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Minocycline relieves myocardial ischemia-reperfusion injury in rats by inhibiting inflammation, oxidative stress and apoptosis

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Abstract. – OBJECTIVE: Myocardial ischemia-reperfusion (I/R) injury (MIRI) is an important cause of irreversible injury to the myocardium in patients with acute myocardial infarction. The purpose of this study was to investigate the effects of minocycline (MC) on inflammation, oxidative stress and apoptosis of myocardial tissues.

MATERIALS AND METHODS: We used rats to establish MIRI model by ligating coronary arteries. The structure and function of rat myocardium were determined by 2, 3, 5-triphenyl tetrazolium chloride (TTC) staining, hematoxylin-eosin (HE) staining and echocardiography. In addition, we detected the expression of inflammatory factors, antioxidant enzymes and apoptosis-related molecules in rats by enzyme-linked immunosorbent assay (ELISA), immunohistochemical (IHC) staining and reverse transcription-polymerase chain reaction (RT-PCR) to determine the effect of MC on inflammation, oxidative stress and apoptosis in I/R rats. Finally, we studied the effect of MC stimulation on the viability of rat cardiomyocytes (H9c2 cells) *in vitro*.

RESULTS: After I/R, the heart function of rats decreased, and the structure of myocardium was destroyed. The levels of inflammation and oxidative stress in I/R rats also increased significantly, manifested by increased inflammatory factors and decreased antioxidant enzymes in serum and myocardial tissue. After treatment of I/R rats with MC, the structure and function of rat myocardium improved significantly, and MC reduced inflammation and oxidative stress levels in rats, thus inhibiting the apoptosis of cardiomyocytes. MC also improved the viability of H9c2 cells *in vitro*.

CONCLUSIONS: MC reduced inflammation and oxidative stress levels in MIRI rat model or H9c2 cells, thus inhibiting cardiomyocyte apoptosis. Therefore, MC has potential application prospects for the treatment of MIRI.

Key Words:
Minocycline, Myocardial ischemia-reperfusion injury, Inflammation, Oxidative stress, Apoptosis.

Introduction

The mortality and disability rate of acute myocardial infarction (AMI) are very high, and the global morbidity rate is also increasing year by year[1]. The treatment of AMI mainly focuses on the rapid opening of infarct-related arteries and the rapid recovery of blood perfusion of myocardial tissue, resulting in the rescue of myocardial cells on the verge of death, the reduction of infarct size and the reduction in the incidence of adverse cardiovascular events[2]. For some patients, even if the blood supply is restored in the shortest time, the heart function cannot be restored due to the myocardial ischemia-reperfusion (I/R) process, and the heart dysfunction and injury will increase. This is called myocardial ischemia-reperfusion injury (MIRI)[3]. MIRI can lead to decreased myocardial systolic function, arrhythmia, myocardial energy metabolism disorders, ultrastructural changes, and non-reflow of blood vessels[4]. The pathological mechanism of MIRI is complex, involving factors such as oxygen free radicals, calcium overload, neutrophil activation, vascular endothelial cell damage and apoptosis[5]. MIRI during cardiac recovery will increase the original injury of myocardium and the occurrence of arrhythmia[6]. Therefore, how to prevent and alleviate MIRI is still a research hotspot in the medical field.

Minocycline (MC) is a second-generation semi-synthetic tetracycline antibiotic and can kill many Gram-positive or negative bacteria[7]. MC is commonly used in the treatment of infectious diseases such as acne, oral infections, syphilis, Lyme disease and cholera[8]. However, recent studies have found that MC has good anti-inflammatory and anti-oxidant effects. MC can inhibit the chemotaxis of neutrophils and eosinophils and reduce the formation of cytokines interleukin (IL)-6, tumor necrosis factor (TNF)-α and IL-2 during inflammation[9]. MC has also been found to inhibit the production of H_2O_2 and scavenge superoxide, peroxynitrite, and

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oxygen free radicals[10]. In addition, MC can inhibit the formation of activated caspase3 and increase the expression of anti-apoptotic proteins[11]. MIRI is accompanied by inflammatory injury, oxidative injury and cell apoptosis, and the role of MC for MIRI is not yet clear. Therefore, we used Sprague Dawley (SD) rats to make MIRI models and study the effect of MC on cardiomyocytes after I/R.

