



Research Paper

JieDu TongLuo(JDTL) treatment regimen delays myocardial fibrosis in rats by inhibiting chymase activity

Accepted 8th October, 2018

ABSTRACT

In this research, the animal model of myocardial necrosis induced by isoproterenol (ISO) in Wistar rats was applied to inform the mechanism of JieDu TongLuo (JDTL) treatment regimen delays myocardial fibrosis in rats by inhibiting chymase activity. The results showed that after treatment with the JDTL regimen for 7 days, the area of both the necrotic foci and the collagen fibres in the myocardium in Wistar rats was reduced significantly. Meanwhile, after 7-day treatment with the JDTL regimen, the myocardial levels of Ang II in every treatment group were reduced as compared with those in the model group rats ($P < 0.05$). Before treatment, the results of toluidine blue staining showed that the number of toluidine blue-positive cells in the myocardial tissue of the ISO model group were significantly higher than that in the normal control group, indicating that chymase activity was substantially increased. Correspondingly, the mRNA and protein expression levels of chymase in the ISO model group increased significantly according to RT-qPCR and Western Blot results ($P < 0.05$). Moreover, after JDTL treatment for 7 days, the number of toluidine blue-positive cells in the myocardial tissues and myocardial chymase activity in the treated group were significantly reduced. The expression of chymase mRNA and protein decreased significantly compared with that in the rats in the ISO group, and the difference was statistically significant ($P < 0.05$). The expression of chymase mRNA in the myocardium was significantly reduced in the rats treated with the JDTL regimen compared with that in the ISO model group, and the difference was statistically significant ($P < 0.05$). In conclusion, the JDTL regimen (detoxification and improvement of the meridian circulation, or TongLuo JieDu) can effectively inhibit collagen fibre formation in the myocardium of rats with myocardial fibrosis.

Key words: Wistar rats, JieDu TongLuo(JDTL), chymase, myocardial tissues.

Jiajuan Guo¹, Yonggui Zhang², Xiaoyan Ma¹, Bo Sun³, Ju Hui¹, Yiming Zhang¹ and Xiangjun Li³

¹Department of Cardiovascular Disease, School of Chinese Medicine, Changchun University of Chinese Medicine, Changchun, Jilin 130021, China.

²Department of Gastroenterology, China-Japan Union Hospital of Jilin University, Number 126, Xiantai street, Changchun, Jilin 130033, China.

³Department of Experimental Pharmacology and Toxicology, School of Pharmaceutical Science, Jilin University, Changchun, Jilin 130021, China.

*Corresponding author. E-mail: gjj-2005@163.com, kerk2008@163.com, 66682693@qq.com, sunbo-2@qq.com, 1830885975@qq.com, 345524120@qq.com, lxj@jlu.edu.cn.

INTRODUCTION

Myocardial fibrosis refers to a disproportionate increase in fibrillar collagen in the myocardium accompanied by remodelling of the myocardial interstitial network and reduced cardiac function. Hypertension, coronary heart disease, hyperlipidaemia, myocarditis, arrhythmia, cardiomyopathy, chronic pulmonary heart disease and many other cardiovascular illnesses can all lead to myocardial fibrosis, and the severity of myocardial fibrosis

is closely related to the development and prognosis of these diseases (Kong et al., 2014). Therefore, understanding the mechanism of myocardial fibrosis and further prevention of its occurrence and development is crucial in treating cardiovascular disease. Improving the quality of life of patients and reducing the risk of death from cardiovascular disease are also of high significance. Elevation of angiotensin II (Ang II), especially localized elevation, plays

an important role in the development of myocardial fibrosis. Ang II can be generated by hydrolysis of Ang I through the activity of angiotensin-converting enzyme (ACE), thereby accelerating the progression of myocardial fibrosis (Gonzalez et al., 2002). This process can be inhibited by ACE inhibitors (ACEIs). However, recent studies have shown that another Ang II production pathway exists in the heart, the chymase pathway (Bacani and Frishman, 2006). Chymase is a serine protease secreted by mast cells that converts Ang I into Ang II. Some studies have found that myocardial chymase can accelerate the process of myocardial fibrosis by increasing the level of Ang II in local tissue and stimulating the proliferation of extra cellular matrix (ECM) synthesis cells (Kanemitsu et al., 2008). Conversely, the progression of myocardial fibrosis defers significantly by mast cell inhibitors (Wang et al., 2008; Wei et al., 2010).

In traditional Chinese medicine, myocardial fibrosis is comparable to diseases in the category of heart failure. The pathogenesis of myocardial fibrosis is believed to be “weakness in the original organ that appears as solid”. The disease is caused by persistent chronic illness invading into the meridian system, and the key to its pathogenesis is “toxin-induced impairment of blood and the meridian circulation”. Imbalanced yin and yang, dysfunction of the internal organs, and blocked energy (qi) and blood flow all result in the accumulation of physiological or pathological waste products in the body, which can lead to corrupted

Pharmaceutical Technology Development Co., Ltd. (Sichuan, China). All the herb granules met the quality standards set by “Pharmacopoeia of the People’s Republic of China”. The herbs were mixed with warm water at a weight ratio of *P. vulgaris* L 3: *A. bidentata* 2: *L. japonicus* Houtt 1.

