European Review for Medical and Pharmacological Sciences 2020; 24: 6400-6408

Simvastatin relieves myocardial ischemia/reperfusion injury in rats through hedgehog signaling pathway

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Abstract. – OBJECTIVE: The aim of this study was to explore the effect of simvastatin (Sim) on myocardial ischemia/reperfusion (I/R) injury in rats and to elucidate the possible underlying mechanism. Our findings might help to provide a certain reference for the clinical prevention and treatment of myocardial I/R injury.

MATERIALS AND METHODS: A total of 60 male Sprague-Dawley (SD) rats were randomized into three groups using a random number table, including: Sham group (n=20), I/R group (n=20) and I/R + Sim group (n=20). The I/R injury model was successfully established in rats *via* ligation of the left anterior descending coronary artery (LAD), followed by reperfusion. Before operation, the rats in I/R + Sim group were administered with Sim at 10 mg/kg/d through oral gavage for 7 d. Cardiac ejection fraction (EF) (%) and fractional shortening (FS) (%) of rats in each group were detected using echocardiography. 2,3,5-triphenyltetrazolium chloride (TTC) staining was performed to measure the myocardial infarction (MI) area in each group. Collagen deposition in myocardial tissues of rats in each group was detected by Masson's trichrome staining. The apoptosis level of myocardial cells and fibroblasts in myocardial tissues of rats in each group were evaluated *via* terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. The level of reactive oxygen species (ROS) in myocardial tissues of rats in each group was determined using fluorescent probes. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was conducted to measure the expression levels of pro-inflammatory cytokines interleukin (IL)-1β and monocyte chemoattractant protein-1 (MCP-1) in myocardial tissues of rats in each group. Furthermore, the effects of Sim on the hedgehog signaling pathway-associated proteins were detected using Western blotting.

RESULTS: Sim significantly alleviated I/R-induced cardiac dysfunction in rats and increased EF (%) and FS (%) ($p<0.05$). Meanwhile, it also evidently mitigated the MI caused by I/R and reduced the infarction area ($p<0.05$). According to the Masson's trichrome staining results, I/R + Sim group exhibited remarkably declined myocardial interstitial collagen deposition compared with I/R group ($p<0.05$). ROS-sensitive fluorescent staining showed that Sim notably reversed the increase of ROS expression and the decrease of myocardial oxidative stress induced by I/R ($p<0.05$). Finally, Western blotting results revealed that Sim dramatically restrained the protein expressions of sonic hedgehog (SHH), patched 1 (PTC1) and glioma-associated oncogene homolog 1 (GLI1) ($p<0.05$).

CONCLUSIONS: Sim can significantly relieve myocardial I/R injury in rats. The possible underlying mechanism may be related to its inhibition on the hedgehog signaling pathway.

Key Words:
Myocardial ischemia-reperfusion, Simvastatin, Hedgehog.

Introduction

Ischemic heart disease (IHD) is not only the leading cause of deaths, but also a major public health problem worldwide[1]. Reperfusion is an important strategy for the heart to rescue irreversible myocardial injury. However, post-ischemia reperfusion can usually cause certain damage to the myocardium, which is termed myocardial ischemia/reperfusion (I/R) injury[2,3]. I/R injury is an inevitable pathophysiological phenomenon in the treatment of IHD and cardiac surgery for patients undergoing thoracotomy. Meanwhile, it can cause reperfusion arrhythmia, temporary mechanical dysfunction, myocardial stunning, and other pathological changes[4,5]. Therefore, further searching for targeted drugs to prevent myocardial I/R injury is of great importance for the prevention and treatment of IHD.

Morphogen is a substance that governs the pattern of tissue development and the positions of

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various special cells. Hedgehog protein is a pivotal morphogen in the embryonic period, developmental period and adulthood, which is expressed in multiple organs and tissues[6]. The hedgehog protein family comprises sonic hedgehog (SHH), Indian hedgehog and Desert hedgehog, among which SHH is the most extensively expressed and profoundly researched. Currently, the SHH pathway has been widely studied in embryogenesis, especially the epithelial-mesenchymal interaction in the formation of limbs, lungs, intestines, hair follicles and bones[7,8]. Besides, the SHH pathway has a crucial regulatory effect on the key life activities in bone marrows, the central nervous system, peripheral nerves, the cardiovascular system, and epithelial tissues in organism homeostasis or the regeneration and repair of severely damaged tissues[9]. However, this pathway is aberrantly activated in acute or chronic myocardial infarction (MI)[10].

Statins exert multiple pleiotropic effects, including improving endothelial function, increasing the bioavailability of vascular nitric oxide, reducing oxidative stress and enhancing endothelial progenitor cell function[11]. They can significantly protect against MI and stroke in rodent models of MI or stroke. This may be related to increased production of endothelial nitric oxide synthases[12]. The exact role and mechanism of simvastatin (Sim), a novel statin, in myocardial I/R injury in rats remain elusive now.

