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Effect of imatinib on DOCA-induced myocardial fibrosis in rats through P38 MAPK signaling pathway

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Abstract. – OBJECTIVE: To explore the role of imatinib in desoxycorticosterone acetate (DOCA)-induced myocardial fibrosis in rats by the p38 mitogen-activated protein kinase (MAPK) signaling pathway.

MATERIALS AND METHODS: Normal group (n=20), DOCA induction group (n=20), and imatinib treatment group (treatment group, n=20) were set up. Then, the cardiac function was examined via magnetic resonance imaging (MRI) and echocardiography (ECG) on the 21st d after modeling. Alkaline phosphatase (ALP) and myocardial function index creatine kinase-MB (CK-MB) were detected. The enzyme-linked immunosorbent assay (ELISA) was performed to measure tumor necrosis factor-gamma (TNF-γ) and interleukin-6 (IL-6). Hematoxylin-eosin (HE) staining assay was carried out to observe the pathological changes in myocardial tissues. Quantitative Polymerase Chain Reaction (qPCR) and Western blotting were employed to measure the expression levels of important myocardial fibrosis-related genes [checkpoint kinase 1 (Chek1) and alpha-smooth muscle actin (α-SMA)], as well as genes and proteins of the p38 MAPK signaling pathway.

RESULTS: In comparison with the normal group, DOCA induction group had significantly lowered fractional shortening (FS, %) and ejection fraction (EF, %), but overtly increased left ventricular end-diastolic dimension (LVEDd) and left ventricular end-systolic dimension (LVESd), as well as levels of serum ALP, alanine aminotransferase (ALT), and CK-MB. Besides, the levels of TNF-γ, IL-6, and IL-1β were notably raised in the DOCA induction group. HE staining results showed that myocardial injury was more severe in DOCA induction group. The results of the gene detection revealed that the expression levels of Chek1, α-SMA, p38 MAPK, and JNK were evidently higher in DOCA induction group than those in the imatinib treatment group (*p*<0.05), and the expression of p38 MAPK protein in the rat myocardial tissues was remarkably lower in the treatment group than that in the DOCA induction group (*p*<0.05).

CONCLUSIONS: Imatinib can regulate the repair of myocardial injury caused by DOCA-induced myocardial fibrosis in rats by repressing the p38 MAPK signaling pathway.

Key Words:
Imatinib, P38 MAPK signaling pathway, DOCA, Myocardial fibrosis, Rats.

Introduction

Myocardial fibrosis, the result of fibroblast proliferation and collagen deposition in different extracellular matrixes, mainly in myocardial interstitial cells, leads to the remodeling of myocardial tissues and thus diastolic and systolic dysfunction[1]. Cardiomyopathy has a very complex pathogenesis and is correlated with the development of hypertension and coronary artery disease. Myocardial fibrosis is caused by many factors, including enhanced stress, immune damage, ischemia, and hyperglycemia and often involves collagen production and degeneration systems, apoptosis, inflammation, and various cardiovascular active substances and cytokines[2]. Besides, myocardial fibrosis, a common pathological result of many diseases, ultimately leads to aggravated myocardial stiffness, reduced coronary flow reserve, ventricular arrhythmia, and sudden death[3,4]. The unavailable normal systolic and diastolic function of the myocardium and the drastically reduced or forcedly interrupted blood supply to coronary arteries severely affect the normal function of the myocardium[5]. In addition, reduced or blocked blood flow leads to speckled rupture of the coronary arteries, which will cause various secondary reactive diseases and ultimately even necrosis when advancing to serious stages[6]. Myocardial fibrosis plays an important role in cardiac remodeling after myocardial infarction and is a predisposing factor for heart failure[7]. With

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decades of in-depth research, the complexity of myocardial fibrosis that leads to characteristic changes in metabolism and ultrastructure, as well as irreversible damage is well currently understood. Myocardial fibrosis is an important target in the treatment of myocardial infarction and may lead to cell death[8]. Myocardial fibrosis includes ROS production, calcium overload, pro-inflammatory cytokine activation, apoptosis, neutrophil filling, and endothelial dysfunction[9]. Therefore, investigating the pathological characteristics and pathogenesis of myocardial fibrosis and searching for new anti-myocardial fibrosis drugs are of great significance for the prevention of severe clinical cardiovascular events.