Animals and treatments

A total of 40 male Wistar rats weighing 180–200 g, purchased from Liaoning Changsheng Biotechnology Co. Ltd. (Certificate No. SCXK (Liao) 2015–0001, Liaoning, China), were raised at the Changchun University of Chinese Medicine Medical Laboratory Animal Center. The rats were kept in groups of 5–6 per cage on a 12-h light-dark cycle, with lights on at 6 am. The room temperature was maintained at 25°C. All rats had access to water and food *ad libitum* during the entire study. All experimental procedures were approved by the Experimental Animal Ethics Committee of Changchun University of Chinese Medicine. The study was conducted in accordance with the BCPT policy for experimental and clinical studies (Tveden-Nyborg et al., 2018).

After adaptive feeding for 7 days, the rats were divided into 5 groups according to body weight with 10 rats in each group: the normal control group (Ctrl), the isoprenaline-

blood flow due to internal toxins, thus forming a vicious cycle that can eventually cause damage to the heart and the body, along with heart energy weakening or exhaustion, and ultimately initiate the development of myocardial fibrosis. Based on the principle of “seeking treatment for the cause of the illnesses”, we used an herb regimen that focuses on detoxification and improving the meridian circulation (JieDu TongLuo, JDTL) of the body. The prescribed medical herbs are *Prunella vulgaris*, *Achyranthes bidentata*, and *Leonurus japonicus* Houtt. The 3-herb combination was employed to regulate the balance of yin-yang in the liver and kidney and to open up the blood flow to expel toxins, thus improving the meridian circulation and inhibiting myocardial fibrosis. In this study, a rat myocardial fibrosis model was replicated by administering a single high dose of isoprenaline (ISO) to rats through subcutaneous injection, followed by treatment with the JDTL regimen for 7 days. The inhibitory effect of JDTL treatment on myocardial fibrosis progression and its impact on the production of Ang II through the chymase-mediated pathway were investigated.

METHODS

Composition of JDTL regimen

The JDTL regimen consists of 3 herbs: *P. vulgaris* L, *A. bidentata* and *L. japonicus* Houtt. were purchased in granule form from Sichuan Neo-Green treated group (ISO), the low-dose and medium-high-dose JDTL-treated groups (Jie L and Jie H) and the captopril-treated group (Cap). Each group of rats was injected with isoprenaline hydrochloride (CAT. I5627, Sigma-Aldrich, USA) subcutaneously at 5 mg/kg body weight to establish the myocardial fibrosis model, except for the rats in the control group, which were instead injected with an equal volume of normal saline. All groups were given food and water after injection following a normal schedule. The rats in the Jie L and Jie H groups were treated daily with JDTL herb granule suspensions at 10 g or 30 g/kg body weight, respectively. The rats in the Cap group were treated daily with captopril (CAT. C4042, Sigma-Aldrich, USA) solution at 0.005 g/kg body weight, while the Ctrl group rats were given an equal volume of tap water daily. All treatment solutions/suspensions/water were administered via gastric gavage. After 7 days of continued treatment, all the rats were sacrificed under anaesthesia. Some parts of their ventricles were fixed in 4% paraformaldehyde solution and other parts were stored in liquid nitrogen for later use.

Histological staining analysis

Myocardial tissues of rats from each group were paraffin-embedded and 5- μ m paraffin sections were prepared. After

washing with xylene to remove paraffin, haemoxylins and eosin (HE) staining, Masson's trichrome staining and toluidine blue staining were performed. HE staining was used to qualitatively assess the extent of myocardial necrosis and to identify signs of stromal hyperplasia. With Masson's trichrome staining, fibrous areas in myocardial tissues were stained blue. Semi-quantitative analysis of rat myocardial tissue fibrosis was performed in blue-stained areas using ImageJ (1.51t, National Institutes of Health, Maryland, USA). Toluidine blue staining was used to identify mast cells. In our study, we examined the numbers of stained mast cells in the myocardial tissues of each group using ImageJ to assess the extent of mast cell infiltration in areas of myocardial fibrosis in rats.

Hydroxyproline assay

A total of 100 mg of rat ventricular myocardial tissue was collected and the hydroxyproline content was determined using the alkaline water method. The levels of hydroxyproline in myocardial tissues were measured by spectrophotometry at a wavelength of $\lambda = 550$ nm following the kit's instructions. Hydroxyproline was recorded in $\mu\text{g}/\text{mg}$ of wet tissue weight. The hydroxyproline assay kit (CAT: A030, Nanjin Jiancheng Bioengineering Institute, Jiangshu, China) was purchased from the Nanjing Jiancheng Bioengineering Institute.

Determination of Ang II levels

The Ang II level in myocardial tissue was determined following the instructions in the ELISA kit. The major steps are as follows: ① 50 μl of a diluted antigen-containing myocardial tissue sample was added to the wells (in dilution buffer), and 50 μl of distilled water was added to the blank well. Then, the samples were incubated at 30°C for 30 min. ② 1st wash: after removing the coating liquid, the samples were washed 5 times with washing buffer for 1 min each. ③ 50 μl of horseradish peroxidase-labelled antibody solution was added to each well, excluding the blank well, followed by another 30 min of incubation at 37°C. ④ 2nd wash: after removing the coating liquid, the samples were washed with washing buffer 5 times for 1 min each. ⑤ 50 μl of substrate A was added to all the wells and the samples were incubated at 37°C in the dark for 10 min. ⑥ The reactions were terminated by adding 50 μl of stop solution to each well. ⑦ Results observation and recording: sample OD values at 450 nm were measured, and Ang II levels were calculated.

