

Original Article

miR-210 inhibits oxidative stress damage in myocardial cell injury model

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Received October 4, 2016; Accepted November 30, 2016; Epub March 1, 2017; Published March 15, 2017

Abstract: This study is to investigate the mechanism of miR-210 in myocardial cell injury induced by oxidative stress. Viability of H9c2 cell induced by different concentrations of hydrogen peroxide (H_2O_2) was determined by MTT. The expression of miR-210 was detected by RT-PCR. H9c2 cardiomyocytes were transfected with miR-210 mimics or miR-210 negative control (NC) respectively, and were then divided into normal control group, H_2O_2 group, H_2O_2 + miR-210 NC group, and H_2O_2 + miR-210 mimics group. Cell apoptosis was detected by Hoechst staining method. The mitochondrial membrane potential (MMP) was determined under fluorescence microscope. The content of malondialdehyde (MDA), the activity of superoxide dismutase (SOD), L-Glutathione (GSH-Px) and Catalase (CAT) were detected. The activity of Caspase 3 and Caspase 9 were determined by colorimetry. The expression of Bcl-2, Bax, cytochrome (CytC) and apoptotic protease activating factor-1 (Apaf-1) were assayed by western blot. Compared with control group, cell viability decreased ($P < 0.01$), while cell apoptotic ratio and content of MDA increased. Compared with control group, the activity of Caspase 3 and Caspase 9 increased ($P < 0.01$), whereas the activity of SOD, GSH-Px and CAT decreased ($P < 0.01$). Additionally, the expression of Bax, Apaf-1 and CAT were upregulated ($P < 0.01$) while expression of Bcl-2 was downregulated ($P < 0.01$) in both H_2O_2 group and H_2O_2 + miR-210 NC group. Compared with H_2O_2 group and H_2O_2 + miR-210 NC group, H_2O_2 + miR-210 mimics group could change the aforementioned changes. Over-expression of miR-210 could significantly inhibit oxidative stress damage in H9c2 cells.

Keywords: miR-210, H9c2 myocardial cells, oxidative stress, apoptosis, mitochondrial pathway

Introduction

It is well documented that a large amount of oxygen free radicals will be produced during pathological process including ischemic myocardium, myocardial ischemia reperfusion, myocardial infarction, myocardial remodeling, and heart failure [1, 2]. These oxygen free radicals will give rise to oxidative stress injury in myocardial cells, thereby causing cell membrane damage, DNA breaks, and finally resulting in cardiomyocyte apoptosis or necrosis. This in return will worsen the condition of the patients. Therefore, alleviating oxidative stress injury and then reducing the apoptosis of myocardial cells are of great significance for the treatment of cardiovascular diseases. miRNAs are a kind of non-coding RNAs, which are highly conserved in evolution. Previous researches mainly focused on the effect of miRNAs in development regulation and tumor formation, while in recent years, successively studies

found that dozens of miRNAs, such as miR-1, miR-29, miR-21, miR-126, miR-133, miR-199, miR-204 and miR-210 showed specific expression in cardiomyocytes, and are closely related to processes of cardiac hypertrophy, cardiac remodeling, heart failure, myocardial ischemia-reperfusion injury, myocardial infarction and other cardiovascular diseases [3-5]. Costantino et al. argued that miRNAs such as miR-210, miR-34a, miR-221 and miR-146a are closely associated with oxidative stress in cardiac myocytes [6]. Further studies from Shi and associates demonstrated that increased miR-210 expression could resist cellular damage in H_2O_2 induced H9c2 cells [7], indicating that miR-210 can resist cardiomyocytes damage caused by oxidative stress. These studies indicate that miR-210 might be a new target for treatment of cardiovascular disease, however, the exact mechanism remains unknown. In this study, H_2O_2 induced H9c2 was used as an oxidative stress cardiomyocyte model. This model was

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intervened by injecting miR-210 mimics, and the impact and specific mechanisms of miR-210 on myocardial oxidative stress injury in H₂O₂ induced H9c2 cells were thereby illustrated.

