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Fasudil protects against isoproterenol-induced myocardial infarction in mice *via* inhibiting Rho/ROCK signaling pathway

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Abstract. – OBJECTIVE: Myocardial infarction (MI) is one of the most common diseases in cardiovascular medicine, and the risk of MI is very serious. Therefore, the purpose of this study was to explore the effect of fasudil on isoproterenol (ISO)-induced MI in mice.

MATERIALS AND METHODS: Forty C57BL/6 mice were divided into four groups, namely, control group, MI group, low dose fasudil and MI treatment group (low fasudil group), high dose fasudil, and MI treatment Group (high fasudil group). MI group and the fasudil group were injected subcutaneously with ISO (85 mg/kg) twice, and every 24 h MI was induced. Low-dose and high-dose fasudil groups were treated with 3 mg/kg/day and 10 mg/kg/day for 4 weeks before the injection of ISO. Cardiac function measured in the fourth week after ISO injection, and body weight and whole heart weight were weighed. Infarct area and thickness were analyzed by HE staining. Besides, the degree of myocardial damage was measured by detecting serum CK and LDH, and excised heart tissue was detected by Real Time-Polymerase Chain Reaction (PCR) and Western blot.

RESULTS: In MI group, the cardiac function was significantly decreased: the left ventricular short axis shortening rate (FS) and left ventricular ejection fraction (EF) were significantly decreased, the left ventricular volume was significantly increased, and the myocardial injury markers CK and LDH were significantly increased. In addition, fasudil treatment significantly relieved heart function after MI in a dose-dependent manner, reducing cardiomyocytes oxidative damage, inhibiting apoptosis.

CONCLUSIONS: Fasudil can reduce ISO-induced MI, reducing cardiomyocytes oxidative damage, inhibiting apoptosis by inhibiting the Rho/ROCK signaling pathway.

Key Words:
Isoproterenol, Myocardial Infarction, Fasudil, Rho/ROCK.

Introduction

Myocardial infarction (MI) is the most common cause of heart failure in ischemic heart disease[1]. According to cardiovascular disease report, MI mortality rate is rising rapidly, and it is also one of the major diseases that threaten people's lives. It brings serious economic burden to families and society and has become a major public health problem[2]. MI is mainly caused by coronary ischemia, which leads to continuous ischemia and hypoxia of cardiomyocytes, thus triggering MI[3]. It is mainly involved in oxidative stress and inflammatory response, and continuous hypoxia of cardiomyocytes leads to impaired mitochondrial function. Besides, a large number of inflammatory cells are activated and accumulate in the infarct zone. Hypoxanthine and xanthine oxidase production increases and Ca^{2+} overload further stimulates oxidative stress and eventually leads to massive myocardial cell necrosis and apoptosis[4]. ISO is a synthetic catecholamine and adrenergic agonist that is reported to induce MI in rats[5]. Yu et al[6] found that rosuvastatin protects against MI induced by ISO in rats. Sharmila et al[7] confirmed that Diosmin prevents ISO-induced mitochondrial oxidative stress in rat hearts. Therefore, reducing oxidative stress, and reducing myocardial cell necrosis and apoptosis is of great significance for relieving ventricular remodeling, improving cardiac function, and inhibiting the development of heart failure.

Fasudil is a novel drug with a wide range of pharmacological effects. Its molecular structure is a 5-isoquinoline sulfonamide derivative, which is a Rho kinase inhibitor. Fasudil expands blood vessels by increasing the activity of myosin light chain phosphatase and reducing the tension of

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endothelial cells, thereby improving microcirculation[8]. At the same time, Xie et al[9] showed that fasudil has antagonistic inflammatory factors, protects cells, and inhibits apoptosis. Fasudil has a protective effect on the heart as it has protective effects on doxorubicin-induced dilated heart disease[10]. In addition, fasudil can improve heart function in patients with heart failure[11].

Rho GTPase is a small molecular weight binding protein with GTPase activity and belongs to the Ras superfamily protein. The Rho/ROCK pathway is associated with inflammatory responses, apoptosis, and oxidative stress. Previous research confirmed that Rho kinase plays an important role in regulating intracellular calmodulin and Ca^{2+} concentrations, while Ca^{2+} plays a role as a second messenger, which induces the excitatory-contraction coupling mechanism and excitatory-transcription mechanism of PASMCs, to regulate the contraction of pulmonary vessels and increase the expression of cytokines, which is directly related to blood vessel and cardiac remodeling[12]. In addition, the downstream molecule of Rho, Myosin phosphatase target subunit 1 (MYPT-1), is related to vascular and cardiac regulation. However, Rho/ROCK signaling pathway has little research on ISO-induced MI. Therefore, this report mainly discusses the effect of fasudil on ISO-induced MI in mice.

