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ORIGINAL ARTICLE

Effect of Gypenosides on Myocardial Ischemia-Reperfusion Injury and its Mechanism

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Key words: **Gypenosides; myocardial ischemia-reperfusion; apoptosis; rats; MPTP**

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Abstract

OBJECTIVES: The present study aimed to clarify the effects of Gypenosides on myocardial ischemia-reperfusion injury. Using rat H9c2 cardiomyocytes as the research object, the model of cardiomyocyte hypoxia and reoxygenation was established to observe the protective effects of Gypenosides on myocardial ischemia-reperfusion injury, revealing the key targets and possible mechanisms for Gypenosides to exert myocardial protection.

MATERIAL AND METHODS: A model of cardiomyocyte hypoxia and reoxygenation was prepared. The activity of cardiomyocytes was detected by CCK-8 method. The cardiomyocyte injury was evaluated by LDH assay. The cardiomyocyte apoptosis rate was detected by flow cytometry. The mitochondrial membrane potential of cardiomyocytes was detected by JC-1 staining. Western blot was used to detect the expression of MPTP downstream apoptotic pathways and MPTP opening-related regulatory factors.

RESULTS: The cell survival rate of each Gypenosides pretreatment group was significantly higher than that of the hypoxia-reoxygenation group, indicating that Gypenosides could inhibit cell apoptosis and the decrease of mitochondrial membrane potential of hypoxia-reoxygenation cells. The expressions Cytochrome C, APAFl, Caspase-9, and Caspase-3 proteins were significantly lower than those of the hypoxia-reoxygenation group, the expression of Bax was significantly lower than those of the hypoxia-reoxygenation group, while the expression of Bcl2 was significantly higher than those of the hypoxia-reoxygenation group.

CONCLUSION: Gypenosides can effectively reduce myocardial ischemia-reperfusion injury in rats. By regulating Bax and Bcl2, Gypenosides can inhibit MPTP opening and the activation of downstream apoptotic pathways, thereby reducing myocardial ischemia-reperfusion injury.

Abbreviations:
HR - Hypoxia/reoxygenation group

INTRODUCTION

Acute myocardial infarction is one of the leading disabling and fatal diseases in the world today (Neri *et al.* 2017). In clinical practice, timely restoration of blood supply to ischemic myocardium, that is,

reperfusion therapy, is recognized as the most effective treatment for patients with acute myocardial infarction. Percutaneous coronary intervention and coronary bypass surgery have been widely used in clinical treatment (Hausenloy and Yellon 2016). However, the myocardial ischemia-reperfusion injury caused by reperfusion therapy cannot be ignored. The exacerbation of ischemic myocardium during reperfusion is mainly manifested by myocardial stunning, severe lethal ventricular arrhythmias, coronary no-reflow, and a further increase in the area of myocardial infarction, all of which seriously threaten the patient's life (Perez and Quintanilla 2017). Moreover, studies have shown that abnormalities of vagus nerve activity and function after myocardial infarction are closely related to the occurrence of ventricular arrhythmia. After left ventricular myocardial infarction, there are obvious abnormalities in the density and range of vagus nerve innervation in the infarct week, ventricular septum, and right ventricle. Although it is clear that ischemic preconditioning and ischemic postconditioning can reduce MIRI (Izzo *et al.* 2016), both of them have limitations in clinical application. At present, there is no effective therapeutic intervention to reduce or avoid MIRI. Therefore, the exploration of cardiac protective interventions that can prevent and treat MIRI and improve the clinical prognosis of patients with myocardial ischemia has become a hotspot in cardiovascular disease research (Shoshan Barmatz *et al.* 2017).

Gypenosides have the functions of lowering blood lipids, lowering blood sugar, antitumor, anti-aging, protecting the liver, and enhancing the immune function of the body. But the role of Gypenosides in MIRI has not been studied yet, so there is some innovative significance.