Materials and Methods

Animal and Grouping

A total of 100 healthy male SD rats (8 weeks old, 200±20 g) were used in this study. Rats were purchased from the Hunan University of Chinese Medicine Laboratory Animal Center and were housed in Specified Pathogen Free barrier facilities. The ambient temperature was 22-24°C and the ambient humidity was 40-60%. The light in the rat room was set to alternate between light and dark for 12 hours. Rats were fed normal rat food and water. The rats were divided into sham group, I/R group, I/R+low-dose MC (LMC, 20 mg/kg) group and I/R+high-dose MC (HMC, 40 mg/kg) group[12]. One week before the model was made, MC (Selleck, Shanghai, China) was used to inject rats intraperitoneally once a day. This study was approved by the Animal Ethics Committee of Hunan University of Chinese Medicine Animal Center.

Procedure of Ischemia-Reperfusion (I/R) Model

After measuring the body weight of the rats, the rats were anesthetized by intraperitoneal injection with 2% sodium pentobarbital (40 mg/kg). The rats were then fixed on the operating table in a supine position. We used scissors to remove the fur from the chest of the rats and disinfected the skin with iodophor. Small animal ventilator (CWE SAR-830, Orange, CA, USA) was used to maintain rats breathing. Then, we cut the skin on the left side of the sternum of the rats and cut off the 3-4th ribs. After the rat heart was exposed, we used scissors to cut the open envelope and found the anterior descending coronary artery between the left atrial appendage and the arterial cone. Then, we use sutures to ligate the coronary arteries. The ST segment elevation and the darkening of the corresponding area of myocardium indicated the success of the model establishment of myocardial ischemia. We then loosened the ligature after half an hour and left myocardial tissue reperfused for 4 hours. Finally, we examined the rats heart function and took the rats blood and myocardial tissue for subsequent experiments.

2, 3, 5-Triphenyl Tetrazolium Chloride (TTC) Staining

After collecting the rat heart, we washed the heart with normal saline. 2% agar at 37°C was used to perfuse the heart and place in a 4°C refrigerator for coagulation. The heart was then cut into 2 mm thick tissue slices. 2% TTC staining solution (Sigma-Aldrich, St. Louis, MO, USA) was used to stain myocardial tissue for 20 minutes. The dark red area was the active myocardium, while the area not stained by TTC was the necrotic myocardium. The degree of myocardial ischemia was expressed as the ratio of area at risk (AAR) and left ventricular area (LVR).

Cardiac Function Detection

After the MIRI model was finished, the rats were anesthetized with 2% sodium pentobarbital (40 mg/kg). Then, we used a 30 MHz high-resolution Vevo 770 small animal ultrasound instrument (Visual Sonics, Toronto, ON, Canada) for echocardiography. We took parameters from three consecutive cardiac cycles and calculated the average. Left ventricular ejection fraction (LVEF) and left ventricular short axis shortening rate (LVFS) were used to evaluate the cardiac function of rats.

Enzyme Linked Immunosorbent Assay (ELISA)

ELISA was used to detect the concentration of inflammatory factors, lactate dehydrogenase (LDH), glutathione peroxidase (GSH-px), catalase (CAT), SOD and malondialdehyde (MDA) in rat serum. The concentration and absorbance of the standard substance in the ELISA kits (R&D Systems, Emeryville, CA, USA) were used for a standard curve. Then, we calculated the concentration of each indicator based on the absorbance of the sample.

Haematoxylin-Eosin (HE) Staining

The rat heart was fixed in 4% paraformaldehyde and used to make paraffin blocks. We used a microtome to make paraffin blocks into 5 μm thick paraffin slices. The slices were placed in a 37°C incubator and baked for 3 days. Hematoxylin and eosin staining solution was used to stain the nucleus and cytoplasm, respectively. Through HE staining (Boster, Wuhan, China), we can observe the changes of rat myocardial structure.

Table I. RT-PCR primers sequences.

Name	Sense sequences (5′-3′)	Anti-sense sequences (5′-3′)
caspase3	TGGACAACAACGAAACCTC	ACACAAGCCCATTTCAGG
caspase9	GGAGTTGACTGAGGTGGGA	AGCAAGGAAGACACTGGGA
Bax	CGGCTGCTTGTCTGGAT	TGGTGAGTGAGGCAGTGAG
Bcl2	GTCACAGAGGGGCTACGA	GTCCGGTTGCTCTCAGG
GAPDH	GTTGTGGCTCTGACATGCT	CCCAGGATGCCCTTTAGT

