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Non-invasive limb ischemic pre-conditioning reduces oxidative stress and attenuates myocardium ischemia-reperfusion injury in diabetic rats

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Abstract

This study was to explore whether repeated non-invasive limb ischemic pre-conditioning (NLIP) can confer an equivalent cardioprotection against myocardial ischemia-reperfusion (I/R) injury in acute diabetic rats to the extent of conventional myocardial ischemic pre-conditioning (MIP) and whether or not the delayed protection of NLIP is mediated by reducing myocardial oxidative stress after ischemia-reperfusion. Streptozotocin-induced diabetic rats were randomized to four groups: Sham group, the I/R group, the MIP group and the NLIP group. Compared with the I/R group, both the NLIP and MIP groups showed an amelioration of ventricular arrhythmia, reduced myocardial infarct size, increased activities of total superoxide dismutase (SOD), manganese-SOD and glutathione peroxidase, increased expression of manganese-SOD mRNA and decreased xanthine oxidase activity and malondialdehyde concentration (All $p < 0.05$ vs I/R group). It is concluded that non-invasive limb ischemic pre-conditioning reduces oxidative stress and attenuates myocardium ischemia-reperfusion injury in diabetic rats.

Keywords: *Repeated non-invasive limb ischemic pre-conditioning, oxidative stress, diabetic*

Introduction

According to the World Health Organization, coronary heart disease (CHD) is now the leading cause of death worldwide, with 3.8 million men and 3.4 million women dying each year. Following an acute myocardial infarction (AMI), timely myocardial reperfusion to restore blood flow in the infarct-related coronary artery remains the most effective intervention for limiting myocardial infarct size, preserving left ventricular (LV) ejection fraction, preventing LV remodelling and improving clinical outcomes. However, ischemia-reperfusion (I/R) injury is a major contributory factor to cardiac dysfunction and infarct size that determines patient prognosis after AMI. New treatment strategies are required to reduce myocardial injury and improve clinical outcomes in patients with coronary heart disease. Ischemic pre-conditioning (IP) is the phenomenon whereby brief episodes of myocardial ischemia render the ischemic territory tolerant to subsequent, sustained insult and limit myocardial necrosis [1]. However, IP necessitates an invasive treatment being applied directly to the myocardium in order to achieve cardioprotection, which in some clinical settings is impractical and harmful. An alternative more amenable strategy is to apply the cardioprotective stimulus to an organ or tissue remote from the heart, an approach encapsulated by the phenomenon of remote ischemic pre-conditioning (RIP). This idea was first conceived by Przyklenk et al. [2] in 1993 and had been demonstrated in many models

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[3–5]. These early experimental studies had depended on using a non-cardiac organ as the remote pre-conditioning stimulus, which requires a vasive operative procedure to apply the pre-conditioning ischemia. However, a less invasive method for applying the remote pre-conditioning strategy is more clinically applicable. Transient hind-limb ischemia as a remote pre-conditioning stimulus introduced by Oxman et al. [6] made it clinically practical. They showed that 10 min of hind-limb ischemia immediately preceding the indexed myocardial ischemia can reduce reperfusion arrhythmias in rat hearts following a sustained ischemic insult. Since delayed cardioprotection is much more practically feasible and of clinical value, we had designed a repeated non-invasive limb ischemic pre-conditioning (NLIP) protocol based on modification of non-invasive animal blood pressure detector with an attempt to perform repeated non-invasive and uniform pre-condition stimuli. Our pilot study showed that repeated NLIP augmented ischemic tolerance in healthy rat myocardium [7].

However, the translation of basic research findings learned from IP into clinical practice has often been inadequate because the majority of basic research findings have stemmed from healthy animals. In the clinical settings, the diseased hearts that are the primary target of viable therapies aimed to activate cardioprotective mechanisms in order to limit post-ischemic myocardial infarct size and attenuate cardiac dysfunction. In particular, diabetic hearts are less resistant to ischemic insults and hyperglycaemia can enhance oxidative stress and compromise the cardioprotective effects of ischemic or pharmacological pre-conditioning. Therefore, the need for effective cardioprotective strategies is particularly pertinent in patients with diabetes mellitus (DM) and its precursor, the 'metabolic syndrome', as these patients experience both increased cardiovascular mortality and worse clinical outcomes following AMI [8]. Whether or not the diabetic myocardium can be protected by the cardioprotective manoeuvres of IP is currently unclear. Although some studies have reported that the diabetic myocardium is amenable to cardioprotection elicited by IP, others have reported no protective effect [9–11]. Interestingly, it appears that the protective effect of IP may be restored by increasing the intensity of the IP stimulus in DM animals [10,12]. We, therefore, postulated that repeated NLIP may confer cardioprotection in diabetes.

