European Review for Medical and Pharmacological Sciences 2019; 23: 6330-6338

Effect of propofol on myocardial ischemia/reperfusion injury in rats through JAK/STAT signaling pathway

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Abstract. – OBJECTIVE: The aim of this study was to investigate the influences of propofol on myocardial ischemia/reperfusion injury in rats through the Janus kinase/signal transducers and the activators of transcription (JAK/STAT) signaling pathway.

MATERIALS AND METHODS: A total of 48 Sprague-Dawley (SD) rats were randomly divided into four groups, including: the sham-operation group (n=12), the model group (n=12), the propofol group (n=12) and the inhibitor group (n=12). The rats in the sham-operation group only received thoracotomy, without the modeling of the ischemia/reperfusion injury. The model of myocardial ischemia/reperfusion injury was established in the rats of the model group, and the rats were given normal saline for intervention. The rats in the propofol group were utilized to prepare the model of myocardial ischemia/reperfusion injury and were intervened with propofol. Meanwhile, the rats in the inhibitor group received intervention with AG490 after the establishment of myocardial ischemia/reperfusion injury model. Immunohistochemistry was applied to detect the expressions of B-cell lymphoma-2 (Bcl-2) and Bcl-2-associated X protein (Bax). Western blotting was utilized to measure the relative protein expressions of phosphorylated JAK2 (p-JAK2) and p-STAT3. The messenger ribonucleic acid (mRNA) expressions of Bax and Bcl-2 were determined via quantitative Polymerase Chain Reaction (qPCR). Furthermore, cell apoptosis was examined using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay.

RESULTS: Immunohistochemistry results showed that compared with the sham-operation group, the positive expression of Bax remarkably increased ($p<0.05$), while Bcl-2 notably decreased ($p<0.05$) in the model group, propofol group, and inhibitor group. The propofol group and inhibitor group showed a significant lower positive expression of Bax ($p<0.05$) and evident higher positive expression of Bcl-2 ($p<0.05$) when compared with the model group. However, there were no significant differences in the positive expressions of Bax and Bcl-2 between the propofol group and inhibitor group ($p>0.05$). According to the results of Western blotting, the relative protein expression levels of p-JAK2 and p-STAT3 proteins were remarkably elevated in the model group, propofol group and inhibitor group in comparison with those in the sham-operation group ($p<0.05$). Propofol group and inhibitor group exhibited remarkably lower protein expression levels of p-JAK2 and p-STAT3 compared with the model group ($p<0.05$). However, no significant differences were observed in the protein expressions of p-JAK2 and p-STAT3 between propofol group and inhibitor group ($p>0.05$). The results of qPCR manifested that the mRNA expression of Bax was notably higher ($p<0.05$), whereas Bcl-2 was significantly lower ($p<0.05$) in the model group, propofol group and inhibitor group than those of the sham-operation group. Compared with the model group, the mRNA expression of Bax was evidently declined ($p<0.05$), while Bcl-2 was significantly elevated ($p<0.05$) in the propofol group and inhibitor group. Meanwhile, there were no evident differences in the mRNA expressions of Bax and Bcl-2 between the propofol group and inhibitor group ($p>0.05$). Subsequent TUNEL assay indicated that the model group, propofol group, and inhibitor group showed remarkably higher apoptosis rate than the sham-operation group ($p<0.05$). Moreover, the apoptosis rate was remarkably reduced in the propofol group and inhibitor group in comparison with the model group ($p<0.05$). However, no significant difference was observed in the apoptosis rate between propofol group and inhibitor group ($p>0.05$).

CONCLUSIONS: Propofol inhibits myocardial cell apoptosis after myocardial ischemia/reperfusion injury by repressing the JAK/STAT signaling pathway.

Key Words
Ischemia/reperfusion, Propofol, JAK/STAT signaling pathway, Apoptosis.

