European Review for Medical and Pharmacological Sciences 2020; 24: 6371-6379

Oxycodone inhibits myocardial cell apoptosis after myocardial ischemia-reperfusion injury in rats *via* RhoA/ROCK1 signaling pathway

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Abstract. – OBJECTIVE: The purpose of this study was to investigate the effect of oxycodone on myocardial ischemia-reperfusion injury in rats through the Ras homolog gene family member A (RhoA)/Rho-associated coiled-coil containing protein kinase 1 (ROCK1) signaling pathway.

MATERIALS AND METHODS: A total of 48 Sprague-Dawley (SD) rats were randomly divided into sham operation group, model group, oxycodone group, and inhibitor group, with 12 rats in each group. The rats in the sham operation group only underwent thoracotomy without ischemia-reperfusion injury, those in the model group were used to prepare the myocardial ischemia-reperfusion model with normal saline intervention, those in the oxycodone group were used to prepare the myocardial ischemia-reperfusion model with oxycodone intervention, and those in the inhibitor group were utilized to prepare the myocardial ischemia-reperfusion model with AG490 intervention. Then, the expressions of B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (BAX) were detected by immunohistochemistry, the relative protein expressions of RhoA and ROCK1 were examined via Western blotting, and the messenger ribonucleic acid (mRNA) expressions of Bcl-2 and BAX were measured by quantitative Polymerase Chain Reaction (qPCR). Thereafter, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was adopted for apoptosis detection, and the levels of creatine kinase-muscle/brain (CK-MB), and cardiac Troponin I (cTnI) in serum were detected using an automatic biochemical analyzer.

RESULTS: Immunohistochemistry results showed that compared with those in the sham operation group, the positive expression of BAX was remarkably increased ($p<0.05$), while that of Bcl-2 was significantly decreased ($p<0.05$) in the model group, oxycodone group, and inhibitor group. Compared with the model group, oxycodone group and inhibitor group had an evidently reduced positive expression of BAX ($p<0.05$) and an evidently raised positive expression of Bcl-2 ($p<0.05$). No differences were found in the positive expressions of BAX and Bcl-2 between oxycodone group and inhibitor group ($p>0.05$). According to Western blotting results, the relative protein expressions of RhoA and ROCK1 in the model group, oxycodone group, and inhibitor group were notably increased compared with those in the sham operation group ($p<0.05$). In comparison with those in the model group, the relative protein expressions of RhoA and ROCK1 in the oxycodone group and inhibitor group were predominantly reduced ($p<0.05$). There were no differences in the relative protein expressions of RhoA and ROCK1 between oxycodone group and inhibitor group ($p>0.05$). Moreover, it was discovered from qRT-PCR results that compared with those in the sham operation group, the mRNA expression of BAX was markedly raised ($p<0.05$), whereas that of Bcl-2 was decreased predominantly ($p<0.05$) in the model group, oxycodone group, and inhibitor group. Compared with the model group, oxycodone group, and inhibitor group had an evidently reduced mRNA expression of BAX ($p<0.05$) and a significantly raised mRNA expression of Bcl-2 ($p<0.05$). No differences were found in the mRNA expressions of BAX and Bcl-2 between oxycodone group and inhibitor group ($p>0.05$). In addition, TUNEL assay results manifested that compared with sham operation group, model group, oxycodone group, and inhibitor group had a markedly elevated apoptosis rate ($p<0.05$). In comparison with the model group, the apoptosis rate in oxycodone group and inhibitor group was remarkably reduced ($p<0.05$). There was no difference in the apoptosis rate between oxycodone group and inhibitor group ($p>0.05$). According to biochemical analysis results, the serum levels of CK-MB and cTnI in model group, oxycodone group, and inhibitor group were significantly increased compared with those in the sham operation group, with statistically significant differences ($p<0.05$). The levels of serum CK-MB and cTnI in the oxycodone group and inhibitor group were substantially lowered in comparison with

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those in the model group, displaying statistically significant differences ($p<0.05$). Besides, the levels of serum CK-MB and cTnI in the oxycodone group were not different from those in the inhibitor group ($p>0.05$).

CONCLUSIONS: Oxycodone inhibits myocardial cell apoptosis after myocardial ischemia-reperfusion injury by suppressing the RhoA/ROCK1 signaling pathway.