METHODS

Preparation of the hypoxia-reoxygenation model

H9c2 cardiomyocytes were obtained (Shanghai Beinuo Biotechnology Co., Ltd)., The cells were removed from a normal cell incubator (37°C, 5% CO_2) when the cells reached a density of 80-90%. The original medium was discarded. The cells were washed with PBS twice, and the Sugar-Free Earle's Medium was replaced. After this, the cells were placed in a 3-gas incubator under a low-oxygen environment (37°C, 5% O2, 5% CO2, 90% N_2) and cultured for 12 hours. Following this, the cells were removed, and the culture medium was replaced with a complete medium. The cells were cultured in a normal cell incubator for 1 h. Subsequent experiments can be performed when the hypoxia-reoxygenation process is completed. This article does not contain any studies with human participants or animals performed by any of the authors.

The grouping is as follows:

A) the Control group, in which the cells are cultured in a normal environment and untreated.
B) the Hypoxia/reoxygenation group (HR), in which the cells were put in a hypoxic environment for 12 h culture, and then cultured in a normal environment for 1 h, that is, H12h/Rlh.
C) the 10 μM Gypenosides pretreatment hypoxia/reoxygenation group, in which the cells were pretreated with 10 μM Gypenosides for 12 h before the hypoxia treatment, and then H12h/Rlh.
D) the 20 μM Gypenosides pretreatment hypoxia/reoxygenation group, in which the cells were pretreated with 20 μM Gypenosides for 12 h before hypoxia treatment, and then H12 h/Rlh.
E) the 40 μM Gypenosides pretreatment in hypoxia/reoxygenation group, in which the cells were pretreated with 40 μM Gypenosides for 12 h before hypoxia treatment, and then H12 h/Rlh.

CCK8 method to detect myocardial cell activity

The cells that have completed the corresponding treatment were obtained. The original medium was discarded. The cells were washed twice with PBS. A total of 110 μl CCK8 was added to each well. The cells were placed in the incubator for 1.5 h. The cells were taken out and a spectrophotometer was applied to measure OD value at 450 nm.

The evaluation of LDH activity to assess cardiomyocyte injury

The cells that have completed the corresponding treatment were obtained. The supernatant of each treatment group was obtained. The LDH activity was calculated following the instructions of the Kit.

Flow cytometry to detect myocardial cell apoptosis

The cells that have completed the corresponding treatment were obtained. The cell pellet was resuspended with an appropriate amount of PBS, and gently washed. The cells were centrifuged at 1,000 rpm for 5 minutes. The supernatant was discarded to obtain a washed cell pellet. An appropriate amount of bindingbuffer was added according to the number of collected cells to resuspend the cells. The no-staining control group, the Annexin V-FITC single staining control group and the PI single staining sample control group were set. A total of 5 μl Annexin V-FITC was added to the corresponding sample, mixed and placed in darkness for 15min. Then 5 μl PI was added to the corresponding sample, mixed and placed for 10min in darkness. Flow cytometry was performed on each sample within 1 h.

JC-1 staining to detect changes in mitochondrial membrane potential of cardiomyocytes

The cells that have completed the corresponding treatment were obtained. The original medium was discarded. The cells were washed with PBS once. Then 1 ml of fresh complete medium was added to each well of the six-well plate, and then 1 ml of JC-1 staining working solution was added to each well of the six-well

