Basic Science

The Effects of Fasudil at Different Doses on Acute Myocardial Infarction in Rats

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Purpose: To explore the effects of different doses of fasudil on cardiomyocytes in rats with acute myocardial infarction (AMI).

Methods: A model of rats experiencing AMI was randomly divided into control groups and fasudil treatment groups according to the different doses of fasudil. After four weeks, hemodynamic parameters were measured. Expression levels of Rho kinase mRNA by the reverse transcription polymerase chain reaction method and the expression levels of apoptosis related proteins, Bcl-2 and bax, were determined by the immunohistochemical method.

Results: In the model of AMI in rats, their hemodynamic deteriorated, and the expression level of the Rho kinase mRNA increased in the myocardial tissue; but the expression level of apoptosis-related protein bcl-2 decreased, and Bax increased ($p < 0.01$). After the administration of fasudil, hemodynamic levels improved ($p < 0.05$), expression levels of Rho kinase mRNA and Bax ($p < 0.01$) decreased, expression levels of bcl-2 increased, and with the added element of dosage increase, the effect was significant ($p < 0.05$).

Conclusions: By administration of different doses of fasudil, the expression level of Rho kinase in myocardial tissue decreased and apoptosis reduced in rats with AMI. Fasudil plays an important role in protecting ischemic myocardium and improving cardiac function post AMI in rats, the effects of which were enhanced as the dosage was increased.

Key Words: Apoptosis • Myocardial infarction • Rho kinase

INTRODUCTION

As the general standard of living has continued to improve, an ever-aging population has seen an annual increase in the incidences of coronary heart disease. Acute myocardial infarction (AMI) is a serious threat to human health because of its high morbidity and high mortality rates. Following myocardial infarction, left ventricular function is significantly affected by a left ventricular remodeling in the final progress of heart failure (HF), which has a serious impact on the patient's quality of life and a poor survival prognosis. Currently, there is no effective treatment for HF, so it is an important part of cardiovascular research to know how to effectively protect the viable myocardium, including prevention and reversal of ventricular remodeling after AMI. Studies have indicated that cardiomyocyte apoptosis is also an important form of cardiomyocyte death and played an important role[1] during the process of left ventricular remodeling after AMI. It is reported in some literature that the inhibition of myocardial apoptosis around the infarction area can significantly reduce infarct size.[2] Therefore, it is an important goal to prevent cardiomyocyte apoptosis after the myocardial infarction for prevention of ventricular remodeling, slowing the process of heart failure. Rho kinase is a new target for treatment of cardiovascular diseases, discovered in

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recent years, which possesses the signal transduction and molecular switching function of the signal peptide.[3] Preliminary animal experiments[4] have confirmed that the expression level of Rho kinase in myocardial cells that survival, increased and apoptosis is increased after myocardial infarction in the rats. The application of Rho-kinase, specific inhibitor-fasudil, can protect the ischemic myocardium and reduce the myocardial infarct size and it is related to the role of its anti-cardiomyocyte apoptosis. However, there have been no further studies on the appropriate dose of fasudil. This study shows that experimental evidence for clinical medicine can be provided by observing the efficacy of different doses of fasudil applied to acute myocardial infarction rats.

MATERIALS AND METHODS

Materials

Fasudil (2 ml: 30 mg) was purchased from Tianjin Red Sun Pharmaceutical Co., Ltd., RNA extraction kit and RT-PCR kit was purchased from the Bo Biological Engineering Co., Ltd. in Hangzhou, the DNA ladder kit was purchased from the Beijing BaiTektronix Biological Engineering Co., Ltd. Bax and Bcl-2 antibody was purchased from the Wuhan Boster Biological Engineering Co., Ltd.

Methods

Animal model and groups

Male Wistar rats (body weight 250-300 g) purchased from Qingdao City and the Drug Administration Institute were used in the experiments in a manner consistent with the literature,[5] and were modified to build the AMI model. Once significant ST-segment elevation in electrocardiogram (ECG) was noted, the myocardial wall was found to be off-white below the ligation, and myocardial wall motion hypomotility of the rats were observed, the AMI model was confirmed to have been built successfully. After the rats were given penicillin 200,000 U/d, via intramuscular injection (im) for 3 days, following a further 24 h interval, the survivors were randomly divided into group A (saline group, n = 12), group B, (a small dose fasudil group), group C (in medium dose fasudil group), and group D (high-dose fasudil group). Another randomly selected 10 rats were used as a sham group, namely group E, only threading in the left anterior descending artery ligation. Groups A and E were given normal saline 0.1 ml intraperitoneal injection twice daily; B, C, and D groups were given fasudil in twice daily doses of 1 mg/kg, 5 mg/kg, 20 mg/kg, respectively, by intraperitoneal injection.