Key Words:
Ischemia-reperfusion, Oxycodone, RhoA/ROCK1 signaling pathway, Apoptosis.

Introduction

Myocardial infarction is a critical and severe cardiovascular disease, whose incidence rate shows a year-by-year increasing trend, and patients tend to be younger due to changes in human lifestyle, increasing life pressure, and the accelerating pace of life. It often causes the death of patients and becomes a major killer of human life and health[1,2]. Myocardial ischemia-reperfusion is a pathological reaction of myocardial infarction. Myocardial ischemia-reperfusion injury may result in the apoptosis of myocardial cells, which further aggravates the injury of myocardial tissues and cells to affect the structure and function of the heart[3,4]. Therefore, myocardial cell apoptosis, as a crucial pathological reaction and pathogenesis of myocardial ischemia-reperfusion injury, exerts a pivotal effect on myocardial repair after myocardial ischemia-reperfusion injury.

As a vital signal transduction pathway in cells, the Ras homolog gene family member A (RhoA)/Rho-associated coiled-coil containing protein kinase 1 (ROCK1) signaling pathway exerts significant regulatory effects on cell proliferation, apoptosis, necrosis, and other physiological and pathological reactions[5,6], and is believed to participate in the pathogenesis of multiple cardiovascular diseases. Various cytokines can activate the RhoA/ROCK1 signaling pathway after myocardial ischemia-reperfusion injury[7,8], thus causing downstream calcium overload and further accelerating cell apoptosis and aggravating injury. Hence, the Rhoa/ROCK1 signaling pathway has become one of the vital target pathways for researching cardiovascular diseases.

Oxycodone, an opioid receptor agonist, is extensively applied in treating pain as an effective narcotic drug. Besides, oxycodone has been detected to remarkably improve myocardial ischemia-reperfusion, thus well protecting the myocardium, but its action mechanisms remain not very clear. This study aims to explore the effect of oxycodone on myocardial ischemia-reperfusion injury in rats through the RhoA/ROCK11 signaling pathway, so as to further clarify its mechanism of action.

Materials and Methods

Laboratory Animals

A total of 48 Specific Pathogen Free (SPF)-grade laboratory Sprague-Dawley (SD) rats aged 1 month old were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. [License No.: SCXK (Shanghai, China) 2014-0003]. The above rats were fed in the Laboratory Animal Center with normal feed and sterile filtered water daily under the conditions of a 12:12 h light/dark cycle, as well as normal room temperature and humidity. This study was approved by the Animal Ethics Committee of Xuhui District Central Hospital Animal Center.

Experimental Reagents and Instruments

Oxycodone (NMLH: J20130142), inhibitor: AG490 (CST, Danvers, MA, USA), primary antibodies: anti-B-cell lymphoma 2 (Bcl-2) antibody, anti-Bcl-2-associated X protein (BAX) antibody, anti-RhoA antibody and, anti-ROCK11 antibody and secondary antibody (Abcam, Cambridge, MA, USA), immunohistochemistry kit and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) apoptosis kit (Thermo Fisher Scientific, Waltham, MA, USA), AceQ quantitative Polymerase Chain Reaction (qPCR) SYBR Green Master Mix kit and HiScript II Q RT SuperMix for qPCR (+gDNA wiper) (Vazyme, Nanjing, China), optical microscope (Leica DMI 4000B/DFC425C, Wetzlar, Germany), and fluorescence qPCR instrument (ABI 7500, Waltham, MA, USA).

Animal Grouping and Treatment

The 48 SD rats were randomly allocated into the sham operation group (n=12), model group (n=12), oxycodone (n=12), and inhibitor group (n=12) using a random number table, and they were used for the experiment after 7 days of adaptive feeding in the Laboratory Animal Center.

