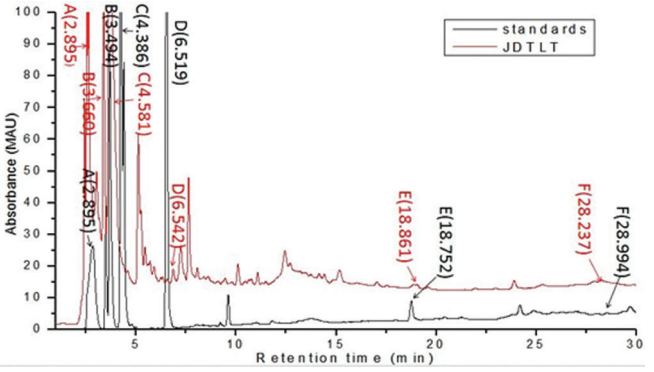
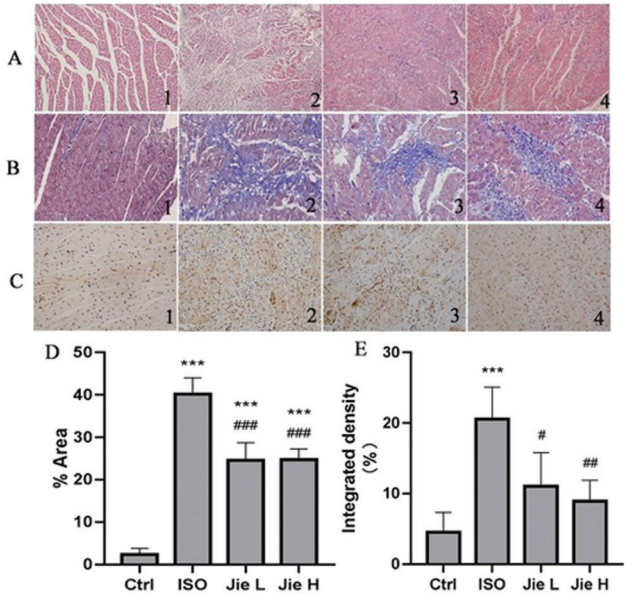


Figure 2. Cardiac histological changes in the different groups. A. Hematoxylin and eosin staining; magnification, 100×; B. Masson trichrome staining; magnification, 200×; C. Immunohistochemical (IHC) staining; magnification, 200×; visualized using DAB; D. Image analysis results of Masson trichrome staining; E. Image analysis results of IHC staining for COL3.



Note: Group 1, the normal control group (Ctrl); Group 2, the model group (ISO); Group 3, the low-dose JDTL group (Jie L); and Group 4, the high-dose JDTL group (Jie H). *** $P < .001$ *vs.* the normal control group; ### $P < .001$, ## $P < .01$, # $P < .05$ *vs.* the model group.

Comparison of vimentin and α-SMA expression in rat myocardial tissue in the different groups

Vimentin is considered a marker of fibroblasts because of its specific expression in fibroblasts. Comparison of the vimentin expression in the rat myocardial tissue in the different groups (Figure 3A, D) showed that the expression intensity of vimentin in the rat myocardial tissue from the model group and the Jie L group was significantly greater than that from the Ctrl group ($P < .001$). However, compared to that from the model group and Jie L group, the expression of vimentin in the rat myocardial tissue from the Jie H group was significantly reduced (Figure 3A, D, $P < .001$).

Although α-SMA is a marker of smooth muscle cells, its expression has been found in myocardial fibroblasts during the period of MF.[11] In this study, α-SMA was used as a marker of myofibroblasts, and its expression in the rat myocardial tissue was detected by IHC. The results (Fig. 3B–C) showed that the expression intensity of α-SMA in the rat myocardial tissue from the model, Jie L, and Jie H groups was significantly greater than that from the Ctrl group ($P < .001$). However, compared to that in the model group, α-SMA expression in the Jie H group was significantly increased ($P < .05$).

