MicroRNA-132 promotes oxidative stress-induced pyroptosis by targeting sirtuin 1 in myocardial ischaemia-reperfusion injury

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Abstract. The present study aimed to investigate the roles of miR-132 in myocardial ischaemia/reperfusion (I/R) injury and the underlying mechanisms. The myocardial I/R model was established using C57BL/J6 mice. Haematoxylin and eosin staining was performed to observe the injury of myocardial tissues. Commercial kits were used to measure the levels of serum myocardial enzymes and inflammatory factors. The *in vitro* I/R model was established by the hypoxia/reoxygenation method using H9C2 cells. A dual luciferase reporter assay was used to confirm the binding of miR-132 and sirtuin 1 (SIRT1). Cell pyroptosis was determined using flow cytometry. Reverse transcription-quantitative PCR was performed to determine the expression of miR-132, SIRT1 and inflammatory factors. The levels of peroxisome proliferator-activated receptor gamma coactivator (PGC)-1α/nuclear factor erythroid-2-related factor 2 (Nrf2) signalling, oxidative stress and pyroptosis-related proteins were detected by western blotting. Apparent histologic injury and elevated levels of serum myocardial enzymes and inflammatory factors were observed in the myocardial I/R model. miR-132 was significantly upregulated and SIRT1 was markedly downregulated in I/R myocardial tissues. miR-132 directly targeted SIRT1 and negatively regulated the expression of SIRT1. PGC-1α, Nrf2, endothelial nitric oxide synthase and superoxide dismutase levels were significantly decreased, while inducible nitric oxide synthase and malondialdehyde levels were significantly increased by I/R induction. The pyroptosis-related proteins NLRP3, caspase-1 and interleukin (IL)-1β were also significantly elevated by I/R induction. Inhibition of miR-132 activated PGC-1α/Nrf2 signalling and inhibited oxidative stress and the expression of the pyroptosis-related proteins NLRP3, caspase-1 and IL-1β, which were all reversed by inhibiting SIRT1 with EX527. The findings of the present study indicated that inhibition of miR-132 may ameliorate myocardial I/R injury by inhibiting oxidative stress and pyroptosis through activation of PGC-1α/Nrf2 signalling by targeting SIRT1.

Introduction

Ischaemia-reperfusion (I/R) injury is the most important and common cause of myocardial damage and subsequent heart failure worldwide (1,2). Myocardial I/R injury may induce cell apoptosis and autophagy by activating oxidative stress and upregulating inflammatory mediators, ultimately resulting in irreversible fibrotic damage (3). However, despite numerous studies on myocardial I/R injury, deeper insight into the underlying mechanisms of myocardial I/R injury is needed.

MicroRNAs (miRNAs) are small endogenous RNAs that are associated with tumourigenesis, cell proliferation and apoptosis (4,5). Generally, the regulation of target genes by miRNAs occurs through binding with the 3'-untranslated region (UTR). Recently, miRNAs were also found to be associated with myocardial I/R injury (6,7). miR-132 was found to be involved in diseases such as epilepsy (8), prostate cancer (9) and hepatocellular carcinoma (10). It was previously demonstrated that targeting of a Na^+-Ca^{2+} exchanger by miR-132 was able to prevent apoptosis of cardiomyocytes under hypoxic conditions (11). It was also reported that miR-132 was upregulated in simulated ischaemic injury in cultured hippocampal neurons (12). However, few studies have focused on the role of miR-132 in myocardial I/R injury and the underlying mechanisms.

Sirtuin 1 (SIRT1), a member of the sirtuin family, regulates the cellular ageing process, participates in metabolic diseases, such as diabetes, and is related to cancer development (13). It has been reported that SIRT1/peroxisome proliferator-activated

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Abbreviations: I/R, ischaemia/reperfusion; miRNA, microRNA; H/R, hypoxia/reoxygenation; LAD, left anterior descending; H&E, haematoxylin and eosin; MDA, malondialdehyde; SOD, superoxide dismutase

Key words: myocardial ischaemia-reperfusion injury, miR-132, sirtuin 1, peroxisome proliferator-activated receptor gamma coactivator-1α/nuclear factor erythroid-2-related factor 2 signalling, oxidative stress

receptor gamma coactivator (PGC)-1α/nuclear factor erythroid-2-related factor 2 (Nrf2) signalling can mediate oxidative stress, which plays an important role in myocardial I/R injury (14,15). SIRT1 and PGC-1α/Nrf2 signalling were also reported to be involved in myocardial I/R injury (16). In addition, miR-132 is also involved in several cancers, such as lymphocytic leukaemia and gastric cancer, as well as in Alzheimer's disease by targeting SIRT1 (17,18). However, no study has yet focused on the role of the miR-132/SIRT1 axis in myocardial I/R injury.

Pyroptosis is considered an inherently inflammatory process of NLRP3/caspase-1-dependent programmed cell death (19,20). Pyroptosis was found to be associated with cell apoptosis, oxidative stress and autophagy (21). An association between pyroptosis and the PGC-1α/Nrf2 signalling pathway was also demonstrated a (22). However, few studies have focused on the role of pyroptosis in myocardial I/R injury and its possible association with the miR-132/SIRT1 axis.

To the best of our knowledge, the present study was the first to investigate the role of miR-132 in myocardial I/R injury and the underlying mechanisms. The aim of the study was to provide deeper insight into the role of miR-132 in myocardial I/R and identify new treatment targets for myocardial I/R injury.

Materials and methods

Animals and myocardial I/R model establishment. A total of 14 C57BL/J6 mice were obtained from the SJA Laboratory Animal Company (Hunan, China). All mice were aged >8 weeks and weighed 20-30 g. All animals were housed under controlled temperature conditions (23-25˚C) and had free access to food and water. Animal handling conformed to the Guidelines for the Care and Use of Laboratory Animals. The study protocol was approved by the Institutional Animal Care Committee at Tongji Medical College of Huazhong University of Science and Technology (Hubei, China).

