

Rosuvastatin inhibits inflammatory response and resists fibrosis after myocardial infarction

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Abstract. – OBJECTIVE: To study the effect of rosuvastatin on myocardial infarction in rats and its mechanism of action.

MATERIALS AND METHODS: 24 Sprague-Dawley (SD) rats were randomly divided into 3 groups: intensive statin group (n=8), myocardial infarction control group (n=8) and sham-operation group (n=8). The left anterior descending coronary artery was ligated to establish myocardial infarction models. Rats in intensive statin group were treated with gavage via rosuvastatin (1 mg × kg) and 1.5 mL distilled water suspension at 3 d before operation, while rats in the other two groups received gavage via the same amount of distilled water till 4 weeks after operation. Venous blood was collected using capillary glass tubes at 3 d before operation (before medication) and the last day in the 4th week after operation. Interleukin-6 (IL-6) was detected via chemiluminescence assay, and tumor necrosis factor- α (TNF- α) was detected via immunofluorescence assay. Hematoxylin and eosin (HE) staining and Masson staining were performed for myocardium to detect the inflammation and fibrosis. Finally, the expressions of inflammatory protein p65, peroxisome proliferator-activated receptor (PPAR) and fibrin were detected via Western blotting, and the Snail expression was detected by immunohistochemical assay.

RESULTS: The survival rate and cardiac function of rats in intensive statin group were superior to those in control group. HE staining and detection of blood IL-6 and TNF- α , and p65 and PPAR protein expressions revealed that the inflammatory levels in the body and myocardium of rats in intensive statin group were decreased compared with those in control group. Masson staining and detection of fibrin level showed that the myocardial fibrosis level of rats in intensive statin group was reduced compared with that in control group.

CONCLUSIONS: Rosuvastatin can reduce the level of myocardial fibrosis through alleviating the inflammatory response in rats with myocardial infarction.

Key Words

Myocardial infarction, Rats, Inflammation, EMT, Myocardial fibrosis.

Introduction

Acute myocardial infarction (AMI) refers to the myocardial necrosis caused by the acute and persistent ischemia and hypoxia. The myocardial tissue ischemia-hypoxia and increased ventricular wall tension after MI can induce the inflammatory response¹. The abnormal increase of inflammatory factors promotes the excessive accumulation, significantly increased concentration or composition change of collagen fibers. Studies have shown that the inflammatory response plays an important role in the occurrence and development of myocardial fibrosis², but the exact mechanism of inflammatory response-induced myocardial fibrosis remains unclear yet at present.

Epithelial-mesenchymal transition (EMT) refers to the biological processes in which epithelial cells lose the close intercellular connection and polarity, and are transformed into mesenchymal cells in a specific environment³. In adults, EMT is closely related to the formation of fibroblasts during damage repair, tissue regeneration and organ fibrosis. Besides, EMT related to tissue repair, regeneration and fibrosis is defined as type 2 EMT, which is an important part in injury-related events. Its main biological effect is to produce fibroblasts to repair the tissue damage caused by wounds and inflammatory responses⁴. With the continuous activation of inflammatory response, the EMT process persists, eventually resulting in organ fibrosis. In the EMT process, the transcription factor Snail is a very important biomarker⁵.

It is widely involved in the development, tumorigenesis and fibrosis as a common downstream target of multiple signaling pathways. At present, the research on EMT mainly focuses on the renal fibrosis, pulmonary fibrosis and tumor metastasis, but whether EMT is involved in the myocardial fibrosis is not fully studied.

