

## Original Article

# Effects of microRNA-139 on myocardial cell injury induced by oxidative stress

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**Abstract:** Objective: This study aims to explore the effects of miR-139 on myocardial cell injury induced by oxidative stress and its mechanisms. Methods: H9c2 cells were used in this study. They were divided into control group, H<sub>2</sub>O<sub>2</sub> group, H<sub>2</sub>O<sub>2</sub>+miR-139-5-p NC group and H<sub>2</sub>O<sub>2</sub>+miR-139-5-p mimics group. Cell activity was detected by MTT method. ROS level was detected by DCFH-DA probe method. MDA and SOD levels and Caspase 3 activity were detected by spectrophotometry. The cell apoptosis was detected by Hoechst 33342 and Annexin V-FITC/PI staining methods. The expression levels of AKT, GSK-3 $\beta$ , Bax and Bcl-2 were determined by Western blotting methods. Results: It showed that the activity of H9c2 cells decreased with the increase of the dose of H<sub>2</sub>O<sub>2</sub>. The activity of miR-139-5-p in H9c2 cells decreased after treatment of H<sub>2</sub>O<sub>2</sub> for 6 h ( $P<0.01$ ). Compared with control group, cell activity in H<sub>2</sub>O<sub>2</sub> group and H<sub>2</sub>O<sub>2</sub>+miR-139-5-p NC group decreased ( $P<0.01$ ), ROS fluorescence intensity increased ( $P<0.01$ ), MDA content increased ( $P<0.01$ ), SOD content decreased ( $P<0.01$ ), apoptosis degree, Caspase 3 activity and Bax levels increased ( $P<0.01$ ), Bcl-2, AKT and GSK-3 $\beta$  decreased ( $P<0.01$ ). However, they were opposite in H<sub>2</sub>O<sub>2</sub>+miR-139-5-p mimics compared with H<sub>2</sub>O<sub>2</sub> group and H<sub>2</sub>O<sub>2</sub>+miR-139-5-p NC group. Conclusions: miR-139-5-p expressed low in oxidative stress of H9c2 cells induced by H<sub>2</sub>O<sub>2</sub> and the oxidative stress injury could be inhibited after increasing the expression of miR-139-5-p, which could be related with the elimination of intracellular oxidative stress products and the resistance to apoptosis through AKT/GSK-3 $\beta$  signaling pathway.

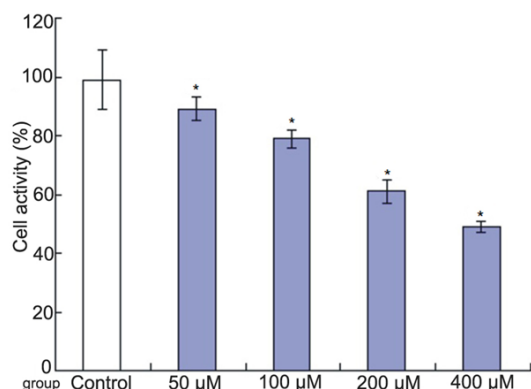
**Keywords:** miR-139-5-p, H9c2 cells, Oxidative stress, ROS, apoptosis, AKT/GSK-3 $\beta$  signaling pathway

## Introduction

microRNA (miRNAs) is a kind of highly conserved endogenous non encoding small molecule RNA. It can bind to the 3'-UTR region of target gene mRNA and participate in the pathophysiology of many diseases by stopping the translation of target genes and regulating its expression. In recent years it was found that miRNAs was involved in the physiological and pathological processes of heart development, cardiac hypertrophy, cardiac remodeling, heart failure, arrhythmia and cardiac ischemia/reperfusion [1]. There were many miRNAs expressed in cardiac muscle cells specifically and participated in the pathophysiology of cardiovascular disease, such as miR-1, miR-29, miR-21, miR-126, miR-133, miR-199 and miR024 [2]. Oxidative stress is one of the important mechanisms of cardiovascular diseases such as ischemia reperfusion injury. Reactive oxygen spe-

cies (ROS) not only can lead to oxidative stress injury, but also induce apoptosis of cardiomyocytes [3, 4]. Therefore, resistance to oxidative stress can inhibit cell damage and apoptosis in a certain extent.

miR-139 was located in chromosome 11q13.4 consisting of 21 amino acids. The primary transcripts were cleaved by RNase and generated two 22-nt mature miRNAs miR-139-5-p and miR-139-3p. The expression of miR-139-5p was down regulated in a variety of tumor cells such as colorectal cancer, gastric cancer, and ovarian cancer and participated in the development of cancer [5]. Saddic found 21 miRNAs abnormal expression in myocardial tissue in left ventricular ischemia patients and down-regulation of miR-139-5p [6]. In this study we detected the expression of miR-139-5p in oxidative stress injury of H9c2 cells and explored its mechanism.