Comparison of TGF-β1 expression and the p-Smad2/3 level in rat myocardial tissues from the different groups

The IHC results (Figure 4A, C) showed that compared to that from the normal control group, TGF-β1 expression in the rat myocardial tissue from the model, Jie H, and Jie L groups was increased significantly ($P < .001$, $P < .01$, $P < .05$, respectively). However, TGF-β1 expression in both the Jie L and Jie H groups was significantly less than that in the model group ($P < .01$, $P < .001$).

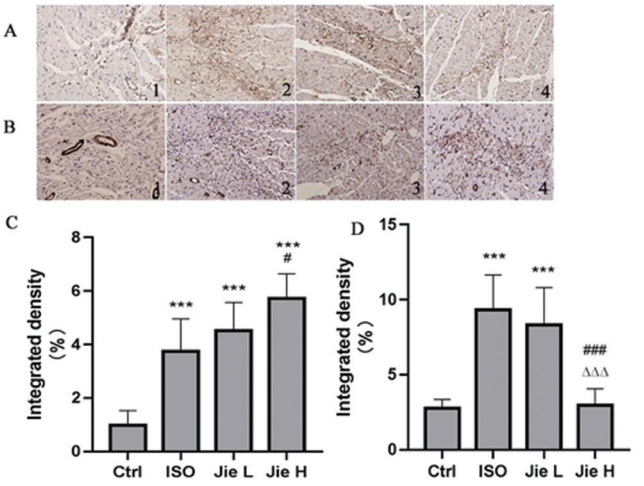
The IHC results (Figure 4B, D) also revealed that the p-Smad2/3 level of rat myocardial tissue from the model group and the Jie L group was significantly greater than that from the normal control group ($P < .001$, $P < .05$). However, compared with the model group, the p-Smad2/3 level from the Jie H group was significantly reduced ($P < .05$). There was no statistically significant difference in TGF-β1 expression or the p-Smad2/3 level between the Jie L and Jie H groups.

Effects of JieDuTongLuo decoction on the proliferation, migration, and transformation of RCFs

RCFs were used to investigate the effects of JDTL decoction on the proliferation, migration, and transformation of RCFs in vitro. SB-431542 is a potent and selective ALK5/TGF-β type I Receptor inhibitor.[12-13] Our study revealed that the cell proliferation activity in the TGF-β1 stimulation group was significantly increased compared with the normal control group ($P < .001$). However, treatment of JDTL decoction attenuated TGF-β1-induced growth of RCF cells, especially at a high dose of JDTL ($P < .05$), similar to the inhibition of SB431542 ($P < .001$, Figure 5A).

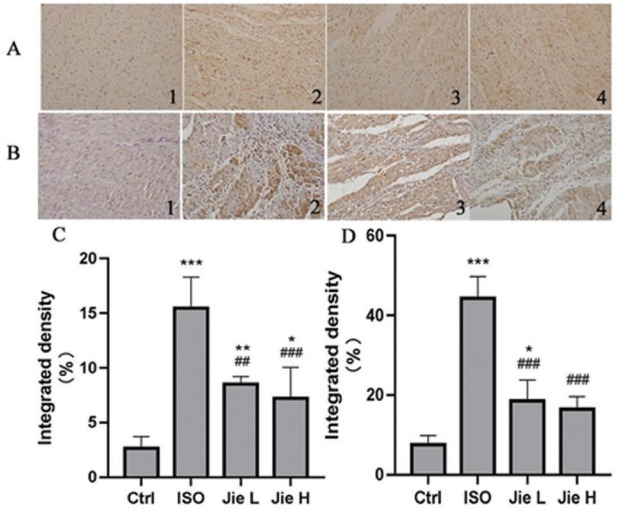
The hydroxyproline content in the cell culture supernatants is shown in Figure 5B. Similar to the results of the cell viability assay, the hydroxyproline content in the TGF-β1 stimulation group was significantly greater than that in the normal control group ($P < .001$); however, high-dose

Figure 3. Immunohistochemistry (IHC) results of vimentin and α-SMA expression in the different groups. A. IHC results of vimentin expression; magnification, 200×; B. IHC results of α-SMA; magnification, 200×; visualized using DAB; C. Image analysis results of IHC staining for α-SMA; D. Image analysis results of IHC staining for vimentin.