Moreover, many substances are produced in case of myocardial damage due to myocardial fibrosis, activating intracellular signaling pathways, such as mitogen-activated protein kinases (MAPKs)[10]. These stimuli may trigger molecular cascades, thereby activating one or more MAPKs. Currently, the known MAPKs can be divided into three classes: extracellular signal-regulated kinases (ERKs), p38 MAPKs, and c-jun N-terminal kinases (JNKs). These three classes can be further divided into different subclasses, including the ERK 1/2 subtype and different JNK proteins, JNK 1, 2, and 3[11]. The p38 MAPK subfamily stress kinases such as p38 and JNK induce inflammation, apoptosis, and cell death, while pro-survival kinases regulate the differentiation and proliferation of cells, facilitate the survival of cells, and protect tissues[12], participating in the regulation of myocardial infarction and ventricular remodeling. The p38 MAPK subfamily is an important signal transduction in myocardial modulation and plays a powerful role in the development and progression of myocardial infarction[13,14]. Subsequently, ERK 1/2 is activated, thus leading to phosphorylation of the downstream cascades and different sensory receptors, attenuating oxidative stress and inflammation, maintaining cytoskeletal structure, and protecting against myocardial injury[15]. Thus, the relative activity of these pro-apoptotic and pro-survival kinase pathways will determine the survival or death of cells. The understanding of the mechanism by which these pathways participate in myocardial fibrosis-induced injury and diseases is incomplete. Therefore, precisely controlling the time, location, and duration of stimulation of the p38 MAPK pathway is important for proper physiological function[16].

This study aims to investigate the influence of imatinib on desoxycorticosterone acetate (DOCA)-induced myocardial fibrosis in rats *via* the p38 MAPK signaling pathway. The analyses on whether the p38 MAPK signaling pathway participates in the pathogenesis of myocardial fibrosis in rats remains rare. Therefore, in this work, the potential role of p38 MAPK in myocardial fibrosis-induced injury in rats was investigated, and *in vivo* experiments and diversified molecular biology techniques were conducted to elucidate the effect on myocardial fibrosis-induced injury in rats. After interference in rat models of DOCA-induced myocardial fibrosis with imatinib, the cardiac function and pathway-related protein expression were detected, and the impact of imatinib on myocardial injury in rats through the p38 MAPK pathway was observed, providing important experimental support and theoretical references for the treatment of myocardial fibrosis with imatinib.

Materials and Methods

Establishment of Animal Models

A total of 60 male Sprague-Dawley (SD) rats were unrestrained, with free access to drinking water. After one week, the rats were anesthetized with pentobarbital sodium (40 mg/kg), ventilated (50 times/min), and subjected to thoracotomy under aseptic condition to remove the right kidney. One week later, DOCA was subcutaneously injected once every three days (twice every week) at 65 mg/kg, and then, the rats were treated with imatinib *via* gavage at 55 mg/kg once a day. The rats in the normal group were given the same dose of normal saline *via* gavage. Each group included 20 rats. Subsequent assays were performed at three weeks after drug intervention. This study was approved by the Animal Ethics Committee of the Changzhi Medical College Animal Center.

Detection of Cardiac Physiological Function Indexes in Rats

After intervention with experimental drug, the left ventricular function indexes, including left ventricular end-diastolic dimension (LVEDd), left ventricular end-systolic dimension (LVESd), fractional shortening (FS), and ejection fraction (EF) of all rats were examined using Philips 7500 ultrasound machine (Philips Medical, Amsterdam, the Netherlands) and magnetic resonance imaging (MRI) and echocardiography (ECG) systems in accordance with the requirements of the instrument manual. Each group of rats to be

examined was fixed in the supine position, and then, electrocardiogram was performed with a probe frequency of 10 MHz.