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Introduction

Myocardial ischemia/reperfusion is an important pathological response of heart diseases. It can induce multiple secondary pathological responses, including cell apoptosis, necrosis, and inflammation. Meanwhile, it further aggravates the damage to myocardial tissues and cells, thereby affecting cardiac structure and function[1,2]. Therefore, how to effectively control myocardial ischemia/reperfusion injury after the attack of myocardial diseases, reduce the destruction of pathological responses triggered by myocardial ischemia/reperfusion to myocardial tissues and cells, as well as to accelerate the recovery of cardiac structure and function after myocardial ischemia/reperfusion injury has become a hotspot in the current research.

Numerous studies have suggested that myocardial ischemia/reperfusion injury can mediate various crucial secondary pathological responses. In particular, mediated myocardial cell apoptosis is one of the vital mechanisms leading to the death of myocardial cells and the destruction of myocardial tissue structure[3,4]. Scholars[5,6] have revealed that extensive and persistent apoptosis of myocardial cells may occur after myocardial ischemia/reperfusion injury. This may further result in changes in myocardial tissue structure and function, which is unfavorable for post-injury tissue repair. Hence, the effective regulation of cell apoptosis after myocardial ischemia/reperfusion injury is considered as a new direction for in-depth research. As an important cell signal transduction pathway, the Janus kinase/signal transducers and the activators of the transcription (JAK/STAT) signaling pathway play a critical regulatory role in cell growth, development, proliferation, apoptosis, and necrosis[7]. Previous authors[8,9] have illustrated that the JAK/STAT signaling pathway is activated under the control of many cytokines after myocardial ischemia/reperfusion injury. Particularly, the phosphorylation of JAK2 and STAT3 is initiated to mediate myocardial cell apoptosis, which is regarded as an important pathological mechanism of apoptosis after myocardial ischemia/reperfusion injury.

As one of the most common intravenous anesthetics in the clinic, propofol is widely used for the anesthesia induction and maintenance in surgery, as well as postoperative sedation in patients with cardiovascular diseases. Jia et al[8] have proved that propofol shows preferable protective effects on myocardial cells, which can also effectively reduce myocardial ischemia/reperfusion injury. The underlying mechanism involves repressing oxidative stress responses after myocardial injury, lipid peroxidation, and cell apoptosis after myocardial ischemia/reperfusion injury and decreasing calcium overload. However, the mechanism of propofol in inhibiting cell apoptosis after myocardial ischemia/reperfusion injury has not been fully clarified. Considering the vital regulatory role of the JAK/STAT signaling pathway in cell apoptosis after myocardial ischemia/reperfusion injury, the aim of this study was to investigate the effects of propofol on myocardial ischemia/reperfusion in rats through the JAK/STAT signaling pathway. Furthermore, we investigated the possible underlying mechanism.

Materials and Methods

Laboratory Animals

A total of 48 SPF laboratory Sprague-Dawley (SD) rats aged 1-month-old were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. [license number: SCXK (Shanghai, China) 2014-0003]. All rats were fed in the Laboratory Animal Center with normal diet and sterile filtered water every day under the conditions of 12/12 h light/dark cycle, conventional room temperature, and humidity. This study was approved by the Animal Ethics Committee of Dalian Medical University Animal Center.

Experimental Reagents and Instruments

Inhibitor: AG490 (CST, USA); primary antibodies: anti-B-cell lymphoma-2 (Bcl-2) antibody, anti-Bcl-2-associated X protein (Bax) antibody, anti-phosphorylated JAK2 (p-JAK2) antibody (Abcam, Cambridge, MA, USA), anti-p-STAT3 antibody (Abcam, Cambridge, MA, USA), secondary antibodies (Abcam, Cambridge, MA, USA), immunohistochemistry kit, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) apoptosis kit, AceQ quantitative Polymerase Chain Reaction (qPCR) SYBR Green Master Mix kit (Vazyme, Nanjing, China), HiScript II Q RT SuperMix for qPCR (+gDNA wiper) kit (Vazyme, Nanjing, China), light microscope (Leica DMI 4000B/DFC425C, Wetzlar, Germany), (Thermo Fisher, Waltham, MA, USA), fluorescence qPCR instrument (ABI 7500, Applied Biosystems, Foster City, CA, USA).

Animal Grouping and Treatment

All 48 SD rats were divided into four groups using a random number table, including: the sham-operation group (n=12), model group (n=12), propofol group (n=12) and inhibitor group (n=12). The rats were fed adaptively in the Laboratory Animal Center for 7 d for subsequent experiments.

