Original Paper

MicroRNA-374 Exerts Protective Effects by Inhibiting SP1 Through Activating the PI3K/Akt Pathway in Rat Models of Myocardial Ischemia-Reperfusion After Sevoflurane Preconditioning

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Key Words

MicroRNA-374 • SP1 • PI3K/Akt pathway • myocardial ischemia-reperfusion • Sevoflurane

Abstract

Background/Aims: Ischemia-reperfusion injury is a leading cause of death in cardiovascular diseases, and microRNAs (miRNAs) have been reported to be potential therapeutic targets in heart disease. Herein, this study investigated the effects of microRNA (miR)-374 on myocardial ischemia-reperfusion (I/R) injury in rat models pretreated with sevoflurane by targeting SP1 through the PI3K/Akt pathway. **Methods:** SD rats were grouped into sham, I/R and sevoflurane + I/R, sevoflurane preconditioning and I/R groups. The biochemical indicators, pathological changes, positive expression of SP1 protein, and apoptosis rates were measured using biochemical detection, Evans blue-TTC staining, immunohistochemistry and TUNEL staining. Real-time PCR and Western blotting were used to investigate the expression of miR-374 mRNA and the protein expression of SP1, PI3K, HO-1, p53, iNOS, c-fos, Akt/p-Akt, and GSK-3β/p-GSK-3β. Cardiomyocyte proliferation and cycle distribution and apoptosis were studied by flow cytometry. **Results:** Compared with the I/R group, in the sevoflurane + I/R group, SOD and IL-10 increased, while MDA, LDH, CK, TNF-α, IL-6 and IL-10 decreased, as did the percentage of infarct area, the positive rate of SP1 and the apoptosis index. The expression of SP1, p53, iNOS and c-fos decreased, and the miR-374 expression of PI3K, HO-1, Akt/p-Akt, GSK-3β/p-GSK-3β increased. With the upregulation of miR-374 and the downregulation of SP1, the expression of SP1, p53, iNOS and c-fos decreased, as did the proportion of cells in G1 phase and the apoptosis rate; the expression of PI3K, HO-1, Akt/p-Akt, GSK-3β/p-GSK-3β increased. The results in the miR-374 inhibitor group contrasted with the above results. **Conclusion:** The results indicated that miR-374 could alleviate myocardial I/R damage in rat models pretreated with sevoflurane by targeting SP1 by activating the PI3K/Akt pathway.

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Introduction

Ischemic heart disease is the main cause of death in cardiovascular diseases, and blood supply restoration, such as reperfusion therapy, which is believed to be the most effective treatment for myocardial ischemia, although it may exacerbate or even cause irreversible damage [1]. Occurring at the time of reperfusion of ischemic myocardium with oxygen and substrate-rich blood, reperfusion could sometimes paradoxically deteriorate heart function [2]. Anesthetics such as sevoflurane and isoflurane are reported to be valid therapeutic drugs for I/R injury [3]. Except for its fast induction, rapid recovery and low possibility of “coronary arterial steal”, sevoflurane has proved to protect the heart against myocardial I/R injury, enhancing recovery of cardiac function, reducing myocardial enzyme release and lowering infarct size when utilized as pretreatment or posttreatment agents [4]. However, the possible mechanism that is responsible for sevoflurane mediating cardioprotection is still unknown [5].

Endogenous 18-22-nucleotide microRNAs (miRs), such as miR-374a and miR-374b, are considered to play significant roles in cell differentiation, proliferation and apoptosis [6-8]. According to a recent study, miR-22 with its target GADD45 could suppress apoptosis of cardiomyocytes, providing a new, valid therapeutic target for alleviating myocardial I/R injury [9]. Furthermore, another study showed that up-regulation of miR-15a and miR-15b were in response to cardiac I/R injury, indicating that the down-regulation of miR-15a or miR-15b might be a practical way to inhibit myocardial apoptosis caused by myocardial I/R injury [10]. Lately, it was shown that miRs could be potential therapeutic targets for alleviating I/R injury through altering key signaling elements [11]. It has been demonstrated that by suppressing CCND1, miR-374a could inactivate the PI3K/AKT axis, inducing the inhibition of colon cancer progression [12]. As an important part of the protective mechanism of ischemic preconditioning, the phosphoinositide 3-kinase (PI3K)/Akt pathway plays a major role in controlling cell growth, survival, proliferation and migration, with an improving function in I/R through activating Akt [13]. As a series of heterodimeric enzymes, PI3Ks are composed of a p110 catalytic subunit and a regulatory subunit that could induce the formation of 3-phosphoinositides, mediating cell division, migration and survival [14]. Additionally, LINC00152 was reported to participate in the PI3K/Akt signaling pathway, with the transcription factor SP1 inducing its overexpression [15]. However, the role of miR-374 in myocardial I/R was not explored. Therefore, this study estimated the effects of miR-374 in rat models of myocardial I/R pretreated with sevoflurane by targeting the transcription factor SP1 through activating the PI3K/Akt pathway.

Materials and Methods

Experimental animals

A total of 40 male Sprague-Dawley (SD) rats, weighing 180 to 250 g and aged from 6 to 8 weeks, were purchased by the North China University of Science and Technology Affiliated Hospital, bred in cages individually with humidity of 55% at 25°C for two weeks with free diet and water intake in dark and under light (12 h/12 h); all mice were fed adaptively for 2 weeks before experiments. This experiment procedure and animal operation protocols were approved by the animal ethics committee.

Model establishment, identification and grouping

Forty-five SD rats were randomly selected and then assigned into the sham group, I/R group and sevoflurane + I/R group (2.5% sevoflurane [Shanghai Yongye Biotechnology Co., Ltd., Shanghai, China] preconditioning and I/R). The rats in each group were anesthetized by intraperitoneal injection of 1% pentobarbital sodium at 45 mg/kg (Sigma-Aldrich Chemical Company, St. Louis, MO, USA) and given ventilator-assisted ventilation through endotracheal intubation (RSP1002, Pushengda Teksystems Ltd., Beijing, China). Then, left thoracotomy was conducted. The model of myocardial I/R injury was established by ligating the left anterior descending branch (LAD) at 3 mm from the inferior border of the left atrial

appendage by using 6/0 suture, and then releasing the suture line and reperfusion for 2 h [16]. Rats in the sham group underwent thread ligation only, and rats in the sevoflurane + I/R group received 2.5% sevoflurane for 30 min before ischemia. Fifteen min later, the I/R model was established as described above. Rats that died in the surgery were immediately replaced. When the anterior wall in the distal end of the ligation site was observed to have cyanosis or pale with naked eyes, ECG (SP2006, Beijing Senxi Technology Co., Ltd., Beijing, China) showed that the S-T segment was elevated and the T wave was ascended, which meant myocardial ischemia. The decrease of the S-T segment, recovery of the R wave in ECG or the ischemic region turning red were the indications of successful reperfusion. Intraoperative temperature of the rats was maintained at 36~38°C.

Cardiac function test

The left ventricular end diastolic pressure (LVEDP), left ventricular developed pressure (LVEDP), left ventricular pressure rising maximum rate ($+dp/dt_{max}$), left ventricular pressure dropping maximum rate ($-dp/dt_{max}$), heart rate (HR) and coronary flow (CF) were observed and recorded at the time of 30, 60, 90 and 120 min after reperfusion using the 16-channel physiological recorder biological signal analysis system (MP100, BIOPAC Systems, Inc., Santa Barbara, CA, USA).

