

## Original Article

# Targeted regulation of Bcl2 by miR-16 for cardiomyocyte apoptosis after cardiac infarction

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Received December 2, 2016; Accepted February 8, 2017; Epub April 1, 2017; Published April 15, 2017

**Abstract:** Apoptosis of cardiomyocytes is pathology basis for ischemia-reperfusion (I/R) damage after cardiac infarction. B-cell lymphoma 2 (Bcl-2) can inhibit cell apoptosis, and may participate in regulation of cardiomyocyte apoptosis after I/R. Previous study showed the role of microRNA (miR)-16 in both cardiomyocyte apoptosis of I/R injury. As Bcl-2 is one target gene of miR-16, this study aimed to investigate if miR-16 plays a role in modulating Bcl-2 expression and post-I/R apoptosis. Rat cardiomyocyte I/R model was generated and divided into Sham and I/R group. Cell apoptosis, infarction area, and Bcl-2/miR-16 expression was tested. *In vitro* cultured H9C2 cells were divided into un-transfected, antagomir-control, antagomir-16 groups. I/R model was mimicked by 6-h hypoxic incubation followed by re-oxygenation for 6 h. Bcl-2 and miR-16 expression was compared, along with TUNEL assay. Antagomir-16 or controlled sequences were injected into cardiomyocytes of I/R rats, followed by quantification of Bcl-2/miR-16 expression, TUNEL assay, and Evens blue staining for infarction area. I/R rats had higher miR-16 expression, cardiomyocyte apoptosis and infarction area, whilst Bcl-2 was down-regulated compared to control ones. I/R treatment also elevated miR-16 expression and apoptosis in H9C2 cells, and decreased Bcl-2 expression. Transfection of antagomir-16 elevated Bcl-2 expression in H9C2 cells and decreased cell apoptosis. Compared to model or control group, antagomir-16 injection decreased cardiomyocyte apoptosis and infarction area, and enhanced Bcl-2 expression. Inhibition of miR-16 expression weakens the inhibitory effect on Bcl-2, decreases apoptosis of cardiomyocyte post-I/R, and suppresses infarction area.

**Keywords:** MicroRNA-16, B-cell lymphoma 2, ischemia-reperfusion, cardiomyocyte, apoptosis

## Introduction

Acute myocardial infarction (AMI) is one necrosis of myocardial tissues caused by acute hypoxia of coronary artery [1]. Reperfusion treatment of coronary after post-AMI is the most effective way to save hypoxic cardiomyocytes, protect cardiac functions and save patient's lives [2]. Reperfusion after ischemia, however, may inevitably lead to ischemia-reperfusion (I/R) injury on hypoxic cardiomyocytes. During such process, apoptosis is the major pathological feature, and plays a critical role in post-AMI cardiac remodeling [3]. B-cell lymphoma 2 (Bcl-2) is one oncogene that can inhibit cell apoptosis induced by multiple factors. Enhanced Bcl-2 expression can potentiate cell resistance against apoptosis [4]. Previous studies showed the involvement of abnormal expression or function of Bcl-2 in modulation of

cardiomyocyte apoptosis after AMI and I/R injury, as its expression level directly affects cardiomyocyte apoptosis and cardiac functions [5, 6]. MicroRNA (miR) is one type of important epigenetic regulator. It can bind onto 3'-untranslated region (3'-UTR) of target gene mRNA via complete or incomplete pairing, to degrade mRNA or impede mRNA translation, thus negatively regulating target gene expression at post-transcriptional level [7]. Previous studies showed the role of miR-16 in various pathological conditions of cardiac issues including AMI [8], I/R [9], and myocardial hypertrophy [10]. Bcl-2 is one definitive target gene of miR-16, which participates in various biological processes including tumor formation [11] and cell apoptosis [12] via targeting Bcl-2 expression. This study thus investigated if miR-16 played a role in regulating Bcl-2 expression and I/R induced myocardial apoptosis.

## Materials and methods

### *Major reagents and materials*

Healthy male adult Wistar rats (6~8 weeks, body weight 220~240 g) were purchased from Shandong University (China). Rat myocardial cell line H9C2 was purchased from ScienCell (US). DMEM culture medium was purchased from Gibco (US). FBS was purchased from Biological Industries (Israel). Trizol was purchased from Invitrogen (US). ReverTra Ace qPCR RT Kit was purchased from Toyobo (Japan). MicroFTM antagomir-16, and MicroFTM antagomir-control were designed and synthesized by Ruibo (China). Mouse anti-Bcl-2 and anti- $\beta$  antibody were purchased from CST (US). One-step TUNEL cell apoptosis test kit was purchased from Beyotime (China). Horseradish peroxidase (HRP) labelled goat anti-rabbit secondary was purchased from Jackson ImmunoResearch (US).