After 4 days of feeding, the animals were randomized into two groups (7 mice per group): The sham and myocardial I/R groups. To establish the myocardial I/R model, the well-established left anterior descending (LAD) coronary artery ligation model was used as described previously (23). Briefly, the mice were anaesthetized using 4% isoflurane with oxygen for 2 min and then maintained with 1.5% isoflurane with oxygen throughout the following surgery, and an incision was made at the fourth intercostal space. The mice were then subjected to 45 min of transitory ligation on the LAD coronary artery followed by reperfusion for 3 h. Occlusion was confirmed by blanching of the left ventricular myocardium below the suture. The sham operation was conducted using the same procedure, except for the placement of the ligature. The animals were then sacrificed by cervical dislocation, and the myocardial tissues and blood samples were collected and stored.

Histological analysis. For histological analysis, all myocardial tissues were fixed with 10% formalin for 24 h at room temperature, embedded in paraffin, and then cut into 5-μm sections. The sections were stained with haematoxylin and eosin (H&E) for 15 min at room temperature and then scanned and photographed using a Leica DFC280 light microscope (Leica Micros Imaging Solutions Ltd.).

Measurement of myocardial enzymes, inflammatory and oxidative stress factors. The serum levels of the myocardial enzymes creatine kinase (CK), CK myocardial band isoenzyme (CK-MB), lactate dehydrogenase (LDH), hydroxybutyrate dehydrogenase (HBDH) and ischaemia-modified albumin (IMA) were determined using commercial kits purchased from Maccura Biotechnology. The serum levels of the inflammatory factors interleukin (IL)-1β, IL-6, and tumour necrosis factor (TNF)-α were all determined using commercially available ELISA kits (IL-1β, ab197742; IL-6, ab100713; and TNF-α, ab208348) according to the manufacturer's instructions (Abcam). The levels of malondialdehyde (MDA) and superoxide dismutase (SOD) in myocardial tissues were measured using MDA and SOD kits (Nanjing Jiancheng Bio-Technology Co., Ltd.), respectively, as described previously (24).

Cell culture and transfection. The myocardial cell line H9C2 was purchased from ATCC. Briefly, cells were cultured in RPMI-1640 (Thermo Fisher Scientific, Inc.) supplemented with 10% Gibco® foetal bovine serum (FBS; Thermo Fisher Scientific, Inc.) and 100 μg/ml penicillin-streptomycin (Sigma-Aldrich; Merck KGaA) at 37˚C and 5% CO_2. Cells were divided into the following groups: i) Control group, ii) hypoxia/reoxygenation (H/R) model group, iii) H/R + inhibitor NC group, iv) H/R + miR-132 inhibitor group, and v) H/R + miR-132 inhibitor + EX527 group. To establish the *in vitro* myocardial I/R model, the H/R method was used. Briefly, cells were placed in a hypoxia cabin under 2% O_2, 93% N_2 and 5% CO_2 for 2 h, and then cultured under normoxic conditions (21% O_2, 5% CO_2 and 74% N_2) for 4 h. For cell transfection, the cells were transfected with miR-132 mimics or inhibitor (5 nM, GeneChem Corp.) or their negative control (NC) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. To inhibit the expression of SIRT1, the SIRT1 inhibitor EX527 (10 μM, Sigma-Aldrich; Merck KGaA) was used to treat the cells. After 48 h of transfection, the transfected cells were harvested and subjected to further experiments. The sequences of miR-132 mimics, mimics NC, miR-132 inhibitor and inhibitor NC were as follows: miR-132 mimics: Sense, 5'-UAACAGUCUACAGCCAUGGUCG-3' and antisense, 5'-CGACCAUGGCUGUAGACUGUUU-3'; mimics NC: Sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACACGUUCGGAGAATT-3'. miR-132 inhibitor: 5'-AGUAACAAUCGAAAGCCACGGU-3'; inhibitor NC: 5'-CAGUACUUUUGUGUAGUACAA-3'.

Cell pyroptosis analysis. For cell pyroptosis analysis, the *in vitro* FAM-FLICA Caspase-1 Detection kit (ImmunoChemistry Technologies, LLC) was used according to the manufacturer's instructions. Briefly, the cells were harvested and washed with PBS. Subsequently, the cells were stained with 2 μg/ml PI and 10 μl FAM-FLICA. In each analysis, 20,000 gated events were recorded. The fluorescence intensity was measured using a FACSCalibur II flow cytometer and CellQuest software, version 5.1 (BD Biosciences).

Dual luciferase reporter assay. To confirm the SIRT1 3'-UTR as a target of miR-132, a dual luciferase reporter assay was conducted. Briefly, the wild-type (WT) or mutant (MUT)

Table I. Primers used in reverse transcription-quantitative PCR.