Hemodynamics

The rats were weighed after 4 weeks and were anesthetized by 10% chloral hydrate. The right external carotid artery was separated, the distal end ligated, the proximal end was clipped by an artery clip, and an intravenous catheter (20 g) was inserted into the vascular centripetal direction of the artery clip. Thereafter, the metallic part of the needle was pulled out, leaving the plastic casing inside, and connected with the blood pressure transducer and the polygraph, a BL-420 biological and functional experimental system (Chengdu Thai Union Technology Co., Ltd.). It was necessary to fill the entire pipeline and blood pressure transducer with saline containing heparin, and the plastic casing was descended into the left ventricle whereafter measurement was taken of left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure LVEDP), and left ventricular pressure maximum rise and fall rate ($\pm$dp/dt max). The above statistics were continuously recorded and stable for 5 min, and the hemodynamics were recorded respectively at 5, 10, and 15 min. intervals, and the average from the 3 respective records was used as the experimental values for this experiment.

Determination of Rho-kinase mRNA expression

Total RNA was isolated from each rat specimen's frozen myocardial tissue (50 mg) using TRIzol reagent, according to the manufacturer's instructions. Total RNA was processed to cDNA by reverse transcription process, using the high-capacity cDNA reverse transcription kit (Applied Biosystems). The genes were amplified through the Taq enzyme and cDNA was used as the template. The specificity of PCR primers were designed and synthesized by the Shanghai Sangon Biological Engineering Technology and Service Co., Ltd. The sequences of β-actin (493 bp) primers were as follows: forward, 5-TGTTTGAGACCTTCAACACCCC-3, reverse, 5-ACGTCACACTTCATGATGGAATTGA-3. Primers for Rho kinase (297 bp) were as follows: forward primer,

5-GGTGATGGCTATTATGGACGAGA-3, reverse primer, 5-TCGGAGCGTTTCCCAAGC-3. The following conditions were created to amplify the gene, including initial denaturation at 94 °C for 3 min, denaturation at 94 °C for 30 s, annealing for 30 s (temperature Rho kinase 58 °C, beta-actin 55 °C), extension 72 °C for 45 s, 35 cycles, 72 °C terminal extension for 5 min to terminate the reaction. Then, we took 10 μl of the PCR product of 1% agarose gel electrophoresis. The relative expression level of Rho kinase mRNA was normalized to that of β-actin as an internal reference.

Observation of the expression of apoptosis-related proteins Bax and Bcl-2 using immunohistochemical method

All specimens were fixed in 10% formalin, paraffin-embedded, and serial 4-μm paraffin sections were dewaxed and rehydrated. Endogenous peroxidase activity was inhibited by incubation with 3% hydrogen peroxide. After blocking sections with 20% (v/v) goat serum in phosphate-buffered saline, sections were incubated overnight at 4 °C with Bax and Bcl-2 antibody agents. Sections were then incubated with the appropriate secondary antibodies. Positive areas were counted and expressed as a percentage of the myocardial tissue. A negative control, where the primary antibody was replaced with either mouse or rat IgG at the same dilution, was always included within the experiment.

In the positive areas, the Bax and Bcl-2 proteins were identified as yellow or brown particles, and diffused within a localized area in the membrane or the cytoplasm. All of the preceding steps were viewed under microscopy. Image acquisition was obtained by digital photomicrography, wherein each was sliced and randomly selected in 5 high power fields. Blinded analysis of positive immuno-stained sections was performed with an image-analysis program (Image Pro Plus, Media Cybernetics). This was used to calculate the sum of the positive points of the tissue slices of each field and integrated optical density values. The mean optical density value represents a semi-quantitative analysis of Bax, Bcl-2 protein expression intensity.

Statistical analysis

Data were processed using statistical software SPSS 17 and count data using the χ^2 test. The results are expressed as means ± SEM; t-test was used between the two groups, and ANOVA analysis q test was used for multiple comparisons between groups. Two groups were evaluated by t-test, and $p < 0.05$ was considered statistically significant.