In the sham operation group, the rats only underwent thoracotomy without ischemia-reper-

fusion injury. In the model group, the rats were instilled dropwise with normal saline through the femoral vein at 5 min before the myocardial ischemia-reperfusion model was prepared, followed by 30 min of myocardial ischemia, and the samples were obtained after reperfusion for 120 min. In oxycodone group, the rats were instilled dropwise with oxycodone (0.5 mg/kg) through the femoral vein at 30 min before the modeling of myocardial ischemia-reperfusion model, followed by 30 min of myocardial ischemia, and the samples were collected after 120 min of reperfusion. In the inhibitor group, the rats were instilled with AG490 (8 mg/kg) in drops through the femoral vein, and 30 min later they were instilled with oxycodone (0.5 mg/kg) through the femoral vein, followed by myocardial ischemia for 30 min. Then, the samples were harvested after 120 min of reperfusion.

Preparation of the Myocardial Ischemia-Reperfusion Model

A total of 5 mL/kg of 7% chloral hydrate was injected intraperitoneally into rats. After successful anesthesia, the rat chest was depilated to expose the skin. Following disinfection, the rats were firmly fixed, and the chest of the rats was cut open with scissors to expose the heart. Then, the anterior descending branch of the left coronary artery was searched, ligation was performed at 1/2 of the left coronary artery. It could be seen that the left anterior wall of the heart of the rat turned pale and the heart beats weakened. At the same time, the electrocardiogram monitoring of the rats showed ST segment elevation and a high T wave, indicating myocardial ischemia. After 45 min of ischemia, the ligature was removed to restore the blood supply of the anterior descending branch of the left coronary artery, and the rat state was closely observed. After the rats were in a stable state, the wounds were sutured layer by layer, and they rats were fed in a separate cage.

Sampling

After successful anesthesia, the abdominal aorta blood was collected, and then, the samples were directly extracted from 6 rats in each group. In brief, the heart tissues of the rats were taken out directly, washed with normal saline, and stored in an Eppendorf (EP; Hamburg; Germany) tube at -80°C for detection *via* Western blotting and quantitative Rea Time-Polymerase Chain Reaction (qRT-PCR). Subsequently, samples were collected from the remaining 6 rats fixed by perfusion. More specifically, the thoracic cavity of the rats was cut open to expose the heart, 400 mL of 4% paraformaldehyde was perfused from the left atrial appendage, and the heart tissues were taken out and soaked and fixed in 4% paraformaldehyde solution for immunohistochemistry and TUNEL assay.

Immunohistochemistry

The tissues embedded by paraffin in advance were cut into 5 μm-thick sections, followed by section flatting, fishing, and baking in 42°C warm water to prepare into paraffin-embedded tissue sections. Then, these sections were sequentially put into xylene solution and gradient alcohol for soaking, and conventional deparaffinization was carried out until hydration. After that, the sections were immersed in citric acid buffer solution, placed in a microwave oven for repeatedly heating for 3 times, with 3 min each time, and braised for 5 min for antigen retrieval. After rinsing, endogenous peroxidase blockers were dripped onto the samples, reacted for 10 min, and then, rinsed again. Subsequently, goat serum was dripped for sealing for 20 min, and after the goat serum sealing solution was removed, anti-BAX primary antibody (1:200), and anti-Bcl-2 primary antibody (1:200) were added and placed in a refrigerator at 4°C overnight. On the next day, the samples were rinsed and dripped with secondary antibody solution for reaction for 10 min. After fully rinsing, streptomyces avidin-peroxidase solution was added for reaction for 10 min. Finally, diaminobenzidine (DAB) was added dropwise for color development, and hematoxylin was applied to counterstain the nucleus, followed by mounting and observation.

Detection Via Western Blotting

The heart tissues preserved at ultra-low temperature were added with lysis buffer, subjected to ice bath for 1 h, and centrifuged for 10 min (14,000 g) in a centrifuge, followed by protein quantification using bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). The absorbance value and the standard curve obtained by protein detection through an enzyme labeling instrument were utilized for calculation of the protein concentration in tissues. Then, protein denaturation was carried out, dodecyl sulfate sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis was used to separate the proteins in tissue samples, and

the position of Marker proteins were observed. When Marker proteins were in a straight line at the bottom of a glass plate, and the electrophoresis was terminated. Subsequently, the proteins were transferred onto a polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), and anti-RhoA primary antibody (1:1000), anti-ROCK1 primary antibody (1:1000), and secondary antibody (1:1000) were sequentially added after the blocking solution was added for 1.5 h of reaction. After rinsing, the color was developed in the dark, and the chemiluminescent reagent was applied for reaction for 1 min to achieve complete color development.

