Fasudil on Myocardial Injury in Rats with Myocardial Ischemia and Reperfusion through Rho-ROCK Signal Pathway

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ABSTRACT

Cardiovascular diseases are very harmful to human life and health. Reperfusion therapy is a standard method to treat cardiovascular diseases and has achieved high clinical effects. However, this treatment method is likely to cause myocardial ischemia-reperfusion injury. It has been reported that the Rho kinase inhibitor fasudil can interfere with cardiomyocyte apoptosis through the Rho-ROCK signaling pathway, so it is often used to treat cardiovascular diseases. The essay aims to research this specific influence of fasudil on cardiac damage in myocardial ischemia-reperfusion mouses through the Rho-ROCK signal path and its related mechanisms. Forty rats were taken as the research object, and the mouses were separated into control clusters. In the observation cluster of fasudil, the rat heart device was perfused by surgery. The rat coronary artery was ligated for 20 minutes to make the rat myocardial ischemia. Then, the ligation was loosened for myocardial perfusion to create a rat myocardial ischemia-reperfusion model. Observation group rats were perfused with quantitative fasudil, 80 minutes after ischemia-reperfusion, the ultrastructural changes and myocardial ischemic area of the rat myocardium were observed under a microscope, and the dynamic changes of the mouse heart were examined by flow cytometry. The PCR fluorescence method was used to explore the outlook layer of Rho-ROCK kinase activity to detect rat cardiomyocyte apoptosis. It is shown that under this intervention of fasudil, this expression level of Rho-ROCK kinase activity in the observation group was reduced by 18.3%, the myocardial cell apoptosis rate was decreased by 26.4%, and one area of myocardial ischemia can be reduced by 32.5%. The ultrastructure of the new object in rats is improved, and the left ventricular diastolic and systolic effect is enhanced. Therefore, the fasudil may decrease cardiac ischemia and focus on injured Rho-ROCK signal path activity.

Introduction

Cardiac ischemia injection damage is a common pathophysiological phenomenon during acute myocardial infarction and reperfusion (1). Studies have found that the area of myocardial infarction continues to increase due to myocardial reperfusion, suggesting that myocardial reperfusion injury may be mediated by apoptosis and inflammation (2). How to reduce or eliminate damage has always been a hot spot in clinical research.

Studies have found that the Rho-ROCK route is an essential signal electron conduction pathway in the normal survival of colloidal cells (3). Various vascular neurons and nutrient metabolism factor receptors can inhibit the abnormal apoptosis of vascular cells by directly activating the signal synthesis pathway in the vascular Rho-ROCK, and can play a role as a cardiovascular health-protective agent (4). Experiments show that long-term use of ROCK inhibitors can effectively treat many related diseases. In clinical practice, fasudil can effectively treat coronary artery spasticity after coronary artery bypass graft surgery, and significantly upgrade the blood flow of this forearm in patients with heart failure (5).

To observe the particular influence of fasudil on cardiac ischemia-injection rat myocardial injury and related mechanisms through the Rho-ROCK signal route, multitudes of relevant materials became
questioned. In the midst of them, Ahmed et al. reviewed the pharmacological effects of fasudil, emphasized the medical application value of fasudil, and pointed out that fasudil has a good influence between therapy and cerebrovascular damage (6). Fu et al. introduced it in detail, and put up the relevance among Rho-ROCK signaling pathway and many diseases, including hypertension, myocardial infarction, and heart failure, the research status of ROCK signaling pathway and the current technical problems (7).

Shimokawa and Satoh pointed out in his article that fasudil is a new type of Rho-kinase receptor inhibitor, which can effectively block the activity in the pathway that inhibits the entire signal of Rho-ROCK, emphasizing the effect of high and low doses of fasudil, inhibitors are even said to be able to effectively block the pathway that exists in the all Rho-ROCK signal, thereby decreasing cell apoptosis, pointing out that this is also the principle of fasudil may have a lot of therapy (8).

Wei et al. depicted the damage; they noticed that nevertheless, the reperfusion cure is of great importance, the disease can be extremely simple to back (9). Sharanek et al. found through research that fasudil can effectively treat various chronic cardiovascular and cerebrovascular diseases by continuously inhibiting its signal synthesis pathway in Rho-ROCK, including acute atherosclerosis, coronary heart disease, and senile disease, cardio-cerebrovascular muscle spasm, ischemic reperfusion injury, hypertension, myocardial hypertension (10).