In the present study, therefore, we investigated the role of Sim in rats with myocardial I/R injury. Its potential underlying mechanism in resisting myocardial I/R injury was further explored. Our findings might help to provide a certain reference for the future clinical treatment of myocardial I/R injury.

Materials and Methods

Laboratory Animal Grouping and Treatment

A total of 60 12-14-week-old male Sprague-Dawley (SD) rats weighing (285.61±10.66) g were randomly assigned into three groups using a random number table, including: Sham group (n=20), I/R group (n=20) and I/R + Sim group (n=20). No statistically significant differences were observed in general information among the three groups of rats, such as age and weight. The rats in each group were first anesthetized by intraperitoneal injection of pentobarbital at 50 mg/kg. Then, a catheter was inserted into the left carotid artery to detect the blood pressure of rats. Heart rate was measured using 2-lead ECG. Subsequently, thoracotomy was performed at the fourth intercostal space to remove the pericardium and expose the heart. Next, the left anterior descending coronary artery was ligated using a 6-0 silk thread at 2 mm above the left atrial appendage to induce local myocardial ischemia. After ischemia for 30 min, the silk thread was loosened, followed by 2 h of reperfusion. The rats in the Sham group underwent the same surgery except the ligation using silk threads. Following reperfusion, the rats were sacrificed, and myocardial tissues of the left ventricular anterior wall were collected. After rinsing with normal saline to remove blood, these tissues were preserved in a refrigerator at -80°C for later use (Figure 1). At 7 d before operation, the rats in I/R + Sim group started to be intragastrically administered with Sim at a dose of 10 mg/kg/d, while those in Sham and I/R groups were injected with an equal volume of normal saline. This investigation was approved by the Animal Ethics Committee of Zhejiang Provincial People's Hospital Animal Center.

Echocardiography

To detect the cardiac function of rats in each group, echocardiography was performed using the MyLab 30CV ultrasound system (Esaite S.P.A Genoa, Italy) and 10-MHz linear ultrasonic transducer. Briefly, the rats were first depilated at the anterior chest and anesthetized. Next, they were placed on a heating plate at 37°C with the left upwards. Parameters, including ejection fraction (EF) (%), fractional shortening (FS) (%), and heart rate (bpm), were finally measured.

2,3,5-Triphenyltetrazolium Chloride (TTC) Staining

Fresh myocardial tissues were first placed into a rat heart slicer and cryopreserved in the refrigerator at -20°C for 30 min for sectioning. Myocardial tissues were then sliced into no more than 6 pieces of about 2 mm-thick sections. The resulting sections were thoroughly soaked in fresh 2% TTC solution (Oxoid, Hampshire, UK), followed by 30 min of incubation. Afterwards, the sections were taken out, fixed with 4% paraformaldehyde, and photographed.

Masson's Trichrome Staining

Paraffin-embedded sections were first de-paraffinized and dehydrated, followed by chromation or removal of mercury salt precipitates. The resulting sections were washed successively with running water and distilled water. Subsequently, the nuclei were stained with Regaud's hematoxylin solution or Weigert's iron hematoxylin solution for

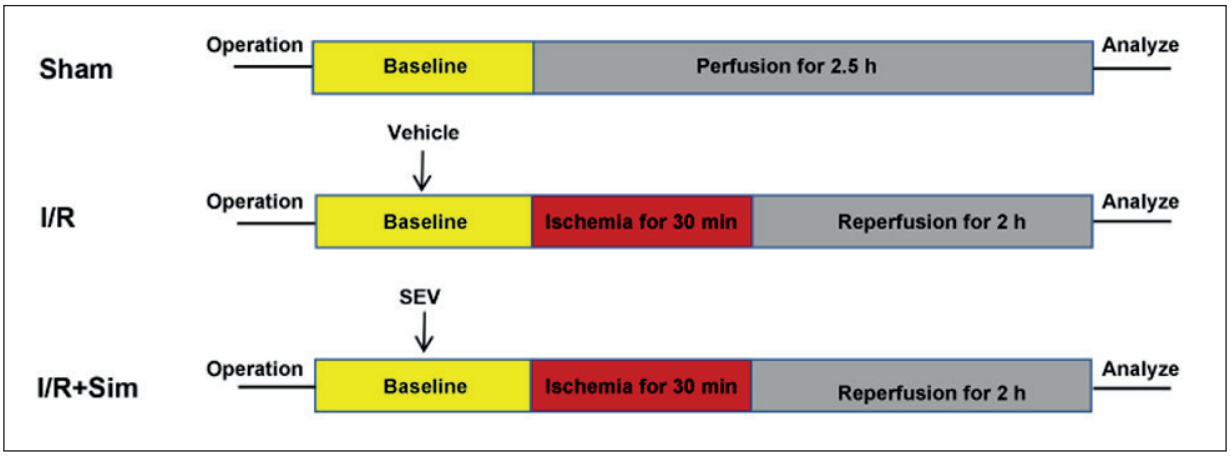

Figure 1. Flow chart of treatment of rats in all groups: Sham group: sham operation group, I/R group: ischemia/reperfusion group and I/R + Sim group: ischemia/reperfusion + simvastatin preconditioning group.