The rats in the sham-operation group only received thoracotomy, without the modeling of the ischemia/reperfusion injury. The rats in model group were given femoral intravenous instillation of normal saline at 3 min before the establishment of myocardial ischemia/reperfusion injury model. This was stopped at 5 min after myocardial ischemia/reperfusion, followed by specimen acquisition. Femoral intravenous infusion of propofol (2 mg/kg) and AG490 (8 mg/kg) was started at 3 min before the modeling of myocardial ischemia/reperfusion injury and stopped at 5 min after myocardial ischemia/reperfusion in the model group and inhibitor group, respectively. Subsequently, the specimens were collected.

Establishment of Myocardial Ischemia/Reperfusion Injury Model in Rats

The rats were first intraperitoneally injected with 7% chloral hydrate (5 mL/kg). After successful anesthesia, the hair on the chest of rats was removed to expose the skin. Subsequently, the rats were disinfected and fixed firmly, and the chest was cut open using a pair of scissors to expose the heart. Next, the left anterior descending coronary artery was found and ligated at 1/2. Myocardial ischemia was induced when the left anterior wall of the heart turned pale, the heartbeat was attenuated, and the electrocardiographic monitoring showed ST-segment elevation and high-amplitude T wave. 45 min later, the ligation was removed, and the blood supply to the left anterior descending coronary artery was restored. Meanwhile, the state of the rats was closely observed. After the rats were in a stable state, the wound was sutured layer by layer. Finally, all rats were raised in separate cages.

Specimen Acquisition

After successful anesthesia, the specimens were directly obtained from 6 rats in each group. Briefly, the cardiac tissues were taken out directly, washed with normal saline, put into Eppendorf (EP) tubes (Hamburg, Germany) and stored at -80°C for Western blotting and qPCR. As for the remaining 6 rats in each group, the specimens were obtained via perfusion-fixation. The thoracic cavity of rats was cut open to expose the heart. Meanwhile, 400 mL 4% paraformaldehyde was perfused from the left atrial appendage. After that, the cardiac tissues were taken out and soaked in 4% paraformaldehyde solution for fixation, followed by immunohistochemistry and TUNEL.

Immunohistochemistry

Tissues embedded in paraffin were sliced into 5 μm-thick sections, followed by spreading in warm water at 42°C, collection, and baking. Paraffin-embedded sections were then successfully prepared. Next, the sections were soaked in xylene solution and graded alcohol, followed by a routine deparaffinization until rehydration. After immersed in citric acid buffer solution, the sections were heated in a microwave oven repeatedly for 3 times (3 min/time) and braised for 5 min, to achieve adequate antigen retrieval. The endogenous peroxidase blocker was then added dropwise onto the specimens and reacted for 10 min after rinsing. After that, the specimens were rinsed and added with goat serum in drops for 20 min of sealing. After the goat serum blocking buffer was shaken off, anti-Bax primary antibody (1:200) and anti-Bcl-2 primary antibody (1:200) were added, followed by incubation in a refrigerator at 4°C overnight. On the next day, the specimens were rinsed and added dropwise with the corresponding secondary antibody for 10 min of incubation. After rinsing adequately, the streptavidin-peroxidase solution was added for 10 min of reaction, followed by color development with diaminobenzidine (DAB), the counterstaining of the nucleus with hematoxylin, mounting, and observation.

Western Blotting Assay

Lysis buffer was first added into cryopreserved cardiac tissues for an ice bath for 1 h, Then the tissues were centrifuged at 14,000 g for 10 min. The concentration of extracted protein was quantified by the bicinchoninic acid (BCA) method (Abcam, Cambridge, MA, USA). Next, the absorbance and standard curve of protein were obtained by a microplate reader. The concentration of proteins in tissues was then calculated. Subsequently, the proteins were separated via sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The position of Marker proteins was observed, and the electrophoresis was stopped when Marker proteins reached the bottom of the glass

plate in a straight line. After that, the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland) and reacted with blocking buffer for 1.5 h. Subsequently, the membranes were incubated with primary antibodies of anti-p-JAK2 (1:1000) and anti-STAT3 (1:1000) overnight. On the next day, the membranes were incubated with corresponding secondary antibodies (1:1000). The immunoreactive bands were developed with a chemiluminescent reagent in the dark for 1 min.