Among the study of the specific influences of fasudil onto myocardial injury among cardiac ischemia-injection rats pass the Rho-ROCK signal route and related mechanisms (11). What’s more, the essay is studying many innovations that have been made in content and detection methods (12). The specific innovations are as follows: First, this article considers the approach to research the project of Rho-ROCK signal pathway after fasudil drug intervention and uses SPT software to perform statistics on related detection results (13).

Data collection and analysis explored the membership between fasudil kinase inhibitor and Rho-ROCK signaling pathway based on scientific objectiveness. Secondly, use the prototype lab to take after the foundation of a rat model cardiomyocytes in the late ischemia-reperfusion myocardial injury inhibition model, to explore whether the inhibition of Rho-kinase can promote apoptosis inhibition in the late ischemia-reperfusion of rat cardiomyocytes (14). Function, and study whether the Rho kinase-specific apoptosis inhibitor fasudil model can effectively inhibit the acute apoptosis of cardiomyocytes and make it have the role of cardiomyocyte protector (13).

Therefore, in the current study, we tried to investigate the effect of fasudil on myocardial injury in rats with myocardial ischemia and reperfusion via the Rho-ROCK signal pathway.

Materials and methods

Lab animals and related equipment

At first, 40 male mice 220±30.0g were picked as the research objects. They were kept at 18-26°C and they were divided into capsules with the worldwide baseline mocking-dent animal rearing species. Explore the goodness of the mouths in front of the tests and rule out rats with ill health. Many rats were separated into other clusters and the fasudil observation group. The main equipment and reagents used in this test can be seen in Table 1.

| A                  | B                      | C                      |
|--------------------|------------------------|------------------------|
| DYY-8C electrophoresis instrument | Beijing Linyi Instrument Factory |                     |
| Electronic analytical balance | Olympus Japan |                     |
| Absolute ethanol | Jimi Electronic Technology Company |                     |
| DGB-20 electric drying oven | Caused by Jiangsu Feng Hua |                     |
| Low temperature refrigerator | Japan Sanyo |                     |
| Collagenase II | American SGH |                     |
| Trypsin | Gibco Corporation United States |                     |
| Fasudil Injection | Sailing Company |                     |
| Trypan blue | Caused by Jiangsu Feng Hua |                     |
| Sulfuric acid | Japan Sanwa Kimono |                     |
| Sodium chloride | American SGH |                     |
| Agarose | American Sigma firm |                     |
| Goat serum | American KPL firm |                     |
Myocardial ischemia reperfusion model

The rat myocardial ischemia-reperfusion model was established by balloon ligation. Weigh the animal anesthetized with 5% pentobarbital (4ml/kg), fix it on the back of the rat, perform an incision on the rat’s abdomen, insert the catheter to the heart until a characteristic left ventricular pressure wave appears, and then. It is connected to the pressure sensor and inputs the signal into the power lab physiological experiment system. Shave, disinfect the surgical area, quickly open the chest, and cut the 4th and 5th ribs to expose the heart. The pericardium was torn apart and the heart was gently squeezed, 2mm below the left ear and 0.6mm beside the pulmonary artery cone. The insertion depth is 1-1.8mm, the width is 3-5mm, and the large heart vein and high-pressure balloon are ligated. The pressure pump was quickly adjusted to 15ATM (1ATM=101.325kPa), and ST-segment elevation and/or t-wave elevation or inversion appeared in the ECG, indicating that the left anterior descending coronary artery was completely occluded. Gently lift the incised skin, return the heart to the chest cavity, and clamp the chest cavity with hemostats. After 30 minutes of ligation, the pressure of the pressure pump was quickly adjusted to 0 atm, and the improved ST-segment returned to normal or decreased by 40%, which was considered to be the successful reperfusion.

Medication method and specimen collection

After successful modeling, mice in the fasudil observation clusters were infused intra-peritoneally with fasudil hydrochloride injection 15 mg/kg for 80 minutes each time, and the control cluster could be infused with a consistent amount of general saline.