Biochemical testing

Two hours after reperfusion, 3 mL thigh arterial blood was collected. After centrifugation at 3500 r/min for 10 min at 4°C, the serum was collected from each group. An automatic biochemical analyzer (bs-330e, Wuhan Shengshida Medical Equipment Co., Ltd., Wuhan, China) was employed to detect the content of superoxide dismutase (SOD), malondialdehyde (MDA), lactate dehydrogenase (LDH) and creatine kinase (CK). The expression of tumor necrosis factor- α (TNF- α) (IL-16, Beyotime Biotechnology Co., Shanghai, China), interleukin-10 (IL-10) (QY-BM10156, Shanghai Qiyao Biological Technology Co., Ltd., Shanghai, China) and interleukin-6 (IL-6) (PI328, Beyotime Biotechnology Co., Shanghai, China) in serum was investigated using ELISA according to the kit operation.

Detection of the area of myocardial infarction

Two hours after perfusion, the hearts of each group were collected and washed with physiological saline. The large vessels, adipose tissue, atrium and right ventricle were removed, and three left ventricles were collected and placed at -80°C for storage. In addition, some left ventricular tissues were collected and fixed with 4% paraformaldehyde for HE staining, immunohistochemical staining and TUNEL staining. The remaining left ventricular tissues were preserved at -80°C. The left ventricles were cut along the long axis into 2 mm slices and then placed in 1% TCC (pH 7.4) dye liquor and bathed at 37°C for 10 min. A digital microscope (Nipkow, Nikon, Tokyo, Japan) was used to take photos, and a single-blind method was used to distinguish normal areas (blue), ischemic areas (pink) and infarct areas (gray). Sigma Scan Pro area measurement software was used to calculate the percentage of infarct size in total myocardium area.

Pathological observation of ventricular tissues

Interventricular tissue specimens were fixed with 4% paraformaldehyde, embedded with paraffin, dehydrated with gradient alcohol, embedded in paraffin and sliced into 4 μ m sections. Some sections were prepared for immunohistochemical and TUNEL staining. The paraffin sections were dewaxed and placed in hematoxylin solution, dehydrated in gradient alcohol, cleared twice in xylene, and then sealed with neutral balsam. Finally, an optical microscope (XSP-36, Boshida Optical Instrument Co., Shenzhen, China) was used to observe the morphological variations.

Immunohistochemistry

The prepared ventricular tissue slices were baked at 60°C for 1 h, conventionally dewaxed with xylene and dehydrated in gradient alcohol, followed by high pressure antigen retrieval, blocking with normal sheep serum (Ns01-01, Bio-technology Co., Ltd., Luoyang, China) at 37°C for 10 min, incubation with primary rabbit anti-rat polyclonal antibody SP1 (1:200) (ABP50404, Abbkine, CA, USA) at 4°C overnight and rinsing with PBS. After adding a 1:1000 dilution of HRP-labeled goat anti-rabbit secondary antibody (A21020, Abbkine, CA, USA), the sections were incubated at room temperature for 30 min. DAB (CAS: 7411-49-6, Suzhou Industrial Park Yacoo Chemical Reagent Co., Ltd., Suzhou, China) was used for staining for 3 min,

followed by washing with water for 3 min, counterstaining with hematoxylin, dehydration, clearing and mounting. PBS was used as negative control, and a known positive slice was used as a positive control. Positive cells were those with over 25% dyeing degree with brown cell nucleus or cytoplasm. Slices were placed under an optical microscope and examined: 5 high magnification fields were randomly selected for each slice, with 100 cardiomyocytes in each view. The average value of the percentage of positive cells per slice was the positive rate.

TUNEL detection

The prepared ventricular tissue sections were embedded with conventional paraffin, and dehydrated with gradient alcohol. The TUNEL detection procedures were in strict accordance with the requirements of the TUNEL kit. Under the fluorescence microscope, 365 nm ultra violet (UV) light was used to stimulate DAPI in the same field of view, and pictures were taken when blue light was observed under microscope. Then the 365 nm UV light was replaced with 565 nm UV light to stimulate TUNEL. When green fluorescence was observed, pictures were taken. Five non-overlapping views were randomly selected in the high magnification microscope in the ischemic zone (i.e., the region of apoptotic positive cells or the anterior wall of the infarcted myocardium). Apoptotic index (AI) was the percentage of apoptotic positive cells in total cellular score per unit area.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA was collected from the parts of the left ventricular tissue and extracted with RNeasy Mini Kit (217004, QIAGEN GmbH, Hilden, Germany). A_{260}/A_{280} and A_{260}/A_{280} ratios were detected on NanoDrop 2000 micro UV spectrophotometer (1011U, NanoDrop Technologies, Wilmington, DE, USA) to determine the total RNA concentration and purity. The total RNA was reverse transcribed into cDNA according to TaqMan MicroRNA Assays Reverse Transcription Primer (4440907, Applied Biosystems, California, USA). Reverse transcribed cDNA was diluted to 50 ng/ μ L, and 1 μ L was added in the 25 μ L reaction amplification system each time. The reverse transcription reaction conditions were 37°C for 30 min and 85°C for 5 s. MiR-374, SP1, PI3K, Akt, GSK-3 β , HO-1, p53, iNOS and c-fos primers were designed and submitted to TaKaRa for synthesis (Table 1). ABI7500 (7500, Applied Biosystems, Foster City, USA) quantitative real-time PCR, reverse transcription quantitative polymerase chain reaction was used for RT-qPCR. The RT-qPCR reaction was one cycle of initial denaturation at 95°C for 30 s, 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 20 s and extension at 72°C for 34 s. The RT-qPCR reaction system was SYBR Premix Ex Taq™ II, 10 μ M forward primer (10 μ M), 0.8 μ L; PCR Reverse Transcriptase (0.8 μ M), 0.8 μ L; ROX Reference Dye (0.4 μ M), 2.0 μ L; and distilled water (6.0 μ L) was used as the internal reference. miR-374 and β -actin for SP1, PI3K, Akt, GSK-3 β , HO-1, p53, iNOS and c-fos. $2^{-\Delta\Delta Ct}$ indicated the relative expression multiple proportions between experimental group and control group. The formula was as follows: $\Delta\Delta Ct = \Delta Ct_{\text{experiment group}} - \Delta Ct_{\text{control group}}$ and $\Delta Ct = Ct_{\text{target gene}} - Ct_{\beta\text{-actin}}$. Ct was the number of amplified cycles when the real time fluorescence intensity of the reaction reached the threshold value. Each experiment was repeated 3 times.

Western blot analysis

The left ventricular tissues were placed in a glass grinder, and 1 mL tissue lysate (BB-3209, Bestbio, Shanghai, China) was added. Then, the grinder was put into ice bath and ground to form a homogenate. The total proteins were routinely

Table 1. Primer sequences for RT-qPCR. Notes: RT-qPCR, reverse transcription quantitative polymerase chain reaction; F, Forward; R, Reverse; SP1, transcription factor SP1; PI3K, phosphatidylinositol 3 kinase; Akt, threonine protein kinase; GSK-3 β , glycogen synthase kinase-3 β ; HO-1, heme oxygenase 1; iNOS, inducible nitric oxide synthase; c-fos, cellular oncogene fos; β -actin, cytoskeletal beta actin