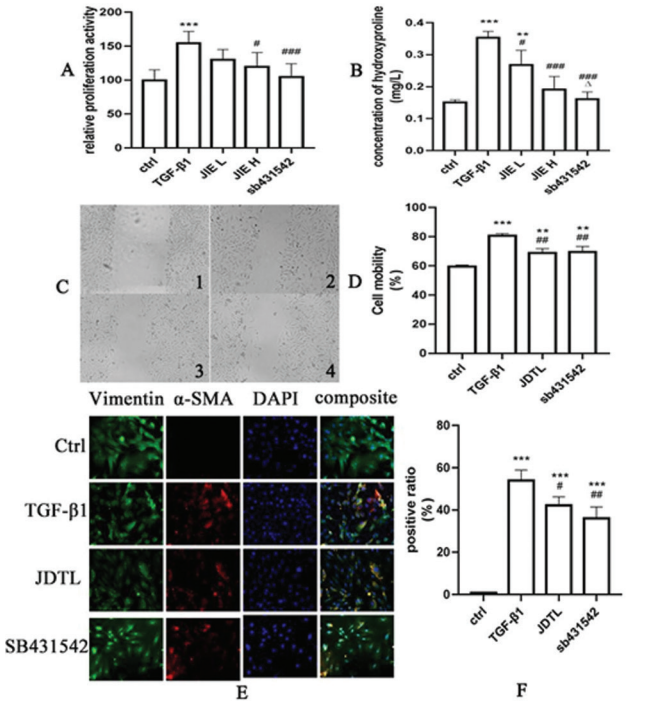
Note: Group 1, the normal control group (Ctrl); Group 2, the model group (ISO); Group 3, the low-dose JDTL group (Jie L); and Group 4, the high-dose JDTL group (Jie H). *** $P < .001$ *vs.* the normal control group; ### $P < .001$, # $P < .05$ *vs.* the model group; $^{\Delta\Delta\Delta}P < .001$ *vs.* the model group.

Figure 4. Immunohistochemistry (IHC) results of TGF-β1 expression and the p-Smad2/3 level in the different groups. A. IHC results of TGF-β1 expression, magnification, 200×; B. IHC results of the p-Smad2/3 level; magnification, 200×; visualized using DAB; C. Image analysis results of IHC staining for TGF-β1 expression; D. Image analysis results of IHC staining for the p-Smad2/3 level.



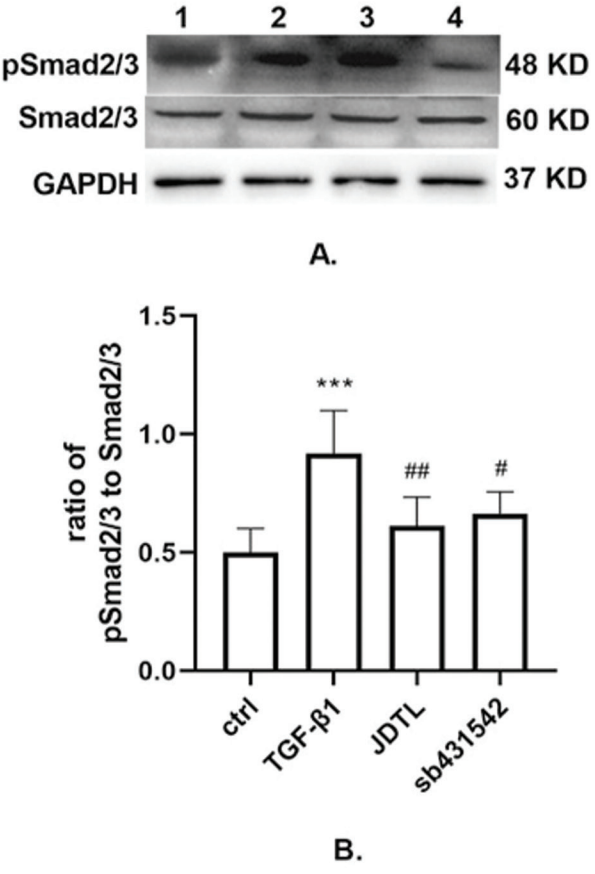
Note: Group 1, the normal control group (Ctrl); Group 2, the model group (ISO); Group 3, the low-dose JDTL group (Jie L); and Group 4, the high-dose JDTL group (Jie H). *** $P < .001$, ** $P < .01$, * $P < .05$ *vs.* the normal control group; ### $P < .001$, ## $P < .01$ *vs.* the model group.