Genes	Species	Sequences
miR-132	Mouse	Forward 5'-CGGTGACTCAGCCTAGATGG-3'
		Reverse 5'-GGACGGGACAGGGAAGGG-3'
	Rat	Forward 5'-GACTGGTCCCGTGGCTTTC-3',
		Reverse 5'-GTGCAGGGTCCGAGGTATTC-3'
SIRT1	Mouse	Forward5'-GCCTCACATGCAAGCTCTAGTGAC-3'
		Reverse 5'-TCGAGGATCTGTGCCAATCATAA-3'
	Rat	Forward 5'-GATCTCCCAGATCCTCAAGCC-3'
		Reverse 5'-TAGTCCATCAAGGAGCCAC-3'
IL-1β	Mouse	Forward 5'-CATGGAATCCGTGTCTTCCT-3'
		Reverse 5'-GAGCTGTCTGCTCATTCACG-3'
	Rat	Forward 5'-CCAGGATGAGGACCCAAGCA-3'
		Reverse 5'-TCCCGACCATTGCTGTTTCC-3'
IL-6	Mouse	Forward 5'-TGACAAAAGAGTTGTGCAATGGC-3'
		Reverse 5'-GAATGTCCACAAACTGATATGCTT-3'
	Rat	Forward 5'-GACTTCCAGCCAGTTGCCTTCTTG-3'
		Reverse 5'-TGGTCTGTTGTGGGTGGTATCCTC-3'
TNF-α	Mouse	Forward 5'-TCCCCAAAGGGATGAGAAGTTC-3'
		Reverse 5'-TCATACCAGGGTTTGAGCTCAG-3'
	Rat	Forward 5'-AAGCCCGTAGCCCACGTCGTA-3'
		Reverse 5'-GCC-CGCAATCCAGGCCACTAC-3'
GAPDH	Mouse	Forward 5'-CACCCACTCCTCCACCTTTG-3'
		Reverse 5'-CCACCACCCTGTTGCTGTAG-3'
	Rat	Forward 5'-GGAGTCCACTGGCGTCTTC-3'
		Reverse 5'-GGCATTGCTGATGATCTTGAGG-3'
U6	Mouse	Forward 5'-ATGGGTCGAAGTCGTAGCC-3'
		Reverse 5'-TTCTCGGCGTCTTCTTTCTCG-3'
	Rat	Forward 5'-CTCGCTTCGGCAGCACA-3'
		Reverse 5'-AACGCTTCACGAATTTGCGT-3'

SIRT1, sirtuin 1; IL, interleukin; TNF, tumour necrosis factor.

3'-UTR of SIRT1 was sub-cloned into the pGL4.10 luciferase reporter vector. Cells were then co-transfected with the vectors, the miR-132 mimics, miR-132 inhibitor, mimics NC or inhibitor NC (5 nM, GeneChem Corp.) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After transfection for 48 h, a luciferase assay was performed using the Bright-Glo™ Luciferase Assay System (Promega Corporation). The luciferase activity was normalized to the Renilla luciferase activity.

Reverse transcription-quantitative PCR (RT-qPCR) assay. To determine the expression of miR-132, SIRT1, IL-1β, IL-6 and TNF-α, RT-qPCR was performed. Total RNA was extracted from myocardial tissues or H9C2 cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). cDNA was synthesized by reverse transcription using the PrimerScript RT reagent kit (Takara Bio, Inc.). qPCR was performed to quantify relative mRNA levels using SYBR-Green RT-qPCR SuperMix kit (Thermo Fisher Scientific, Inc.) with customized primers (Table I) on an AB7300 thermo-cycler (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR reaction conditions were as follows: Initial activation step at 95˚C for 10 sec, followed by 40 cycles of denaturation at 95˚C for 15 sec, annealing at 55˚C for 25 sec, and extension at 72˚C for 10 sec. GAPDH and U6 small nuclear RNA (U6 snRNA) were used as internal references for mRNA and miRNA, respectively. Relative expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (25).

Western blot assay. Western blotting was performed to determine the expression of SIRT1, PGC-1α, Nrf2, endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), NLRP3, caspase-1 and IL-1β. GAPDH was used as a control. Briefly, proteins were extracted by RIPA buffer (Vazyme Biotech Co., Ltd.) and quantitated with a bicinchoninic acid assay kit (Thermo Fisher Scientific, Inc.). Subsequently, 30 μg proteins were loaded on 10% SDS-PAGE, transferred to PVDF membranes and then blocked with 5% non-fat milk. Then, membranes were incubated with the primary antibodies anti-SIRT1 (cat. no. ab110304, 1:2,000), anti-PGC-1α (cat. no. ab54481, 1:10,000), anti-Nrf2 (cat. no. ab62352, 1:1,000), anti-eNOS (cat. no. ab76198, 1:500), anti-iNOS

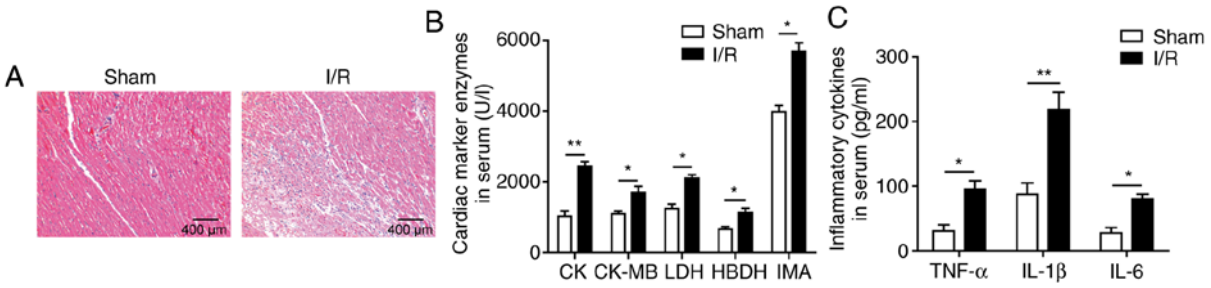

Figure 1. Effects of I/R on myocardial injury and levels of serum myocardial enzymes and inflammatory factors. (A) Histological analysis by haematoxylin and eosin staining; scale bar, 400 μm. (B) Serum levels of the myocardial enzymes CK, CK-MB, LDH, HBDH and IMA. (C) Levels of the inflammatory factors IL-1β, IL-6 and TNF-α as determined by ELISA. The results are representative of three independent experiments. Error bars represent the mean ± standard deviation. *P<0.05 and **P<0.01. I/R, ischemia/reperfusion; CK, creatine kinase; CK-MB, CK myocardial band isoenzyme; LDH, lactate dehydrogenase; HBDH, hydroxybutyrate dehydrogenase; IMA, ischaemia-modified albumin; IL, interleukin; TNF, tumour necrosis factor.