Genes	Primer sequences (5'-3')
miR-374	F: 5'-ATATAATACAACCTGC-3' R: 5'-CACTTAGCTGGTGTGA-3'
U6	F: 5'-CTCGCTTCGGCAGCACA-3' R: 5'-AACGCTTCACGAATTTGCGT-3'
SP1	F: 5'-GGCTACCCCTACCTCAAAGG-3' R: 5'-CACAACATACTGCCACCAG-3'
PI3K	F: 5'-CAGCACCTCGTATGGCTCAAT-3' R: 5'-GTCCTCACTCTTGATCCCCAG-3'
Akt	F: 5'-CCACGCACACTCGGGCCG-3' R: 5'-CAATGCAGAGGGGTGACG-3'
GSK-3 β	F: 5'-GCTTCAACCCCTTCAAATGC-3' R: 5'-GACGCAGAGCGGTGTATTG-3'
HO-1	F: 5'-TCTATCGTGCATGAAC-3' R: 5'-CAGCTCCTCAACAGCTCAA-3'
p53	F: 5'-GTCGGCTCCGACTATACCACTATC-3' R: 5'-CTCTCTTTGCACTCCCTGGGG-3'
iNOS	F: 5'-GGAGAGATTTTTCACGACACCC-3' R: 5'-CCATGCATAAATTGGACTTGCA-3'
c-fos	F: 5'-ACTGCGAGAACCAAGCTACTGCTG-3' R: 5'-GTACGTCCATTGACATGTTGCTCAG-3'
β -actin	F: 5'-CCCTGGCTCCTAGCACCAT-3' R: 5'-GATAGAGCCACCAATCCACACA-3'

extracted, and the protein concentration of each sample was determined by BCA Kit (20201ES76, Yeasen Biological Technology Co., Ltd., Shanghai, China). After protein denaturation, protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then the protein on the gel was transferred to the nitrocellulose membrane. A total of 5% skimmed milk powder was added at 4°C to block the nitrocellulose membrane overnight. Then, 1:500 diluted primary rabbit anti-rat polyclonal antibody SP1 (bs-0975R), PI3K (bs-5570R), phosphorylated (p)-Akt (bs-5194R), iNOS (bs-2072R), HO-1 (bs-2075R), p-GSK-3 β (bs-5368R) and c-fos (bs-10172R) (all purchased from Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China), 1:500 diluted primary rabbit anti-rat monoclonal antibody GSK-3 β (ab93926; Abcam, Inc., Cambridge, MA, USA) and 1:1000 diluted primary rabbit anti-rat polyclonal antibody Akt (ABP50387, Abbkine, CA, USA), p53 (ABP50389 Abbkine, CA, USA) and β -actin (ABP57456, Abbkine, CA, USA) were added and incubated overnight. PBS was used to wash three times at room temperature, once every 5 min. Then, 1:10000 dilution of horse-radish peroxidase (HRP)-labeled IgG goat anti-rabbit secondary antibody (A21020, Abbkine, CA, USA) was added, avoiding exposure to light, and incubated at 37°C for 1 h, followed by PBS washing 3 times, once every 5 min at room temperature. The membrane reacted with DAB (SCL008-25, Biomiga, San Diego, CA, USA) for 1 min at room temperature. After absorbing the liquid, the sections were covered with the plastic wrap, and X ray was conducted (36209ES01, Qianjiao Biological Technology Co., Ltd., Shanghai, China) for observation. β -actin was used as internal reference. The ratio of the gray value between the target band and the internal reference strip was used to evaluate the expression of the protein. Each experiment was repeated 3 times.

Cell extraction

The apex of the left ventricular tissues of the sham and sevoflurane I/R groups that were frozen previously were cut into pieces of approximately 3 mm³, washed with D-Hanks' liquid (HMK0011, Beijing Hua Maiké Biotechnology Co., Ltd., Beijing, China). After discarding the supernatant, low sugar DMEM (SH30021, Beijing North Tongzheng Biotechnology Development Co. Ltd., Beijing, China) with 0.15% collagenase was added. The mixture was transferred to a 50 ml centrifuge tube and digested for 30 min using an electromagnetic stirrer (1008011909, Tianjin Heng Trading Co. Ltd., Tianjin, China) at 37°C. Next, the mixture was centrifuged at 700 rpm/min at 4°C for 5 min to remove the supernatant. Suitable amounts of DMEM low sugar medium containing 10% fetal bovine serum were added, followed by centrifugation at 700 rpm/min at 4°C for 5 min. The debris was added to the medium and blown into single cell suspensions. The suspensions were transferred to a 6-well culture dish to be evenly distributed and washed with D-Hanks' liquid. Then, a proper volume of 0.25% trypsin (25200056, Gibco, Grand Island, USA) was added to the culture dish, which was then put into an incubator with 5% CO₂ for digestion at 37°C for 7 ~ 9 min. DMEM containing a volume fraction of 20% fetal bovine serum was added to terminate digestion. Then, the suspensions were centrifuged at 1200 rpm/min at 4°C for 5 min, with the supernatant discarded. The sections were resuspended in DMEM medium containing 20% fetal bovine serum and then inoculated into a new culture dish at 37°C. Then, the sections were put in an incubator with volume fraction of 5% CO₂ for further culture for subsequent cell experiments. Later, the culture solution was replaced every 3 days.

Dual luciferase reporter gene assay

Target gene analysis of miR-374 was performed using the biological prediction website miRanda. Dual luciferase reporter gene test was used to test whether SP1 was the direct target gene of miR-374. The full-length 3'UTR region of the SP1 gene was cloned and amplified (Forward: 5'-GATCTTTGCTAGGACATCCTAAATTTATATACCTT-3'; Reverse: 5'-GATATATAAATTTAGGATGTCCTAGCAAAGATC-3'). The plasmid was extracted in accordance with the Omega plasmid small amount of extraction kit (D6943-01, Beijing Think-Far Technology Co. Ltd., Beijing, China). After restriction endonuclease Xba I and Xho I double enzyme digestion, the target fragment was connected with the luciferase reporter vector pmirGLO (E1330, Promega Corporation, Madison, USA) and transformed into *Escherichia coli* DH5H cells for amplification. The plasmids were named pmirGLO-SP1-3'UTR-wt and pmirGLO-SP1-3'UTR-mut. Wt and Mut with correct sequencing were co-transfected with miR-374 into HEK-293T cells (K1223, Invitrogen Life Technologies, Carlsbad, CA, USA). Then, the cells were plated at a density of 2×10^5 /well in 6-well plates and transfected after cell adherence. After transfection, the cells were cultured for 48 h and collected. The fluorescence intensity was measured by a fluorescence detector (Glomax20/20, Promega, Madison, Wisconsin, USA). Each experiment was repeated 3 times.

Cell transfection and grouping

The cardiomyocytes in logarithmic growth phase were placed in 6-well plates until the cell density increased to 50% and then transfected with Lipofectamine 2000 (11668019, Thermo Fisher Scientific, San Jose, California, USA). In this procedure, 250 μ L of Opti-MEM without serum (31985, Gibco, Grand Island, USA) was used to dilute 100 pmol miR-374 mimics, miR-374 inhibitors, siRNA-SP1, miR-374 inhibitors + siRNA-SP1 and negative control (the final concentration was 50 nM), followed by gently mixing and incubating at room temperature for 5 min. Next, 250 μ L Opti-MEM without serum was used to dilute 5 μ L Lipofectamine 2000, followed by gently mixing and incubating at room temperature for 5 min. The above two mixtures were combined, incubated at room temperature for 20 min and added to culture plates. After culture in 5% CO₂ at 37°C for 6 ~ 8 h, new medium (INV-00002, INNOVATE, Wuxi, China) was used for incubation for 24 ~ 48 h. Cell grouping was as follows: the normal group (cardiomyocytes of rats in the sham group were not transfected with any sequence), the blank group (cardiomyocytes of rats in the sevoflurane + I/R group were not transfected), the NC group (cardiomyocytes of rats in the sevoflurane + I/R group were transfected with miR-374 negative control sequences), the miR-374 mimics group (miR-374 mimics were transfected in cardiomyocytes of rats in the sevoflurane + I/R group), the miR-374 inhibitors group (miR-374 inhibitors were transfected into cardiomyocytes of rats in the sevoflurane + I/R group), the siRNA-SP1 group (siRNA-SP1 were transfected into cardiomyocytes of rats in the sevoflurane + I/R group) and the miR-374 inhibitors + siRNA-SP1 group (miR-374 inhibitors and siRNA-SP1 were co-transfected into cardiomyocytes of rats in the sevoflurane + I/R group).

