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Effect of miR-26a targeting GSK-3β/β-catenin signaling pathway on myocardial apoptosis in rats with myocardial ischemia-reperfusion

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Abstract. – OBJECTIVE: The aim of this study was to evaluate the effect of micro ribonucleic acid (miR)-26a on myocardial ischemia-reperfusion (I/R) injury in rats and to explore its potential mechanism. Our findings might help to provide references for clinical prevention and treatment of myocardial I/R.

MATERIALS AND METHODS: A total of 60 male Sprague-Dawley (SD) rats were randomly divided into three groups using a random number table, including: Control group (n=20), I/R group (n=20) and I/R + miR-26a siRNA group (n=20). I/R model was established *via* recanalization after ligation of left anterior descending coronary artery (LAD). The model of miR-26a knockdown was established in rats of I/R + miR-26a siRNA group *via* tail intravenous injection of miR-26a siRNA. Ejection fraction (EF%) and fractional shortening (FS%) of rats in each group were detected *via* echocardiography. The infarction area of each group was detected *via* 2,3,5-triphenyltetrazolium chloride (TTC) assay. Subsequently, morphological changes in myocardial cells of each group were detected *via* hematoxylin-eosin (H&E) staining. Myocardial apoptosis level was measured *via* terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. At the same time, the expression levels of pro-apoptotic proteins Bcl-2 associated X protein (Bax) and cleaved (C)-caspase3 in myocardial tissues of the three groups were determined using Western blotting. Finally, the effects of miR-26a knockdown on the expressions of glycogen synthase kinase (GSK)-3β/β-catenin signaling pathway-related proteins were detected *via* Western blotting and immunohistochemistry.

RESULTS: The expression of miR-26a in myocardial tissues of I/R group increased significantly when compared with that in Control group ($p<0.05$). Knockdown of miR-26a significantly improved cardiac insufficiency caused by I/R, which also obviously increased both EF% and FS% in rats ($p<0.05$). In addition, knockdown of miR-26a significantly inhibited myocardial infarction caused by I/R injury, and reduced infarction area from (43.08±2.43) to (21.54±1.82) ($p<0.05$). The results of H&E staining revealed that in I/R + miR-26a siRNA group, myofilaments were arranged more orderly, the degree of degradation and necrosis was significantly lower, and cellular edema was significantly alleviated when compared with I/R group. Subsequent TUNEL staining demonstrated that rats in I/R + miR-26a siRNA group showed remarkably lower level of myocardial apoptosis than I/R group ($p<0.05$). Meanwhile, the protein expression levels of Bax and C-caspase3 were remarkably declined in I/R + miR-26a siRNA group ($p<0.05$). Furthermore, the results of Western blotting showed that miR-26a siRNA could significantly reverse the inhibition of GSK-3β/β-catenin signaling pathway induced by I/R injury ($p<0.05$).

CONCLUSIONS: Knockdown of miR-26a could significantly improve I/R-induced myocardial injury and promote cardiac function in rats. The possible underlying mechanism might be related to targeted regulation of miR-26a on GSK-3β/β-catenin signaling pathway. Therefore, miR-26a was expected to be a new therapeutic target for myocardial I/R injury.

Key Words:
MiR-26a, Myocardial ischemia-reperfusion, Myocardial cells, Apoptosis, GSK-3β/β-catenin.

Introduction

Myocardial infarction (MI) is the major cause of cardiovascular disease death, which is al-

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so a major public health problem worldwide[1,2]. Currently, the recovery of coronary blood flow (reperfusion) and/or early percutaneous coronary intervention (PCI) is the most effective treatment for MI[3]. Paradoxically, reperfusion itself can cause myocardial cell damage and death. Its mechanism is related to locally increase oxidative stress and inflammatory response in myocardial cells. This phenomenon is called "reperfusion injury". In animal experiments, ischemia/reperfusion (I/R) injury is considered as the leading cause of final MI area[4,5]. To improve the clinical efficacy of acute MI, it is of great importance to develop new drugs or search for novel targets in inhibiting reperfusion injury and protecting cardiac function.

Micro ribonucleic acids (miRNAs) are a group of single-stranded non-coding RNAs with 20-24 nucleotide (nt) in length. They exist in eukaryotes and have regulatory functions[6]. MiRNAs can regulate various gene expressions through targeted binding to specific genes, thus playing important roles in physiological activities such as cell proliferation, differentiation and apoptosis[7]. For example, inhibition of miR-155 can significantly improve neurological impairment, reduce cerebral infarction volume and promote the angiogenesis in the ischemic region in rats with cerebral infarction. The underlying mechanism may be mediated through the angiotensin II type 1 receptor (AT1R)/vascular endothelial growth factor receptor 2 (VEGFR2) pathway[8]. Another study[9] has demonstrated that miR-195 inhibits VEGFA in a targeted manner. Therefore, inhibiting miR-195 level can significantly facilitate the angiogenesis in the ischemic region. However, the exact role of miR-26a in I/R injury-induced myocardial apoptosis has not been fully elucidated.