**Figure 1.** The effects of  $H_2O_2$  on activity of H9c2 cells. Compared with control, \* $P < 0.01$ .

## Materials and methods

### Cell lines and cell culture

Rat cardiac muscle cell line H9c2 was supplied by the cell center of Chinese Academy of Sciences (GMR5, Shanghai, China). H9c2 cells were cultured in DMEM (GIBCO) supplemented with 10% fetal bovine serum (GIBCO) at 37°C with 5%  $CO_2$ . They were treated with 50 μM, 100 μM, 200 and 400 μM  $H_2O_2$  respectively and selected treatment of 200 μM  $H_2O_2$  to do other experiment. The H9c2 cells were divided into control group,  $H_2O_2$  group,  $H_2O_2$ +miR-139-5-p NC group and  $H_2O_2$ +miR-139-5-p mimics group. Transfection of cells was performed using lipofectamine 2000 and miR-139-5-p mimics and miR-139-5-p negative control (NC) according to the manual.

### Detection of cell activity by MTT assay

H9c2 cells were harvested and adjusted their concentration, they were seeded at density 5000 cells/well in 96-well plates and cultured at 37°C with 5%  $CO_2$  for 48 h. The cells were added 20 μl MTT (5 mg/ml) and incubated for an additional 4 h at 37°C. Then culture medium was removed and 150 μL of DMSO were added to each well with shaking at low speed for 10 min. The MTT solution was aspirated and optical densities (OD) of the supernatant were read at 570 nm using a Microplate Reader (Thermo, Molecular Device Co). The experiments were repeated three times and the negative control was conducted using only cell-free culture medium (means  $\pm$  SEM).

### RNA extraction and real-time PCR

Total RNA was extracted using Trizol Kit (Invitrogen) according to the manufacturer's protocol. 1 μg total RNA was subjected to reverse transcription using reverse transcription system (Promega). Real-time PCR were performed using SYBR Green PCR Master Mix (Qigen). Primers were as follows: miR-139-5p Forward: 5'-CCTCTACAGTGCACGTGTCTC-3', miR-139-5p Reverse: 5'-CGCTGTTCTCATCTGTCCTCGC-3'; GADPH Forward: 5'-AGCCACATCGCTCAGACA-3', Reverse: 5'-TGGACTCCACGACGTACT-3'. At the end of each reaction, a melting curve analysis was performed to confirm the absence of primer dimers. Glyceraldehyde 3-phosphate dehydrogenase (GADPH) gene was used as an internal control for normalization of RNA quantity and quality differences in all samples. Quantifications of target genes mRNA was performed using the  $2^{-\Delta\Delta Ct}$  method. The PCR products underwent electrophoresis on 2.5% agarose gel and were then visualized under UV illumination using ethidium bromide staining.

