

# Postconditioning attenuates myocardial ischemia-reperfusion injury by inhibiting complement activation and upregulation of miR-499

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**Abstract.** The complement system plays a vital role in myocardial ischemia/reperfusion (I/R) injury. microRNA (miR)-499 is involved in the cardioprotection of ischemic postconditioning (IPostC). The present study aimed to study the role of the complement system and miR-499 in IPostC. Rat hearts were subjected to coronary ligation for 30 min, followed by reperfusion for 2 h. IPostC was introduced at the onset of reperfusion with three cycles of reperfusion for 30 sec and coronary artery occlusion for 30 sec. To study the role of miR-499 in IPostC, adeno-associated virus (AAV) vectors of miR-499-5p (AAV-miR-499-5p) and miR-499-5p-sponge (AAV-miR-499-5p-sponge) were transfected via tail vein injection, followed by IPostC protocols. Cardiac injury as well as the status of local and systemic complement activation and inflammation were assessed. IPostC significantly attenuated I/R-induced rat cardiomyocyte apoptosis and

the myocardial infarct size. These beneficial effects were accompanied by decreased local and circulating complement component (C)3a and C5a levels, decreased inflammatory marker expression, decreased NF- $\kappa$ B signaling and increased cardiac miR-499 expression. AAV-miR-499-5p prevented local and systemic complement activation and inflammation as well as enhanced the cardioprotection of IPostC, whereas AAV-miR-499-5p-sponge produced the opposite effects. In summary, IPostC protected the rat myocardium against I/R injury, by inhibiting local and systemic complement activation; inflammation; NF- $\kappa$ B signaling; and upregulation of miR-499. As such, miR-499 may have a critical role in IPostC-mediated cardioprotection against I/R injury.

## Introduction

Reperfusion following acute myocardial infarction is a double-edged sword. On one hand, reperfusion is the only option to salvage a dying myocardium (1). On the other hand, reperfusion can initiate myocardial ischemia/reperfusion (I/R) injury (MIRI), which produces additional serious tissue damage and may even become fatal to some patients receiving revascularization therapy (2).

The mechanisms underlying MIRI are poorly understood. Recently, complement activation has been found to play a pivotal role in MIRI (3-5). As part of the innate immune defense, the complement system consists of over 30 plasma and cell membrane proteins that are activated in a sequential manner, producing both local and systemic manifestations (6). For instance, excessive complement activation products complement component (C)3a and C5a are aggravating factors for myocardial necrosis and inflammatory cell infiltration (7). Conversely, inhibition of the complement cascade has been shown to significantly reduce MIRI (8,9). Therefore, targeting the complement cascade may be a promising therapeutic strategy for treating I/R injury. Ischemic postconditioning (IPostC), defined as rapid, intermittent interruptions of blood flow in early reperfusion, has been shown to alleviate I/R injury in animal models and patients with acute myocardial infarction (10-12). However, the mechanisms of IPostC-related

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**Abbreviations:** AAV, adeno-associated virus; C3a, complement component 3a; C5a, complement component 5a; I/R, ischemia/reperfusion; IPostC, ischemic postconditioning; miRNA/miR, microRNA; MIRI, myocardial I/R injury; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; TTC, triphenyl tetrazolium chloride

**Key words:** postconditioning, miR-499; myocardial ischemia/reperfusion injury, the complement system, inflammation

cardioprotection are not well understood. To the best of our knowledge, the role of critical novel pathways of I/R injury, such as activation of the complement system, have not been studied in myocardial IPostC.

MicroRNAs (miRNAs/miR) play a vital role in regulating ischemic injury (13). miR-499, a cardiac-enriched miRNA, participates in protection of the ischemic myocardium (14,15). A recent study has reported that miR-499 has an antiapoptotic effect against MIRI (16). However, the potential role and mechanism of action underlying the cardioprotective effect of miR-499 in IPostC are poorly understood. The present study investigated the role of complement activation and the possible involvement of miR-499 in IPostC of the rat myocardium during I/R injury.

## Materials and methods

**Animals.** A total of 105 male adult Sprague-Dawley rats (8 weeks old, weighing 240-280 g) were collected from the Laboratory Animal Center of Guangxi Medical University. The rats were housed under standard laboratory conditions (temperature,  $25\pm 2^{\circ}\text{C}$ ; relative humidity,  $50\pm 15\%$ ; 12 h dark/light cycle) and rats were permitted *ad libitum* access to food and water. All animal protocols were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, USA) and were approved by the Animal Care and Use Committee of Guangxi Medical University.

**MIRI model.** The rat MIRI model was established using a previously described method (17). Briefly, the rats were intraperitoneally injected with sodium pentobarbital (2%, 50 mg/kg) for general anesthesia and mechanically ventilated using a small animal respirator. A left parasternal incision was made in the fourth intercostal space to expose the heart. The left anterior descending coronary artery (LAD) was temporarily ligated at 2-3 mm below the lower edge of the left auricle using an 8-0 silk suture. A small plastic tube was inserted through the ligature to form a snare to enable reperfusion by reversing occlusion. Cardiac ischemia was induced by tightening the ligature around the plastic tube for 30 min. Successful ligation was visually confirmed when the anterior wall of the left ventricle turned pale and by elevation of the ST segment on precordial leads of the electrocardiogram. Reperfusion was induced by loosening the ligation via the plastic tube, which lasted for 2 h. IPostC was performed at the onset of reperfusion with three cycles of reperfusion for 30 sec, followed by coronary artery occlusion for 30 sec. Immediately after reperfusion, the rats were euthanized by cervical dislocation. Death of the rats was confirmed by the lack of a heart beat and respiration, and then the left ventricle of rats was harvested for further analysis.