Immunohistochemical (IHC) Staining

The slices were put into citrate buffer and heated to 95°C for 20 minutes to repair the antigen. 3% H_2O_2 was used to inactivate peroxidase. Then, 10% goat serum was used to block non-specific antigens. After incubating the myocardial tissue with the primary antibody dilution (SOD1, ab13498, Abcam, Cambridge, MA, USA; Prdx1, ab109506, Abcam, Cambridge, MA, USA; caspase3, 9662S, Cell Signaling Technology, Danvers, MA, USA; caspase9, ab184786, Abcam, Cambridge, MA, USA; Bcl2, ab59348, Abcam, Cambridge, MA, USA; Bax, ab32503, Abcam, Cambridge, MA, USA), we placed the slices in a 4°C refrigerator overnight. After washing the slices with phosphate-buffered saline (PBS), we incubated them with secondary antibody dilution (GeneTech, Shanghai, China) for 1 hour. Then, we used diaminobenzidine (DAB) for color development and stained the cell nucleus with hematoxylin. Then, we used an optical microscope to observe the staining results.

Cell Culture and Hypoxia-Reoxygenation (H/R) Model

The rat myocardial cell line, H9c2 cells, was used in this study. 10% fetal bovine serum (Gibco, Rockville, MD, USA) and 1% double antibody (Gibco, Rockville, MD, USA) was added into the Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Rockville, MD, USA) to configure the complete medium. H9c2 cells were cultured in a sterile incubator with 37°C and 5% CO_2. When the cell density reached 90%, we replaced the complete medium with phosphate-buffered saline (PBS) and filled the incubator with 95% N_2. After 4 hours, we replaced the PBS with the complete medium and put the cells back in the incubator with 37°C and 5% CO_2.

RNA Isolation and Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The total RNA in myocardial tissue and H9c2 cells was extracted by the TRIzol method (Invitrogen, Carlsbad, CA, USA). A spectrophotometer was used to detect the concentration of total RNA. HiScript Reverse Transcriptase (Vazyme, Nanjing, Jiangsu, China) was used for reverse transcription. We configured the reverse transcription system to obtain cDNA according to the manufacturer's instructions. Then, SYBR Green Master Mix (Vazyme, Nanjing, Jiangsu, China) was used for PCR. GAPDH expression was used as a reference. The relative expression level of RNA was represented as $2^{-\Delta\Delta CT}$. The primer sequences were shown in Table I.

Cell Counting kit-8 (CCK8) Assay

H9c2 cells were seeded into 96-well plates and treated according to experimental requirements. 10 μL of CCK8 reagent (Dojindo Molecular Technologies, Kumamoto, Japan) was added to each well. The cells were then placed in an incubator and incubated for 2 hours. Then, we used a microplate reader to detect the absorbance (OD) of each well of the 96-well plate at a wavelength of 450 nm in the dark. The cells in the control group were not treated. Cell viability = $(OD_{sample}-OD_{control}) / OD_{control}$.

5-Ethynyl-2′-Deoxyuridine (EdU) Cell Proliferation Assay

EdU cell proliferation assay was used to detect the proliferation ability of H9c2 cells. After the cells were treated according to experimental requirements, we used EdU (Sigma-Aldrich, St. Louis, MO, USA) to label proliferating H9c2 cells and 4',6-diamidino-2-phenylindole (DAPI) to label cell nucleus. We analyzed the proliferation ability of H9c2 cells by calculating the percentage of positive cells.

Terminal Deoxynucleotidyl Transferase (TdT) -Mediated dUTP-Biotin Nick End Labeling (TUNEL) Assay

TUNEL assay was used to detect the apoptosis level of H9c2 cells. After treating cells with 4% paraformaldehyde and 0.1% Triton X-100, we used TUNEL detection solution (Sigma-Aldrich,