Statins are methylglutaryl coenzyme A reductase inhibitors, which are currently widely used anti-atherosclerotic basic drugs in clinical practice⁶. It is generally accepted that the great decline in low-density lipoprotein cholesterol (LDL-C) is a basis for the benefit of statins, but with the deepening of study on statins, people have found that statins also have several effects independently of their lipid-lowering effect⁷. A large number of studies have suggested that there is no association between the multiple effect mechanism of statins and their lipid-lowering effect, because it is found that their multiple effects have been produced prior to the lipid-lowering effect, including improving the endothelial function, anti-inflammation, anti-platelet, stabilizing atherosclerotic plaque and anti-arrhythmia⁸. This study aimed to study the inflammatory response, myocardial fibrosis and changes in EMT biomarkers in rats after MI, and evaluate the effects of intensive statin therapy on inflammatory response and myocardial fibrosis in MI rats.

Materials and Methods

Experimental Materials

Healthy male Sprague-Dawley (SD) rats were provided by Laboratory Animal Center of Chongqing Medical University (Chongqing, China). This study was approved by the Animal Ethics Committee of Yantai Yuhuangding Hospital Animal Center. Tumor necrosis factor- α (TNF- α) assay kits were purchased from Yanyu Chemical Reagent Co., Ltd. (Shanghai, China), and interleukin-6 (IL-6) chemiluminescence assay kits were purchased from Beckman Coulter (Miami, FL, USA). Rabbit anti-rat Snail polyclonal antibodies, rabbit anti-rat cadherin polyclonal antibodies and goat anti-rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

MI Modeling and Grouping

Under the anesthesia via intraperitoneal injection of 2.5% pentobarbital sodium (30 mg/kg), the rat's neck was separated layer by layer and

connected to the animal ventilator (tidal volume of about 4 mL/time, respiratory rate of 60 times/min); then, a parasternal longitudinal incision was made on the left, and separated layer by layer until the heart was exposed. The left anterior descending branch was ligated using 6-0 ophthalmic non-invasive needle below the inferior margin of left auricle at about 3-4 mm away from the aortic root; the chest wall was sutured firmly using the 0# wire. Finally, 400,000 units of penicillin were injected to prevent infection. After waking, rats were fed in independent cages. The left anterior descending branch was not ligated in sham-operation group, but only threading; the remaining operations were the same as above.

24 SD rats were randomly divided into 3 groups: intensive statin group (n=8), MI control group (n=8) and sham-operation group (n=8). Rats in intensive statin group were treated with gavage via rosuvastatin (1 mg $\times$ kg) and 1.5 mL distilled water suspension at 3 d before operation, while rats in the other two groups received gavage via the same amount of distilled water till 4 weeks after operation.

Echocardiography

The cardiac functions of rats were detected before operation and at 1 and 4 weeks after operation using the high-frequency ultrasound (Philips, Eindhoven, The Netherlands). After anesthesia using inhalation of 1-2% isoflurane, the cardiac systolic and diastolic functions were detected on the long axis and short axis, respectively, and the respiratory rate and heart rate were recorded. Left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) were obtained via left ventricular end-systolic diameter (LVESd), left ventricular end-diastolic diameter (LVEDd), left ventricular posterior wall thickness at end-systole (LVPWTs), left ventricular posterior wall thickness at end-diastole (LVPWTD) and interventricular septal thickness at end-systole (IVSs).

TNF- α Radioimmunoassay

Venous blood was collected using the capillary glass tube from the fundus venous plexus of rats in each group at 3 d before operation (before medication) and the last day in the 4th week after operation. The serum was separated via centrifugation to detect TNF- α via radioimmunoassay. According to instruction of kit, the buffer solution, standard samples, samples, antiserum and

125I-TNF were added and mixed evenly at 4°C overnight. After PR reagent and separating agent were added, the mixture was placed at room temperature for 20 min, followed by centrifugation at 3500 rpm and 4°C for 25 min. Further detection methods were according to the instructions of the TNF- α radioimmunoassay Kit (Beyotime, Shanghai, China)

IL-6 chemiluminescence Detection

The level of IL-6 in serum or plasma was measured by streptavidin-biotin method. The highly specific and highly-sensitive mouse anti-human IL-6 monoclonal antibodies were used as the coating antibodies. After the IL-6 calibrator or serum to be tested was added, another biotin-labeled mouse anti-human IL-6 monoclonal antibody and horseradish peroxidase (HRP)-labeled streptavidin were added for incubation, forming the solid-phase antibody-antigen-biotin-labeled antibody-enzyme-labeled streptavidin complex. After full washing, luminescent substrate was added to determine the relative light unit (RLU). The standard curve obtained by the calibrator could be used to calculate the content of IL-6 in the sample.