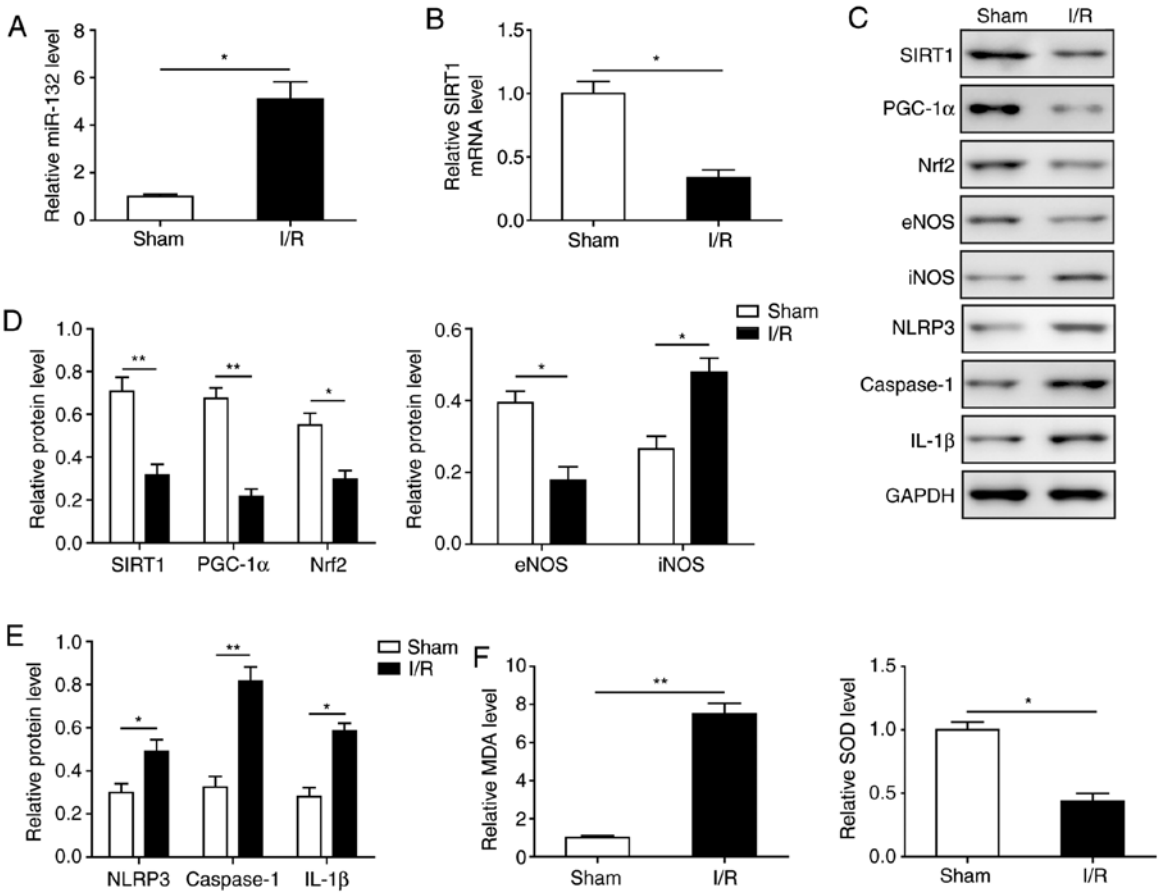

Figure 2. Effects of I/R on Nrf2-mediated oxidative stress and pyroptosis. (A) Expression of miR-132 in myocardial tissues in the I/R group and sham group by RT-qPCR. (B) SIRT1 mRNA level in myocardial tissues in the I/R group and sham group by RT-qPCR. (C) Protein levels of SIRT1, PGC-1α, Nrf2, eNOS, iNOS, NLRP3, caspase-1 and IL-1β in myocardial tissues by western blotting. (D) Quantification of protein expression of SIRT1, PGC-1α, Nrf2, eNOS and iNOS in C. (E) Quantification of protein expression of NLRP3, caspase-1, and IL-1β in C. (F) Levels of SOD and MDA in myocardial tissues. The results are representative of three independent experiments. Error bars represent the mean ± standard deviation. *P<0.05 and **P<0.01. I/R, ischemia/reperfusion; Nrf2, nuclear factor erythroid-2-related factor 2; RT-qPCR, reverse transcription-quantitative PCR; SIRT1, sirtuin 1; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator-1α; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; IL, interleukin; SOD, superoxide dismutase; MDA, malondialdehyde.

(cat. no. ab15323, 1:500), anti-NLRP3 (cat. no. ab214185, 1:1,000), anti-caspase-1 (cat. no. ab62698, 1:500), anti-IL-1β (cat. no. ab200478, 1:1,000) and anti-GAPDH (cat. no. ab8245, 1:2,000) (all purchased from Abcam) at 4˚C overnight, followed by the corresponding secondary horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody (cat. nos. ab205719 and ab205718, Abcam) for 1 h at room temperature. The films were scanned using the Pierce ECL Western Blotting Substrate (Pierce; Thermo Fisher Scientific, Inc.). The proteins were quantified using Quantity One software, version 4.2.1 (Bio-Rad Laboratories, Inc.).

Statistical analysis. All experiments were repeated at least three times, and one representative result is presented. All