Measurement of chymase activity

A total of 50 mg of fresh myocardial tissue was

homogenized in 2 ml of phosphate buffer at 10 ml/L (pH 7.4) and the homogenized solution was kept overnight at 4°C. After centrifugation on the following day, 20 μl of supernatant was collected and mixed with 80 μl of incubation solution for chymase assays. At the same time, 100 μl of chymostatin was added to an empty test tube as the control. The samples were then incubated at 37°C for 10 min before the addition of 0.15 ml of 15% trichloroacetic acid to stop the reaction. The samples were subjected to centrifugation for 10 min before the supernatant was collected, which was then mixed with 10% o-phthalaldehyde to detect His-Leu, a metabolite of Ang I. The reaction generates a fluorescence signal, which is stabilized by adding 6 mol/L of HCl to the samples. The fluorescence signals were then read at 340-nm excitation and 450-nm emission. Chymase activity was calculated in nmol/(min • mg tissue) according to the signal intensity.

RT-qPCR detection of chymase expression

Two-step RT-qPCR was used to detect the expression level of chymase in myocardial tissues from each group of rats.

Total RNA extraction from myocardial tissue: Total RNA from myocardial tissues was extracted by following the instructions of TRIzol® (Gibco, USA). The main steps were as follows: 100 mg of apical myocardial tissue was homogenized in 1 ml of TRIzol® reagent. Then, 200 μl of chloroform was added, and the mixture was shaken vigorously before allowing it to stand on ice for 2 min. The samples were then centrifuged at 12,000 × g for 15 min at 4°C. The supernatant was transferred to a new tube and then an equal volume of isopropanol was added to precipitate the total RNA. Thereafter, the RNA pellet was washed with 70% ethanol and resolved in RNase-free water. The purity of the RNA samples was determined by OD260 and OD280 ratios; samples with an OD260/OD280 > 1.8 were selected for reverse transcription.

Quantitative PCR assay to determine the expression level of chymase: The chymase gene expression level was detected using the TransScript® Green Two-step qRT-PCR Supermix Kit (Cat#: Aq201, Beijing TransGen Biotech Co., Ltd., Beijing, China) following the manual of the kit. First-strand cDNA synthesis and gDNA removal were performed. The reaction solution was 20 μl in total, including 1 μg of total RNA, 4 μl of 5X TransScript® All-in-one Supermix and 1 μl of gDNA remover. The solution was incubated at 42°C for 15 min and then 85°C for 5 s to inactivate reverse transcriptase and the gDNA remover before initiating the qPCR assay.

The total volume of the qPCR solution was also 20 μl , which contained 1 μl of cDNA template, 0.4 μl each of upstream and downstream primers, and 10 μl of 2X TransStart® Tip Green qPCR Supermix. The steps for PCR

amplification were as follows: after pre-denaturing the cDNA at 94°C for 30 s, the samples were subjected to 45 cycles of amplification: 94°C for 5 s, 60°C for 15 s, and 72°C for 10 s. After the experiment was completed, the CT values for each sample were calculated using the $2^{-\Delta\Delta CT}$ method to represent the relative expression level of the target gene; $\Delta\Delta CT = (CT_{\text{target}} - CT_{\text{reference}})_{\text{experiment}} - (CT_{\text{target}} - CT_{\text{reference}})_{\text{control}}$ (Livak and Schmittgen, 2001) (Bubner and Baldwin, 2004). In this study, the primer sequences used to amplify a 99-bp fragment of chymase (GI: 6978666) were 5'-GAGAATGTGCAGGGCAGTTG-3' (upstream) and 5'-GGGCTCCTGGAGTCTCATCT-3' (downstream). A 177-bp GAPDH sequence was also amplified as the internal reference (NM_017008.4) using the primer sequences 5'-GTTACCAGGGCTGCCTTCTC-3' (upstream) and 5'-GATGGTGATGGGTTTCCCGT-3' (downstream).

Western blot detection of chymase protein expression

The protein levels of chymase in myocardial tissues from each group were detected using Western Blot. The main procedures were as follows: 50 mg of apical myocardial tissue was fully lysed in 300 μ l of RIPA buffer (CAT#: P0031B, Beyotime Biotechnology Co. Ltd., Shenzhen, China), and protein quantification was performed using the Bradford method. Then, 100 μ g of each protein sample was used to run SDS-PAGE electrophoresis, and the proteins were then transferred to a 0.22- μ m PVDF membrane. The membrane was blocked with 5% non-fat dry milk and then incubated with rabbit anti-chymase polyclonal antibody (CAT#: A5668, AB clonal Biotechnology Co. Ltd., Wuhan, China) at 1:1000 dilution. The hybridization signal was detected using an imaging system (Shanghai Tianneng Biotechnology Co., Ltd., Shanghai, China) and the results were analysed.

