

## Original Article

# Effect of metformin on myocardial cell apoptosis induced by hypoxia/reoxygenation injury

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Received July 7, 2018; Accepted August 24, 2018; Epub December 15, 2018; Published December 30, 2018

**Abstract:** Objective: To investigate the effect and underlying mechanism of metformin on myocardial cell apoptosis induced by myocardial ischemia-reperfusion injury in rats. Methods: Forty suckling Wistar rats were selected, and the hearts were removed under aseptic condition. Rat myocardial cells were primarily cultured to establish hypoxia/reoxygenation injury (H/R) models of myocardial cells *in vitro* and the models were randomly classified into four groups: blank control group, metformin group, H/R group and metformin + H/R group. The apoptotic rate of primary rat myocardial cells in each group was tested with the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. The cell counting kit-8 (CCK-8) assay was utilized to measure the viability of primary rat myocardial cells in each group. The relative expression of HIF-1 $\alpha$  mRNA of primary rat myocardial cells in each group was assessed by using real-time PCR. The relative expression of Bcl-2 and Bax protein of primary rat myocardial cells in each group was tested by using Western Blot assay. Results: As compared with the blank control group, the apoptotic rate of primary rat myocardial cells in the H/R group was significantly increased ( $P < 0.001$ ) and the cell viability was obviously reduced ( $P < 0.001$ ). When compared with hypoxia and reoxygenation culture, metformin pre-treatment significantly reduced the apoptotic rate of myocardial cells ( $P < 0.001$ ) and promoted cell survival in rats ( $P < 0.001$ ). Meanwhile, real-time PCR results showed that the relative expression of HIF-1 $\alpha$  mRNA was significantly decreased in the metformin + H/R group. Western Blot results showed that the relative expression of Bcl-2 protein was significantly increased and relative expression of Bax protein was significantly decreased in the metformin + H/R group, with statistical differences. Conclusion: Metformin can reduce the apoptosis of rat myocardial cells following ischemia-reperfusion injury. The underlying mechanism may be associated with an improvement in cell viability, suppression in HIF-1 $\alpha$  expression, increase in the apoptosis inhibitor Bcl-2e expression and decrease in the Bax expression.

**Keywords:** Metformin, myocardial cells, apoptosis, myocardial ischemia-reperfusion injury

## Introduction

Acute myocardial infarction is an acute critical disease commonly occurred in clinics and the morbidity is increasing year by year, which seriously threatens human life and health [1, 2]. The primary goal of treatment of acute myocardial infarction is to restore myocardial blood flow perfusion as soon as possible and rescue moribund myocardial cells [3]. However, myocardial ischemia-reperfusion injury may occur during this process, leading to myocardial stunning, arrhythmia, no-reflow phenomena and other adverse events that affect prognosis in patients with acute myocardial infarction. Recent studies have indicated that, the pathogenesis of myocardial ischemia-reperfusion injury is relatively complex and involves multiple links,

among which apoptosis is one of the key links [4, 5]. Therefore, how to effectively inhibit the apoptosis of myocardial cells and accordingly relieve myocardial ischemia-reperfusion injury plays a key role in improving the prognosis of such patients [6].

Metformin is a clinically widely used oral antidiabetics. Since its introduction in the 1950s, metformin has been extensively used in the treatment of patients with type 2 diabetes mellitus [7]. Recent studies have reported that, metformin has anti-inflammatory effects and improves energy metabolism and endothelial function, in addition to its hypoglycemic effect [8, 9]. El Messaoudi et al. have found that, metformin significantly reduced the incidences of great vessel disease and acute myocardial in-

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fraction in diabetic patients after administration, compared with those without metformin administration [10]. Solskov et al. have shown that metformin exerts a cardioprotective effect and attenuates myocardial ischemia/reperfusion injury. However, few studies reported the role of myocardial cell apoptosis in the metformin-induced reduction of ischemia-reperfusion injury [11]. Therefore, this study was aimed to explore the effect of metformin on myocardial cell apoptosis and its possible mechanism by establishing myocardial hypoxia/reoxygenation models, and to provide experimental evidence for clinical treatment.

## Materials and methods

### *Experimental animals*

Forty SPF suckling Wistar rats, 2 days old, irrespective of gender, were provided by the Experimental Animal Center.