QPCR

Cardiac tissues preserved for standby use were added with ribonucleic acid (RNA) extraction reagent to extract total RNA in specimens. Extracted total RNA was then reverse transcribed into complementary deoxyribose nucleic acid (cDNA) using a reverse transcription kit, with a reaction system of 20 μL. Specific reaction conditions were as follows: 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 a total of 35 cycles. ΔCt was first calculated, and the expression of target genes was then calculated. The primer sequences used in this study were shown in Table I.

TUNEL Apoptosis Assay

The tissues embedded in paraffin were first sliced into 5 μm-thick sections, followed by spreading in warm water at 42°C, collection, and baking. After that, paraffin-embedded sections were successfully prepared. Then, the sections were soaked in xylene solution and graded alcohol, followed by a routine deparaffinization until rehydration. TdT solution was added in drops for reaction in the dark for 1 h. Subsequently, deionized water was added dropwise, followed by incubation for 15 min to terminate the reaction. Next, hydrogen peroxide was added in drops to block the activity of the endogenous peroxidase, followed by the addition of the working solution for 1 h of reaction. After rinsing, the DAB solution was added for color development, followed by mounting for the observation.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM Corp., Armonk, NY, USA) was used for all statistical analysis. The enumeration data were expressed as mean ± standard deviation. The *t*-test was performed for data meeting the normal distribution and homogeneity of variance. A corrected *t*-test was used for data meeting the normal distribution and heterogeneity of variance and non-parametric test for data not meeting normal distribution and homogeneity of variance. One-way ANOVA was used to compare the differences among different groups, followed by the post-hoc test (Least Significant Difference). The rank sum test was utilized for ranked data, and a Chi-square test was utilized for enumeration data. $p<0.05$ was considered statistically significant.

Results

Expressions of Bax and Bcl-2 Detected Via Immunohistochemistry

As shown in Figure 1, the cells with positive expressions were sepia. The sham-operation group showed a significant lower positive expression level of Bax and an evident higher positive expression level of Bcl-2. In contrast, the model group exhibited evident higher positive expression level of Bax and lower positive expression level of Bcl-2. According to the statistical results (Figure 2), the average optical density of the positive expression of Bax remarkably increased in the model group, propofol group, and inhibitor group when compared with the sham-operation group ($p<0.05$). However, Bcl-2 notably decreased in the model group, propofol group, and inhibitor

Table I. Primer sequences.

Name	Primer sequence
Bax	Forward primer: 5'TGTTTGATTCCTCGTCGCT 3' Reverse primer: 5'GGCACCGTAATGGCACTG 3'
Bcl-2	Forward primer: 5'TGGCAGTGTCTTAGCTGGTTGT 3' Reverse primer: 5'TTGGTTCAGCCACTGCCGAT 3'
GAPDH	Forward primer: 5'ACGGCAAGTTCAACGGCACAG 3' Reverse primer: 5'GAAGACGCCAGTAGACTCCACGAC 3'

