



Original Article

Protective Effects on Hypoxia Reoxygenation Cardiomyocytes by GLP-1R Agonists via PI3K/AKT Signaling Pathway

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SUMMARY

Objective: The aim of the current study was to observe the protective effect of GLP-1R agonist on hypoxia reoxygenation cardiomyocytes and to explore the role of GLP-1R/PI3K/AKT signaling pathway. **Methods:** H9c2 myocardial cells were randomly divided into four groups: control group, hypoxia reoxygenation model group (H/R), Exenatide group and PI3K/AKT signaling pathway inhibitor group. CCK-8 method was used to detect myocardial enzymes, and the expression of Caspase-3, Bcl-2 and Bax proteins was detected by Western blot.

Results: The apoptosis rate in exenatide group was decreased than the H/R group. The levels of LDH and CK-MB in H/R group were higher than control group, which compared with the H/R group, exenatide group were decreased, and it in PI3K/AKT pathway inhibitor group were increased than exenatide group. The expression of Caspase-3 and Bax in H/R group was higher than that in control group group, and the level of Bcl-2 was decreased. Compared with H/R group, the levels of Caspase-3 and Bax in exenatide group were decreased, and the level of Bcl-2 was increased. Compared with exenatide group, the levels of Caspase-3 and Bax were significantly increased in the PI3K/AKT pathway inhibitor group, and the level of Bcl-2 was decreased.

Conclusion: Exenatide has protective effects on the cardiomyocytes during hypoxia reoxygenation injury. The GLP-1R/PI3K/AKT signaling pathway may be involved in the process that exenatide inhibiting cardiomyocyte apoptosis.

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1. Introduction

Acute myocardial infarction (AMI) is currently an important public health problem. The incidence of AMI is increased year by year. Its prevention is one of the most difficult tasks that placed in front of medical personnels. The main intervention measures for the treatment of AMI is the rapid opening of the infarct-related artery and reperfuse myocardium. But in clinical practice we found that in the acute myocardial ischemia reperfusion, these treatments are accompanied by myocardial ischemia reperfusion injury (MIRI) and affect the therapeutic effect, and may cause serious adverse consequences,¹ so the clinical need effective strategy to alleviate MIRI. Through drug pretreatment² or post-reperfusion drug intervention³ effectively alleviate the ischemia-reperfusion injury is a hotspot in cardiovascular research. Earlier researches have shown that cardiomyocyte apoptosis is involved in the early pathophysiological changes of MIRI. To a certain extent determines the severity of infarction and prognosis. Effective control of myocardial cell, can better improve the ischemic reperfusion injury. Glucagon-like peptide 1 (GLP-1) agonist and GLP-1 receptor (GLP-1R) mutual recognition, inhibiting

the secretion of glucagon by promoting insulin synthesis to exert complex physiological functions.⁴ In recent years, studies have found that GLP-1 receptor agonists not only have the significance of diabetes treatment, but also have a good protective effect on myocardial ischemia reperfusion. But the specific mechanism is not clear, need to be further studied. Exenatide is a GLP-1 receptor agonist analogues. In this study, H9c2 cardiomyocytes were used as the research object and establish a hypoxia reoxygenation model to simulate the ischemia-reperfusion environment, to explore the effect of GLP-1 agonist exenatide on the apoptosis of hypoxia reoxygenation cardiomyocytes and the possible mechanism that provide new ideas for the clinical treatment of myocardial ischemia reperfusion injury.

2. Materials and methods

2.1. Cells and culture

The H9c2 cardiomyocyte cell line was taken out from liquid nitrogen and dissolved in a 37 °C water bath. Be careful not to allow water to come into contact with the frozen tube seal. The solubilized cells were transferred to a centrifuge tube, and 4 ml of DMEM/F12 medium prepared with 10% fetal bovine serum (FBS), penicillin (100 µg/ml) and streptomycin (100 µg/ml) was added, and centrifuged at 1500 rpm. minute. After discarding the supernatant, 1 mL of the me-

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dium was added and repeatedly blown. A culture dish was prepared, 5 ml of the medium was added to the culture dish, and the cell liquid was transferred to a Petri dish and repeatedly blown until uniform. Subsequently, the culture dish was placed in an incubator containing 5% CO₂ at 37 °C, and the liquid exchange or passage was determined according to the growth condition, and the cells after 3 passages were used for the experiment.