### ROS content determination

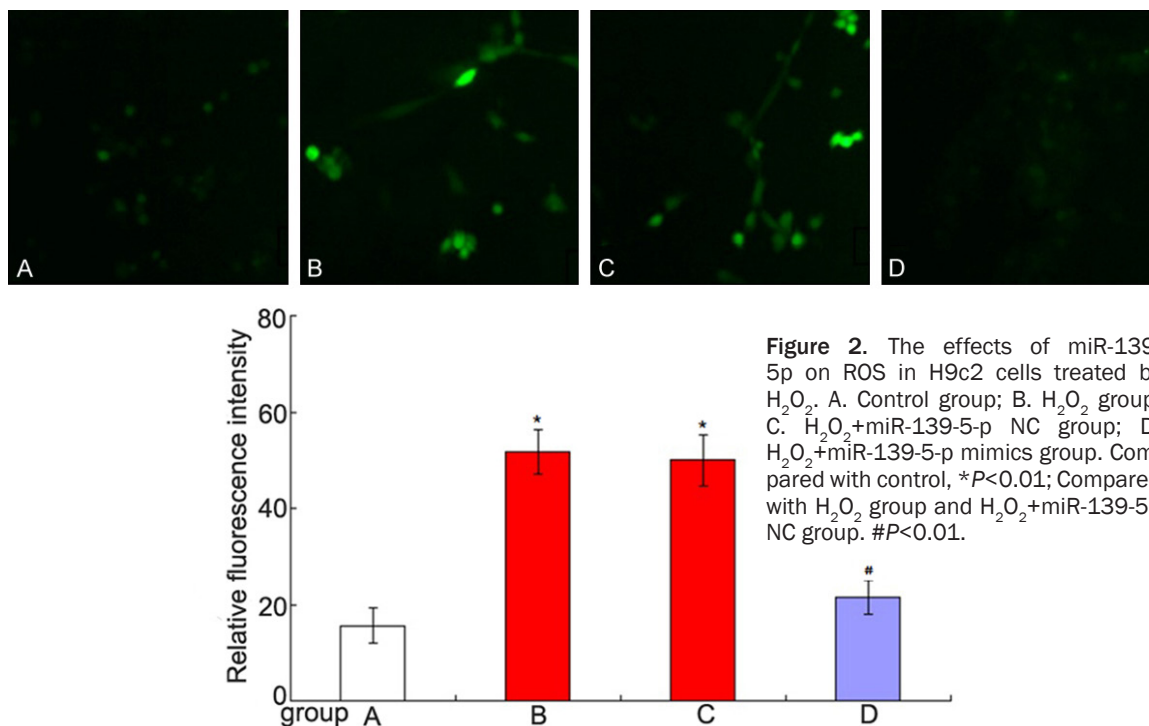
H9c2 cells were harvested and adjusted their concentration, they were seeded at density  $1 \times 10^4$  cells/well in 6-well plates and cultured at 37°C with 5%  $CO_2$  for 48 h. The cells were added 20 μM DCFH-DA (PBS dilution) and incubated for an additional 2 h at 37°C. Then they were observed under fluorescence microscope.

### Determination of MDA and SOD content

H9c2 cells were harvested and adjusted their concentration, they were seeded at density  $1 \times 10^4$  cells/well in 6-well plates and cultured at 37°C with 5%  $CO_2$  for 48 h. The cells were frozen and thawed repeatedly and the supernatant was collected to determine the MDA and SOD content respectively according to the manual.

### Flow cytometry analysis

Fluorescein Annexin V-FITC/PI double labeling was performed with the Annexin V-FITC apoptosis detection kit (Beckman) to detect the apoptosis of H9c2 cells. They were seeded in 6-well plates and cultured at 37°C with 5%  $CO_2$  for 48



**Figure 2.** The effects of miR-139-5p on ROS in H9c2 cells treated by H<sub>2</sub>O<sub>2</sub>. A. Control group; B. H<sub>2</sub>O<sub>2</sub> group; C. H<sub>2</sub>O<sub>2</sub>+miR-139-5-p NC group; D. H<sub>2</sub>O<sub>2</sub>+miR-139-5-p mimics group. Compared with control, \**P*<0.01; Compared with H<sub>2</sub>O<sub>2</sub> group and H<sub>2</sub>O<sub>2</sub>+miR-139-5p NC group. #*P*<0.01.

**Table 1.** The effects of miR-139-5p on MDA and SOD in H9c2 cells treated by H<sub>2</sub>O<sub>2</sub> ( $\bar{x} \pm s$ )

| group   | MDA (mmol/L protein) | SOD (U/L protein) |
|---|----------------------|-------------------|
| control   | 1.68±0.20            | 19.41±3.40        |
| H <sub>2</sub> O <sub>2</sub>                     | 4.30±0.35*           | 9.80±1.33*        |
| H <sub>2</sub> O <sub>2</sub> +miR-139-5-p NC     | 4.22±0.62*           | 9.76±1.57*        |
| H <sub>2</sub> O <sub>2</sub> +miR-139-5-p mimics | 2.05±0.25**          | 15.63±1.63**      |

Compared with control, \**P*<0.01; Compared with H<sub>2</sub>O<sub>2</sub> group and H<sub>2</sub>O<sub>2</sub>+miR-139-5p NC group. \*\**P*<0.01.

h. Then cells were stained with Annexin V-FITC and PI according to the manual of the kit.