Materials and methods

Cell transfection and grouping

H9c2 rat rdiomyocytes provided by Cell Bank of Chinese Academic of Science were cultured in DMEM medium (Gibco company, USA) containing 10% fetal bovine serum (Gibco company, USA) and kept at 37°C, under the condition of 5% CO₂. The miR-210 mimics and miR-210 mimics negative control (NC) (RiboBio Company, Guangzhou, China) were transfected into H9c2 cells by Lipofectamine (Thermo Fisher Scientific Inc., Waltham, USA), with the concentration of 200 nm. The cells were then cultured in 96-well plates or 6-well plates for 6 hours, followed by replacement of medium with DMEM medium containing 10% fetal bovine serum. After cultured in 37°C in carbon dioxide incubator for 48 hours, the cells were divided into four groups: the normal group, H₂O₂ group, H₂O₂ + miR-210 negative control group (NC), and H₂O₂ + miR-210 mimics group. Each group was taken in parallel copies and applied to the following detections.

Real time polymerase chain reaction (RT-PCR)

Once harvested, total RNA was extracted by using the trizol method (Takara Biotechnology (Dalian) Co., Ltd., Dalian, China) and the concentration of RNA was measured by spectrophotometer later. Reverse transcription of RNA was executed by one-step RT-PCR kit (Takara Biotechnology (Dalian) Co., Ltd., Dalian, China) and the products were used as templates. PCR amplification of miR-210 was performed and GADPH was taken as internal reference. The amplified products were then applied to 2% agarose gel electrophoresis. The primers used in this study were as follows: miR-210 upstream primer: 5'-AGCGTGCTGTGCGTGTGAC-3'; miR-210 downstream primer: 5'-CAGTGCAGGGTCCGAGGTATT-3'; GADPH upstream primer: 5'-AGCCACATCGCTCAGACA-3'; GADPH downstream primer: 5'-TGGACTCCACGACGTACT-3'. The RT-PCR was performed in a 25 µl reaction mixture for each tube, and the program was as follows: denaturing at 94°C for 5 min, followed by 35

cycles of denaturing at 94°C for 45 s, annealing at 59°C for 45 s, extension at 72°C for 60 s.

MTT assay

The H9c2 cells were treated with H₂O₂ at concentrations of 0, 25, 100, 200, and 400 µM for 6 hours respectively and cell viabilities were detected with the method of MTT (Gibco Company, USA). After incubated with 20 µL MTT at the concentration of 5 mg/mL for 4 hours, the supernatant was discarded, and 150 µL DMSO was added to each well for 10 minutes. The OD values were then measured at 570 nm by microplate reader (BioTek, Vermont, USA).

Hoechst staining

Hoechst staining was performed when the cells were harvested using Hoechst staining kit (Beyotime Biotechnology, Shanghai, China) strictly according to the operation manual. Briefly, after the culture medium was abandoned, the cells were washed with PBS for 3 times and added with 1 mL dye. Once fixed for 5 min, staining solution was abandoned and washed with PBS for 3 times. Morphology of the cells was observed and photographed under a microscope, and cells with white nucleus were considered as apoptotic cells.

Mitochondrial membrane potential detection

Mitochondrial membrane potential (MMP) of the cells were detected with MMP assay kit (Shanghai Beyotime Biotechnology company, Shanghai, China). After cultured for 48 h, the transfected cell were harvested, followed by washing with PBS for 3 times and adding with 500 µL JC-1 working fluid generated by mixing 1 µL JC-1 to 499 µL 1 × incubation buffer. Afterwards the mixture was incubated for 15 min at 37°C, and then washed with 1 × incubation buffer. Finally, the cells were observed and photographed by inverted microscope with the excitation wavelength of 525 nm and emission wavelength of 590 nm. Red fluorescence indicates normal cell mitochondrial membrane, while green fluorescence demonstrates serious damage to the mitochondrial membrane. The ratio of red fluorescence to green fluorescence was taken as MMP ratio, and the smaller the ratio, the heavier the mitochondria were damaged.