12h after the last administration, the rat was decapitated and put to death. The chest cavity was opened on ice, the rat myocardial tissue was taken out, the external tissue was removed, and then the heart mass was measured to calculate the cardiac hypertrophy index (HWI=heart mass/weight). Part of the left ventricular tissue is divided into two, one is fixed with 20% formaldehyde and after dehydration, and it is routinely embedded and sliced for myocardial tissue pathological examination and collagen area measurement. One part is fixed in 5% glutaraldehyde and routine after dehydration. Embedding is used to prepare ultra-thin sections, observe with an electron microscope, grind another part of the myocardium and ice cubes and homogenize, collect the supernatant after centrifugation at low temperature 3000RPM for 20 minutes, put it in liquid nitrogen and quick-frozen it in the refrigerator test at a high temperature of 60°C. Put a small amount of myocardial tissue into a sterilized RF-F mortar, then grind it into powder by adding liquid nitrogen, and then store it in a centrifuge tube of RNF. After rapid freezing, store liquid nitrogen in an ultra-low temperature refrigerator at 60°C for subsequent testing.

Microscopic observation of ultrastructural changes in rat myocardial tissue

We explore the pathological differences of rat myocardial tissue behind an electron lens and take the prepared adult rat new-type myocardial vascular tissue valve slices, the cells are conventionally stained, dehydrated and waxed, and then the cells are stained with HEE type staining. The H-Limp optical transmission microscope is used to continue to observe the rat myocardial valve tissue slices. Morphological and ultra-microstructure, use the ultra-thin structure microtome to make super-thin structure slices of 2μm and perform positioning observation. After positioning, continue to ultra-thin slices to obtain ultra-thin structural slices of 60 nm thickness, using triple acetic acid. After dyeing with uranyl dihydrogen and triple citrate hydrogen peroxide, continue to observe the changes in ultrastructure morphology under the h-7500 optical transmission electron microscope and take pictures at the same time for recording.

Myocardial ischemia area detection

At 12h after reperfusion, the rat ventricles were isolated from the removed specimens. Cut a slice about 1mm thick from the apex of the heart 2cm in the direction perpendicular to the left room long axis; after taking the picture, put 3g/L TTC staining for 10min at 30 °C. The black area indicates the area of myocardial ischemia and the infarct area. No coloring picture uses the image analysis system to measure and calculate the ischemic area according to the formula.

Cardiomyocyte apoptosis Detection

Immediately fix the central ventricular muscle of the free wall of the left ventricle in 5%
paraformaldehyde (pH 7.4) for 15 hours (60°C), and then fix it with 75% ethanol. Carry out conventional paraffin embedding and make a cross-section of the heart. Bake the slides in the oven and keep them at room temperature. Under a fluorescence microscope, all heart nuclei are blue at an excitation wavelength of 350 to 380 nm. Apoptosis-positive cardiomyocyte nuclei are green at excitation wavelengths from 470nm to 490nm. According to the distribution of apoptotic cells, four positive visual acuity images were taken from each slice, four times were randomly selected from each specimen slice, 300 times, and the number of apoptotic cells per 200 cells was calculated on average.

**Rho-ROCK signal pathway activity detection**

Calciol’s Rho-ROCK Activity Kit (Rock AK) was used to detect the Rho-ROCK activity of the sample. The method is as follows: each aliquot of supernatant containing 10g of protein is added to loops of the 93-flat disk, and everyone is pre-coated muscle Globulin phosphorylates the target subunit (MYPT1). The Rho-ROCK nutrition may phosphorylate the Thr696 location of MYPT1 after incubating at a high temperature of 300 degrees for 40 minutes. Next, a 200 bay horseradish peroxidase-conjugated anti-phosphorylated MYPT650 antibody was added and reacted at centigrade for 2 minutes. Measure it at 350nm situated in bookshelf, and calculate the motivation layer of the Rho-ROCK signal route according to the even loophole and the formula accepted.