#### Hoechst33342/PI staining

The cells were seeded in 6-well plates and cultured at 37°C with 5% CO<sub>2</sub> for 48 h. Then they were stained with Hoechst33342/PI staining kit according to the manual of the kit. They were observed under fluorescence microscope.

#### Activity detection of caspase 3

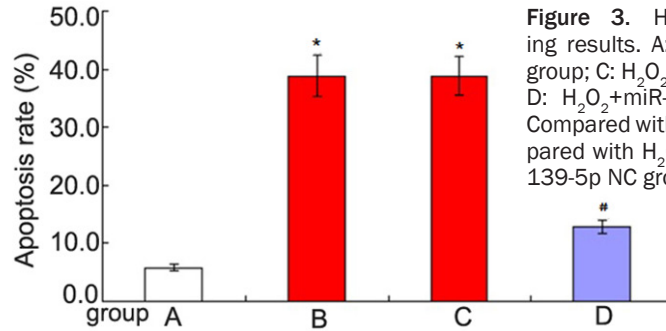
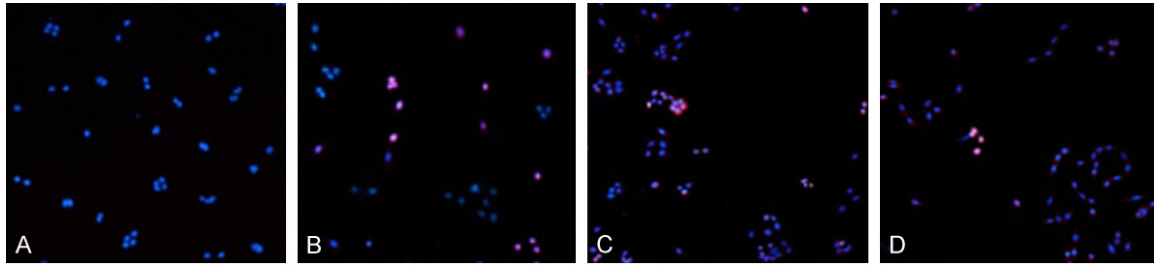
The cells were seeded in 6-well plates and cultured at 37°C with 5% CO<sub>2</sub> for 48 h. The activity of Caspase-3 was detected using Caspase-3 spectrophotometric detection kit according to the manual. OD values at 405 nm were determined.

#### Western blotting

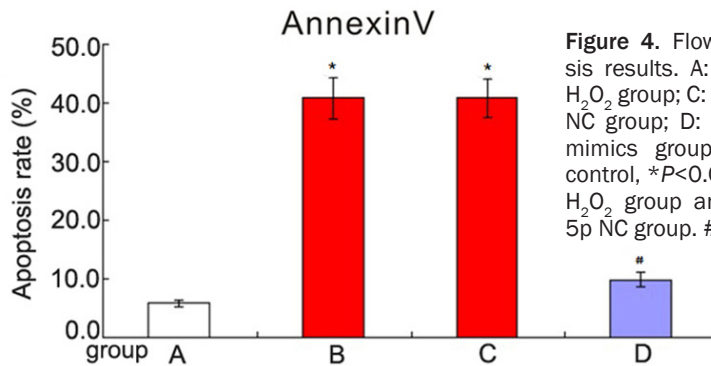
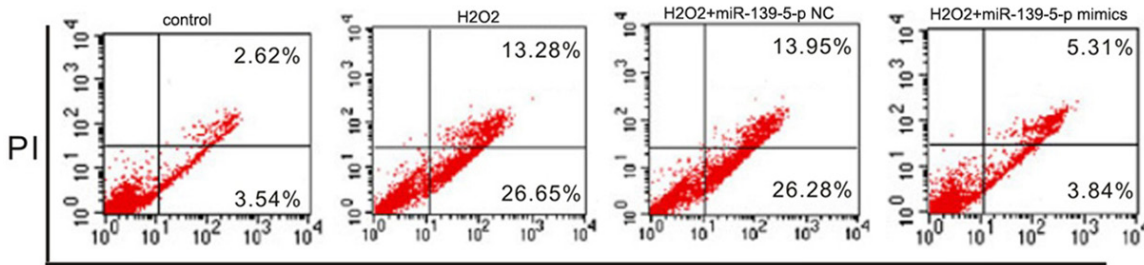
The cells were lysed with RIPA lysis buffer. Total proteins were isolated and their concentration was determined by BCA kit according to the manual. Proteins were separated on 12% polyacrylamide gels and transferred to PVDF membrane. After the transmembrane, PVDF membrane was rinsed with TBS for 10 to 15 min, placed in TBS/T blocking buffer containing 5% (w/v) skimmed milk powder and shook at room temperature for one hour. It was incubated at room temperature for two hours after added with appropriate primary antibodies. Then the membrane was rinsed with TBST for three times (5 to 10 minutes one time). The membrane was incubated at room temperature for one hour with HRP labeled secondary antibody (1:10000) and rinsed for three times with TBST (5 to 10 minutes at a time). The protein bands were scanned and quantified as a ratio to GAPDH.