Examination of Myocardial Function and Liver Function

Blood was routinely collected from rat tail vein after experiments, let stand at room temperature for 30 min, and centrifuged at 1000 g for 10 min. Next, the supernatant was collected to determine myocardial function index creatine kinase-MB (CK-MB), liver function indicators alanine aminotransferase (ALT), and alkaline phosphatase (ALP) in case of myocardial fibrosis. Moreover, we observed whether these indexes were changed and affected the progression of myocardial fibrosis, so as to provide important references for early diagnosis of myocardial injury and predict the occurrence of disease in advance.

Determination of Inflammatory Cytokines in Each Group of Rats

The rats were intraperitoneally anesthetized and then killed, and the heart was taken and washed with normal saline. Thereafter, the ischemic myocardial tissues (0.5 g) were taken, broken at a low-temperature environment using a homogenizer containing prepared tissue lysis solution and centrifuged at 1200×g at 4°C for 30 min. Next, a pipette was used to collect the supernatant. The level of the serum tumor necrosis factor-gamma (TNF-γ) was measured using an Enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA), and a microplate reader was utilized to read the absorbance in each group. Relevant assays were performed according to actual situations and the instructions.

Hematoxylin-Eosin (HE) Staining

The heart tissues were fixed in 10% neutral buffered formalin for 7 d, washed with running water for 24 h, and dehydrated with gradient alcohol, followed by conventional section preparation using a microtome (Leica RM 2125, Wetzlar, Germany). Then, the tissues were sliced into sections (5 μm in thickness). Next, the sections were deparaffinized, hydrated with 95%, 90%, 80%, 75%, and 50% ethanol, respectively, permeabilized, dipped, and embedded in paraffin. Thereafter, the embedded blocks were prepared into pathological sections. Lastly, the dried thin sections were stained with HE, mounted and histologically observed using a light microscope.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

The total ribonucleic acids (RNAs) were extracted from the rat myocardial tissues in each group with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and, then, the RNAs meeting relevant purity and concentration were reversely transcribed into complementary deoxyribose nucleic acid (cDNA) strands (the use of isopropanol was paid attention to). Primer amplification was performed using a 20 μL system (2 μL of cDNA, 10 μL of mix, 2 μL of primer and 6 μL of ddH_2O, for 40 cycles), and then the conventional PCR amplification was carried out. The primer sequences of the target genes and the internal reference β-actin were designed based on the sequences of GenBank (Table I). The expression levels of the target genes were detected by qRT-PCR. The mRNA expression levels in rat myocardial tissues in each group were calculated by $2^{-\Delta\Delta Ct}$.

Western Blotting

The rat heart tissues were cut into pieces, weighed, added with radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) at a ratio of 100 mg: 1 mL, and then homogenized. Next, the tissues were centrifuged at low temperature and 3000 g for 10 min, and the supernatant was collected and stored at ultra-low temperature for later use. The proteins were extracted using strong lysis buffer, and then, the total protein concentration in the rat of the myocardial tissues in each group was detected *via* a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA), in accordance with the instructions. Then, the samples were prepared, the gel plate was installed, and the gels were pre-

Table I. PCR primers.