Immunohistochemistry

Morphological changes in cardiac fibrosis were detected. At 21 d after MI, heart specimens were fixed with phosphate buffered saline (PBS) and 4% paraformaldehyde, and embedded in paraffin overnight. Specimens were cut into 4 mm-thick sections and fixed on the glass slide. Tissue sections were dewaxed, dehydrated and transparentized, followed by hematoxylin and eosin (HE) staining and Masson staining. The image of each section was recorded with a microscope (Olympus Optical Co., Ltd, Tokyo, Japan).

Immunohistochemical Staining

After the heart was fixed in 10% formalin solution, embedded in paraffin, sectioned, dewaxed, hydrated and repaired with antigen, the Snail antibody was used to incubate sections at room temperature for 2 h. After sections were

washed with phosphate buffered saline (PBS) for 3 times, the biotin-labeled secondary antibody was used to incubate sections for 10 min. Next, sections were washed again, stained with hematoxylin dye for 3 min and rinsed with tap water for 1 min. The peripheral area around MI was detected in each group, and the staining intensity was observed under a light microscope.

Western Blotting

Heart tissues were taken, cut into pieces, and ground in lysis buffer via ultrasound. After centrifugation, the supernatant was extracted to determine the protein concentration. After the electrophoresis plate was installed, the protein was added into the loading well, followed by electrophoresis under constant voltage. After that, the gel was paved onto the polyvinylidene difluoride (PVDF) membrane and placed into the membrane transfer solution for membrane transfer under constant voltage at 0°C. The PVDF membrane was sealed using 5% skim milk powder at room temperature for 1 h, and then added with primary antibody for incubation at 4°C overnight. After the membrane was washed for 3 times with PBST on the next day, the secondary antibody was incubated at room temperature for 1 h, and the membrane was washed again for 3 times. Finally, developing liquid was added for exposure, followed by gray scan of bands and data analysis.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (SPSS IBM, Armonk, NY, USA) was used for the data processing and analysis. Fisher's exact test (sample size: $n \leq 40$) was adopted. $p < 0.05$ suggested that the difference was statistically significant.

Results

Basic Data

There were no significant differences in body weight, heart rate and LVEF among groups (Table I).

Table I. Comparisons of baseline data.

	Sham-operation	MI	Treatment	<i>p</i>
Weight (g)	260.3 $\pm$ 5.8	263.5 $\pm$ 6.36	260.8 $\pm$ 4.34	0.927
Heart rate	356.6 $\pm$ 26.1	352.7 $\pm$ 26.1	366.8 $\pm$ 15.3	0.837
LVEF (%)	69.7 $\pm$ 4.5	67 $\pm$ 8.5	67.4 $\pm$ 3.8	0.817

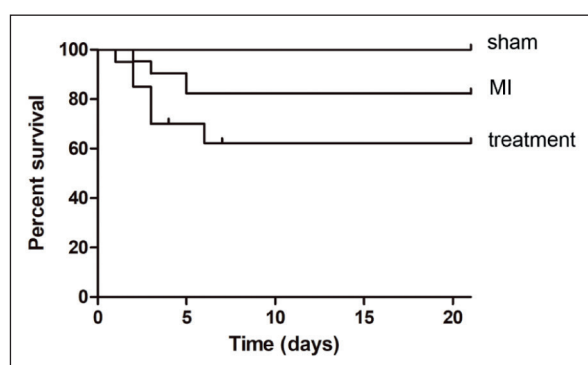


Figure 1. Survival curve of rats in each group.