#### Statistical analysis

The data are expressed as mean ± SD and analyzed with SPSS 17.0 software, t test was used to evaluate the differences among groups. A value of *P*<0.05 was taken to denote statistical significance.



**Figure 3.** Hoechst33342/PI staining results. A: Control group; B: H<sub>2</sub>O<sub>2</sub> group; C: H<sub>2</sub>O<sub>2</sub>+miR-139-5p NC group; D: H<sub>2</sub>O<sub>2</sub>+miR-139-5p mimics group. Compared with control, \*P<0.01; Compared with H<sub>2</sub>O<sub>2</sub> group and H<sub>2</sub>O<sub>2</sub>+miR-139-5p NC group. #P<0.01.



**Figure 4.** Flow cytometry analysis results. A: Control group; B: H<sub>2</sub>O<sub>2</sub> group; C: H<sub>2</sub>O<sub>2</sub>+miR-139-5p NC group; D: H<sub>2</sub>O<sub>2</sub>+miR-139-5p mimics group. Compared with control, \*P<0.01; Compared with H<sub>2</sub>O<sub>2</sub> group and H<sub>2</sub>O<sub>2</sub>+miR-139-5p NC group. #P<0.01.

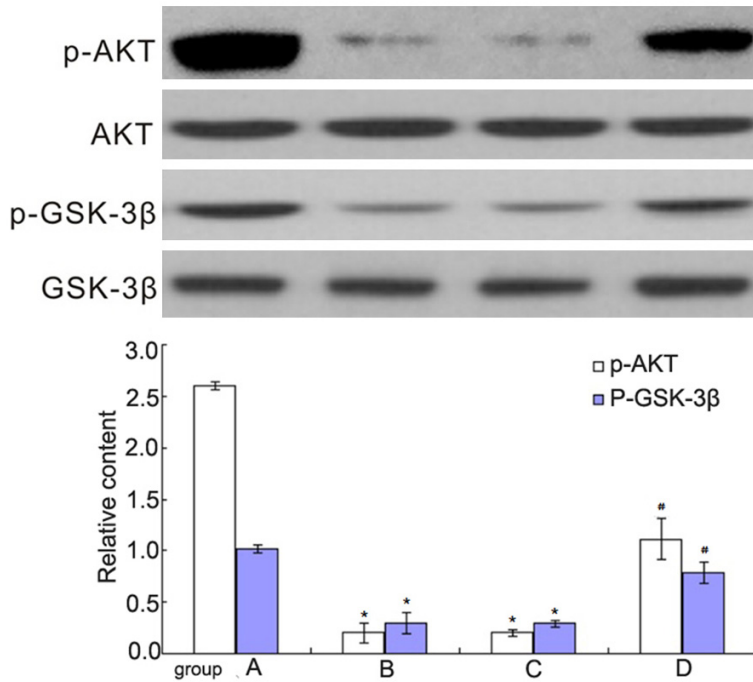
**Results**

*The effects of H<sub>2</sub>O<sub>2</sub> on activity of H9c2 cells*

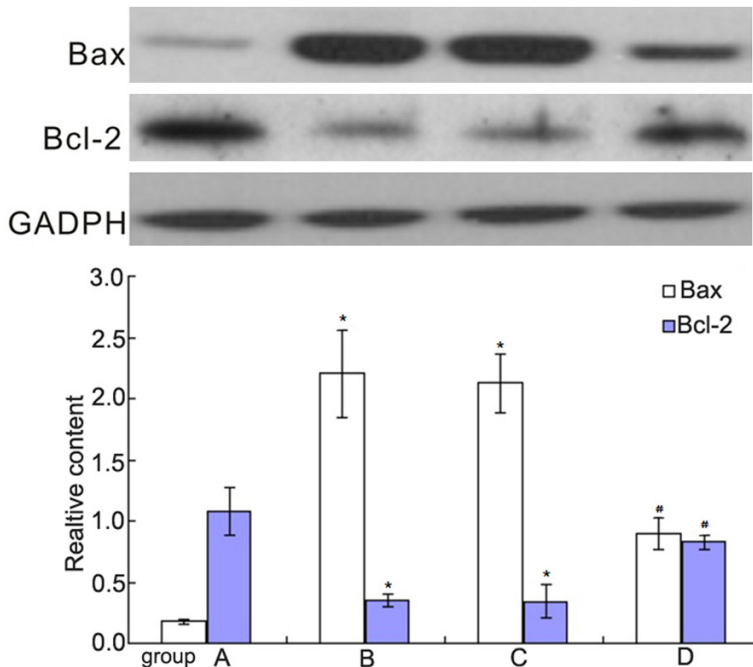
The effects of H<sub>2</sub>O<sub>2</sub> on activity of H9c2 cells were shown in **Figure 1**. It showed that the activity of H9c2 cells decreased gradually with the content of H<sub>2</sub>O<sub>2</sub> increased. We selected 200 μM H<sub>2</sub>O<sub>2</sub> to do other experiments.