It has been well accepted that increased reactive oxygen species (ROS) and the subsequent oxidative damage is one of the major causes of I/R injury. The present study proposed to test the hypotheses that repeated NLIP can confer an equivalent cardioprotection against I/R injury in acute diabetic, DM type 1 (DM1) rats, to the extent of conventional myocardial ischemic pre-conditioning (MIP) and that the delayed protection of NLIP is mediated by attenuating oxidative stress in myocardium after ischemia-reperfusion. Thereby, this intervention will be both non-invasive and virtually cost-free to facilitate its implementation in clinical settings.

Methods

Animals

Healthy adult Wistar male rats (230–260 g) were supplied by Experimental Animals Research Institute of Medical Science Academic of China (SPF grade, SCXK11-00-0007). All animals were housed in cages in groups of four, maintained at 20–30°C with a natural light–dark cycle. The animals had free access to standard pelleted diet and tap water. The care and use of the animals were conducted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, China. This study was approved by the Laboratory Animal Care and Use Ethical Committee, Tianjin Medical University (Tianjin, China).

Experimental models

Induction of experimentally acute diabetes mellitus. A chemical model of DM1 was used. The rats were given i.v. streptozotocin (STZ, MERCK Co., California, USA) solution citric acid-citrate buffer, pH = 4.3, at a dose of 45 mg/kg. Blood glucose levels were measured 3 days after STZ injections and immediately before modelling of myocardial ischemia-reperfusion injury using a 1GM-0001A glucometer (B.Braun Co., Melsungen, Germany). Animals with glucose levels of at least 16.7 mmol/L were used in this study. One week after the injection of STZ, the following procedures were performed.

Induction of myocardial ischemia-reperfusion injury. After thoracotomy and pericardiotomy, a braided silk suture (3–0, 1.0 metric) was passed superficially into the myocardium, around the left anterior descending (LAD) coronary artery 2 mm proximal from its origin in the atrioventricular sulcus and placed there. The heart rate (HR) and blood pressure (BP) were then allowed to stabilize for 15 min. The suture was passed through a small tube to form a snare to occlude the LAD coronary artery. The presence of myocardial ischemia was confirmed by elevation of the ST-segment in the ECG and an immediate fall in mean arterial pressure (MAP) by 15~30 mmHg. For reperfusion, the snare was released and reperfusion was visually confirmed by epicardial blushing, gradual resolution of the ECG. A total of 30 min ligation and 2 h reperfusion was completed. Blood samples were taken through femoral vein before LAD occlusion and at the end of reperfusion for biochemical analysis or ELISA assay.

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Induction of NLIP. The technique of NLIP was standardized in our laboratory and was performed by animal non-invasive tester for blood pressure (Taimeng Technology Co., Chengdu, China). The thigh of the left hind-limb of the rat was encased in the bolster connected with an aerocyst and the pulse sensor was placed on the dorsal artery of the foot. The repeated NLIP was achieved by three episodes of 5 min occlusion of the left femoral artery followed by 5 min of reperfusion for 3 consecutive days. The ischemia was confirmed by the disappearance of pulse wave when the saccule is pressurized, while the pulse reappearances denote reperfusion when reducing pressure. During the ischemic period, the skin of the left leg became purple and the temperature decreased. The skin colour returned gradually to rose and temperature increased to normal after reperfusion.

Experimental protocol

Animals were randomly divided into the following four groups (Figure 1). All groups underwent a 30 min LAD occlusion followed by 120 min reperfusion.

I. *Sham*. Rats subjected to anaesthesia by sodium pentobarbital solution (3%, 1.5 ml/kg per day, ip.) for 3 days. On the next day, after thoracotomy and pericardiotomy, the rats were set aside without further intervention.

II. *Ischemia-reperfusion injury (I/R)*. Rats were subjected to 30 min LAD occlusion followed by 120 min reperfusion. The other procedures were the same as in group I.

III. *Myocardial ischemic pre-conditioning (MIP), as a positive control*. Rats were subjected to three episodes of ischemic pre-conditioning as a 5 min period of myocardial ischemia with subsequent 5 min reperfusion immediately before 30 min LAD ischemia. The other procedures were the same as in group II.