Data analysis

All the data were analysed using GraphPad Prism 5.0 software. The data were expressed as the mean \pm standard deviation. Differences between all the groups were compared using one-way analysis of variance (ANOVA). Comparisons between every 2 groups were performed via Newman-Keuls analysis. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Effects of JDTL treatment on myocardial histological structures in rats

After 7 days of ISO injection, paraffin sections of myocardial tissues from each group were stained with HE. In the tissues

from the ISO model group, myocardial necrotic lesions of various sizes with distinct boundaries were noted. These lesions contained empty necrotic spaces filled with lysed/ruptured myocardial cells, proliferating macrophages, fibroblasts and collagen components. The necrotic lesions were mainly distributed in the papillary muscles and the inner layers of the myocardium, while lesions in the middle and outer myocardial layers were rare. In the JDTL-treated groups and the Cap-treated group, the characteristics of myocardial necrosis were similar to those in the model group, but the numbers and areas of necrosis were reduced by varying degrees, and large areas of necrosis in the myocardium were rarely observed in the treatment groups, as shown in **Figure 1**.

To further evaluate collagen proliferation in the myocardial necrotic foci, we performed Masson's trichrome staining on the paraffin sections of myocardial tissues from each group. The results showed that a large amount of fibrous tissue filled the empty spaces left behind by myocardial necrosis, as shown in **Figure 2A**. The image analysis showed that, as compared with the normal control group, the areas of collagen fibres in the ISO group were significantly increased ($P < 0.0001$); however, as compared with the ISO group, the areas of collagen fibres were significantly decreased in the myocardium in the high-dose JDTL-treated group and the Cap-treated group ($P < 0.001$), whereas no statistically significant difference was observed in the collagen fibre areas between the low-dose JDTL treatment group and the model group. The differences in the sizes of the collagen fibre-filled, in the myocardial tissues of each JDTL-treated group and the Cap-treated group, were not statistically significant as shown in **Figure 2B**.

To assess the expression of collagen in the myocardial tissues of each group quantitatively, we used the alkaline hydrolysis method to determine the hydroxyproline (HYP) content in rat ventricular tissues from all groups. The results indicated that the hydroxyproline levels in the ventricular tissues of the model group were significantly greater than those in the normal control group, and the difference was statistically significant ($P < 0.0001$). Consistently, the hydroxyproline content in the myocardial tissues of the JDTL groups at all doses was higher than that in the control group but lower than that in the model group, and the differences were statistically significant ($P < 0.001$). The hydroxyproline content in the myocardial tissues of the Cap-treated group was lower than that in the model group, but the difference was not statistically significant as shown in **Figure 2C**.

Effect of JDTL treatments on Ang II levels in rat myocardial tissue

The results of the Ang II assay, shown in the **Figure 3**, demonstrated that the level of Ang II in the myocardium of

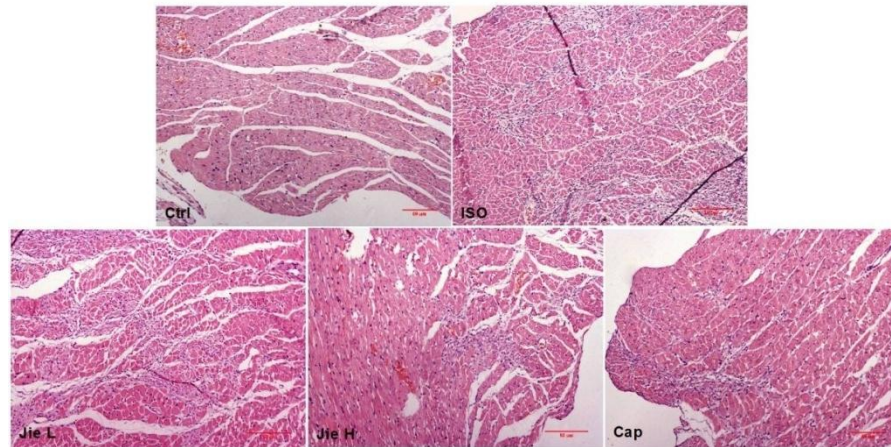
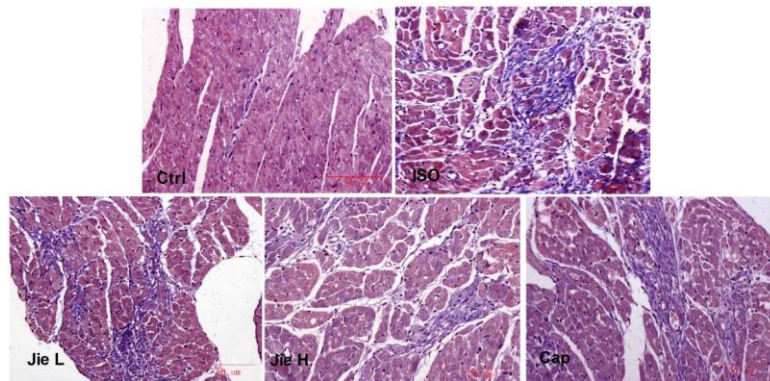


Figure 1: HE staining results of myocardial tissues in each group (x100). In the Ctrl group, the myocardium appeared orderly without fracture and necrosis. In the ISO group, myocardial necrotic lesions of various sizes with clear boundaries were detected; myocardial cells were lysed and ruptured and many empty necrotic spaces were filled with hyperplastic macrophages, fibroblasts and collagen. Although the features of myocardial tissue necrosis are similar among the model group, the JDTL treatment groups (Jie L and Jie H), and the Cap-treated group, the numbers and areas of necrosis were reduced by varying degrees in all the treatment groups, and large areas of integrated necrosis were rare in the hearts of the rats after all the treatments. Ctrl: The normal control group; ISO: the model group; Jie L and Jie H: the low-dose and high-dose JDTL groups, respectively; Cap: the captopril-treated group, the same as other figures.