5-10 min. After fully washing with water, the sections were stained with ponceau-acid fuchsin solution for 5-10 min. Next, they were immersed in 2% glacial acetic acid solution for a moment, followed by 3-5 min of differentiation using 1% phosphomolybdic acid aqueous solution. Finally, the sections were transparentized using 95% alcohol, absolute alcohol and xylene, and mounted in neutral resin.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) Staining

Sliced myocardial tissue sections were first baked in an oven at 60°C for 30 min, and de-paraffinized using xylene for 5 min ×3 times. Subsequently, the sections were dehydrated in 100% alcohol, 95% alcohol and 70% alcohol for 3 times, respectively. The resulting sections were incubated with protein kinase K for 30 min and rinsed with phosphate-buffered saline (PBS). After reaction with TdT and Luciferase-labeled dUTP at 37°C for 1 h, the sections were further incubated with the horseradish peroxidase-labeled specific antibodies in an incubator at 37°C for 1 h. Then, the cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO, USA). Finally, the cells were photographed and counted under a fluorescence microscope.

Detection of Messenger Ribonucleic Acid (mRNA) Expressions of Inflammation-Associated Genes by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNAs were extracted from myocardial tissues of rats in each group using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The concentration and purity of extracted RNAs were measured using an ultraviolet spectrophotometer. Absorbance $(A)_{260}/A_{280}$=1.8-2.0 indicated eligible for subsequent procedures. Next, the mRNAs were synthesized into complementary deoxyribonucleic acids (cDNAs) and stored in the refrigerator at -80°C. RT-PCR was conducted in the system composed of 2.5 μL of 10× Buffer, 1 μL of cDNAs, 0.5 μL of both forward primers and reverse primers at 20 μmol/L, 10 μL of LightCycler® 480 SYBR Green I Master (2×) and 5.5 μL of ddH_2O. RT-PCR amplification for all the indicators was conducted in the same system (Table I).

Western Blotting

Myocardial tissues of rats in each group were first fully ground in lysis buffer and ultrasonicated. The resulting lysate was centrifuged, and the supernatant was aspirated and aliquoted into Eppendorf (EP; Hamburg, Germany) tubes. The concentration of total protein was measured using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). All protein samples were diluted into the same constant concentration, aliquoted, and preserved in a refrigerator at -80°C for use. Subsequently, the proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto cellulose acetate/polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). Next, the membranes were incubated with primary antibodies at 4°C overnight. On the next day, the membranes were incubated with the goat anti-rabbit secondary antibody in dark for 1 h. Immuno-reactive bands were finally scanned using an Odyssey scanner (Seattle,

Table I. Primer sequences of all indicators in RT-PCR.

Target gene		Primer sequence
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Forward	5'-GACATGCCGCCTGGAGAAACCC-3'
	Reverse	5'-AGCCCAGGATGCCCTTTAGTCCA-3'
Interleukin (IL)-1β	Forward	5'-AGCGCTGATAGTCGATCGGGTC-3'
	Reverse	5'-ACGTGTCCCGTGATCGTAGTTCA-3'
Monocyte chemoattractant protein-1 (MCP-1)	Forward	5'-ACGTAGTTGCTGATTGCTAGTCA-3'
	Reverse	5'-ACGATGCTAGTGCTGATGCTAGG-3'

WA, USA) and quantified. The level of proteins to be detected was corrected using glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Detection of Reactive Oxygen Species (ROS) by Fluorescent Probe Staining

A circle was first drawn using an immunohistochemical pen around the myocardial tissues to avoid the running of antibodies. Then, ROS-sensitive dye (MedChem Express, Monmouth Junction, NJ, USA) diluted by PBS at 1:200 was added dropwise into the circle, followed by incubation in a dark place for 30 min. The resulting sections were rinsed with PBS for 5 min ×3 times. Finally, the sealed sections were photographed under a fluorescence microscope, and the fluorescence intensity in each group was quantified using ImageJ software.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM Corp., Armonk, NY, USA) was used for all statistical analysis. Measurement data were presented as mean ± standard deviation (SD). *t*-test was conducted to compare the difference between two groups. $p<0.05$ was considered statistically significant.

Results

Effect of Sim Preconditioning on the Cardiac Function of Rats In Each Group

According to echocardiograms (Figure 2), there was no statistically significant difference in the heart rate of rats among the three groups ($p>0.05$). Therefore, the differences in EF (%) and FS (%) among the three groups were not caused by the difference in heart rate. Compared with the Sham group, I/R group exhibited significantly larger heart chambers and thinner heart wall ($p<0.05$). However, Sim preconditioning substantially improved the I/R-induced aberrant changes in the heart structure of rats. Subsequently, FS (%) and EF (%) of rats were further measured in each group. It was found that Sim preconditioning significantly inhibited the decrease of FS (%) and EF (%) in I/R group. The above results suggest that Sim preconditioning can prevent the of deterioration reperfusion-induced cardiac function in rats.