Figure 5. Inhibitory effects of JDTL on the proliferation, migration, and transformation of rat cardiac fibroblasts (RCFs). A. Proliferation activity of RCFs as determined by the MTT assay; B. Concentration of hydroxyproline in the cell culture supernatant as measured by a kit; C. Microscopy results of the wound-healing test; magnification, 200×; D. Comparison of cell mobility between groups; E. Image analysis results of double immunofluorescence staining for both vimentin and α-SMA; F. Comparison of the results of double immunofluorescence staining between groups; magnification, 200×.



Note: Group 1, the normal control group (Ctrl); Group 2, the model group (ISO); Group 3, the low-dose JDTL group (Jie L); Group 4, the high-dose JDTL group (Jie H). *** $P < .001$, ** $P < .01$ *vs.* the normal control group; ### $P < .001$, ## $P < .01$, # $P < .05$ *vs.* the TGF-β1 group, $^{\Delta}P < .05$ *vs.* the low-dose JDTL-treated group.

Figure 6. Inhibitory effect of JDTL on TGF-β1-promoted activation of the Smad signaling pathway in rat cardiac fibroblasts (RCFs). A. Western blot results of the p-Smad, Smad, and GAPDH levels in RCFs. Lane 1, the normal control group; Lane 2, the TGF-β1 group; Lane 3, the JDTL group; Lane 4, the SB431542 group. B. The ratio of p-Smad/Smad in the RCFs from the different groups. *** $P < .001$, vs. the normal control group; ## $P < .01$, # $P < .05$ vs. the TGF-β1 group.



JDTL treatment (Jie H group) could significantly reduce TGF-β1-enhanced hydroxyproline accumulation in the cell culture supernatant ($P < .05$), the inhibition degree of which was close to that of SB431542 ($P < .001$).

Although there was no statistically significant difference in the cell viability and ECM secretion capacity between the Jie L and Jie H groups, the high-dose JDTL treatment was reasonably selected for the subsequent experiments due to its excellent inhibitory effects. The additional experiments included the wound-healing test. As shown in Figure 5C–D, the cell migration rate of RCFs enhanced by TGF-β1 was remarkably reduced in the Jie H and SB431542 groups ($P < .01$), although cell migration in these two groups was still faster than that in the normal control group ($P < .01$).

Furthermore, the expression levels of vimentin and α-SMA in the cells treated with TGF-β1 were detected by double immunofluorescence staining. As shown in Figure 5E–F, compared with the normal control group, the rate of co-expression of vimentin and α-SMA in the cells treated with TGF-β1 (TGF-β1 group) was dramatically increased ($P < .001$). However, treatment with either high-dose JDTL or SB431542 reduced TGF-β1-enhanced vimentin and α-SMA expression significantly ($P < .05$, $P < .01$).

JieDuTongLuo decoction treatment decreased the ratio of Smad/p-Smad in Rat myocardial fibroblasts.