A

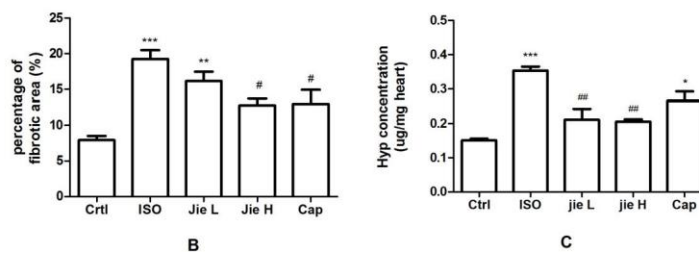


Figure 2: Masson's trichrome staining and Hyp detection results of myocardial tissues in each group (x 100). A). The sample of masson's trichrome staining picture in each group; B). image analysis results of masson's trichrome staining in each group, compared with the Ctrl group, the collagen fibre areas of myocardial tissues in the ISO group and the low-dose JDTL-treated groups were increased significantly ($P < 0.0001$, $P < 0.01$), while that in the high-dose JDTL and the Cap group were decreased significantly vs that in the ISO group ($P < 0.05$); C). the Hyp concentration in the ISO group and the Cap group was increased statistically ($P < 0.0001$, $P < 0.05$), while that in the low- and high-dose JDTL group was decreased significantly compare with that in the ISO group ($P < 0.01$).

vs the Ctrl group, *** $P < 0.0001$, ** $P < 0.01$, * $P < 0.05$; vs the ISO group, ### $P < 0.0001$, ## $P < 0.01$, # $P < 0.05$, the same as other figures.

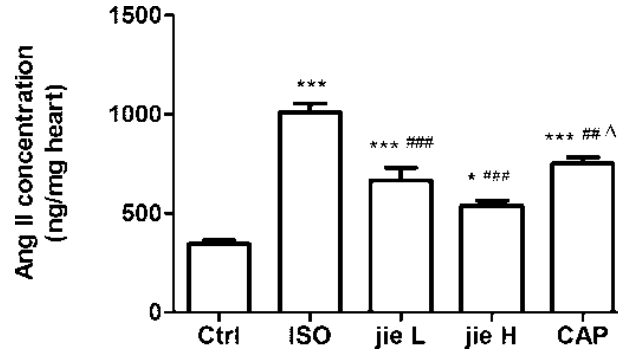
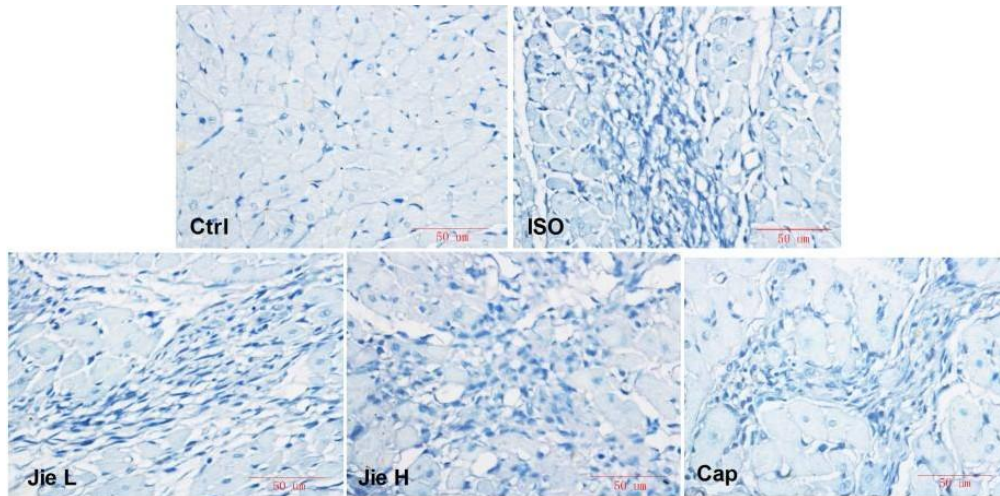
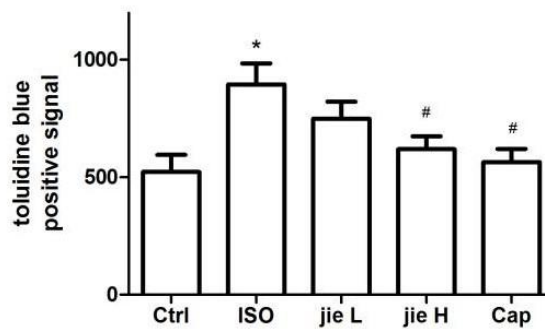


Figure 3: Comparison of Ang II concentrations in the hearts of rats of various groups vs the Jie H group, Δ $P < 0.05$, the same as other figures below.



A



B

Figure 4: Comparison of toluidine blue staining results for the rats in each group. A). The sample of toluidine blue staining picture in each group; B). image analysis results of toluidine blue staining results in each group, compared with the control group, the number of toluidine blue staining positive cell in the ISO group was increased significantly ($P < 0.05$), while that in the high-dose JDTL and the Cap group were decreased significantly vs that in the ISO group ($P < 0.05$).

rats in the ISO group was significantly increased ($P < 0.0001$). Though the Ang II concentration in the low-,

high-dose JDTL-treatment group and the Cap-treatment group were higher than that in the control group

significantly, while lower statistically than that in the model group, the difference was statistically significant ($P < 0.0001$, $P < 0.01$). On the other hand, the myocardial Ang II level in the Cap-treated group was noticeably higher than that in the high-dose JDTL-treated group, with statistical significance ($P < 0.01$), but no statistically significant difference in Ang II levels were found between the Cap-treated group and the low-dose JDTL group.