### *Main reagents and equipment*

DMEM culture medium was bought from Hyclone (USA), flow cytometry kits from BD (USA), fetal bovine serum from Gibco (USA), CCK-8 reagents and crystal violet from Sigma (USA), the kits for the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay from BD Biosciences (USA), rabbit anti-mouse HIF-1 $\alpha$ , Bcl-2, and Bax monoclonal antibodies from Santa (USA), secondary antibody (goat anti-rabbit) from Boster Biological Technology Co., Ltd. (Wuhan, China), automatic microplate readers from Rayto Life and Analytical Sciences Co., Ltd. (USA) and real-time PCR apparatus from ABI company (Germany).

### *Culture of primary myocardial cells in neonatal rats*

Suckling Wistar rats were selected, and the hearts were aseptically removed and placed in pre-cooled DMEM culture medium. After bloodstain was thoroughly washed, the atriums and aorta were dissected, then the ventricles were isolated. Myocardial tissue was cut into 1-mm<sup>3</sup> pieces and transferred to a centrifuge tube, and slowly digested in 0.125% trypsin and 0.125 collagenase II until myocardial tissue was completely digested. Tissue was centrifuged at 1000 r/min for 10 min, then the supernatant was aspirated and discarded, and DMEM culture medium containing 10% fetal

bovine serum was added to prepare cell suspension. In the meantime, 0.1 mmol/L 5-BrdU was added to inhibit the growth of non-ventricular myocytes. Myocardial cells were transferred to a culture flask and placed in a 37°C incubator for primary culture.

### *Establishment of hypoxia/reoxygenation injury model of primary myocardial cells*

A hypoxia/reoxygenation injury model of primary myocardial cells was established according to the method reported by Buja [12]. Serum-free, glucose-free DMEM culture medium was used as hypoxic solution, and hypoxic solution balanced with a mixed gas (containing 95% N<sub>2</sub> and 5% CO<sub>2</sub>) for over 2 hours was used as saturated hypoxic solution. Normal culture medium of myocardial cells was discarded and quickly replaced by saturated hypoxic solution, then myocardial cells were cultured in a hypoxic chamber (95% N<sub>2</sub> and 5% CO<sub>2</sub>) at 37°C for 3 hours. In this way, hypoxic model was established. After myocardial cells were subjected to hypoxia for 10 hours, the culture medium was replaced with DMEM culture medium containing glucose and 10% fetal bovine serum. Myocardial cells were placed in a 37°C incubator (95% air and 5% CO<sub>2</sub>) and cultured for additional 3 hours. In this way, a reoxygenation model was established.

### *Grouping of myocardial cells*

Primary myocardial cells were randomly classified into four groups after 72-hour culture. Blank control group: myocardial cells were cultured under normoxia condition. Metformin group: myocardial cells were cultured under normoxia conditions with metformin at a final concentration of 100  $\mu$ mol/L. H/R group: myocardial cells were subjected to 3 hours of hypoxia culture and 3 hours of reoxygenation culture. Metformin + H/R group: myocardial cells were cultured with metformin at final concentration of 100  $\mu$ mol/L for 12 hours, followed by 3 hours of hypoxia culture and then 3 hours of reoxygenation culture [13].

### *TUNEL assay for apoptotic rate of myocardial cells*

Myocardial cells in each group were seeded onto 12-well plates at 4\*10<sup>4</sup>/mL and cultured for 72 hours, then fixed in 4% neutral formalin for 10 minutes. Subsequently cells were hydrat-

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**Table 1.** Apoptotic rates of primary rat myocardial cells in each group

Group	Case (n)	Apoptotic rate (%)
Blank control group	10	6.8±1.4
Metformin group	10	7.2±1.5
H/R group	10	38.7±3.4*
Metformin + H/R group	10	22.5±2.3#
F		435.7
P		P<0.001

Note: \*P<0.001, compared with the blank control group, metformin group and metformin + H/R group; #P<0.001, compared with the blank control group and metformin group.

ed with 100%, 95% and 70% gradient alcohol, for 5 minutes at each concentration, and washed with PBS for 10 minutes. TUNEL staining was conducted following the instructions of TUNEL kit (Sigma, USA). Eight fields of vision were randomly selected under high power field to calculate the apoptotic rate of myocardial cells in each group. Apoptosis rate = number of apoptotic cells/total number of cells \*100%.

### CCK-8 assay for the viability of myocardial cells

In each group, myocardial cells were seeded onto 96-well plates at a density of  $4 \times 10^4$ /ml and cultured with culture medium at 37°C in a 5% CO<sub>2</sub> incubator for 72 hours. After 10 µL CK-8 was added to each well, cells were incubated for additional 4 hours in the incubator. The absorbance of each well at 450 nm was measured using a microplate reader, and the average value of three wells was calculated.