RESULTS

Living conditions of rats

There were 48 rats that survived after AMI 24 h, and 10 died in the next four weeks during the intervention period, respectively. The deceased rats had been assigned to the following groups: 2 in group A, 3 in group B, 2 in group C, and 3 in group D; with mortality rates of 16.67%, 25%, 16.67%, and 25% respectively. Statistically, there was no difference. There was no death in the sham group, and the rats included in the final data analysis consisted of 10 in group A, 9 in group B, 10 in group C, 9 in group D and 10 in group E.

Comparison of hemodynamic

Compared with the sham group, rats with myocardial infarction LVSP and ±dp/dt max had significantly lower scores ($p < 0.01$). LVEDP was significantly increased ($p < 0.01$) compared with the group with application of saline myocardial infarction, the application of fasudil treatment with rats with LVSP and ±dp/dt max were significantly ($p < 0.01$) increased, and LVEDP was significantly lower ($p < 0.01$) in the group with a higher dose. Overall, there was a significant pronounced effect (Table 1).

Pathological changes

HE staining showed that the stripes of the left ventricular area were clearly visible, and myocardial fibers were arranged in neat rows; there was no granulation tissue and proliferation of fibrous tissue, and no inflammatory cell infiltration in the myocardial interstitial space in the sham group. Among the AMI groups, within the infarct area the myocardial fibers were dissolved, fractured, and myocardial stripes disappeared. Myocardial tissue was replaced by granulation tissue and proliferation of fibrous tissue, and the infarction area was infiltrated by a large number of inflammatory cells. The border of the infarction areas was composed of

Table 1. Comparison of hemodynamic parameters in 5 groups ($\bar{x} \pm s$)

Group	Number	LVSP (mmHg)	LVEDP (mmHg)	+dp/dt$_{max}$ (mmHg·s^{-1})	-dp/dt$_{max}$ (mmHg·s^{-1})
A	10	117.16 ± 6.45*	2.87 ± 1.61*	4753.10 ± 488.72*	-4757.25 ± 510.93*
B	9	128.52 ± 4.21*†	-3.56 ± 2.16*†	5660.92 ± 572.15*†	-5938.03 ± 576.45*†
C	10	140.19 ± 6.47*†	-6.14 ± 2.15*†‡	6468.63 ± 494.80*†‡	-6675.15 ± 629.62*†§
D	9	146.99 ± 3.91*$^{\dagger\#}$	-8.16 ± 2.32*†**	7283.22 ± 564.37*$^{\dagger\#}$	-7619.61 ± 472.23*$^{\dagger\#}$
E	10	159.31 ± 4.56	-10.78 ± 1.95	8901.98 ± 698.88	-8735.90 ± 785.20

* Compared with the sham operation group, $p < 0.01$; † Compared with group A, $p < 0.01$; group C compared with group B, ‡ $p < 0.01$, § $p < 0.05$; group D compared with group C, $^{\#}$ $p < 0.01$, ** $p < 0.05$.

myocardial fibers, fibrous tissue and granulation tissue. Myocardium fibers arranged in neat rows and myocardial stripes were clearly visible in the non-infarcted areas. The clear boundary between the infarction area and non-infarcted area indicated that the myocardial infarction model was a success (see Figure 1).

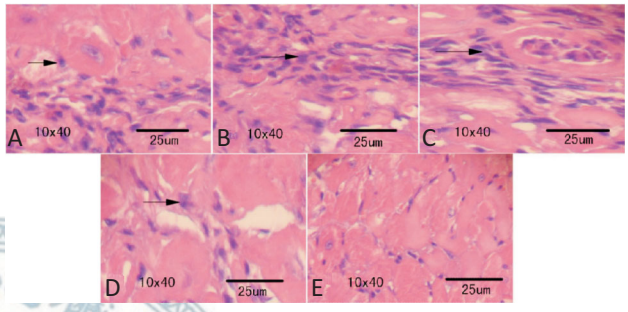

Figure 1. *HE staining, in groups A, B, C and D (the AMI group), there were a large number of inflammatory cells around the infarction area; in group E (the sham group), there was no inflammatory cell infiltration into the myocardial interstitial space.*

Expression changes of Rho kinase mRNA and Bcl-2 and Bax in myocardial cells

In a statistical overview of the readings on the Rho kinase mRNA and the expression levels in apoptosis-related protein Bax within rats with myocardial infarction compared with the sham cardiomyocytes, the readings were significantly increased and the expression levels of bcl-2 were significantly reduced ($p < 0.01$, $p < 0.05$). Compared with the saline control group, the administration of fasudil treatment of myocardial cells, Rho kinase mRNA and Bax expressions were all decreased ($p < 0.01$, $p < 0.05$). However, bcl-2 expression was increased ($p < 0.01$, $p < 0.05$) and the high dose group is more significant in Table 2, and Figures 2 and 3.