Target gene	Primer sequence (F-R)
β-actin	5-CAGTGCCAGCCTCGTCTCAT-3' 5-AGGGCCATCCACAGTCTTC-3'
Alpha-smooth muscle actin (α-SMA)	5-GTCCCAGACATCAGGGAGTAA-3' 05-TCGGATACTTCAGCGTCAGGA-3'
P38 MAPK	5'-CCAGATGCCGAAGATGAACT-3' 5'-GGGCTGCTGTGATCCTCTTAT-3'
JNK	5'-TTCCATTGTGGGTAGGTGG-3' 5'-CTTACAGCTTCCGCTTCAG-3'
Collagen I	5-GGCAGTGCCTTTTGTGGAAG-3' 5-TCTATGGCCCGCTTCATGTC-3'
Chek1	5-ATCAGCCCAAACCCCAAGGAGA-3' 5-CGCAGGAAGGTCAGCTGGATAG-3'

pared. Thereafter, the samples were added with loading buffer, loaded and electrophoresed, followed by wet/semi-dry membrane transfer. Next, the membrane was blocked at room temperature, incubated with the primary antibody in a box overnight and then, the secondary antibody for 1 h, added with freshly prepared enhanced chemiluminescence (ECL) mixture. After development in a dark room, the bands were processed using the software. An Odyssey scanner was employed to scan and quantify the protein bands, and the level of the proteins to be tested was corrected with glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Western blotting bands were quantified using Image Lab software. The expression levels of the relevant proteins in each group were calculated.

Statistical Analysis

All raw experimental data recorded were processed using the Statistical Product and Service Solutions (SPSS) 20.0 analysis software (IBM Corp., Armonk, NY, USA) and subjected to multiple comparisons. The obtained experimental results were expressed as mean ± standard deviation ($\bar{\chi}$±SD), and $p<0.05$ suggested that the difference was statistically significant. GraphPad Prism 8.0 (La Jolla, CA, USA) was used for histograms.

Results

Cardiac Function Indexes in Each Group Determined

DOCA induction group exhibited markedly reduced FS and EF and distinctly elevated LVEDd and LVESd in comparison with the normal group ($p<0.05$), indicating that the models are successfully established (Figure 1).

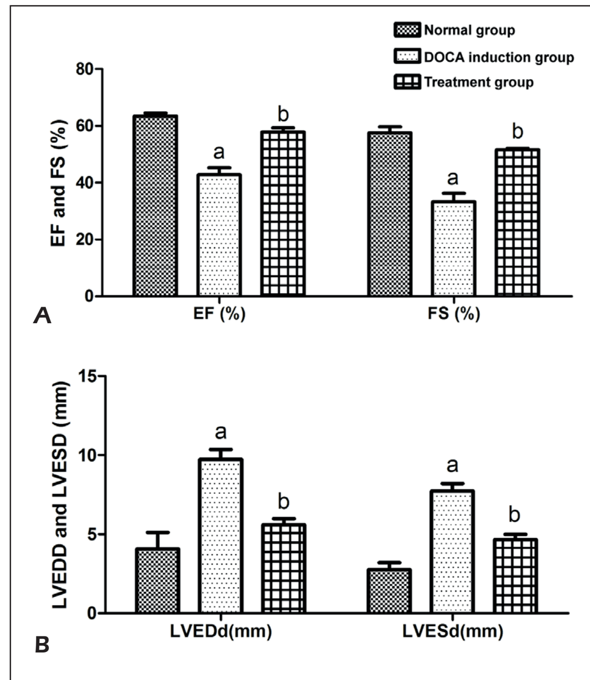

Figure 1. Rat cardiac function indexes detected via MRI and ECG. The FS and EF are significantly lower in DOCA induction group than those in the normal group, while the LVEDd and LVESd are notably higher in DOCA induction group than those in the normal group. **A**, $p<0.05$ *vs.* normal group. **B**, $p<0.05$ *vs.* DOCA induction group.

Myocardial Function and Liver Function Detected

The detection results of liver function indexes ALT and ALP and myocardial function indicator CK-MB (Figure 2) revealed that the levels of ALP, ALT, and CK-MB were clearly higher in DOCA induction group than those in the normal group ($p<0.05$), and they were evidently decreased in the treatment group ($p<0.05$), implying that the liver function and myocardial function indexes will dramatically increase during the development and progression of DOCA-induced myocardial fibrosis, which provides an important reference for early diagnosis.