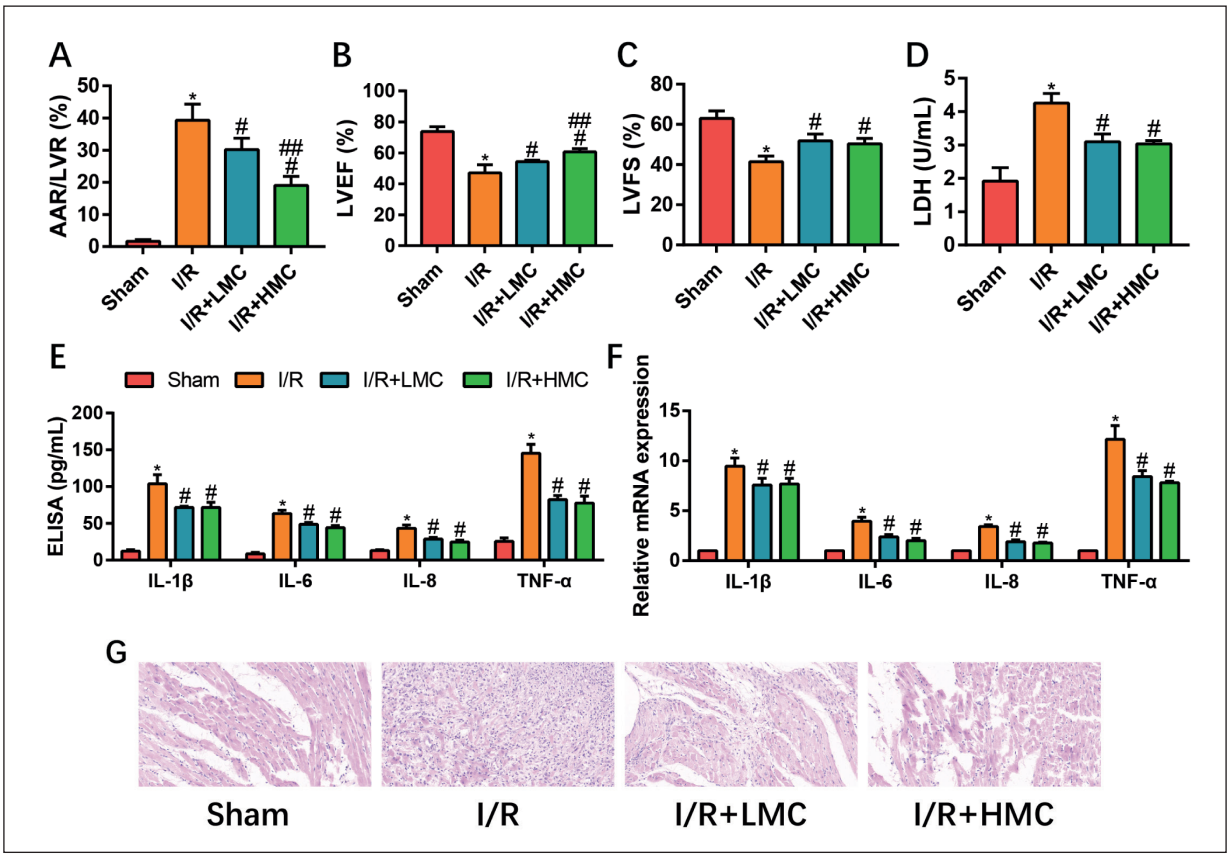

Figure 1. MC relieved I/R-induced myocardial injury and inhibited inflammation. **A**, The ratio of area at risk (AAR) and left ventricular area (LVR) represented the range of myocardial ischemia; **B**, **C**, Cardiac function of rats (LVEF and LVFS) was examined by echocardiography; **D**, LDH level in rat serum was examined by ELISA; Inflammatory cytokines (IL-1β, IL-6, IL-8 and TNF-α) in rat serum was examined by ELISA (**E**) and RT-PCR (**F**); **G**, HE staining of rat myocardium (200×). ("*" means $p<0.05$ *vs.* Sham group; "#" means $p<0.05$ *vs.* I/R group; "##" means $p<0.05$ *vs.* I/R+LMC group).

St. Louis, MO, USA) to incubate the cells in the dark for 1 hour. DAPI was used to stain the cell nucleus. Finally, we used a fluorescence microscope to observe the staining results and calculated the percentage of apoptotic cells.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 21.0 statistical software (IBM, Armonk, NY, USA) and GraphPad Prism 7.0 software (La Jolla, CA, USA) were used to analyze the results of this study. All continuous variables were expressed as mean ± standard deviation. Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ indicated that the difference was statistically significant. All experiments were repeated three times.

Results

MC Relieved I/R-Induced Myocardial Injury and Inhibits Inflammation

We first examined the effect of MC on myocardial structure and function in I/R rats. TTC staining was used to detect the myocardial ischemic area in rats. The AAR/LVR of I/R rats was significantly higher than that of the sham group. MC was found to improve the myocardial ischemia, while the effect of high and low concentration MC on AAR/LVR was not significantly different (Figure 1A). We used echocardiography to detect changes in rat cardiac function. After I/R, the LVEF (Figure 1B) and LVFS (Figure 1C) of the rats decreased. MC increased the LVEF and FS of rats, and higher concentrations of MC had better effects. In addition, we examined the expression of myocardial injury marker LDH. MC was also found to inhibit the increase in LDH in I/R rats (Figure 1D). ELISA (Figure 1E) and RT-PCR (Figure 1F) detected