MTT assay

After cell transfection for 12 h, the cells were collected, washed twice with PBS, and added with 0.25% trypsin to prepare single cell suspension. After counting, the suspensions were placed in 96-well plates (0.1 mL/well) at a density of $3 \times 10^4 \sim 5 \times 10^4$ /well (cells from each group) and incubated in an incubator. The culture plates were removed separately at 0 h, 48 h and 72 h after cell adherence. Each well had 20 μ L 0.5% MTT solution added (M2128, Sigma, St. Louis, USA) and was cultured for 4 h with the supernatant removed. Each well had 100 μ L dimethyl sulfoxide (DMSO) added (D5879-100ML, Sigma, St. Louis, USA), followed by gentle shaking for 10 min to fully dissolve the formazan crystals. ELISA (BS-1101, De Tie Laboratory Equipment Co., Nanjing, China) was used to detect the absorbance value of each well at 490 nm. Each experiment was repeated 5 times. With time as the abscissa and the OD value as the ordinate, the curve of cell viability was drawn.

Flow cytometry

PI single staining method: after transfection, the cells were collected, treated with 0.25% trypsin and centrifuged at 400 g for 5 min at 4°C for 5 min, and the supernatant was discarded. Then, the cells were washed with cold PBS, centrifuged, and the supernatant was discarded. After resuspension with PBS, the density of the cells was adjusted to approximately 1×10^5 /mL. Then, 2 mL 75% ethanol pre-cooled at -20°C was added to the cells at 4°C for 30 min. The ice-cold ethanol was discarded after centrifugation, and PBS was added for washing after the supernatant was discarded. After incubation with 100 μ L RNase A (R7000, ThermoFisher, USA) for 30 min without exposure to light, 400 μ L PI dye (P4170, Sigma, St. Louis, USA) was added to the cells after mixing and avoiding exposure to light at 4°C for 30 min, flow cytometry (CytoFLEX, Beckman Coulter, USA) was employed to record the red fluorescence at a 488 nm stimulation wavelength to detect the cell cycle.

Annexin V/PI double parameter: after transfection for 48 h, the cells were treated by 0.25% trypsin (containing EDTA) (PYG0107, Boster Biological Technology Co., Ltd., Wuhan, China) and collected in a tube, followed by centrifugation, and then the supernatant was discarded. Then, the cells were washed 3 times by cold PBS, after which they were centrifuged; the supernatant was discarded. According to the Annexin-V-FITC cell apoptosis detection kit (K201-100, BioVision, Milpitas, CA, USA), Annexin-V-FITC/PI dye was made of Annexin-V-FITC, PI and HEPES dye in proportion of 1: 2: 50. A total of 1×10^6 cells were resuspended in every 100 μ L dye, after which they were evenly mixed. After incubation at room temperature for 15 min, 1 mL HEPES dye was added and then mixed. FITC and PI fluorescence were detected by excitation at 515 nm and 620 nm bandpass filters, respectively, at 488 nm, and then cell apoptosis was detected. The experiment was repeated 3 times. Flow cytometry scatter diagrams showed that the early apoptotic cells were in the lower right quadrant, necrotic and advanced apoptotic cells were in the upper right quadrant, mechanically

damaged or necrotic cells were in the left upper quadrant, and living cells were in the lower left quadrant. Apoptosis rate (%) = percentage of early apoptotic cells + percentage of late apoptotic cells.

Statistical analysis

All statistical tests were performed with SPSS 21.0 (IBM Corp, Armonk, NY, USA). Measurement data were presented as means $\pm$ standard deviation. The *t* test was used for comparison between two groups and one-way analysis of variance (ANOVA) for comparisons among groups, with $P < 0.05$ considered statistically significant.

Results

The sevoflurane + I/R group has decreased LVEDP and increased LVDP, $+dp/dt_{max}$, $-dp/dt_{max}$, HR and CF

The indexes of cardiac function (LVEDP, LVDP, $+dp/dt_{max}$, $-dp/dt_{max}$ and HR and CF) were detected at 30, 60, 90 and 120 min after I/R. The results showed that compared with the sham group, LVEDP in the I/R and sevoflurane + I/R groups increased significantly at 30, 60, 90 and 120 min ($P < 0.05$), and LVDP, $+dp/dt_{max}$, $-dp/dt_{max}$, HR and CF obviously decreased ($P < 0.05$). Compared with the I/R group, in the sevoflurane + I/R group at 30, 60, 90 and 120 min, the LVEDP decreased observably ($P < 0.05$), and LVDP, $+dp/dt_{max}$, $-dp/dt_{max}$ and HR increased significantly ($P < 0.05$). At 30 min, there was no significantly change in CF ($P > 0.05$), and CF increased significantly at 60, 90 and 120 min ($P < 0.05$) (Fig. 1). These results suggested that the sevoflurane + I/R group has decreased LVEDP and increased LVDP, $+dp/dt_{max}$, $-dp/dt_{max}$, HR and CF.

The sevoflurane + I/R group has increased serum SOD content and decreased MDA, LDH and CK contents

Biochemical examination was conducted to measure the contents of SOD, MDA, LDH and CK. Compared with the sham group, after reperfusion for 120 min, the I/R and sevoflurane + I/R groups had remarkably increased content of serum SOD ($P < 0.05$) and evidently increased contents of MDA, LDH and CK ($P < 0.05$). Compared with the I/R group, in the sevoflurane + I/R group, the content of serum SOD was observably increased ($P < 0.05$), and the contents of MDA, LDH and CK decreased significantly ($P < 0.05$) (Table 2). The content of LDH in serum of rats in the I/R and sevoflurane + I/R groups increased significantly,

Fig. 1. Results of cardiac function tests after I/R at 30, 60, 90, and 120 min. A, LVEDP; B, LVDP; C, $+dp/dt_{max}$; D, $-dp/dt_{max}$; E, HR; F, CF; *, $P < 0.05$ compared with the sham group; #, $P < 0.05$ compared with the I/R group; Sham group ($n = 15$); I/R group ($n = 15$); Sevoflurane + I/R group ($n = 15$); LVEDP, left ventricular end diastolic pressure; LVDP, left ventricular developed pressure; $+dp/dt_{max}$, left ventricular pressure rising maximum rate; $-dp/dt_{max}$, left ventricular pressure dropping maximum rate; HR, heart rate; CF, coronary flow; I/R, ischemia and reperfusion.