In the present study, the model of myocardial I/R was established in rats using miR-26a knockdown. The effects of miR-26a knockdown on cardiac function, myocardial apoptosis and infarction area were detected. Furthermore, the potential molecular mechanism of miR-26a in affecting myocardial I/R injury was analyzed.

Materials and Methods

Laboratory Animal Grouping and Modeling

A total of 60 male Sprague-Dawley (SD) rats aged 10-12 weeks old and weighing (267.56±11.52) g were enrolled as research subjects. All rats were randomly divided into three groups using a random number table, including: Control group (n=20), I/R group (n=20) and I/R + miR-26a siRNA group (n=20). This study was approved by the Ethics Committee of Dalian Municipal Central Hospital (Dalian, China). No statistically significant differences were found in basic data, such as age and body weight, among the three groups. A certain dose (4 mL/kg) of miR-26a siRNA was injected into rats in I/R + miR-26a siRNA group *via* tail vein. After 7 d, the I/R injury model was established as follows. Rats in each group were anesthetized *via* intraperitoneal injection of 50 mg/kg pentobarbital sodium. The cannula was inserted into the left carotid artery to measure the blood pressure of rats. Heart rate was monitored using two-lead electrocardiograph (ECG). Subsequently, the thorax was cut open in the 4th intercostal space, and the pericardium was excised to expose the heart. The left anterior descending coronary artery (LAD) was ligated at 2 mm above the left auricle using 6-0 silk thread to induce local myocardial ischemia. 30 min after ischemia, the silk thread was loosened, followed by reperfusion for 2 h. Rats in Control group underwent the same operation except for LAD ligation with silk thread. After reperfusion, rats were executed, and myocardial tissues were collected from the left ventricular anterior wall. After rinsing blood residues with normal saline, the tissues were placed in a refrigerator at -80°C for subsequent use.

Detection of Expression of Related Genes via Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

(1) Total RNA was extracted from myocardial tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). The concentration and purity of extracted RNA were detected using an ultraviolet spectrophotometer. RNA samples with absorbance $(A)_{260}/A_{280}$ of 1.8-2.0 could be used. (2) Extracted messenger RNA (mRNA) was synthesized into complementary deoxyribonucleic acid (cDNA) through RT and stored in a refrigerator at -80°C. (3) Specific RT-PCR system included: 2.5 μL 10 × Buffer, 2 μL cDNA, 0.25 μL forward primer (20 μmol/L), 0.25 μL reverse primer (20 μmol/L), 0.5 μL dNTPs (10 mmol/L), 0.5 μL Taq enzyme (2×10^6 U/L) and 19 μL ddH_2O. The amplification system of RT-PCR was the same as above. Primer sequences used in this study were shown in Table I.

Table I. Primer sequences in RT-PCR.

Target gene		Primer sequence
GAPDH	Forward	5'-GACATGCCGCCTGGAGAAAC-3'
	Reverse	5'-AGCCCAGGATGCCCTTTAGT-3'
MiR-26a	Forward	5'-ATTTCGCTGCTTTTTCTGTTCCTT-3'
	Reverse	5'-CCGTAGCTGGCTGGCCCCGT-3'

2,3,5-Triphenyltetrazolium Chloride (TTC) Staining

(1) Fresh myocardial tissues were sliced into sections by grinding tool and frozen in a refrigerator at -20°C for 30 min. (2) Myocardial tissues were sliced into 2 mm-thick sections (not more than 6 sections for each tissue). (3) The sections were incubated with fresh TTC solution (2%) (Oxoid, Hampshire, UK) for not less than 0.5 h. (4) After 0.5 h, the sections were taken out, fixed with 4% paraformaldehyde and photographed.

Echocardiography

To detect the cardiac function of rats in each group, echocardiography was performed using Mylab 30CV ultrasound system (Esaote S. P.A, Genoa, Italy) and 10-MHz linear ultrasound transducer. After shaving the chest hair, the rats were anesthetized and placed on a heating plate at 37°C, with the left side upwards. Ejection fraction (EF%), fractional shortening and heart rate (bpm) were detected.

Hematoxylin-Eosin (H&E) Staining

Heart tissue obtained in each group was placed in 10% formalin overnight, which was then dehydrated and embedded in paraffin. Subsequently, myocardial tissues were sliced into 5 μm-thick sections, fixed on a glass slide and baked dry, followed by staining. According to relevant instructions, the sections were soaked in xylene, ethanol in gradient concentration and hematoxylin, respectively. After sealing with resin and air dried, the sections were observed and photographed under a light microscope. Finally, the morphology of myocardial cells, cardiac interstitium and myofilament was observed.