### Real-time PCR detection of HIF-1α expression in myocardial cells of each group

Trizol reagent was used to extract total RNA in each group. Total RNA was synthesized into cDNA by reverse transcriptase. β-actin was employed as an internal control. β-actin upstream primer: 5'-CATTAAAGGAGAAGCTGTGCT-3', downstream primer: 5'-GTTGAAGGTAGTTTCGTGGA-3'. HIF-1α upstream primer: 5'-GGACGATGAACA TCAAGCA-3', downstream primer: 5'-ACGTTT-AGCTCCACATCAGGAC-3'. Bcl-2 upstream primer: 5'-GGCATCTTCTCCTCCAG-3', downstream primer 5'-CATCCCAGCCTCCGTTAT-3'; Bax upstream primer: 5'-TTTGTTACAGGGTTTC ATCCAGG-3', downstream primer: 5'-TTCCAGATGGTG-AGCGAGGC-3'. Reaction system (20 µL) included cDNA 2 µL, upstream primer (0.8 µL), down-

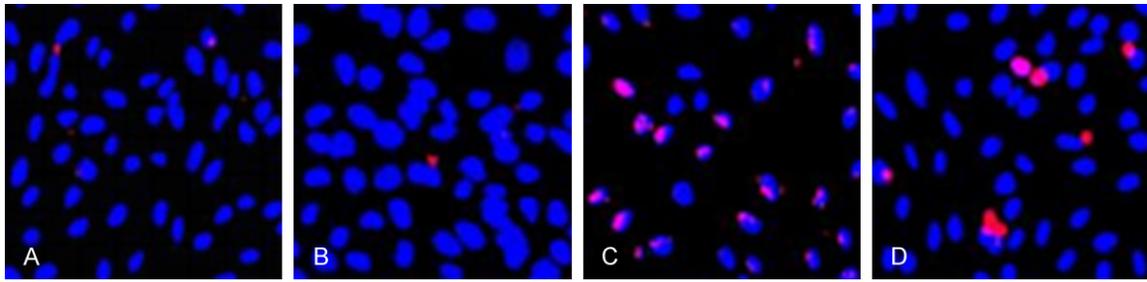
stream primer (0.8 µL), SYBR Green PCR Master Mix (2X, 10 µL), and ddH<sub>2</sub>O (7.2 µL). After centrifugation, cells were amplified in an Applied Biosystems 7500 Quantitative PCR instrument. Reaction conditions: pre-denaturation at 94°C for 1 min, denaturation at 92°C for 40 sec, renaturation at 56°C for 25 sec and extension at 75°C for 30 sec, totally 30 cycles. Using β-actin as the reference gene, the relative expression levels of HIF-1α, Bcl-2, and Bax mRNA in each group were calculated with 2<sup>-ΔΔCt</sup> method.

### Western blot assay for Bcl-2 and Bax protein expression in myocardial cells of each group

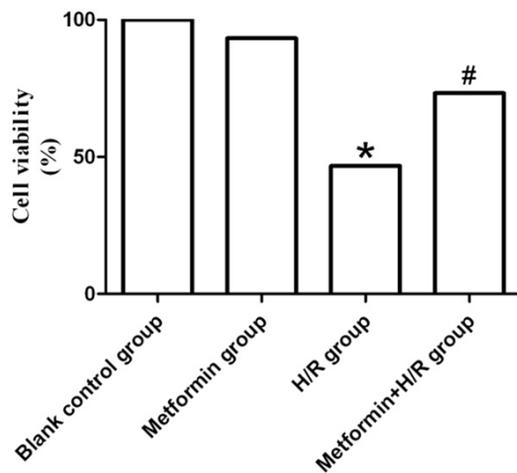
In each group, myocardial cells were lysed and then protein concentration was assayed by using a microplate reader. The sample volume of total protein was 30 µg. Proteins were transferred to a PVDF membrane by electroblotting transfer method following SDS-PAGE gel electrophoretic separation. Membrane was then cut based on the desired target strips. After blocked with TBS-T solution containing 5% skimmed milk powder for 1 hour at room temperature, cells were incubated overnight with rabbit anti-mouse Bcl-2 (1:200 dilution) or Bax (1:800 dilution) monoclonal antibodies and rabbit anti-mouse β-actin antibodies (1:200 dilution) at 4°C. Membrane was washed with TBS-T solution at room temperature and incubated with goat anti-rabbit secondary antibody for 30 minutes, followed by another wash of TBS-T solution. Membrane was reacted with chromogenic reagent, exposed and scanned. The Image one software was employed for calculating the optical density of the target strips.

