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MiR-208a participates with sevoflurane post-conditioning in protecting neonatal rat cardiomyocytes with simulated ischemia-reperfusion injury *via* PI3K/AKT signaling pathway

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Abstract. – OBJECTIVE: We aimed to evaluate the role of miR-208a in sevoflurane post-conditioning protecting neonatal rat cardiomyocytes with simulated ischemia reperfusion injury (SI/RI) *via* PI3K/AKT signaling pathway.

MATERIALS AND METHODS: The cardiomyocytes of healthy neonatal rats were extracted as the Normal group. Other cardiomyocytes were used to establish the SI/RI model. Sevoflurane post-conditioning, miR-208a inhibitor, or PI3K/AKT pathway activator were used in the treatment of cardiomyocytes. The cell viability, cell cycle, apoptosis, levels of superoxide dismutase (SOD), malondialdehyde (MDA), miR-208a and mRNA, expression of PI3K, AKT, and autophagy-related factors in each group were measured and compared. Monodansylcadaverine (MDC) was used to measure the fluorescence intensity of autophagosomes.

RESULTS: In neonatal rat cardiomyocytes with SI/RI, the expression of miR-208a and MDA, apoptosis and the expression of autophagy-related factors increased, with PI3K/AKT pathway inhibited, SOD level decreased, cell viability reduced, and the fluorescence intensity of autophagosomes enhanced (all $p<0.05$). Sevoflurane post-conditioning can promote the increase of SOD and the decrease of MDA in cardiomyocytes with SI/RI, with PI3K/AKT pathway activated, viability of cardiomyocytes enhanced, apoptosis reduced, the expression of autophagy-related factors and the fluorescence intensity of autophagosomes inhibited (all $p<0.05$). Sevoflurane post-conditioning combined with miR-208a inhibitor or PI3K/AKT pathway activator further promoted the above-mentioned effects (all $p<0.05$).

CONCLUSIONS: Our results indicate that the inhibited expression of miR-208a suppresses the expression of autophagy-related factors, enhances cell viability of cardiomyocytes, and reduces apoptosis, thereby protecting neonatal rat cardiomyocytes with SI/RI.

Key Words:
MiR-208a, Sevoflurane post-conditioning, Ischemia reperfusion injury.

Introduction

Neonatal asphyxia is an important cause of disability and even death in neonates. Asphyxia-induced ischemia and hypoxia often result in heart, liver, kidney, and brain injuries, and reoxygenation after hypoxia can cause ischemia-reperfusion injury[1]. Organ systems are immature in neonates as compared with adults. Ischemia reperfusion injury in the cardiac myocytes of neonates is prone to cause decreased systolic function and impaired vascular reactivity, and may even lead to heart failure, myocardial infarction, and death[2]. The simulated ischemia-reperfusion injury (SI/RI) model is an *in vitro* model of ischemia reperfusion injury in the cardiac myocytes, which enables not only the observation of effect of active substances on the morphology, metabolism, and physiological function of cardiac myocytes, but also the exploration of the mechanism of action of drugs[3].

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Sevoflurane is an inhalational anesthetic commonly used in the clinic setting. It is widely used in general anesthesia because of its low blood-gas partition coefficient, rapid induction, and little impact on circulation and respiration. A growing number of studies showed that sevoflurane can protect cardiac myocytes from ischemia-reperfusion injury by inhibiting their apoptosis and improving myocardial mitochondrial respiratory function[4,5]. Sevoflurane post-conditioning refers to inhalation of sevoflurane for anesthesia during reperfusion/myocardial reoxygenation. A previous study[6] showed that sevoflurane post-conditioning significantly alleviated reperfusion injury in rats with myocardial infarction, promoted the reduction of myocardial infarct volume, and played a protective role in myocardial ischemia-reperfusion injury (MIRI), but that protective effect against MIRI disappeared after the administration of phosphoinositide 3-kinase inhibitor (PI3K inhibitor). The results suggested that the PI3K signaling pathway mediates the repair of MIRI after sevoflurane post-treatment, but it remains unclear how sevoflurane activates the PI3K signaling pathway.

MicroRNAs (miRNAs) can affect myocardial injury after sevoflurane post-treatment by regulating the related pathways[7,8]. As a cardiac-specific miRNA, miR-208a has been confirmed to be closely associated with myocardial pathological changes[9]. Wang et al[10] found that miR-208a aggravates cardiac hypertrophy and myocardial fibrosis by inhibiting the expression of fibroblast growth factors and thyroid hormone receptor associated proteins. Montgomery et al[11] also confirmed that the inhibition of miR-208a expression suppresses the progression of heart failure. Besides, miR-208a is found to be associated with the progression of MIRI[12]. However, it remains unclear whether miR-208a will have an impact on the process of sevoflurane post-conditioning activating the PI3K pathway after hypoxia for myocardial protection. The aim of this study was to investigate the role of miR-208a on the effect of sevoflurane post-conditioning against SI/RI on the cardiomyocytes of neonatal rat, and its potential regulatory mechanisms.

Materials and Methods

Ethics Statement

All animal researches in this study were approved by the Animal Protection Committee of Children's Hospital of Fudan University. All surgical and experimental procedures were consistent with the Guide for the Care and Use of Laboratory Animals by National Institutes of Health. Moreover, we applied measures to minimize the number of animals used in this study and the pain of these animals during the research.

Primary Cardiomyocyte Culture and Establishment of SI/RI Model

We purchased 15 neonatal Sprague-Dawley (SD) rats (aged 1-2 days) from the Shanghai Lab. Animal Research Center (Pudong New Area, Shanghai City, China). Five of them were assigned to the Normal group with their cardiomyocytes extracted. The rest of the SD rats were used to establish the SI/RI model using cardiomyocytes. The rat was first disinfected with 75% ethanol and euthanized by decapitation. The chest cavity was opened before the pericardium was stripped off. The ventricular myocardium was extracted and then placed in serum-free Dulbecco's Modified Eagle's Medium (DMEM) before it was cut into 1 mm^3 pieces with ophthalmic scissors. The cells were dissociated 3 times for 3 min each, with 0.125% trypsin about 10 times the volume of cells. After dissociation, the cells were placed in DMEM medium containing 10% fetal bovine serum and centrifuged at 1,500 r/min for 3 min before the supernatant was discarded. The cells were resuspended in DMEM containing 10% fetal bovine serum. The medium was then placed in an incubator for 2 h for cell attachment. Following incubation, the purification of cardiomyocytes was performed. Unattached cells were filtered through a 200-mesh screen to remove undissociated pieces of tissue. The cell suspension was collected and counted, seeded in a 96-well plate at a density of $5x10^5$ cells/well, and adjusted to the concentration of 0.1 mmol/L with 5-Bromo-2'-deoxyuridine, the thymidine analogue. The cells were maintained in a 37°C incubator with 5% CO_2 and a relative humidity of 90%. The medium was changed once every 24 hours, and the cells were cultured for 3 consecutive days before experimental use. The SI/RI model was established according to the reference: first, the cells were placed in nitrogen-saturated hypoxic solution (NaCl: 137 mmol/L; KCl: 12 mmol/L; $MgCl_2$: 0.49 mmol/L; $CaCl_2{\cdot}2H_2O$: 0.9 mmol/L; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid: 4 mmol/L; sodium lactate: 20 mmol/L; deoxyglucose: 10 mmol/L; sodium sulfite: 0.75 mmol/L; adjusted to pH 6.5) for 2 h[13]. The

96-well plate was then washed twice with phosphate-buffered saline and cultured for 4 h in pure oxygen-saturated solution (containing 95% O_2 and 5% CO_2) to induce reoxygenation injury.