**Experimental grouping.** The rats were randomly divided into following groups ( $n=15$  per group): i) Sham, in which the ligature was passed under the LAD, but not tied, and maintained for 150 min; ii) I/R, in which the rats were subjected to 30 min of ischemia followed by 2 h of reperfusion; iii) IPostC, in which the rats underwent three cycles of 30 sec of reperfusion and 30 sec of ischemia, initiated

immediately at the onset of reperfusion; iv) adeno-associated virus (AAV), in which the empty AAV vector ( $1\times 10^{12}$  v.g./rat) was injected into the tail vein and did not receive any other treatment; v) AAV + IPostC, in which the empty AAV vector ( $1\times 10^{12}$  v.g./rat) was injected into the tail vein, followed by the IPostC procedure after 4 weeks; vi) sponge + IPostC, which received the AAV vector of miR-499-5p-sponge (AAV-miR-499-5p-sponge,  $1\times 10^{12}$  v.g./rat) via tail vein injection, followed by the IPostC procedure after 4 weeks; vii) miR-499 + IPostC, which received the AAV vector of miR-499-5p (AAV-miR-499-5p,  $1\times 10^{12}$  v.g./rat) followed by the IPostC procedure after 4 weeks. In total, 5 of the 105 rats used in this study were excluded: A total of 2 in the I/R group and 1 in the IPostC group died due to ventricular fibrillation; 1 in the AAV + IPostC group died due to cardiogenic shock during reperfusion; and 1 in the sponge + IPostC group died due to viral delivery failure. The results described are for the remaining 100 rats.

**AAV transfection.** The AAV-miR-499-5p (5'-GCTGTTAAG ACTTGCAGTGATGTTTAGCTCCTCTCCATGTGAACA TCACAGCAAGTCTGTGCTGC-3'), AAV-miR-499-5p-sponge (5'-AAACATCACTGCAAGTCTTAATATACAAAC ATCACTGCAAGTCTTAAACATCAAACATCACTGCAAGTCTTAATCTTCAAAACATCACTGCAAGTCTTAA-3'), and empty AAV vector (pHBAAV-U6-MCS-CMV-EGFP; Hanbio Biotechnology Co., Ltd.) as AAV control were constructed using previously described methods (18). The AAV vectors were injected through the tail vein at a dose of  $1.0\times 10^{12}$  genome copies per rat. Successful transfections were confirmed by detection of miR-499-5p expression in myocardial tissue at 4 weeks after injection.

**Reverse transcription-quantitative PCR (RT-qPCR).** Total RNA of ischemic myocardium was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and cDNA was synthesized from 1  $\mu\text{g}$  of total RNA using the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, Inc.) according to the manufacturer's instructions. qPCR was performed in 96-well plates using 2X SYBR Green qPCR ProMix (Guangzhou Yingzan Biological Technology Co., Ltd.) using an ABI 7300 Real-Time PCR System (Applied Biosystems). The thermocycling conditions were as follows:  $95^{\circ}\text{C}$  for 2 min, 40 cycles of  $95^{\circ}\text{C}$  for 5 sec, and  $60^{\circ}\text{C}$  for 30 sec. All reactions were performed in triplicate. U6 was used as the reference gene. The relative expression of miRNA was determined using the  $2^{-\Delta\Delta\text{Cq}}$  method (19). The primer sequences used for RT-qPCR are listed in Table I.

**Western blotting.** Frozen rat myocardial tissue was lysed in radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology) containing 1% phenylmethylsulfonyl fluoride (Beyotime Institute of Biotechnology) on ice. The protein concentration was measured using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). Normalized protein samples (30  $\mu\text{g}$ ) were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to PVDF (EMD Millipore). The membranes were blocked with 5% nonfat milk at room temperature for 1 h, followed by incubation with primary antibodies against C3a (Abcam;

Table I. The primer sequences for reverse transcription-quantitative PCR.

Gene	Sequence
U6 forward	5'-CTCGCTTCGGCAGCACAC-3'
U6 reverse	5'-AACGCTTCACGAATTTGCGT-3'
miR-499 forward	5'-TTAAGACTTGACAGTGATGTTT-3'
miR-499 reverse	5'-CAGTGCAGGGTCCGAGGTAT-3'
miRT Random	5'-GTCGTATCCAGTGCAGGGTCC
Primer <sup>a</sup>	GAGGTATTTCGACTGGATACGACNNNNN-3'

miR, microRNA. <sup>a</sup>Used in reverse transcription for cDNA synthesis.

cat. no. 171080; 1:1,000), C5a (Abcam; cat. no. 202039; 1:1,000), NF- $\kappa$ B p65 (Cell Signaling Technology, Inc.; cat. no. 8242; 1:1,000), TNF- $\alpha$  (Cell Signaling Technology, Inc.; cat. no. 11948; 1:1,000), IL-1 $\beta$  (Abcam; cat. no. 200478; 1:1,000), IL-6 (Abcam; cat. no. 208113; 1:1,000) and  $\beta$ -actin (Abcam; cat. no. 8226; 1:1,000) overnight at 4°C. The membranes were washed three times with Tris-buffered saline containing 0.1% Tween-20 and then incubated with infrared dye-conjugated secondary antibodies (LI-COR Biosciences; cat. no. 926-32211; 1:10,000) for 1 h at room temperature. Protein bands were detected using an Odyssey Infrared Imaging System (LI-COR Biosciences) and the relative intensity of the bands was quantified using ImageJ v1.8.0 software (National Institutes of Health).

**ELISAs.** A total of 2 h after reperfusion of the myocardium, 2 ml of venous blood was collected into EDTA-coated tubes. After centrifugation at 2,000  $\times$  g and 4°C for 10 min, the plasma samples were collected and stored at -80°C for further analysis. The plasma levels of C3a (cat. no. 08510r), C5a (cat. no. 08513r), TNF- $\alpha$  (cat. no. 11987r), NF- $\kappa$ B p65 (cat. no. 08788r), IL-1 $\beta$  (cat. no. 08055r) and IL-6 (cat. no. 04640r) were detected using commercial ELISA kits (Cusabio Technology LLC).