IV. *Repeated non-invasive limb pre-conditioning (NLIP)*. Rats were subjected to three episodes of 5 min occlusion of left femoral artery followed by 5 min of reperfusion for 3 consecutive days. On the next day, the procedures were the same as in group II.

Haemodynamic measurements

The animals were weighed and anaesthetized with urethane (1 g/kg, i.p.; Tianjin Chemicals Co., Tianjin City, China) and were prepared for surgery. The left carotid artery was cannulated with a heparin-filled polyethylene catheter for BP monitoring via a pressure transducer connected to a BL-420 data acquisition and analysis Bio-Lab system (Taimeng Scientific and Technologic Co. Ltd., Chengdu, China). A left thoracotomy was then performed to expose the heart at the fifth intercostal space. After removing the pericardium,

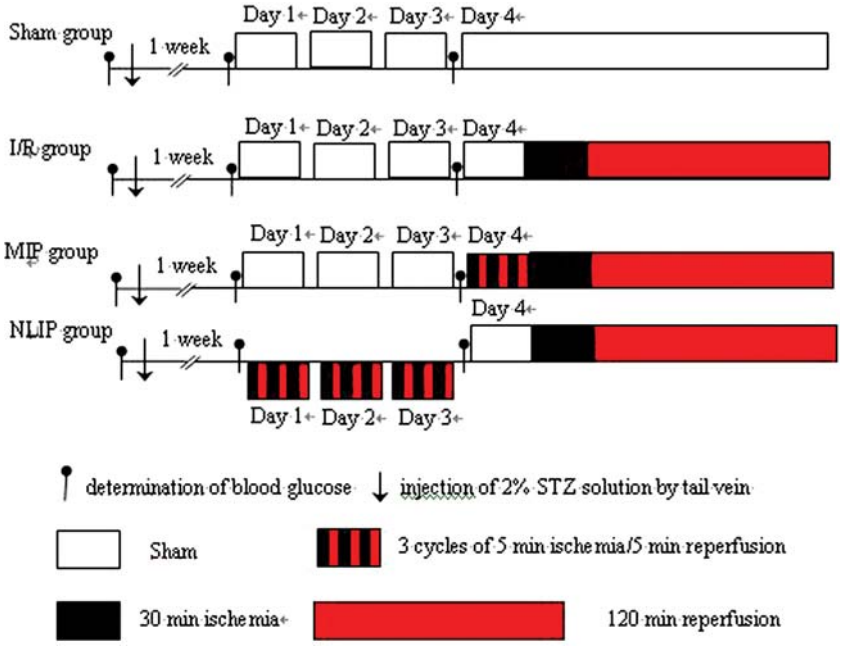

Figure 1. Experimental protocol used to determine the protective effect of repeated non-invasive limb ischemic pre-conditioning (NLIP) on myocardium after ischemia/reperfusion in streptozotocin (STZ)-induced diabetic rats. In the Sham group (n = 10) there was no intervention; ischemia-reperfusion (I/R, n = 16) included 30 min occlusion of left anterior descending (LAD) coronary artery followed by 2 h of reperfusion; myocardium ischemic pre-conditioning (MIP, n = 16) was elicited by three circles of 5 min ischemia followed by 5 min reperfusion before 30 min LAD occlusion; repeated non-invasive limb ischemic pre-conditioning (NLIP, n = 16) was performed by three circles of 5 min ischemia followed by 5 min reperfusion for 3 days before I/R surgery.

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MAP and pressure rate index (PRI, as a marker for myocardial oxygen consumption) were derived online. Animals were ventilated artificially with room air at a rate of 55~60 breaths/min, with tidal volume 8~10 ml/kg using an animal ventilator (HX-300, Taimeng Scientific and Technologic Co. Ltd., Chengdu, China). The body temperature of the animal was maintained at 37 ± 0.5°C. Standard lead II ECG and HR were recorded throughout the experiment.

Analysis of rhythm disturbances

Haemodynamically significant rhythm disturbances arising during 30 min ischemia were analysed. Ventricular tachyarrhythmia (VA), including ventricular premature contraction (VPC), ventricular tachycardia (VT) and ventricular fibrillation (VF), were assessed using the international accord of the Lambeth Conventions [13] using the following criteria:

1) The onset of each type of VA.
2) The number of animals in the group in which one or more episodes of VA arose.