Establishment of Sevoflurane Post-Conditioning Model and Cell Grouping

The cardiomyocytes in this study were divided into 6 groups. In the Normal group, the normal cardiomyocytes were used. In the SI/RI group, cardiomyocytes were subjected to hypoxia for 2 h and then reoxygenation for 1 h. In the SI/RI+SP group, cardiomyocytes were subjected to hypoxia for 2 h, and received 20 min of 3% sevoflurane along with 40 min of reoxygenation. In miR-208a inhibitor+SI/RI+SP group, the miR-208a inhibitor was transfected into the cardiomyocytes 24 h before the establishment of the SI/RI model, followed by the same procedures as those in the SI/RI+SP group. In SI/RI+SP+IGF-1 group, cardiomyocytes were incubated 20 min with insulin-like growth factor 1 (IGF-1) dissolved in 0.1% DMSO (to ensure a concentration of 5 μmol/L) at the same time of sevoflurane post-conditioning, followed by reoxygenation, and the rest of the procedures were the same as those in the SI/RI+SP group. In miR-208a inhibitor+SI/RI+SP+IGF-1 group, cardiomyocytes were transfected with miR-208a inhibitor 24 h before the establishment of the SI/RI model, and subjected to 2 h of hypoxia and the same sevoflurane post-conditioning and incubation with IGF-1 at the time of the 1 h reoxygenation.

Cell transfection was performed according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The empty plasmids and 100 pmol miR-208a inhibitor (GenePharma, Pudong New Area, Shanghai City, China) were diluted with 250 μL serum-free media Opti-MEM (Gibco, Grand Island, NY, USA) (the final concentration of cells: 50 nM), and gently mixed before an incubation of 5 min at room temperature. Then, 5 μL of Lipofectamine 2000 was diluted and mixed with serum-free medium Opti-MEM and incubated for 5 min at room temperature. The diluted miR-208a inhibitor was evenly mixed with the diluted Lipofectamine 2000 and kept at room temperature for 20 min, followed by incubation at 37°C for 6 h in an incubator containing 5% CO_2. After incubation, the medium was changed to a complete medium with 10% fetal bovine serum and the mixture was cultured for 48 h before subsequent procedures.

Implementation of sevoflurane post-conditioning: DMEM was exposed to a mixed gas flow of 97.5% O_2 and 2.5% sevoflurane (Maruishi Pharmaceutical Co., Ltd., Chome Fushimimachi, Chuoku, Osaka, Japan) to prepare a sevoflurane-saturated DMEM. The sevoflurane-saturated DMEM was then sampled at 37°C to measure the concentration of sevoflurane using the gas chromatograph (HP5890, Hewlett-Packard, CA, USA), ensuring a sevoflurane concentration of 0.46 to 0.54 mmol/L. The cells were incubated in sevoflurane-saturated DMEM for 20 min during reoxygenation and exposed to the mixed gas flowed in from the gas inlet port and out from the gas outlet port. The gas outlet port was monitored using an anesthetic gas monitor (Vamos, Draeger, Lubeck, Germany) to ensure a gas flow rate of 5 L/min. After grouping, each group of cells was placed under a light microscope (BX53, Olympus, Ishikawamachi, Hachioji, Tokyo, Japan) to observe the cell morphology.

Measurement of MDA and SOD Levels in Cell Culture Media

After reoxygenation, cell culture medium of each group was collected to centrifuge at 2,500 r/min for 5 min. The supernatant was discarded, and the cells were resuspended in double distilled water and vortexed for 1 min to ensure the complete cell lysis. Malondialdehyde (MDA) assay kit (A003-1, Jiancheng Bio-Engineering Institute, Xuanwu District, Nanjing City, China) and superoxide dismutase (SOD) assay kit (A001-3, Jiancheng Bio-Engineering Institute, Xuanwu District, Nanjing City, China) were used to determine the levels of MDA and SOD in each cell culture medium. The procedure was carried out in strict accordance with the kit instructions.

Cell Proliferation by MTT Assay

After reoxygenation, the cells were collected and counted before they were inoculated into a 96-well plate at $3x10^3$-$5x10^3$ cells per well. One hundred microliter of DMEM medium and 10 μL of 0.5% MTT solution (SenBeiJia Biological Technology Co., Ltd., Nanjing City, China) were added into each well. The cells were then incubated for 4 h in a 37°C incubator containing 5% CO_2. After incubation, the supernatant was discarded and 100 μL of dimethyl sulfoxide (DMSO) stock solution was added into each well, followed by 10 min of shaking to completely dissolve the crystals. Optical density was then measured by the microplate reader (Multiskan MK3, Thermo

Fisher Scientific, Waltham, MA, USA) at a wavelength of 490 nm. The cell viability curve was plotted with the time point as abscissa and the OD value as ordinate.

Cell Cycle and Apoptosis by Annexin V-FITC/PI Staining

Cell suspension of each group was collected at a concentration of $1x10^6$ cells/mL. One milliliter of cells was centrifuged at 2,500 r/min for 10 min, and the supernatant was discarded. Two milliliters of phosphate-buffered saline (PBS) were added to per milliliter of cell suspension before centrifugation. The supernatant was then discarded, and the cells were fixed by adding pre-cooled 70% ethanol and incubated at 4°C overnight. By the next day, the cells were washed twice with PBS, and 100 μL of the cell suspension was separated and added with 50 μg of propidium iodide (PI) containing RNAase. The cell suspension was kept at dark environment for 30 min before filtering through a 100-mesh nylon mesh net. The cell cycle was detected by flow cytometry for red fluorescence at an excitation wavelength of 488 nm.