2.2. Cardiomyocytes simulate model of ischemia reperfusion injury preparation

H9c2 cardiomyocytes grow to 80% in culture dishes, then cultured in 6-well plates (Corning) after suspending with pancreatin, when the cells were adhered the plates wall and filled to 80%, the culture was continued for 1 h; N₂ and CO₂ were mixed in a certain proportion, inleted the hypoxia model container (America) for a period of time, to create a hypoxic environment; cultured cells with N₂ saturated D-Hank for some time, then put it into the hypoxia reoxygenation model container ventilated for 6 h, 8 h, 10 h, 12 h each time, observing cells injury, and replaced the D-Hank for DMEM synthesis medium containing 10% FBS cultured for 4 h, to determine the success of modeling.

The CCK-8 method measures cell viability and determines the optimal concentration of exenatide. 100 µl of different concentrations of exenatide (0, 100, 200, 400, 600 nM) pretreated cells were added to each well in a 96-well plate. After incubation for 24 hours in the incubator, 10 µl of CCK 8 solution was added to each well, followed by incubating the plate in an incubator. Hours, cell viability was determined based on OD values, and the optimal concentration of exenatide was explored. Repeat the above steps, add 100 µl of H9c2 cardiomyocytes with hypoxia for 6.80.12.12.14 hours in a 96-well plate, add 10 µl of CCK 8 solution after incubator incubation, incubate for another 2 hours, and determine the survival rate according to the OD value, choose the right hypoxia reoxygenation time.

2.3. IRB statement

Our experiment does not involve human subjects. It is a cell experiment and does not require ethical approval.

2.4. Grouping

Control group: no treatment, normal culture. H/R group: H/R model was established with H9c2 cells. Exenatide group: added 200 nM Exenatide before H/R model was established. PI3K/AKT pathway inhibitor group: addition of API-2 dilution to 8 µmol/L at 10 min prior to addition of 200 nM Exenatide.

2.5. The survival rate of the cells

Cell suspension was collected. The survival rate of the cells was detected by CCK-8.

2.6. The level of the myocardial enzymes

The level of lactate dehydrogenase (LDH), creatine phosphokinase isoenzymes (CK-MB) of culture supernatant in each group was detected by enzyme linked immunosorbent assay (ELISA).

2.7. The apoptosis rate of the cells

The apoptosis rate of the cells was detected by flow cytometry.

2.8. The expression of Caspase-3, Bcl-2 and Bax proteins

The expression of Caspase-3, Bcl-2 and Bax proteins in cardiomyocytes was detected by western blot, immunofluorescent test.

2.9. Fluorescence direactance and DAPI staining

To identify the expression of the anisotropy of cells and to observe the localization of protein in cells.

2.10. Statistic analysis

The data analysis was carried out using statistical software SPSS17.0. T-test was used for the mean comparison of measurement data between two groups. The $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Comparison of cardiomyocyte survival rates

CCK-8 Kit found that H9c2 cell survival rate has been declining when prolonged the H/R time. Compared with the control group, the survival rate of H9c2 cells was 79.59% when the H/R time was 10/4 h ($p < 0.01$). So determine H/R time 10/4 h as the best hypoxia reoxygenation time (Table 1).

And, exenatide increased the survival rate of H9c2 cells under the condition of H/R. At 200 nM, the survival rate of H9c2 cells was the highest, so exenatide 200 nM concentration was selected as the intervention condition for subsequent experiments (Table 2).

3.2. The levels of LDH and CK-MB in supernatant

The change of LDH and CK-MB also represents the degree of myocardial injury. The results showed that compared with control group, LDH and CK-MB levels have increased significantly in H/R group ($p < 0.01$); compared with H/R group, LDH and CK-MB levels were significantly decreased in exenatide group, the difference was statistically significant ($p < 0.01$) (Table 3, Figure 1).

Table 1

Hypoxia reoxygenation (H/R) time the influence of the survival rate of H9c2 cells ($\bar{x} \pm s$).