Myocardial infarct size assessments

At the end of 2 h of reperfusion, the LAD coronary artery was re-occluded and 2 ml of 0.6% Evans Blue (Sigma, Missouri, USA) was injected through the femoral vein to distinguish the non-ischemic area from area at risk (AAR), which was delineated by the area of myocardium not dyed. The heart was excised, rapidly frozen and then cut into 1 mm slices, perpendicular to the septum from the apex to the base. The slices were incubated at 37°C with TTC; triphenyl tetrazolium chloride (Yinghai Fine Chemicals Co., Shanghai, China) solution (1% in phosphate buffer, pH 7.4) for 25 min. In the presence of dehydrogenase enzymes in the viable myocytes, NADH reacts with TTC to stain deep red while infracted myocardium remains pale owing to the absence of dehydrogenases. The slices were fixed in 10% formalin for 12 h to obtain three colours: blue (non-ischemic area); deep and pale red (AAR as a whole); and unstained pale, representing the size of infarcted area (IA). Three parts of the slices were weighed, respectively. Infarct size (IS) was expressed as the percentage of IA relative to AAR.

Assay for creatine kinase-MB (CK-MB) activity and cardiac troponin I (cTnI) content in the serum

Blood samples were collected before the 30 min of LAD occlusion and at the end of reperfusion. The serum was separated and stored at -70°C until analysed. The CK-MB activity was determined in duplicate with a colourmetric method using a CK-MB immunoinhibition kit (Fuxing Changzheng Co., Shanghai, China) according to the manufacturer's instructions. cTnI was assessed using immunoassay with a goat anti-rat cTnI ELISA kit (Adlitteram diagnostic laboratories, California, USA) referring to the instructions.

Measurement of activities of total superoxide dismutase (T-SOD), manganese SOD (Mn-SOD), glutathione peroxidase (GSH-PX) and xanthine oxidase (XOD) and determination of malondialdehyde (MDA) content in the myocardium

Myocardial tissues in AAR were sampled at the end of 2 h of reperfusion and stored at -70°C for T-SOD, Mn-SOD, GSH-PX, XOD and MDA detections. All samples were homogenated and centrifuged. The extracted supernatants were reacted with tested reagents in the detection kits (Jiancheng Co., Nanjing, China) according to the manufacture's directions. The resultant mixture were analysed spectrophotometrically (751-GW spectrophotometer, BIO-RAD Laboratories, Italy) at 550 nm, 412 nm, 530 nm and 532 nm absorbance, respectively, for T-SOD, Mn-SOD, GSH-Px and XOD. T-SOD, Mn-SOD and GSH-PX activities were expressed as units per milligram protein, XOD activity was expressed as units per gram protein and MDA values were given as micromoles per milligram protein.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis of Mn-SOD gene transcription

Mitochondria is a major source of superoxide production during TCA and mitochondrial oxidative damage plays a critical role in I/R injury. We therefore aimed to study whether or not NLIP can revert acute myocardial I/R mediated down-regulation of the gene expression of Mn-SOD, a major antioxidant enzyme in the mitochondria. Total RNA was isolated from the myocytes with TRIzol reagent (Invitrogen, California, USA), according to the manufacture's directions. Single-strand cDNA templates were prepared from 1 μg total RNA using oligo (dT)18 and M-MLV reverse transcriptase (Promega, California, USA). Specific cDNA were then amplified by PCR using the following primers (Baobio, Dalian, China): Mn-SOD sense primer: 5'-GACCTGCCTTACGA CTATGG-3', anti-sense primer: 5'-GACCT TGCTCCTTATTGAAGC-3' and β-actin sense primer: 5'-TGACGGGGTCACCCACACTGTG CCCAT CTA-3', anti-sense primer: 5'-CTAGAA GCATTG CGGTGGACGATGGAGGG-3'. PCR amplification (Mastercycler PCR System, Eppendorf AG, Germany) from cDNA was performed in a final volume of 40 μL containing buffer, Takara Taq and specific primers Takara, Dalian, China (invested and owned by Japan). The cycling conditions were: denaturation at 94°C for 45 s, annealing at 64°C for 45 s, and elongation

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at 72°C for 60 s. The optimum cycle number was 31 cycles for Mn-SOD and β-actin. All PCR products were determined by 2% agarose gel electrophoresis by ethidium bromide staining and visualized by UV transillumination. Gel images were analysed by densitometry using UNIVERSAL HOOD II (BIO-RAD Laboratories). Mn-SOD gene expression data are presented as normalized to β-actin expression.