Annexin V-FITC/PI staining was used to detect apoptosis. The cell suspension was collected to incubation in a 37°C incubator with 5% CO_2 for 48 h. After incubation, the cells were rinsed with PBS, added with 5 mL of 70% ethanol, and incubated at 4°C for 24 h. After that, the cells were centrifuged at 10,000 r/min for 5 min and the cell pellet was dispersed with 1 mL PBS. Five microliter of RNAase (10 $mg \cdot mL^{-1}$) was added into the cells and the mixture was kept at room temperature for 1 h. Five microliter of annexin V-FITC (M3012, Shanghai Majorbio, New Area, Shanghai City, China) and 2.5 μL of PI were added into the 100 μL cell and evenly mixed before incubating for 15 min in the dark. Cell apoptosis was detected by the flow cytometer (CytoFLEX®, Beckman, CA, USA) at an excitation wavelength of 490 nm. The percentage of apoptotic cells was calculated by data acquisition and analysis using CellQuest 3.0 analysis software.

Quantitative Real Time-PCR

The total RNA was extracted using RNA extraction kit (D203-01, Beijing GenStar Biosolutions Co., Ltd., Changping District, Beijing, China). Primers for miR-208a, PI3K, AKT, p62, Beclin1, U6, and GAPDH in this study were synthesized by TaKaRa Bio (Dalian, China) (Table I). Reverse transcription was performed using EasyScript First-Strand cDNA Synthesis SuperMix kit (AE301-02, Beijing TransGen Biotech, Haidian District, Beijing, China). For reverse transcription, a 20 μL reaction was used, which contained 5 μL of total RNA, 5 μL of Mix reagent, 9 μL of RNase-Free Water, and 1 μL of Random Hexamer Primers. The reaction was incubated at 37°C for 10 min, then at 85°C for 5 s, and terminated at 4°C. The cDNA was kept at 20°C. After 24 h, the cDNA was used to perform quantitative Real Time-PCR (qRT-PCR) using SYBR® Premix Ex Taq™ II kit (RR820A, TaKaRa Biotechnology Co., Ltd., Jinzhou District, Dalian, China). The procedure was carried out according to the kit instructions. For qRT-PCR, a 20 μL reaction was used, which contained 10 μL of SYBR Premix, 6 μL of sterile water, 2 μL of cDNA template, 0.4 μL of ROX reference Dye, and 0.8 μL of upstream and downstream primers, respectively.

Table I. Primer sequence of qRT-PCR.

Name	Sequences
miR-208a	F: 5'-CGCGGCATAAGACGAGCAAAAAGC-3' R: 5'-ACGACAGTTCAACGGCAGCACCG-3'
PI3K	F: 5'-TGCAGTTCAACAGCCACACACTAC-3' R: 5'-TAAACAGGTCAATGGCTGCATCA-3'
AKT	F: 5'-GCTGAGTACGAGAACTGGGGAAA-3' F: 5'-TCACACCCACACCAGGTATTTG-3'
p62	F: 5'-GCCCTGTACCCACATCTCC-3' R: 5'-CCATGGACAACATCTGAGAG-3'
Beclin1	F: 5'-AGGAGCAGTGCACAAAGG-3' R: 5'-AGGGAAGAGGGAAAGGAC-3'
GAPDH	F: 5'-CCCCCATTGTATCCGTTGTG-3' R: 5'-TAGCCCAGGATGCCCTTTAGT-3'
U6	F: 5'-ATCGCCTTCGGCAGCACA-3' R: 5'-CACGCTGCACGAATTCGCGT-3'

qRT-PCR, quantitative real-time PCR.

The reaction was amplified for 45 cycles, each cycle consisting of initial denaturation of 30 s at 95°C, denaturation of 30 s at 95°C, annealing of 30 s, extension of 30 s at 72°C. U6 was used as the internal control of miR-208a. GAPDH was used as the internal control of PI3K, AKT, p62, and Beclin 1. ABI 7500 Real-Time PC System (7500, Applied Biosystems, Waltham, MA, USA) was used to conduct qRT-PCR. The relative expression of a target gene was calculated using the $2^{-\Delta\Delta CT}$ method. $\Delta CT = CT_{\text{target gene}} - CT_{\text{reference gene}}$; $\Delta\Delta CT = \Delta CT_{\text{the experimental group}} - \Delta CT_{\text{the control group}}$.

Western Blot

The total protein of the cells was extracted using RIPA reagent kit (R0010, Solarbio Science & Technology Co., Ltd., Tongzhou District, Beijing, China). The cells were washed 3 times with PBS, and lysed with an appropriate amount of lysis buffer (60% RIPA buffer + 39% SDS + 1% protease inhibitor) on ice for 30 min. After lysis, the lysates were centrifuged at 3,000 r/min for 30 min at 4°C. The supernatant was used to determine the levels of the proteins by BCA Protein Assay Kit (Jining Shiye Co., Ltd., Jinshan District, Shanghai, China). Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) Gel Preparation Kit (89888, Thermo Fisher Scientific, Waltham, MA, USA) was used to prepare 10% separation gel and 5% stacking gel. After gel electrophoresis, the proteins were transferred onto the polyethylene difluoride (PVDF) membranes by wet transfer, and the membranes were blocked with 5% bovine serum albumin (BSA) at room temperature for 1 h. After the blocking process, the membranes were incubated at 4°C overnight with primary antibodies of PI3K (1:600, ab86714, Abcam, Cambridge, UK), AKT (1:600, ab8805, Abcam, Cambridge, UK), p-PI3K (1:1,000, ab182651, Abcam, Cambridge, UK), p-AKT (1:1,000, ab38449, Abcam, Cambridge, UK), p62 (1 μg/mL, ab91526, Abcam, Cambridge, UK), Beclin 1 (1 μg/mL, ab62557, Abcam, Cambridge, UK), and GAPDH (1 μg/mL, ab37168, Abcam, Cambridge, UK). After incubation, the membranes were washed with PBS for 5 times, with 5 min each, and incubated on a shaker for 1 h with secondary goat anti-rabbit polyclonal antibody that had been diluted with 5% skim milk (1:5,000, ab205718, Abcam, Cambridge, UK). The membranes were then rinsed with PBS for 3 times, 10 min each. The membranes were immersed in the enhanced chemiluminescence (ECL) substrate solution (WBKLS0500, Pierce, CA, USA) and were processed for imaging using Bio-Rad gel imaging system (MG8600, ThMorgan, Changping District, Beijing, China). The fluorescent bands were used to analyze absorbance values using E-Gel Imager gel documentation system (Thermo Fisher Scientific, Waltham, MA, USA). The mean absorbance values of the samples were divided by the mean absorbance values of the corresponding internal reference, and the results were the relative levels of proteins in samples. The experiment was repeated 3 times and the mean values were calculated.

Fluorescence Intensity of Autophagosomes by Monodansylcadaverine (MDC) Staining

Each group of cell suspension was inoculated into a 24-well plate with 3×10^4 cells/well. After cell adhesion of 24 h, the cells were incubated in an incubator at 37° C and 5% CO_2 for 30 min. After washing with PBS, 50 μL of cell suspension was aspirated to mix with 10 μL of MDC solution (G0170-100T, Beijing Solebo Technology Co., Ltd., Beijing, China). After 30 min of staining, the cell suspension was centrifuged at 2000 r/min for 5 min. After collection, the cells were washed with PBS, placed on a glass slide, and fixed with 50% glycerophosphoric acid. The slide was observed and filmed with 355 nm emission filter and 512 nm blocking filter under the fluorescence microscope using a 355 nm emission filter and a 512 bandpass filter (XSP-63B, Shanghai Optical Instrument Factory, Shanghai, China).