Table 2. Biochemical examination of the SOD, MDA, LDH and CK contents in serum of rats in each group after reperfusion for 120 min. Notes: SOD, superoxide dismutase; MDA, malondialdehyde; LDH, lactate dehydrogenase; CK, creatine kinase; *, $P < 0.05$ compared with the sham group; #, $P < 0.05$ compared with the I/R group; sham group (n = 15); I/R group (n = 15); Sevoflurane + I/R group (n = 15); I/R, ischemia and reperfusion

Groups	SOD (U/mL)	MDA (nmol/mL)	LDH (U/L)	CK (U/mL)
Sham group	262.00 ± 30.12	4.93 ± 0.51	72.09 ± 7.40	73.29 ± 8.01
I/R group	176.50 ± 19.32*	8.48 ± 0.90*	281.09 ± 27.19*	205.83 ± 23.12*
Sevoflurane + I/R group	204.00 ± 22.11#	6.58 ± 0.70*#	201.13 ± 22.19*#	122.63 ± 14.52*#

Table 3. The levels of TNF- α , IL-6 and IL-10 in serum of rats in each group. Notes: TNF- α , tumor necrosis factor- α ; IL-6, interleukin-6; IL-10, interleukin-10; *, $P < 0.05$ compared with the sham group; #, compared with the I/R group, $P < 0.05$; sham group (n = 15); I/R group (n = 15); Sevoflurane + I/R group (n = 15); I/R, ischemia and reperfusion

Groups	TNF- α (ng/L)	IL-6 (ng/L)	IL-10 (ng/L)
Sham group	15.35 ± 1.60	63.48 ± 6.12	8.83 ± 0.92
I/R group	127.72 ± 13.00*	151.01 ± 15.00*	47.61 ± 5.07*
Sevoflurane + I/R group	92.45 ± 9.40*#	88.60 ± 9.21*#	60.91 ± 6.52*#

suggesting the injury to myocardial tissue and successful modeling.

Rats in the sevoflurane + I/R group have reduced contents of TNF- α and IL-6 and increased content of IL-10

ELISA was performed to determine the levels of TNF- α , IL-6 and IL-10 in serum of rats in each group, and results demonstrated that after reperfusion 120 min, the levels of TNF- α , IL-6 and IL-10 in serum of rats in the I/R and sevoflurane + I/R group were raised observably compared with the sham group ($P < 0.05$). Compared with the I/R group, contents of TNF- α and IL-6 in serum of rats in the sevoflurane + I/R group were reduced evidently ($P < 0.05$), and content of IL-10 increased significantly ($P < 0.05$) (Table 3). The abovementioned results demonstrated that rats in the sevoflurane + I/R group have reduced contents of TNF- α and IL-6 and increased content of IL-10.

The percentage of infarct size after reperfusion for 120 min in the sevoflurane + I/R group is decreased markedly

Next, the left ventricular infarct size in each group was observed. The percentage of infarct size in the I/R and sevoflurane + I/R groups increased significantly compared with the sham group after reperfusion for 120 min ($P < 0.05$). Compared with the I/R group, the percentage of infarct size after reperfusion for 120 min in the sevoflurane + I/R group decreased markedly ($P < 0.05$) (Fig. 2).

The myocardial tissue damage and leukocyte infiltration is decreased in the sevoflurane + I/R group

Histopathological examination for left ventricular myocardial tissues of rats in each group was further observed. In the sham group, the structure of myocardial fiber of the

Fig. 2. Percentage of infarct size (%) after reperfusion for 120 min. Notes: *, $P < 0.05$ compared with the sham group; #, $P < 0.05$ compared with the I/R group; sham group (n = 15); I/R group (n = 15); Sevoflurane + I/R group (n = 15); I/R, ischemia and reperfusion.

Fig. 3. HE staining of myocardial tissue (400 ×) in each group. Notes: sham group (n = 15); I/R group (n = 15); Sevoflurane + I/R group (n = 15); I/R, ischemia and reperfusion.

Fig. 4. The positive expression of SP1 protein in myocardial tissues in each group (400 ×). Notes: A, pictures of immunohistochemical staining; B, positive rates of SP1 protein in each group, the arrows refer to SP1-positive stained cells; *, $P < 0.05$ compared with the sham group; #, $P < 0.05$ compared with the I/R group; sham group (n = 15); I/R group (n = 15); Sevoflurane + I/R group (n = 15); I/R, ischemia and reperfusion.

left ventricular myocardial tissue was normal, there was no congestion or edema in mesenchyme. In the I/R and sevoflurane + I/R groups, there was edema in the interstitial tissue of myocardial fiber, the connective tissue was loose, and a few leukocytes were infiltrated around the vessels. The myocardial tissue damage and leukocyte infiltration decreased in the sevoflurane + I/R group (Fig. 3).

Positive rate of SP1 protein in myocardial tissue of the sevoflurane + I/R group is evidently lowered

The SP1 protein positive rate in myocardium of rats in each group was determined by immunohistochemical staining. All cells were stained blue in the nuclei. SP1 protein was localized in the nucleus or cytoplasm and was brown when positively expressed. In the sham group, the positive rate of SP1 protein was $(13.31 \pm 1.52)\%$ ($P < 0.05$); a small amount of brown granules could be seen in the cardiomyocytes. In the I/R group, the positive rate of SP1 protein was $(32.35 \pm 3.25)\%$ ($P < 0.05$). In the sevoflurane + I/R group, the positive rate of SP1 protein was $(22.56 \pm 1.56)\%$ ($P < 0.05$), and more scattered brown granules could be seen. Compared with the sham group, in the I/R and sevoflurane + I/R groups, the positive rate of SP1 protein in myocardial tissue was significantly increased ($P < 0.05$). Compared with the I/R group, the positive rate of SP1 protein in myocardial tissue of sevoflurane + I/R group was significantly lowered ($P < 0.05$) (Fig. 4).

Apoptosis index is evidently decreased in the sevoflurane + I/R group

After DAPI staining, fluorescence microscopy showed that all cell nuclei were blue, and green fluorescence of TUNEL staining represented positive staining. Compared with the sham group ($5.03 \pm 0.52\%$), the apoptosis indexes of myocardial tissues of rats in the I/R ($28.63 \pm 2.91\%$) and sevoflurane + I/R ($16.11 \pm 1.75\%$) groups increased sharply ($P < 0.05$). Compared with the I/R group, the apoptosis index decreased evidently in the sevoflurane + I/R group ($P < 0.05$) (Fig. 5).

The sevoflurane + I/R group has increased expression of miR-374, PI3K, HO-1, Akt, and GSK-3 β and protein phosphorylation levels of p-Akt and p-GSK-3 β but decreased expression of SP1, p53, iNOS and c-fos

As revealed RT-qPCR and Western blot analysis, compared with the sham group, after reperfusion in the I/R and sevoflurane + I/R groups, the relative expression of miR-374 in

Fig. 5. Myocardial tissue apoptosis indexes of rats in each group. Notes: A, TUNEL fluorescence staining map; B, apoptotic indexes in each group; *, $P < 0.05$ compared with the sham group; #, $P < 0.05$ compared with the I/R group; sham group ($n = 15$); I/R group ($n = 15$); Sevoflurane + I/R group ($n = 15$); I/R, ischemia and reperfusion.

Fig. 6. MiR-374 expression and relative mRNA and protein expressions of related genes. Notes: A, miR-374 expression and mRNA expressions of related genes; B, protein band graph; C, protein expression of related genes; *, $P < 0.05$ compared with the sham group; #, $P < 0.05$ compared with the I/R group; sham group ($n = 15$); I/R group ($n = 15$); Sevoflurane + I/R group ($n = 15$); I/R, ischemia and reperfusion.

myocardium of rats was significantly lowered ($P < 0.05$). The relative mRNA and protein expression of SP1, PI3K, p53, iNOS and c-fos, the mRNA expression of Akt and GSK-3 β and the protein phosphorylation levels of p-Akt and p-GSK-3 β significantly increased ($P < 0.05$), while there was no significant change in the expression of Akt and GSK-3 β proteins ($P > 0.05$). Compared with the I/R group, in the sevoflurane + I/R group, the relative expression of miR-374 in myocardium of rats was evidently increased ($P < 0.05$), the mRNA and protein expression of SP1, p53, iNOS and c-fos was markedly reduced ($P < 0.05$) and the mRNA and protein expression of PI3K and HO-1, the mRNA expression of Akt and GSK-3 β and protein phosphorylation levels of p-Akt and p-GSK-3 β were remarkably increased ($P < 0.05$), while there was no significant change in the expression of Akt and GSK-3 β proteins ($P > 0.05$) (Fig. 6). In brief, the sevoflurane + I/R group has increased expression of miR-374, PI3K and HO-1, Akt, and GSK-3 β and protein phosphorylation levels of p-Akt and p-GSK-3 β , but decreased expression of SP1, p53, iNOS and c-fos.