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Affiliated Hospital of Jining Medical University.

### *Establishment of I/R model and grouping*

10% hydrate chloral (5 mg/g) was applied by intraperitoneal injection on Wistar rats for anesthesia. Rats were fixed in a supine position. Monitor electrode was fixed on four limbs for ECG recording. Ventilation machine was connected to tracheal intubation for assisted respiration. The chest cavity was opened at the fourth left inter-rib site. The left descending branch of coronary artery was identified and ligated at the boundary between pulmonary artery conical and aorta. ECG monitor showed elevated ST segment in Q-lead by 0.1 mV or T wave peak, plus whitening of myocardial tissues and weakening pulsation, indicating successful generation of AMI model. 60 min after blocking blood supply, flow in the left descending branch of coronary artery was recovered. Parallel sham group was prepared by placing 6-0 suture without ligation.

I/R model rats were divided into three groups (N=5 each): (1) I/R model group, which received 50  $\mu$ l saline on myocardial surface 24 h before surgery. (2) I/R+antagomir-control group, which received injection of  $2 \times 10^3$  mol/L antagomir-control (50  $\mu$ l) 24 h before surgery. (3) I/

R+antagomir-16 group, which received injection of  $2 \times 10^3$  mol/L antagomir-16 (50  $\mu$ l) 24 h before surgery.

### *TUNEL assay for myocardial apoptosis*

All rats were sacrificed 3 days after I/R treatment. Myocardial tissues were sectioned and tested for apoptosis using TUNEL assay kit. In brief, frozen sections were firstly fixed for 60 min in 4% paraformaldehyde, and were treated in PBS containing 0.1% Triton X-100. After iced incubation for 2 min, slices were rinsed twice in PBS, followed by adding 50  $\mu$ l TUNEL testing buffer, and was incubated at 37°C for 60 min. Apoptotic cells were labelled by fluorescent probes, which can be excited at 488 nm wave length.

### *Quantification of infarction area*

All rats were sacrificed 3 days after I/R surgery. 2 mL 1% Evans blue was injected into left ventricles via apex. Left ventricular wall tissues were removed, sectioned and placed into 2% TTC. After 37°C incubation for 15 min, saline was applied to wash excess dyes. Tissues were fixed in 4% paraformaldehyde followed by observation. Infarcted myocardial tissues presented as grey-white whilst non-infarcted area showed red color. Infarction ratio = infarction area/total area $\times$ 100%.

### *Cardiomyocytes transfection and I/R processing*

Rat myocardial H9C2 cells were cultured in DMEM medium containing 10% FBS, and were kept in a 37°C chamber with 5% CO<sub>2</sub>. Cells at log-phase with satisfactory growth status were collected for transfecting antagomir-control or antagomir-16. Original culture medium was removed 48 h later, with the addition of low-glucose, serum-free DMEM medium to mimic hypoxia conditions. Cells were placed in a chamber with 5% CO<sub>2</sub> and 95% N<sub>2</sub>. 6 h later, normal serum-containing medium was applied for 6 h incubation under 5% CO<sub>2</sub> and 95% O<sub>2</sub>. Cells were then collected for further assays.

### *qRT-PCR for gene expression*

RNA was extracted by Trizol method. cDNA was synthesized from RNA by ReverTra Ace qPCR. Using cDNA as the template, PCR amplification was performed under the action of TaqDNA

**Table 1.** Apoptotic rate of cardiomyocytes and infarction area between two groups

Group	Myocardial infarction area (%)	Cardiomyocyte apoptotic rate (%)
Sham	0.0±0.0	4.9±1.3
AMI	45.9±5.2*	27.3±6.1*

Note: \*, P<0.05 compared to Sham group.