Detection Via qRT-PCR

The reserved heart tissues were added into a ribonucleic acid (RNA) extraction reagent to extract the total RNA in samples. Then, reverse transcription was carried out on the extracted total RNA using a reverse transcription kit to obtain the complementary deoxyribonucleic acids (cDNAs), and the reaction system was designed to be 20 μL. Reaction conditions: reaction at 53°C for 5 min, pre-denaturation at 95°C for 10 min, denaturation at 95°C for 10 s, and annealing at 62°C for 30 s for 35 cycles. The Δ^{Ct} value was calculated first, and then, the difference in the expression of the target gene was calculated. Specific primer sequences are shown in Table I.

Apoptosis Detection Via TUNEL

The tissues embedded in paraffin in advance were cut into 5 μm-thick sections, placed into 42°C warm water for section flatting, fishing and baking, and prepared into paraffin-embedded tissue sections. Then, these paraffin-embedded tissue sections were put into xylene solution and gradient alcohol for soaking in sequence, and conventional deparaffinization was carried out until hydration. Thereafter, TdT enzyme reaction solution was added dropwise for reaction for 1 h in the dark, and deionized water was dripped for incubation for 15 min to terminate the reaction. Next, hydrogen peroxide was added in drops to block the endogenous peroxidase activity, working solution was dripped for 1 h of reaction, and diaminobenzidine (DAB) solution was dripped for color development after rinsing. Finally, mounting and observation were conducted after rinsing.

Detection of the Activity of Creatine Kinase-Muscle/Brain (CK-MB) and Cardiac Ttroponin I (cTnI)

The collected abdominal aorta blood was put into a centrifuge for centrifugation at 1500 rpm for 15 min. The supernatant was taken and put into a full-automatic biochemical analyzer to detect the activity of CK-MB and cTnI in serum.

Statistical Analysis

In this study, Statistical Product and Service Solutions (SPSS) 20.0 (IBM Corp., Armonk, NY, USA) software was adopted for statistical analysis. Count data were expressed as mean ± standard deviation. Data conforming to normal distribution and homogeneity of variance were detected by the *t*-test, those conforming to normal distribution and heterogeneity of variance were examined *via* the corrected *t*-test, and those not conforming to normal distribution and homogeneity of variance were detected using the non-parametric test. Comparison between multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). The rank-sum test was carried out for ranked data, and the chi-square test for count data. $p<0.05$ was considered statistically significant.

Table I. Primer sequences.

Name	Primer sequence
BAX	Forward primer: 5'TGTTTGATTCCTCGTCGCT3' Reverse primer: 5'GGCACCGTAATGGCACTG3'
Bcl-2	Forward primer: 5'TGGCAGTGTTGTTGT3' Reverse primer: 5'TTGGTTCAGCCACTGCCGAT3'
GADPH	Forward primer: 5'ACGGCAAGTTCAACGGCACAG3' Reverse primer: 5'GAAGACGCCAGTAGACTCCACGAC3'