Statistical analysis of data

All values were expressed as means ± standard deviation. Differences in infarct size, biochemical values and haemodynamic data at a particular time point among the groups were compared by one-way ANOVA followed by Tukey–Kramer *post hoc* test for multiple comparisons. Haemodynamic data within the groups were compared using paired *t*-test. *Chi-square* test or Fisher's exact probability test was used for comparison of incidence of VA. Differences were considered significant or highly significant at p-values < 0.05 or p-values < 0.01. All analysis was done using SPSS 11.5 software.

Results

General characteristics of the experimental animals

A total of 78 adult rats were used. Eight animals (four in the I/R group, two in the MIP group, two in the NLIP group) were excluded owing to certain experimental limitations, such as failure to occlude the coronary artery, severe hypotension resulted from excessive bleeding, intractable ischemia-induced ventricular fibrillation which did not recover within 2 min of occurrence and poor delineation of the risk zone. Twelve animals were excluded before starting the protocol owing to failure to form hyperglycaemia or death from hypoglycaemia.

After injection of STZ, the animals exhibited polydipsia, polyphagia, urorrhagia and weight loss. On the day of surgery, the body weights in the four groups obviously reduced compared with that before STZ injection (229.9 ± 17.6 vs 248.7 ± 11.6, 238.0 ± 17.7 vs 249.2 ± 10.9, 227.7 ± 22.1 vs 250.1 ± 10.1, 231.5 ± 19.0 vs 249.3 ± 11.3 g, $p < 0.01$). The blood glucose concentrations in the four groups before the injection of STZ were 5.76 ± 0.89, 5.63 ± 0.57, 5.57 ± 0.82 and 5.89 ± 0.71 mmol/L, respectively. At the third day of STZ injection, levels of glycaemia significantly increased to 19.86 ± 3.77, 21.15 ± 3.75, 20.82 ± 3.23 and 20.75 ± 3.50 mmol/L (all $p < 0.01$ vs before STZ injection). On the day of surgery, the glucose levels remained high (21.00 ± 4.80, 21.73 ± 4.66, 21.62 ± 3.80 and 21.17 ± 5.24 mmol/L, respectively), while there were no statistical differences among the groups.

Haemodynamics

The haemodynamic parameters were measured before the 30 min occlusion on LAD, at 15 and 30 min of LAD coronary artery occlusion and at 30 and 120 min of reperfusion for analysis. Values of HR, MAP and PRI in the Sham group did not change significantly throughout the experiment. These parameters showed a significant decrease in the other three groups on LAD occlusion and improved to a certain extent on reperfusion but did not recover to baseline (Table I). Intervention of myocardial or limb ischemic pre-conditioning elicited no significant changes in HR, MAP and RPI at all the time points compared with that in the I/R group (Table I).

Ischemic arrhythmias

The primary criterion in assessing the efficacy of MIP or NLIP in this study was the infarct size. However, an additional criterion was provided by the incidence and severity of ischemic VA. No animal in the Sham group had any type of VA throughout the experiments. The onset time of VPC and VT in the I/R group were earlier as compared with that in the MIP and the NLIP groups ($p < 0.01$, Table II). After MIP or NLIP the incidence of VPC, VT and VF were significantly lower than that in the I/R group ($p < 0.05$, Table II).

Myocardial infarct size

There were no significant differences in the mass of AAR and the ratio of AAR to total mass among the groups, so this factor could not influence the size of the resulting infarct. However, MIP and repeated NLIP led to significant reduction in mass of infarct area ($p < 0.05$ vs I/R group). Thirty minutes of LAD coronary artery occlusion followed by 2 h of reperfusion in I/R group produced an infarct size of 34.14 ± 8.27% of AAR. Repeated NLIP reduced the infarct size by 44% compared with I/R group ($p < 0.01$), suggesting that the cardioprotective effect of NLIP is comparable to that of MIP ($p < 0.01$, Figure 2).

Serum CK-MB and cTnI

The serum CK-MB activities and cTnI concentrations before ischemia did not differ among the groups. Myocardial I/R resulted in significant increases of CK-MB activities and cTnI concentrations in the I/R group (all $p < 0.01$ vs pre-ischemia or Sham) that were significantly attenuated by MIP or NLIP (all $p < 0.05$ vs I/R group). These two parameters did not differ between MIP and NLIP groups (Figure 3).

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Table I. Systemic haemodynamic variables during I/R injury in DM rats ($\bar{x}\pm S$).