Statistical Analysis

All data were processed using Statistical Product and Service Solution (SPSS) 21.0 software package (IBM Corp., Armonk, NY, USA). The measurement data were expressed as mean ± standard deviation ($\bar{x}$ ± SD). The comparison between the two groups was based on the *t*-test. The comparison among multiple groups was based on One-way analysis of variance (One-way ANOVA). The Kolmogorov-Smirnov test was used to test the normality assumption. For normally distributed data, the comparison among multiple groups was based on Tukey's HSD test as the post-hoc test of One-way ANOVA. For non-normally distributed data, the comparison among multiple groups was based on Dunn's multiple comparison test as the post-hoc test of Kruskal-Wallis test. $p<0.05$ was considered statistically significant.

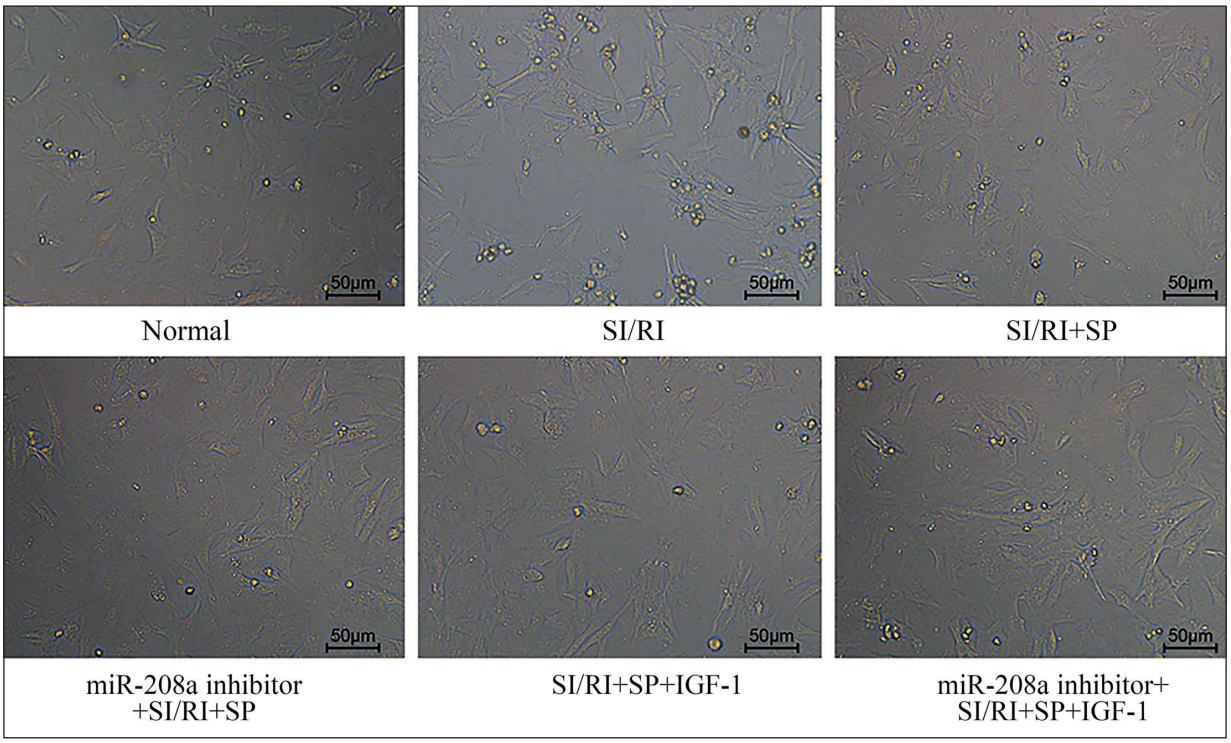

Figure 1. Morphology of cardiomyocytes under inverted microscope (×200). SI, simulated ischemia; RI, reperfusion injury; IGF-1, insulin-like growth factor 1.

Results

Morphology of Cardiomyocytes

The morphology of cardiomyocytes in each group was observed. The cardiomyocytes in the Normal group were in fusiform shape. After modeling of SI/RI, the cardiomyocytes were in round shape. Sevoflurane post-conditioning, transfection of miR-208a inhibitor, and incubation with IGF-1 could significantly improve the morphology of cardiomyocytes after SI/RI and, when combined together, they would improve the morphology of cardiomyocytes to a greater degree (Figure 1).

SOD and MDA Levels of Cardiomyocytes

Compared with the Normal group, SOD levels decreased but MDA levels increased in the cardiomyocytes of the other five groups (all $p<0.05$). Compared with the SI/RI group, SI/RI+SP group, miR-208a inhibitor+SI/RI+SP group, and SI/RI+SP+IGF-1 group showed increased SOD levels but decreased MDA levels in cardiomyocytes after SI/RI. These changes were greater in miR-208a inhibitor+SI/RI+SP+IGF-1 group (all $p<0.05$) (Table II).

Cell Viability of Cardiomyocytes

Compared with the Normal group, the cell viability of cardiomyocytes of the other five

Table II. Measurement of MDA and SOD levels in each group.

Group	MDA (nmol/mL)	SOD (U/mL)
Normal group	1.44 ± 0.59	224.05 ± 22.16
SI/RI group	12.74 ± 1.48*	106.84 ± 8.73*
SI/RI+SP group	10.31 ± 1.15*#	135.46 ± 12.77*#
miR-208a inhibitor+SI/RI+SP group	7.65 ± 0.98*#$	168.53 ± 14.21*#$
SI/RI+SP+IGF-1 group	7.24 ± 1.12*#$	166.22 ± 16.35*#$
miR-208a inhibitor+SI/RI+SP+IGF-1 group	4.63 ± 0.47*#$^	195.23 ± 19.82*#$^

SOD, Superoxide dismutase; MDA, malondialdehyde; SI, simulated ischemia; RI, reperfusion injury; IGF-1, insulin-like growth factor 1. Compared with the Normal group, *$p < 0.05$; compared with SI/RI group, #$p < 0.05$; compared with SI/RI+SP group, $$p < 0.05$; for comparison between miR-208a inhibitor+SI/RI+SP group and SI/RI+SP+IGF-1 group, ^$p < 0.05$.

groups significantly decreased (all $p<0.05$). Compared with the cardiomyocytes in the SI/RI group, the cell viability of cardiomyocytes in those groups with sevoflurane post-conditioning significantly improved (all $p<0.05$). Sevoflurane post-conditioning combined with miR-208a inhibitor or PI3K/AKT pathway activator further promoted the increase of cell viability of cardiomyocytes and the combination of the three treatments delivered an even better effect (all $p<0.05$) (Figure 2).