Cytokines in Each Group Detected

In this report, the levels of the inflammatory factors NF-γ, interleukin-6 (IL-6), and IL-1β were measured. It was found that the levels of these three indexes were clearly higher in the DOCA in-

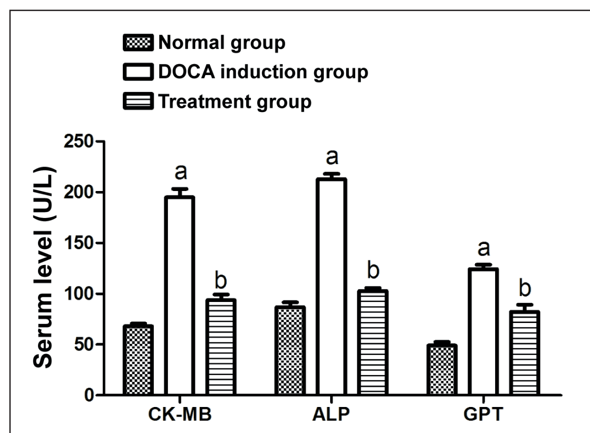

Figure 2. Biochemical results. The content of ALP, ALT, and CK-MB remarkably declines in the treatment group, indicating that the liver function and myocardial function indicators are abnormal. **A**, $p<0.05$ *vs.* normal group. **B**, $p<0.05$ *vs.* DOCA induction group.

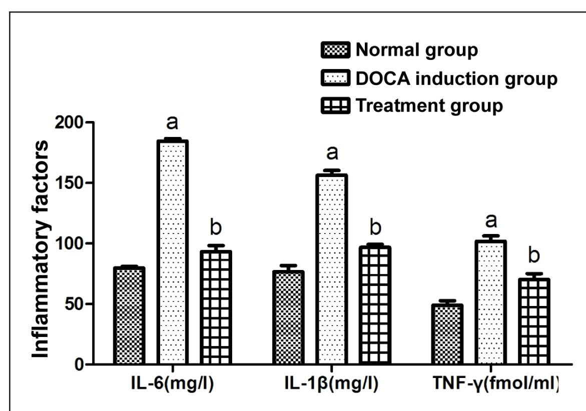

Figure 3. Content of the inflammatory factors. The levels of TNF-γ, IL-6, and IL-1β are significantly raised in DOCA induction group but distinctly decline in the treatment group.

duction group than those in the other two groups, and they were overtly lowered in the treatment group (p<0.05), suggesting that a large number of inflammatory factors are produced in case of myocardial fibrosis, which further indicates the progression of myocardial fibrosis (Figure 3).

Results of HE Staining

The results of HE staining (Figure 4) showed that in the DOCA induction group, the structure of the myocardial cells was disordered, and the muscle fibers were thickened (Figure 4A), while the myocardial injury after ischemia-reperfusion was alleviated after the treatment with imatinib (Figure 4B).

Apoptosis-Related and Pathway-Related Gene Expressions Detected via qRT-PCR

Based on the results of the qRT-PCR assay shown in Figure 5, the levels of Collagen, Chek1, α-SMA, p38 MAPK, and JNK were distinctly lowered in the treatment group (p<0.05), but they were overtly elevated in the DOCA induction group, suggesting that the treatment of imatinib suppresses the expression of myocardial fibrosis-related molecules and the development of severer myocardial fibrosis.

Pathway-Related Protein Expression Determined Through Western Blotting

The protein results are shown in Figure 6. The protein expression level of p38 MAPK was remarkably downward in the treatment group (p<0.05), while that in the myocardial fibrosis tissues in the DOCA inducting group showed an opposite tendency, implying that the treatment with imatinib promotes the repair of injury.