Grouping	Sample size	Cell survival rate (%)
The normal group	4	98.60 ± 0.45
H/R (6/2 h)	4	90.72 ± 3.60*
H/R (8/3 h)	4	85.65 ± 4.42*
H/R (10/4 h)	4	79.59 ± 4.96**
H/R (12/5 h)	4	75.46 ± 8.08*
H/R (14/6 h)	4	58.90 ± 12.34**

Note: * $p < 0.05$, compared with the normal group; ** $p < 0.01$, compared with the normal group.

Table 2

Enatide H9c2 on the survival rate of myocardial cell effect ($\bar{x} \pm s$).

Grouping	Sample size	Cell survival rate (%)
The normal group	4	98.47 ± 1.09
H/R (0 nM)	4	78.53 ± 6.61
H/R (100 nM)	4	75.14 ± 5.01*
H/R (200 nM)	4	81.32 ± 5.93*
H/R (400 nM)	4	79.22 ± 6.39*
H/R (600 nM)	4	70.21 ± 6.72*

Note: * $p < 0.01$, compared with H/R group (0nM).

Table 3

Effect of exenatide on the supernatant LDH and CK-MB concentration of culture ($\bar{x} \pm s$).

Grouping	Sample size	LDH (U/L)	CK-MB (U/L)
A	4	99.57 ± 1.33 [#]	1.60 ± 0.29 [#]
B	4	135.71 ± 10.72*	10.69 ± 1.67*
C	4	109.35 ± 7.59 ^{#*}	5.82 ± 1.40 ^{#*}
D	4	128.12 ± 6.50* [^]	8.92 ± 0.96* [^]

Note: * $p < 0.01$, compared with the normal group; [#] $p < 0.01$, compared with H/R group; [^] $p > 0.05$, compared with the H/R group. Group A is normal group; Group B is H/R group; Group C is Exenatide group; Group D is the pathway inhibition group.

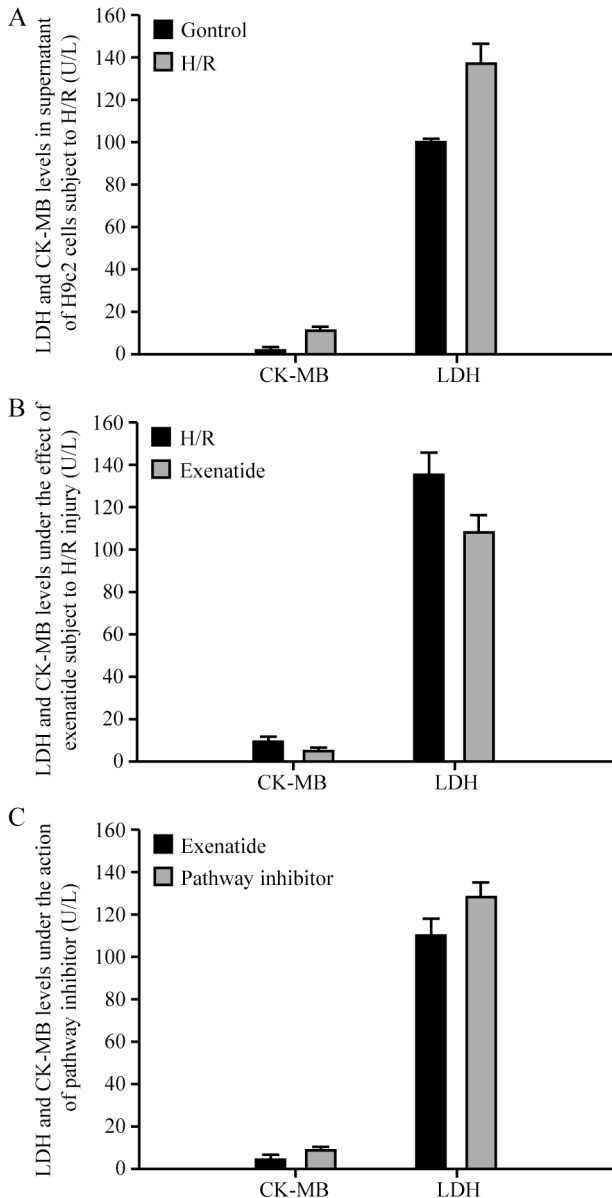


Figure 1. Effect of exenatide on the supernatant LDH and CK-MB concentration of culture.