Materials and Methods

Experimental Animal

C57BL/6 mice (Fujian Medical University, Fujian), aged 6-8 weeks and weighing 25±2 g, were cultured at the Specific Pathogen Free (SPF) Experimental Animal Center of Fujian Medical University, with average temperature of 22±2°C, relative humidity of 60±10%, and regular illumination. The mice were fed with the pellets and drunk freely. Keep the cage clean every day. This investigation was approved by the Ethics Committee of Dongying People's Hospital Animal Center.

Building the MI Model

Forty mice were divided into control group, MI group, low fasudil group, and high fasudil group. Mice in MI group and fasudil group were injected subcutaneously with ISO (85 mg/kg) twice and induce MI every 24 h. Control group was injected subcutaneously with normal saline. Low fasudil group and high fasudil group were treated with fasudil 3 mg/kg/day and 10 mg/kg/day for 4 weeks before the injection of ISO. At the same time, control group and the MI group were treated with the same dose of normal saline.

Detecting Heart Function

In the fourth week after the injection of ISO, the cardiac function of the four groups of mice was examined. The cardiac function indexes, including left ventricular end-diastolic volume (EDV), left ventricular end-systolic volume (ESV), left ventricular end diastolic diameter (LVIDd), left ventricular systolic diameter (LVIDs), left ventricular mass (LV Mass), and calculated left ventricular short axis shortening rate (LVFS), left ventricular ejection fraction (LVEF) were detected by echocardiography.

Collecting Sample

Four groups of mice were weighed after measuring cardiac function and were sacrificed by intraperitoneal injection. Blood was collected from the abdominal aorta. The blood sample was naturally coagulated, centrifuged for 15 min, and the supernatant was stored at –20°C. Then, the heart tissue was separated, and the indwelling needle was inserted from the apex of the heart. At the same time, the right atrial appendage was cut with a surgical scissors, and perfused with physiological saline, the circulating blood was completely rinsed, and the heart of the mouse was cut from the aortic root to remove the surface water. Next, the heart weight (mg) was weighed, and the heart was placed in liquid nitrogen, stored at –80°C, and subjected to Western blot, real-time PCR, and section staining analysis.

Hematoxylin-Eosin Staining (HE staining)

Free mouse heart tissue was fixed with 4% paraformaldehyde (Fujian Medical University, Fujian). First, the heart tissue was dehydrated with alcohol and xylene, embedded in paraffin, and sliced with a microtome. Finally, the sections were sealed by HE staining, and then observed under a microscope, and images were collected.

Detect Myocardial Damage: Creatine Kinase (CK), Lactate Dehydrogenase (LDH), Superoxide Dismutase (SOD) and Malondialdehyde (MDA)

The collected mouse serum was collected, and the supernatant liquid was collected after centrifugation. The working solution (Jiancheng, Nanjing, China) was dispensed and mixed with an appro-

priate amount of the upper layer liquid, and the absorbance was measured at a wavelength of 450 nm using a microplate reader. Finally, calculation was conducted according to the standard curve.

Western Blot

Protein was extracted from cardiac tissue, and the protein concentration was measured by bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA) and stored at –80°C. An equal amount of protein sample was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitro membrane. After blocking with 5% skim milk for 2 h, protein was incubated overnight at 4°C with diluted specific antibody. Next day, the membrane was washed three times with Phosphate-Buffered Saline and Tween-20 (PBST) for 10 min each time, and coupled with horseradish peroxidase (HRP) with the corresponding secondary antibody, followed by incubation for 1 h and washing with PBST and observed in an enhanced chemiluminescence (ECL) system. Specific antibody: Collagen I (1:1000, Abcam, Cambridge, MA, USA, Rabbit). SOD1 (1:2000, Abcam, Cambridge, MA, USA, Rabbit), SOD2 (1:2000, Abcam, Cambridge, MA, USA, Rabbit), Caspase3 (1:3000, Abcam, Cambridge, MA, USA, Rabbit), Caspase9 (1:3000, Abcam, Cambridge, MA, USA, Rabbit), Bcl-2 (1:2000, Abcam, Cambridge, MA, USA, Mouse), Bax (1:1000, Abcam, Cambridge, MA, USA, Mouse), ROCK1 (1:2000, Abcam, Cambridge, MA, USA, Mouse), ROCK2 (1:2000, Abcam, Cambridge, MA, USA, Mouse), MYPT-1 (1:1000, Abcam, Cambridge, MA, USA, Rabbit), and GAPDH (US 1:1000 CST, Danvers, MA, USA).