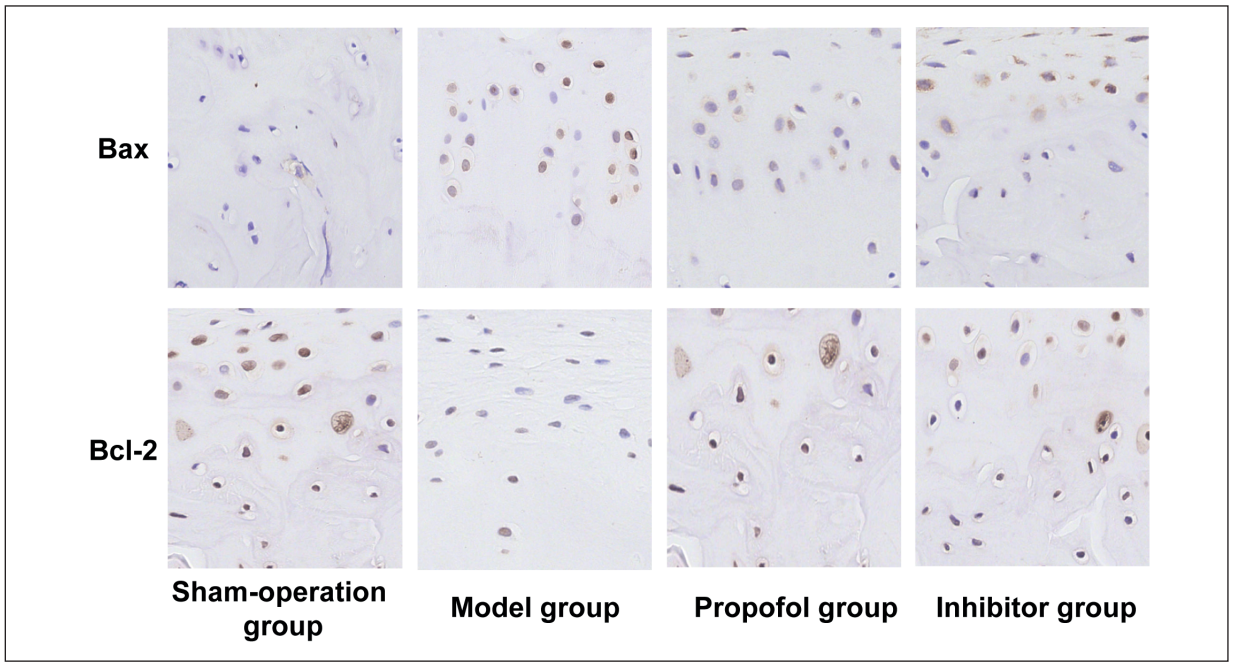

Figure 1. Expressions of Bax and Bcl-2 detected via immunohistochemistry (×200).

group, displaying statistically significant differences (p<0.05). In comparison with the model group, the propofol group and inhibitor group exhibited (evidently) an evident reduced average optical density of the positive expression of Bax and up-regulated the average optical density of the positive expression of Bcl-2 (p<0.05). However, no significant differences were observed in the positive expressions of Bax and Bcl-2 between propofol group and inhibitor group (p>0.05).

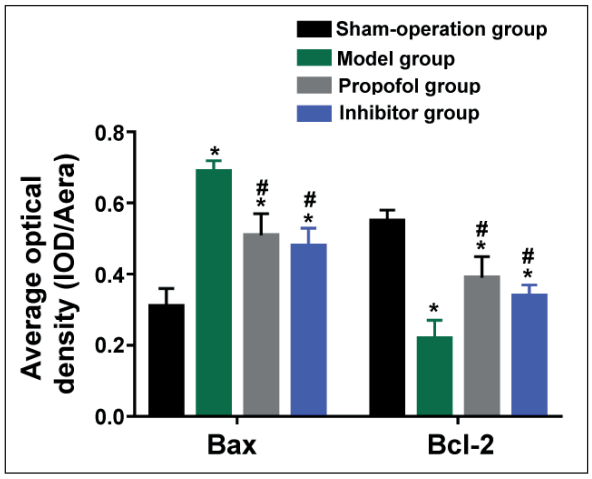

Figure 2. Average optical density of positive expression in each group. Note: p*<0.05 *vs.* sham-operation group, $p^{\#}$<0.05 *vs.* model group.

Relative Expressions of Proteins Detected Via Western Blotting

The protein expressions of p-JAK2 and p-STAT3 were significantly lower and higher in the sham-operation group and model group, respectively (Figure 3). The statistical results (Figure 4) showed that the relative expression levels of p-JAK2 and p-STAT3 proteins were markedly elevated in the model group, propofol group, and inhibitor group in comparison with the sham-operation group, with statistically significant differences (p<0.05). The propofol group and inhibitor group manifested significantly lower protein expression levels of p-JAK2 and p-STAT3 than the