### Statistical analysis

SPSS statistical software, version 21.0 was utilized for statistical analysis of all experimental data. Measurement data were presented as mean ± standard deviation, comparisons between two groups were performed using a t-test, and comparisons among groups were performed using one-way analysis of variance and post-hoc Bonferroni test. Count data were described as percentages. Chi-square test was employed for inter-group comparison and chi-squared partition test was used for comparison between two groups. The difference was deemed statistically significant at P<0.05.



**Figure 1.** TUNEL assay for apoptosis of primary rat myocardial cells in each group. A: Blank control group; B: Metformin group; C: H/R group; D: Metformin + H/R group.



**Figure 2.** Comparison of the viability of primary rat myocardial cells in each group. \* $P < 0.001$ , compared with the blank control group, metformin group and metformin + H/R group; # $P < 0.001$ , compared with the blank control group and metformin group.

## Results

### *Apoptosis of primary rat myocardial cells each group*

After primary myocardial cells were cultured for 72 hours, TUNEL assay results showed that the apoptotic rate of primary myocardial cells in the H/R group was  $(38.7 \pm 3.4)\%$ , which was significantly higher than that  $(6.8 \pm 1.4\%)$  of blank control group, with remarkable differences ( $P < 0.001$ ). The apoptotic rate of primary cells in metformin + H/R group was  $(22.5 \pm 2.3)\%$ , which was markedly lower than that of H/R group, with statistical difference ( $P < 0.001$ ). Results are shown in **Table 1** and **Figure 1**.

### *Viability of primary myocardial cells in rats*

After primary myocardial cells were cultured for 72 hours, CCK-8 detection revealed that, using the viability of myocardial cells in the blank con-

trol group as the reference, the viability of primary myocardial cells in the H/R group was  $46.7\%$  ( $OD = 0.7 \pm 0.1$ ), which was significantly lower than that in the blank control group ( $OD = 1.5 \pm 0.4$ ); there was a statistical difference between the two groups ( $P < 0.001$ ). The viability of primary myocardial cells in the metformin + H/R group was  $73.3\%$  ( $OD = 1.1 \pm 0.2$ ), which was strikingly higher than that in the H/R group ( $P < 0.001$ ). Results are shown in **Figure 2**.

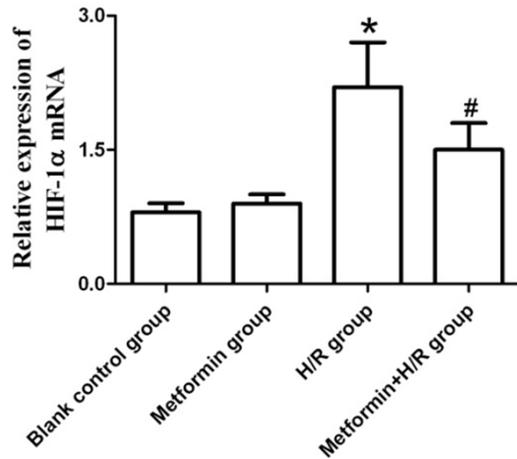
### *HIF-1 $\alpha$ , Bcl-2 and Bax mRNA expression in primary rat myocardial cells in each group*

Real-time PCR detection showed that, the expression levels of HIF-1 $\alpha$  mRNA and Bax mRNA in myocardial cells were remarkably increased in the H/R group, and the Bcl-2 mRNA expression was significantly decreased as compared with the blank control group and metformin group (all  $P < 0.001$ ). Compared with the H/R group, the expression levels of HIF-1 $\alpha$  mRNA and Bax mRNA in myocardial cells were decreased, but the expression of Bcl-2 mRNA was elevated in the metformin + H/R group, with statistical difference (all  $P < 0.001$ ). Results are shown in **Figures 3-5**.