Sim Preconditioning Decreased I/R-Induced MI Area In Rats

MI area was evaluated using TTC staining in each group. The results showed that MI area was 0% *vs.* (65.19±2.04) % *vs.* (37.29±1.39)% in Sham group, I/R group and I/R + Sim group, respectively (Figure 3), with statistically significant differences ($p<0.05$). Therefore, it can be inferred that Sim preconditioning effectively decreases the I/R-induced MI area in rats.

Sim Preconditioning Reduced I/R-Induced Myocardial Fibrosis

To evaluate the effect of Sim on myocardial fibrosis, the expression of collagen in myocardial tissues of rats was detected using Masson's trichrome staining. According to the results (Figure 4), Sim distinctly reduced post-I/R myocardial interstitial fibrosis ($p<0.05$), further restraining cardiac remodeling after MI.

Sim Preconditioning Reduced I/R-Induced Myocardial Oxidative Stress

Oxidative stress is an important mechanism of I/R injury. Therefore, the level of the oxidative stress marker ROS in myocardial tissues of rats was determined in each group. Based on the results (Figure 5), the level of ROS declined significantly in myocardial tissues of rats with I/R injury after Sim preconditioning ($p<0.05$).

Effect of Sim Preconditioning on Myocardial Cell Apoptosis In Rats

The apoptosis level of myocardial cells in the three groups of rats was determined *via* TUNEL staining. It was found that myocardial cell apoptosis remarkably rose in myocardial tissues of

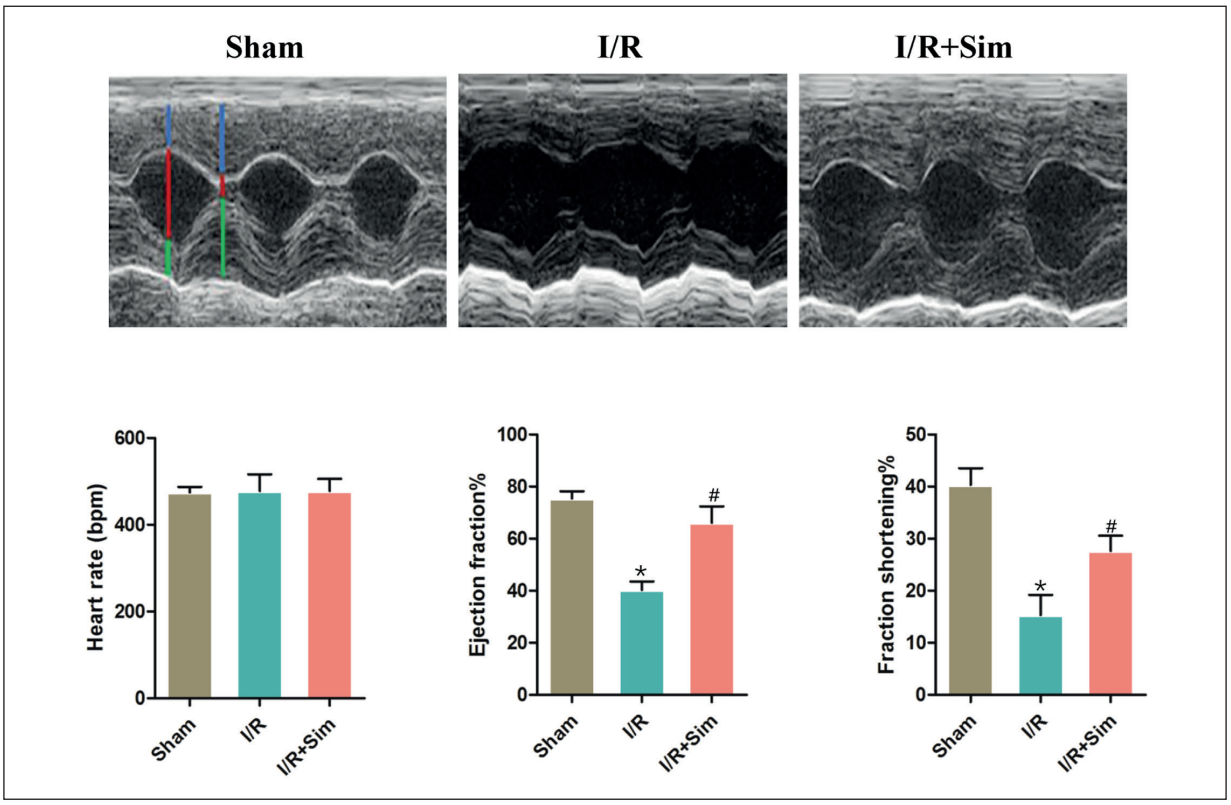

Figure 2. Effect of Sim preconditioning on the cardiac function of rats in each group: Sham group: sham operation group, I/R group: ischemia/reperfusion group, and I/R + Sim group: ischemia/reperfusion + simvastatin preconditioning group. *p<0.05 *vs.* Sham group, #p<0.05 *vs.* I/R group.

rats with myocardial I/R injury (p<0.05). Meanwhile, the number of apoptotic myocardial cells decreased notably after Sim preconditioning (p<0.05) (Figure 6). These findings imply that Sim preconditioning can significantly inhibit myocardial cell apoptosis in rats with I/R injury.