polymerase. Primer sequences were: miR-16P<sub>F</sub>: 5'-GGCGG TAGCA GCACG TAAAT A-3'; miR-16P<sub>R</sub>: 5'-GTGCA GGGTC CGAGG T-3'; U6P<sub>F</sub>: 5'-GCTTC GGCAG CACAT ATACT AAAAT-3'; U6P<sub>R</sub>: 5'-CGCTT CACGA ATTTG CGTGT CAT-3'; Bcl-2P<sub>F</sub>: 5'-CGGGA GAACA GGGTA TGA-3'; Bcl-2P<sub>R</sub>: 5'-CAGGC TGGAA GGAGA AGAT-3'; β-actin P<sub>F</sub>: 5'-TACAA CCTCC TTGCA GCTCC-3'; β-actin P<sub>R</sub>: 5'-GGATC TTCAT GAGGT AGTCA GTC-3'. In a total 10 μl reaction system, 5.0 μl 2×SYBR Green Mixture; 0.5 μl forward/reverse primer (5 μm/L), 1 μl cDNA and ddH<sub>2</sub>O. Reaction conditions were: 95°C denature for 5 min, followed by 40 cycles each containing 95°C 15 s, 60°C 60 s. PCR was performed on a fluorescent quantitative PCR cyclers (ABI ViiA7).

#### Western blot for protein expression

Proteins were extracted and quantified by BCA method. 40 μg supernatant was separated in SDS-PAGE and was transferred to PVDF membrane, which was blocked in 5% defatted milk powder for 60 min at room temperature. Primary antibody (Bcl-2 at 1:200, β-actin at 1:800) was added for 4°C overnight incubation. Unbounded primary antibody was washed away. Secondary antibody (anti-mouse at 1:8000) was added for 60 min room temperature incubation, followed by ECL development. The film was exposed and scanned for data collection.

#### Statistical analysis

SPSS18.0 was used for statistical analysis. Measurement data were presented as mean ± standard deviation (SD). Student t-test was employed for comparing between-group data. A statistical significance was defined when P<0.05.

### Results

#### Up-regulation of miR-16 and down-regulation of Bcl-2 in I/R myocardial tissues

Compared to sham group, I/R model rats had significantly elevated infarction area, indicating

successful generation of I/R model (Table 1). TUNEL assay showed no significant apoptosis of cardiomyocytes, whilst I/R group had significantly elevated cell apoptosis in myocardial tissues (Table 1). qRT-PCR results showed significantly elevated miR-16 expression in I/R rat myocardial tissues compared to Sham group, whilst Bcl-2 mRNA level was significantly lower (Figure 1A). Western blot results showed remarkably decreased Bcl-2 protein expression level in cardiomyocytes of I/R group (Figure 1B). Results showed that miR-16 up-regulation played a role in suppressing Bcl-2 expression and facilitating I/R inducing apoptosis.

#### MiR-16 down-regulation decreased I/R induced H9C2 cell apoptosis

In vitro hypoxic treatment followed by re-oxygenation remarkably induced miR-16 expression in H9C2 cells compared to normal cultured cells (Figure 2A). Bcl-2 expression remarkably decreased (Figure 2B) and enhanced cell apoptosis (Figure 2C). Transfection of antagomir-16 elevated Bcl-2 expression in H9C2 cells, and suppressed the sensitivity of I/R induced apoptosis, thus decreasing cell apoptosis.

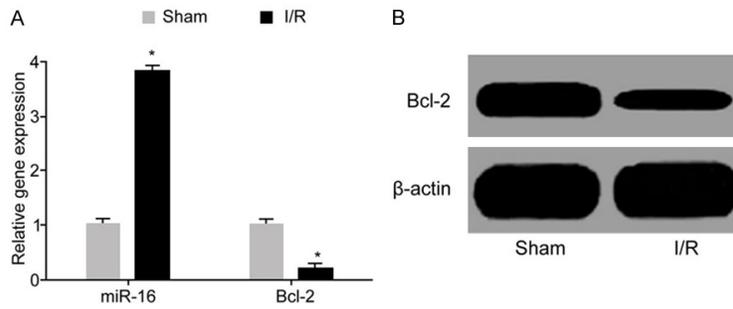
#### Intra-muscular injection of antagomir-16 decreased post-I/R myocardial apoptosis and infarction area

Compared to I/R or I/R+antagomir-control group, intramuscular injection of antagomir-16 into myocardial tissues significantly decreased miR-16 expression (Figure 3A) and elevated Bcl-2 expression (Figure 3A and 3B). The injection of antagomir-16 also significantly decreased post-I/R myocardial apoptosis (Table 2) and lowered infarction area (Table 2). These results showed that inhibition of miR-16 expression weakened the inhibitory effects of miR-16 on Bcl-2 expression, decreased the severity of Bcl-2 down-regulation post-I/R, thus protecting against myocardial apoptosis after I/R and decreased infarction area.