			LAD occlusion			LAD oeperfusion	
Group	*n*	Baseline	0 min	15 min	30 min	30 min	120 min
MAP (mmHg)							
Sham	10	90.6 ± 11.1	90.6 ± 9.2	87.4 ± 11.2	85.0 ± 9.4	82.8 ± 12.0	80.3 ± 12.2
I/R	16	93.0 ± 15.3	67.3 ± 14.3$^{**\Delta\Delta}$	58.4 ± 13.3$^{**\Delta\Delta}$	65.7 ± 13.3$^{**\Delta\Delta}$	77.8 ± 10.7**	74.7 ± 12.2**
MIP	16	85.8 ± 17.8	63.7 ± 13.8$^{**\Delta\Delta}$	58.3 ± 13.1$^{**\Delta\Delta}$	62.0 ± 11.9$^{**\Delta\Delta}$	73.8 ± 9.4**	72.6 ± 12.5**
NLIP	16	89.6 ± 15.1	64.5 ± 16.5$^{**\Delta\Delta}$	58.4 ± 16.3$^{**\Delta\Delta}$	60.1 ± 13.2$^{**\Delta\Delta}$	71.5 ± 14.8**	72.0 ± 12.9**
HR (beat/min)							
Sham	10	378 ± 25	380 ± 22	378 ± 34	375 ± 31	377 ± 32	369 ± 34
I/R	16	380 ± 37	377 ± 40	344 ± 38**	340 ± 40**	358 ± 38**	358 ± 48**
MIP	16	369 ± 29	373 ± 28	348 ± 25**	341 ± 26**	359 ± 27**	355 ± 34**
NLIP	16	377 ± 39	376 ± 39	343 ± 44**	334 ± 45**	353 ± 43**	367 ± 29**
PRI (beat/min mmHg $\times 10^3$)							
Sham	10	34.2 ± 6.8	34.4 ± 5.7	33.0 ± 5.5	31.8 ± 5.7	31.2 ± 5.4	29.6 ± 5.9
I/R	16	35.8 ± 6.1	25.6 ± 6.4$^{\Delta\Delta}$	20.5 ± 6.3$^{**\Delta\Delta}$	22.4 ± 5.5$^{**\Delta\Delta}$	28.4 ± 5.9**	27.3 ± 4.8**
MIP	16	31.6 ± 6.0	23.6 ± 4.9$^{\Delta\Delta}$	20.6 ± 5.4$^{**\Delta\Delta}$	21.4 ± 5.0$^{**\Delta\Delta}$	26.6 ± 4.2**	25.6 ± 5.0**
NLIP	16	33.7 ± 5.6	24.4 ± 3.8$^{\Delta\Delta}$	20.1 ± 5.5$^{**\Delta\Delta}$	20.0 ± 4.6$^{**\Delta\Delta}$	25.3 ± 6.6**	26.9 ± 5.2**

Sham, Sham group; I/R, ischemia-reperfusion; MIP, myocardial ischemic pre-conditioning; NLIP, repeated non-invasive limb ischemic pre-conditioning; MAP, mean arterial pressure; HR, heart rate; PRI, pressure-rate index; LAD, left anterior descending; *n*, number of animals per group $^{**}p < 0.01$ vs pre-ischemia; $\Delta\Delta p < 0.01$ vs Sham group.

Myocardial activities of T-SOD, Mn-SOD, GSH-PX and XOD and MDA content

The activities of T-SOD and Mn-SOD were markedly decreased in animals in I/R, MIP and NLIP groups compared with that in Sham group (all $p < 0.01$). However, the levels in the MIP and NLIP groups were significantly higher than that in the I/R group ($p < 0.01$, Figure 4A). Myocardial I/R resulted in an obvious reduction of GSH-PX activity in the I/R group ($p < 0.01$ vs Sham) and repeated NLIP as well as MIP prevented this reduction ($p > 0.1$ vs Sham, Figure 4B). The activity of XOD and content of MDA increased significantly in the I/R group ($p < 0.01$ vs Sham) and was attenuated by either MIP or NLIP ($p < 0.01$, Figures 4C and D).

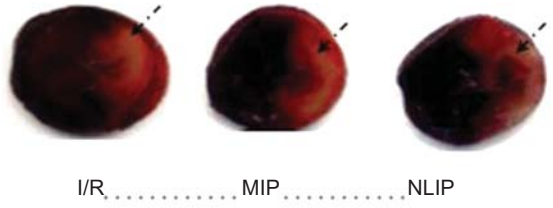
Mn-SOD gene transcription

The level of Mn-SOD gene expression relative to β-actin in the myocardium underwent 30 min of LAD occlusion followed by 2 h of reperfusion in the I/R group was low. NLIP significantly up-regulated mRNA expression of Mn-SOD in the myocardium ($p < 0.01$ vs I/R group), indicative of a delayed protection against ischemia-reperfusion induced injury. This effect attained to the extent of MIP ($p < 0.01$, Figure 5).