Enzyme-Linked Immunosorbent Assay (ELISA)

Mouse serum and heart tissue were taken. The serum was centrifuged, and the supernatant was extracted. An appropriate amount of heart tissue was taken in the Eppendorf (EP, Hamburg, Germany) tube, PBS, and steel balls were added, and the supernatant was collected by homogenization for 10 min. After that, the absorbance (OD value) was measured at a wavelength of 450 nm using a commercial kit (Jianglai, Shanghai, China), as prompted by the instructions. The standard product concentration was plotted on the abscissa, and the optical density (OD) value was plotted on the ordinate. Finally, the sample concentration was calculated after the standard curve was drawn.

RNA Isolation and Quantitative Real Time-PCR

A small amount of heart tissue was taken from an enzyme-free EP tube, and RNA was extracted using an RNA kit (Thermo Fisher Scientific, Waltham, MA, USA). Then, RNA was dissolved in water without ribonuclease. Then, the RNA concentration was measured by a calculation analyzer, and the absorbance at 260 nm, 280 nm, and 320 nm was measured. If the A260 / A280 was between 1.8 and 2.1, the RNA quality was considered standard quality and can be used in subsequent experiments.

MRNA quantitative analysis was achieved using Prism 7300 Sequence Detection System, 25 μL reaction System was used including SYBR Green (12.5 μL; Thermo Fisher Scientific, Waltham, MA, USA), 1 μL of primers (0.5 mL each from the stock, Thermo Fisher Scientific, Waltham, MA, USA), 10.5 μL of water and 0.5 μL of template. PCR conditions: 10 min denaturation at 95°C, 40 cycles of denaturation at 95°C for 15s, 60°C annealing for 30 s and 72°C extension for 30 s. The data was analyzed by SDS software and the results were then output to EXCEL for further analysis. U6 was used as the internal reference for miR-383-5p, and GAPDH as the internal reference for the other mRNAs. Finally, the fold amplification was calculated using the $2^{-\Delta\Delta Ct}$ method, and the comparative threshold cycle (Ct) was compared. All the primers were listed in Table I.

Statistical Analysis

All statistical results were presented as mean ±SD (standard deviation). GraphPad Prism 5 Software (La Jolla, CA, USA) was used. Student's *t*-test was used to analyze the comparison between the two groups. *p*-values less than 0.05 were considered statistically significant results.

Results

Fasudil Relieves Structural Changes in the Heart Caused by ISO-Induced MI

By HE staining, compared with control group, the myocardial fibrosis was evident in MI group, the cell arrangement was disordered, the cell gap was enlarged, but the myocardial fibrosis was remarkably reduced in high fasudil group, and the cells were arranged neatly. The difference in low fasudil group was not significant (Figure 1A). In addition, Western blot

Table I. Real time PCR primers.

Gene name	Forward (5′ > 3′)	Reverse (5′ > 3′)
Bax	CAGTTGAAGTTGCCATCAGC	CAGTTGAAGTTACCATCAGC
Bcl-2	GACTGAGTACCTGAACCGGCATC	CTGAGCAGCGTCTTCAGAGACA
Caspase3	TGGAACAAATGGACCTGTTGACC	AGGACTCAAATTCTGTTGCCACC
Caspase9	GGCTGTTAAACCCCTAGACCA	TGACGGGTCCAGCTTCACTA
Collagen I	TCTCCACTCTTCTAGTTCCT	TTGGGT CATTTCCACATGC
SOD1	GGTGAACCAGTTGTGTTGTC	CCGTCCTTTCCAGCAGTC
SOD2	CAGACCTGCCTTACGACTATGG	CTCGGTGGCGTTGAGATTGTT
ROCK1	AGATATGGCAAACAGGATT	CTTCACAAGATGAGGCAC
ROCK2	CTAGAGTGCCGTAGATGCCA	GGTTCTAGGGGATGATCGGG
MYPT-1	GACCAAGGTGAAGTTCGACGA	ACATTGGCGTAATTGATGTCGG
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

RT-PCR, quantitative Reverse Transcription-Polymerase Chain Reaction.

and Real-time PCR confirmed the protein and mRNA expression levels of Collagen I (Figure 1B-1D), which in MI group were also remarkably increased compared with control group. This suggests that fasudil treatment can effectively alleviate cardiac structural disorders and inhibit the expression of Collagen I.