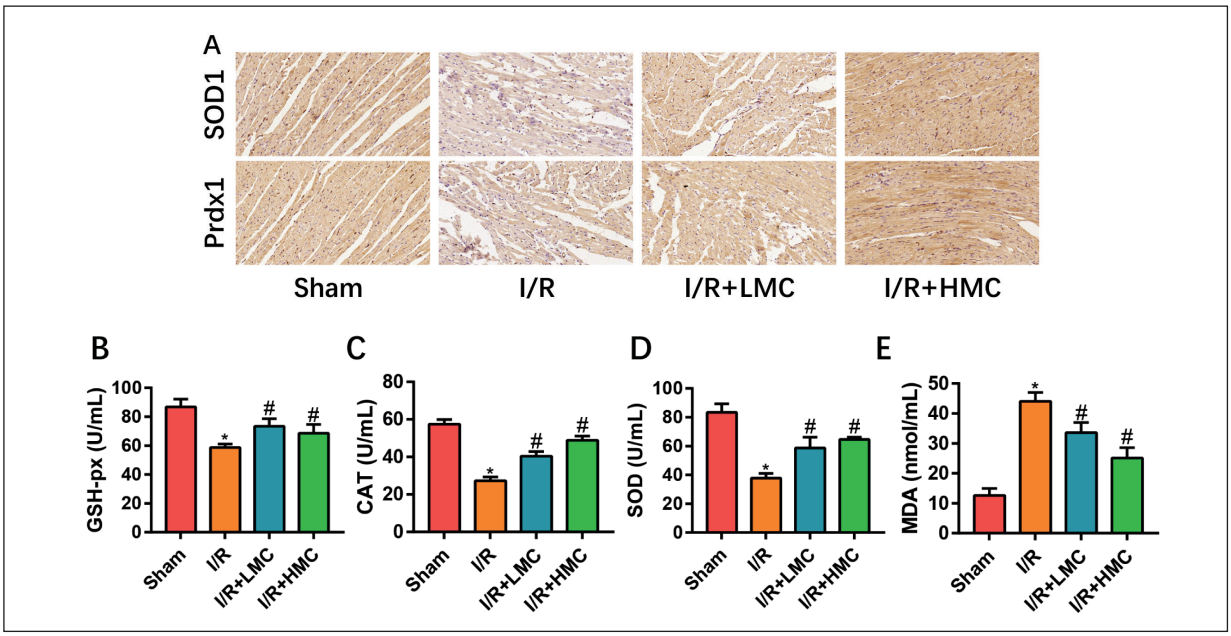

Figure 2. MC reduced I/R-induced oxidative stress in rat myocardium. **A**, IHC staining results of SOD1 and Prdx1 in rat myocardium (200×); **B-E**, The level of GSH-px, CAT, SOD and MDA in rat serum. ("*" means $p<0.05$ *vs.* Sham group and "#" means $p<0.05$ *vs.* I/R group).

protein and RNA expression of inflammatory factors in rat serum and myocardial tissue. IL-1β, IL-6, IL-8 and TNF-α increased in I/R rats, while MC reduced their expression. The results of HE staining also showed that the myocardial tissue of I/R rats was significantly destroyed, and MC can improve the structure of myocardial tissue (Figure 1G).

MC Reduced I/R-Induced Oxidative Stress in Rat Myocardium

Oxidative stress injury is also one of the important mechanisms of I/R-induced myocardial injury in rats, so we detected the changes in the oxidative stress level in I/R rat myocardial tissue. IHC staining detected the antioxidant enzymes SOD1 and Peroxiredoxin 1 (Prdx1) in myocardial tissue. SOD1 and Prdx1 in myocardial tissue of I/R rats were significantly reduced. After using MC to treat I/R rats, we found SOD1 and Prdx1 increased in rat myocardium (Figure 2A). In addition, we used ELISA to detect the levels of GSH-px (Figure 2B), CAT (Figure 2C), SOD (Figure 2D) and MDA (Figure 2E) in rat serum. MC was found to increase the concentration of GSH-px, CAT and SOD in I/R rats, while decreasing the level of MDA.

MC Inhibited I/R-Induced Cardiomyocyte Apoptosis in Rats

We detected the expression of caspase3, caspase9, Bcl2 and Bax in rat myocardial tissue by IHC (Figure 3A). The expression of pro-apoptotic molecules caspase3/9 and Bax in myocardial tissue of I/R rats increased, while the anti-apoptotic molecule Bcl2 was opposite. After treatment with MC, the apoptosis level of rat cardiomyocytes decreased. In addition, we detected the changes in mRNA levels of caspase3/9, Bcl2 and Bax by RT-PCR (Figure 3B). MC was also found to decrease the mRNA expression of caspase3/9 and Bax, and increase the mRNA expression of Bcl2.