*The effects of miR-139-5p on ROS in H9c2 cells treated by H<sub>2</sub>O<sub>2</sub>*

As shown in **Figure 2**, the fluorescence intensity of ROS increased in H<sub>2</sub>O<sub>2</sub> group and H<sub>2</sub>O<sub>2</sub>+miR-139-5p NC group compared with control group (P<0.01), while it decreased in H<sub>2</sub>O<sub>2</sub>+miR-139-5p mimics group compared with H<sub>2</sub>O<sub>2</sub> group and H<sub>2</sub>O<sub>2</sub>+miR-139-5p NC group (P<0.01).



**Figure 5.** The western blotting results of AKT and GSK-3β. A: Control group; B: H<sub>2</sub>O<sub>2</sub> group; C: H<sub>2</sub>O<sub>2</sub>+miR-139-5-p NC group; D: H<sub>2</sub>O<sub>2</sub>+miR-139-5-p mimics group. Compared with control, \**P*<0.01; Compared with H<sub>2</sub>O<sub>2</sub> group and H<sub>2</sub>O<sub>2</sub>+miR-139-5p NC group. #*P*<0.01.



**Figure 6.** The effects of miR-139-5p on Bax and Bcl-2 in H9c2 cells treated by H<sub>2</sub>O<sub>2</sub>. A: Control group; B: H<sub>2</sub>O<sub>2</sub> group; C: H<sub>2</sub>O<sub>2</sub>+miR-139-5-p NC group; D: H<sub>2</sub>O<sub>2</sub>+miR-139-5-p mimics group. Compared with control, \**P*<0.01; Compared with H<sub>2</sub>O<sub>2</sub> group and H<sub>2</sub>O<sub>2</sub>+miR-139-5p NC group. #*P*<0.01.