Table II. Effects of MIP and NLIP on ventricular arrhythmia during ischemia in diabetic rats.

		Onset time ($\bar{x}\pm S$, min)		Incidence (%)		
Group	*n*	VPC	VT	VPC	VT	VF
Sham	10	—	—	0	0	0
I/R	16	6.56 ± 0.94	8.69 ± 4.34	15$^{\Delta\Delta}$	10$^{\Delta}$	7
MIP	16	12.84 ± 5.73$^{\#\#}$	17.77 ± 5.31$^{\#\#}$	8$^{\#}$	4$^{\#}$	2$^{\#}$
NLIP	16	10.36 ± 2.85$^{\#\#}$	14.60 ± 5.36$^{\#}$	9$^{\#}$	3$^{\#}$	2$^{\#}$

$^{\#}p < 0.05$.
$^{\#\#}p < 0.01$ vs I/R group.
$^{\Delta}p < 0.01$.
$^{\Delta\Delta}p < 0.01$ vs Sham group.



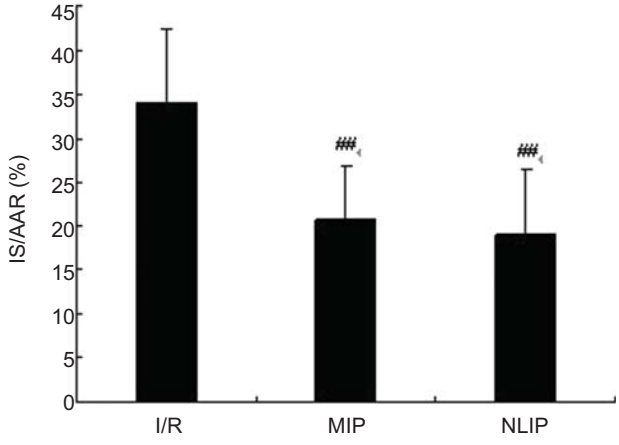


Figure 2. Determination of infarct size (IS) by triphenyltetrazolium chloride (TTC) staining in diabetic rats ($n = 8$). Representative macroscopic figures of myocardium tissue showing myocardial infarction (pale white colour, dotted arrow) and non-infarction (red colour) area within the area-at-risk (AAR) or the non-ischemic area (dark colour). IS is expressed as a percentage of the infracted area (IA) relative to AAR. Repeated NLIP significantly reduced the ratio by 44% compared with I/R group, showing equivalent cardioprotection to that of MIP, $^{\#\#}p < 0.01$ vs I/R group. Values are group means ± standard deviation.

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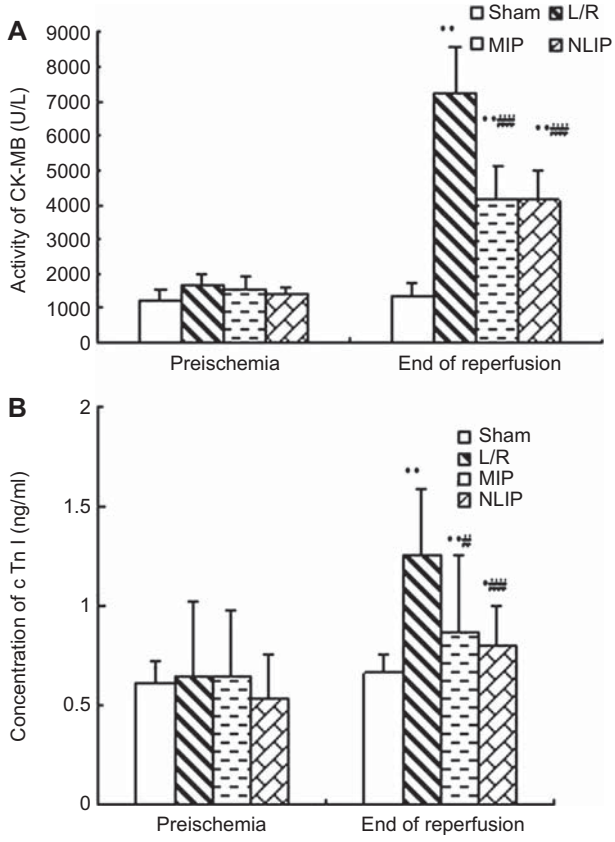

Figure 3. The effects of MIP and NLIP on activity of CK-MB (A) and content of cTnI (B) in serum in diabetic rats ($n = 6, 8$). $^{*}p < 0.05$, $^{**}p < 0.01$ vs pre-ischemia; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs I/R group; $p < 0.01$ vs Sham group. Values are group means ± standard deviation.