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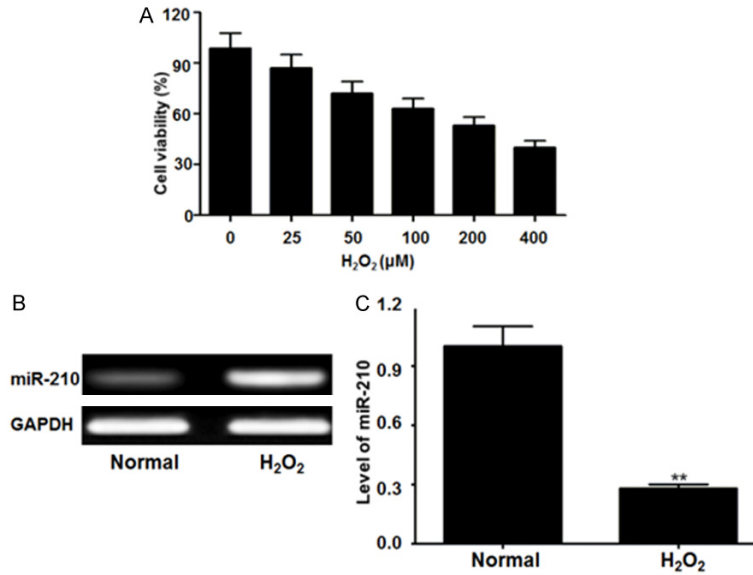


Figure 1. Effect of different H₂O₂ concentration on H9c2 cells (A) and the expression of miR-210 on cell expression in H₂O₂ induced cells (B, C), **P < 0.01.

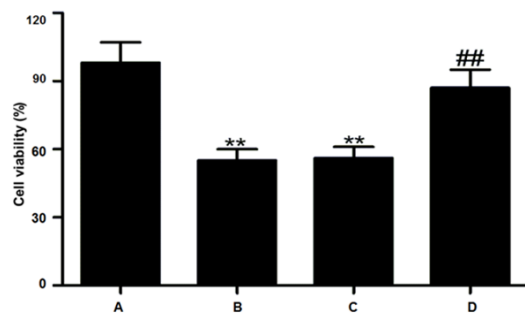


Figure 2. Effect of miR-210 on cell viability in H₂O₂ induced H9c2 cells detected by MTT assay. A: Normal group; B: H₂O₂ induced group; C: H₂O₂ + miR-210 NC group; D: H₂O₂ + miR-210 mimics group. **Compared with normal group, P < 0.01; ##Compared with H₂O₂ induced group and H₂O₂ + miR-210 NC group, P < 0.01.

Viability assay of MDA contents, SOD, GSH-Px and CAT

Once the cells were harvested, treatment of freeze-thaw cycle was undertaken for several times to release the cell contents. The lysate was centrifuged at 1500 rpm/min for 10 min at 4°C and the supernatant was taken for oxidative stress related enzyme activity detection. Detection kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) were used for detection of malondialdehyde (MDA contents), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) activity.

Apoptosis detection

As for apoptosis detection, Caspase 3 and Caspase 9 activity assay kits (Shanghai Beyotime Biotechnology Company, Shanghai, China) were adopted. The collected cells were digested with trypsin without EDTA, centrifuged at 1500 rpm/min for 10 min, then the cells were collected and lysed for 30 min with 50 μL precooling lysis buffer, followed by centrifuging at 10 000 rpm/min for 1 min at 4°C. Protein concentration was detected with the supernatant. Each 50 μL supernatant with the concentration of about 100~200 proteins was sequentially added with 50 μL reaction liquid and 5 μL substrate.

After kept away from the light and incubated for 4 h, the absorbance was measured at 405 nm using spectrophotometer. Equivalent absorbance value (OD/mg) per milligram protein was calculated.

Western blot

After collected, the cells were suspended in cell lysis buffer for 30 minutes, and then centrifuged to obtain protein samples. Protein concentration was tested using BCA protein quantitative kit (Shanghai Beyotime Biotechnology Company, Shanghai, China). For western blot detection, protein samples were denatured by boiling for 10 minutes and loaded on a SDS gel for polyacrylamide gel electrophoresis. The samples were then transformed to a positive charged nylon membrane. After transformation, the membrane was blocked in 5% skim milk for 1 h, followed by incubation with first antibodies (1:100) at 4°C overnight. These antibodies included rabbit anti-Bax, Bcl-2, CytC, Apaf-1 and GAPDH monoclonal antibody independently (Epitomics company, Hangzhou, China). Second antibody was added and incubated at room temperature for 1 hour. The membrane was developed with enhanced chemiluminescence. Protein bands were detected using FluorChem®Q imaging system (Cell Biosciences, Santa Clara, CA, USA) and the integral absorbance (IA) value of each band was analyzed.