Fasudil Relieves Cardiac Function Changes Caused by ISO-Induced MI

Echocardiography was used to examine the cardiac function of each group. The left ventricular diameter and volume of MI group were significantly increased, while EF and FS were significantly reduced, compared with control group. EF and FS of low fasudil group were also significantly lower but were significantly elevated in high fasudil group compared with MI group (Figure 2A, 2B). Compared with control group, the weight and heart mass of mice in MI group increased, and the HW/BW ratio increased significantly. Fasudil treatment could inhibit the weight and heart mass increased of mice after MI and inhibited the increase of HW/BW ratio in a

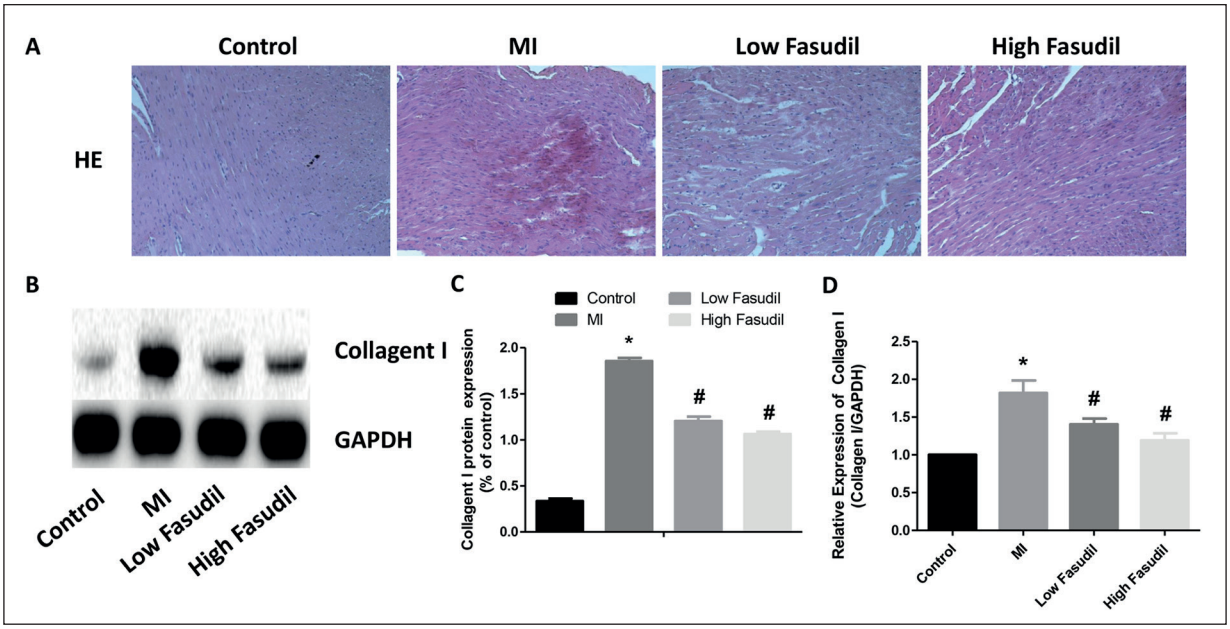

Figure 1. Fasudil relieves structural changes in the heart caused by ISO-induced myocardial infarction. **A,** HE staining (magnification: 200×. **B, C,** Western blot detects Collagen I expression. GAPDH is used as an internal control. **D,** Real Time-PCR is used to detect Collagen I mRNA expression. ("*" indicates statistical difference from the control group $p<0.05$, "#" indicates statistical difference from MI group $p<0.05$, "&" indicates statistical difference from the High fasudil group).