Statin Therapy Improved the Cardiac Function After MI

The analysis of survival rate of rats in each group after operation showed that the postoperative survival rate in intensive statin group was higher than that in control group (Figure 1). The echocardiography of rats in each group before operation and at 1 week and 4 weeks after operation showed that LVEF and LVFS in intensive statin group were significantly superior to those in control group, and the differences were statistically significant (Table II).

Statin Therapy Alleviated the Inflammation And Fibrosis After MI

The heart of rats in each group was collected at 1 week and 4 weeks after operation for HE staining and Masson staining. The results showed that the number of inflammatory cells infiltrating the peripheral area of MI in intensive statin group was significantly lower than that in control group, and the inflammatory infiltration area was also smaller than that in control group ($p<0.05$) (Figure 2A-C). Masson staining at 4 weeks revealed that the myocardial fibrosis area was significantly smaller than that in control group (Figure 2B-D), suggesting that statin therapy can alleviate the myocardial inflammatory cell infiltration and subsequent fibrosis in MI rats.

Statin Therapy Alleviated the Inflammatory Response

Venous blood of rats in each group was detected before and after operation, and it was found that there were no differences in the TNF- α and IL-6 concentrations among three groups before operation. The detection after operation revealed that the levels of inflammatory cytokines in rats in three groups were increased after operation ($p<0.05$). Moreover, the intergroup comparison showed that the TNF- α and IL-6 concentrations in intensive statin group were significantly decreased compared with those in control group ($p<0.05$) (Figure 3A-B), indicating that the oral administration of statin can reduce the inflammatory response in rats. To further study the myocardial inflammatory level, the p65 and peroxisome proliferator-activated receptor (PPAR) levels in myocardial tissues were detected by Western blotting (Figure 3C-D). It was found that the activation of p65 and PPAR in control group was significantly higher than that in intensive statin group, and the ratio of activated p65 and PPAR to non-activated p65 and PPAR was also significantly increased ($p<0.05$), proving that the oral administration of statin can reduce the expressions of myocardial inflammatory factors in rats after MI.

Statin Therapy Alleviated the Myocardial Fibrosis Level

Snail protein staining of myocardial tissues in each group after operation showed that the expression of Snail protein in myocardial tissues after MI was increased significantly, while the Snail staining was decreased significantly after statin therapy, and the difference was significant compared with that in control group ($p<0.05$) (Figure 4A-B). Results of Western blotting of fibrosis in type I collagen and α -smooth muscle actin (α -SMA) revealed the type I collagen and α -SMA expressions after MI, and the expressions of type I collagen and α -SMA after statin therapy, were decreased ($p<0.05$) (Figure 4C-D), suggesting that statin therapy can reduce the Snail protein expression in tissues and inhibit the formation of fibrosis-related proteins.

Table II. Comparisons of LVEF and LVFS among three groups.

	Sham-operation	MI	Treatment
LVEF (%)	66.8 $\pm$ 2.9	32.5 $\pm$ 2.5*	41.1 $\pm$ 2.6**
LVFS (%)	35.5 $\pm$ 1.7	20 $\pm$ 1.2*	25.8 $\pm$ 0.7**

*Compared with sham-operation group, $p<0.05$; **compared with MI group, $p<0.05$.