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Table 1. Effect of MDA content, SOD, GSH-Px and CAT viability of miR-210 on H₂O₂ induced H9c2 cells ($\bar{x} \pm s$)

| Groups | MDA (nmol/mg protein) | SOD (U/mg protein) | GSH-Px (U/mg protein) | CAT (U/mg protein) |
|--|--------------------------|-----------------------|--------------------------|-----------------------|
| Normal group | 1.18 ± 0.10 | 89.24 ± 8.23 | 3.58 ± 0.35 | 36.29 ± 3.63 |
| H ₂ O ₂ induced group | 3.50 ± 0.35** | 29.88 ± 2.83** | 1.51 ± 0.15** | 13.87 ± 1.33** |
| H ₂ O ₂ + miR-210 NC group | 3.51 ± 0.34## | 29.79 ± 2.77## | 1.51 ± 0.14## | 13.89 ± 1.90## |
| H ₂ O ₂ + miR-210 mimics group | 1.35 ± 0.14## | 75.50 ± 7.52## | 3.32 ± 0.34## | 29.58 ± 2.99## |

Note: Compared with normal group, **P < 0.01; compared with H₂O₂ induced group and H₂O₂ + miR-210 NC group, ##P < 0.01.

Data analysis

All experiments were performed at least three times and the data were analyzed by SPSS17.0 statistical analysis software (SPSS Inc, Chicago, IL, USA). The data were expressed as $\bar{x} \pm s$. T test was used to compare the differences between different groups, while P < 0.05 was considered as statistically significant.

Results

H₂O₂ concentration optimization

To identify a suitable H₂O₂ concentration that can induce oxidative stress injury to H9c2 cells without incurring H9c2 cell death, MTT was performed after treatment with H₂O₂. As shown in **Figure 1A**, as the induced concentration increased, the activity of H9c2 cells decreased. Additionally, it was found that induction with 200 μ M H₂O₂ could both significantly inhibit the vitality of H9c2 cells and would not induce a large number of cell death. In total, the observation strongly suggest that 200 μ M H₂O₂ was the optimal concentration for oxidative stress injury cell model construction. Therefore, this concentration was chosen as an induction dose in the follow-up experiments.

Expression of miR-210 in H₂O₂ induced cells

In order to characterize the expression of miR-210 in H₂O₂ induced cells, RT-PCR was performed. As illustrated in **Figure 1B** and **1C**, compared with the normal group, expression level of miR-210 increased significantly in H₂O₂ induced group. It is possible to assume that miR-210 expression increased after oxidative stress injury.

Effect of miR-210 on cell viability in H₂O₂ induced H9c2 cells

To assess the function of miR-210 on cell viability in H₂O₂ induced H9c2 cells, MTT assay was

undertaken. Compared with the normal group, viabilities of H9c2 cells in both H₂O₂ induced group and H₂O₂ + miR-210 NC group decreased significantly (P < 0.01) (**Figure 2**). Compared with H₂O₂ induced group and H₂O₂ + miR-210 NC group, the viability of H9c2 cells in H₂O₂ + miR-210 mimics increased significantly (P < 0.01). In summary, the results indicated that expression of miR-210 could enhance viability of H9c2 cells.

Effect of miR-210 on the viabilities of MDA content, SOD, GSH-Px and CAT in H₂O₂ induced H9c2 cells

To unravel the effect of miR-210 on the viabilities of MDA content, SOD, GSH-Px and CAT viability of miR-210 on in H₂O₂ induced H9c2 cells, related enzyme activity detections were performed. As listed in **Table 1**, compared with the normal group, MDA content in H₂O₂ induced group and H₂O₂ + miR-210 NC group increased, while viabilities of SOD, GSH-Px and CAT decreased significantly (P < 0.01). Compared with H₂O₂ induced group and H₂O₂ + miR-210 NC group, MDA content in H₂O₂ + miR-210 mimics group decreased, while viabilities of SOD, GSH-Px and CAT increased significant (P < 0.01). In line with this, it is possible to draw a conclusion that miR-210 could improve antioxidant capacity by removing the oxidative stress, thereby reducing H₂O₂ induced H9c2 cell damage.