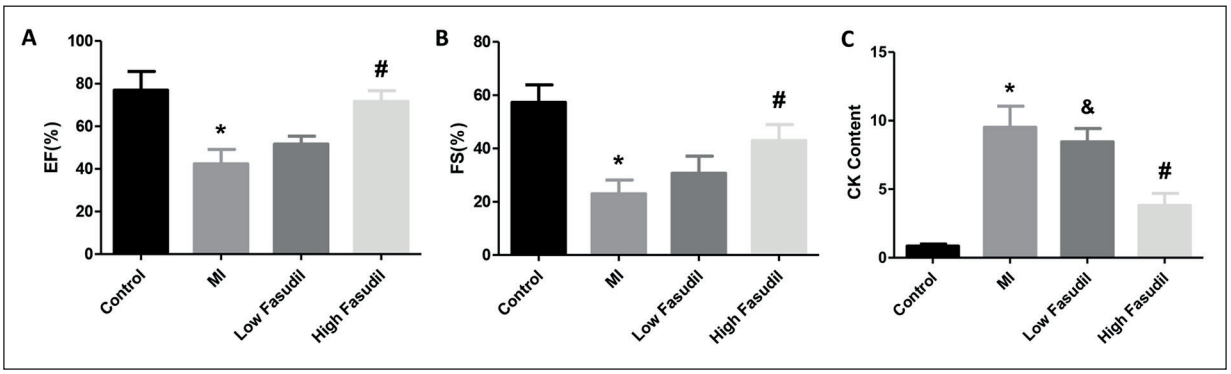

Figure 2. Fasudil relieves cardiac function changes induced by ISO-induced myocardial infarction. **A, B,** Two-dimensional ultrasonography is used to detect EF and FS values in each group of mice. **C,** Serum CK content of each group of mice. ("*" indicates statistical difference from the control group $p<0.05$, "#" indicates statistical difference from the MI group $p<0.05$, "&" indicates statistical difference from the High fasudil group).

dose-dependent manner (Table II). Moreover, the serum CK level test indicated that the CK content in MI group was significantly increased, and the CK content in low fasudil group was not significantly decreased, but in high fasudil group was significantly decreased (Figure 2C).

Fasudil Inhibits Oxidative Stress Caused by ISO-Induced MI

SOD1 and SOD2 expressions were detected by Western blot and Real time PCR (Figure 3A-3C). It was observed that the protein expression and mRNA content of SOD1 and SOD2 were significantly decreased in MI group. Compared with MI group, fasudil treatment promoted the expression of SOD1 and SOD2 significantly increased in a dose-dependent manner. Secondly, kits were used to detect the levels of MDA and LDH (Figure 3D, 3E). In MI group LDH and MDA were significantly increased, while the levels of MDA and LDH were significantly decreased in a dose-dependent manner after fasudil treatment. Serum SOD content was significantly inhibited in MI group, and fasudil could effectively promote the expression of SOD, which was positively correlated with the dose (Figure 3F).

Fasudil Inhibits Apoptosis Caused by ISO-Induced MI

First, apoptosis-related indicators, including Caspase3, Caspase9, Bax, and Bcl-2 were detected by Western blot (Figure 4A, 4B). It was found that apoptotic molecules such as Caspase3, Caspase9, and Bax were significantly elevated in MI group, while the anti-apoptotic molecule Bcl-2 was significantly inhibited. Low dose fasudil treatment could inhibit the expression of apoptotic proteins and promote Bcl-2 expression, while high dose fasudil treatment could promote Bcl-2 expression more significantly. At the same time, the content of Caspase3, Caspase9, Bax, and Bcl-2 mRNA was detected by Real-time PCR (Figure 4C-4F). The mRNA results also detected that fasudil could increase the expression of Bcl-2 in cardiomyocytes and inhibit the expressions of Caspase3, Caspase9, and Bax, thereby inhibiting cardiomyocyte apoptosis.

Fasudil Inhibits Activation of Rho/ROCK Pathway

The Rho/ROCK pathway regulates myocardium and blood vessels. By Western blotting (Figure 5A), it was found that ROCK1 and ROCK2 and its downstream molecule MYPT-1 were re-

Table II. Body weight, heart weight and heart specific gravity of each group of mice.

	Control	MI	Low Fasudil	High Fasudil
BW (g)	23.76 ± 1.62	26.27 ± 1.04*	25.83 ± 1.13$^{\#\&}$	24.63 ± 1.82$^{\#}$
HW (mg)	112.1 ± 7.8	144.5 ± 10.5*	136.1 ± 12.4$^{\#\&}$	128.2 ± 10.8$^{\#}$
HW/BW (mg/g)	4.70 ± 0.18	5.550 ± 0.27*	5.20 ± 0.41$^{\#\&}$	5.21 ± 0.29$^{\#}$