Detection of Glycogen Synthase Kinase (GSK)-3Ð Expression in Myocardial Tissues via Immunohistochemistry

Myocardial tissue sections were first baked in an oven at 60°C for 30 min. Then they were deparaffinized with xylene (5 min × 3 times), and dehydrated with 100% ethanol, 95% ethanol and 70% ethanol for 3 times. Endogenous peroxidase activity was then inhibited by 3% hydrogen peroxide methanol. After sealing with goat serum for 1 h, the tissues were incubated with anti-GSK-3β antibody (diluted at 1:200 with PBS) at 4°C overnight, followed by washing with phosphate-buffered saline (PBS) for 4 times on a shaker. After that, fluorescein isothiocyanate (FITC)-secondary antibody was added for incubation at 37°C for 1 h. The nucleus was then stained with 4',6-diamidino-2-phenylindole (DAPI) (Beyotime, Shanghai, China). After color development, photography was performed under a light microscope (Magnification ×200). 6 samples were randomly selected in each group, and 5 fields of view were randomly selected for each sample.

Western Blotting

Myocardial tissues of rats in each group were fully ground in lysis buffer, followed by ultrasonic lysis. After centrifugation, the supernatant was taken and placed into Eppendorf (EP) tubes. The concentration of extracted protein was detected *via* ultraviolet spectrometry, and protein samples were quantified to the same concentration. After that, protein samples were sub-packaged and placed in a refrigerator at -80°C for use. Total protein was then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). After incubation with primary antibodies at 4°C overnight, the membranes were incubated again with goat anti-rabbit secondary antibody in dark for 1 h. Immuno-reactive bands were scanned and quantified using the Odyssey scanner. The expression levels of proteins were calculated, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal reference.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was used for all statistical analysis. Measure-

ment data were expressed as mean ± standard deviation. *t*-test was used for the comparison of data between two groups. $p<0.05$ was considered statistically significant.

Results

Expression of miR-26a in Myocardial Tissues in Each Group

As shown in Figure 1, the results of RT-PCR revealed that miR-26a expression in myocardial tissues was significantly up-regulated in I/R group when compared with that in Control group ($p<0.05$). After injection of miR-26a siRNA *via* tail vein, the expression level of miR-26a in the infarction region was significantly inhibited ($p<0.05$), indicating successful establishment of miR-26a knockdown model in rats.

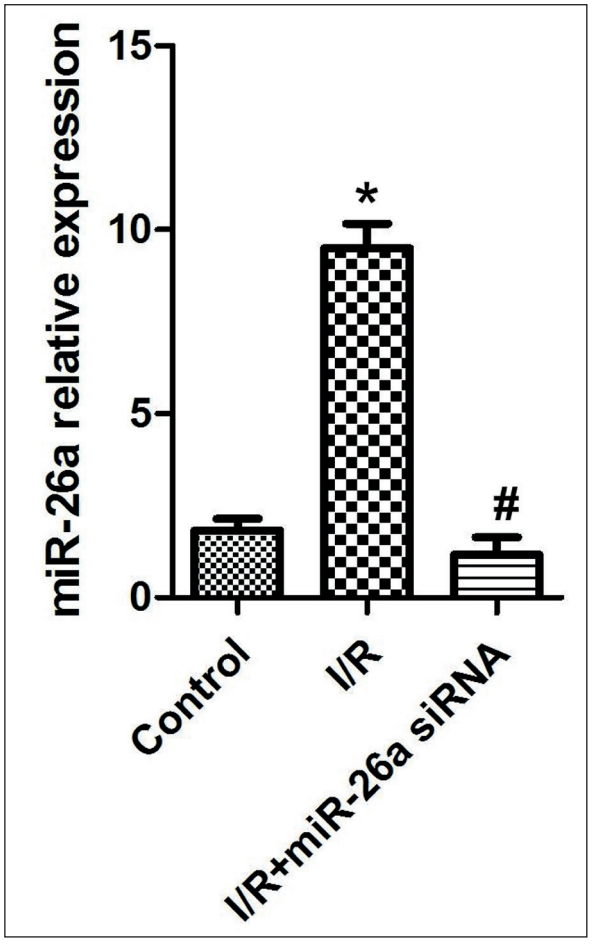

Figure 1. Expression of miR-26a in myocardial I/R injury. Control: Control group, I/R: ischemia-reperfusion group, I/R + miR-26a siRNA: ischemia-reperfusion + miR-26a knockdown group. The difference was statistically significant * *vs.* Control group and # *vs.* I/R group ($p<0.05$).