**Triphenyl tetrazolium chloride (TTC) staining.** The myocardial infarct area was assessed using TTC staining. Briefly, rat heart tissues were quickly isolated and washed with cold saline, then immediately frozen at temperature of -70°C for 10 min and finally sliced into 2-mm cross-sections from 2 mm below the ligation line. The slices were stained in 2% TTC solution (Beijing SolarbioScience and Technology Co., Ltd.) at 37°C for 20 min and then fixed in 4% paraformaldehyde at room temperature for 20 min. The infarct size was quantified using ImageJ software.

**TUNEL staining.** TUNEL staining was performed on the myocardial sections to detect apoptotic cardiomyocytes, according to the manufacturer's instructions (Roche Diagnostics). Myocardial samples were fixed in 4% paraformaldehyde at 4°C for 24 h and embedded in paraffin. The dewaxed myocardial tissue sections were immersed in 3% hydrogen peroxide in methanol for 10 min at room temperature, and washed 3 times with PBS; then the tissue sections and proteinase K working solution incubated at 37°C for

30 min, which the tissue sections and TdT reaction mixture are incubated at 37°C for 2 h. After washing 3 times with PBS, the nuclei were counterstained with DAPI solution (5  $\mu$ g/ml) at room temperature for 5 min. A total of 50  $\mu$ l anti-fade mounting medium was added to TUNEL-positive cells, which were observed in five randomly selected visual fields using a fluorescence microscope (magnification,  $\times$ 200). The index of apoptosis was expressed as follows: Index of apoptosis=(number of apoptotic cardiomyocytes/total number of cardiomyocytes)  $\times$ 100%.

**Statistical analysis.** The data were presented as the mean  $\pm$  SD and analyzed using SPSS 13.0 (SPSS Inc.) software. One-way ANOVAs were performed using post hoc Tukey's multiple comparison tests. A P-value <0.05 was considered statistically significant.

## Results

***IPostC reduces I/R-induced myocardial complement activation.*** Generation of C3a and C5a is commonly involved in all three complement activation pathways (20). Therefore, the present study examined the effects of IPostC on complement activation by detecting the expression of C3a and C5a in the rat myocardium. Western blot analysis showed that the expression levels of C3a and C5a were significantly increased in the rat myocardium following I/R injury, whereas IPostC attenuated the C3a and C5a upregulation following I/R (Fig. 1).

***IPostC reduces the I/R-induced myocardial inflammatory response.*** Previous studies have demonstrated that activation of the complement system promotes inflammation in heart, kidney and brain tissues (21-23). To determine whether the inhibitory effect of IPostC on the complement system affects the inflammatory response in the myocardium the expression of the proinflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$  and IL-6 was examined. I/R significantly increased the expression levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6, as measured by western blot analyses compared to the sham group. IPostC then significantly reduced the expression levels of these inflammatory cytokines in the rat myocardium, compared with the I/R group, although this did not reach the expression levels observed in the sham group (Fig. 2).

***IPostC reduces I/R-induced NF- $\kappa$ B activation.*** To explore the underlying mechanism of action behind the anti-inflammatory effect of IPostC, the present study examined the expression levels of NF- $\kappa$ B p65, a transcription factor critical for the activation of the complement system and proinflammatory cytokines. I/R induced a significant increase in the NF- $\kappa$ B p65 expression levels in the rat myocardium compared with the sham group, which was partially attenuated by IPostC (Fig. 3).

***miR-499 reduces the expression of complement factors and inflammatory cytokines in the IPostC rat myocardium.*** Previous studies have suggested that miR-499 has a protective effect against myocardial ischemia (14,15,24). In order to further understand the role of miR-499 in myocardial IPostC, the present study transfected rats with empty AAV,

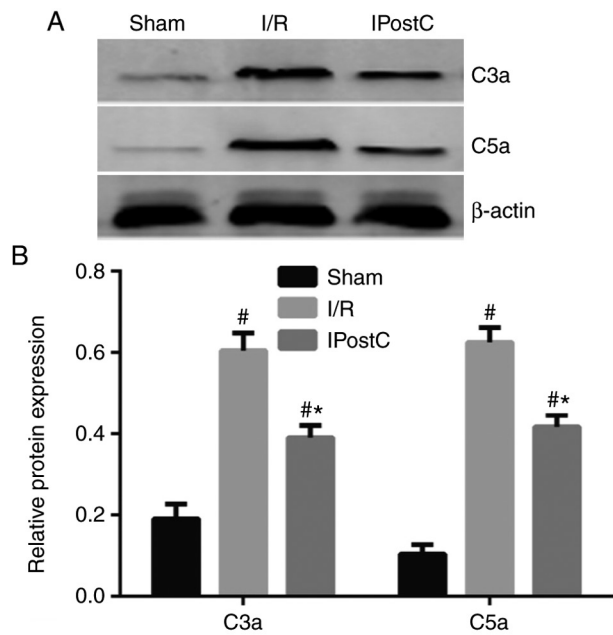


Figure 1. IPostC reduces I/R-induced expression of C3a and C5a in the rat myocardium. (A) Western blotting showing the expression of C3a and C5a under I/R injury and IPostC, and (B) quantification of C3a and C5a expression. Data are presented as the mean  $\pm$  SD,  $n=4$ . The experiments were repeated three times. <sup>#</sup> $P<0.05$  vs. Sham; <sup>#</sup> $P<0.05$  vs. I/R. C, complement component; I/R, ischemia/reperfusion; IPostC, ischemic postconditioning.