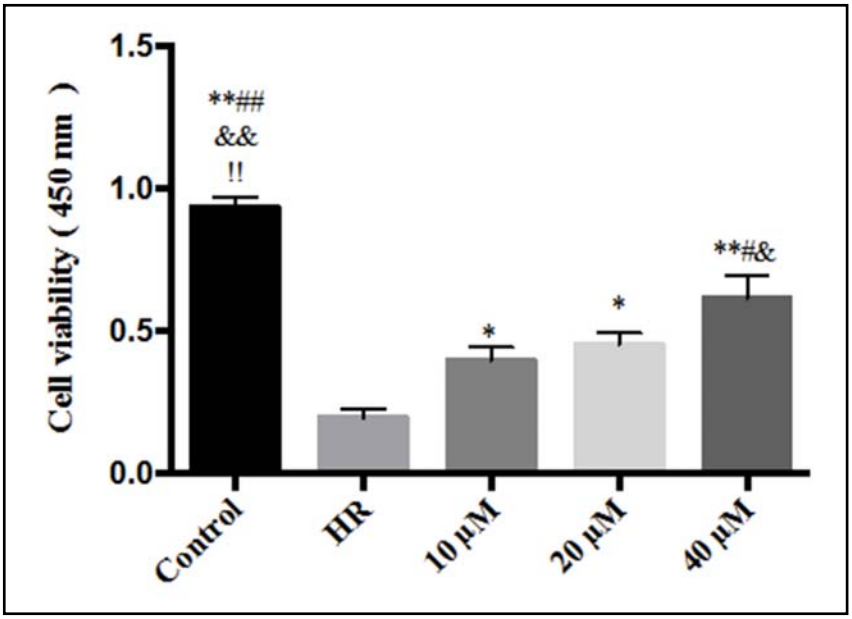

Fig. 1. CCK8 method to detect the effect of drugs on the viabilities of cardiomyocytes.
** *P*<0.01 vs HR * *P*<0.05 vs HR ## *P*<0.01 vs 10 μM # *P*<0.05 vs 10 μM
&& *P*<0.01 vs 20 μM & *P*<0.01 vs 20 μM !! *P*<0.01 vs 40 μM

plate. After mixing thoroughly, the cells were put in an incubator at 37°C for 20 min. An appropriate amount of 5 JC-1 staining buffer was diluted to 1 with deionized water, mixed and placed in an ice bath for use. The stained cells were removed. The supernatant was discarded. The cells were washed 2 times with JC-1 staining buffer (1), and then 2 ml of fresh complete medium was added to each well of the six-well plate. The cell slides of each treatment group were taken out of the plate, and corresponding slide samples were made. The cells were observed under a fluorescence microscope and images were collected.

Western-blot to measure the expressions of various proteins

After protein extraction and concentration determination, SDS-PAGE electrophoresis, wet transfer, blocking, immunization, and finally chemiluminescence ECL were all conducted. The protein expressions were determined.

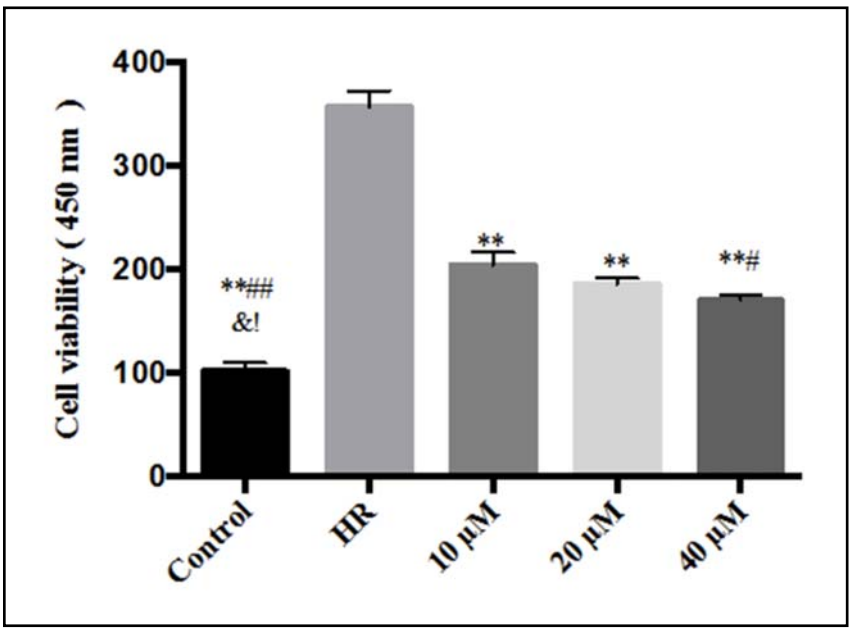

Fig. 2. Effect of drugs on LDH viability.
** *P*<0.01 vs HR ## *P*<0.01 vs 10 μM # *P*<0.05 vs 10 μM & *P*<0.01 vs 20 μM
! *P*<0.05 vs 40 μM

Statistical analysis

Each experiment was repeated three times. The Bonferroni Test in One-way ANOVA in SPSS13.0 was used to make a pair-wise comparison between multiple samples. $P<0.05$ was a significant difference.

RESULTS

CCK8 method to detect cardiomyocyte viability

The results showed that the cell viability of the HR group was significantly lower than that of the Control group, and the cell viabilities of the drug pretreatment groups were all significantly higher than that of the HR group, as shown in Figure 1.