Effect of miR-210 on cell apoptosis in oxidative stress injury H9c2 cells

To examine the effects of miR-210 played during oxidative stress injury, Hoechst staining was performed and cell morphology was observed. As illustrated in **Figure 3**, compared with normal group, the apoptosis ratio of H9c2 cells in H₂O₂ induced group and H₂O₂ + miR-210 NC group increased significantly (P < 0.01). And the apoptosis ratio in H₂O₂ + miR-210 mimics

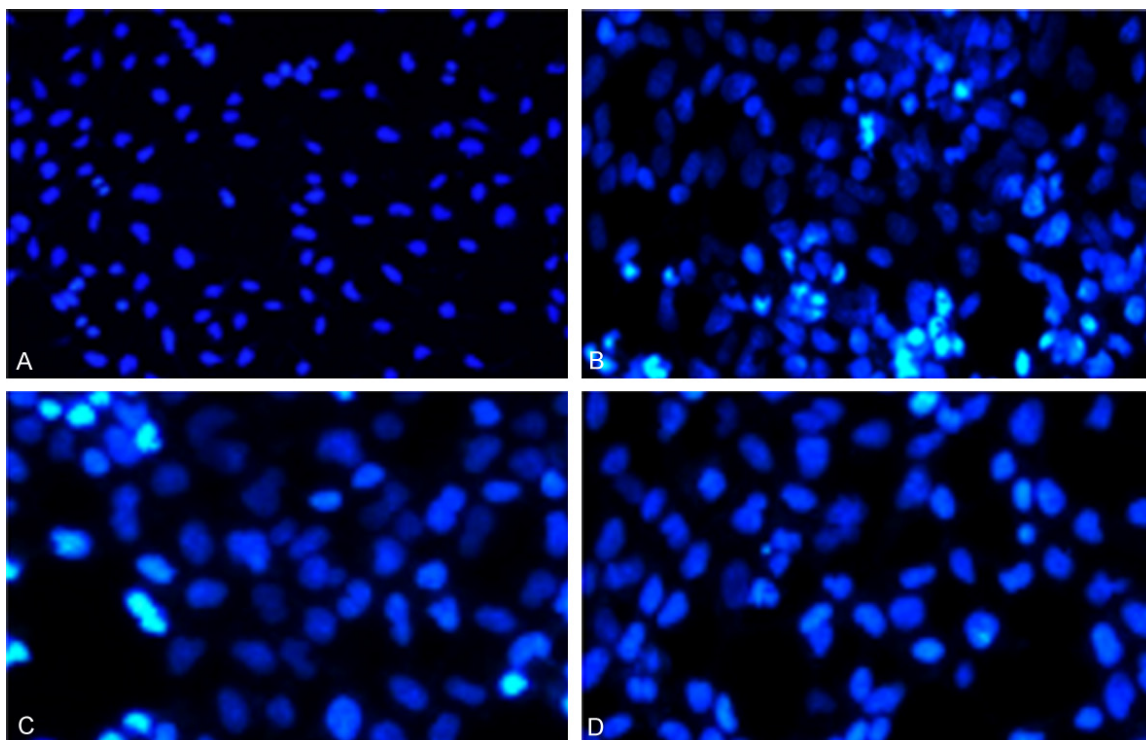


Figure 3. Effect of miR-210 on cell apoptosis in H₂O₂ induced H9c2 cells showed by Hoechst staining. A: Normal group; B: H₂O₂ induced group; C: H₂O₂ + miR-210 NC group; D: H₂O₂ + miR-210 mimics group.

Table 2. Effect of miR-210 on viabilities of MMP, caspase 3 and caspase 9 in H₂O₂ induced H9c2 cells ($\bar{x} \pm s$)

| Groups | MMP | Caspase 3 (U/mg protein) | Caspase 9 (U/mg protein) |
|--|---------------|-----------------------------|-----------------------------|
| Normal group | 3.32 ± 0.33 | 0.79 ± 0.08 | 3.13 ± 0.31 |
| H ₂ O ₂ induced group | 1.58 ± 0.16** | 4.36 ± 0.47** | 7.36 ± 0.74** |
| H ₂ O ₂ + miR-210 NC group | 1.59 ± 0.15** | 4.36 ± 0.44** | 7.40 ± 0.60** |
| H ₂ O ₂ + miR-210 mimics group | 2.98 ± 0.29## | 1.35 ± 0.14## | 4.58 ± 0.46## |

Note: Compared with normal group, **P < 0.01; compared with H₂O₂ induced group and H₂O₂ + miR-210 NC group, ##P < 0.01.

induced H9c2 cells decreased significantly when compared with H₂O₂ induced group and H₂O₂ + miR-210 NC group (P < 0.01). In light of this, we can conclude that increasing the expression of miR-210 in H₂O₂ induced H9c2 cells could significantly improve cell viability, and effectively inhibit the apoptosis of the cells.