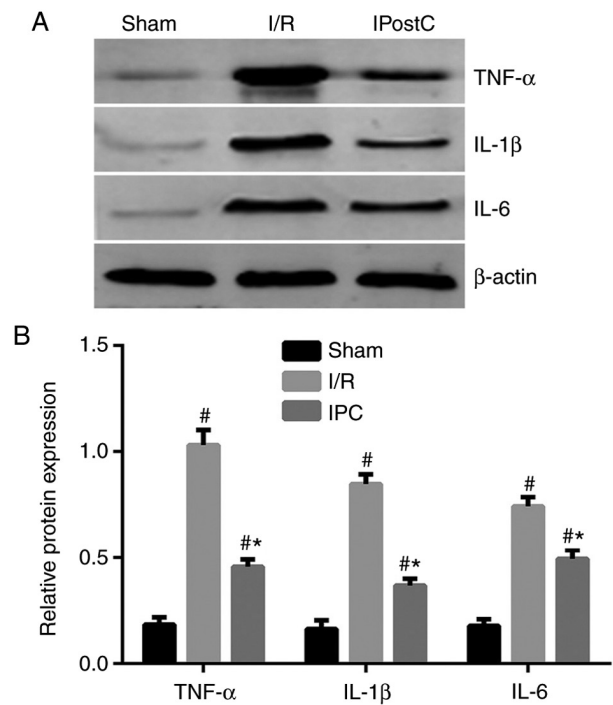


Figure 2. IPostC reduces the I/R-induced expression of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in the rat myocardium. (A) Western blotting of rat myocardial tissue showing the expression of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 under Sham, I/R injury and IPostC conditions, and (B) quantification of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 expression. Data are presented as the mean  $\pm$  SD,  $n=4$ . The experiments were repeated three times. <sup>#</sup> $P<0.05$  vs. Sham; <sup>#</sup> $P<0.05$  vs. I/R. I/R, ischemia/reperfusion; IPostC, ischemic postconditioning.

AAV-miR-499-5p and AAV-miR-499-5p-sponge, and then subjected the rats to MIRI protocols with or without IPostC.

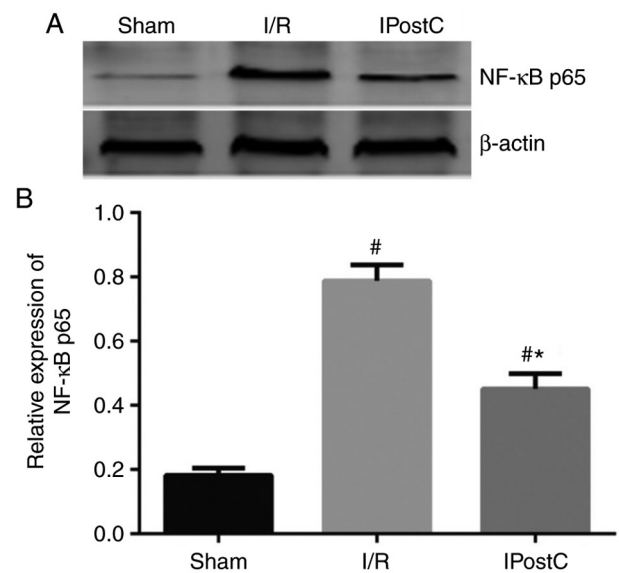


Figure 3. IPostC reduces I/R-induced NF- $\kappa$ B activation. (A) Western blotting showing the expression of NF- $\kappa$ B p65 under I/R injury and IPostC, and (B) quantification of NF- $\kappa$ B p65 expression. Data are presented as the mean  $\pm$  SD,  $n=4$ . The experiments were repeated three times. <sup>#</sup> $P<0.05$  vs. Sham; <sup>#</sup> $P<0.05$  vs. I/R. I/R, ischemia/reperfusion; IPostC, ischemic postconditioning.

None of the rats showed behavioral abnormalities following injection of the AAV vectors. RT-qPCR confirmed the successful AAV transfection of the agents in the rat myocardium in all animals, treated either with or without IPostC (Fig. 4A). Compared with the sham group, the expression of miR-499 was significantly decreased in the I/R group, but it was significantly increased in the IPostC group (Fig. 4A). Notably, in rats receiving IPostC, AAV-miR-499-5p-sponge significantly increased the expression levels of C3a and C5a (Fig. 4B) as well as the proinflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$  and IL-6 (Fig. 4B), compared with the rats receiving empty AAV. In contrast, AAV-miR-499-5p significantly reversed the upregulation of complement factors and inflammatory cytokines in the IPostC rat myocardium (Fig. 4B). Taken together, these results strongly support a protective effect of miR-499 in the IPostC rat myocardium, in part, by inhibiting the upregulation of complement factors and inflammatory cytokines.

**miR-499 inhibits NF- $\kappa$ B activation in the rat myocardium treated with IPostC.** The present study further examined the effect of miR-499 on NF- $\kappa$ B activation during IPostC. The expression of NF- $\kappa$ B p65 was not affected by the AAV (Fig. 5; AAV + IPostC). However, transfection with AAV-miR-499-5p-sponge significantly increased NF- $\kappa$ B p65 expression (Fig. 5; sponge + IPostC vs. IPostC), whereas transduction with AAV-miR-499-5p significantly inhibited NF- $\kappa$ B p65 expression (Fig. 5; miR-499 + IPostC vs. IPostC). Together, these results further indicated a protective role of miR-499 in myocardial IPostC, in part by inhibiting NF- $\kappa$ B p65 pathway activation.