Since the Smad signaling pathway is mainly regulated by TGF-β1, our study focused on the effect of JDTL on TGF-β1-mediated activation of the Smad signaling pathway. Western blot analysis showed that compared with that in the normal control group, the ratio of p-Smad/Smad in the TGF-β1 group was significantly increased ($P < .001$). But compared to the TGF-β1 group, the ratio of p-Smad/Smad in both the JDTL and SB431542 groups was significantly decreased ($P <$

.01, $P < .05$, Figure 6), suggesting the inhibitory effect of JDTL on TGF-β1-promoted activation of the Smad signaling pathway. Our study also found that there was no statistically significant difference between the inhibitory effects of JDTL and SB431542.

DISCUSSION

TCM believes that the occurrence of MF is caused by emotional factors that bring out liver dysfunction so that the maintenance of the normal flow of qi fails and stagnation of qi occurs, eventually causing qi depression, which leads to blood stasis as well as the stasis of veins and collateral vessels. At the same time, qi stagnation can turn into fire, blood stasis can turn into heat, and heat polarization can turn into fire. After experiencing illness for a long period of time, the fire stasis in the body becomes poisonous, which blocks the venation, damages the venation and nutrient-qi, causes damage to the heart blood, and then develops myocardial remodeling. Therefore, it is necessary to develop a novel therapeutic approach for MF based on TCM. On the other hand, ISO belongs to the group of catecholamine drugs that have been used to establish an animal MF model. Injection of ISO into the abdominal cavity of an animal can increase the myocardial contractility and frequency by stimulating cardiac β-adrenergic receptors, destroying the balance of the myocardial oxygen supply, and increasing the concentration of angiotensin II in the circulatory system and myocardium, thereby promoting massive accumulation of collagen to reduce the compliance of the ventricular wall and the proliferation of collagen fibers, which in turn leads to the occurrence of MF. The disordered arrangement of myocardial tissue and pyknosis of the nucleus are typical histological features of MF. Other histological features of MF include cardiomyocyte hypertrophy, cytoskeletal rearrangement, and damaged cardiomyocytes, which are replaced by collagen fibers, leading to the accumulation of hydroxyproline in the myocardial tissue and elevated expression of COL1 and COL3.[14]

JDTL decoction is a water decoction containing three types of TCMs: *Prunella Spica*, *Achyranthis Bidentatae Radix*,and *Leonuri Fructus* (at a ratio of 3:2:1, respectively). In this study, HPLC identified the main pharmacological components of JDTL decoction. Finally, we identified nine pharmacological components of JDTL decoction that were selected/judged based on the Chinese Pharmacopoeia and published literature.[15] Among these nine standard compounds, six compounds were previously known to be a part of the JDTLT extract, and the remaining three compounds were newly discovered.

In this study, JDTL decoction was used to treat ISO-induced MF rats. In this rat MF model, after 7 days of treatment, the degree of MF was found to be significantly reduced. In addition, the collagen composition in the myocardial tissue of the necrotic area was also significantly reduced, and the expression level of COL3 in the JDTL-treated group was significantly less than that in the model group (ISO only). Moreover, the *in-vitro* study showed that the treatment of RCFs with

JDTL decoction significantly inhibited cell proliferation activity and reduced the content of hydroxyproline in the cell culture supernatant, indicating that JDTL decoction has a potential inhibitory effect on MF.