Cardiac Function of Rats in Each Group

As shown in Figure 2, two-dimensional echocardiography showed that no statistically significant difference was found in heart rate among the three groups. Therefore, the effects of heart rate on EF% and FS% in each group could be eliminated. Compared with Control group, ventricular cavity was enlarged and heart wall became significantly thinner in I/R group. These abnormal changes in heart structure induced by I/R could be significantly improved after miR-26a knockdown. Furthermore, FS% and EF% were detected in each group as well. The results found that FS% and EF% significantly increased by miR-26a knockdown in I/R injury rats ($p<0.05$).

Effect of miR-26a Knockdown on Pathological Structure of Heart

To evaluate the microstructural changes in myocardial cells in the cross section of heart, H&E staining was performed on myocardial tissues. As shown in Figure 3, obvious edema occurred in myocardial cells of I/R group, and myofilaments were arranged disorderly. Meanwhile, there were varying degrees of degradation and necrosis, accompanied by inflammatory cell infiltration. After miR-26a knockdown, myocardial tissue edema was significantly alleviated. Moreover, abnormalities in myofilaments were significantly improved as well, indicating that miR-26a knockdown could alleviate myocardial I/R injury.

Effect of miR-26a Knockdown on Infarction Area in Each Group

The infarction area in each group was evaluated *via* TTC staining. The results showed that the infarction area in the three groups was 2.02±1.21 *vs.* 44.39±1.50 *vs.* 20.09±2.92, respectively, showing statistically significant differences ($p<0.05$) (Figure 4). The above results suggested that inhibiting miR-26a could reduce the infarction area of I/R injury rats.

Myocardial Apoptosis Level in Each Group

To explore the effect of miR-26a knockdown on myocardial apoptosis in rats, myocardial apoptosis level was detected *via* TUNEL staining. The