MC Improved H/R-Induced H9c2 Cell Injury In Vitro

To determine the effect of MC on cardiomyocytes, we used MC to stimulate H9c2 cells *in vitro*. We examined the effects of 1, 3, 5, 7, 10, 15 and 20 μmol/L of MC on the viability of H9c2 cells through the CCK8 assay. 10 μmol/L was found to be the optimal concentration for MC stimulation of H9c2, so we used 10 μmol/L of MC to stimulate H9c2 cells in subsequent experiments (Figure 4A). We induced the I/R of H9c2 cells by H/R *in vitro*. ELISA detected LDH in H9c2 cells. The H9c2 cells in the H/R group expressed more LDH, while the stimulation of MC reduced LDH (Figure 4B). We also detected the change in the proliferation capacity of H9c2 cells through the EdU cell proliferation assay and found that MC increased the proliferation capacity of H9c2 cells

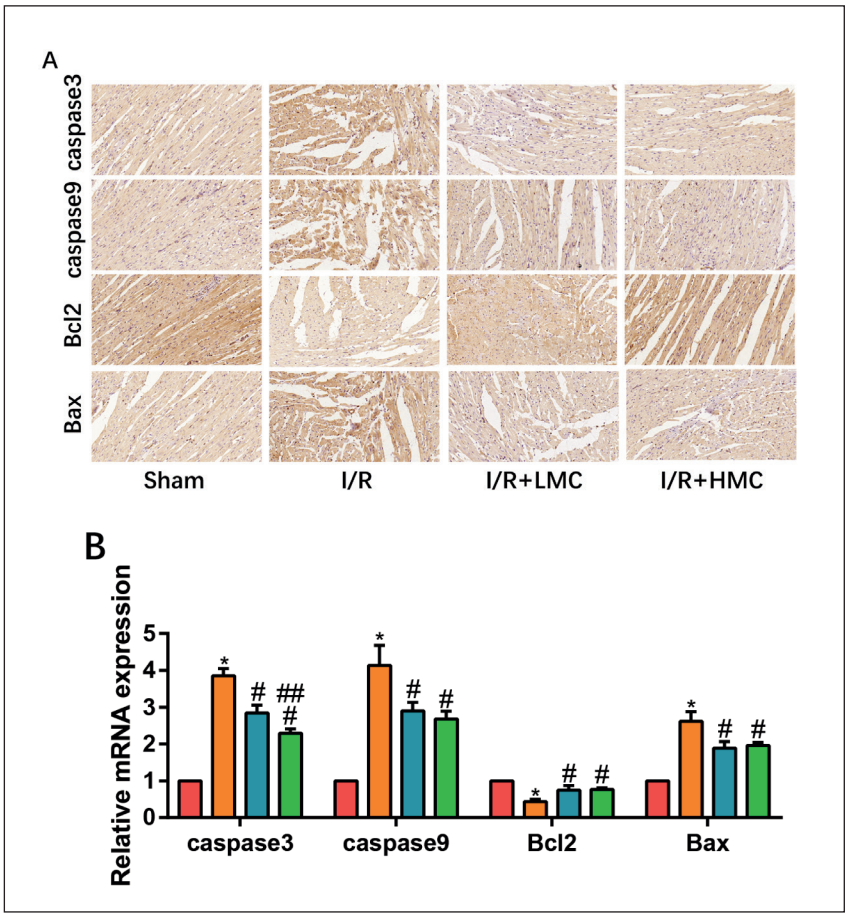

Figure 3. MC inhibited I/R-induced cardiomyocyte apoptosis in rats. **A**, IHC staining results of caspase3, caspase9, Bcl2 and Bax in rat myocardium (200×); **B**, mRNA of caspase3, caspase9, Bcl2 and Bax was examined by RT-PCR. ("*" means $p<0.05$ *vs.* Sham group; "#" means $p<0.05$ *vs.* I/R group; "##" means $p<0.05$ *vs.* I/R+LMC group).

in vitro (Figure 4C). In addition, we detected the apoptosis level of H9c2 cells by TUNEL assay. The induction of H/R increased the apoptosis rate of H9c2 cells, while the stimulation of MC inhibited the apoptosis of H9c2 cells (Figure 4D). The percentage of proliferating cells (Figure 4E) and the percentage of apoptotic cells (Figure 4F) were calculated according to staining results.