*The effects of miR-139-5p on MDA and SOD in H9c2 cells treated by H<sub>2</sub>O<sub>2</sub>*

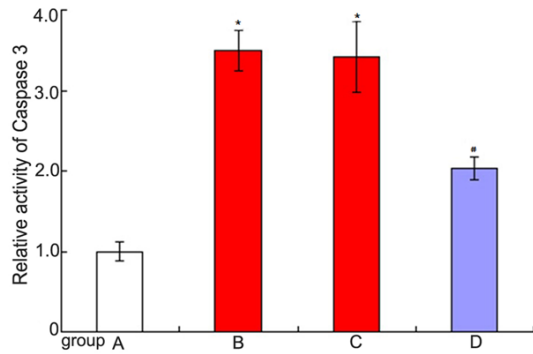
The effects of miR-139-5p on MDA and SOD in H9c2 cells treated by H<sub>2</sub>O<sub>2</sub> were shown in **Table 1**. It showed that the MDA content increased and the SOD content decreased significantly in H<sub>2</sub>O<sub>2</sub> group and H<sub>2</sub>O<sub>2</sub>+miR-139-5p NC group compared with control group (*P*<0.01), while they were contrary in H<sub>2</sub>O<sub>2</sub>+miR-139-5-p mimics group compared with H<sub>2</sub>O<sub>2</sub> group and H<sub>2</sub>O<sub>2</sub>+miR-139-5p NC group (*P*<0.01).

*The effects of miR-139-5p on H9c2 cell apoptosis treated by H<sub>2</sub>O<sub>2</sub>*

The effects of miR-139-5p on H9c2 cell apoptosis treated by H<sub>2</sub>O<sub>2</sub> were shown in **Figures 3 and 4**. We could find that there were more apoptosis cells in H<sub>2</sub>O<sub>2</sub> group and H<sub>2</sub>O<sub>2</sub>+miR-139-5p NC group compared with control group (*P*<0.01), while apoptosis cells decreased in H<sub>2</sub>O<sub>2</sub>+miR-139-5-p mimics group compared with H<sub>2</sub>O<sub>2</sub> group and H<sub>2</sub>O<sub>2</sub>+miR-139-5p NC group (*P*<0.01).

*The effects of miR-139-5p on AKT/GSK-3β pathway in H9c2 cells treated by H<sub>2</sub>O<sub>2</sub>*

The western blotting results of AKT and GSK-3β were shown in **Figure 5**. It showed that the phosphorylation level of AKT and GSK-3β decreased in H<sub>2</sub>O<sub>2</sub> group and H<sub>2</sub>O<sub>2</sub>+miR-139-5p NC group compared with control group (*P*<0.01), while they were contrary in H<sub>2</sub>O<sub>2</sub>+miR-139-5-p mimics group compared with



**Figure 7.** The effects of miR-139-5p on Caspase 3 activity in H9c2 cells treated by H<sub>2</sub>O<sub>2</sub>. A: Control group; B: H<sub>2</sub>O<sub>2</sub> group; C: H<sub>2</sub>O<sub>2</sub>+miR-139-5-p NC group; D: H<sub>2</sub>O<sub>2</sub>+miR-139-5-p mimics group. Compared with control, \*P<0.01; Compared with H<sub>2</sub>O<sub>2</sub> group and H<sub>2</sub>O<sub>2</sub>+miR-139-5p NC group. #P<0.01.

H<sub>2</sub>O<sub>2</sub> group and H<sub>2</sub>O<sub>2</sub>+miR-139-5p NC group (P<0.01).

*The effects of miR-139-5p on Bax and Bcl-2 in H9c2 cells treated by H<sub>2</sub>O<sub>2</sub>*

The effects of miR-139-5p on expression of Bax and Bcl-2 in H9c2 cells treated by H<sub>2</sub>O<sub>2</sub> were shown in **Figure 6**. It showed that the Bax expression levels increased and the Bcl-2 expression levels decreased significantly in H<sub>2</sub>O<sub>2</sub> group and H<sub>2</sub>O<sub>2</sub>+miR-139-5p NC group compared with control group (P<0.01), while they were contrary in H<sub>2</sub>O<sub>2</sub>+miR-139-5-p mimics group compared with H<sub>2</sub>O<sub>2</sub> group and H<sub>2</sub>O<sub>2</sub>+miR-139-5p NC group (P<0.01).

*The effects of miR-139-5p on Caspase 3 activity in H9c2 cells treated by H<sub>2</sub>O<sub>2</sub>*

The effects of miR-139-5p on Caspase 3 activity in H9c2 cells treated by H<sub>2</sub>O<sub>2</sub> were shown in **Figure 7**. It showed that the activity of Caspase 3 increased in H<sub>2</sub>O<sub>2</sub>+miR-139-5-p NC group compared with control group (P<0.01), while it decreased in H<sub>2</sub>O<sub>2</sub>+miR-139-5-p mimics group compared with H<sub>2</sub>O<sub>2</sub> group and H<sub>2</sub>O<sub>2</sub>+miR-139-5p NC group (P<0.01).