### Discussion

AMI caused by myocardial hypoxia severely threatens people's health, and is one common reason causing heart failure and cardiac death [13]. Currently the most effective way targeting AMI is to recover blood supply for cardiac tissues to engage re-perfusion, but frequently leading to I/R injury [14]. I/R injury can aggra-

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**Figure 1.** Up-regulation of miR-16 and down-regulation of Bcl-2 expression. A. qRT-PCR for miR-16 and Bcl-2 gene expression; B. Western blot for Bcl-2 protein expression. \*,  $P < 0.05$  compared to Sham group.

vate injury of myocardial structure/function, and severely compromise cardiac functional recovery after re-perfusion [15]. Study has demonstrated that I/R injury induced myocardial cell apoptosis or necrosis is the most critical patho-physiological process in cardiac dysfunction post-AMI. Myocardial necrosis mainly occurs at late stage whilst cell apoptosis spans the whole process [16]. Apoptosis is one featured change of I/R injury and largely determines the degree of I/R injury, thus affecting patient's prognosis. Therefore, effective alleviation or rescue of I/R injury induced apoptosis is one research focus and has importance for improving post-AMI cardiac function and retarding myocardial remodeling.

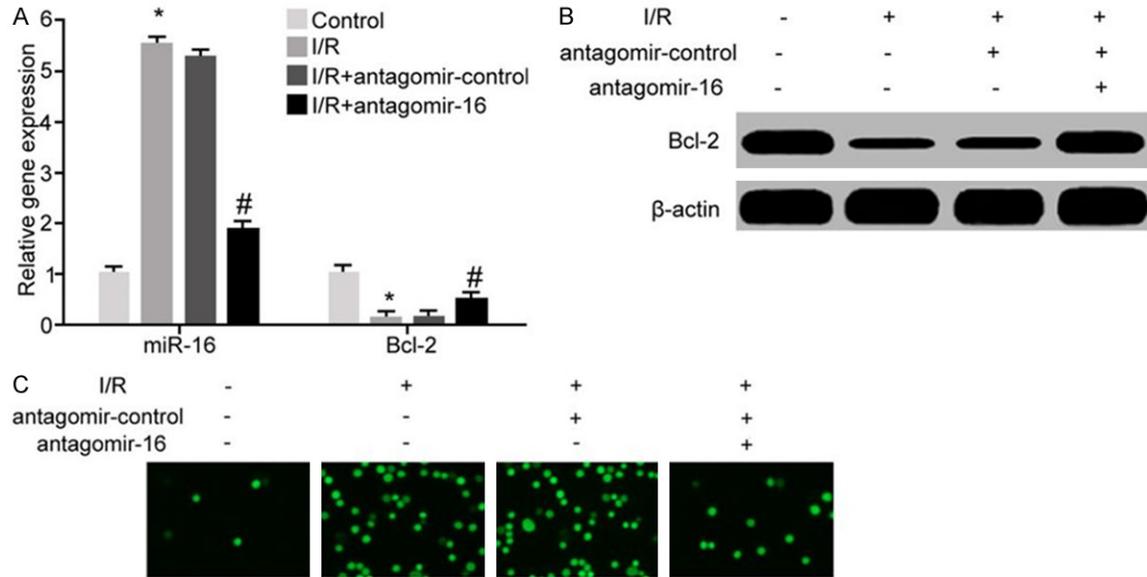
As one oncogene, Bcl-2 plays a critical role in cell apoptosis, and is one important regulatory gene in mitochondria dependent apoptotic pathway [17]. Bcl-2 can antagonize Bax protein within the same gene family to co-regulate cell apoptosis, during which Bcl-2 exerts anti-apoptotic roles whilst Bax facilitates cell apoptosis [18]. Up-regulation of Bcl-2 expression may form Bcl-2 homodimer or Bcl-2/Bax heterodimer, both of which can inhibit cell apoptosis. When Bcl-2 expression was decreased, Bax function may override it to exert anti-apoptotic roles [19]. Therefore, Bcl-2 expression level directly affects cell apoptotic process. Bcl-2 can also exert anti-apoptotic roles via multiple mechanisms including inhibition cytochrome C (Cyt C) release, decreasing production of free oxygen radicals or lipid peroxides, suppressing transmembrane  $Ca^{2+}$  flow or impeding apoptotic signal pathway transduction [20]. Previous studies showed that abnormality of Bcl-2 expression or function participated in modulating myocardial apoptosis after AMI or I/R injury,