**miR-499 reduces circulating inflammatory cytokines in rats treated with IPostC.** To further assess the effects of miR-499



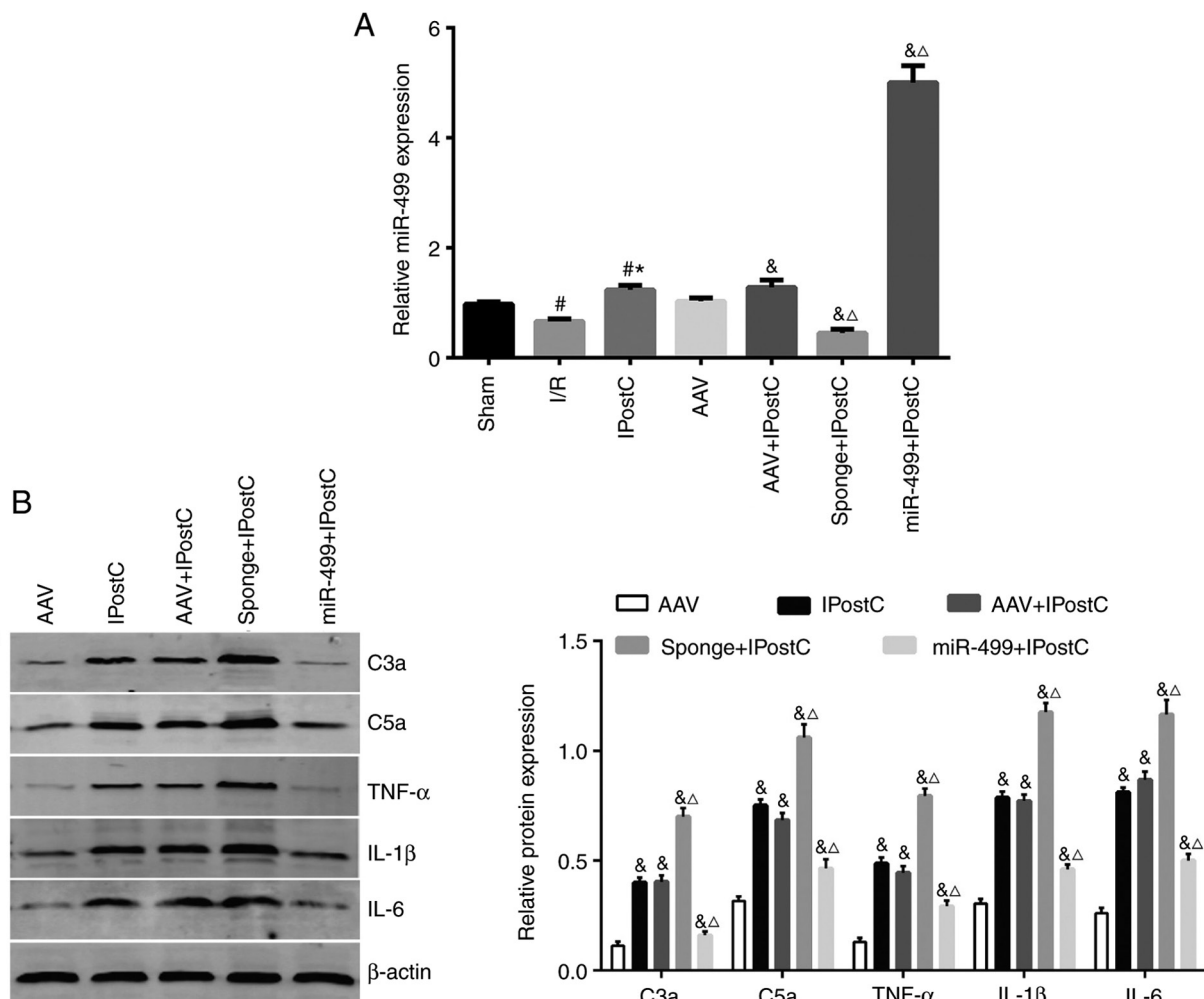


Figure 4. The effects of miR-499 on the expression of complement factors and inflammatory cytokines in the rat myocardium. (A) Reverse transcription-quantitative PCR analysis of miR-499 expression levels in the rat myocardium receiving transduction of AAV-miR-499-5p, AAV-miR-499-5p-sponge and AAV control, with and without IPostC. The AAV injection was performed at 4 weeks prior to the PCR analysis. (B) Western blot analysis of C3a, C5a, TNF- $\alpha$ , IL-1 $\beta$  and IL-6 expression in the treated rat myocardium. Quantification of the band intensity is presented beside the representative blots. Data are presented as the mean  $\pm$  SD, n=4. The experiments were repeated three times. \*P<0.05 vs. Sham; #P<0.05 vs. I/R; &P<0.05 vs. AAV; &ΔP<0.05 vs. AAV + IPostC. AAV, adeno-associated virus; C, complement component; I/R, ischemia/reperfusion; IPostC, ischemic postconditioning; miR, microRNA.

on the systemic inflammatory status during IPostC, the present study transfected rats with empty AAV, AAV-miR-499-5p and AAV-miR-499-5p-sponge and then measured the plasma levels of circulating complement factors and proinflammatory cytokines, including C3a, C5a, TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and NF- $\kappa$ B p65, using ELISAs. There were no significant differences in the plasma measurements between the IPostC and AAV + IPostC groups (Table II). In comparison with the IPostC and AAV + IPostC groups, the plasma levels of C3a, C5a, TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and NF- $\kappa$ B p65 were all significantly higher in the rats receiving AAV-miR-499-5p-sponge and they were all significantly lower in the rats receiving AAV-miR-499-5p (Table II).

**miR-499 reduces I/R-induced cardiomyocyte apoptosis during IPostC.** The present study assessed cell apoptosis in the rat myocardium using TUNEL assays. Compared with the sham group, a significantly higher number of TUNEL-positive cells was detected in the I/R group (Fig. 6; brown nuclei in I/R vs. Sham). IPostC treatment produced significantly fewer

apoptotic cells than the I/R group (Fig. 6; IPostC vs. I/R). Transfection of the empty AAV had a minimal effect on cell apoptosis (Fig. 6; IPostC vs. AAV + IPostC). In contrast, AAV-miR-499-5p further reduced the number of apoptotic cardiomyocytes in the IPostC rats (Fig. 6; miR-499 + IPostC vs. IPostC), whereas AAV-miR-499-5p-sponge significantly increased the number of TUNEL-positive cells in the IPostC myocardium (Fig. 6; sponge + IPostC vs. IPostC). Taken together, these results indicated a protective role of miR-499 against apoptosis in the IPostC rat myocardium.