**Results and discussion**

**Analysis of the detection results of Rho-ROCK signal pathway activity, myocardial ischemic area and cardiac capsule velocity under the action of fasudil**

The observation found after infusion of fasudil, the rats in the observation cluster increased their LUSP, LUEDP, and their heart rate slowed down, which were statistically different from the controllable cluster (p<0.05). Dim sum function indicators of the observation group were stable at each time, and the stability of heart function was bigger than the certain cluster. LUSP decreased after myocardial ischemia-reperfusion in the control group. LUEDP increased, and dip/dt decreased, which was statistically significant relative to the observation cluster (p<0.05). The rats in the observation cluster were perfused for 30 minutes with fasudil. The left ventricular systolic function was significantly improved after filtrate, which was longer than that of the control cluster, with statistical disparity (P<0.05). After the intervention of fasudil in the observation cluster, the LUSP and LUEDP increased, and the heart rate slowed down. The changes in cardiac function of the two groups are shown in Table 2.

| A    | B     | C      | D      | E      |
|------|-------|--------|--------|--------|
| CG   | 75.2±4.68 | 98.3±4.15 | 67.5±5.58 | 227.4±6.36 |
| OG   | 78.4±4.92 | 95.6±5.21 | 72.4±4.95 | 195.3±5.75 |

The results of the study showed that under the intervention of fasudil, the activity level of the Rho-ROCK signaling pathway in the observation group was significantly lower than that in the control group. The activity level of the Rho-ROCK signaling pathway in the cardiac problem of mice in the control group was (47.3±4.25). The activity level of the Rho-ROCK signal pathway in myocardial tissue was (26.7±3.68), and it has a disparity between the two clusters (P<0.05).

In the observation group, the activity level of the Rho-ROCK signal route decreased under the action of fasudil, indicating that fasudil can prohibit the expression of Rho-ROCK kinase protein. The study noticed that the apoptosis index of cardiomyocytes in the control cluster was (15.3±2.36), and that of the observation group was (11.4±1.86). Measuring the observation cluster and the rat cluster, the quick velocity of cardiomyocytes can be significantly reduced, indicating that the use of razonodil can directly inhibit the rat Rho-ROCK reflex signal to make its pathway activity significantly reduce and improve. Myocardial cells apoptosis rapidly when rats undergo myocardial ischemia and reperfusion. The results of the researchers show that fasudil therapy can effectively inhibit the pathway activity of r and Rho-ROCK in the signal, and it can also effectively reduce the cardiomyocyte nucleus in male rats during
myocardial ischemia and reperfusion. The death rate, the specific data is shown in Figure 1.

**Figure 1.** The effect of fasudil on Rho-ROCK signal route movement and cardiac unit apoptosis velocity during myocardial ischemia-reperfusion

Learned from the statistic in Figure 1, we can know that fasudil may inhibit the activity of the Rho-ROCK signaling pathway, and may efficiently decrease the myocardial unit apoptosis velocity among myocardial ischemia injection in rats. The observation group rats under the intervention of fasudil. The expression level of Rho-ROCK kinase activity can reduce to 18.3%, and the apoptotic velocity of cardiomyocytes was decreased to 26.4% linked to the control cluster.

The consequences of the observation depicted that the observation experiment cluster and the hospital cluster respectively weighted the total left room cross-sectional district and the ischemic danger district. The two percentages of the ischemic risk area and the total left ventricular cross-sectional area were the regional ischemic area and the risk of infarction. The two percentages of the area and regional ischemic risk area are the regional risk area after cardiac infarction. The two measurement combinations compare the ischemic area risk area and the difference is large without any statistical factual significance (p>0.05). it has zero apparent pale ischemic cardiac structure death district in the control cluster. The pale myocardial infarction death area was the most obvious in the ischemia-reperfusion group, and the pale myocardial infarction death area in the fasudil cluster was better than that of the other ischemia-reperfusion groups. Significantly reduced, the difference is large and there is no significant statistical significance (p<0.05). The fasudil results observed that the area of myocardial ischemic infarction in the control cluster could be also apparently decreased compared with other controls. The conclusions of the experiment found that Fasudil may effectively decrease the district of cardiac ischemia in mice by reducing the motivation of the Rho-ROCK signal route. The relevant statistic is depicted in Figure 2.

**Figure 2.** The effect of fasudil on the myocardial ischemic area during injection in rats by reducing the motivation of Rho-ROCK signal route

We can learn in Figure 2, we can see that fasudil may effectively decrease the cardiac ischemic district through injection in mice by decreasing the motivation of the Rho-ROCK signal route, and reducing the myocardial ischemic area by 32.5%.