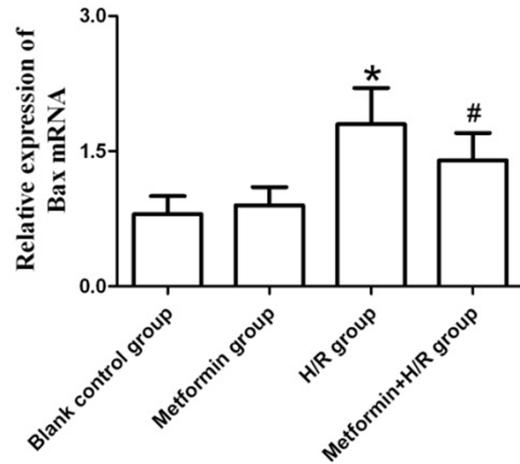
### *HIF-1 $\alpha$ , Bcl-2 and Bax expression in primary myocardial cells of each group*

Western blot assay demonstrated that, when compared with the blank control group and metformin group, the expression levels of HIF-1 $\alpha$  protein and Bax protein in myocardial cells were significantly increased in the H/R group, and the expression of Bcl-2 protein was significantly decreased (all  $P < 0.001$ ). As compared with the H/R group, the levels of HIF-1 $\alpha$  protein and Bax protein in myocardial cells were decreased, while the level of Bcl-2 protein was elevated in the metformin + H/R group, with statistical difference (all  $P < 0.001$ ). Results are shown in **Table 2** and **Figure 6**.

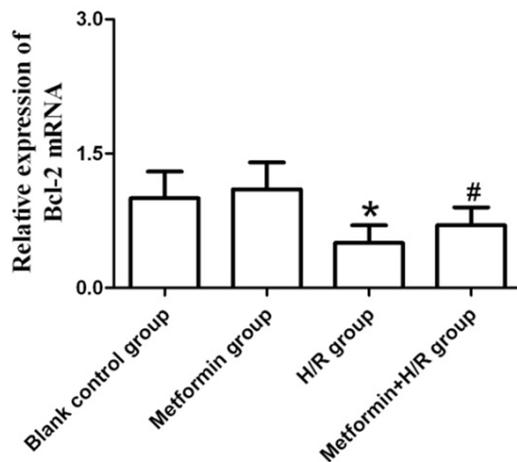
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**Figure 3.** Comparison of HIF-1 $\alpha$  mRNA expression in primary myocardial cells of each group ( $F=46.3$ ,  $P<0.001$ ). \* $P<0.001$ , compared with the blank control group, metformin group and metformin + H/R group; # $P<0.001$ , compared with the blank control group and metformin group.



**Figure 5.** Comparison of Bax mRNA expression in primary myocardial cells of each group ( $F=32.1$ ,  $P<0.001$ ). \* $P<0.001$ , compared with blank control group, metformin group and metformin + H/R group; # $P<0.001$ , compared with blank control group and metformin group.



**Figure 4.** Comparison of Bcl-2 mRNA expression in primary myocardial cells of each group ( $F=26.8$ ,  $P<0.001$ ). \* $P<0.001$ , compared with the blank control group, metformin group and metformin + H/R group; # $P<0.001$ , compared with the blank control group and metformin group.

### Discussion

The most effective treatment for acute myocardial infarction is rapid restoration of blood supply in the ischemic myocardia. However, restoration of blood flow perfusion following myocardial ischemia fails to relieve or eliminate the damage to ischemic myocardial cells; instead, it aggravates myocardial injury, namely, myocardial ischemia/reperfusion injury [12]. Studies have reported that the essence of isch-

emia/reperfusion injury is inflammatory response, oxidative stress and calcium overload that may damage myocardial cells [13]. In recent years, increasing scholars have found the important role of apoptosis in ischemia/reperfusion injury, and apoptosis can reduce the number of myocardial cells and weaken cardiac function [14]. Myocardial cells have shown to undergo less apoptosis during sustaining ischemia, and cell apoptosis increased significantly after reperfusion [15]. Lee et al. and Chen et al. have demonstrated that, lowering cell apoptosis during myocardial ischemia/reperfusion injury can reduce approximately 50-70% of myocardial injury area [16, 17]. In this study, the apoptotic rate of myocardial cells in the H/R group was substantially increased as compared with blank control group. Obviously, an effective inhibition of myocardial cell apoptosis can relieve the damage to myocardia and cardiac function after reperfusion.