LDH viability measurement to evaluate myocardial cell injury

The results showed that the LDH viability of the HR group was significantly higher than that of the Control group, and the cell viabilities of the drug pretreatment groups were all significantly lower than that of the HR group, as shown in Figure 2.

Flow cytometry detection of myocardial cell apoptosis rate

The results showed that the apoptosis rate of the HR group was significantly higher than that of the Control group, and the cell viabilities of the drug pretreatment groups were all significantly lower than that of the HR group, as shown in Figure 3.

JC-1 staining to detect mitochondrial membrane potential changes in cardiomyocytes

In the study, JC-1 staining was used to detect the change of DYm in cells of each experimental group. When DYm is high, red fluorescence can be generated. When DYm is low, JC-1 is in the form of a monomer, and green fluorescence is generated at this moment. Therefore, the conversion of JC-1 from red fluorescence to green fluorescence indicates a decrease in DYm. The results showed that the DYm of the HR group was significantly lower than that of the control group. Drugs with different concentrations could inhibit the decrease of DYm in hypoxic-reoxygenated cells, as shown in Figure 4.

Western-blot measurement of the expressions of various proteins

The results show that the drug can reduce the expressions of Cytochrome C, APAF1, Caspase9 and Caspase3 proteins, see Figure 5.

DISCUSSION AND CONCLUSIONS

It is currently believed that the ROS generation bursts during reperfusion, and excessive ROS further leads to the irreversible opening of MPTP, which is one of the important mechanisms for the occurrence of MIRI (Di Lisa and Bernardi 2015). MPTP is a transmembrane structure on mitochondria. The outer membrane of mitochondria has high permeability, and substances with a molecular weight of less than 1500 kDa can pass through, while the inner membrane of mitochondria only allows substances with a molecular weight of less than 1.5 kDa to pass. The proton chestnut on the inner membrane of the mitochondria transfers protons from the mitochondrial matrix into the outer chamber during the electron transfer process, thereby forming a potential difference between the inside and outside of the

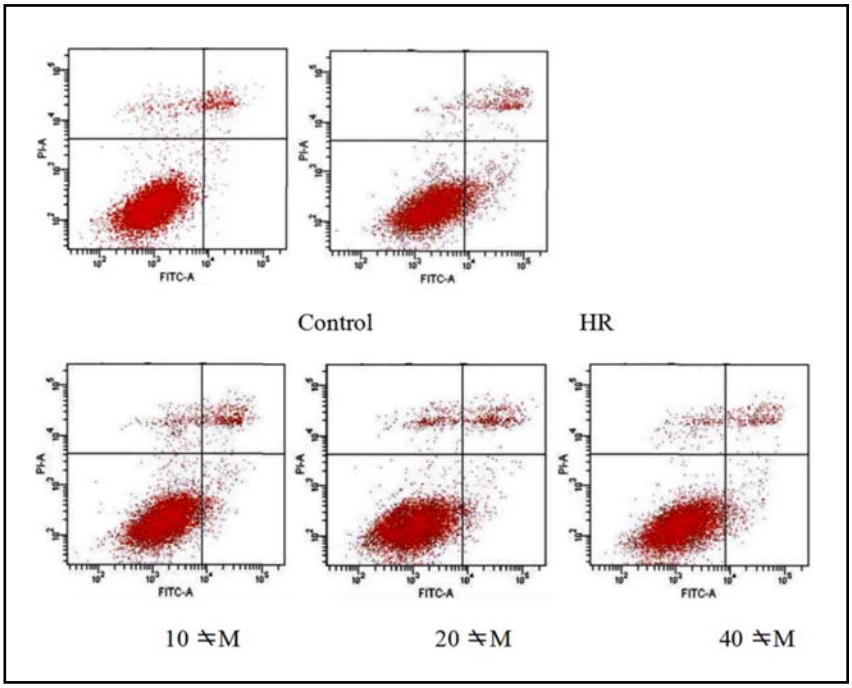

Fig. 3. Effect of drugs on myocardial apoptosis.