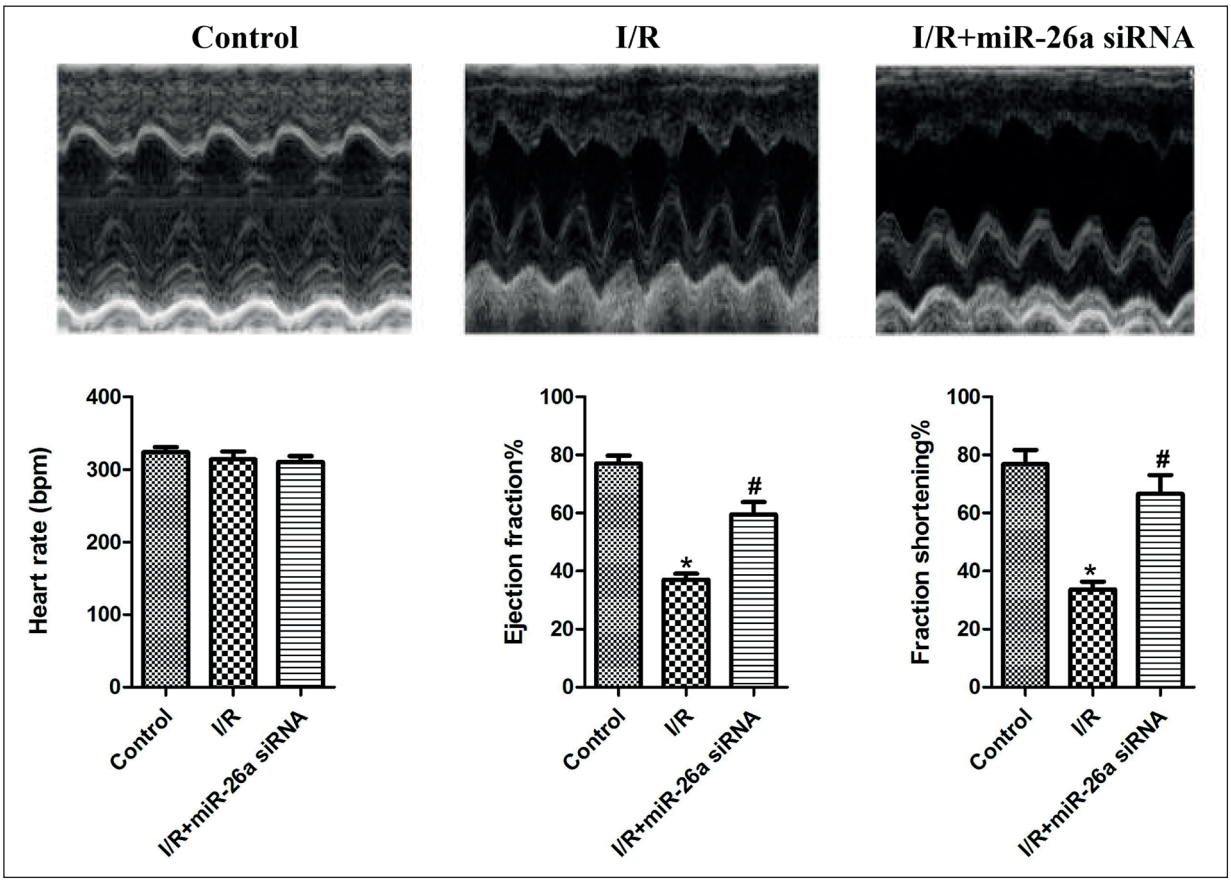

Figure 2. Cardiac function of rats in each group. Control: Control group, I/R: ischemia-reperfusion group, I/R + miR-26a siRNA: ischemia-reperfusion + miR-26a knockdown group. The difference was statistically significant * *vs.* Control group and # *vs.* I/R group ($p<0.05$).

results manifested that after I/R injury occurred, the apoptosis of myocardial cells and fibroblasts in myocardial tissues increased significantly ($p<0.05$), which was about (39.09±2.66) times higher than that of Control group. After miR-26a knockdown, the number of apoptotic myocardial cells declined

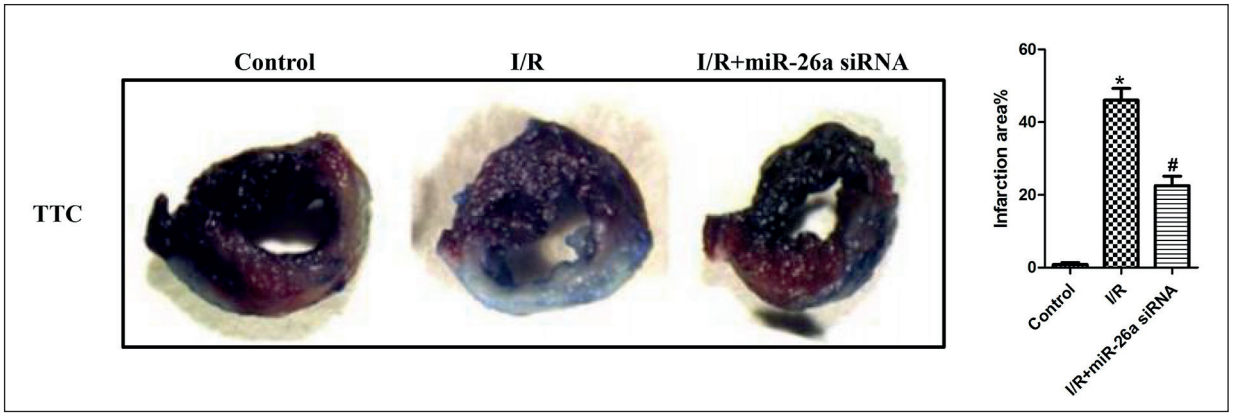

Figure 3. TTC staining of heart in each group. Control: Control group, I/R: ischemia-reperfusion group, I/R + miR-26a siRNA: ischemia-reperfusion + miR-26a knockdown group. The difference was statistically significant * *vs.* Control group and # *vs.* I/R group ($p<0.05$).