as its expression level directly affects myocardial apoptosis and cardiac functions [5, 6]. Increasing evidences showed that, as one critical component of epigenetic regulation, miR expression/function abnormality is closely correlated with cardiovascular diseases. MiR participates in multiple patho-physiology processes including AMI [21], I/R injury [15] or cardiac remodeling [22], with possible roles of aggravating injury [6] and decreasing damage [15].

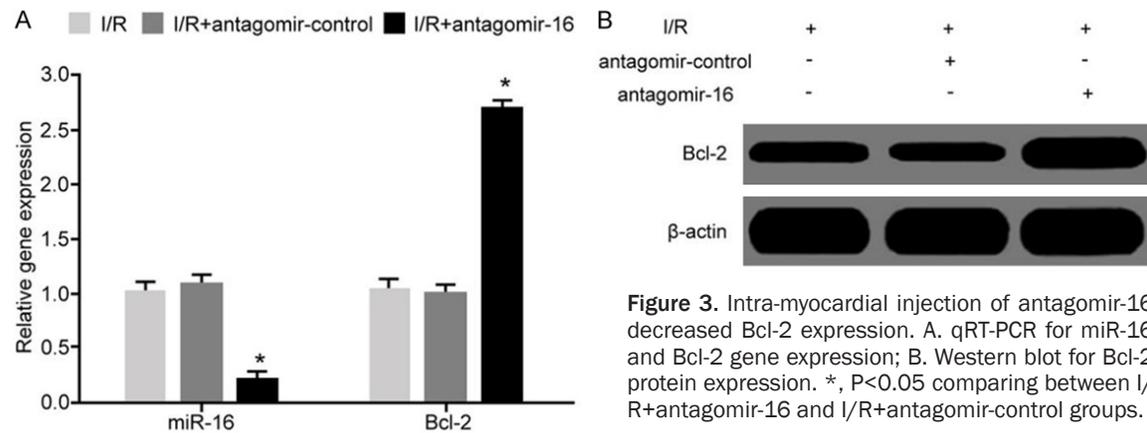
Previous study showed the role of miR-16 in various cardiac patho-physiological processes such as AMI [8], I/R injury [9] or myocardial hypertrophy [10]. As one definitive target gene of miR-16, Bcl-2 expression is under tight regulation of miR-16, and is involved in regulation of tumor formation, cell proliferation and apoptosis. This study thus investigated if miR-16 played a role in modulating Bcl-2 expression or I/R injury induced cardiomyocyte apoptosis.

Results of this study showed significantly higher myocardial infarction area and cell apoptosis in I/R model rats compared to Sham group, indicating successful generation of model for further experiments. I/R rats also had significantly higher miR-16 expression than that in Sham group, whilst Bcl-2 expression was significantly lower. Results indicated that miR-16 up-regulation might play a role in suppressing Bcl-2 expression and facilitating I/R induced cell apoptosis. Chinda et al showed remarkably higher myocardial infarction area and cell apoptosis in I/R model rats, plus lower Bcl-2 expression [5]. Indicating the correlation between Bcl-2 down-regulation and I/R induced apoptosis, as similar to our observation. *In vitro* cultured H9C2 cells were induced for apoptosis after re-oxygenation after hypoxic treatment, accompanied with elevated miR-16 expression and lower Bcl-2 expression. Ertracht et al found significantly lower Bcl-2 expression and higher apoptosis in H9C2 cells after I/R treatment [23], as consistent with our observation showing higher Bcl-2 expression in I/R model H9C2 cells. Devaux et al found elevated serum miR-16 expression in AMI patients, and suggesting that to be one auxiliary parameter reflecting contraction function of left ventricle, as higher expression indicated worse left ventricular

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**Figure 2.** MiR-16 down-regulation weakened I/R induced H9C2 cell apoptosis. A. qRT-PCR for miR-16 and Bcl-2 expression; B. Western blot for Bcl-2 protein expression; C. TUNEL for H9C2 apoptosis. \*, P<0.05 comparing between I/R and control group; #, P<0.05 comparing between I/R+antagomir-16 and I/R+antagomir-control group.