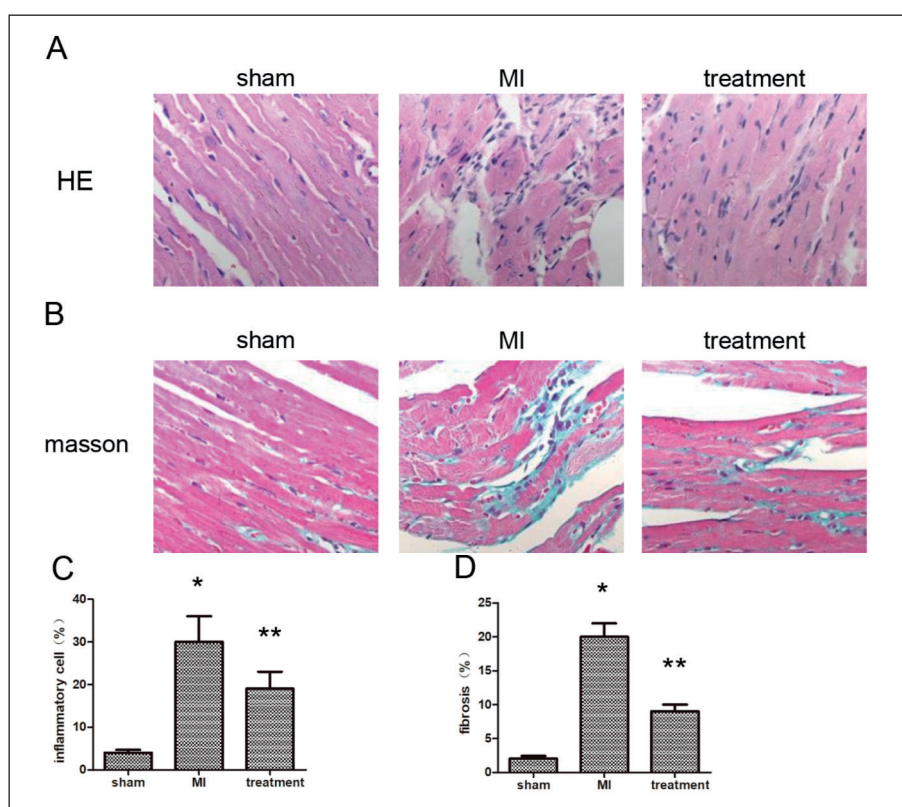


Figure 2. Changes in myocardial tissues after MI. **A**, HE staining of heart of rats in sham-operation group, MI group and treatment group, shows the myocardial inflammatory cell infiltration (40 $\times$). **B**, Masson staining of heart of rats in sham-operation group, MI group and treatment group, shows the myocardial fiber tissue filling (40 $\times$). **C**, Statistics of inflammatory cell infiltration in each group. **D**, Statistics of fiber tissue filling range in each group. * $p < 0.05$ vs. sham-operation group; ** $p < 0.05$ vs. MI group.

Discussion

At present, various studies have proved that the inflammatory response will inevitably occur after MI. Ridker et al⁹ and Takahashit et al¹⁰ confirmed the increases in inflammatory factors after MI via clinical and animal experiments. Inflammatory response is crucial for removal of irreversible lesions and healing of lesions, but it can also induce the myocardial damage and even fibrosis in and around the infarct region. Excessive myocardial necrosis or apoptosis and myocardial fibrosis may eventually lead to acute cardiac rupture or chronic cardiac dilatation¹¹, so the anti-inflammatory therapy after MI is crucial. Statins have become the basic therapeutics in treatment of cardiovascular diseases, which are not only due to the outstanding lipid-lowering effects, but also some other biological effects. A number of studies have shown that statins can significantly inhibit a variety of inflammatory factors and reduce the level of CRP as well as the expressions of chemokines and

adhesion molecules¹². In this experiment, the detection of TNF- α and IL-6 in SD rats at 3 d before operation and 4 weeks after operation proved the close relationship between MI and inflammatory response again.

Inflammation plays an important role in the occurrence and development of myocardial fibrosis. Some studies, through establishing MI model, have shown that mice with TNF- α knockout have less serious inflammation and myocardial fibrosis¹³. In the *in vitro* study on myocardial cells, the decrease in TNF- α , MMP-2 and MMP-9 can alleviate the deposition of myocardial collagen and improve the left ventricular function¹⁴. So far, there have been many studies on inflammation and myocardial fibrosis^{13,15}, but all of them are in the exploratory stage, and its exact mechanism has not been found. We found that rosuvastatin therapy can significantly alleviate the inflammatory response after MI, and reduce the expressions of inflammatory factors in the body and myocardium of rats. EMT refers to the biological processes in