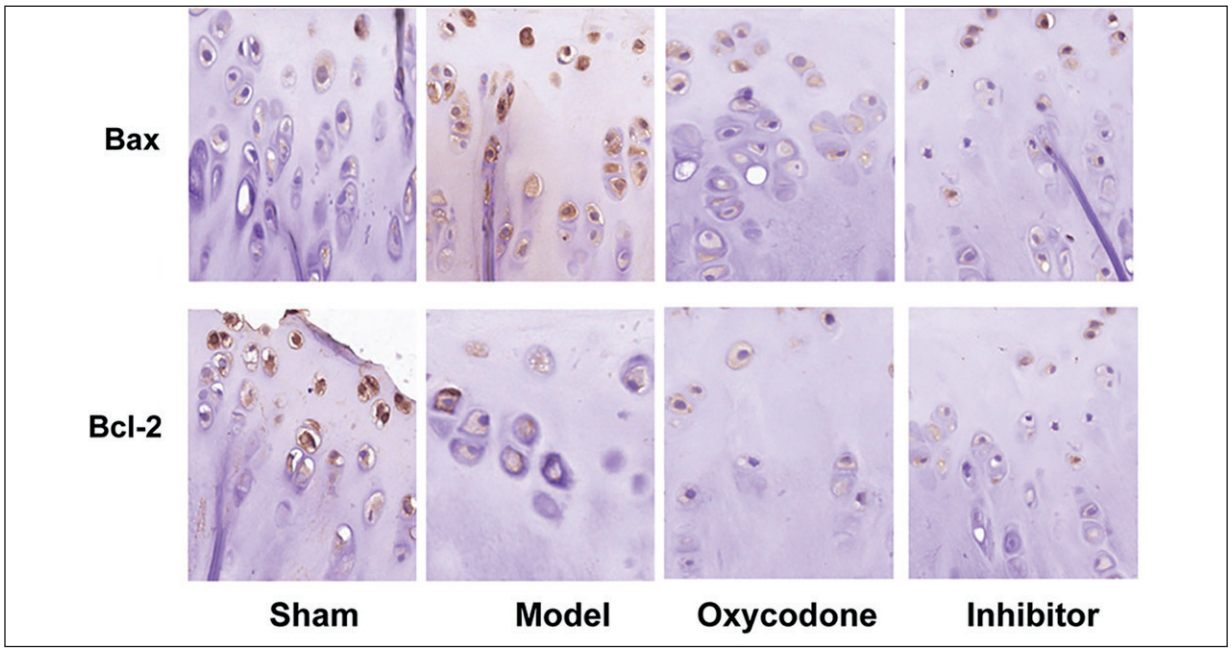

Figure 1. Expressions of BAX and Bcl-2 detected *via* immunohistochemistry (200×).

Results

Expressions of BAX and Bcl-2 Detected Via Immunohistochemistry

As shown in Figure 1, the positive expression was manifested as tan. Sham operation group had a lower positive expression level of BAX and a higher positive expression level of Bcl-2. On the contrary, model group had a higher positive expression level of BAX and a lower positive expression level of Bcl-2. According to the statistical results (Figure 2), compared with those in the sham operation group, the average optical density value of the positive expression of BAX was significantly increased, while that of the positive expression of Bcl-2 was prominently decreased in the model group, oxycodone group, and inhibitor group, with statistically significant differences ($p<0.05$). Additionally, in comparison with those in the model group, the average optical density value of the positive expression of BAX was remarkably reduced, but that of the positive expression of Bcl-2 was notably elevated in the oxycodone group and inhibitor group, showing statistically significant differences ($p<0.05$).

Protein Relative Expression Detected Via Western Blotting

It was found in Figure 3 that the protein expression levels of RhoA and ROCK1 were lower in the sham operation group and higher in the other three groups. The statistical results (Figure 4) manifested that compared with those in the sham operation group, the relative protein expression levels of RhoA and ROCK1 in the model group, oxycodone group, and inhibitor group were evidently increased, and the differences were statistically significant ($p<0.05$). Moreover,

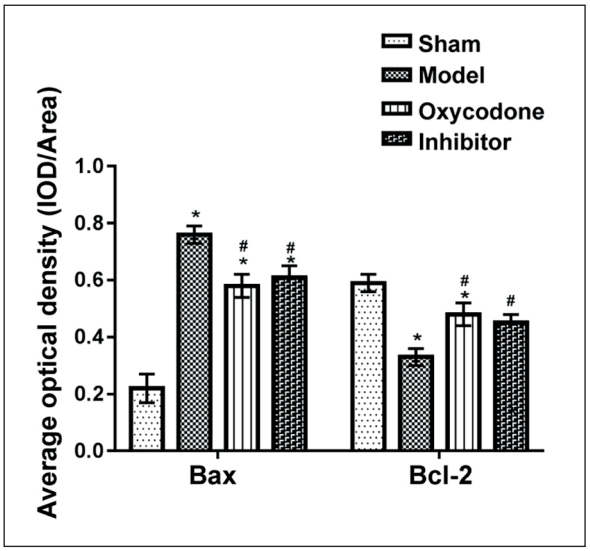

Figure 2. Average optical density values of the positive expressions of BAX and Bcl-2 in each group. *Note:* *$p<0.05$ *vs.* sham operation group, and $^{\#}p<0.05$ *vs.* model group.