3.3. Comparison of apoptosis rate

Flow cytometry results showed that compared with the control group, the apoptosis rate of H9c2 cells increased significantly in H/R group ($p < 0.05$). Compared with the H/R group, the apoptosis rate in exenatide group decreased significantly ($p < 0.01$). Compared with exenatide group, the apoptosis rate of PI3K/AKT pathway inhibitor

group increased significantly ($p < 0.01$) (Table 4, Figure 2).

3.4. Comparison of the expression of Caspase-3, Bcl-2, Bax

Western blot results showed that compared with control group, the H/R group has high protein expression significantly ($p < 0.01$); compared with H/R group, the expression of caspase-3 in exenatide group was significantly decreased ($p < 0.01$). Compared with control group, the expression of Bax was significantly increased and Bcl-2 decreased significantly in H/R group ($p < 0.01$); compared with H/R group, the expression of Bax in exenatide group was significantly decreased, but Bcl-2 increased significantly ($p < 0.01$); compared with exenatide group, the expression of Bax in PI3K/AKT pathway inhibitor group was significantly increased and Bcl-2 decreased significantly ($p < 0.01$) (Figure 3).

3.5. Fluorescence directance and DAPI staining

Caspase-3: Observation and discovery under fluorescence microscope: The expression of protein in normal group was lower than that in normal group. Compared with normal group, the expression of Caspase-3 in H9c2 cells was significantly higher in H/R group. Increased fluorescence intensity in cytoplasm, nuclear DAPI staining was blue. Compared with group H/R, the expression of Caspase-3 decreased significantly in H/R exenatide group. Cytoplasmic fluorescence intensity decreased significantly. Comparison with the H/R + exenatide group, the level of Caspase-3 expression in the pathway inhibitor group was significantly increased in cytoplasmic fluorescence intensity (Figure 4A).

Bax: Observation and discovery under fluorescence microscope: The expression of proteins in the normal group were lower compared with the normal group; H/R group H9c2 cells Bax expression levels were significantly higher, increased cytoplasm visible fluorescence intensity, DAPI nuclear staining was blue; compared with H/R group, the expression of H/R + exenatide in group Bax was significantly lower, and cytoplasm fluorescence intensity decreased H/R; and group + exenatide pathway inhibitor group Bax expression levels increased significantly, cytoplasmic fluorescence intensity increased significantly (Figure 4B).

Bcl-2: The expression of protein in normal group was lower than that in normal group under fluorescence microscope. Compared with the normal group, the expression of Bcl-2 in H9c2 cells in H/R group was significantly decreased, the intensity of cytoplasmic fluorescence staining was significantly decreased, and the nucleus of nuclear DPI staining was blue. Compared with H/R group, Bcl-2 expression and cytoplasmic fluorescence staining intensity in H/R exenatide group were significantly higher than those in H/R group. Compared with the H/R Exenatide group, the expression of Bcl-2 and the intensity of cytoplasmic fluorescence staining were significantly decreased in the pathway inhibitor group (Figure 4C).

Table 4

Comparison of the rate of apoptosis level ($\bar{x} \pm s$).

Grouping	Sample size	Apoptosis rate (%)
A	4	8.05 ± 0.46
B	4	48.95 ± 1.20*
C	4	17.80 ± 0.45 [#] *
D	4	37.50 ± 0.66 [#] *

Note: * $p < 0.01$, compared with the normal group; [#] $p < 0.01$, compared with H/R group. Group A is normal group; Group B is H/R group; Group C is exenatide group; Group D is the pathway inhibition group.