SP1 is a target gene of miR-374

MicroRNA.org showed that miR-374 was able to target SP1, proving that SP1 was a direct target gene of miR-374. A dual luciferase reporter gene assay was used to confirm that SP1 was a target gene of miR-374. Compared with the NC group, the luciferase activity of the SP1 wild-type 3'-UTR was significantly inhibited by miR-374 ($P < 0.05$), while the luciferase activity of the mutant 3'-UTR was not inhibited (Fig. 7). These results indicated that miR-374 could specifically bind SP1-3'-UTR and downregulate the expression of the SP1 gene at the post-transcriptional level.

Fig. 7. Verification of the target relation between miR-374 and SP1. Notes: A, validation results of biological prediction site; B, results of luciferase activity; *, $P < 0.05$ compared with the NC group; I/R, ischemia and reperfusion; wt, wild type; mut, mutant.

miR-374 inhibits the expression of SP1 through activating the PI3K/Akt pathway

As suggested by the results of RT-qPCR and Western blot analysis, compared with the normal group, in the blank, NC, miR-374 mimics, miR-374 inhibitors, siRNA-SP1 and miR-374 inhibitors + siRNA-SP1 groups, the relative expression of miR-374 in cardiomyocytes significantly decreased ($P < 0.05$). The mRNA and protein expression of SP1, PI3K, HO-1, p53, iNOS and c-fos, the mRNA expression of Akt and GSK-3 β and the protein phosphorylation level of p-Akt and p-GSK-3 β evidently increased ($P < 0.05$), while there was no significant change in the expression of Akt and GSK-3 β ($P > 0.05$). Compared with the NC group, in the blank group, the relative expression of miR-374 in cardiomyocytes was not significantly different ($P > 0.05$). Compared with the blank and NC groups, the relative expression of miR-374 in cardiomyocytes of the miR-374 mimics group were notably increased ($P < 0.05$), and there was no significant difference in the relative expression of miR-374 in cardiomyocytes of the siRNA-SP1 group ($P > 0.05$). In the miR-374 mimics and siRNA-SP1 groups, the mRNA and protein expression of SP1, p53, iNOS and c-fos in cardiomyocytes decreased markedly ($P < 0.05$), while the mRNA and protein expression of PI3K and HO-1, mRNA expression of Akt and GSK-3 β and protein phosphorylation levels of p-Akt and p-GSK-3 β evidently increased ($P < 0.05$), with no obvious change of protein expression in Akt and GSK-3 β ($P > 0.05$). In the miR-374 inhibitors group, the relative expression of miR-374 in cardiomyocytes decreased significantly ($P < 0.05$), the mRNA and protein expression of SP1, p53, iNOS and c-fos decreased significantly ($P < 0.05$), and the mRNA and protein expression of PI3K and HO-1, mRNA expression of Akt and GSK-3 β and protein phosphorylation levels of p-Akt and p-GSK-3 β were evidently lowered ($P < 0.05$), with no evident change in the protein expression of Akt and GSK-3 β ($P > 0.05$). In the miR-374 inhibitors + siRNA-SP1 group, the relative expression of miR-374 in cardiomyocytes decreased significantly ($P < 0.05$), and the mRNA and protein expression of other related genes and the levels of protein phosphorylation of p-Akt and p-GSK-3 β did not change significantly ($P > 0.05$) (Fig. 8). These results demonstrated that miR-374 inhibits expression of SP1 through activating the PI3K/Akt pathway.

Upregulation of miR-374 and downregulation of SP1 promotes proliferation of cardiomyocytes after transfection

The MTT assay was used to detect cell proliferation. The proliferation of cardiomyocytes in each group accelerated at 24 h, 48 h and 72 h. The proliferation ability of cardiomyocytes in each group at 0 h was not significantly different but became significantly different at 24 ~ 72 h. Compared with the normal group, in the blank, NC, miR-374 mimics, miR-374 inhibitors, siRNA-SP1 and miR-374 inhibitors + siRNA-SP1 groups, the proliferation abilities of cardiomyocytes decreased ($P < 0.05$). There was no significant difference in cell proliferation ability between the blank group and the NC group and between the miR-374 mimics group and the siRNA-SP1 group ($P > 0.05$). Compared with the blank and NC groups,

Fig. 8. The relative expression of miR-374 and the mRNA and protein expression of related genes in cardiomyocytes after transfection. Notes: A, the relative expression of miR-374 and the mRNA expressions of related genes in cardiomyocytes after transfection; B, protein band diagram; C, the protein expressions of related genes; *, $P < 0.05$ compared with the normal group; #, $P < 0.05$ compared with the blank and NC groups; the experiment was repeated three times; I/R, ischemia and reperfusion.

Fig. 9. Proliferation of cardiomyocytes after transfection. Notes: *, $P < 0.05$ compared with the normal group; #, $P < 0.05$ compared with the blank and NC groups; the experiment was repeated three times; I/R, ischemia and reperfusion.

the proliferation rate of cardiomyocytes increased significantly in the miR-374 mimics and siRNA-SP1 groups ($P < 0.05$) and notably decreased in the miR-374 inhibitors group ($P < 0.05$), with no significant difference in the miR-374 inhibitors + siRNA-SP1 group ($P > 0.05$) (Fig. 9). These results suggested that upregulation of miR-374 and downregulation of SP1 promotes proliferation of cardiomyocytes after transfection.

Upregulation of miR-374 and downregulation of SP1 arrests more cells at G1 phase and inhibits apoptosis of cells after transfection

A single staining was used to detect the cell cycle distribution. In comparison with the normal group, in the blank, NC, miR-374 mimics, miR-374 inhibitors, siRNA-SP1 and miR-374 inhibitors + siRNA-SP1 groups, the percentage of G1 phase cells increased evidently ($P < 0.05$), and the percentage of S phase cells decreased significantly ($P < 0.05$). There was no significant difference in cell cycle distribution between the blank group and the NC group ($P > 0.05$). Compared with the blank and NC groups, in the miR-374 mimics and siRNA-SP1 groups, the percentage of G1 phase cells decreased significantly ($P < 0.05$) and the percentage of S phase cells increased remarkably ($P < 0.05$). In the miR-374 inhibitors group, the percentage of G1 phase cells increased evidently ($P < 0.05$) and the percentage of S phase cells decreased significantly ($P < 0.05$). There was no significant difference in cell cycle distribution in the miR-374 inhibitors + siRNA-SP1 group ($P > 0.05$). There was no significant difference in G2 phase ratio between any two groups ($P > 0.05$) (Fig. 10). It can be concluded that upregulation of miR-374 and downregulation of SP1 arrests more cells at G1 phase.

Fig. 10. The cycle distribution of cardiomyocytes in each group after transfection. Notes: A, flow cytometry; B, comparison of cell cycle distribution; *, $P < 0.05$ compared to the normal group; #, $P < 0.05$ compared to the blank and NC groups; the experiment was repeated three times; I/R, ischemia and reperfusion.