Effect of JDTL treatments on Toluidine blue-positive cells in rat myocardial tissue

The results of toluidine blue staining showed, in the **Figure 4A**, that in the myocardial tissues of normal rats, only a small amount of cells were toluidine blue-positive, and they were evenly distributed in the myocardial interstitium. Image analysis results showed that the number of toluidine blue-positive cells in the myocardial tissues of the model group was significantly higher than that of the control group ($P < 0.05$). The numbers of toluidine blue-positive cells in the myocardial tissues of the high-dose JDTL-treated groups and the captopril-treated group were lower than that of the model group, the difference was statistically significant ($P < 0.05$).

Effect of JDTL treatments on Chymase changing in rat myocardial tissue Chymase activity

According to the chymase activity assays for each group of rats, chymase activity in myocardial tissues was significantly elevated in the ISO group as compared with that in the normal group ($P < 0.05$). After JDTL treatment, myocardial chymase activity was significantly reduced in the high-dose group as compared with that in the ISO group, and the difference was statistically significant ($P < 0.05$). The result of chymase activity in rats from each group is shown in **Figure 5A**.

Chymase mRNA and protein expression levels

According to the RT-qPCR results shown in **Figure 5B**, the expression of chymase mRNA in the myocardial tissues of the ISO group was significantly higher than that in the normal group ($P < 0.05$). A significant decrease in the expression of chymase mRNA was observed in myocardial tissues of the high-dose JDTL group as compared with that in the ISO group and the low-dose JDTL group, and the differences were statistically significant ($P < 0.05$).

The Western Blot results, as shown in **Figure 5C**, indicated a marked increase in the expression of chymase protein in the myocardial tissues of the ISO group as compared with that in the normal group ($P < 0.05$). After treatment with the JDTL regimen, chymase protein expression levels in

myocardial tissues from both the low-dose and high-dose JDTL groups were significantly reduced as compared with those in the ISO group. Chymase protein expression was also reduced in the Cap-treated group as compared with that in the model group. All the differences were statistically significant ($P < 0.05$). Further analysis showed that the expression of chymase protein in myocardial tissues from the high-dose JDTL group was considerably lower than that in the low-dose JDTL group, and the difference was statistically significant ($P < 0.05$).

DISCUSSION

Chinese medicine believes that the key to the pathogenesis of myocardial fibrosis is “toxin-induced impairment of the blood and meridian circulation”. The production of toxins is as a result of yin-yang imbalance, organ dysfunction and disrupted circulation of blood and energy, impeding timely release of physiological or pathological waste products in the body. Accumulation of these waste products causes damage to the body, leading to “toxification due to poor meridian flow”. Endogenous toxins, corruption of the blood and meridian network, and illnesses in the meridian system can cause disorders in blood and energy circulation, which may lead to a vicious cycle that eventually causes damage to the heart, weakens the heart and circulation, and results in heart failure and exhaustion. ISO belongs to the chemical family of catecholamines. Subcutaneous injection of ISO into rats can accelerate the heart rate, augment myocardial contractility, and increase myocardial oxygen consumption by inducing cardiac β -adrenergic receptors, thereby causing myocardial microcirculatory disturbances and myocardial damage. Myocardial necrotic lesions of various sizes with clear boundaries can be observed under a microscope in ISO-injected rat tissues. In these lesions, myocardial cells appear lysed and fractured, and as the disease progresses, the empty necrotic spaces gradually become filled with proliferating macrophages, fibroblasts, and collagen components. Therefore, the ISO-induced myocardial fibrosis model has been widely used by scholars as an animal model for “toxin-induced impairment of the blood and meridian circulation” in traditional Chinese medicine (Allawadhi et al., 2018).

In this study, with toxin elimination from the system as the focus of treatment, a 3-herb regimen was used to rebalance the yin-yang of the liver and kidney and to open up the blood circulation. We recommend using this JDTL regimen to treat myocardial fibrosis. The regimen is composed of *P. vulgaris*, *A. bidentata* and *L. japonicus* Houtt. Among these, *P. vulgaris* is a bitter herb with a cold nature that has the function of opening up and improving energy and blood circulation, thus serving as the main ingredient in the regimen facilitating the movement of energy and materials and blockage removal in the meridian system to eliminate toxins. *A. bidentata* has a sweet and sour taste with