Discussion

In this investigation, the myocardial fibrosis was induced by DOCA in rats, and whether there was myocardial fibrosis-induced injury was further observed. The pathogenesis of myocardial fibrosis is explored to find potential treatment methods. Myocardial fibrosis in rats has a similar physiological process to that in humans so that rats can serve as study subjects as well. In this work, it was found that the FS and EF in DOCA induction group were significantly lower than those in the normal group, while the LVEDd and LVESd were evidently raised. The results of the HE staining revealed that the thickening of the cardiac muscle fibers and fibrosis of myocardial cells were relatively evident in DOCA induction group. These results indicated that the animals used in this investigation were appropriate.

The repair for myocardial fibrosis easily occurs in its early stage, which maintains the structural integrity of the necrotic area by forming connective tissue scars. However, in the long term, the

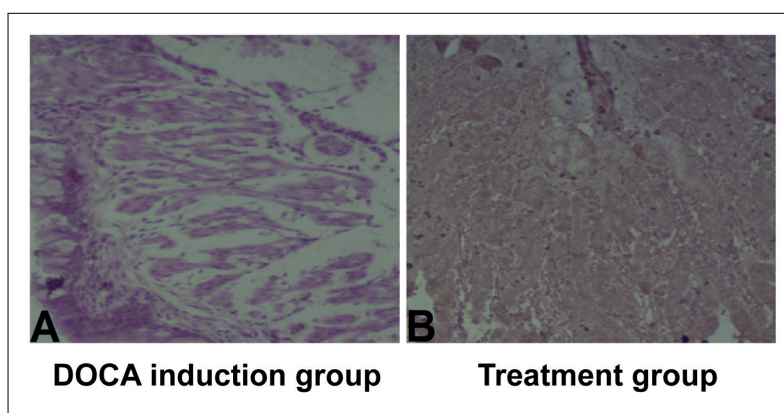

Figure 4. HE staining of rat heart. **A**, DOCA induction group (×10). **B**, Treatment group (×10). DOCA induction group has disordered the structure of myocardial cells and thickened the muscle fibers, and the treatment group displays almost undetectable myocardial injury.

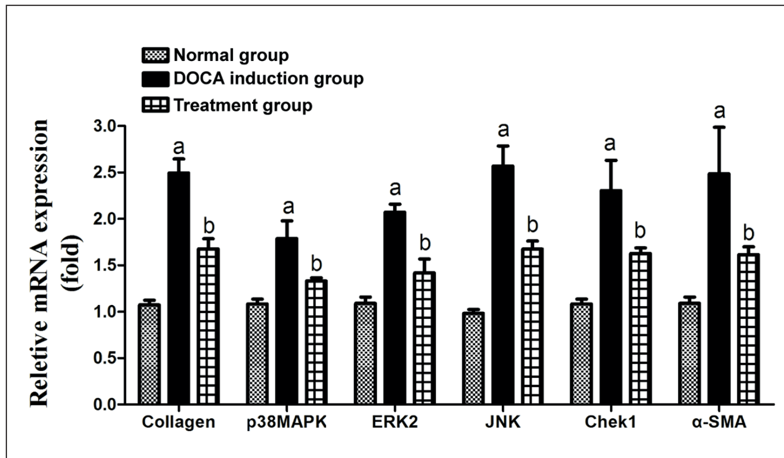

Figure 5. Expression levels of genes. The levels of Collagen, Chek1, α-SMA, p38 MAPK, and JNK are markedly reduced in the treatment group but elevated in the DOCA induction group. [a]$p<0.05$ *vs*. normal group, and [b]$p<0.05$ *vs*. DOCA induction group.