Effect of miR-210 on MMP, viabilities of caspase 3 and caspase 9 in H₂O₂ induced H9c2 cells

In order to extend effects of miR-210 on cell apoptosis, MMP and viabilities of caspase 3

and caspase 9 were measured. As shown in **Table 2**, compared with normal group, MMP reduced whereas viabilities of caspase 3 and caspase 9 increased significantly in H₂O₂ + miR-210 NC group (P < 0.01). In H₂O₂ + miR-210 mimics group, MMP increased and the caspase 3 and caspase 9 activities decreased when compared with that

in H₂O₂ + miR-210 NC group, and the differences were statistically significant (P < 0.01). Taken together, the results suggested that miR-210 had the effect of anti-apoptosis during oxidative stress injury in H9c2 cells.

Expression of Bax, Bcl-2, CytC and Apaf-1 in H₂O₂ induced H9c2 cells

To reveal the underlying mechanism of miR-210 on cell apoptosis at molecular level, western blot was performed. As shown in **Figure 4A**, compared with normal group, expression of Bax raised whereas Bcl-2 reduced in H₂O₂ induced and H₂O₂ + miR-210 NC groups. The differences were statistically significant (P <

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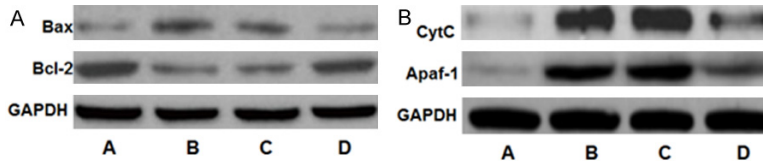


Figure 4. Effect of miR-210 on expression of Bax, Bcl-2 (A), CytC, and Apaf-1 (B) in H_2O_2 induced H9c2 cells illustrated by western blot. A: Normal group; B: H_2O_2 induced group; C: H_2O_2 + miR-210 NC group; D: H_2O_2 + miR-210 mimics group.

0.01). The expression of Bax and Bcl-2 were just the opposite in H_2O_2 + miR-210 mimics groups in comparison with that in H_2O_2 induced group and H_2O_2 + miR-210 NC group ($P < 0.01$). Compared with the normal group, the expression of Apaf-1 and CytC increased significantly ($P < 0.01$), while the expression of Apaf-1 and CytC decreased significantly in H_2O_2 + miR-210 mimics group in comparison with H_2O_2 + miR-210 NC group ($P < 0.01$) (Figure 4B). These studies further supported the hypothesis that miR-210 had the effect of anti-apoptosis during oxidative stress injury in H9c2 cells.

Discussion

Many studies have found that pathological processes of heart failure, myocardial ischemia, myocardial infarction, heart remodeling, ischemia-reperfusion injury and other cardiovascular diseases can produce a large number of ROS [8, 9]. Large amounts of ROS can cause oxidative stress and myocardial cell damage, resulting in myocardial lipid peroxidation, DNA damage and mitochondrial damage, finally leading to myocardial apoptosis and necrosis which in return worsen the condition and create a vicious cycle [10]. Therefore, inhibiting cardiac oxidative stress and reducing myocardial apoptosis have significant implications for the treatment of cardiovascular diseases. H_2O_2 is one of ROS and has different inducing concentrations for different cells [11]. Many reports have proved that H_2O_2 can induce oxidative stress injury in myocardial cells, therefore H_2O_2 is frequently used in myocardial ischemia and ischemia reperfusion injury simulations. Based on studies reported previously [12, 13], we used H_2O_2 with different concentrations to produce oxidative stress injury in myocardial cells in this study. Finally we found that the viabilities of H9c2 cells can be inhibited significantly under the condition of 200 μM H_2O_2 through

MTT assay, and H_2O_2 at this concentration can damage myocardial cells perfectly without affecting cell function seriously. Our finding was in consistent with studies reported previously [12, 13].