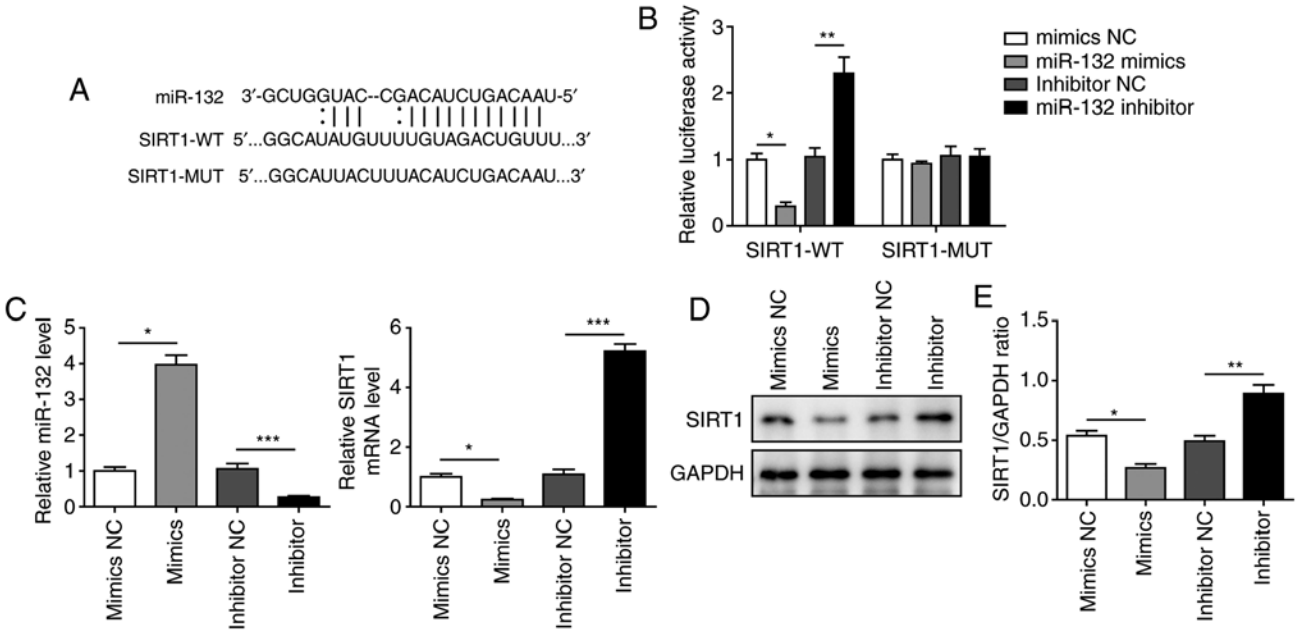

Figure 3. miR-132 directly targeted SIRT1 and negatively regulated SIRT1 expression. (A) The predicted binding site of miR-132 and SIRT1. (B) The relative luciferase activity of SIRT1-WT and SIRT1-MUT groups by dual luciferase reporter assay. (C) Expression of miR-132 and SIRT1 in cells transfected with mimics NC, miR-132 mimics, inhibitor NC or miR-132 inhibitor by RT-qPCR. (D) Protein level of SIRT1 in cells transfected with mimics NC, miR-132 mimics, inhibitor NC or miR-132 inhibitor by western blotting. (E) Quantification of SIRT1 protein expression. The results are representative of three independent experiments. Error bars represent the mean ± standard deviation. *P<0.05, **P<0.01 and ***P<0.001. SIRT1, sirtuin 1; WT, wild-type; MUT, mutant; RT-qPCR, reverse transcription-quantitative PCR; NC, negative control.

experimental data are expressed as the mean ± standard deviation. Student's t-test (two-tailed) was used for comparison between two groups. One-way analysis of variance followed by Tukey's post hoc test was used for multiple comparisons. SPSS version 13.0 (SPSS, Inc.) was used for statistical processing. P<0.05 was considered to indicate statistically significant differences.

Results

Effects of I/R on myocardial injury and levels of serum myocardial enzymes and inflammatory factors. First, alterations in myocardial tissues and serum levels of myocardial enzymes and inflammatory factors following I/R were demonstrated. As shown in Fig. 1A, marked histological myocardial structural abnormalities were observed in myocardial tissues of the I/R group, such as tissue necrosis, massive inflammatory infiltration, and perinuclear vacuolization. In addition, the serum levels of the myocardial enzymes CK, CK-MB, LDH, HBDH and IMA were all significantly upregulated after I/R induction compared with the sham group (Fig. 1B). The serum levels of IL-1β, IL-6 and TNF-α were also significantly increased in the I/R group (Fig. 1C). These results indicated successful establishment of the myocardial I/R injury model.

Effects of I/R on Nrf2-mediated oxidative stress and pyroptosis. The effects of I/R on oxidative stress and pyroptosis were next investigated. First, miR-132 was found to be significantly increased, while the mRNA level of SIRT1 was significantly decreased in myocardial tissues of I/R mice compared with the sham group (Fig. 2A and B). Western blotting demonstrated that the expression of the PGC-1α/Nrf2 signalling-related proteins PGC-1α, Nrf2 and eNOS was significantly inhibited by I/R induction, while the expression of iNOS was significantly upregulated (Fig. 2C and D), suggesting that the PGC-1α/Nrf2 signalling pathway was inhibited and oxidative stress was enhanced by I/R. Furthermore, MDA levels were significantly upregulated and SOD levels were downregulated in myocardial tissues of I/R mice (Fig. 2F), indicating the increased oxidative stress in I/R mice. In addition, the pyroptosis-related proteins NLRP3, caspase-1, and IL-1β were all significantly upregulated in myocardial tissues of I/R mice, indicating that pyroptosis was activated (Fig. 2C and E). These results indicate that I/R induced PGC-1α/Nrf2 signalling pathway-mediated oxidative stress and downstream signalling-associated pyroptosis in myocardial tissues.

miR-132 directly targets SIRT1 and negatively regulates the expression of SIRT1. To further investigate the roles of miR-132 and SIRT1 in I/R-induced myocardial injury, a dual luciferase reporter assay was conducted to confirm the binding between miR-132 and SIRT1. A binding site for miR-132 was identified in the 3'-UTR of SIRT1 (Fig. 3A). As shown in Fig. 3B, the luciferase activity was significantly decreased when cells were transfected with miR-132 mimics and significantly elevated when miR-132 was inhibited in the SIRT1-WT group. However, no significant difference was observed in the SIRT1-MUT groups after transfection with miR-132 mimics or inhibitor, suggesting that SIRT1 is a direct target of miR-132. miR-132 mimics markedly increased the level of miR-132, and miR-132 inhibitor significantly reduced the level of miR-132 (Fig. 3C). The mRNA and protein levels of SIRT1 were found to be significantly upregulated in H9C2 cells transfected with miR-132 inhibitor and significantly downregulated when