**miR-499 reduces the rat myocardial infarct size during IPostC.** Finally, the present study assessed the role of miR-499 on IPostC by measuring the myocardial infarct size. There was virtually no visible myocardial infarction in the sham group (Fig. 7). In contrast, a large infarct zone was visible on the left ventricle of the I/R group (Fig. 7; I/R). Interestingly, IPostC significantly reduced the infarct myocardium area, with or without introduction of AAV compared with the I/R group (Fig. 7; IPostC vs. I/R; AAV + IPostC vs. I/R).

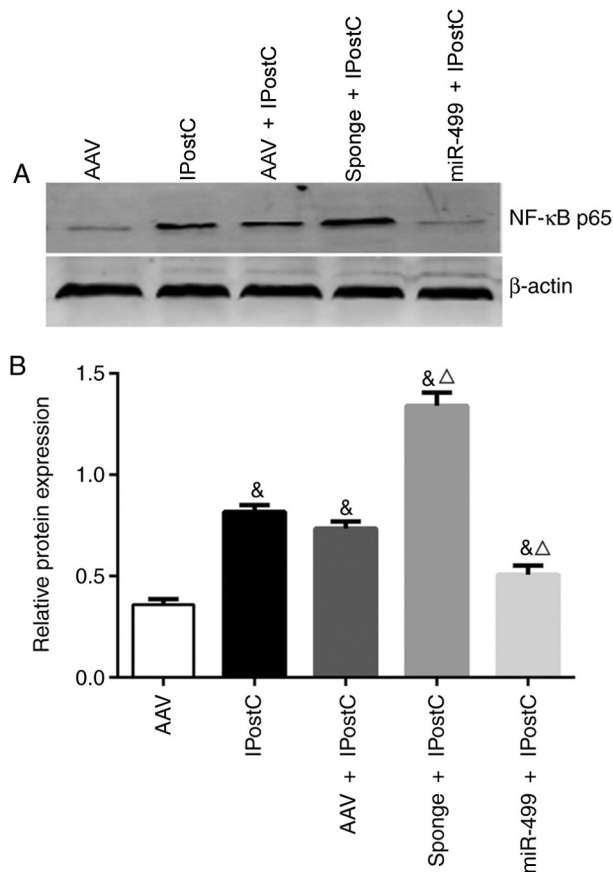


Figure 5. miR-499 inhibits NF- $\kappa$ B activation in the rat myocardium treated with IPostC. (A) Western blotting of NF- $\kappa$ B p65 expression in the rat myocardium treated with IPostC and the various AAV vectors. (B) Quantification of NF- $\kappa$ B p65 expression in the rat myocardium in (A). Data are presented as the mean  $\pm$  SD,  $n=4$ . The experiments were repeated three times. \* $P<0.05$  vs. AAV;  $\Delta P<0.05$  vs. AAV + IPostC. AAV, adeno-associated virus; IPostC, ischemic postconditioning; miR, microRNA.

Notably, overexpression of the miR-499 further reduced the myocardial infarct zone following IPostC (Fig. 7; IPostC and AAV + IPostC vs. miR-499 + IPostC). Similarly, inhibition of miR-499 abolished the protective effect of IPostC on the myocardium and resulted in an increased infarct size (Fig. 7; IPostC and AAV + IPostC vs. sponge + IPostC).

## Discussion

The major finding of the present study was that IPostC attenuated I/R-induced myocardial injury in a miR-499-dependent manner. IPostC effectively reduced I/R-induced complement activation, local and systemic inflammation, cardiomyocyte apoptosis and the myocardial infarct size. To the best of our knowledge, the present study is the first to demonstrate that miR-499 is an essential regulator of IPostC-mediated protection against I/R-induced myocardial injury, which is in part through local and systemic inhibition of complement activation, inflammation and NF- $\kappa$ B signaling. Taken together, these results revealed a novel mechanism of action for miRNA-mediated IPostC protection.

Complement activation plays an important role in MIRI. Hill and Ward (25) first reported C3 deposition in an infarcted myocardium and revealed the relevance of complement

activation in MIRI. In addition, Yasojima *et al* (26) have shown that endogenous C3 produced by the heart contributes to the degree of MIRI. Furthermore, it has been shown that suppressing specific components of the complement cascade protects against MIRI (27-30) and that ischemic preconditioning attenuates MIRI by inhibiting complement activation (31). However, to the best of our knowledge, the effect of IPostC on the complement system has not been reported. The present study found that the local and circulating levels of C3a and C5a were higher in the I/R group and lower in the IPostC group, cementing a critical role of complement factors in IPostC-induced cardioprotection.

Inflammatory responses represent a major pathological process leading to MIRI (32). An unchecked local inflammatory response has been observed in tissues subjected to I/R, whereas IPostC appears to inhibit the inflammatory response by reducing the expression or activation of local inflammatory mediators, or by decreasing inflammatory cytokine release (33,34). Approaches that aim to inhibit these inflammatory responses following I/R could potentially lead to the attenuation of MIRI. Previous studies have shown that IPostC inhibits inflammation in renal tissues following I/R (35-37). The present study demonstrated a significant inflammatory response in rat cardiomyocytes following I/R injury and that IPostC significantly attenuated the inflammatory responses, partially due to the effect of miR-499 on reducing the expression of proinflammatory mediators in the IPostC rat myocardium.