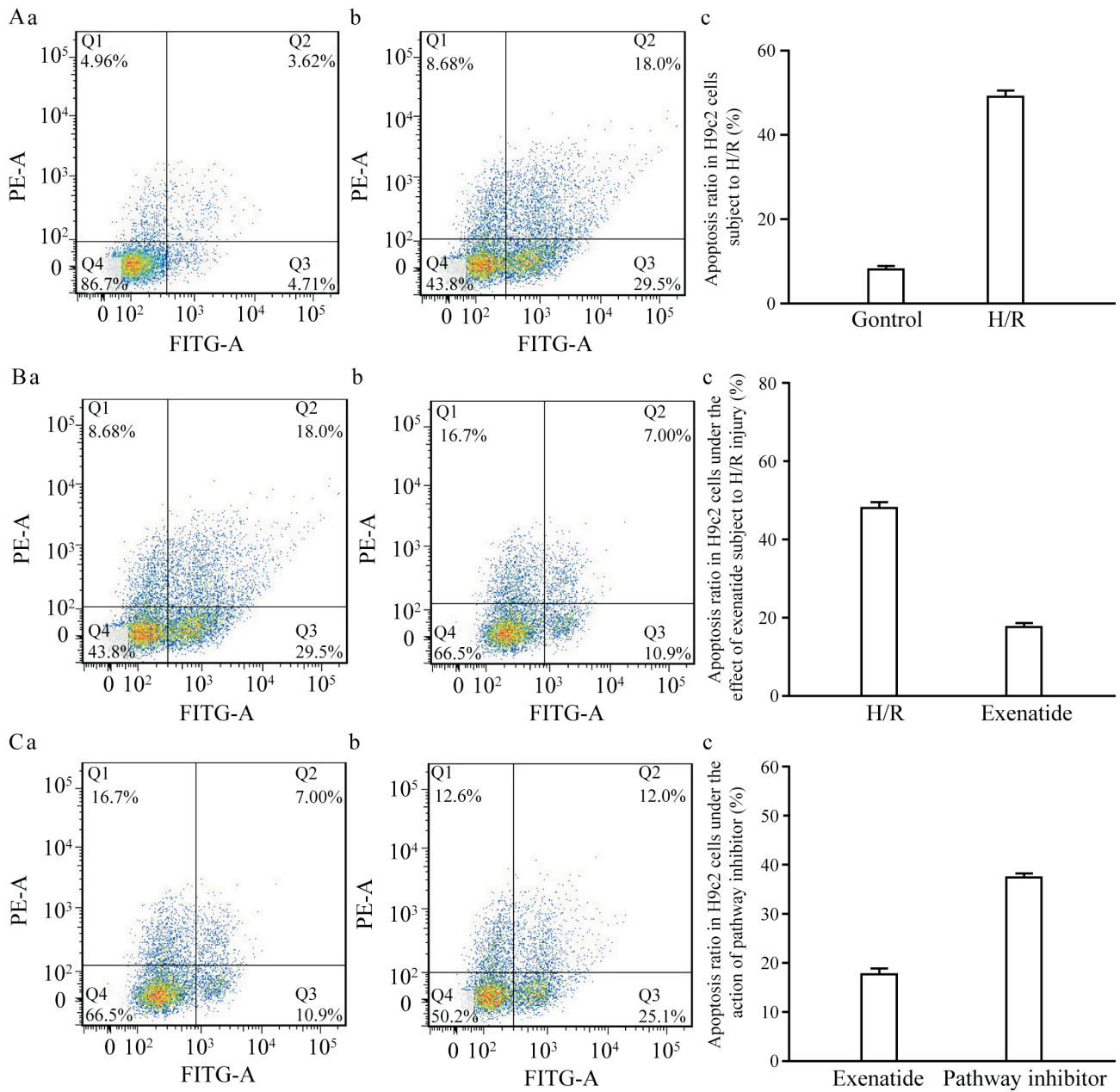


Figure 2. Comparison of the rate of apoptosis level. A: Compared with the group control, the apoptosis rate of H9c2 cells increased significantly in group H/P ($p < 0.05$). B: Compared with the group H/P, the apoptosis rate in exenatide group decreased significantly ($p < 0.01$). C: Compared with exenatide group, the apoptosis rate of the PI3K/AKT pathway inhibitor group increased significantly ($p < 0.01$).

4. Discussion

Studies have confirmed that GLP-1 receptor is widely distributed in myocardial vascular smooth muscle and vascular endothelial cells.⁵ We hypothesized that GLP-1 receptors present in the cardiovascular system may be resistant to myocardial apoptosis in some way, thereby improving the adverse effects of myocardial ischemia-reperfusion injury. In this study, the use of mixed gas access method for cardiomyocyte hypoxia and reoxygenation operation to establish the myocardial ischemia-reperfusion injury model in vitro.⁶ In this study, the H9c2 cardiomyocytes of rat as the research object, by using hypoxia reoxygenation device to simulate the pathophysiological changes of myocardial ischemia reperfusion injury, and to study the protective effect of exenatide on cardiomyocytes injured and to explore its possible mechanism. The experimental results suggest that: the survival rate of H/R car-

diomyocytes in the exenatide-treated group was significantly higher than that in the normal untreated group and H/R group, whereas the apoptosis rate shows the opposite trend, which proves that Exenatide has protective effect in hypoxia-reoxygenation cardiomyocytes, and the specific mechanism can be further explored by experiments.