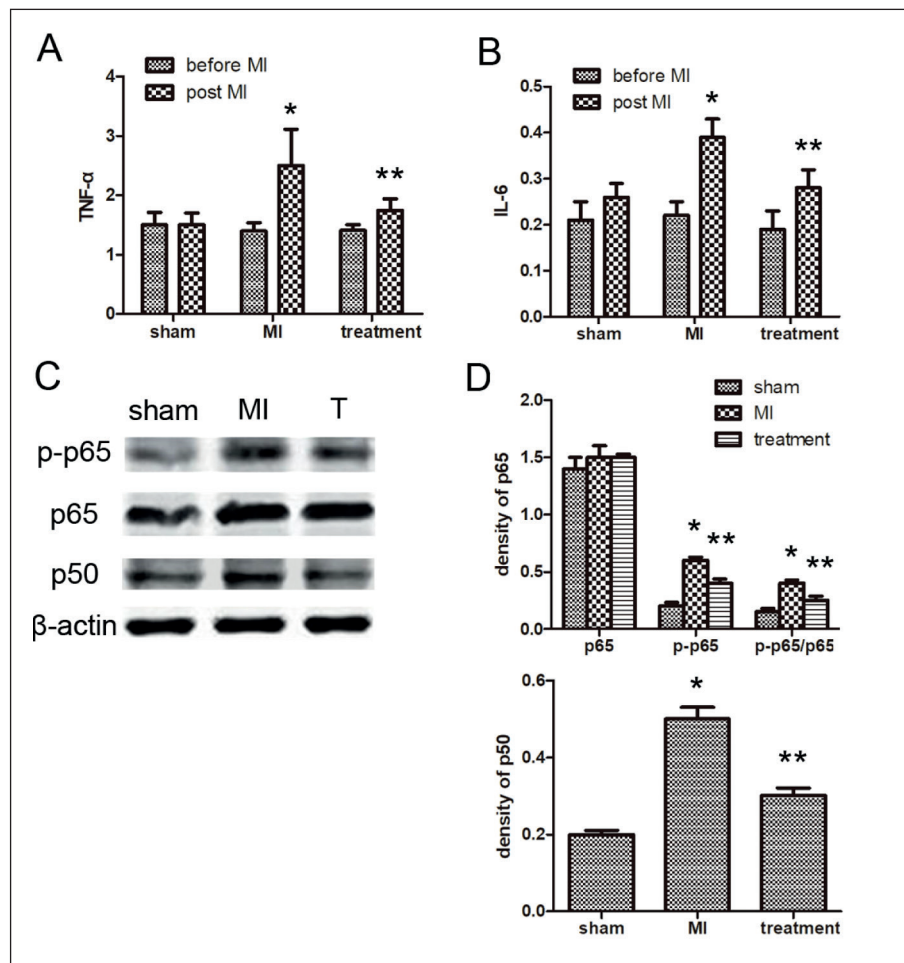


Figure 3. Inflammatory level in rats in each group. **A**, Serum TNF- α levels in rats in sham-operation group, MI group and treatment group before and after MI. **B**, Serum IL-6 levels in rats in sham-operation group, MI group and treatment group before and after MI. **C**, Detection of p65, p-p65 and p50 expressions via Western blotting. **D**, Statistics of p50 gray value and p-p65/p65 ratio in each group. * $p < 0.05$ vs. sham-operation group; ** $p < 0.05$ vs. MI group.

which epithelial cells lose the close intercellular connection and the polarity, thus leading to be transformed into mesenchymal cells. The main biological effect of EMT is repairing the tissue damage caused by wounds and inflammatory responses¹⁶. Under pathological conditions, the inflammatory response continues to be activated and EMT process continues to exist, eventually causing organ fibrosis⁷. In EMT process, the transcription factor Snail is an important molecule, which is a kind of DNA-binding protein containing a zinc finger structure, and it can specifically recognize and bind to target genes, thereby inhibiting the expression of E-cadherin gene and transforming epithelial cells into mesenchymal cells. This study showed that rosuvastatin therapy can significantly reduce the Snail expression

in myocardial tissues of MI rats, and lower the expressions of fibrosis in collagen I and α -SMA.