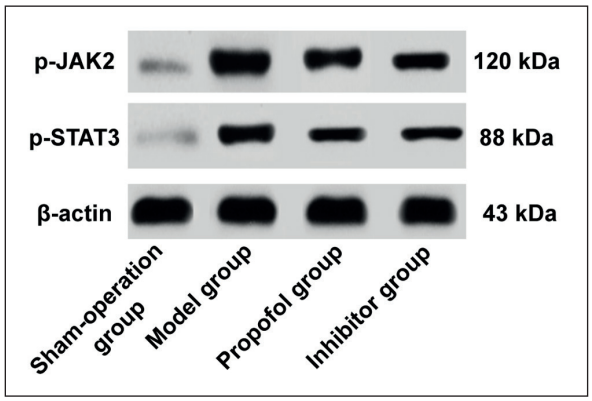

Figure 3. Protein expressions detected via Western blotting.

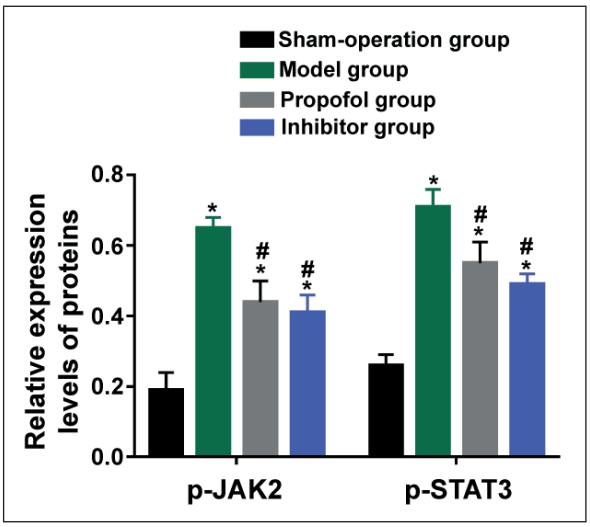

Figure 4. Relative expression levels of proteins in each group. Note: $p^{*}<0.05$ *vs.* sham-operation group, $p^{\#}<0.05$ *vs.* model group.

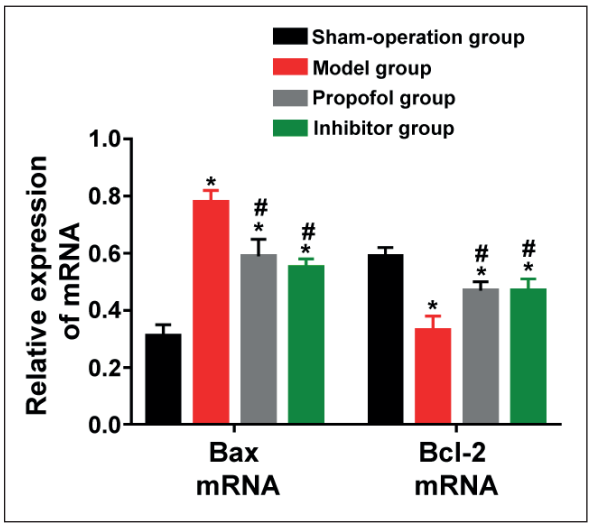

Figure 5. Relative expression of mRNA in each group. Note: $p^{*}<0.05$ *vs.* sham-operation group, $p^{\#}<0.05$ *vs.* model group.

model group, and the differences were statistically significant ($p<0.05$). However, no evident differences were found in the protein expression levels of p-JAK2 and p-STAT3 between the propofol group and inhibitor group ($p>0.05$).

Relative Expressions of mRNAs Detected Via qPCR

As shown in Figure 5, compared with the sham-operation group, the messenger RNA (mRNA) expression of Bax was evidently higher, while Bcl-2 was prominently lower in the model group, propofol group, and inhibitor group ($p<0.05$). In comparison with the model group, the mRNA expression of Bax remarkably decreased, while Bcl-2 notably increased in the propofol group and inhibitor group, showing statistically significant differences ($p<0.05$). However, there were no significant differences in the mRNA expressions of Bax and Bcl-2 between the propofol group and inhibitor group ($p>0.05$).