**Discussion**

A large number of ROS can be produced in the course of the cardiovascular disease, which can cause oxidative stress damage and eventually lead to apoptosis and necrosis of myocardial cells [3, 4]. Therefore, resistance to oxida-

tive stress damage is of great significance for the treatment of cardiovascular disease. H<sub>2</sub>O<sub>2</sub> is a kind of ROS and can induce oxidative stress injury in myocardial cells. It was often used to simulate myocardial ischemia, ischemia reperfusion injury, etc. In this study we found that 200 μM H<sub>2</sub>O<sub>2</sub> could inhibit the activity of H9c2 cells, we selected this content to do the experiment, the content was similar with the references [7, 8].

MDA is a typical byproduct of lipid peroxidation in the cell membrane, its content was positively correlated with the extent of cell damage. SOD is a class of endogenous antioxidant enzymes and can remove the active oxygen species in the cells. Therefore, the degree of oxidative damage of the cells can be indirectly reflected by detecting the changes of MDA and SOD in the cell. In this study we found that over-expression of miR-139-5-p could increase SOD content and decrease MDA content in H9c2 cells treated by H<sub>2</sub>O<sub>2</sub>, which suggested that miR-139-5-p could resist to oxidative stress damage in H9c2 cells induced by H<sub>2</sub>O<sub>2</sub> through eliminating the oxidative stress products in the cells.

It was confirmed that increased ROS could lead to the decrease of myocardial cell survival rate and induce apoptosis [9]. miR-139-5-p agonist could relieve the cerebral injury and neuron apoptosis in rats with hypoxic ischemic brain damage [10]. In this study we found that over expression of miR-139-5-p could inhibit apoptosis of H9c2 cells treated by H<sub>2</sub>O<sub>2</sub>, which suggested that miR-139-5-p could resist to oxidative stress damage in H9c2 cells induced by H<sub>2</sub>O<sub>2</sub> through inhibiting cell apoptosis. Bcl-2 family proteins play a key role in the process of cell apoptosis and Bcl-2 is the most important anti-apoptotic protein. Caspase is the executor of apoptosis and Caspase 3 is the main effect factor in the process of cell apoptosis. It was found that Bcl-2 expression decreased and Bax expression and Caspase 3 activity increased in cell apoptosis, it could significantly inhibit the apoptosis when these changes were reversed [11, 12]. We also confirmed these in this study.

ROS has the roles of activating transcription factors and gene expression, inhibiting AKT, ERK signal pathway and leading to cell apoptosis [13, 14]. miR-139-5-p could induce apoptosis of Tca8113 cells by AKT signaling pathway

[15]. PI3K/AKT signaling pathway can regulate cell proliferation, adhesion and other biological functions. It was confirmed that PI3K/AKT signal pathway was a survival signal of cardiomyocytes treated by H<sub>2</sub>O<sub>2</sub> and also upstream survival signal of Bcl-2 and Bax [16, 17]. The apoptosis of H9c2 cells induced by H<sub>2</sub>O<sub>2</sub> was accompanied by decreased phosphorylation levels of p-AKT and p-GSK-3β [12, 18, 19]. The apoptosis of H9c2 cells induced by H<sub>2</sub>O<sub>2</sub> could be resisted by activating AKT/GSK-3β pathway [20-22]. In this study we found that over expression of miR-139-5-p could increase phosphorylation levels of p-AKT and p-GSK-3β in H9c2 cells treated by H<sub>2</sub>O<sub>2</sub>. We also found that miR-139-5-p could increase Bcl-2 expression and decrease Bax expression, which suggested that miR-139-5-p could inhibit apoptosis by activating AKT/GSK-3β pathway and regulating apoptosis related proteins expression.

In a word, over-expression of miR-139-5-p could significantly reverse oxidative stress damage of H9c2 cells induced by H<sub>2</sub>O<sub>2</sub>, which could be related with eliminating the oxidative stress products in the cells and resisting apoptosis by AKT/GSK-3β signal pathway.

#### Disclosure of conflict of interest

None.

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