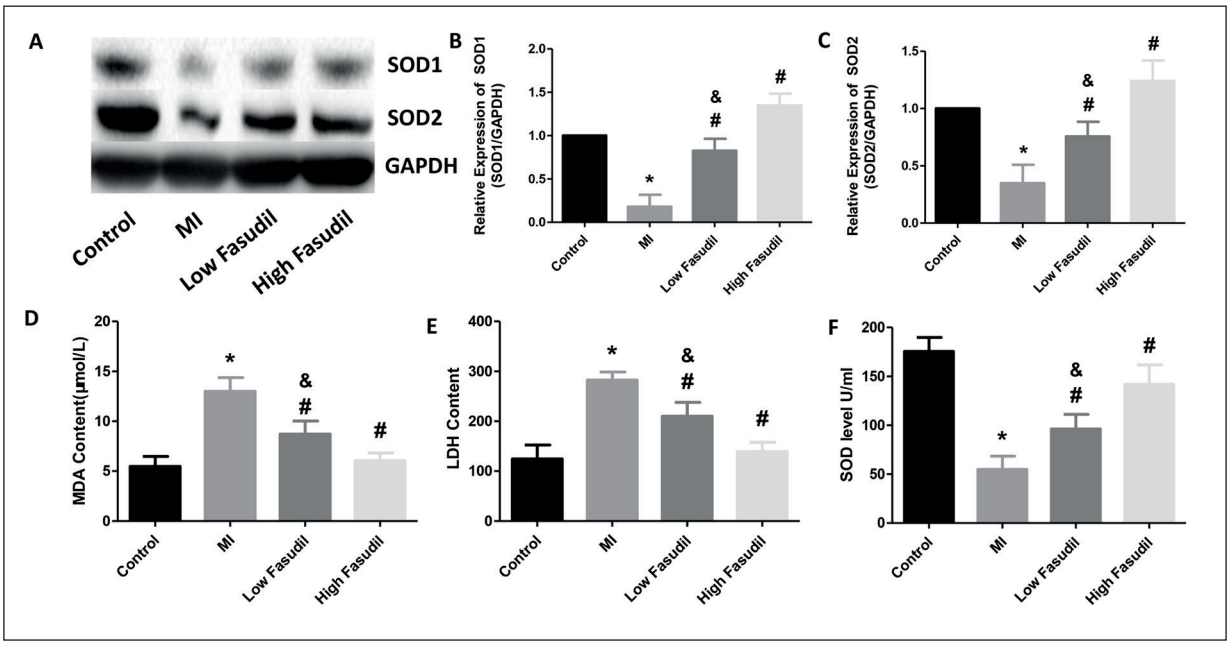

Figure 3. Fasudil inhibits oxidative stress induced by ISO in myocardial infarction. **A,** Western blotting detects the expressions of SOD1 and SOD2. GAPDH is used as an internal control. **B, C,** Real-time PCR is used to detect SOD1 and SOD2 mRNA expression. **D-F,** Serum SOD, MDA and LDH levels are measured in each group. ("*" indicates statistical difference from the control group $p<0.05$, "#" indicates statistical difference from the MI group $p<0.05$, "&" indicates statistical difference from the High fasudil group).

markably elevated in MI group, while in fasudil group, ROCK1, ROCK2, and MYPT-1 levels were effectively inhibited and dose dependent. Similar results were obtained for mRNA (Figure 5B-5D). ELISA results showed that compared with control group, the levels of ROCK1 and ROKC2 were significantly increased in MI group, and fasudil treatment was effective in inhibiting ROCK1 and ROCK2 expression and was positively correlated with the dose (Figure 5E, 5F).

Discussion

MI refers to the necrosis of cardiomyocytes caused by ischemia and hypoxia, which is one of the most important causes of death in patients with cardiovascular disease[13]. Acute myocardial infarction (AMI) has the characteristics of rapid onset, rapid development, and poor prognosis, which seriously threaten human health. Therefore, the occurrence and development of prevention and treatment of AMI is imminent, and it is urgent[14].

Echocardiography is often used clinically to aid in the diagnosis of cardiac function in patients[15]. In this study, the echocardiography showed that EF and FS in MI group were significantly decreased, and the left ventricular hypertrophy was evident. In the high fasudil group, EF and FS were significantly increased, and the ventricular structure was significantly improved, but the change in low fasudil group was not significant. This indicates that fasudil treatment can effectively relieve heart function caused by ISO-induced MI. HE staining showed that fasudil treatment can alleviate cardiac structural abnormalities caused by ISO-induced MI. In conclusion, fasudil treatment improved the structural and functional abnormalities of the heart caused by ISO-induced MI.