Apoptosis Rate Detected Via TUNNEL

As shown in Figure 6, the apoptotic cells were sepia. The sham-operation group had fewer apoptotic cells, while the model group had more apoptotic cells. The model group, propofol group, and inhibitor group showed significantly higher apoptosis rates than the sham-operation group ($p<0.05$) (Figure 7). Compared with the model group, the apoptosis rate was evidently declined in the propofol group and inhibitor group, and the differences were statistically significant ($p<0.05$). However, no evident difference was observed in the apoptosis rate between the propofol group and the inhibitor group ($p>0.05$).

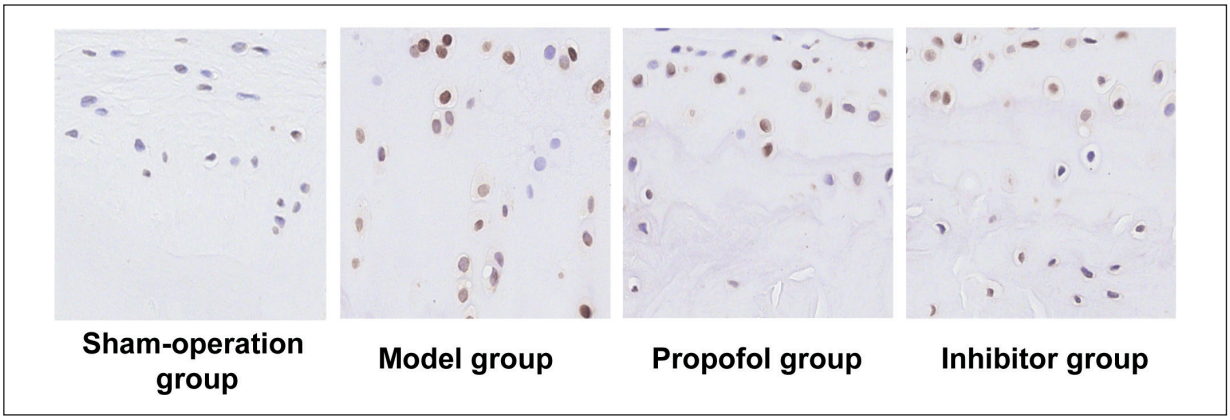

Figure 6. Cell apoptosis detected via TUNNEL (×200).

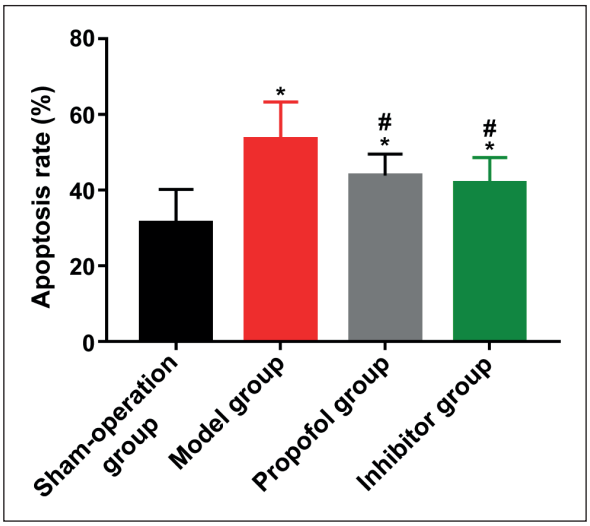

Figure 7. Apoptosis rate in each group. Note: $p^{*}<0.05$ *vs.* sham-operation group, $p^{\#}<0.05$ *vs.* model group.