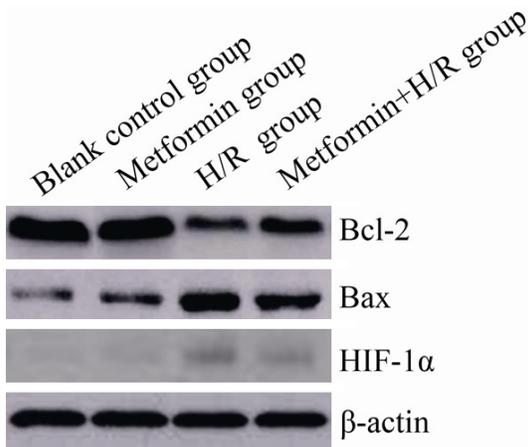
Apoptosis is a process of gene-regulated programmed cell death that is regulated by intracellular apoptosis regulatory proteins, and it is caused by loss of balance due to the confrontation between anti-apoptotic proteins and proapoptotic proteins [18]. Bcl-2 is an important regulator of apoptosis, and Bax is a Bcl-2 related X protein. The expression levels of Bcl-2 and Bax proteins are directly related to the regulation of apoptosis, with elevated Bcl-2 level inhibiting apoptosis and elevated Bcl-2 level pro-

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**Table 2.** Comparison of Bcl-2 and Bax protein expression in primary rat myocardial cells in each group

Group	Case (n)	HIF-1 $\alpha$ relative expression	Bcl-2 relative expression	Bax relative expression	Bcl-2/Bax ratio
Blank control group	10	1.0 $\pm$ 0.1	1.0 $\pm$ 0.3	0.8 $\pm$ 0.2	1.2 $\pm$ 0.3
Metformin group	10	0.9 $\pm$ 0.1	1.1 $\pm$ 0.3	0.9 $\pm$ 0.2	1.1 $\pm$ 0.3
H/R group	10	2.2 $\pm$ 0.5*	0.5 $\pm$ 0.2*	1.8 $\pm$ 0.4*	0.3 $\pm$ 0.1*
Metformin + H/R group	10	1.5 $\pm$ 0.3#	0.7 $\pm$ 0.2#	1.4 $\pm$ 0.3#	0.5 $\pm$ 0.2#
F		39.8	16.85	26.16	34.06
P		<0.001	<0.001	<0.001	<0.001

Note: \*P<0.001, compared with the blank control group, metformin group and metformin + H/R group; #P<0.001, compared with the blank control group and metformin group.



**Figure 6.** Western blot assay of the relative expression levels of HIF-1 $\alpha$ , Bcl-2 and Bax proteins in myocardial cells of each group.

moting apoptosis [19]. Another study reported that HIF-1 $\alpha$  is an important protein induced by hypoxia environment and a key protein that mediates apoptosis [20]. In our present study, when compared with the blank control group, HIF-1 $\alpha$  mRNA expression was significantly elevated in the H/R group, Bcl-2 protein expression was substantially reduced, Bax protein expression was significantly increased, and the Bcl-2/Bax ratio was significantly decreased. This evidence indicates that during ischemia/reperfusion injury, hypoxia upregulated HIF-1 $\alpha$  expression and inhibited the expression of Bcl-2, an apoptosis inhibitor, leading to myocardial cell apoptosis.

Metformin is a biguanide drug that lowers blood glucose mainly by reducing food absorption and gluconeogenesis and promoting glucose

uptake in tissues. Metformin has a cardiovascular protective effect and prevents cardiovascular disease, but the specific mechanism is still unclear [21]. In the present study, when compared with the H/R group, the apoptotic rate of myocardial cells in the metformin + H/R group was remarkably lower, and the viability of myocardial cells was enhanced, with statistically significant difference. In addition, the

expression of HIF-1 $\alpha$  in myocardial cells was strikingly decreased in the metformin + H/R group, while the expression of Bcl-2 protein was remarkably elevated and the expression of Bax protein was lowered; the difference was statistically significant. This evidence demonstrated that metformin can reduce the HIF-1 $\alpha$  expression and improve hypoxia of myocardial cells, accordingly increase the ratio of Bcl-2/Bax protein expression and reduce myocardial cell apoptosis, which was consistent with the findings of Wang et al. and Du et al. [22, 23].

In summary, metformin can inhibit myocardial cell apoptosis induced by myocardial ischemia/reperfusion injury, and the mechanisms may be related to down-regulation of HIF-1 $\alpha$  and Bax expression, up-regulation of Bcl-2 expression, and enhancement of the Bcl-2/Bax ratio. However, in-depth studies are needed for further confirmation of the dose-dependence of metformin, its effects and action mechanism on other apoptosis-related proteins.

### Disclosure of conflict of interest

None.

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