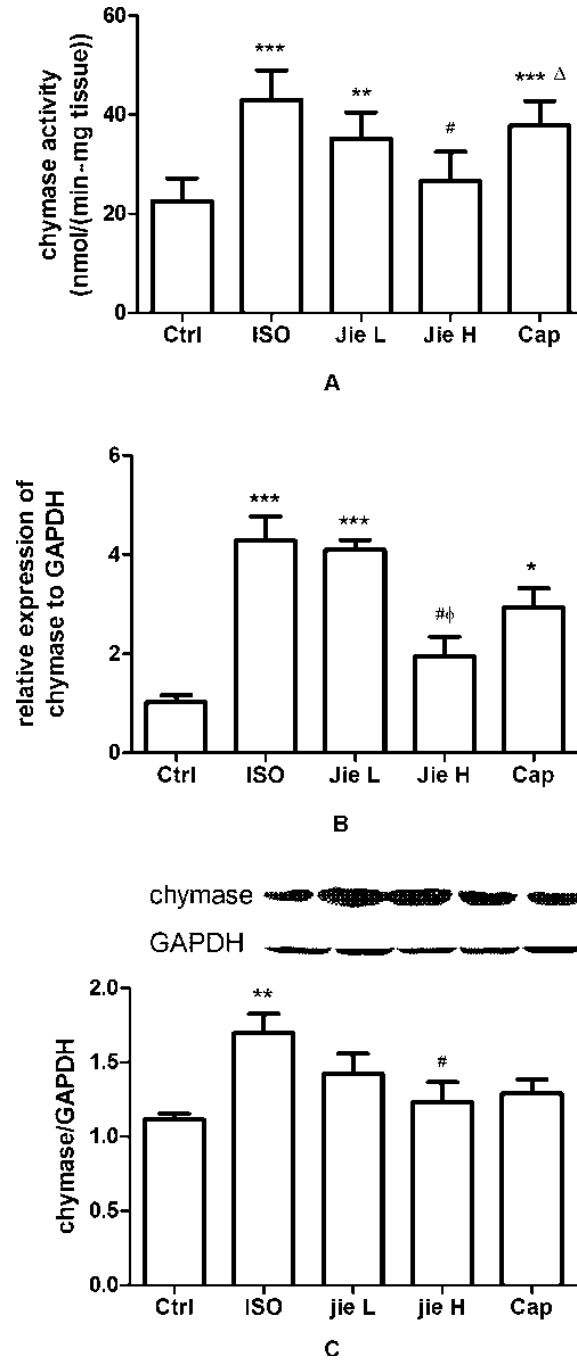


Figure 5: Effect of JDTL treatments on Chymase changing in rat myocardial tissue. A). Comparison of chymase activity in rats from each group; B). Comparison of chymase mRNA expression in various groups of rats; C). Comparison of chymase mRNA expression in various groups of rats. Ctrl: normal control group; ISO: model group; Jie L, Jie M, and Jie H: low-dose, middle-dose, and high-dose JDTL groups, respectively; Cap: captopril-treated group. Compared with the Jie L group, ^φ P<0.05.

a neutral nature and is a good tonic for the liver and kidney. As a secondary herb in the regimen, it can help with the yin-yang balance by nurturing the yin and masking the yang. *L.*

japonicus Hoult has a spicy yet sweet taste with a hint of bitterness. With a slightly cold and cooling nature, it can facilitate energy flow and blood circulation and remove

local blockage, thus improving the meridian circulation. In this study, a rat model of myocardial fibrosis was generated by a single ISO injection, and then the rat models were treated with the JDTL regimen for 7 days. The results showed that after treatment with different doses of JDTL, both the numbers of myocardial necrotic lesions and the areas of necrosis were reduced by various degrees. The levels of hypertrophy of the myocardial interstitium were also significantly reduced as compared with the model group.

Heart mesenchymal cells such as mast cells, endothelial cells, and fibroblasts are the major extracellular matrix-producing cells in the process of myocardial fibrosis (Duffy, 2011; Piera-Velazquez et al., 2011). Among them, mast cells and cardiac fibroblasts can be stained by toluidine blue. In this study, myocardial tissues were stained with toluidine blue and the numbers of rat myocardial mesenchymal cells were examined. The results showed that the numbers of these cells increased significantly 7 days after ISO injection. After treatment with JDTL, an obvious decrease in toluidine blue-positive cells in the ISO-treated rat myocardium was observed. Masson's trichrome staining is a method used to identify the fibre composition in tissues by staining collagen in blue colour. Hydroxyproline is a unique amino acid that accounts for approximately 13% of all amino acids in collagen. The hydroxyproline content in tissue is an important quantitative indicator to evaluate the extent of organ fibrosis. In this study, we used Masson's trichrome staining and hydroxyproline assays to qualitatively and quantitatively assess the collagen content of myocardial tissue in each group. The results showed that 7 days after subcutaneous injection of ISO, myocardial necrotic lesions were filled with a large amount of fibrous tissue, indicating that myocardial fibrosis had developed. The results from the image analysis demonstrated that the collagen-filled areas in the rats treated with middle-high-dose JDTL were significantly reduced as compared with those in the ISO model group. The hydroxyproline assay suggested that the collagen content in JDTL-treated rats was also significantly lower than that in the ISO model group, indicating that JDTL treatment can significantly inhibit the development of myocardial fibrosis and delay its progression.

Locally released Ang II is an important myocardial fibrosis-stimulating factor. Both *in vitro* and *in vivo* studies have demonstrated that Ang II can promote myocardial fibrosis by stimulating fibroblast proliferation and enhancing collagen synthesis activity (Sadoshima and Izumo, 1993; Schorb et al., 1993; Ma et al., 2017). It was also found in the present study that 7 days after injecting isoproterenol into rats, myocardial Ang II levels were significantly elevated, but after treatment with the JDTL regimen, the Ang II levels in the myocardial tissues of ISO rats were significantly reduced. This effect is similar to that of Cap treatment, indicating that the treatment mechanism of JDTL therapy for myocardial fibrosis is associated with inhibition of the renin angiotensin aldosterone system

(RAAS) pathway. Studies have shown that in addition to the classical RAAS, Ang II can also be produced by the chymase pathway (Kennedy et al., 2013). Chymase is a serine protease originally found in mast cells. Chymase has been reported to be the most powerful known Ang I-converting enzyme in local tissues till date. The biological activity of Ang II derived from the chymase pathway is 20-times stronger than that of Ang II derived from the RAAS pathway (Kofford et al., 1997). In this study, the activity of chymase in rats in each group was examined. The results showed that chymase activity in myocardial tissues of the ISO group increased significantly. Using Western Blot and RT-qPCR, we found that the expression of chymase in myocardial tissues of the ISO group also increased significantly. However, after JDTL treatment, myocardial chymase activity in the rats that received ISO injection was significantly decreased, and chymase mRNA and protein expression levels were also notably reduced.