Effect of Sim Preconditioning on Myocardial Inflammation In Each Group of Rats

Additionally, the mRNA expression levels of pro-inflammatory factors IL-1β and MCP-1 in myocardial tissues of rats were determined using

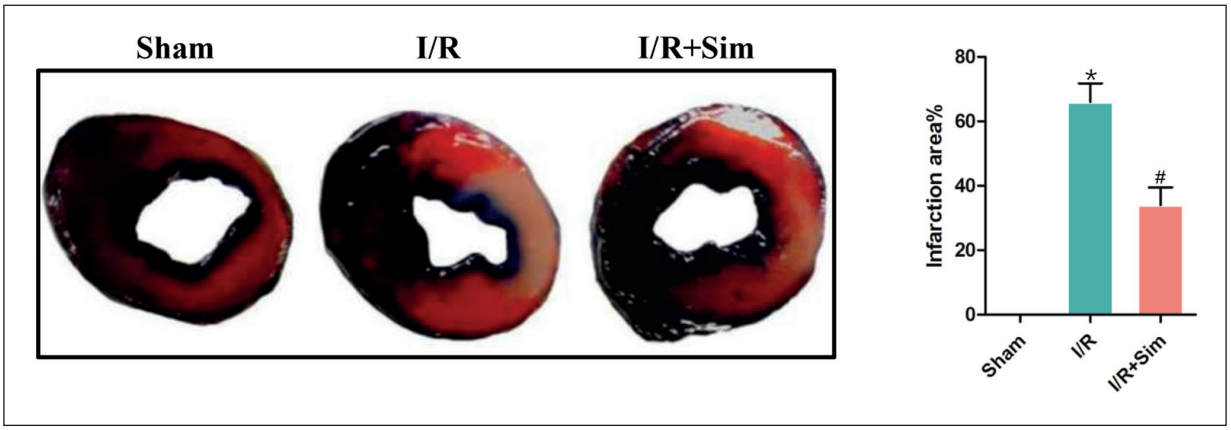

Figure 3. Effect of Sim preconditioning on I/R-induced MI area in rats: Sham group: sham operation group, I/R group: ischemia/reperfusion group, and I/R + Sim group: ischemia/reperfusion + simvastatin preconditioning group. *p<0.05 *vs.* Sham group, #p<0.05 *vs.* I/R group.

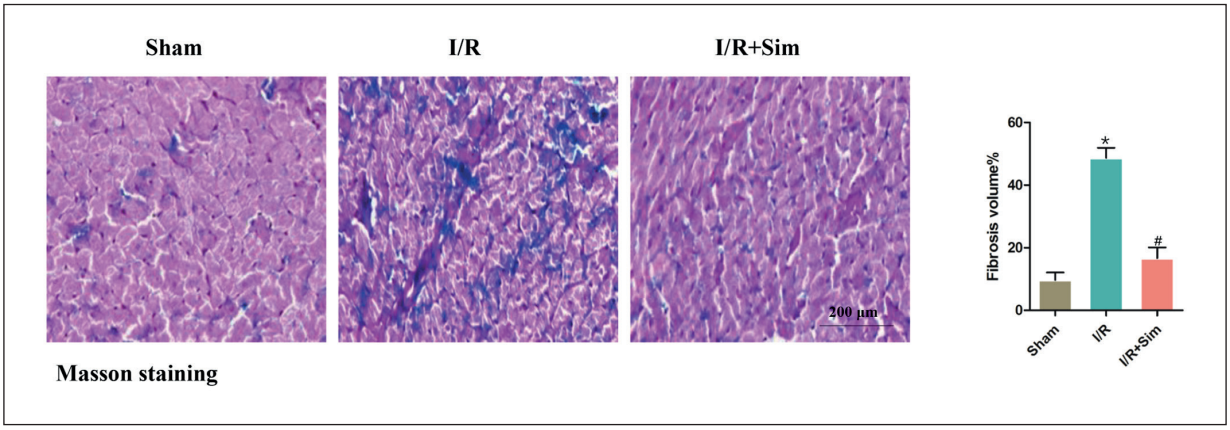

Figure 4. Effect of Sim preconditioning on I/R-induced myocardial fibrosis: Sham group: sham operation group, I/R group: ischemia/reperfusion group, and I/R + Sim group: ischemia/reperfusion + simvastatin preconditioning group, (magnification: 200×) *p<0.05 *vs.* Sham group, $^{\#}p$<0.05 *vs.* I/R group.

RT-PCR. According to the results (Figure 7), the levels of IL-1β and MCP-1 were evidently elevated in the I/R group compared with those in the Sham group (p<0.05). However, their levels distinctly declined in I/R + Sim group in comparison with I/R group (p<0.05).