continuous remodeling, due to myocardial fibrosis is able to lead to progressive ventricular dilatation, decreased cardiac function, and chronic heart failure[17]. During fibrosis, the accumulation of collagen materials away from the infarct site results in downward myocardial elasticity and impaired contractility. The normal structure of the heart is composed of several different types of cells like myocardial cells, fibroblasts, extracellular matrix (ECM), including collagens[18]. The excessive accumulation of ECM is an important pathological change in heart diseases. Collagen is a major component of ECM, acts as an important participant in supporting and protecting myocardial cells and maintains the normal structural and functional components of the myocardial tissues[19]. Cardiomyopathy has very complicated pathogenesis, which is related to the development of hypertension and coronary artery disease. Oxidative stress, inflammatory factors, and growth factors play key roles in the development and progression of cardiomyopathy. Interstitial fibrosis refers to excessive deposition of ECM in tissues[19], and normal cardiac ECM maintains the homeostasis between matrix protein synthesis and degradation[20]. The sustained acute and cumulative long-term changes impair heart structure and function. These pathways trigger oxidative stress and inflammation, weakening the integrity of the cardiovascular wall, and facilitating the progression of myocardial fibrosis[21]. Currently, studying the specific mechanisms of myocardial fibrosis may be conductive to understanding the pathogenesis and treatment mechanism of the disease, providing theoretical support for subsequent studies on myocardial ischemia-related diseases. Inflammation impairs the integrity of cell membrane and results in the release of intracellular myocardial enzymes such as LDH and CK-MB in extracellular fluid[22]. In this analysis, the liver function and the myocardial function indexes including CK-MB were measured, and the results showed that the serum ALP, ALT, and CK-MB levels were markedly higher in DOCA induction group than

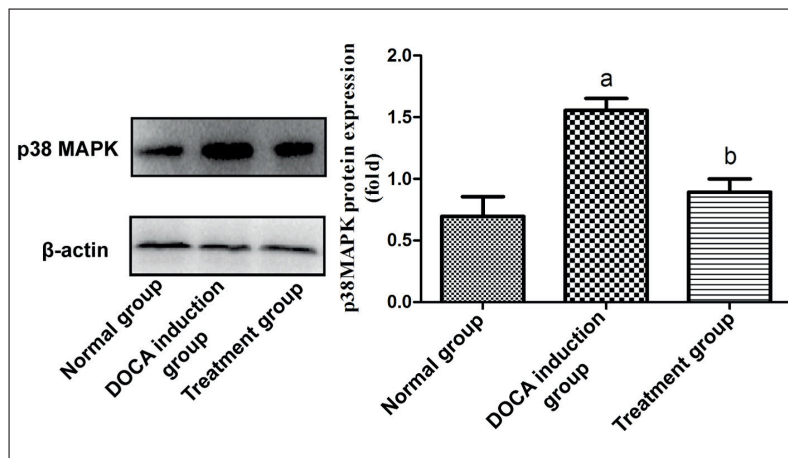

Figure 6. Protein expression. The treatment group exhibits a remarkably declined protein expression level of p38 MAPK ($p<0.05$). [a]$p<0.05$ *vs*. normal group, and [b]$p<0.05$ *vs* DOCA induction group.

those in the normal group, suggesting that the liver function and myocardial function indexes display evident upward trends in the development and progression of myocardial fibrosis, which provides an important reference for early diagnosis. Furthermore, the rheology of the heart manifested that the myocardial ischemia-reperfusion models were successfully selected. Moreover, the HE results demonstrated that DOCA resulted in disordered myocardial cells and thickened muscle fibers, which is in line with the results of the biochemical index test, implying that imatinib is capable of alleviating myocardial injury.