Discussion

Ischemia/reperfusion is a critical pathological process of heart diseases. It poses great impacts on the physiological and pathological conditions of myocardial cells. In particular, it shows important regulatory effects on the apoptosis of myocardial cells[10,11]. Apoptosis is a crucial programmed cell death, which can lead to the destruction and dysfunction of the myocardial tissue structure. Currently, it is believed that the multiple cytokines released after injury can activate several cell signal transduction pathways, thereby mediating pathological damage secondary to injury. The JAK/STAT signaling pathway, especially the JAK2/STAT3 signaling pathway, is an essential cell signal transduction pathway. It has been demonstrated to be involved in modulating the ischemia/reperfusion injury, especially cell apoptosis after injury[12,13]. There are 4 proteins in the JAK family namely JAK1, JAK2, JAK3, and Tyk2. Meanwhile, there are 7 members in the STAT family, including STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6. Among them, JAK2 and STAT3 constitute the JAK2/STAT3 signaling pathway, playing a pivotal role in apoptosis. After the ischemia/reperfusion injury, the cytokines released by injured tissues can stimulate JAK2 to be phosphorylated into p-JAK2 by binding to its receptors. Meanwhile, they can expose the STAT binding sites on JAK that are originally in a conservative state. STAT3 can be activated and phosphorylated into p-STAT3 immediately after conjugating with the binding site on JAK2. This may further induce the transcription of the downstream target genes closely associated with cell apoptosis, including Bax and Bcl-2, as well as mediate the occurrence of cell apoptosis[14,15]. Previous studies have demonstrated that both Bax and Bcl-2 play important roles in the process of cell apoptosis. They can regulate the process to maintain normal physiological functions of cells. Bax positively regulates the apoptosis and promotes its occurrence[16,17]. However, Bcl-2 exerts a negative regulation on cell apoptosis and inhibits its occurrence[18]. The abnormal changes in the expression levels of Bax and Bcl-2 alter Bax/Bcl-2 ratio, eventually leading to an imbalance between apoptosis and anti-apoptosis and triggering the apoptotic response in cells. According to the results in this study, significantly abnormal expression levels of Bax and Bcl-2 in myocardial cells were observed in cardiac tissues of rats with myocardial ischemia/reperfusion injury. This was manifested as remarkably elevated Bax expression and significantly reduced Bcl-2 expression. All these results suggested that the myocardial cell apoptosis immediately occurred after myocardial ischemia/reperfusion injury, with notably increased apoptosis rate.

As a common intravenous anesthetic drug used in clinical surgeries, propofol shows vital functions, such as anesthesia induction and maintenance as well as sedation. Besides, it can effectively protect the heart, brain, and other organs and tissues and ameliorate myocardial ischemia/reperfusion injury. In particular, it has preferable inhibitory effects on cell apoptosis induced by myocardial ischemia/reperfusion injury[19,20]. In this research, it was observed that the expression of the pro-apoptotic gene Bax was evidently up-regulated, while the anti-apoptotic gene Bcl-2 was notably reduced in the myocardial tissues after myocardial ischemia/reperfusion injury. This might eventually trigger widespread myocardial cell apoptosis in myocardial tissues and increase the apoptosis rate of myocardial cells. However, after the intervention with propofol, the expression level of pro-apoptotic gene Bax was markedly down-regulated, while the anti-apoptotic gene Bcl-2 was prominently up-regulated. Meanwhile, the apoptosis rate significantly decreased, implying that the propofol was able to repress myocardial cell apoptosis induced by myocardial ischemia/reperfusion injury. To further explore the related mechanism of propofol, p-JAK2 and p-STAT3

were detected in this research. They have been considered as key molecules in the JAK/STAT signaling pathway that is closely correlated with apoptosis. According to the results in this study, the expressions of both p-JAK2 and p-STAT3 in myocardial tissues were remarkably up-regulated after myocardial ischemia/reperfusion injury. The results indicated that the JAK/STAT signaling pathway was activated, which might be one of the possible mechanisms of myocardial cell apoptosis. Moreover, both propofol and inhibitor of the JAK/STAT signaling pathway could remarkably lower the expression levels of p-JAK2 and p-STAT3 in myocardial tissues after myocardial ischemia/reperfusion injury. This suggested that they could inhibit myocardial cell apoptosis by repressing the JAK/STAT signaling pathway. In addition, the effect of propofol was similar to that of the JAK/STAT signaling pathway inhibitor. It could be concluded that propofol inhibited the myocardial cell apoptosis after myocardial ischemia/reperfusion injury by repressing the JAK/STAT signaling pathway.

Conclusions

We demonstrated that propofol inhibits myocardial cell apoptosis after myocardial ischemia/reperfusion injury by repressing the JAK/STAT signaling pathway.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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