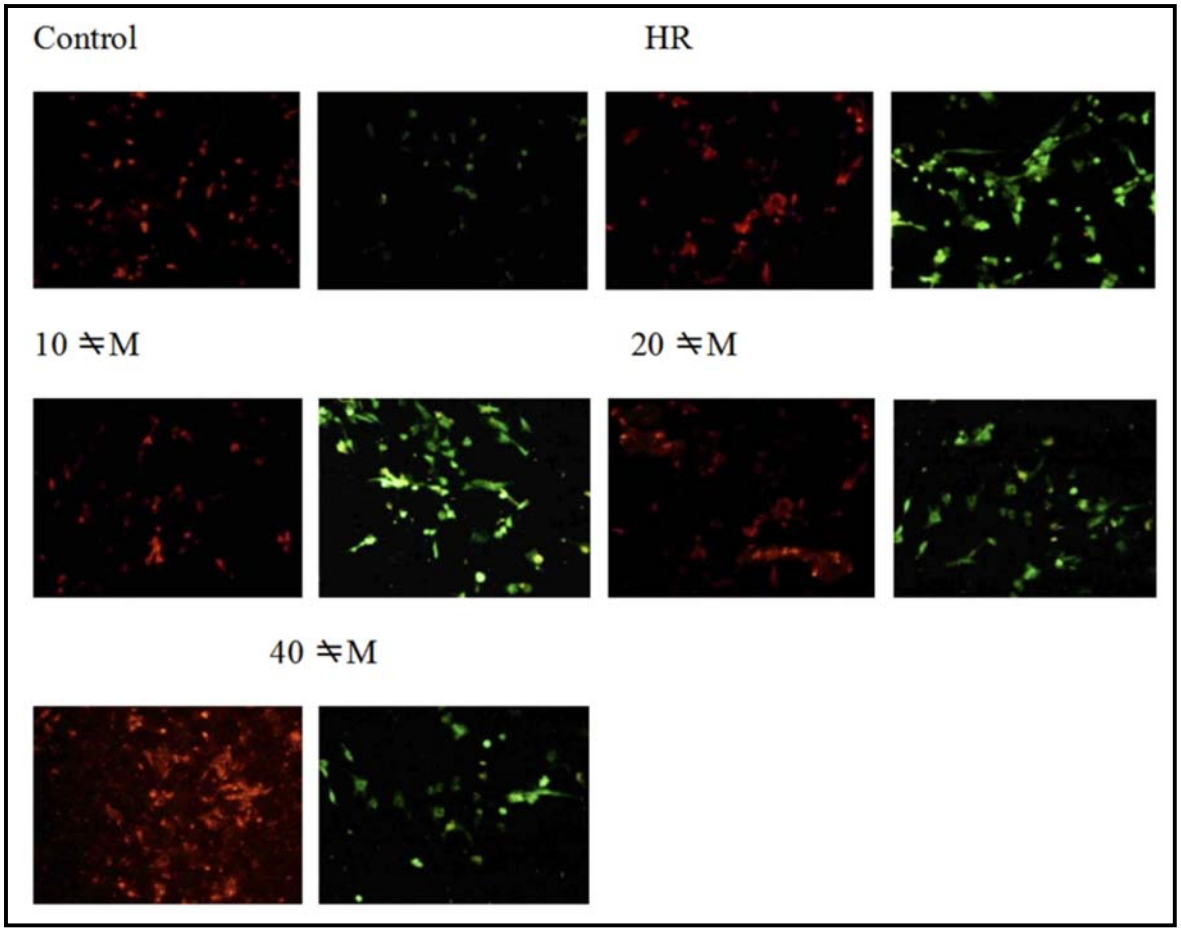

Fig. 4. Effect of drugs on mitochondrial membrane potential of cardiomyocytes.

mitochondria. Under physiological conditions, MPTP opens intermittently, and protons or positive ions in the outer chamber enter the inner chamber with a potential difference, preventing excessive accumulation of positive ions in the outer chamber. When Ca2 + overload, persistent oxidative stress, adenosine deficiency, increased phosphate concentration, and mitochondrial depolarization occur, MPTP is in an irreversible open state at a high level, and a large number of substances with a molecular weight greater than 1.5 kDa would pass through the mitochondrial inner membrane, making ΔΨm decreases rapidly, causing imbalanced ions, mitochondrial swelling, and ATP depletion, which eventually leads to cell death (Ong *et al.* 2015).