The importance of complement activation is highlighted by its interaction with the inflammatory response and the NF- $\kappa$ B pathway, which together profoundly determine the outcome of I/R-induced tissue injury (38). Activated C3a and C5a attract proinflammatory leukocytes and promote the release of inflammatory cytokines during I/R (39). Once stimulated, such an inflammatory process tends to be self-propagative, resulting in sustained apoptosis and necrosis following MIRI (40). Recently, a correlation between complement activation and the NF- $\kappa$ B signaling pathway has been reported (38,41). NF- $\kappa$ B belongs to a family of transcription factors that plays a key role in regulating inflammatory responses and cell survival (42). Prior work has shown that IPostC inhibits I/R-induced inflammation and organ injury through NF- $\kappa$ B signaling (35,43). However, the exact role of the complement-NF- $\kappa$ B interaction in myocardial IPostC is unclear. The present results showed, for the first time, that IPostC simultaneously blocked complement activation and NF- $\kappa$ B signaling, supporting the notion that inhibition of both complement and NF- $\kappa$ B signaling may be involved in IPostC-induced cardioprotection.

miR-499 is most abundantly expressed in the heart and plays important roles in myocardial infarction and MIRI (44,45). It has been shown that the miR-499 expression levels correlate with cardiomyocyte apoptosis and the severity of the infarction induced by I/R (15). For example, reperfusion reduces the expression levels of miR-499 in canine left ventricles and the miR-499 expression levels are negatively correlated with troponin T and creatine kinase-muscle/brain levels (24). However, the role of miR-499 in IPostC was unknown until a report in 2016 (16). In agreement with this previous report, the present study found that MIRI resulted in a reduction of miR-499, which was correlated with increased cardiomyocyte

Table II. The plasma levels of complement factors and inflammatory cytokines in rats (mean  $\pm$  SD).

Group	C3a ( $\mu$ g/ml)	C5a (ng/ml)	TNF- $\alpha$ (ng/l)	IL-1 $\beta$ (ng/ml)	IL-6 (pg/ml)	NF- $\kappa$ B p65 (pg/ml)
Sham	887.5 $\pm$ 67.6	116.4 $\pm$ 7.8	276.4 $\pm$ 5.7	25.9 $\pm$ 1.2	70.3 $\pm$ 4.1	644.4 $\pm$ 26.6
I/R	1941.0 $\pm$ 47.1 <sup>a</sup>	235.3 $\pm$ 11.0 <sup>a</sup>	540.3 $\pm$ 6.9 <sup>a</sup>	60.1 $\pm$ 1.5 <sup>a</sup>	181.8 $\pm$ 4.5 <sup>a</sup>	1282.0 $\pm$ 26.6 <sup>a</sup>
IPostC	1333.9 $\pm$ 69.8 <sup>a,b</sup>	180.6 $\pm$ 8.8 <sup>a,b</sup>	355.7 $\pm$ 13.2 <sup>a,b</sup>	36.2 $\pm$ 1.1 <sup>a,b</sup>	130.1 $\pm$ 7.7 <sup>a,b</sup>	813.4 $\pm$ 20.0 <sup>a,b</sup>
AAV + IPostC	1366.7 $\pm$ 51.2 <sup>a,b</sup>	188.3 $\pm$ 9.5 <sup>a,b</sup>	360.0 $\pm$ 14.4 <sup>a,b</sup>	40.6 $\pm$ 1.6 <sup>a,b</sup>	131.0 $\pm$ 8.1 <sup>a,b</sup>	849.6 $\pm$ 19.2 <sup>a,b</sup>
Sponge + IPostC	1735.0 $\pm$ 42.6 <sup>c,d</sup>	212.3 $\pm$ 9.4 <sup>c,d</sup>	445.1 $\pm$ 7.4 <sup>c,d</sup>	46.9 $\pm$ 1.3 <sup>c,d</sup>	161.8 $\pm$ 3.5 <sup>c,d</sup>	975.5 $\pm$ 15.8 <sup>c,d</sup>
miR-499 + IPostC	1085.6 $\pm$ 64.4 <sup>c,d</sup>	154.8 $\pm$ 9.7 <sup>c,d</sup>	308.3 $\pm$ 5.9 <sup>c,d</sup>	31.5 $\pm$ 0.7 <sup>c,d</sup>	89.3 $\pm$ 3.3 <sup>c,d</sup>	742.4 $\pm$ 17.8 <sup>c,d</sup>

n=4. The experiments were repeated four times. <sup>a</sup>P<0.05 vs. Sham; <sup>b</sup>P<0.05 vs. I/R; <sup>c</sup>P<0.05 vs. IPostC; <sup>d</sup>P<0.05 vs. AAV + IPostC. AAV, adeno-associated virus; I/R, ischemia/reperfusion; IPostC, ischemic postconditioning; miR, microRNA.