TNF-γ and IL-6, pro-inflammatory cytokines, participate in myocardial fibrosis-related injury. TNF-γ increases the inflammatory cascade by elevating the expression of other pro-inflammatory cytokines, including ILs[23]. Besides, TNF-γ is able to induce myocardial apoptosis and ventricular remodeling[24]. Some reports have manifested that myocardial fibrosis is involved in both inflammatory reactions and gene activation in cells. TNF-γ is indispensable for the progression of inflammation in myocardial rats. IL-6 is capable of resulting in the excessive production of other inflammatory mediators[25]. We found that the content of IL-6, IL-1β, and TNF-γ was increased in the DOCA induction group, indicating that the elevated levels of IL-6 and TNF-γ further promote the progression of myocardial fibrosis and aggravate the inflammatory response. Such content was decreased after treatment with imatinib, suggesting that the disease is mitigated after treatment with imatinib. This implies that imatinib achieves good efficacy in treating myocardial fibrosis. The results of this work are consistent with the findings of the above previous studies. It shows that imatinib can prevent irreversible damage to cells caused by its excessive production and represses the damage, due to inflammation. Myocardial fibrosis causes heart failure, enhances left ventricular stiffness, and declines the ventricular wall compliance, thereby resulting in systolic dysfunction, especially diastolic dysfunction[26]. Excessive ECM caused by an imbalance between synthesis and degradation exerts an important function in heart failure. Collagen has good toughness and keeps the elasticity of the heart[27]. Therefore, the excessive deposition of Collagen destroys the structure of the heart, thus giving rise to cardiac dysfunction. In addition, Chek1 and α-SMA play important roles in myocardial fibrosis. Chek1 is a key molecule for the regulation of the mitotic proliferation of cardiomyocytes, and α-SMA is an important component of cardiac fibroblast expression. In the case of myocardial fibrosis, its expression will be remarkably increased[28]. We discovered that Collagen, Chek1, and α-SMA were lowly expressed in the imatinib treatment group, while the expression levels of Collagen, Chek1, and α-SMA in the DOCA induction group were clearly raised and higher than those in the normal group, indicating that imatinib inhibits the expression of myocardial fibrosis-related molecules, which is consistent with the above findings.

The regulation mechanism on the severity of myocardial fibrosis remains unclear. The p38 MAPK signaling pathway is important for pancreatic fibrosis, tubulointerstitial fibrosis, and fibronectin expression in human peritoneal mesothelioma cells. Recently, Liu et al[29] have reported that the p38 MAPK signaling pathway alters the development of myocardial fibrosis, forming feedback loop regulation. MAPK plays a part in many biological processes, including the migration and death of cells and the development of cardiomyopathy[30]. Its downstream JNK and p38 are stress protein kinases activated by stress stimuli. The activation of JNK and p38 triggers various cellular responses, like inflammatory responses and apoptosis. These changes may directly or indirectly activate the MAPK signaling pathway. Activated MAPK induces apoptosis and destroys the inflammatory state. Yu et al[15] have shown that suppressed p38/JNK maintains the cytoskeletal structure and protects against myocardial injury by relieving oxidative stress and inflammation. It can participate in modulating myocardial infarction and ventricular remodeling, which is an important signal transduction in myocardial regulation and plays a powerful role in the development and progression of myocardial fibrosis[14]. In this report, the expression levels of p38 MAPK and JNK genes were significantly decreased in the treatment group, and the protein detection results revealed that the content of MAPK was clearly decreased in the treatment group. The inhibition of MAPK and JNK expression after treatment with imatinib suppresses the development of myocardial fibrosis, which is in line with the results of other different drugs[31,32]. Therefore, imatinib is capable of mitigating DOCA-induced myocardial fibrosis by repressing the MAPK pathway. In short, we demonstrated that imatinib participated in the development of myocardial fibrosis *via* the p38 MAPK signaling pathway. Such an experimental result enriches and complements the theoretical basis of p38 MAPK in myocardial fibrosis

and treatment with imatinib. Subsequent molecular experiments such as immunofluorescence, flow cytometry, and EMSA can be conducted to study multiple aspects and perspectives, hoping to provide an important theoretical and experimental basis for following relevant research.

Conclusions

Based on a series of *in vivo* animal experiments and gene protein experiments in this study, we found that imatinib modulates the progression of DOCA-induced myocardial fibrosis in rats by repressing the p38 MAPK signaling pathway. Therefore, imatinib can be applied to regulate the progression of myocardial fibrosis. In summary, the results of this work provide experimental evidence and certain theoretical basis for the treatment of myocardial fibrosis and the role of p38 MAPK signaling pathway.

Conflict of Interests

The authors declared no that they have no conflict of interests.

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