Myocardial ischemia reperfusion can lead to damage of myocardial cells, manifested as serum myocardial injury markers LDH, CK-MB increased and myocardial cell apoptosis increased.⁷ In this study, by comparing the levels of myocardial enzymes in the supernatant of each experimental group to determine whether the damage of myocardial cells and assess the extent of myocardial injury, so as to provide evidence that Exenatide has protective effect in cardiomyocytes hypoxia-reoxygenation injury.

Among apoptosis regulation genes, Bcl-2 family was found to have a close relationship with apoptosis earliest. According its func-

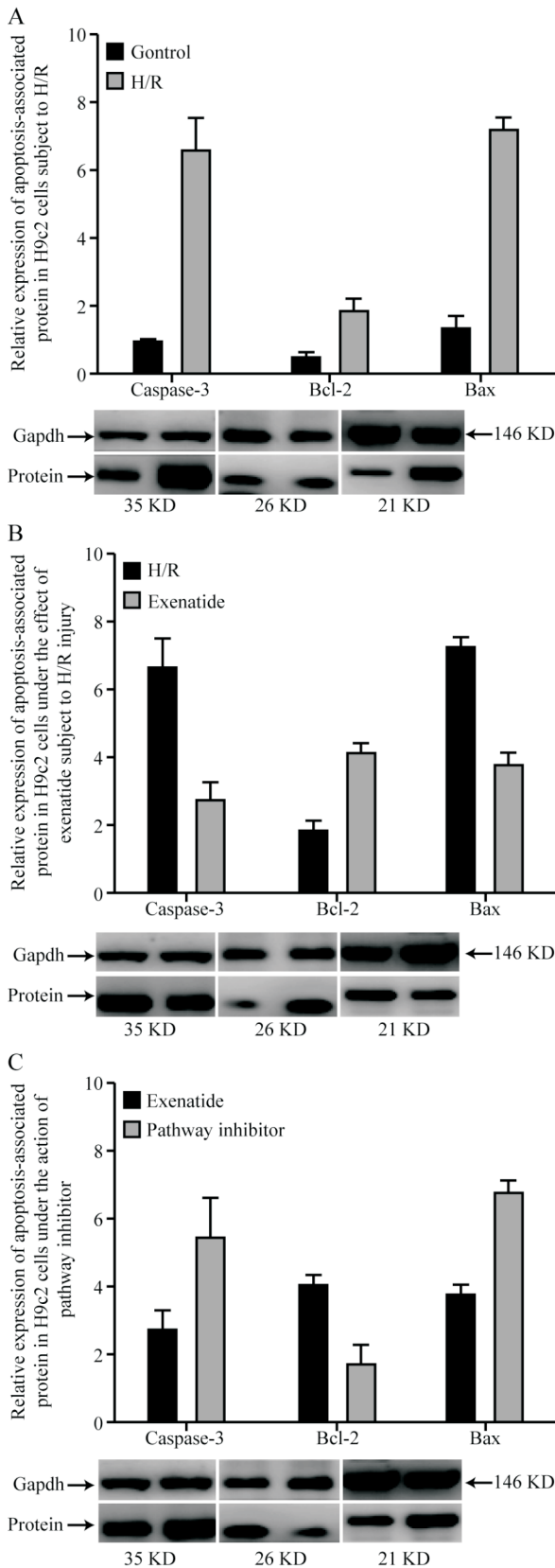


Figure 3. Effect on Caspase-3, Bax and Bcl-2 expression. A: Compared with the group control, the expression of Caspase-3 and Bax in H9c2 cells increased significantly and Bcl-2 decreased significantly in group H/P ($p < 0.01$). B: Compared with the group H/P, the expression of Caspase-3 and Bax in H9c2 cells increased significantly and Bcl-2 decreased significantly in exenatide group ($p < 0.01$). C: Compared with exenatide group, the expression of Caspase-3 and Bax increased significantly and Bcl-2 decreased significantly in the PI3K/AKT pathway inhibitor group ($p < 0.01$).