Cell Cycle After Transfection by Flow Cytometry

Compared with the Normal group, the other five groups showed a significant increase in the G0/G1 phase cells and a decrease in the S-phase cells (all $p<0.05$). Compared with the SI/RI group, the other groups that received sevoflurane post-conditioning presented a significant decrease in G0/G1 phase cells and increase in S-phase cells (all $p<0.05$). Sevoflurane post-conditioning combined with miR-208a inhibitor or PI3K/AKT pathway activator further reduced the number of G0/G1 phase cells and increased the number of S-phase cells, and the combination of the three treatments produced an even better effect (all $p<0.05$) (Figure 3).

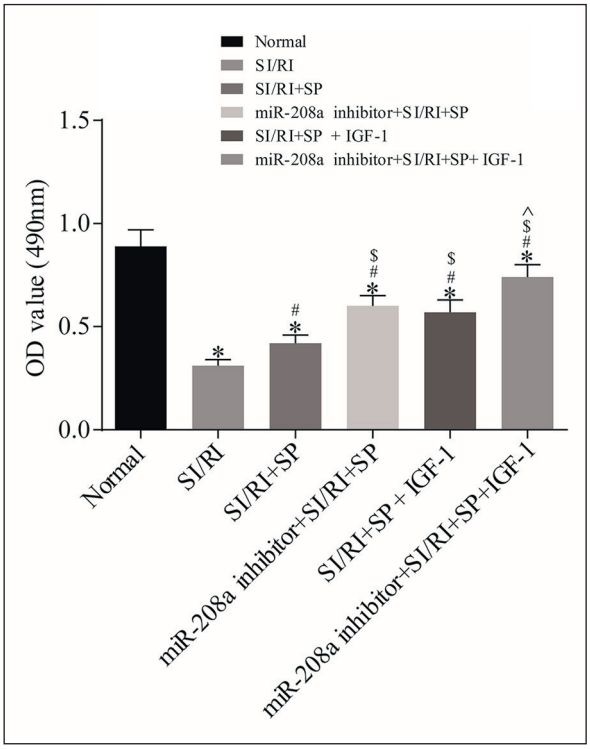

Figure 2. Cell viability of cardiomyocytes in each group by MTT assay. Compared with the Normal group, *$p<0.05$; compared with the SI/RI group, $^{\#}p<0.05$; compared with SI/RI+SP group, $^{\$}p<0.05$; for comparison between miR-208a inhibitor+SI/RI+SP group and SI/RI+SP+IGF-1 group, $^{\wedge}p<0.05$. OD value, optical density value; SI, simulated ischemia; RI, reperfusion injury; IGF-1, insulin-like growth factor 1.

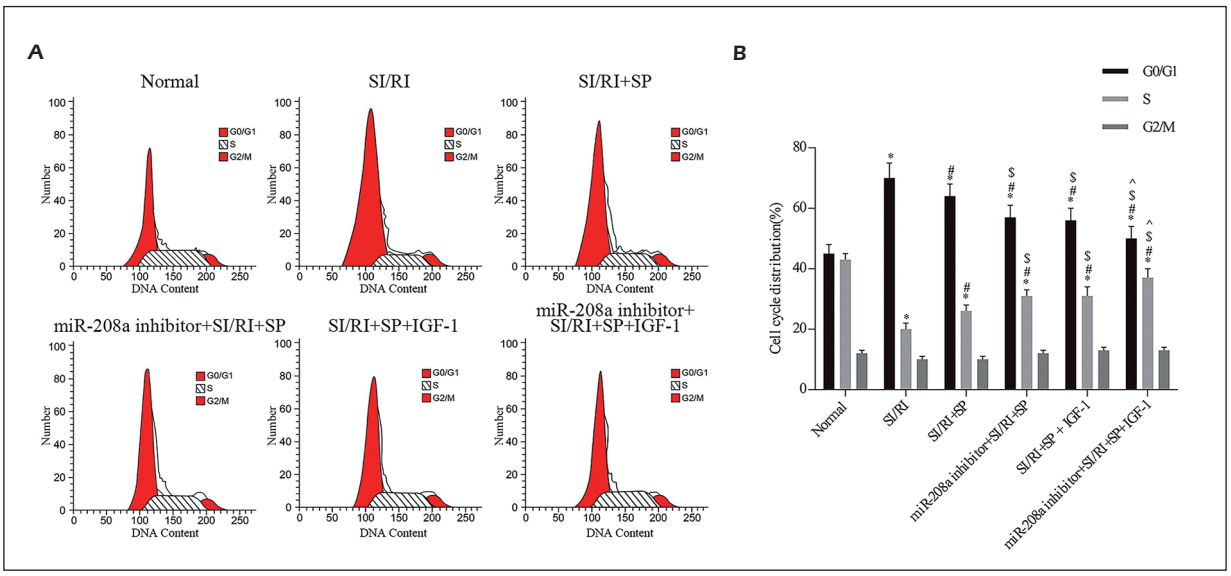

Figure 3. Cell cycle after transfection in each group by flow cytometry. **A**, Cell cycle distribution in each group. **B**, Statistics of cell cycle distribution in each group. Compared with the Normal group, *$p<0.05$; compared with SI/RI group, $^{\#}p<0.05$; compared with SI/RI+SP group, $^{\$}p<0.05$; for comparison between miR-208a inhibitor+SI/RI+SP group and SI/RI+SP+IGF-1 group, $^{\wedge}p<0.05$. SI, simulated ischemia; RI, reperfusion injury; IGF-1, insulin-like growth factor 1.

Cardiomyocyte Apoptosis by Flow Cytometry

Compared with the Normal group, the apoptosis of cardiomyocytes in the other five groups significantly increased (all $p<0.05$). Compared with the SI/RI group, the other groups that received sevoflurane post-conditioning demonstrated a significant decrease in the apoptosis of cardiomyocytes (all $p<0.05$). Sevoflurane post-conditioning combined with miR-208a inhibitor or PI3K/AKT pathway activator further reduced the apoptosis of cardiomyocytes and the combination of the three treatments delivered an even better effect (all $p<0.05$) (Figure 4).