Effect of Sim Preconditioning on the Hedgehog Signaling Pathway In Myocardial Tissues of Rats with I/R Injury

Western blotting results demonstrated that Sim preconditioning remarkably repressed the

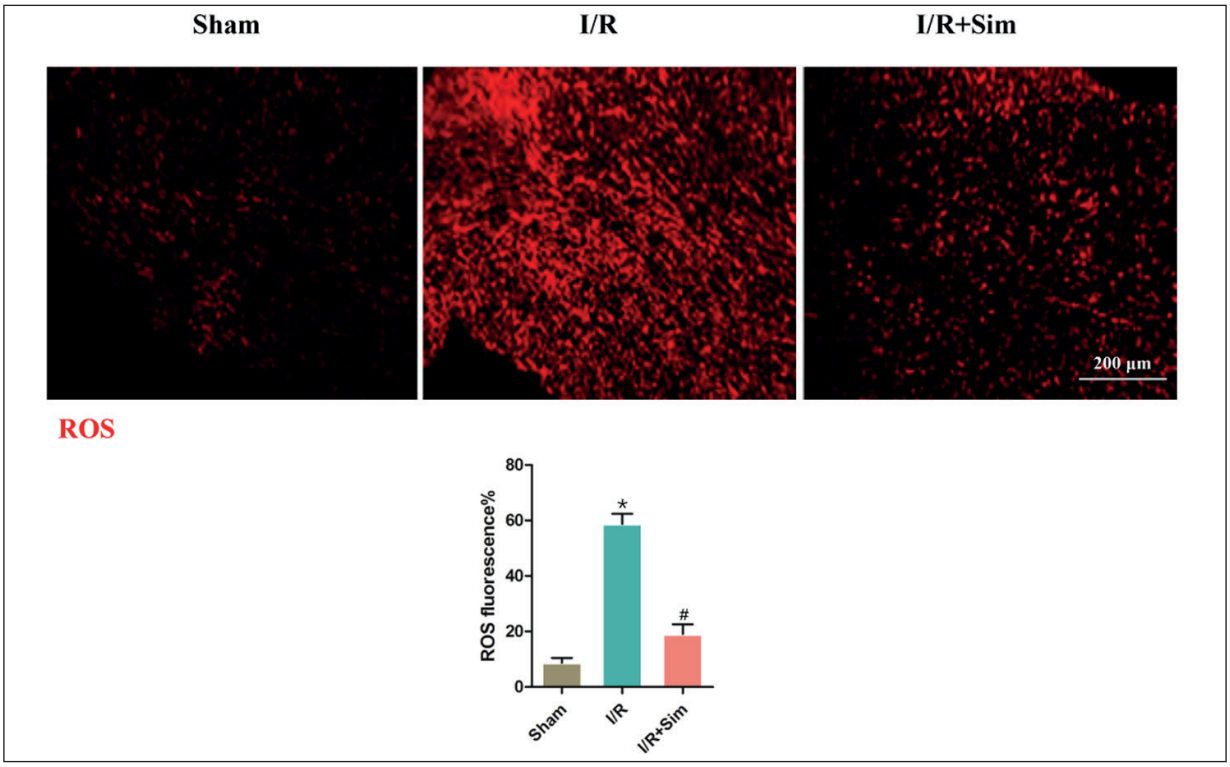

Figure 5. Effect of Sim preconditioning on I/R-induced myocardial oxidative stress: Sham group: sham operation group, I/R group: ischemia/reperfusion group, and I/R + Sim group: ischemia/reperfusion + simvastatin preconditioning group, (magnification: 200×) *p<0.05 *vs.* Sham group, $^{\#}p$<0.05 *vs.* I/R group.