The diagnosis, intervention, and treatment of MI have always been the focus of global researchers. Previous studies have demonstrated that thrombopoietin can improve ventricular function in rats with MI, thereby regulating the remodeling gene[16]. In addition, Luo et al[17] found that synthetic mesenchymal stem cells were beneficial for the treatment of AMI. Moreover, McDonald et al[18] proposed that healin treatment can preserve myocardial function and reduce myocardial fibrosis in a mouse model of AMI. However, previous studies have shown little effect on fasudil in the treatment of ISO-induced MI, so it is speculated that fasudil can alleviate ISO-induced MI by inhibiting Rho/

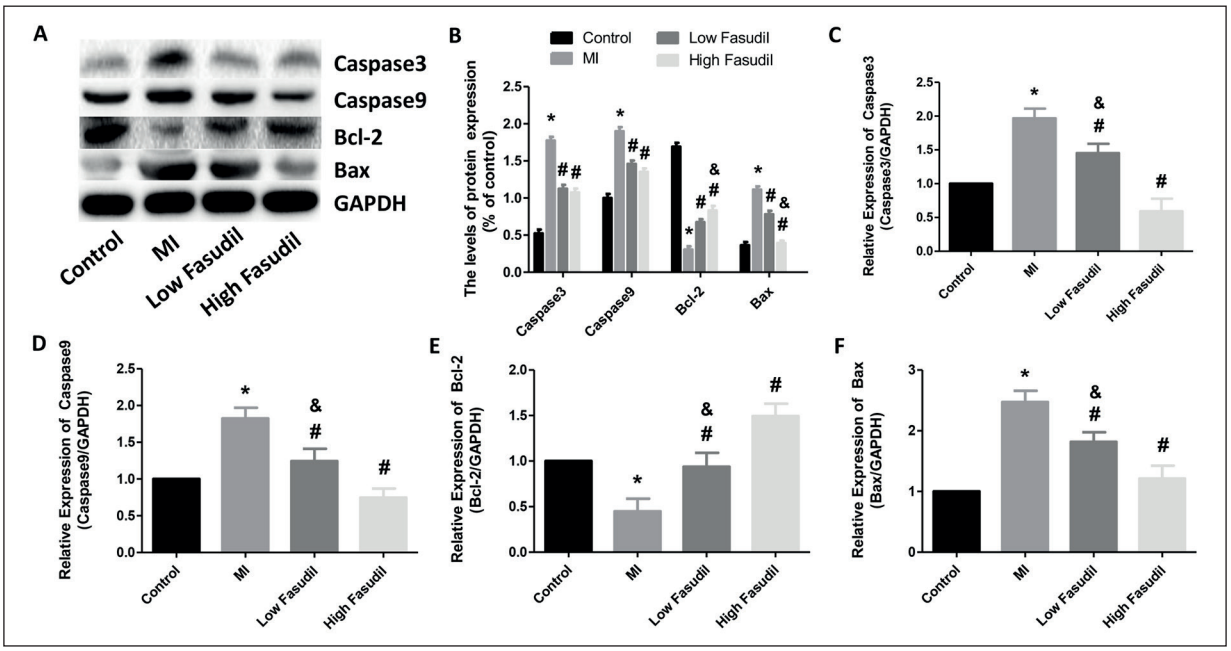

Figure 4. Fasudil inhibits ISO-induced apoptosis induced by myocardial infarction. **A, B,** Western blot is used to detect the expressions of Caspase3, Caspase9, Bcl-2, and Bax. GAPDH is used as an internal control. **C-F,** Real-time PCR is used to detect the expressions of Caspase3, Caspase9, Bcl-2, and Bax mRNA. ("*" indicates statistical difference from the control group $p<0.05$, "#" indicates statistical difference from the MI group $p<0.05$, "&" indicates statistical difference from the High fasudil group).

ROCK signaling pathway, inhibit oxidative stress, thereby improving cardiac function and preventing cells apoptosis.