Expression of MiR-208a and mRNA Expression of PI3K, AKT, and Autophagy-Related Factors

The results of qRT-PCR showed that the expression of miR-208a significantly increased in all groups compared with the Normal group, and significantly decreased in all groups that received sevoflurane post-conditioning compared with the SI/RI group (all $p<0.05$); the expression of miR-208a was not significantly different between SI/RI+SP group and SI/RI+SP+IGF-1 group ($p>0.05$); the expression of miR-208a was significantly lower in the miR-208a inhibitor+SI/RI+SP group and miR-208a inhibitor+SI/RI+SP+IGF-1 group than that in SI/RI+SP group (both $p<0.05$) (Figure 5). Compared with the Normal group, all other groups presented a significant decrease in the mRNA levels of PI3K and AKT but a significant increase in the mRNA levels of p62 and Beclin1 (all $p<0.05$). Compared with the SI/RI group, the other groups that received sevoflurane post-conditioning showed a significant increase in the mRNA levels of PI3K and AKT, but a decrease in the mRNA levels of p62 and Beclin1 (all $p<0.05$). Sevoflurane post-conditioning combined with miR-208a inhibitor or PI3K/AKT pathway activator further promoted the increase in the mRNA levels of PI3K and AKT and the decrease in the mRNA levels of p62 and Beclin1; moreover, the combination of the three treatments delivered an even better effect (all $p<0.05$). It indicated that low expression of miR-208a can activate the PI3K/AKT signaling pathway and inhibit the expression of autophagy-related factors in the myocardial SI/RI cells.

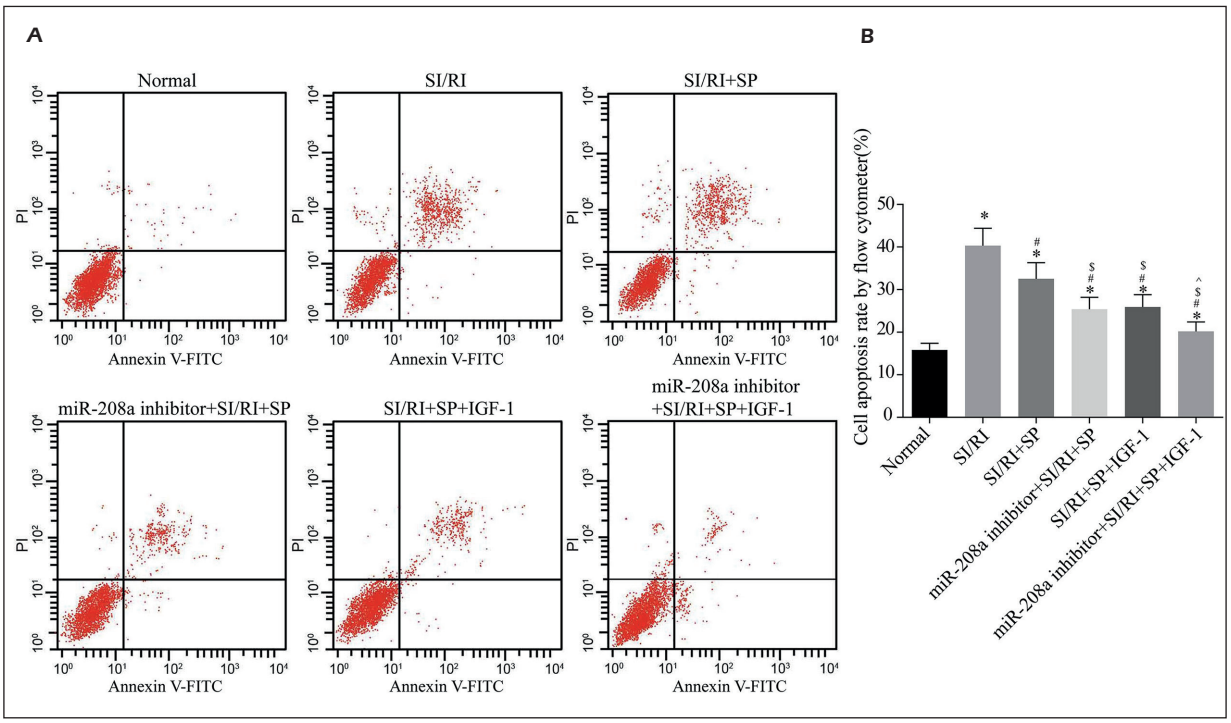

Figure 4. Apoptosis of cardiomyocytes in each group by flow cytometry. **A**, Flow cytometry scatter plot of each group. **B**, Apoptosis of cardiomyocytes in each group by annexin V-FITC/PI staining in each group. Compared with the Normal group, *$p<0.05$; compared with SI/RI group, $^{\#}p<0.05$; compared with SI/RI+SP group, $^{\$}p<0.05$; for comparison between miR-208a inhibitor+SI/RI+SP group and SI/RI+SP+IGF-1 group, $^{\wedge}p<0.05$. SI, simulated ischemia; RI, reperfusion injury; IGF-1, insulin-like growth factor 1.

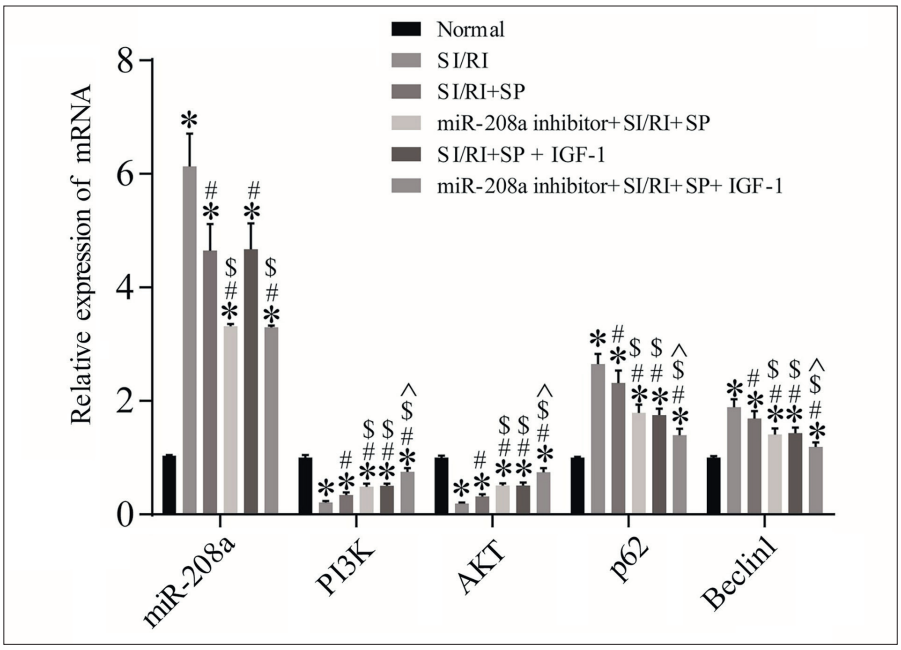

Figure 5. mRNA levels of the factors related to PI3K/AKT signaling pathway and autophagy by qRT-PCR. Compared with the Normal group, *$p<0.05$; compared with SI/RI group, $^{\#}p<0.05$; compared with SI/RI+SP group, $^{\$}p<0.05$; for comparison between miR-208a inhibitor+SI/RI+SP group and SI/RI+SP+IGF-1 group, $^{\wedge}p<0.05$. SI, simulated ischemia; RI, reperfusion injury; IGF-1, insulin-like growth factor 1.