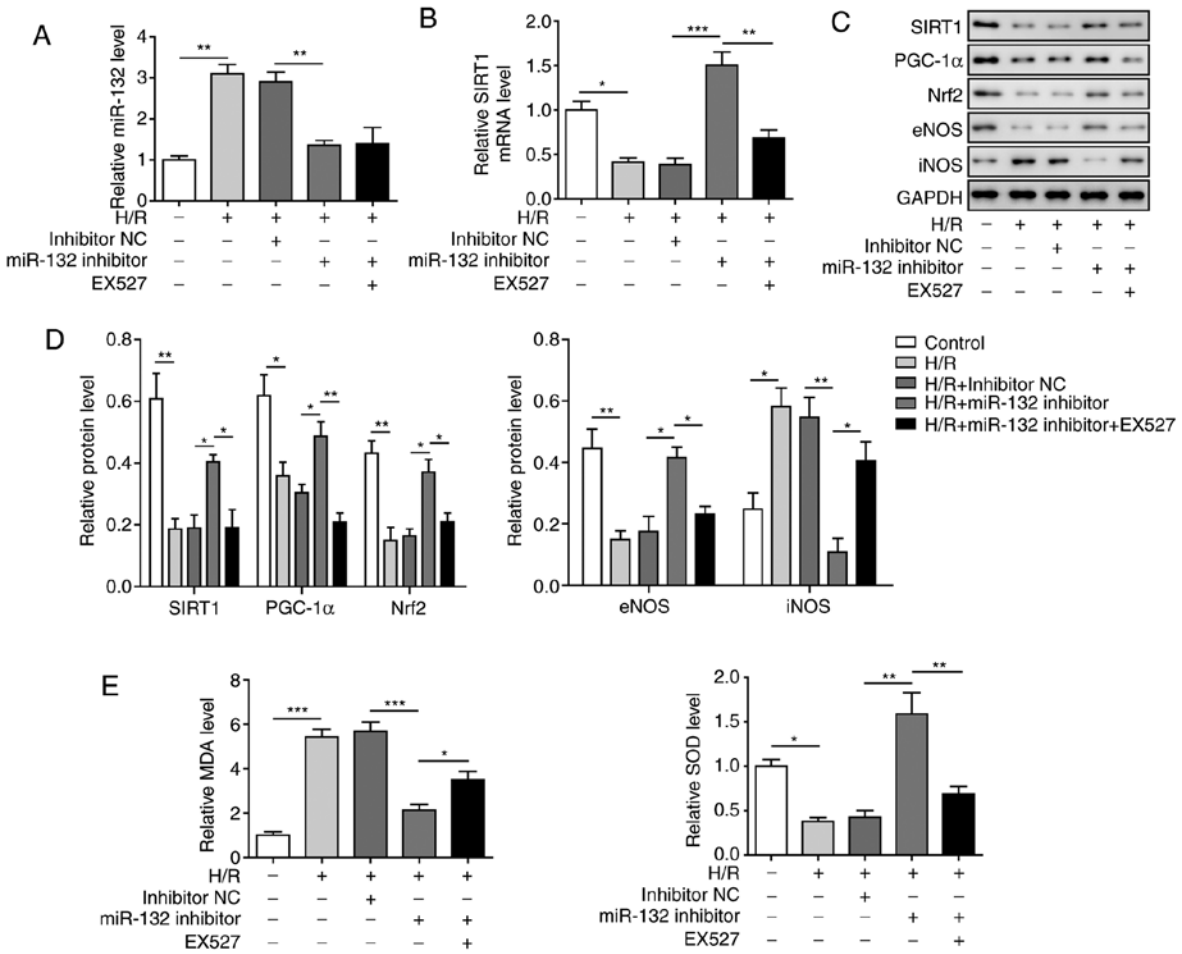

Figure 4. Inhibition of miR-132 suppressed H/R-induced oxidative stress by activation of PGC-1α/Nrf2 signalling through upregulation of SIRT1. (A) Expression of miR-132 in different groups of cells by RT-qPCR. (B) SIRT1 expression in different groups of cells by RT-qPCR. (C) Protein levels of SIRT1, PGC-1α, Nrf2, eNOS and iNOS in different groups of cells by western blotting. (D) Quantification of SIRT1, PGC-1α, Nrf2, eNOS and iNOS protein expression. (E) Levels of MDA and SOD in different groups of cells. The results are representative of three independent experiments. Error bars represent the mean ± standard deviation. *P<0.05, **P<0.01 and ***P<0.001. H/R, hypoxia/reoxygenation; Nrf2, nuclear factor erythroid-2-related factor 2; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator-1α; SIRT1, sirtuin 1; RT-qPCR, reverse transcription-quantitative PCR; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; SOD, superoxide dismutase; MDA, malondialdehyde.

miR-132 was overexpressed (Fig. 3C-E). These results indicated that SIRT1 is a direct target of miR-132 and is negatively regulated by miR-132.

Inhibition of miR-132 inhibits H/R-induced oxidative stress by activation of PGC-1α/Nrf2 signalling through upregulation of SIRT1. To investigate the effects of miR-132 on H/R-induced oxidative stress *in vitro*, a miR-132 inhibitor was applied, and SIRT1 was inhibited by the SIRT1 inhibitor EX527. The results demonstrated that the miR-132 inhibitor significantly decreased the expression of miR-132, which was induced by H/R treatment, while the expression of SIRT1 was significantly increased compared with that of the H/R group. Moreover, the aforementioned effects were markedly reversed by EX527 treatment (Fig. 4A and B), further confirming the regulatory effect of miR-132 on SIRT1. Analysis of PGC-1α/Nrf2 signalling revealed that PGC-1α, Nrf2 and eNOS were all significantly upregulated and that iNOS was significantly downregulated by inhibiting miR-132 compared with the H/R group (Fig. 4C and D). However, the inhibition of SIRT1 by EX527 was able to markedly reverse the effects induced by the miR-132 inhibitor. Similar results were also observed with MDA and SOD levels. The increased level of MDA and decreased level of SOD induced by H/R were significantly inhibited by the miR-132 inhibitor, and these effects were recovered by EX527 treatment (Fig. 4E). These results suggest that the miR-132 inhibitor suppressed H/R-induced oxidative stress via activation of PGC-1α/Nrf2 signalling by upregulating SIRT1.