To investigate the effect of fasudil on ISO-induced MI, four groups, namely, control group, ISO group, low fasudil group and high fasudil

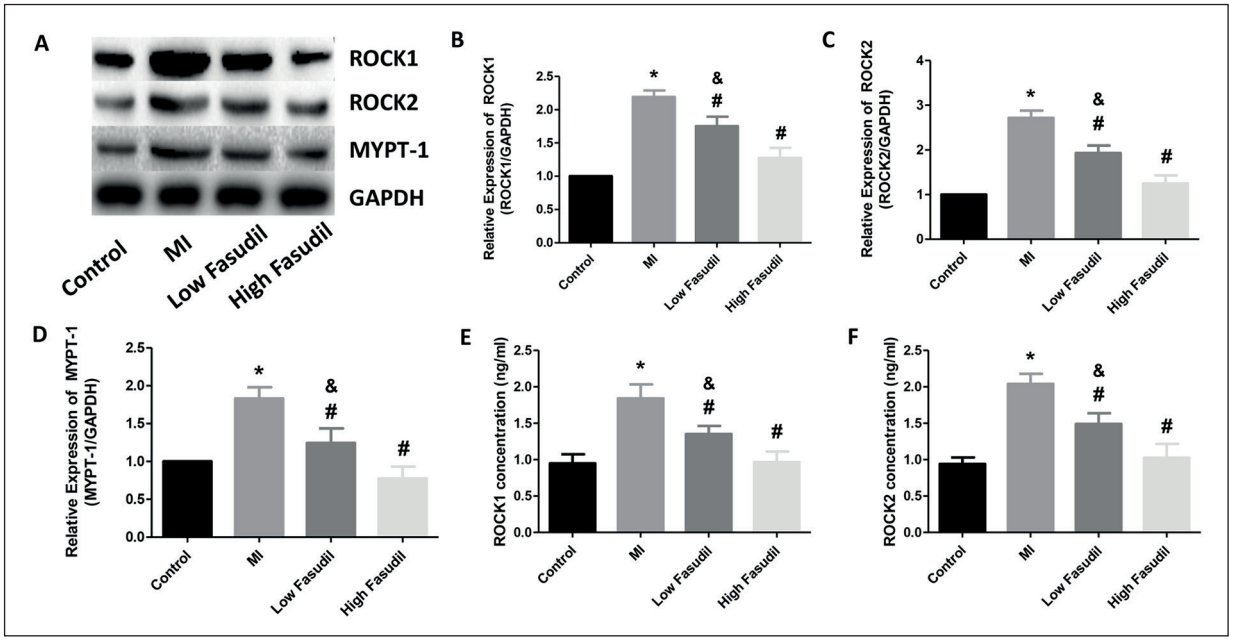

Figure 5. Fasudil inhibits activation of Rho/ROCK signaling pathway. **A,** Western blot is used to detect ROCK1, ROCK2, and MYPT-1 expressions. GAPDH is used as an internal control. **B-D,** Real-time PCR is used to detect ROCK1, ROCK2, and MYPT-1 mRNA expressions. **E, F,** Elias detect ROCK1 and ROCK2 levels. ("*" indicates statistical difference from the control group $p<0.05$, "#" indicates statistical difference from the MI group $p<0.05$, "&" indicates statistical difference from the High fasudil group).

group were set up. Firstly, echocardiography was used to detect the EF and FS values of ISO group, and HE staining showed that our modeling was successful. Second, we validated molecules involved in oxidative stress and apoptosis by Western blot and Real-time PCR. In MI group, the expression of SOD1 and SOD2 was significantly inhibited, while the content of MDA and LDH was significantly increased. This indicates that the ISO-induced MI destroys the redox balance. However, the expression of SOD1 and SOD2 in low fasudil group and high fasudil group showed increased, and the contents of MDA and LDH were significantly decreased. This indicates that fasudil can increase the antioxidant capacity of cardiomyocytes. Furthermore, the expressions of Caspase3, Caspase9, and Bax in MI group were significantly increased, and the expression of Bcl-2 was inhibited. This demonstrates a significant increase in cardiomyocyte apoptosis after ISO treatment. In fasudil group, apoptosis protein was inhibited, and Bcl-2 expression was promoted in a dose-dependent manner. The results demonstrate that fasudil inhibits ISO-induced cardiomyocyte oxidative stress and apoptosis.

Krijnen et al[19] found that Rho/ROCK signaling can induce cardiomyocyte apoptosis by inhibiting flappase. Also, Choraniopoulos et al[20] found that Rho/ROCK signaling pathway was involved in NF-κB signaling pathway and regulated cardiomyocyte inflammatory responses. This study results showed that fasudil can significantly inhibit the Rho/ROCK signaling pathway. Besides, ISO-induced MI resulted in increased expression of ROCK1, ROCK2, and MYPT-1, while different concentrations of fasudil inhibited the downstream effector molecule of Rho and were positively correlated at dose.

Conclusions

In summary, fasudil inhibits ISO-induced MI, as well as inhibits redox imbalance and apoptosis in cardiomyocytes by inhibiting the Rho/ROCK signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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