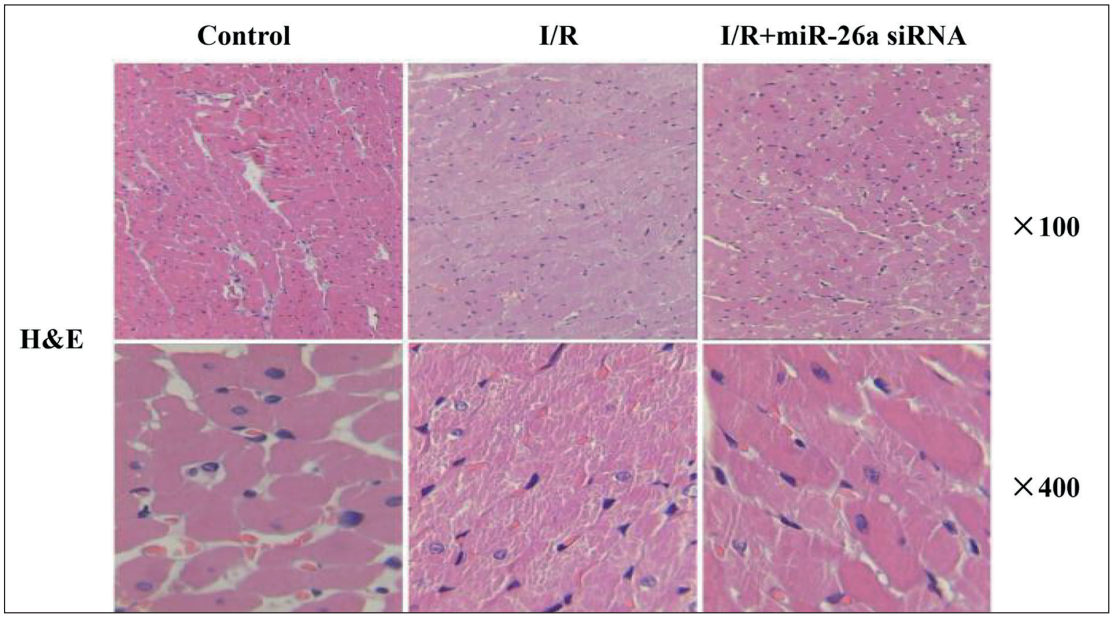

Figure 4. Effect of miR-26a knockdown on infarction area in each group. Control: Control group, I/R: ischemia-reperfusion group, I/R + miR-26a siRNA: ischemia-reperfusion + miR-26a knockdown group. The difference was statistically significant * *vs.* Control group and # *vs.* I/R group ($p<0.05$).

to (12.53±1.26) times when compared with that in Control group ($p<0.05$) (Figure 5).

Expression of Apoptosis-Related Genes in Myocardial Tissues in Each Group

The expression levels of apoptosis-related genes Bax and C-caspase3 in the myocardium were detected in each group. The results showed that both the expressions of Bax and C-caspase3 increased significantly in I/R group, indicating increased myocardial apoptosis level ($p<0.05$). Compared with I/R group, the expression levels of the above two proteins in the myocardium declined significantly in I/R + miR-26a siRNA

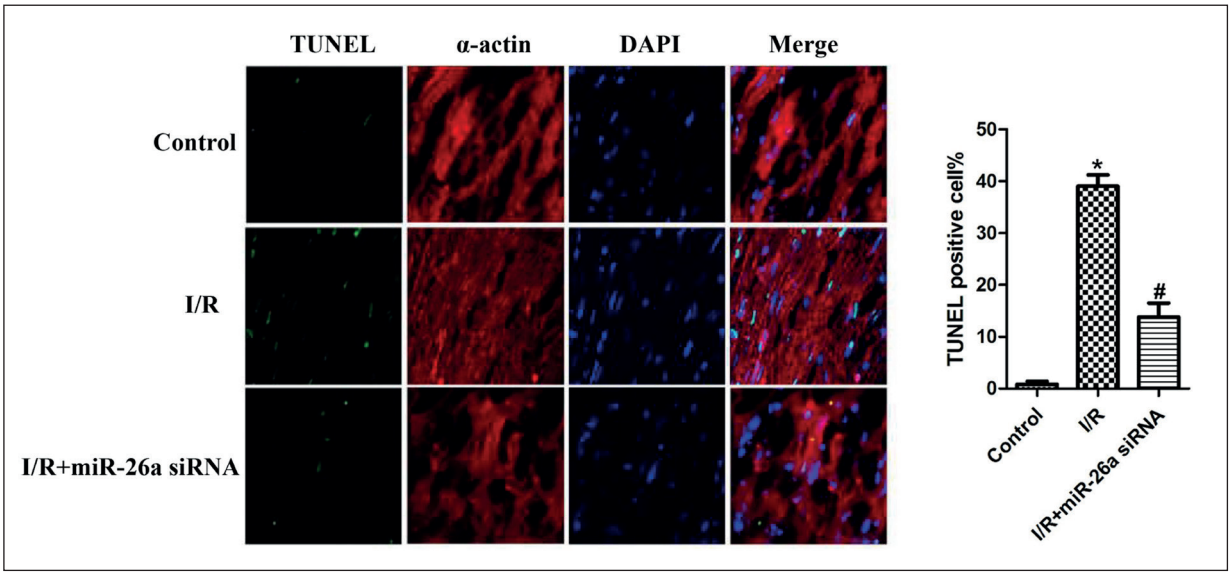

Figure 5. Myocardial apoptosis level in each group. Control: Control group, I/R: ischemia-reperfusion group, I/R + miR-26a siRNA: ischemia-reperfusion + miR-26a knockdown group (Magnification ×200). The difference was statistically significant * *vs.* Control group and # *vs.* I/R group ($p<0.05$).