Discussion

MIRI is a complex pathological process involving multiple factors. Calcium overload, oxidative stress injury, inflammatory response and myocardial cell apoptosis are the main mechanisms of MIRI after reperfusion[13]. MC has been found to have pharmacological properties such as anti-inflammatory, anti-oxidation and anti-apoptosis in addition to good anti-microbial effects[14]. Our results revealed the potential therapeutic effect of MC on the rat MIRI model. The myocardium of rats with coronary artery ligation showed obvious inflammation. In addition, the anti-oxidant capacity of I/R rat myocardium was reduced and the level of cardiomyocyte apoptosis was increased. MC reduced inflammatory cytokines in myocardium and serum and increased the expression of anti-oxidant enzymes, thereby improving myocardial injury caused by inflammation and oxidative stress. The apoptosis of rat cardiomyocytes was thus decreased, and cardiac function was significantly improved. In addition, MC also improved H/R-induced H9c2 cell injury *in vitro*. Therefore, MC has potential application prospects for the treatment of MIRI.

Inflammation is an important pathophysiological process of MIRI. During myocardial I/R, activated NF-κB enters the cell nucleus as a nuclear transcription factor and initiates the expression of

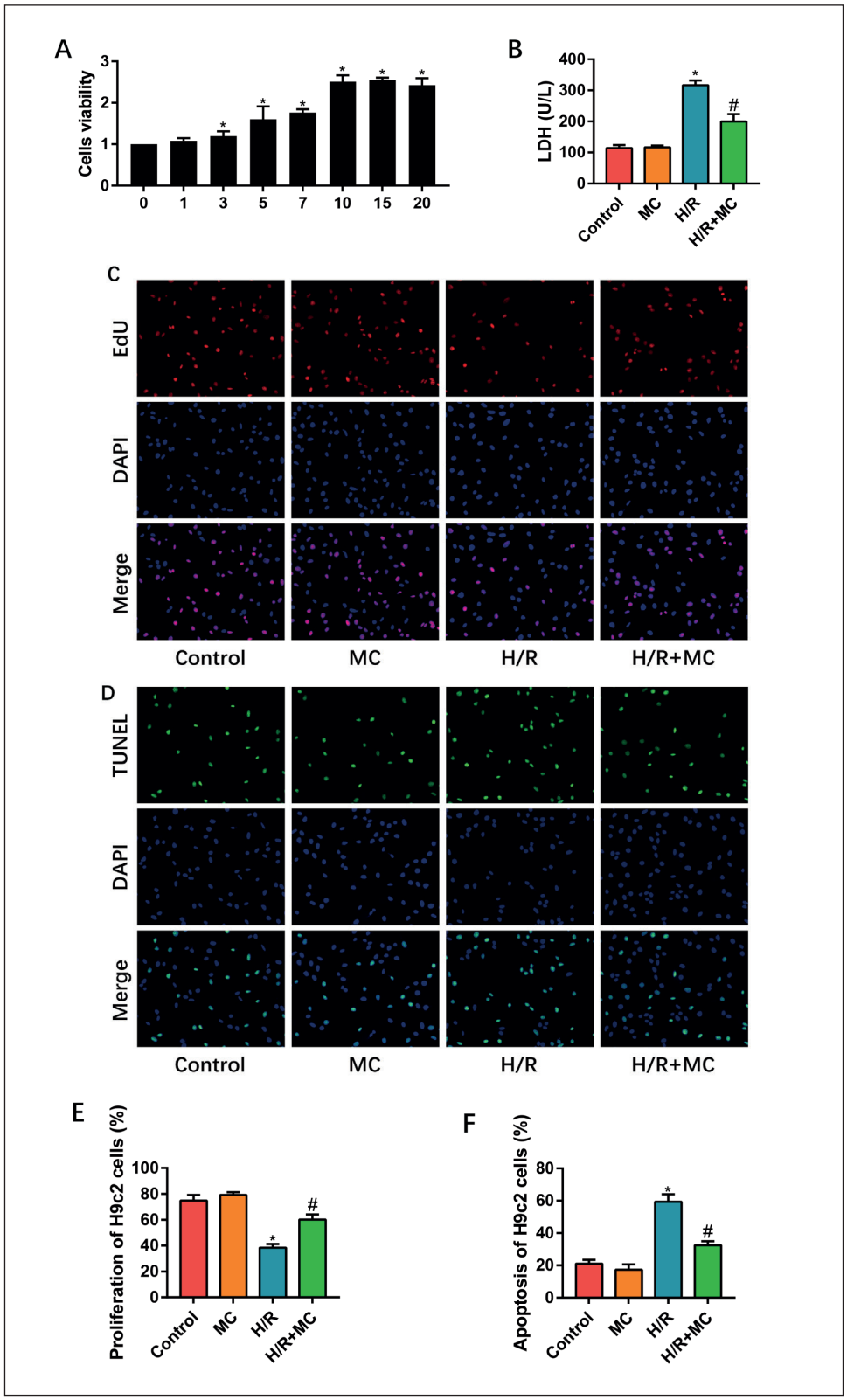

Figure 4. MC improved H/R-induced H9c2 cell injury *in vitro*. **A**, Cells viability of H9c2 cells was examined by CCK8 assay; **B**, LDH level in H9c2 cells was examined by ELISA; **C**, EdU staining of H9c2 cells (200×); **D**, TUNEL staining of H9c2 cells (200×); **E**, **F**, Results of EdU and TUNEL staining was quantified. ("*" means $p<0.05$ *vs.* control group and "#" means $p<0.05$ *vs.* H/R group).

inflammation-related genes such as TNF-α and IL-6, thereby promoting myocardial injury[15]. The myocardial inflammation in MIRI is characterized by infiltration of neutrophils in myocardial interstitium and increased expression levels of proinflammatory factors such as TNF-α and IL-1β in myocardial tissue[16]. Previous studies have found that inhibiting the activation of the inflammatory pathway NF-κB and down-regulating the expression of TNF-α and IL-6 can relieve MIRI[17]. Therefore, it can be considered that the inflammatory pathway mediated by the activated inflammatory pathway NF-κB plays an important regulatory role in MIRI. In our study, the expressions of various interleukins and TNF-α in serum and myocardial tissue were detected by ELISA and RT-PCR. The treatment with MC was found to significantly reduce inflammatory factors in I/R rats and the therapeutic effect was positively correlated with the concentration of MC. This showed that MC has a good anti-inflammatory effect on rats after I/R.