**Figure 3.** Intra-myocardial injection of antagomir-16 decreased Bcl-2 expression. A. qRT-PCR for miR-16 and Bcl-2 gene expression; B. Western blot for Bcl-2 protein expression. \*, P<0.05 comparing between I/R+antagomir-16 and I/R+antagomir-control groups.

**Table 2.** Comparison of myocardial infarction area and apoptotic rate

Group	Myocardial infarction area (%)	Cardiomyocyte apoptotic rate (%)
I/R	42.7±5.5	25.2±6.2
I/R+antagomir-control	44.5±6.1*	24.8±5.6*
I/R+antagomir-16	29.6±4.8#	15.6±3.1#

Note: \*, P<0.05 comparing between I/R group and I/R+antagomir-control group; #, P<0.5 comparing between I/R+antagomir-16 group and I/R+antagomir-control group.

function [8]. Li et al also showed remarkably elevated miR-16 expression in myocardial tis-

sues of AMI rats [24]. All these above studies supported the participation of miR-16 expression abnormality in AMI related cardiac injury. Its role in I/R induced myocardial injury, however, is still unclear yet. This study showed significantly elevated miR-16 expression in both H9C2 cell culture and myocardial tissues of I/R rats, suggesting possible correlation between miR-16 and I/R induced myocardial injury. Chen et al found hundred-folds increase of miR-16 in I/R-induced acute kidney injury (AKI) patients, suggesting the involvement of miR-16 in I/R injury, as consistent with Chen et al [9]. Further assay showed that transfection of antagomir-16 significantly decreased Bcl-2 expression in

H9C2 cells, and decreased sensitivity for I/R induced cell apoptosis, suppressed cell apoptosis. Intramuscular injection of antagomir-16 also significantly decreases myocardial apoptosis after I/R treatment, and shrinks infarction area. Results showed that during I/R processing, up-regulation of miR-16 can decrease Bcl-2 expression and weaken its antagonizing effects against myocardial infarction area, thus enhancing cell apoptosis and aggravating I/R induced myocardial injury. The inhibition of miR-16 expression, on the other hand, improves I/R injury. Chinda et al showed that dipeptidyl peptidase-4 (DDP-4) inhibitor vildagliptin treatment significantly up-regulated Bcl-2 expression in I/R model rats, decreased infarction area and decreased myocardial apoptosis [5]. Ertracht et al showed that the replenishment of TVP1022 effectively maintained Bcl-2 protein expression in *in vitro* cultured H9C2 cells, maintain mitochondrial membrane potential and inhibited cytochrome C release, thus effectively protecting myocardial cells from apoptosis [23]. This study up-regulated Bcl-2 expression in myocardial cells by epigenetic approaches and observed the role of Bcl-2 in protecting myocardial cells from apoptosis. Chen et al showed that over-expression of miR-16 in I/R model mice significantly facilitated apoptosis of renal glomerular epithelial cells, aggravated I/R induced kidney injury and impaired renal function [9]. In this study, inhibition of miR-16 expression can decrease cell apoptosis, aggravate I/R induced injury, further supporting results of Chen et al [9]. Moreover, Huang et al found significantly lower miR-16 expression in myocardial hypertrophy model rats [10]. MiR-16 can participate in regulating hypertrophic phenotype of cardiomyocytes via targeted inhibition on expression of cell cycle protein D1, D2 and E1. In details, miR-16 inhibits the acquirement process of hypertrophic phenotype of cardiomyocytes, indicating potential role of miR-16 as one antagonizing factor for myocardial function, as similar to this study. Our research for the first time revealed the role of miR-16 up-regulation in inducing myocardial apoptosis post-I/R, which has not been reported previously. This study is based on *in vitro* cell study and animal model, but leaving the expression of miR-16 in clinical patients and implications to be further elucidated.

### Conclusion

During I/R injury of cardiomyocytes, miR-16 is up-regulated whilst Bcl-2 expression is sup-

pressed. The inhibition of miR-16 expression can weaken the inhibitory function of miR-16 on Bcl-2, decreasing myocardial apoptosis post-I/R, and shrinking infarction area.

### Acknowledgements

This project supported by the Shandong Province education science and technology project (NO.J07YE10).

### Disclosure of conflict of interest

None.

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