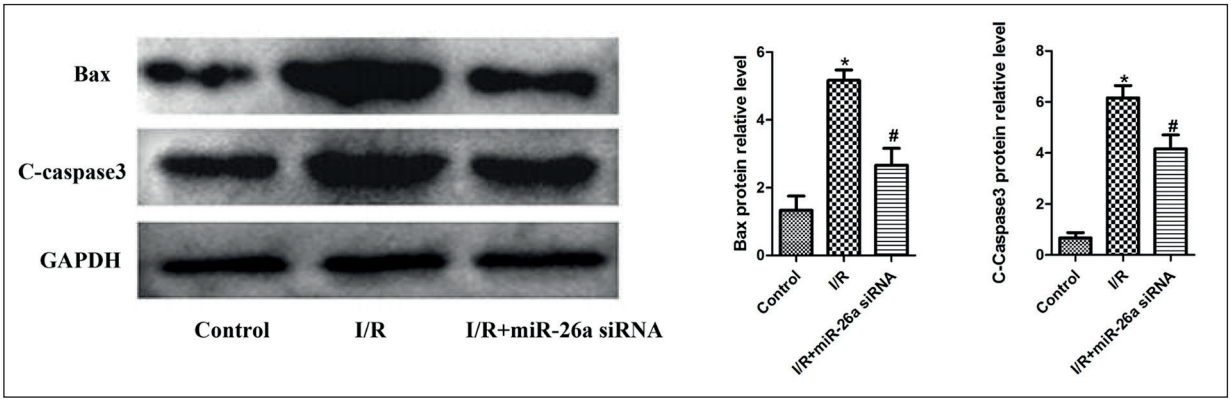

Figure 6. Expression of apoptosis-related genes in myocardial tissues in each group. Control: Control group, I/R: ischemia-reperfusion group, I/R + miR-26a siRNA: ischemia-reperfusion + miR-26a knockdown group. The difference was statistically significant * *vs.* Control group and # *vs.* I/R group ($p<0.05$).

group ($p<0.05$) (Figure 6). These findings further confirmed the inhibitory effect of miR-26a siRNA on I/R injury-induced myocardial apoptosis in rats.

Effect of miR-26a on GSK-3β/β-Catenin Signaling Pathway in Myocardium

Considering the mechanism of GSK-3β/β-catenin signaling pathway in myocardial I/R injury, we explored whether the protective effect of miR-26a on myocardial I/R injury was mediated by GSK-3β/β-catenin signaling pathway. The expression levels of GSK-3β and β-catenin in myocardial tissues were detected in the three groups. Results indicated that the expressions of GSK-3β and β-catenin in myocardial tissues of I/R + miR-26a siRNA group were significantly higher than those of I/R group (Figure 7A). This suggested that inhibiting miR-26a could activate the GSK-3β/β-catenin signaling pathway. Moreover, the potential target genes of miR-26a were analyzed online using the bioinformatics analysis

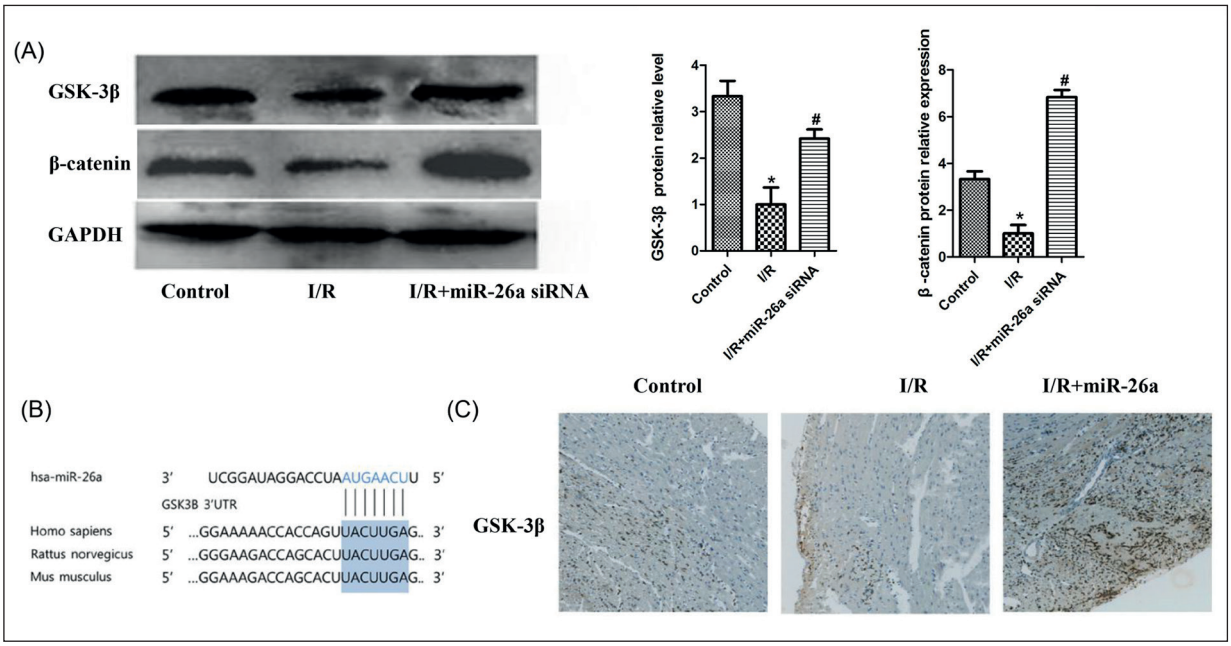

Figure 7. Effect of miR-26a on GSK-3β/β-catenin signaling pathway in myocardium (Magnification ×200). Control: Control group, I/R: ischemia-reperfusion group, I/R + miR-26a siRNA: ischemia-reperfusion + miR-26a knockdown group. The difference was statistically significant * *vs.* Control group and # *vs.* I/R group ($p<0.05$).