DISCUSSION

Rho/Rho kinase is widely distributed in mammalian tissues and cells and can interact with a variety of vasoactive substances, affecting the function and structure of the smooth muscle cells through the regulation of the myosin light chain phosphorylation and dephosphorylation processes. Furthermore, it is directly involved in the pathological process of cardiovascular disease and closely related to the development of cardiovascular disease.[6-8] Rho kinase inhibitor was applied to treat cardiovascular disease as a therapy for animals in experimental clinical trials and achieved impressive results.[9] In recent years, fasudil also is becoming more extensively used in the cardiovascular field, where it has been applied successfully in a significant number of cases. It is expected to become the new drug of choice for prevention and treatment for cardiovascular diseases.[10]

Incidences of AMI have increased annually, and the patients who suffer this medical event are becoming ever younger. However, with the development of medical technology, the mortality rate among patients in the acute phase has been greatly reduced. AMI is still a serious challenge in the treatment of cardiovascular disease,[11] with ongoing efforts to ascertain how to best maintain viable myocardium, reduce further loss of myocardium, prevent ventricular remodeling, and delay heart failure for such patients.

This study demonstrated that Rho kinase mRNA expression levels increased in myocardial tissue surrounding the infarct zone in rats which were 4 weeks into myocardial infarction. The LVSP and left ventricular pressure maximum rise and fall rate (±dp/dt max) were lowered, and the LVEDP rate increased ($p < 0.01$) when compared with the sham group. Continuous administration of fasudil treatment with different doses for 4

Table 2. Comparison of Rho mRNA kinase and the expression of Bcl-2 and Bax protein in myocardial ischemic tissue of rat in 5 groups ($\bar{x} \pm s$)

Group	Numbers	Rock	Bax	Bcl-2
A	10	0.18 ± 0.39*	2.25 ± 0.40*	0.51 ± 0.25*
B	9	1.36 ± 0.29*‡	1.82 ± 0.36*§	0.92 ± 0.35*§
C	10	1.04 ± 0.40*$^{‡\#}$	1.40 ± 0.40*$^{‡\#}$	1.40 ± 0.43*$^{‡\#}$
D	9	0.69 ± 0.18†‡**	0.97 ± 0.33*‡**	1.83 ± 0.49*‡**
E	10	0.33 ± 0.11	0.50 ± 0.30	2.95 ± 0.39

Compared with the group E, * $p < 0.01$, † $p < 0.05$; Compared with the group A, ‡ $p < 0.01$, § $p < 0.05$; group C compared with group B, $^{\#}$ $p < 0.05$; group D compared with group C, ** $p < 0.05$.

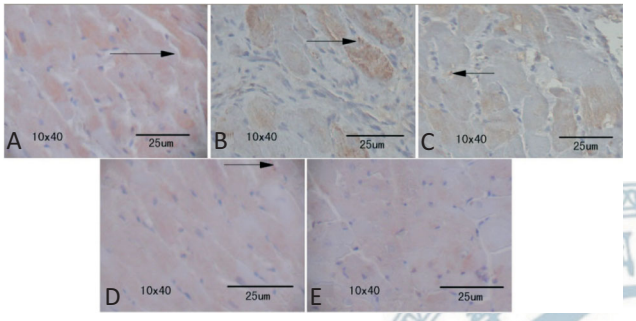

Figure 2. *Expression of Bax in immunohistochemistry staining: compared with E, the expression of Bax in A, B, C and D is increased, but with the increase of fasudil injection,the Expression of Bax dwindled.*