Protein Expression of PI3K, AKT, and Autophagy-Related Factors

The results of Western blot showed that compared with the Normal group, all other groups presented a significant decrease in the protein levels of PI3K, AKT, p-PI3K, and p-AKT but a significant increase in the protein levels of p62 and Beclin1 (all $p<0.05$) (Figure 6). Com-

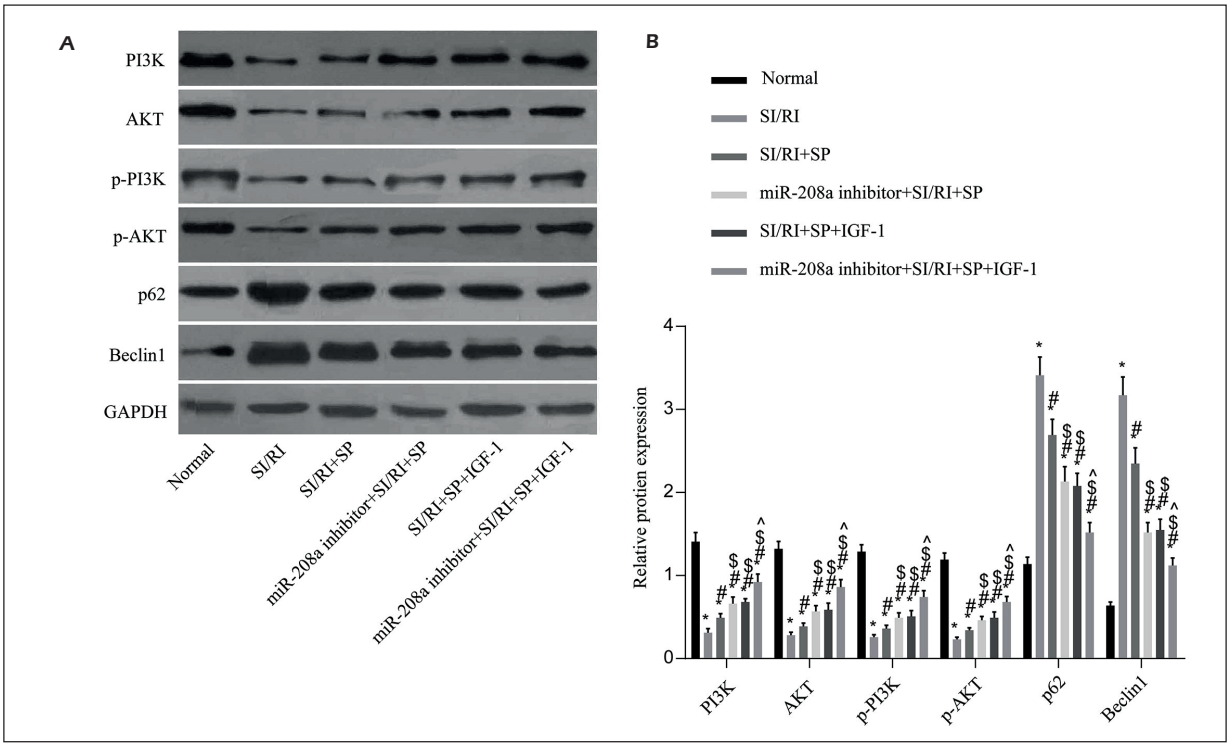

Figure 6. Protein levels of the factors related to PI3K/AKT signaling pathway and autophagy by Western blot. (**A**) Protein bands; (**B**) relative protein levels of the factors related to PI3K/AKT signaling pathway and autophagy. Compared with the Normal group, *$p<0.05$; compared with SI/RI group, $^{\#}p<0.05$; compared with SI/RI+SP group, $^{\$}p<0.05$; for comparison between miR-208a inhibitor+SI/RI+SP group and SI/RI+SP+IGF-1 group, $^{\wedge}p<0.05$. SI, simulated ischemia; RI, reperfusion injury; IGF-1, insulin-like growth factor 1.

pared with SI/RI group, the other groups that received sevoflurane post-conditioning showed a significant increase in the protein levels of PI3K, AKT, p-PI3K, and p-AKT but a significant decrease in the protein levels of p62 and Beclin1 (all $p<0.05$). Sevoflurane post-conditioning combined with miR-208a inhibitor or PI3K/AKT pathway activator further promoted the increase in the protein levels of PI3K, AKT, p-PI3K and p-AKT and the decrease in the protein levels of p62 and Beclin1, and the combination of the three treatments generated an even better effect (all $p<0.05$). It suggested that low expression of miR-208a could further inhibit the increase of autophagy-related factor expression in myocardial SI/RI cells after sevoflurane post-conditioning.

Fluorescence Intensity of Autophagosomes

Fluorescence intensity of autophagosomes was detected by MDC staining. The results showed that compared with the Normal group, the fluorescence intensity of autophagosomes in other groups increased (all $p<0.05$). Compared with the SI/RI group, other groups that received sevoflurane post-conditioning saw the decrease of fluorescence intensity of autophagosomes (all $p<0.05$) (Figure 7). Sevoflurane post-conditioning combined with miR-208a inhibitor or PI3K/AKT pathway activator could further inhibit the fluorescence intensity of autophagosomes and the effect of the three of them combined was more significant (all $p<0.05$). The effect indicated that low expression of miR-208a could activate PI3K/AKT pathway to further inhibit the fluorescence intensity of autophagosomes in myocardial SI/RI cells.

Discussion

Previous studies have shown that sevoflurane can effectively reduce myocardial injury and markedly contribute to cardioprotection potentially by involving in the regulation of autophagy and apoptosis[14]. In recent years, evidence has shown that miRNAs have important association with the cardioprotective role of sevoflurane inhalation. Liu et al[15] found that sevoflurane inhalation plays an important role in cardioprotection in the patients undergoing off-pump coronary artery bypass surgery by effectively reducing myocardial injury in patients and significantly lowering the expression of miR-208. Cardiac-enriched miR-208a is closely associated with the progression of cardiac diseases[16]. Liu et al[17] confirmed that the increased expression of miR-208a in plasma can be an independent risk factor for coronary heart disease. Liu et al[18] explored the relationship between miR-208a expression and MIRI and found that the inhibited expression of miR-208a can improve MIRI by activating the p21 signaling pathway. Scholars have proved the protective role of the PI3K/AKT signaling pathway in the development of MIRI[19]. In this study, the SI/RI model was constructed using neonatal rat cardiomyocytes, and different treatment was performed