**Analysis of the influence of fasudil on cardiac ischemia-injection damage through Rho-ROCK signaling pathway**

Electron microscopic observations depicted linked to the finding cluster. HE staining of myocardial tissue in the control group showed obvious structural disorder, focal necrosis, and adipose tissue infiltration between myocardial cells. Under the electron microscope, myocardial cells showed high edema. Abnormal myofibrils, loss of local muscle filaments, mitochondrial edema, deformation and partial membrane fusion, accompanied by the medulla, visible local fracture of myocardial tissue, part of myocardial fiber hypertrophy or atrophy, myocardial cell infiltration in adipose tissue, reducing myocardial electron microscope. There is mild edema of the cells below, and the mitochondria are reduced and deformed. Under the intervention of fasudil, the
pathological changes of myocardial tissue in the observation group were improved under the microscope. After 10 hours of reperfusion, it was observed that the hepatocyte cords in the control group were arranged disorderly, myocardial cells were swollen and vacuolar, and the interstitial myocardial tissue was changed. Narrow or disappear, visible inflammatory cell infiltration. The consequences of the learning depict that Fasudil may improve the ultrastructure of cardiac tissue through the Rho-ROCK signal route. The related statistic is depicted in Figure 3.

As we can see in Figure 3, Fasudil may improve the ultrastructure of myocardial tissue through this Rho-ROCK signaling pathway, reducing focal necrosis of myocardial tissue by 17.2%, reducing mitochondrial edema by 15.4%, and reducing myocardial fibrosis by 22.6%

The results of this paper show that the dose-dependent inhibitory effect of fasudil on the activity of the Rho-ROCK signaling route improves myocardial injury, amplifies the activity of MLCP, reduces the expression of P-MLC2V in the unit, and inhibits the activity of NOX2, as Rho-ROCK inhibitor of signal activity, fasudil agent blocks the phosphorylation of myosin-regulated light chain (MLC20) mediated by Rho-ROCK signaling pathway. Therefore, fasudil can be used to treat coronary artery spasm. Since the fasudil reaction blocks the phosphorylation of nuclear Mlc2v mediated by Rho-ROCK in the signal input pathway, calabash and cardio may likely be dephosphorylated through the active dephosphorylation reaction of nuclear mlc2v. Effectively inhibit the functional expression of I/nox2, thereby effectively protecting heart cells in rats from various oxidative stress damages during I/R. ROCK plays a role in myocardial I/R injury. In different animal models of I/R injury, the Rho-ROCK signaling pathway is harmful. The consequences of the learning found that fasudil can decrease cardiac ischemia-injection damage through the Rho-ROCK signaling pathway. The particular statistic is depicted in Figure 4.

We can see in Figure 4 that fasudil may decrease myocardial injury during myocardial ischemia-reperfusion through the Rho-ROCK signaling pathway. Linked to the control cluster, the fasudil finding cluster caused less myocardial damage caused by cardiac ischemia-injection. 38.1%.

Myocardial ischemia caused by cardiovascular atherosclerosis is very harmful to human life and health (15). Reperfusion therapy is a common method for the treatment of cardiovascular diseases and has achieved high clinical effects. However, this treatment is likely to cause myocardial ischemia-reperfusion injury (16). The excessively high velocity of myocardial cell apoptosis tends to cause myocardial injury during reperfusion (17). The Rho kinase inhibitor fasudil can reduce the activity of the Rho-ROCK signaling pathway to interfere with myocardial cell apoptosis, so it is often used to treat cardiovascular diseases (18).
Fasudil is a Rho kinase inhibitor. Rho-kinase, also known as non-Rho kinase-related protein kinase and non-Rho kinase-related coiled-coil cell-forming protein kinase (ROCK), is a type of serine/threonine-based protein kinase, which is used for small gamma proteins. It forms the substrate of Rho's upstream and downstream photosynthesis enzymes (19). Rho-kinase itself can also be activated by multiple upstream protein molecular signals, such as kinase rho, sphingosine phosphatidylcholine (SPC), arachidonic acid and protein kinase SPC. Rho-kinase is its main and most direct upstream protein that stimulates molecular signals. The downstream target matrix molecular functions of rho kinase include about 30 kinds, such as family myosin light chain ribose phosphatase (MLCP), myosin, Erm kinase family myosin, adduct myosin and RHO kinase (20). The Rho/Rho-kinase cell signal incremental conduction control pathway can control various biological changes of human cells through this complex phosphorylation and other dephosphorylated kinase signals. Rho/rho kinase is mainly involved in the long-term pathological changes of vascular atherosclerosis, vascular myocardial spasm and arterial vascular stenosis, promotes the normal formation of vascular thrombosis, causes vascular oxidative stress, promotes the normal apoptosis of endothelial cells, and disconnects synapses. Inhibiting the growth of axons and seriously affects the normal formation of the central neural network, and plays an important long-term leading role in pathogenesis and functional development (21).