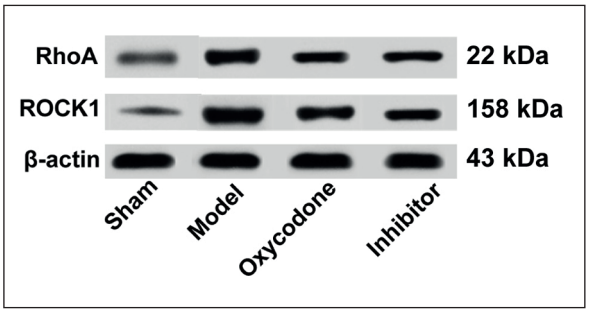

Figure 3. Protein expression detected by Western blotting.

compared with those in the model group, the relative protein expression levels of RhoA and ROCK1 in the oxycodone group and inhibitor group were markedly reduced, showing statistically significant differences (p<0.05).

QRT-PCR Detection Results

According to the statistical results (Figure 5), compared with those in the sham operation group, the relative messenger RNA (mRNA) expression of BAX was significantly increased, while that of Bcl-2 was prominently decreased in the model group, oxycodone group and inhibitor group, displaying statistically significant differences (p<0.05). Besides, in comparison with those in the model group, the relative mRNA expression of BAX was reduced remarkably, but that of Bcl-2 was notably elevated in the oxycodone group and inhibitor group, showing statistically significant differences (p<0.05).

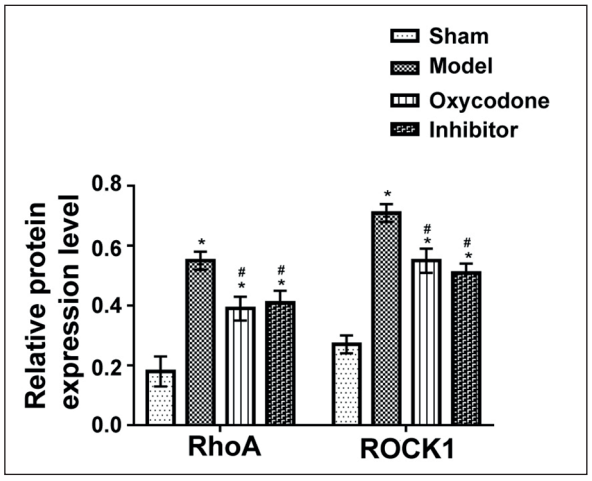

Figure 4. Relative protein expression level in each group *Note:* *p<0.05 *vs.* sham operation group, and #p<0.05 *vs.* model group.

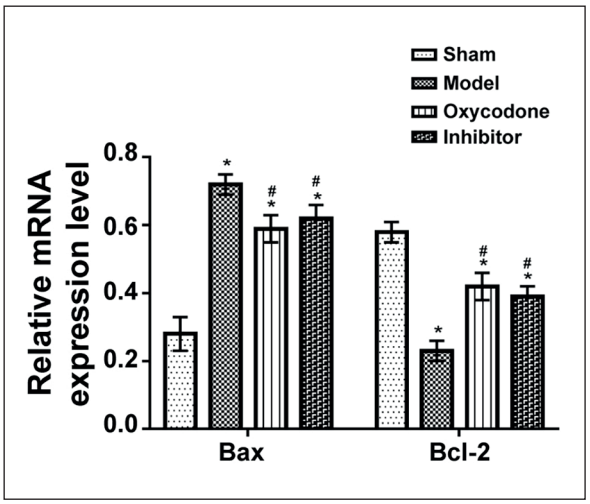

Figure 5. Relative mRNA expression level in each group *Note:* *p<0.05 *vs.* sham operation group, and #p<0.05 *vs.* model group.

Cell Apoptosis Rate Detected by TUNEL

It was discovered that the apoptotic cells were tan, and the number of apoptotic cells in the sham operation group was larger than that in the model group (Figure 6). The results (Figure 7) demonstrated that in comparison with that in sham operation group, the apoptosis rate in the model group, oxycodone group and inhibitor group was significantly increased, and the difference was statistically significant (p<0.05). Besides, compared with that in the model group, the apoptosis rate in oxycodone group and inhibitor group was significantly reduced, displaying a statistically significant difference (p<0.05).