Inhibition of miR-132 suppresses H/R-induced pyroptosis. Finally, the effects of miR-132 on cell pyroptosis in the H/R model were determined. As shown in Fig. 5A and B, the expression of the pyroptosis-related proteins NLRP3, caspase-1 and IL-1β were all significantly suppressed by miR-132 inhibitor treatment compared with the H/R model group. However, when SIRT1 was inhibited by EX527, the inhibitory effects of the miR-132 inhibitor were significantly recovered, suggesting that the inhibitory effects of the miR-132 inhibitor on cell pyroptosis were mediated through regulation of SIRT1. The cell pyroptosis analysis revealed that the cell pyroptosis induced by H/R was also significantly inhibited by the miR-132 inhibitor, which was reversed by treatment with EX527 (Fig. 5C and D). Similar results were observed for the

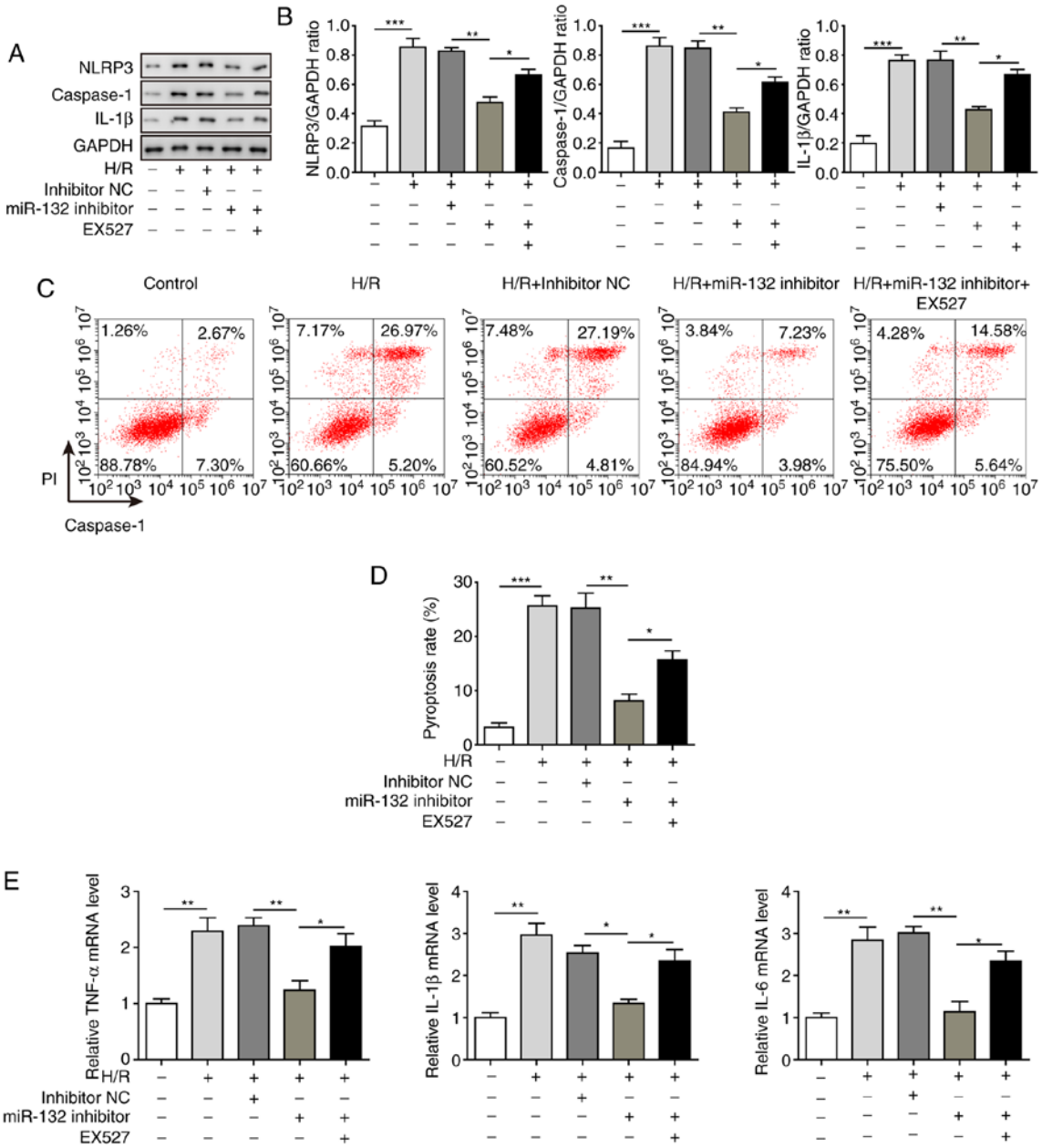

Figure 5. Inhibition of miR-132 suppressed H/R-induced pyroptosis. (A) Expression of pyroptosis-related proteins NLRP3, caspase-1 and IL-1β in different groups of cells by western blotting. (B) Quantification of NLRP3, caspase-1 and IL-1β protein expression. (C) Cell pyroptosis detection by flow cytometry analysis. (D) Quantification of pyroptosis rates in the different groups in C. (E) mRNA levels of IL-1β, IL-6 and TNF-α in different groups of cells by RT-qPCR. The results are representative of three independent experiments. Error bars represent the mean ± standard deviation. *P<0.05, **P<0.01 and ***P<0.001. H/R, hypoxia/reoxygenation; IL, interleukin; TNF, tumour necrosis factor.

mRNA levels of IL-1β, IL-6 and TNF-α in the H/R model, which were also significantly decreased by the miR-132 inhibitor and reversed by EX527 treatment (Fig. 5E). These results suggested that miR-132 inhibition may suppress H/R-induced cell pyroptosis through regulation of SIRT1.