Fig. 11. Apoptosis rate of cardiomyocytes in each group after transfection. Notes: A, apoptotic scatter plot; B, comparisons of apoptosis rate in each group; *, $P < 0.05$ compared to the normal group; #, $P < 0.05$ compared to the blank and NC groups; the experiment was repeated three times; I/R, ischemia and reperfusion.

Annexin V-FITC/PI double staining was used to detect cell apoptosis. Compared with the normal group, the Blank, NC, miR-374 mimics, miR-374 inhibitors, siRNA-SP1 and miR-374 inhibitors + siRNA-SP1 groups, the apoptosis rate of cardiomyocytes increased markedly ($P < 0.05$) between the blank group and the NC group and between the miR-374 mimics group and the siRNA-SP1 group, there was no significant difference in myocardial apoptosis ($P > 0.05$). Compared with the blank and NC groups, in the miR-374 mimics and siRNA-SP1 groups, the apoptosis rate of cardiomyocytes decreased significantly ($P < 0.05$), and the apoptosis rate of cardiomyocytes in the miR-374 inhibitors group increased markedly ($P < 0.05$). There was no significant difference in cell apoptosis rate in the miR-374 inhibitors + siRNA-SP1 group ($P > 0.05$) (Fig. 11). These findings suggest that upregulation of miR-374 and downregulation of SP1 inhibits apoptosis of cells after transfection.

Discussion

As one of the leading causes of death, the deleterious effects of myocardial I/R have been studied over the past 40 years [17]. Therefore, this study was supposed to explore the effects of miR-374 in myocardial I/R rat models that were pretreated with sevoflurane by targeting transcription factor SP1 through the PI3K/Akt pathway. The results showed that miR-374 could be a therapeutic target for myocardial I/R by targeting transcription factor SP1 through the PI3K/Akt pathway in rats after treatment with sevoflurane.

Experimental results of a dual luciferase reporter gene assay showed that compared with the NC group, the luciferase activity of the SP1 wild-type 3'-UTR was significantly inhibited by miR-374 ($P > 0.05$), while the luciferase activity of mutant 3'-UTR was not inhibited ($P < 0.05$). These results indicated that miR-374 could specifically bind SP1-3'-UTR and downregulate the expression of the SP1 gene at the post-transcriptional level. That is to say, SP1 is a direct target gene of miR-374. A recent study has demonstrated that by interacting with p16^{INK4a} through the fourth ankyrin repeat, SP1 could combine with the promoters of miR-141 and miR-146b-5p and their transcriptional activation [18]. Recently, a study showed that within the Bcl-2 and SP1 mRNA 3'UTRs, miR-429 could combine with putative binding sites to lower their expression; through targeting Bcl-2 and SP1, up-regulation of miR-429 could depress invasion and advance apoptosis in esophageal carcinoma cells, indicating that Bcl-2 and SP1 could be major targets of miR-429 [19].

Compared with the normal group, in the blank, NC, miR-374 mimics, miR-374 inhibitors, siRNA-SP1 and miR-374 inhibitors + siRNA-SP1 groups, the relative expression of miR-374 in cardiomyocytes decreased significantly ($P < 0.05$). SP1, which is a major transcription factor in cellular processes, could recruit and interact with some proteins, remodel chromatin and regulate gene expression [20]. Consistent with the results in this study, Li et al. found that SP1 increased miR-182 expression, and knockdown of miR-182 inhibited lung cancer cell growth but improved the invasive and migratory abilities of cells, enhancing N-cadherin expression [21].

Compared with the normal group, in the blank, NC, miR-374 mimics, miR-374 inhibitors, siRNA-SP1 and miR-374 inhibitors + siRNA-SP1 groups, the mRNA and protein expression of SP1, PI3K, HO-1, p53, iNOS and c-fos, the mRNA expression of Akt and GSK-3 β , and the protein phosphorylation levels of p-Akt and p-GSK-3 β were evidently increased ($P < 0.05$). The results indicated that miR-374 activated the PI3K/Akt pathway. In a recent study, through directly binding the 3' untranslated region of the tumor-suppressor genes *PTEN* and *CDKN1A*, miR-93 inhibited their expression and activated the c-Met/PI3K/Akt pathway by regulating c-Met protein expression [22]. Furthermore, Chen et al. found that after overexpression of miR-374a, PI3K/Akt signaling pathway and its downstream cell cycle factors, including c-myc, CDK2, CDK1 and EMT-related genes such as ZEB1, Snail, Vimentin and N-cadherin, were downregulated [23].

MiR-374 may alleviate myocardial I/R damage in rat models pretreated with sevoflurane by targeting transcription factor SP1 through activating the PI3K/Akt pathway. With mammalian target of rapamycin (mTOR), one of its downstream targets, the PI3K/Akt pathway plays a protective role in myocardial I/R injury [23]. As small (22 nt), noncoding, single-stranded RNA molecules that can downregulate gene expressions, miRNAs are considered to regulate a great number of heart diseases, such as heart failure, hypertrophy, remodeling and arrhythmia, and to significantly alleviate I/R injury through altering key signaling elements [11]. In a study conducted by Li Song et al., up-regulation of the expression of miR-320 in the rat model might exacerbate the risk of left ventricular remodeling after myocardial I/R injury, while decreased expression of miR-320 might reduce the risk and even improved cardiac function [24]. We found that compared with the I/R group, in the SR group, the content of SOD and IL-10 increased, while the content of MDA, LDH, CK, TNF- α and IL-6 decreased, as did the percentage of infarct area and the apoptosis index. Increased SOD levels and decreased levels of LDH, CK, MDA, TNF- α , and IL-6 and infarct size induced by I/R indicated alleviation of myocardial I/R injury [25]. Consistent with the results of this study, Tian et al. proved that compared with the I/R group, the levels of CK, LDH, MDA, IL-6, and TNF- α , as well as the infarct size and cell apoptosis, were decreased in the hearts of the rats in the Echinatin-treated group, while the SOD activity increased, suggesting improvement of I/R injury [26]. Additionally, it was observed that compared with the blank and NC groups, in the miR-374 mimics and siRNA-SP1 groups, the cell proliferation ability was enhanced, while the cell apoptosis rate decreased. There were no obvious changes in cell proliferation, cycle distribution and apoptosis rate in the miR-374 inhibitors + siRNA-SP1 group. Enhanced cardiac c-kit⁺ cell survival and proliferation and decreased apoptotic activity were positive

indicators of myocardial infarction [27]. Chen et al. witnessed an evident increase in cell proliferation and TNF-induced apoptosis in neonatal rat cardiac myocytes, suggesting a prevention of M6P/IGF2R against cell death or promotion to mitogenesis for certain heart diseases [28].

Conclusion

Our study provided evidence that miR-374 could alleviate myocardial I/R damage by targeting SP1 through activating the PI3K/Akt pathway. Nevertheless, we suggested more prospective studies with larger sample size should be conducted to provide more precise estimates to confirm the effects of miR-374 on myocardial I/R, to be closer to the goal of developing new genetic therapeutic strategies for the treatment of ischemic heart disease.

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Disclosure Statement

No conflict of interests exist.