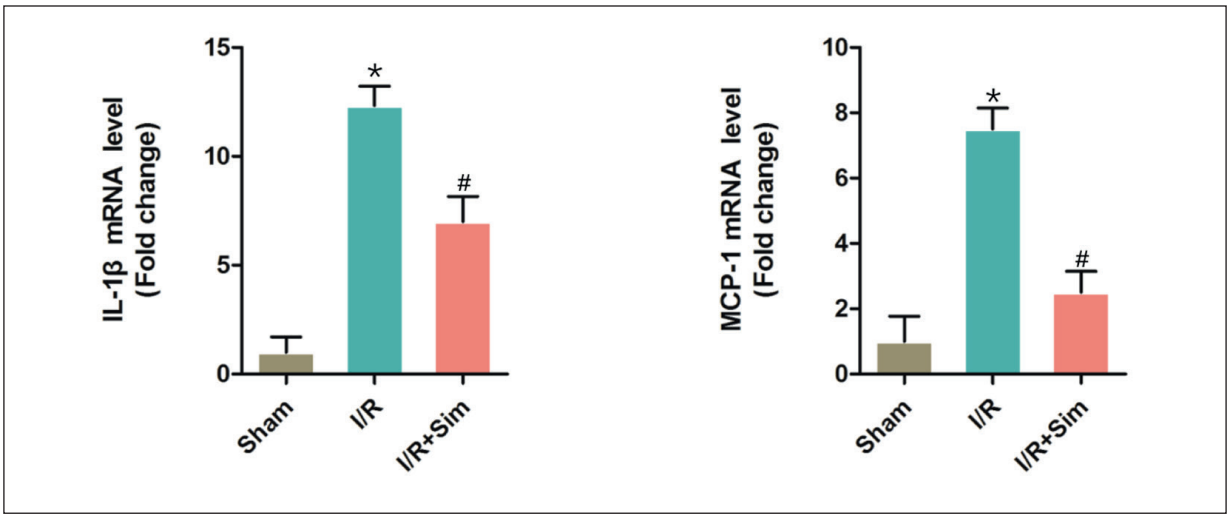

Figure 6. Effect of Sim preconditioning on myocardial cell apoptosis: Sham group: sham operation group, I/R group: ischemia/reperfusion group, and I/R + Sim group: ischemia/reperfusion + simvastatin preconditioning group. *p<0.05 *vs.* Sham group, #p<0.05 *vs.* I/R group.

activation of the hedgehog signals in myocardial tissues of rats with I/R injury. Sim remarkably decreased the protein expressions of SHH, patched 1 (PTC1) and glioma-associated oncogene homolog 1 (GLI1) in myocardial tissues (p<0.05) (Figure 8).

Discussion

Rapid restoration of blood flow to the occluded coronary artery *via* drug intervention or mechanical means is the most efficacious strategy to limit MI area and improve the clinical prognosis of acute

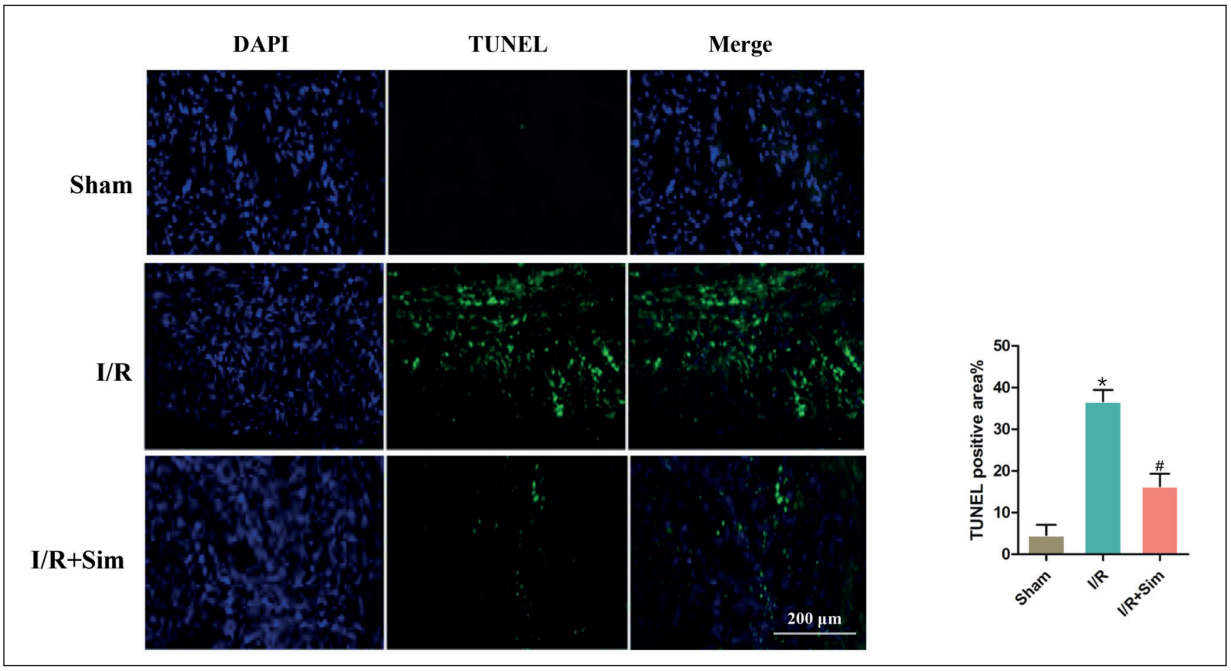

Figure 7. Flow chart of treatment of rats in all groups: Sham group: sham operation group, I/R group: ischemia/reperfusion group, and I/R + Sim group: ischemia/reperfusion + simvastatin group, (magnification: 200×) *p<0.05 *vs.* Sham group, #p<0.05 *vs.* I/R group.