Discussion

Despite numerous studies on myocardial I/R injury, the mechanisms underlying the role of miRNAs in myocardial I/R injury remain unclear. Recently, the role of miRNAs in myocardial ischaemia has been reported in several studies. He *et al* demonstrated that the upregulation of miR-1 and miR-133a decreased the apoptosis of cardiomyocytes in myocardial ischaemic post-conditioning (26). Li *et al* observed that the miR-939-mediated nitric oxide signalling pathway was involved in myocardial ischaemia (27). However, no study had focused on the role of miR-132 and its association with oxidative stress and pyroptosis in myocardial I/R injury. To the best of our knowledge, the present study was the first to demonstrate that inhibition of miR-132 could improve myocardial I/R injury by inhibiting oxidative stress and pyroptosis through activation of PGC-1α/Nrf2 signalling by targeting SIRT1.

A role for miR-132 has been reported in several diseases. Smith *et al* reported that miR-132 was downregulated in Alzheimer's disease, and that deficiency of miR-132 resulted in increased tau expression, phosphorylation and aggregation (28). You *et al* demonstrated that miR-132 inhibited the migration and

invasion of lung cancer cells by targeting ZEB2 (29). miR-132 is also considered to be associated with ischaemia-related diseases. Keasey *et al* demonstrated that miR-132 was upregulated by ischaemic preconditioning in cultured hippocampal neurons (12). Huang *et al* found that the expression of miR-132 was increased in cardiac fibroblasts following ischaemia, and inhibition of miR-132 enhanced angiogenesis and reduced cell apoptosis (30). In the present study, an *in vivo* mouse myocardial I/R injury model was successfully established and miR-132 was found to be upregulated in myocardial I/R injury, which was consistent with previous studies.

A number of studies have demonstrated the role of SIRT1 in myocardial I/R injury. Hsu *et al* demonstrated that SIRT1 could protect against myocardial I/R injury (31). Fan *et al* reported that SIRT1 contributed to the resistance to I/R-induced acute kidney injury (32). It was also observed that melatonin may protect against myocardial I/R injury by activating SIRT1 signalling and inhibiting oxidative stress (33). In diabetic rats, the activation of SIRT1 led to activation of eNOS, ultimately resulting in improvement of myocardial I/R injury (34). The present study demonstrated that SIRT1 was downregulated in myocardial I/R injury and was a direct target of miR-132. It was also observed that the overexpression of miR-132 downregulated SIRT1 levels and that the inhibition of miR-132 increased SIRT1 expression, indicating that miR-132 negatively regulated SIRT1. Previous studies have also reported the association between SIRT1 and miR-132. miR-132 was found to affect aberrant B-cell cytokine regulation by targeting SIRT1 in patients with relapsing-remitting multiple sclerosis (35). Xiong *et al* demonstrated that downregulation of miR-132 suppressed endoplasmic reticulum (ER) stress in colitis by activation of SIRT1 (36). The present study was the first to demonstrate the negative regulatory association between miR-132 and SIRT1 in myocardial I/R injury.

The association between SIRT1/PGC-1α/Nrf2 signalling-mediated oxidative stress and myocardial I/R injury has been demonstrated in several studies. Wang *et al* observed that SIRT1/Nrf2 signalling was inhibited while ER stress was activated in an I/R model (16). Pan *et al* demonstrated that sulforaphane exerted an antioxidant effect on myocardial I/R injury through the activation of the Nrf2/HO-1 antioxidant pathway (37). In the present study, it was also observed that PGC-1α/Nrf2 signalling and SIRT1 were inhibited in myocardial I/R injury. The suppression of PGC-1α/Nrf2 signalling contributed to oxidative stress, which was reflected by the upregulation of MDA and iNOS and downregulation of SOD and eNOS in a myocardial injury model. The effects induced by myocardial injury were reversible by inhibition of miR-132, which caused activation of PGC-1α/Nrf2 signalling and suppression of oxidative stress.

Pyroptosis is associated with oxidative stress. It is considered that exacerbated oxidative stress and inflammation can induce autophagy, apoptosis and pyroptosis (38). Although the role of oxidative stress in I/R-induced injury has been well established, few studies have demonstrated the effects of miR-132 on oxidative stress in myocardial I/R injury and the possible association with pyroptosis. The present study demonstrated that the levels of NLRP3, caspase-1 and IL-1β and the pyroptosis ratio were markedly increased in myocardial I/R injury. We first demonstrated that inhibition of miR-132 may improve myocardial I/R injury by suppressing oxidative stress-induced pyroptosis by targeting SIRT1. Moreover, inhibition of SIRT1 by EX527 markedly reversed the effects of miR-132 inhibition, further confirming the role of the miR-132/SIRT1 axis in myocardial I/R injury. However, further study is required to gain deeper insight into the association between oxidative stress and pyroptosis in myocardial I/R injury.

In conclusion, *in vivo* and *in vitro* studies were conducted to investigate the role of miR-132 in myocardial I/R injury. The results revealed that miR-132 was upregulated in myocardial I/R injury and that inhibition of miR-132 was able to improve myocardial I/R injury by inhibiting oxidative stress and pyroptosis through activation of PGC-1α/Nrf2 signalling by targeting SIRT1. The findings of this study may provide deeper insight into the role of miR-132 in myocardial I/R injury and help identify new treatment targets for myocardial I/R injury.

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Availability of data and materials

All data generated or analysed during the present study are included in this published article.

Authors' contributions

ZY and LSL conceived the study. LKS collected the data. LL analysed the data. ZY and LSL performed the experiments. ZY and LSL provided the resources and supervised the study. ZY, LSL, LKS and LL wrote the original draft of the manuscript. ZY and LSL reviewed and edited the manuscript. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Animal Care Committee at Tongji Medical College of Huazhong University of Science and Technology (Hubei, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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