Detection of the Activity of CK-MB and cTnI in Serum

It was revealed in Figure 8 that in comparison with those in the sham operation group, the levels of CK-MB and cTnI in the model group, oxycodone group, and inhibitor group were remarkably increased, and the differences were statistically significant (p<0.05). In addition, compared with those in the model group, the levels of CK-MB and cTnI in the oxycodone group and inhibitor group were predominantly reduced, displaying statistically significant differences (p<0.05).

Discussion

Ischemia-reperfusion, as a vital pathological process of myocardial infarction, substantially

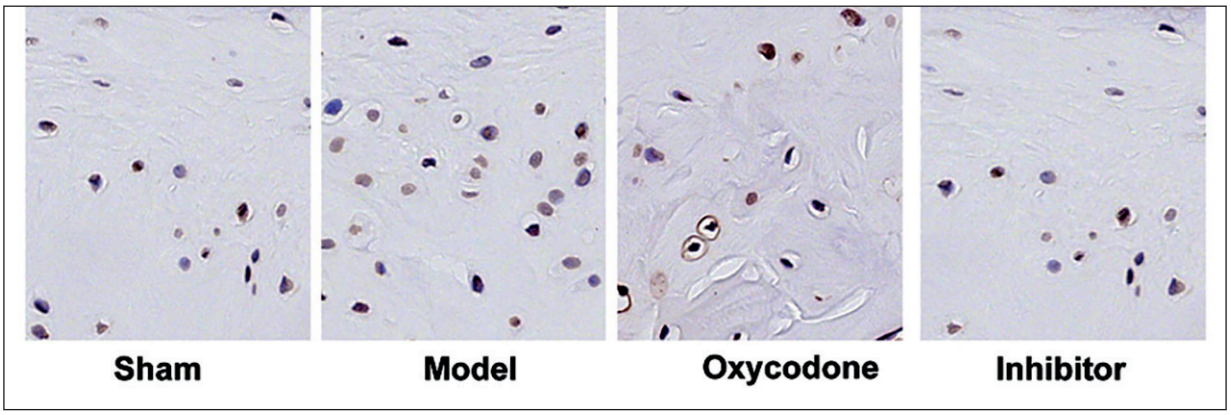

Figure 6. Cell apoptosis rate detected by TUNEL (200×).

affects the physiological and pathological states of myocardial cells, especially the apoptosis of myocardial cells[9,10]. During myocardial infarction, ischemia-reperfusion injury may further lead to myocardial cell apoptosis and other various pathological reactions. The release of various cytokines and inflammatory factors caused by ischemia-reperfusion injury can further activate several downstream signaling pathways[11,12], including the RhoA/ROCK1 signaling pathway. RhoA/ROCK1 signaling pathway is a crucial cell signal transduction pathway and has been proved to participate in the mediation of cell apoptosis after ischemia-reperfusion injury. RhoA, as a Rho protein with the largest quantity in the body, has the activity of GTP enzyme and exerts regulatory impacts on a variety of biological effects by modulating downstream target proteins, including ROCK1. GTP can combine with the Rho domain of downstream ROCK1 protein under the action of RhoA protein, thus activating ROCK1 protein and further regulating transcription and translation of downstream apoptosis-related target genes and proteins[13,14]. BAX and Bcl-2 play pivotal roles in the process of cell apoptosis. They can regulate cell apoptosis and maintain normal physiological functions of cells. BAX positively regulates apoptosis. In other words, it stimulates the occurrence of apoptosis[15,16]. However, Bcl-2 negatively modulates apoptosis. Specifically, it inhibits the occurrence of apoptosis[17]. Abnormal changes in BAX and Bcl-2 expressions can cause changes in the ratio of BAX/Bcl-2, imbalance between apoptosis and anti-apoptosis, and the apoptosis of cells. The results of this study demonstrated that significant abnormalities in the expressions