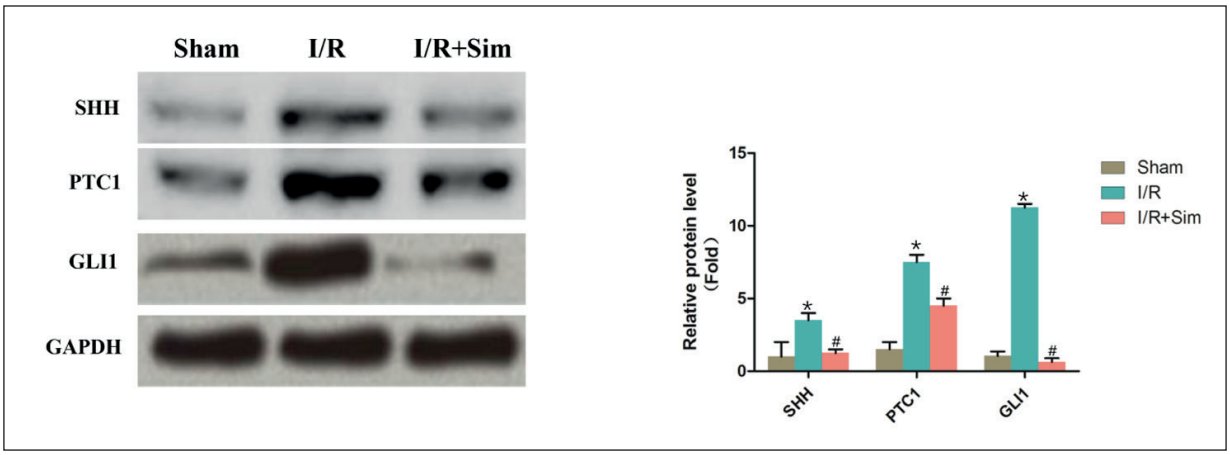

Figure 8. Effect of Sim preconditioning on the hedgehog signaling pathway in myocardial tissues of rats with I/R injury: Sham group: sham operation group, I/R group: ischemia/reperfusion group, and I/R +Sim group: ischemia/reperfusion + simvastatin preconditioning group. *$p<0.05$ *vs.* Sham group, #$p<0.05$ *vs.* I/R group.

MI[13]. However, I/R can cause additional myocardial cell deaths, while increasing the area of MI. The main factors for I/R injury include oxidative stress, inflammation, and apoptosis. During myocardial ischemia, the consumption of ATP in myocardial cells weakens the ability of sarcoplasmic reticulum to take in Ca^{2+}. This further induces massive Ca^{2+} accumulation in the mitochondria, thereby decreasing myocardial cell contractility[14]. In reperfusion, oxygen enters again myocardial cells to damage the electron transport chain and increase ROS in the mitochondria. Mitochondrial Ca^{2+} overload and increased ROS can drive the opening of mitochondrial membrane permeability conversion pore to cause cellular energy disorders. Ultimately, this may result in irreversible myocardial cell necrosis and apoptosis[15]. Therefore, inhibiting myocardial cell apoptosis, inflammation, and oxidative stress during reperfusion can effectively improve I/R-induced abnormalities in cardiac function and reduce MI area.

Hedgehog protein was initially found to be specifically expressed in the artery of zebra fish. From then on, hedgehog has been corroborated by growing studies to play an important role in the vascular development of mammals. Mice lacking smoothened, the sensor of hedgehog, in the embryo exhibit evident angiogenesis defects. *In vitro* investigation has proved that exogenous SHH can promote the formation of endothelial vascular plexus. Meanwhile, hedgehog signals can be activated to drive angiogenesis in many tissues of adult mice[16]. The hedgehog/VEGF/angiopoietin 2 signaling axis plays a pivotal role in the development of coronary arteries[17]. Besides, the SHH pathway needs to be activated in the development of embryonic heart outflow tract, coronary arteries, coronary veins, and ventricular septum in the cardiovascular system[18]. Numerous studies have indicated that hedgehog signals are important for maintaining normal functions of coronary arteries in adults. Specifically, once the adult heart is damaged by ischemia, hedgehog will be immediately activated to repair it. In terms of mechanism, SHH signals can be activated by the interaction between SHH protein and its receptor PTC1, while releasing the binding of SHH to the other receptor smoothened. As a result, the transcription factor GLI is activated to induce the expression of downstream genes PTC1 and GLI. The expressions of SHH, PTC1, and GLI are significantly activated in myocardial tissues of MI rats. Overexpression of SHH gene can further evidently improve MI-induced injury[19]. Notably, the protein expressions of the hedgehog signals hedgehog, PTC1 and GLI are markedly inhibited in the diabetic rat model of MI. However, SHH receptor stimulant SAG1.3 can distinctly relieve MI-induced myocardial injury and cardiac dysfunction in diabetic rats[20]. Besides, the hedgehog signaling pathway is markedly activated in the cartilage in osteoarthritis. Statins can inhibit the activation of the hedgehog signaling pathway to reverse cholesterol accumulation in the cartilage, ultimately delaying cartilage degradation[21]. In the present study, it was found that Sim preconditioning significantly improved the cardiac function of rats with myocardial I/R injury, reduced MI area, and repressed myocardial cell apoptosis, oxida-

tive stress, and inflammation. Moreover, Sim preconditioning evidently downregulated the protein expressions of SHH, PTC1 and GLI.

Conclusions

The present study first corroborates that Simvastatin can relieve myocardial I/R injury. The possible underlying may be related to its inhibition on the hedgehog signaling pathway. The novelty of this study was that Sim is likely to be a potential drug for the future treatment of myocardial I/R injury.

Conflict of Interests

The authors declare that they have no conflict of interests.

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