The opening of MPTP leads to an irreversible decrease in ΔΨm (Kim *et al.* 2014). Cytochrome C is

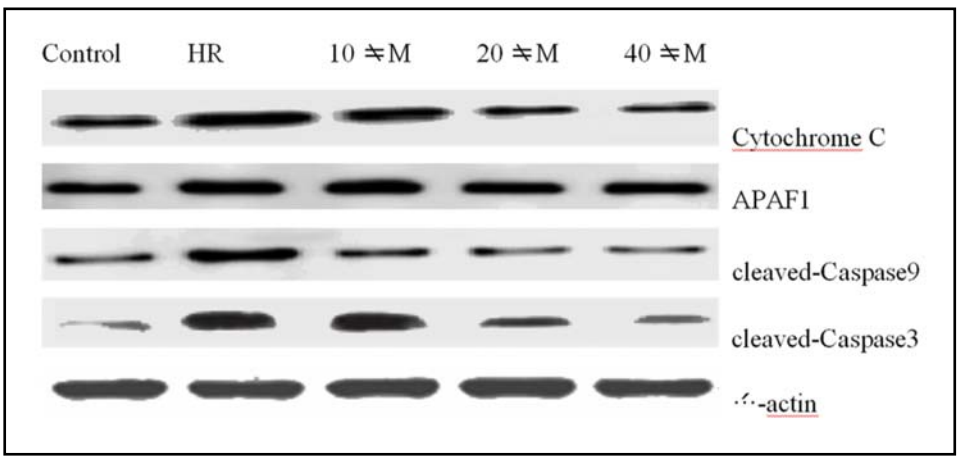

Fig. 5. Effect of drugs on the expression of various proteins.

released into the cell oar and forms apoptotic bodies with the protein precursors of apoptotic protease activating factor and cysteine protease 9, which activated Caspase9 by the help of dATP. Caspase9 continues to digest the Caspase3 precursor, initiate the caspase cascade apoptosis reaction, and eventually cause apoptosis (Wang *et al.* 2017; Ye *et al.* 2016).

At present, it is believed that the components of MPTP mainly include voltage-dependent anion channels, adenine nucleotide transposase, cyclophilin D, phosphate carrier, and F1F0ATP synthetase. Studies have shown that Bcl2 family proteins have important regulatory effects on the opening of MPTP (Perez and Quintanilla 2017). The pro-apoptotic protein Bax can promote the opening of MPTP by binding to ANT or VDAC, while the anti-apoptotic protein Bcl2 can competitively bind ANT and inhibit the binding of Bax to ANT or VDAC, thereby further inhibiting the opening of MPTP. In addition, Bcl2 overexpression can also inhibit the excessive production of mitochondrial ROS, inhibit the release of Cytochrome C and apoptosis-inducing factors, and prevent the activation of the downstream caspase cascade apoptosis response, thus exerting its anti-apoptotic effect (Feng *et al.* 2016; Karch *et al.* 2015). In the study, the expression of the apoptotic pathway downstream of the mitochondria was measured. It is found that the drug can significantly reduce the expression of Cytochrome C, APAF1, Caspase9, and Caspase3 while simultaneously inhibiting the decrease of $\Delta\Psi m$ and maintaining the stability of $\Delta\Psi m$, indicating that the drug can effectively inhibit the activation of mitochondrial downstream apoptotic pathways.

DECLARATIONS

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication

Not applicable.

Availability of data and material

Not applicable.

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None.

Competing interests

There are no potential conflicts of interest to disclose.

Author Contributions

WXL is resposible for the guarantor of integrity of the entire study, study concepts & design, definition of intellectual content, experimental studies, data acquisition & analysis,statistical analysis, manuscript preparation & editing & review; XTD is resposible for the definition of intellectual content, experimental studies, experimental studies, data acquisition & analysis; YMS and HHG are resposible for the literature research, clinical studies; MP is resposible for the guarantor of integrity of the entire study, study design, literature research, literature research, clinical studies, statistical analysis, manuscript preparation & editing & review. All authors read and approved the final manuscript.

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