Fasudil has effective inhibitory effects on cell Rho kinase activity and capillary expansion. ROCK hormone is an important biochemical enzyme that participates in a series of life phenomena in epithelial cells, such as mitosis and membrane adhesion, cytoskeletal movement regulation, and muscle epithelial cell vasoconstriction and skin tumor epithelial cell blood infiltration (22, 23). Rho-kinase is mainly an important cellular component involved in the normal movement of blood cells, intracellular tissue damage, and the control of hypertension and viscosity, and it often plays an important role in the study of the specific pathogenesis of chronic diseases of blood cell dynamics and skin inflammation. Fasudil phosphate is an active rho kinase response inhibitor. It can inhibit the phosphorylation of smooth muscle globulin light connection in the last stage of arterial steady muscle vasoconstriction, and fundamentally effectively inhibit the abnormal occurrence of arterial vasospasm, and effectively relieve the stenosis of the central plaque in coronary atherosclerosis (24). Fasudil may effectively help improve cerebral vascular wall spasm by effectively inhibiting its rho kinase activity and at the same time ensure an increase in cerebral vascular reflux (25). It can be seen that fasudil may effectively decrease the diameter and volume of cardiomycocyte hypertrophy by 14.8% in rats under excessive stress and overload, and reduce the normal index of cardiac cell quality in rats by 25.7%, which can be significantly and effectively improved (26).

Experimental studies have shown that the use of asking aquiline tablets can effectively improve rat myocardial infarction, spontaneous myocardial hypertension, pressure and cardiac overload and rat myocardial hypertrophy caused by the use of rat myocardial vascular tension II and use myocardial hypertrophy in rats induced by valproic adrenal gland II (27, 28). Li et al. believe that it has a certain clinical treatment inducing effect, and can study the clinical mechanism of treating a variety of cardiovascular and cerebrovascular diseases. The inducing effect has the following three main aspects (29). First, inhibiting muscle and plasma cell globulin phosphokinase can activate the phosphorylation and contraction process of muscle and plasma cell globulin light chain, and it is also believed to effectively inhibit other contractile blood vessels, blocking the continuous flow of hydrogen ions in calcium. It prevents the hydrogen ions in calcium from contracting and oxidizing due to the absence of thyroid adrenaline or prostaglandin in the deoxygenation and hydrogenation reaction. Under normal circumstances, it can avoid reducing the continued existence of hydrogen ions in calcium. Secondly, it can increase the oxidative formation of adenine and dodecyl nucleotide phosphate by cellular nicotinic acid, resist peroxyl free radicals, and can inhibit the oxidative degradation of cytoskeletal collagen (30, 31). Third, Rho-kinase as an inhibitor receptor can inhibit inflammatory macrophages, antagonize the activation and secretion of anti-inflammatory cytokines, block the binding effect of inflammatory cytokines, and have a certain anti-inflammatory effect (32).
The consequences of the learning depicted that under the intervention of fasudil, the expression layer of Rho-ROCK kinase activity in the observation group was reduced by 18.3%. The myocardial cell apoptosis rate was reduced by 26.4% compared with the control group, and the area of myocardial ischemia was reduced by 32.5%. The ultrastructure of the new machine in rats is improved, and the left ventricular diastolic and systolic functions are improved. Therefore, we can see that fasudil may decrease cardiac ischemia and focus on injury motivation.

The study found that fasudil can improve the ultrastructure of myocardial tissue through the Rho-ROCK signaling pathway, reducing focal necrosis of myocardial tissue by 17.2%, reducing mitochondrial edema by 15.4%, and reducing myocardial fibrosis by 22.6%. Fasudil can reduce myocardial injury during myocardial ischemia-injection through the Rho-ROCK signaling pathway. Linked to the control cluster, the myocardial damage caused by cardiac ischemia-injection in the fasudil observation group was 38.1% less.

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Interest conflict
The authors declare no conflict of interest.

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