Conclusions

There is an inflammatory process after MI, and rosuvastatin can significantly alleviate the inflammatory response. Significant myocardial fibrosis occurs after MI, in which EMT may be involved. Rosuvastatin has an effect of inhibiting myocardial fibrosis after MI.

Conflict of Interest

The authors declare that they have no conflict of interest.

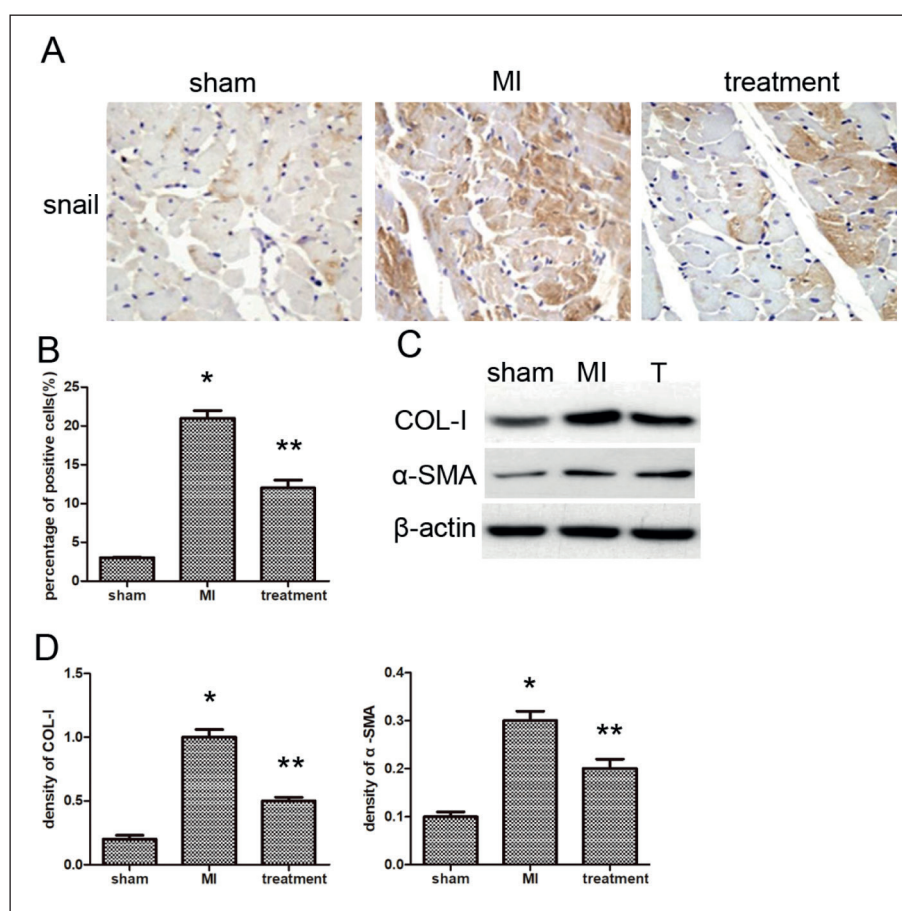


Figure 4. Myocardial fibrosis level in rats in each group. **A**, Snail immunohistochemical staining of myocardium of rats in sham-operation group, MI group and treatment group (40×). **B**, Statistics of positive cells in Snail immunohistochemical staining. **C**, Detection of type I collagen and α -SMA expressions via Western blotting. **D**, Gray value analyses of type I collagen and α -SMA. * p <0.05 vs. sham-operation group; ** p <0.05 vs. MI group.

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