miRNAs are kinds of highly conserved small non-coding RNA molecules (containing

18-25 nucleotides) which were firstly found in *C. elegans* in 1993. miRNAs can inhibit the translation of target genes via base-pairing with complementary sequences in 3'-UTR region of target genes, thereby emerge as regulators of cell growth, proliferation, differentiation, and apoptosis. miRNAs are closely related to the process of development, metabolism and many other disease related progresses [14-18]. Studies in recent years found that miRNAs can also regulate the initiation and development of myocardial infarction, heart failure, cardiac remodeling, myocardial ischemia-reperfusion injury, and other cardiovascular diseases [3, 4]. It is hypothesized that miRNAs play important roles in cardiac myocytes adaptability and survival during the procedure of myocardial ischemia-reperfusion injury [19-21]. In this study, we firstly detected the expression level of miR-210 in H_2O_2 induced H9c2 cells and the result showed that miR-210 expression was significantly reduced, which was consistent with earlier findings [5, 21]. Therefore, investigating the specific mechanism of miR-210 against myocardial cellular oxidative stress is of great significance for miR-210 to become a new therapeutic target for cardiovascular disease.

Oxidative stress can cause lipid peroxidation which greatly reduces the viabilities of SOD, GSH-Px and CAT. MDA is the product of lipid peroxidation and the expression level of MDA is positively correlated with cell damage degree. SOD, CAT and GSH-Px are antioxidant enzymes produced by cells to resist external stimulation and have antioxidant effect. In this study, we invented to investigate the effect of miR-210 on oxidative stress products in H9c2 cells and the results proved that miR-210 mimics could increase the viabilities of SOD, GSH-Px and CAT in H_2O_2 induced H9c2 cells while reduce the MDA content at the same time. All these results

suggested that miR-210 can reduce cell damage induced by H₂O₂ in H9c2 cells by clearing products of oxidative stress and improving antioxidant capacity.

Generation of oxidative stress during cardiovascular diseases will finally induce apoptosis in myocardial cells, and the apoptosis will further worsen the condition [23]. In this study, we examined the effect of miR-210 on cell viabilities and cell apoptosis in H₂O₂ induced H9c2 cells by MTT assay and Hoechst staining, the result revealed that cells transfected with miR-210 mimics showed high cell viability and low ratio of cell apoptosis. The result was in full accordance with previous research [24], which proved that improving expression of miR-210 in H9c2 cells can significantly enhance cell viability and inhibit cell apoptosis.

Cell apoptosis is regulated by endogenous and exogenous pathway, wherein the intrinsic regulation is mediated by inner mitochondrial pathway. During cell apoptosis, both increasing of mitochondrial permeability and reducing of mitochondrial membrane potential could result in CytC releasing from the inner mitochondrial membrane gap and entering cytoplasm [23]. Released CytC will thereby form apoptotic bodies with Apaf-1 and Caspase 9, stimulate the caspase cascade, and finally transmit the apoptosis signal to Caspase 3, resulting in cell apoptosis. Bcl-2 family proteins also play important roles during cell apoptosis, while heterodimer of Bcl-2 and Bax or oligomer formed by Bax can induce channel formation on mitochondrial membrane, prompt CytC release and finally induce cell apoptosis [24]. The increasing of myocardial apoptosis in H₂O₂ induced H9c2 is often associated with decreasing expression of Bcl-2 and increasing expression of Bax, while at the same time the viabilities of Caspase 3 and Caspase 9 increase [23, 24]. The induction of H₂O₂ on PC12 cells can also results in high expression of CytC in cytoplasm and remarkable enhancement of Caspase 3 viability [25]. In this study, we detected the viabilities of Caspase 3 and Caspase 9 through colorimetric detection, the results showed that up-regulation of miR-210 expression level in H₂O₂ induced H9c2 cells can significantly reduce the viabilities of Caspase 3 and Caspase 9, decrease the expression of CytC, Apaf-1 and Bax, and increase MMP and the expression of Bcl-2.

In summary, up-regulation of miR-210 expression in H₂O₂ induced H9c2 cells can significantly increase the antioxidant ability of cells and reduce cell apoptosis, and this representation is connected with the function of miR-210 in increasing of viabilities of SOD, CAT and GSH-Px, reducing MDA contents and inhibition of mitochondrial pathway.

Disclosure of conflict of interest

None.

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