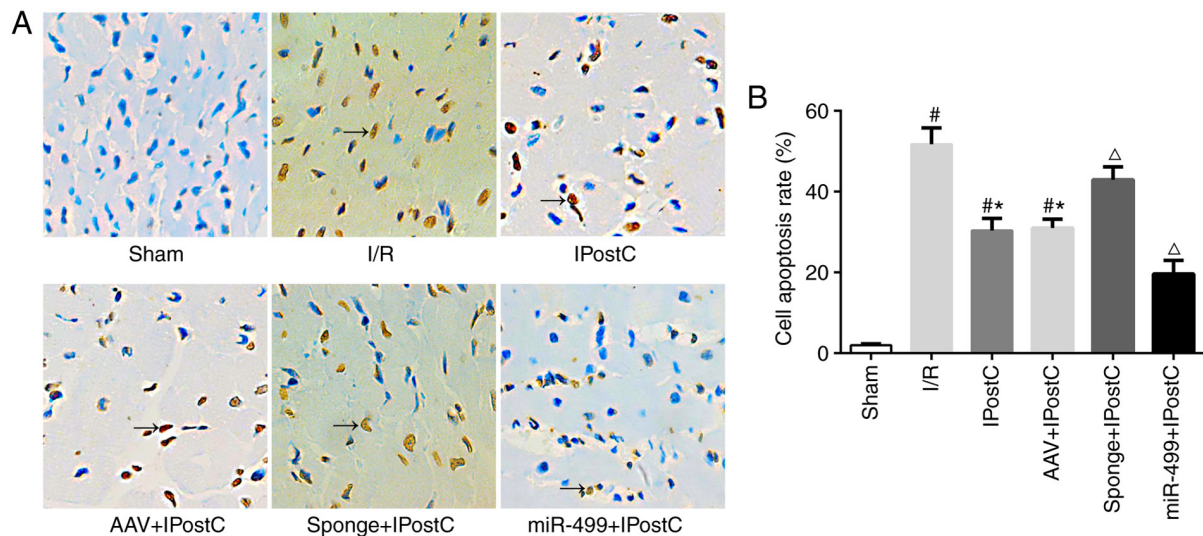


Figure 6. miR-499 protects against cardiomyocyte apoptosis during IPostC. (A) Representative micrographs of TUNEL staining in the rat myocardium (magnification,  $\times$ 200). Arrows indicate TUNEL-positive cells. (B) Quantitative analysis of the percentage of TUNEL-positive cardiomyocytes. Data are presented as the mean  $\pm$  SD, n=4. The experiments were repeated three times. <sup>#</sup>P<0.05 vs. Sham; <sup>\*</sup>P<0.05 vs. I/R; <sup>Δ</sup>P<0.05 vs. AAV + IPostC. AAV, adeno-associated virus; I/R, ischemia/reperfusion; IPostC, ischemic postconditioning; miR, microRNA.

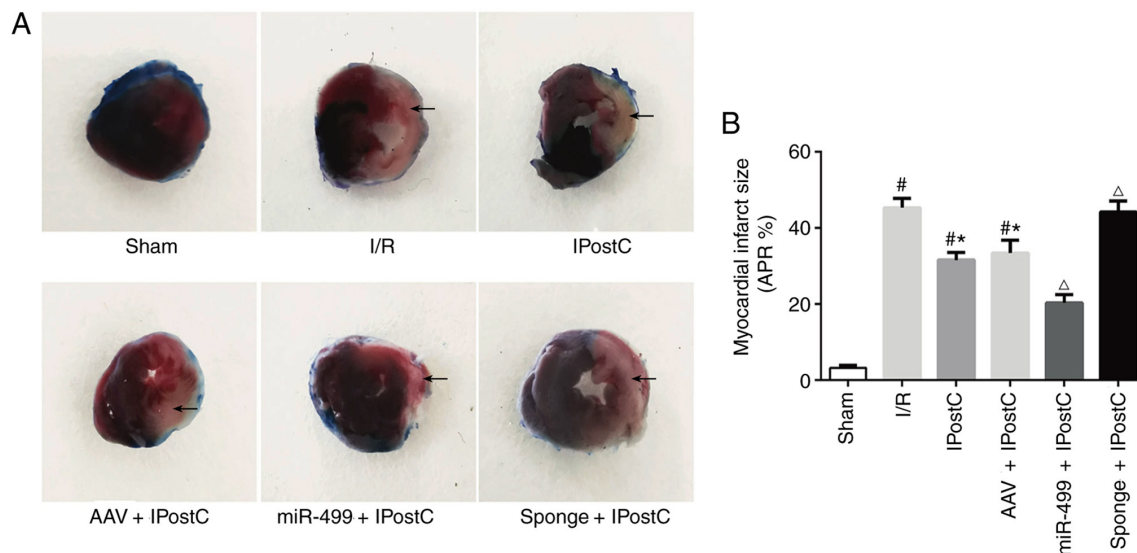


Figure 7. The myocardial infarct size in each group as detected using TTC staining. (A) Representative photographs of TTC staining of myocardial slices. The slices were taken from 2 mm below the ligation line (magnification,  $\times$ 200). (B) Quantitative analysis of the myocardial infarct size. Data are presented as the mean  $\pm$  SD, n=4. The experiments were repeated three times. <sup>#</sup>P<0.05 vs. Sham; <sup>\*</sup>P<0.05 vs. I/R; <sup>Δ</sup>P<0.05 vs. AAV + IPostC. AAV, adeno-associated virus; I/R, ischemia/reperfusion; IPostC, ischemic postconditioning; miR, microRNA; TTC, triphenyl tetrazolium chloride.



apoptosis and an increased myocardial infarct size. Moreover, the converse changes were found in the IPostC myocardium. The present mechanistic studies revealed that IPostC led to an increased miR-499 level and that by manipulating the miR-499 level, critical pathways such as complement activation, inflammation and NF- $\kappa$ B signaling were affected. This resulted in significantly altered outcomes of myocardial protection following IPostC. Therefore, miR-499 plays a central role in IPostC-induced cardioprotection.

A primary limitation of the present study is that the effects of IPostC and miR-499 on cardiac function in ischemia-reperfusion rats were not evaluated. Previous studies have shown that IPostC improves the longitudinal contractile function of the reperfused myocardium in patients with acute myocardial infarction (46) and that miR-499 improves left ventricular function in a rat model of IPostC (16). Considering that cardiomyocyte apoptosis and myocardial necrosis are closely related to cardiac function, it is speculated that miR-499 may improve cardiac function in ischemia-reperfusion rats. Future functional studies using ultrasound or electrocardiography to evaluate the effect of miR-499 on live rats during IPostC are recommended.

In conclusion, miR-499 regulated IPostC-mediated protection against I/R-induced myocardial injury, in part by inhibiting the activation of local and systemic C3a and C5a; inflammation; and NF- $\kappa$ B signaling. The present data provided mechanistic evidence which could support the development of novel therapeutics aimed at harnessing IPostC-mediated cardioprotection against MIRI.

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## Availability of data and materials

The datasets generated and analyzed during the present study are available from the corresponding author on reasonable request.

## Authors' contributions

ZH and YH designed and performed experiments, analyzed data and co-wrote the paper. QJL, HW and XYZ performed experiments. RHT and GQZ designed experiments; provided research funding; and edited and revised the manuscript. All authors have read and provided final approval of the manuscript. ZH and RHT confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

All animal protocols were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National

Institutes of Health, USA) and were approved by the Animal Care and Use Committee of Guangxi Medical University.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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