software Targetscan. The results predicted that GSK-3β was a potential target gene of miR-26a (Figure 7B). Immunohistochemical staining also revealed that miR-26a siRNA could up-regulate the expression of GSK-3β in myocardial tissues (Figure 7C).

Discussion

I/R injury is the major cause of heart failure after MI or cardiac operation, characterized by high morbidity and mortality rates[10]. The way to rescue the viable myocardium and restore its mechanical-electrical function has become the priority in clinical research currently[11,12]. It is accepted that rapid recovery of normal blood supply to coronary artery is considered as the only effective way to reduce heart injury[13]. Besides, cardiac reperfusion itself can also lead to additional myocardial cell death, which further expands the infarction area of heart. Factors leading to reperfusion injury mainly include oxidative stress, inflammation and apoptosis[14-16]. During myocardial ischemia, massive consumption of adenosine triphosphate in myocardial cells reduces the ability of sarcoplasmic reticulum to take in Ca^{2+}. This may result[17] in massive accumulation of mitochondrial Ca^{2+}. During reperfusion, oxygen re-enters myocardial cells, causing damage to mitochondrial electron transport chain and increased production of reactive oxygen species (ROS)[2]. Overload of mitochondrial Ca^{2+} and increased production of ROS promotes the opening of mitochondrial permeability transition pore, thereby leading to cellular energy disorders and irreversible cell necrosis and apoptosis[18]. The above findings suggest that mitochondria, as the organelle for energy supply, are intracellular important Ca^{2+} buffering system and the major source of ROS production. Meanwhile, they are the determinants of cell survival and death as well. Therefore, inhibition of myocardial apoptosis, inflammation and oxidative stress, and improvement of myocardial mitochondrial function during reperfusion can effectively reverse I/R injury-induced cardiac dysfunction and reduce infarction area. Apoptosis, also known as programmed cell death, refers to programmed death controlled by genes to keep homeostasis under physiological or pathological conditions[19]. Studies have demonstrated that a variety of apoptosis-inducing signals are activated during MI. This may induce the edema, apoptosis or necrosis of myocardial cells in the infarction region. In particular, myocardial cells die mainly in the form of necrosis within the first 6 h after MI, followed by the form of apoptosis[20]. In the cardiovascular field, miRNAs are considered as key targets for regulating the expressions of physiological and pathological myocardial stress genes. Suh et al[21] have indicated that miR-26a is highly expressed in the heart of rats, whose expression declines in ischemic preconditioning. MiR-26a can induce the apoptosis of a variety of tumor cells. For example, the expression of miR-26a is significantly declined in liver cancer cells. Meanwhile, exogenous overexpression of miR-26a significantly inhibits the proliferation and promotes the apoptosis of liver cancer cells[22]. Moreover, miR-26a inhibits the proliferation and promotes the apoptosis of porcine immature Sertoli cells through targeted inhibition on PAK2[23]. In addition, miR-26a is up-regulated in HaCaT cells under UV irradiation, which promotes the apoptosis of HaCaT cells through targeting histone methyltransferase EZH2 as well[24]. Previous studies have found that the 3'-UTR of GSK-3β gene contains a potential binding site of miR-26a, which is incomplete complementary to miR-26a. GSK-3β is involved in anti- and pro-apoptotic processes. However, miR-26a induces smooth muscle hypertrophy *via* inhibiting GSK-3β[21]. In the present study, the results found that miR-26a was highly expressed in myocardial tissues of I/R injury rats. Inhibiting miR-26a expression significantly improved cardiac function of I/R rats, which was manifested as significantly increased EF% and FS%. In addition, inhibition of miR-26a could markedly lower the infarction area of I/R rats, reduce the number of apoptotic myocardial cells and decrease the expressions of pro-apoptotic genes of Bax and C-caspase3. Finally, Western blotting and bioinformatics analysis showed that miR-26a could suppress the up-regulation of β-catenin mediated by GSK-3β in a targeted manner. Therefore, inhibiting miR-26a could activate the GSK-3β/β-catenin signaling pathway.

Conclusions

We found for the first time that the miR-26a knockdown could alleviate cardiac dysfunction and myocardial apoptosis induced by I/R injury through activating the GSK-3β/β-catenin signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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