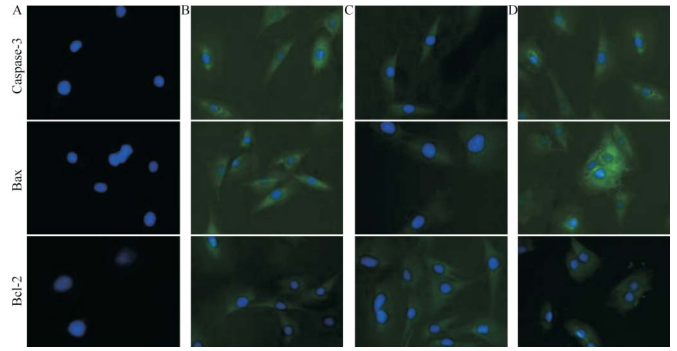


Figure 4. Comparison expression of Caspase-3, Bax and Bcl-2 ($\times 1000$).

tions can be classified as anti-apoptosis Bcl-2 family (including Bcl-2, Bcl-xL, Bcl-w, Mel-1, Nr-13, etc) and Bax subfamily of pro-apoptosis (including Bxa, Bak, Bid, Bad, Bik, Bnips, etc).⁸ The expression of Bax and Bcl-2 protein was examined in this study, the expression of apoptotic gene was significantly changed after exenatide pretreatment, the expression of Bcl-2 protein was increased and the expression of Bax protein decreased, which indicated that Exenatide pretreatment could interfere with the regulation of apoptosis-related gene expression, and thus prevent the occurrence of hypoxia-reoxygenation injury, exert anti-apoptosis and protect cardiomyocytes. Researchers studied the expression of Bcl-2 and Bax in the reperfusion region and non-reperfusion region of rats with ischemia-reperfusion injury, shown that the expression of Bcl-2 in the reperfusion region was extremely difficult to be found and the expression of Bxa was increased, which indicated that the apoptosis of cardiomyocytes occurred in the perfusion area.⁹ Similarly, it was found that Bcl-2 expression was significantly increase in the non-reperfusion region, which may be a self-protection mechanism of surviving myocardium during ischemia reperfusion.¹⁰ The caspase gene can regulate the function of Fas receptors, activates JNK and p38-k, and increases the intracellular Ca^{2+} concentration through the second messenger pathway, and the apoptosis is induced.¹¹ Caspase-3 is generally considered to be a key protease of that promotes apoptosis in Caspase family.¹² In this experiments showed that Caspase-3 almost no expression in normal group, and highest expression in hypoxia-reoxygenated cardiomyocytes. It was proved that Caspase-3 was involved in the process of hypoxia-reoxygenation injury in cardiomyocytes. After Exenatide intervened, the expression of Caspase-3 compared decreased, but still higher than the normal group confirmed that Exenatide could interfere with the process of hypoxia-reoxygenation injury in some way, so as to reduce the reperfusion injury and protect the function of cardiomyocytes. Myat et al.¹³ conducted an experimental study of myocardial ischemia-reperfusion injury in rats, found that activation of Caspase-3 is a mechanism of cardiomyocyte apoptosis after MIRI which leading to decreased cardiac function, so provided a new site that inhibiting the expression of Caspase-3 to prevent MIRI.

There were several research evidences indicated that PI3K-AKT signal transduction play a very important role in myocardial ischemia-reperfusion injury.^{14,15} The expression of Bax and Caspase-3 protein in hypoxia-reoxygenation cardiomyocytes were increased significantly after inhibiting PI3K-AKT signal pathway, and the expression of Bcl-2 protein were significantly down-regulated, confirmed that PI3K-AKT signaling pathway participated in the process of Exenatide inhibition of cardiomyocyte apoptosis and protection of reperfusion myocardial cells. Other mechanisms for the activation of the PI3K-AKT signaling pathway are not covered by this experiment.

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Conflicts of interest

The authors declare no conflict of interest.

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