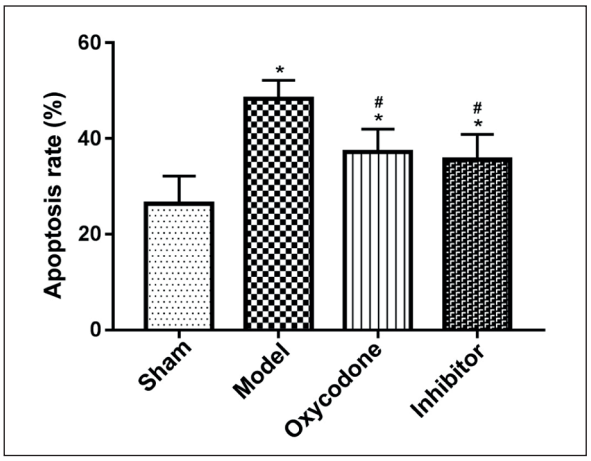

Figure 7. Apoptosis rate in each group. *Note:* **p*<0.05 *vs.* sham operation group, and #*p*<0.05 *vs.* model group.

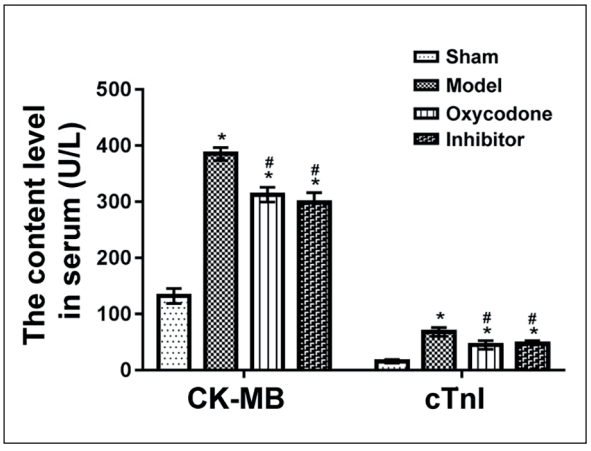

Figure 8. Levels of CK-MB and cTnI in each group. *Note:* **p*<0.05 *vs.* sham operation group, and #*p*<0.05 *vs.* model group.

of ROCK1, RhoA, BAX, and Bcl-2 in myocardial cells could be observed in the heart tissues of rats with myocardial ischemia-reperfusion injury, indicating that the RhoA/ROCK1 signaling pathway is activated, and the degree of myocardial cell apoptosis is aggravated in the heart tissues of rats with myocardial ischemia-reperfusion injury.

Oxycodone, as an analgesic commonly used in the opioid central nervous system in clinic, has a good exciting effect on opioid receptors so as to exert a potent analgesic effect[18]. Besides, oxycodone also well protects the organs and tissues of the body, improves ischemia-reperfusion injury, and especially exerts a good inhibitory effect on apoptosis caused by ischemia-reperfusion injury[19,20]. It was further observed that after myocardial ischemia-reperfusion injury, the expression of BAX facilitating the apoptosis in myocardial tissues was significantly raised, while that of Bcl-2 suppressing the apoptosis was remarkably reduced, which led to widespread myocardial cell apoptosis in myocardial tissues and the markedly elevated apoptosis rate of myocardial cells. However, after oxycodone intervention, the above results were reversed, implying that oxycodone is able to significantly suppress myocardial cell apoptosis caused by myocardial ischemia-reperfusion injury. To investigate the related mechanism of oxycodone, the key molecules RhoA and ROCK1 closely related to apoptosis in the RhoA/ROCK1 signaling pathway were detected in this study. It was discovered that the expressions of RhoA and ROCK1 in myocardial tissues were notably elevated after myocardial ischemia-reperfusion injury, suggesting the activation of the RhoA/ROCK1 signaling pathway, which is one of the possible mechanisms of myocardial cell apoptosis. However, oxycodone and the RhoA/ROCK1 signaling pathway inhibitor could remarkably lower the expressions of RhoA and ROCK1 in myocardial tissues after myocardial ischemia-reperfusion injury, which implies that oxycodone and the RhoA/ROCK1 signaling pathway inhibitor inhibit the RhoA/ROCK1 signaling pathway and further block myocardial cell apoptosis, and oxycodone has a similar effect to the RhoA/ROCK1 signaling pathway inhibitor.

Conclusions

To sum up, oxycodone suppresses myocardial cell apoptosis after myocardial ischemia-reperfusion injury by inhibiting the RhoA/ROCK1 signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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