Conclusions

The results of this study suggest that chymase-derived Ang II plays an important role in the progression of myocardial fibrosis and can promote the synthesis of myocardial tissue matrix components in rats. The JDTL treatment regimen can significantly inhibit the synthesis of extracellular matrix components in rat cardiac muscle and delay the progression of myocardial fibrosis. Although the mechanism is not yet clear, the current results indicate that inhibition of chymase activity and expression in myocardial tissue may be one of the effects of the treatment. Therefore, the chymase production pathway of Ang II may be the therapeutic target of JDTL.

ACKNOWLEDGEMENTS

This study was funded by the National Natural Science Foundation Youth Science Fund Project (81503544) and the Jilin Provincial Department of Science and Technology Project (201801011108JC).

REFERENCES

- Allawadhi P, Khurana A, Sayed N, Kumari P, Godugu C (2018). Isoproterenol-induced cardiac ischemia and fibrosis: Plant-based approaches for intervention. *Phytother Res*. doi: 10.1002/ptr.6152. [Epub ahead of print].
- Bacani C, Frishman WH (2006). Chymase: a new pharmacologic target in cardiovascular disease. *Cardiol. Rev.* 14(4):187-193.
- Bubner B, Baldwin IT (2004). Use of real-time PCR for determining copy number and zygosity in transgenic plants. *Plant Cell Reports*. 23(5):263-271.
- Duffy HS (2011). Fibroblasts, myofibroblasts, and fibrosis: fact, fiction, and the future. *J. Cardiovasc. Pharmacol.* 57(4):373-375.
- Gonzalez A, Lopez B, Querejeta R, Diez J (2002). Regulation of myocardial fibrillar collagen by angiotensin II. A role in hypertensive heart disease?

- J. Mol. Cell. Cardiol. 34(12):1585-1593.
- Kanemitsu H, Takai S, Tsuneyoshi H, Yoshikawa E, Nishina T, Miyazaki M, Ikeda T, Komeda M (2008). Chronic chymase inhibition preserves cardiac function after left ventricular repair in rats. *Eur. J. Cardiothorac. Surg.* 33(1):25-31.
- Kennedy S, Wu J, Wadsworth RM, Lawrence CE, Maffia P (2013). Mast cells and vascular diseases. *Pharmacol. Ther.* 138(1):53-65.
- Kofford MW, Schwartz LB, Schechter NM, Yager DR, Diegelmann RF, Graham MF (1997). Cleavage of type I procollagen by human mast cell chymase initiates collagen fibril formation and generates a unique carboxyl-terminal propeptide. *J. Biol. Chem.* 272(11):7127-7131.
- Kong P, Christia P, Frangiannis NG (2014). The pathogenesis of cardiac fibrosis. *Cellular and Molecular Life Sciences.* 71(4):549-574.
- Ma ZG, Yuan YP, Zhang X, Xu SC, Wang SS, Tang QZ (2017). Piperine attenuates pathological cardiac fibrosis via PPAR-gamma/AKT pathways. *EBioMedicine.* 18:179-187.
- Piera-Velazquez S, Li Z, Jimenez SA (2011). Role of endothelial-mesenchymal transition (EndoMT) in the pathogenesis of fibrotic disorders. *Am. J. Path.* 179(3):1074-80.
- Sadoshima J, Izumo S (1993). Molecular characterization of angiotensin II--induced hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts. Critical role of the AT1 receptor subtype. *Circ. Res.* 73(3):413-423.
- Schorb W, Booz GW, Dostal DE, Conrad KM, Chang KC, Baker KM (1993). Angiotensin II is mitogenic in neonatal rat cardiac fibroblasts. *Circ. Res.* 72(6):1245-54.
- Tveden-Nyborg P, Bergmann TK, Lykkesfeldt J (2018). Basic & Clinical Pharmacology & Toxicology Policy for Experimental and Clinical studies. *Basic Clin. Pharmacol. Toxicol.* 123(3):233-235.
- Wang Y, Tandan S, Cheng J, Yang C, Nguyen L, Sugianto J, Johnstone JL, Sun Y, Hill JA (2008). Ca²⁺/calmodulin-dependent protein kinase II-dependent remodeling of Ca²⁺ current in pressure overload heart failure. *J. Biol. Chem.* 283(37):25524-25532.
- Wei CC, Hase N, Inoue Y, Bradley EW, Yahiro E, Li M, Naqvi N, Powell PC, Shi K, Takahashi Y (2010). Mast cell chymase limits the cardiac efficacy of AngI-converting enzyme inhibitor therapy in rodents. *J. Clin. Invest.* 120(4):1229-1239.