Vimentin, an intermediate filament protein, is often considered a protein marker of fibroblast activation and also a representative factor of mesenchymal cells in the epithelial–mesenchymal transition process. It has been reported that vimentin can interact with actin during cell migration[16] as well as reduce epithelial cell activity via weakening cell adhesion and increasing cell exercise capacity, thereby destroying the dense structure between cells and the original cell cytoskeleton and leading to tissue fibrosis.[17] Furthermore, α-SMA exists in the striated muscle of humans, mice, and other mammals, including the bone, heart, and smooth muscle.[18] In addition, α-SMA is an effector protein downstream of the TGF-β1 signaling pathway and is involved in the activation of fibroblasts induced by TGF-β1. Smad, a protein that is downstream of the TGF-β1 signaling pathway, can regulate the gene expression of α-SMA to induce the transformation of fibroblasts into myofibroblasts. Upregulation of α-SMA expression can promote the formation of the actin cytoskeleton and induce the differentiation of fibroblasts into myofibroblasts. At the same time, the activation of fibroblasts, the synthesis and secretion of ECM components, and the cell proliferation ability are significantly enhanced, which are involved in tissue damage repair[19-20] and fibrotic disease progression.[21] The current study revealed that in a rat MF model, after 7 days of JDTL decoction treatment, the expression level of vimentin in the necrotic area of myocardial tissue was significantly less than that in the model group (ISO only, without JDTL). The expression level of α-SMA in the JDTL treatment group was also significantly greater than that in the control group, suggesting that both myocardial fibroblasts and myofibroblasts are the main cell types that accelerate MF. Previous studies have shown that the conversion of fibroblasts into myofibroblasts and their migration seem to have a more important role in the process of MF.[22-26] In our study, RCFs were used as a tool to investigate the effect of JDTL decoction on cell migration and the phenotypic transformation process. The results of the wound-healing test showed that JDTL decoction treatment could significantly inhibit the migration of myocardial fibroblasts. Moreover, the results of double immunofluorescence staining showed that JDTL decoction treatment significantly reduced the proportion of the vimentin and α-SMA co-expressed cells, suggesting that this treatment could significantly inhibit the production of myocardial fibroblasts by inhibiting the degree of MF as well as cell migration and phenotypic transformation.

According to previous reports, ISO can promote angiotensin II production to induce activation of the classical downstream TGF-β1/Smad signaling pathway.[27] Once TGF-β1 is activated, it promotes the phosphorylation of Smad2

and Smad3, the downstream signaling molecules of TGF-β1, to form heterotrimeric complexes with their common mediator, Smad4, which enters the nucleus, resulting in the transcriptional regulation of target gene expression.[28] The role of TGF-β1 in the development of MF has been reported. TGF-β1 can induce the differentiation of cardiac fibroblasts into myofibroblasts and the activation of myofibroblasts; stimulate the synthesis of ECM components, such as collagen, fibronectin, and proteoglycan; and promote the deposition of ECM.[29] The IHC results in this study showed that JDTL decoction treatment significantly inhibited the expression of TGF-β1 in the myocardial tissue of the rats with MF. In the rat MF model, the phosphorylation of Smad2/3, a key target downstream of the TGF-β1 pathway, was also significantly reduced after JDTL decoction treatment. Furthermore, the *in-vitro* experimental studies demonstrated that JDTL decoction treatment significantly decreased the ratio of p-Smad2/3 in TGF-β1-treated RCFs, implying that this treatment could prevent MF by inhibiting the TGF-β1 pathway and thus downregulating Smad2/3 phosphorylation.

CONCLUSION

Taken together, our study (including the *in-vivo* and *in-vitro* experiments) revealed that JDTL decoction has inhibitory effects on the transformation of fibroblasts into myofibroblasts, the collagen deposition in myocardial tissue, and the MF process. The molecular mechanisms underlying the inhibitory effects of JDTL decoction include inhibition of the TGF-β1/Smad2/3 pathway through downregulation of TGF-β1 expression and phosphorylation of Smad2/3 as well as inhibition of the expression of vimentin and α-SMA. Our findings provide a new theoretical basis and experimental support for the use of JDTL decoction to treat MF.

COMPETING INTERESTS

The authors declare no potential conflicts of interest.

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AUTHOR CONTRIBUTIONS

GJJ and LXJ conceived and designed the experiments. HJ, ZH, LL, WB, SJX, and SJ conducted the experiments. LXJ analyzed the data. HJ wrote the paper. LXJ revised the paper. All authors provided final approval and agreed to be responsible for all aspects of the work, and there are no conflicts of interest.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The Ethics Committee of Laboratory Animals of Changchun University of Chinese Medicine approved all experiments.

CONSENT TO PUBLISH

All authors agree to publish

AVAILABILITY OF DATA AND MATERIALS

All data and materials are available

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