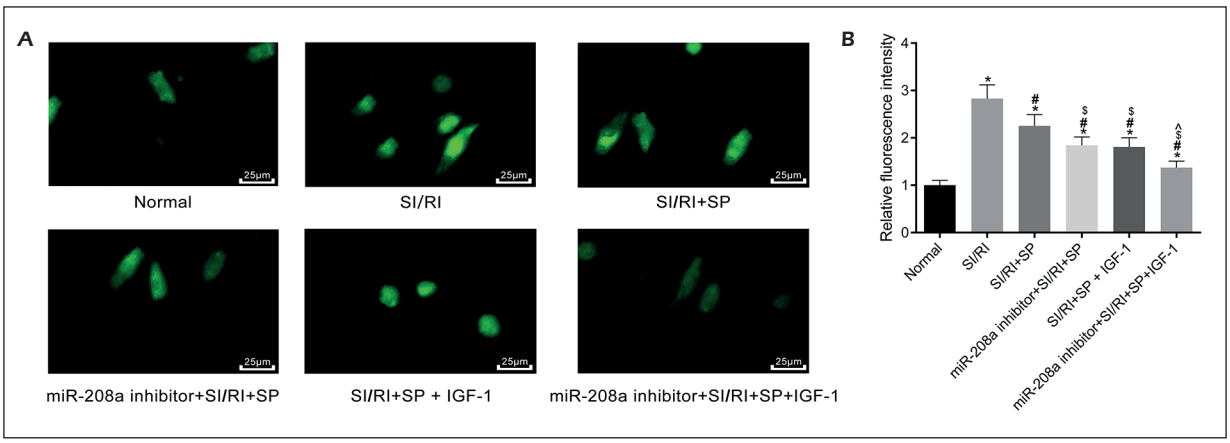

Figure 7. Fluorescence intensity of autophagosomes by MDC staining. **A**, Fluorescence imaging of autophagosomes (×400). **B,** Relative fluorescence intensity of autophagosomes. Compared with the Normal group, $*p<0.05$; compared with SI/RI group, $^{\#}p<0.05$; compared with SI/RI+SP group, $^{\$}p<0.05$; for comparison between miR-208a inhibitor+SI/RI+SP group and SI/RI+SP+IGF-1 group, $^{\wedge}p<0.05$. SI, simulated ischemia; RI, reperfusion injury; IGF-1, insulin-like growth factor 1.

in each group to detect that miR-208a can regulate SI/RI in rat cardiomyocytes after sevoflurane post-conditioning by mediating PI3K/AKT signaling pathway. Moreover, inhibition of miR-208a expression could facilitate the activation of the PI3K/AKT pathway, thereby promoting the protective effect of sevoflurane post-conditioning for SI/RI.

In this study, the neonatal rat cardiomyocytes changed from fusiform shape to round shape after modeling of SI/RI. Sevoflurane post-conditioning, miR-208a knockout and the inhibition of PI3K/AKT signaling pathway could significantly improve the morphology of cardiomyocytes after SI/RI. Liu et al[20] have shown that SOD and MDA are both important in the progression of myocardial injury. As a common metalloenzyme, SOD can promote the elimination of free radicals and protect cells from free radical damage[21]. As a metabolite of lipid peroxidation, MDA production is affected by free radicals. The level of MDA is often used to reflect the body resistance to free radicals and the degree of lipid peroxidation[22]. In this study, we found that, compared with cardiomyocytes in the Normal group, cardiomyocytes with SI/RI had significantly decreased levels of SOD but significantly increased levels of MDA. After sevoflurane post-conditioning, these cardiomyocytes had significantly increased levels of SOD but significantly decreased levels of MDA. The results indicate that sevoflurane post-conditioning promote the elimination of free radicals by enhancing SOD activity and inhibiting the expression of MDA. Compared with SI/RI+SP group, miR-208a inhibitor+SI/RI+SP group, SI/RI+SP+IGF-1 group, and miR-208a inhibitor+-SI/RI+SP+IGF-1 group showed more significant improvements in the levels of SOD and MDA, and the improvements were even more significant in miR-208a inhibitor+SI/RI+SP+IGF-1 group. The results indicate the positive role of miR-208a inhibition and PI3K/AKT pathway activation in the treatment of SI/RI in neonatal rat cardiomyocytes after sevoflurane post-conditioning.

Cardiomyocyte apoptosis mainly occurs during reperfusion after myocardial ischemia and is the major type of cell death in MIRI[23]. Studies[24,25] have confirmed that the inhibition of cardiomyocyte apoptosis can significantly promote the reduction of myocardial infarct size. This study found that the inhibition of miR-208a and the activation of PI3K/AKT pathway can promote cell cycle arrest in the S phase in cardiomyocytes and significantly suppress the apoptosis of neonatal rat cardiomyocytes after sevoflurane post-conditioning. Autophagy is the process of selectively eliminating damaged and denatured proteins in the cells. When the cells are under such oxidative stress as hypoxia and impaired mitochondrial function, the level of autophagy is significantly enhanced[26]. However, excessive level of autophagy can cause damage to normal cells while promoting the phagocytosis of damaged cells[27]. Beclin-1, an important marker of autophagic flux, engages in the regulation of autophagy by promoting the formation of autophagosomes, and is positively correlated with autophagic activity[28]. P62, an ubiquitin-binding protein, has been shown to play an important role in autophagy and the transduction of various signaling pathways[29]. Furthermore, P62 can be used as an important marker reflecting autophagic activity, and its level is closely associated with the clearance of autophagosomes[30]. In recent years, scholars[31,32] have showed that SI/RI is closely related to autophagy, and that SI/RI can lead to autophagic death in a large number of cardiomyocytes[31,32]. It was also found in our study that the expression of Beclin-1 and P62 and the fluorescence intensity of autophagosomes in the SI/RI group were significantly higher than those in the Normal group, but the expression of Beclin-1 and P62 and the fluorescence intensity of autophagosomes were markedly decreased after sevoflurane post-conditioning. The expression of autophagy-related factors and the fluorescence intensity of autophagosomes were further reduced when miR-208a knockout and PI3K/AKT signaling pathway activation were used along with sevoflurane post-conditioning. The results reveal that sevoflurane post-conditioning may regulate the autophagy in rat to inhibit the expression of miR-208a, thereby activating PI3K/AKT signaling pathway to regulate SI/RI in neonatal rat cardiomyocytes.

Conclusions

The inhibition of miR-208a expression can promote the activation of PI3K/AKT signaling pathway, thereby inhibiting the autophagy of cardiac myocytes, enhancing cardiomyocyte viability, and reducing apoptosis. With this mechanism, low expression of miR-208a can protect neonatal rat cardiomyocytes from SI/RI after sevoflurane post-conditioning. MiR-208a is expected to be

an important target for inhaled anesthesia in the treatment of SI/RI in neonatal rat cardiomyocytes, and the combined use of PI3K/AKT pathway activator can deliver better effect. However, more experiments are warranted to confirm these conclusions.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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