A study has shown that oxidative stress is inextricably linked to I/R injury[18]. MDA and SOD are the most commonly used and most sensitive indicators reflecting oxidative stress. Scavenging oxygen free radicals depends largely on SOD, and its activity directly affects the free radical scavenging ability. Scavenging free radicals in the body can help the body avoid oxidative damage, and also avoid the aggravation of I/R injury. When the human body is damaged by oxidative stress, the membrane lipid peroxidation reaction will also increase, and further aggravate the damage. The end product of this process is MDA. MDA increases with oxidative stress and cells can also be severely damaged, leading to increased production of free radicals[19]. We found that GSH-px, SOD and CAT in rats after I/R decreased significantly, while the concentration of MDA increased. This showed that MIRI led to a decrease in the antioxidant capacity of rats. MC improved the anti-oxidant capacity of I/R rats.

Apoptosis is one of the important links of MIRI pathogenesis, and the degree of apoptosis determines the severity of MIRI[20]. Apoptosis genes can be divided into two categories, including pro-apoptotic proteins (such as Bax, etc.) and anti-apoptotic proteins (such as Bcl2, etc.) When Bcl2 is combined with Bax, it can inhibit Bax-induced apoptosis. Under normal physiological conditions, Bcl2 and Bax levels are in dynamic equilibrium. When the body is subjected to external influences and the balance between Bcl2 and Bax is broken, Bax is secreted in large quantities. Bcl2 cannot fully bind Bax, leading to apoptosis[21]. The inhibition of Bax by MC and the promotion of Bcl2 indicated the anti-apoptotic effect of MC. In addition, MC also inhibited the activity of the caspase cascade pathway.

To sum up, this is the first study to investigate the effect of MC on MIRI. MC was first applied to the rat MIRI model and showed good therapeutic effect in the treatment of MIRI rat model. In addition, MC has been used in anti-infective therapy and has good safety. Therefore, MC may have potential preventive and therapeutic effects on MIRI.

Conclusions

The data reported above showed that MC effectively improved the structure and function of myocardium in rats after I/R. In addition, MC reduced the level of inflammation and oxidative stress in I/R rat myocardium, thereby inhibiting the apoptosis of myocardial cells. MC also improved the viability of H9c2 cells *in vitro*. Therefore, MC may become a new therapy for MIRI. Therefore, MC may be a new therapeutic agent for MIRI.

Conflict of Interest

The authors declare that they have no conflict of interests.

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