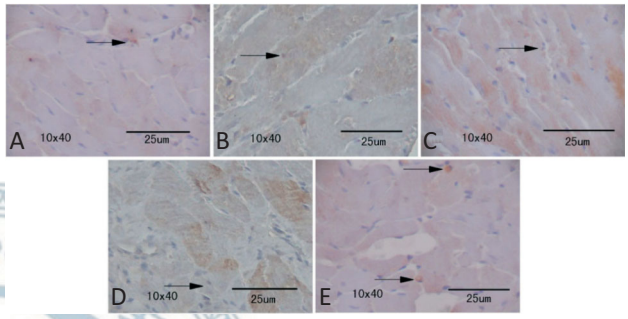

Figure 3. *Expression of Bcl-2 in immunohistochemistry staining: compared with E, the expression of Bcl-2 in A, B, C and D is decreased; but as fasudil injection doses increased, the expression of Bax is gradually increased.*

weeks after myocardial infarction showed reduced the levels of Rho kinase mRNA, lowered left ventricular end diastolic pressure, reduced left ventricular preload and improved cardiac function. These results were consistent with previous studies.[12-14]

This study further found that as fasudil doses increased, Rho kinase mRNA expression levels decreased in myocardial tissue, and there was a corresponding increase in the improvement of cardiac function. The difference was statistically significant.

In recent years, studies have shown that cardiomyocyte apoptosis is an important cause of death of myocardial cells, a significant independent factor influencing the infarction range, and plays an important role in ventricular remodeling and subsequent heart failure after AMI.[15,16]

Apoptosis is the process of cell death by initiating gene regulation in stimulating factors external to the cell, and a variety of other factors start the process of apoptosis by regulating the expression of apoptosis-related genes. Bcl-2 and Bax were recognized as closely apoptosis-related regulatory proteins. Bcl-2 is mainly distributed in the mitochondrial outer membrane and can regulate permeability of the cell. The main function of Bcl-2 is to promote cell survival and inhibit the apoptosis of Bax, which is located in the cytoplasm. When the expression of Bax is high, it can be translocated from the cytoplasm to the mitochondrial membrane and combine with Bcl-2 to form heterodimers. When the Bcl-2 is inactive, permeability of the mitochondrial membrane changes, which causes mitochondrial damage, and apoptosis increases within the myocardial tissue.[16,17] Studies have shown that Rho kinase is involved in all stages of apoptosis, and plays a key role in the development and progression of apoptosis,[18,19] although which specific mechanisms are involved remains unclear. During in vitro experiments conducted to prove the adenoviral over-expression of activated Rho A, it was shown that this over expression can lead to cardiac hypertrophy (targeted to Rho kinase) and caused cardiomyocyte apoptosis by regulation of the Bax mitochondrial pathway.[20] This study also found that the level of Bax in the ischemic myocardial tissue increased, compared with the sham group among the myocardial infarction rats. and there was also a reduction in bcl-2

expression levels as well. However, Bax expression levels decreased and bcl-2 expression levels increased after the injection of fasudil. These indicated that fasudil can protect myocardial cells and reduce apoptosis by affecting the expression of bcl-2 and bax, which was consistent with previous research results.[2,4,13] Further studies have also found that apoptosis was further reduced with the increase of fasudil dosage, and the difference was statistically significant in this experiment.

Concerning limitations of the study, the AMI model in rats was oversimplified. In addition to EKG and HE staining, the serum troponin T (I) levels need to be evaluated. Cardiac echocardiography on left ventricular function would be a good choice during post-surgical follow-up. TUNEL assay or other tests for detecting cell apoptosis around the infarct area should also be performed. Additionally, Western blot analyses for signaling proteins involved in the AMI-mediated apoptosis should be assessed. Finally, in order to most effectively explore Rho activity, PCR for m RNA expression is not sufficient, and a specific Pho activation assay would be important.

CONCLUSIONS

In summary, cardiomyocyte apoptosis plays an important role in the development of acute myocardial infarction in rats with heart failure. Rho kinase is involved in all stages of apoptosis, and at the same time apoptosis can also activate Rho kinase activity. This can increase cell damage, creating a vicious cycle, and this cycle is closely related to heart failure.[21,22] Fasudil, a Rho kinase inhibitor, can decrease apoptosis by inhibiting Rho kinase activity, protecting the ischemic myocardium and improving cardiac function. This beneficial effect increases with the amount of fasudil injected, and the difference (depending upon the amount administered) was statistically significant. The fasudil anti-apoptotic effects may be a target for new research in clinical drug treatments used in treating heart failure after an acute myocardial infarction. However, there are still several characteristics of fasudil use that require further investigation: its mechanism is not very clear, correct dosages need to be ascertained, and the potential for side effects should be clarified.

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