References

- 1 Yang L, Xie P, Wu J, Yu J, Yu T, Wang J, Xia Z, Zheng H: Sevoflurane postconditioning improves myocardial mitochondrial function and reduces myocardial ischemia-reperfusion injury by up-regulating HIF-1. *Am J Transl Res* 2016;8:4415-4424.
- 2 Zhang Y, Ren J: Targeting autophagy for the therapeutic application of histone deacetylase inhibitors in ischemia/reperfusion heart injury. *Circulation* 2014;129:1088-1091.
- 3 Yao YT, Fang M, Li LH: Sevoflurane postconditioning protects isolated rat hearts against ischemia-reperfusion injury. *Perfusion* 2010;25:1320-1328.
- 4 Gong JS, Yao YT, Li LH: Sevoflurane postconditioning attenuates reperfusion-induced ventricular arrhythmias in isolated rat hearts exposed to ischemia/reperfusion injury. *Mol Biol Rep* 2012;39:6417-6423.
- 5 Mao J, Zhang Y, Jiao L, Lau WB, Wang L, Liu B, Gao E, Koch WJ, Ma XL, Wang Y: Sevoflurane postconditioning attenuates myocardial ischemia/reperfusion injury via caveolin-3-dependent PI3K/Akt and p38/JNK2 inhibition. *Circulation* 2013;128:S121-129.
- 6 Wang L, Song G, Liu M, Chen B, Chen Y, Shen Y, Zhu J, Zhou X: MicroRNA-375 overexpression influences C19 cell proliferation, apoptosis and differentiation through the Notch signaling pathway. *Int J Mol Med* 2016;37:47-55.
- 7 Wang Y, Xin H, Han Z, Sun H, Gao N, Yu H: MicroRNA-374a promotes esophageal cancer cell proliferation via Axin2 suppression. *Oncol Rep* 2015;34:1988-1994.
- 8 Gong H, Cao Y, Han G, Zhang Y, You Q, Wang Y, Pan Y: p53/microRNA-374b/AKT1 regulates colorectal cancer cell apoptosis in response to DNA damage. *Int J Oncol* 2017;50:1785-1791.
- 9 Yang J, Chen L, Yang J, Ding J, Li S, Wu H, Zhang J, Fan Z, Dong W, Li X: MicroRNA-22 targeting CBP protects against myocardial ischemia-reperfusion injury through anti-apoptosis in rats. *Mol Biol Rep* 2014;41:555-561.
- 10 Liu LF, Liang Z, Lv ZR, Liu XH, Bai J, Chen J, Chen C, Wang Y: MicroRNA-15a/b are up-regulated in response to myocardial ischemia/reperfusion injury. *J Geriatr Cardiol* 2012;9:28-32.

- 11 Ye Y, Perez-Polo JR, Qian J, Birnbaum Y: The role of microRNA in modulating myocardial ischemia-reperfusion injury. *Physiol Genomics* 2011;43:534-542.
- 12 Chen Y, Jiang J, Zhao M, Luo X, Liang Z, Zhen Y, Fu Q, Deng X, Lin X, Li L, Luo R, Liu Z, Fang W: microRNA-374a suppresses colon cancer progression by directly reducing CCND1 to inactivate the PI3K/AKT pathway. *Oncotarget* 2016;7:41306-41319.
- 13 Zhao MM, Yang JY, Wang XB, Tang CS, Du JB, Jin HF: The PI3K/Akt pathway mediates the protection of SO₂ preconditioning against myocardial ischemia/reperfusion injury in rats. *Acta Pharmacol Sin* 2013;34:501-506.
- 14 Kumar A, Redondo-Munoz J, Perez-Garcia V, Cortes I, Chagoyen M, Carrera AC: Nuclear but not cytosolic phosphoinositide 3-kinase beta has an essential function in cell survival. *Mol Cell Biol* 2010;30:2122-2131.
- 15 Cai Q, Wang ZQ, Wang SH, Li C, Zhu ZG, Quan ZW, Zhang WJ: Upregulation of long non-coding RNA LINC00152 by SP1 contributes to gallbladder cancer cell growth and tumor metastasis via PI3K/AKT pathway. *Am J Transl Res* 2016;8:4068-4081.
- 16 Huang J, Li Y, Zhang J, Liu Y, Lu Q: The Growth Hormone Secretagogue Hexarelin Protects Cardiomyocytes From *in vivo* Ischemia/Reperfusion Injury Through Interleukin-1 Signaling Pathway. *Int Heart J* 2017;58:257-263.
- 17 Benhabbouche S, Crola da Silva C, Abrial M, Ferrera R: [The basis of ischemia-reperfusion and myocardial protection]. *Ann Fr Anesth Reanim* 2011;30 Suppl 1:S2-16.
- 18 Al-Khalaf HH, Mohideen P, Nallar SC, Kalvakolanu DV, Aboussekhra A: The cyclin-dependent kinase inhibitor p16INK4a physically interacts with transcription factor Sp1. Cyclin-dependent kinase 4 to transactivate microRNA-141 and microRNA-146b-5' and simultaneously attenuates response to ultraviolet light-induced DNA damage. *J Biol Chem* 2013;288:35511-35521.
- 19 Wang Y, Li M, Zang W, Ma Y, Wang N, Li P, Wang T, Zhao Y: miR-429 up-regulation induces apoptosis and suppresses invasion by targeting Bcl-2 and SP-1 in nasopharyngeal carcinoma. *Cell Oncol (Dordr)* 2013;36:385-394.
- 20 Li L, Davie JR: The role of Sp1 and Sp3 in normal and cancer cell biology. *Ann Anat* 2010;192:275-283.
- 21 Yang WB, Chen PH, Hsu Ts, Fu TF, Su WC, Hsu WC, Wang WC, Hung JJ: Sp1-mediated microRNA-182 expression regulates lung cancer progression. *Oncotarget* 2014;5:740-753.
- 22 Ohta K, Hoshino H, Wang J, Ohtsuka Y, Kishimoto SK, Colquhoun S, Hoon DS: MicroRNA-93 activates c-Met/PI3K/Akt pathway activity in hepatocellular carcinoma by directly inhibiting PTEN and CDKN1A. *Oncotarget* 2015;6:3211-3224.
- 23 Zhang J, Wang C, Yu S, Liu Y, Chen Y, Liang H, Hua F, Xu G, Yu P: Sevoflurane postconditioning protects rat hearts against ischemia/reperfusion injury via the activation of PI3K/AKT/mTOR signaling. *Sci Rep* 2014;4:7317.
- 24 Song CL, Liu B, Li Y, Shi H, Li YX, Zhang JC, Lu Y, Wang G, Liu J, Yu YP, Guo ZY, Wang JP, Zhao Z, Liu JG, Liu YH, Liu YC, et al: The protective effect of microRNA-320 on left ventricular remodeling after myocardial ischemia-reperfusion injury in the rat model. *Int J Mol Sci* 2014;15:17442-17456.
- 25 Yi C, Zhang C, Huang C, Jiang H, Xu W, Lu J, Liao Y, Ma R, Li X, Wang J: Vagus nerve stimulation attenuates myocardial ischemia/reperfusion injury by inhibiting the expression of interleukin-17A. *Exp Ther Med* 2016;11:1159-1176.
- 26 Chen XH, Li Y, Li CL, Jiang HL, Zhang Y, Han JC, Liu J, Chen M: Cardioprotection provided by Echinatin against ischemia/reperfusion in isolated rat hearts. *BMC Cardiovasc Disord* 2016;16:119.
- 27 Yang J, Zhou Y, Liu B, Wang H, Du X: Trop2 plays a cardioprotective role by promoting cardiac c-kit+ cell proliferation and inhibition of apoptosis in the acute phase of myocardial infarction. *Int J Mol Med* 2013;31:1298-1304.
- 28 Chen Z, Ge Y, Kang JX: Down-regulation of the M6P/IGF-II receptor increases cell proliferation and reduces apoptosis in neonatal rat cardiac myocytes. *BMC Cell Biol* 2004;5:15.