Discussion

Whether or not myocardial IP can confer cardioprotection in subjects with DM1 remains a debate. The study by Ravingerova et al. [14] on isolated hearts from rats with DM1 induced by a single intravenous injection of STZ (45 mg/kg) showed that the anti-arrhythmic effect of IP became clear only at the chronic stage of diabetes (9 weeks of DM1, with blood glucose level of 23.8 ± 0.9 mmol/L), while in the acute stage (1 week of DM1, with blood glucose level of 17.4 ± 0.7 mmol/L) IP did not reduce the incidence of ischemic arrhythmia. Tosaki et al. [15] reported that IP did not confer anti-arrhythmia and anti-myocardial stunning effects in 4-week (blood glucose level above 16.7 mmol/L) and 8-week (blood glucose level above 22.2 mmol/L) diabetic rats induced by intraperitoneal injection of STZ (65 mg/kg). We inferred that different STZ dose or different blood glucose level, different experimental model (*in vivo* or *in vitro*) and different stage and severity of the disease might result in these discrepancies. For the first time, we have demonstrated a non-invasive limb ischemic pre-conditioning technique for delayed protection of myocardium against arrhythmia and infarction in diabetic rats. The major findings are as follows: (1) repeated NLIP for 3 days in diabetic rats induced a similar level of cardioprotection to MIP; (2) the protection offered by repeated NLIP was associated with the attenuation of oxidative stress. Results of the current study demonstrated that both MIP and repeated NLIP did lead to an equivalent decrease in the incidence of ischemic arrhythmia, attenuation in myocardial cellular injury and limitation of infarct size in animals with the acute stage of DM.

Generation of ROS during early reperfusion has been implicated as a major player in the pathogenesis of tissue injury [16]. The burst production of ROS occurs within the first minute and peaks at 4–7 min after reperfusion and increased ROS generation is still detectable during later periods of reperfusion [17]. The sources of ROS during I/R include the respiratory burst of neutrophil, increased activity of XOD on endothelium, dysfunction of mitochondria and the metabolism of catecholamine and arachidonic acid [18,19]. Excessive production of ROS during reperfusion can lead to an imbalance between oxidant and anti-oxidant in tissues and cells, resulting in oxidative stress. ROS react with biomacromolecules directly or by peroxidative chain reactions and destroy biological membranes, resulting in cell dysfunction. SOD converts $O_2^{-}\cdot$ to H_2O_2, which is then reduced by GSH-PX and catalase to water. Pathological conditions lead to modulated expression and function of oxidant and antioxidant enzymes, including NAD(P)H oxidase, XOD, myeloperoxidase (MPO), SOD, catalase and GSH-PX. IP may have limited delivery of oxygen substrate and thereby directly attenuated the generation of ROS. In turn, the reduced generation of ROS may have mitigated myocardial cellular injury [20]. Experimental studies in different animals or clinical observations in patients with AMI indicated that IP exhibited early or late protection by elevating activity of SOD and reducing content of MDA [21,22]. IP can also inhibit the activation of XOD [23] and upregulate the activity of GSH-PX [24]. It was demonstrated that classical ischemic pre-conditioning could result in the reprogramming of gene expression. The most common genes that are expressed by virtually any kind of stress conditioning include antioxidants like SOD and GSH-PX [25]. As an important anti-oxidative enzyme existing in the mitochondrium, Mn-SOD is sensitive to the regulation of IP. Studies reported that levels of both mRNA and protein of Mn-SOD were augmented by IP [26,27]. Only a few researchers involved the effect of limb ischemic pre-conditioning on oxidative and anti-oxidative function in I/R myocardium [28,29]. Chen et al. [29] showed that skeletal ischemic pre-conditioning induced by four-cycle 10 min ischemia-reperfusion elevated the expression of Mn-SOD and GSH-PX in the area of risk in the myocardium of non-diabetic rats. However, reports about the effects of IP on I/R induced oxidative injury is lacking in diabetic rats and no study thus

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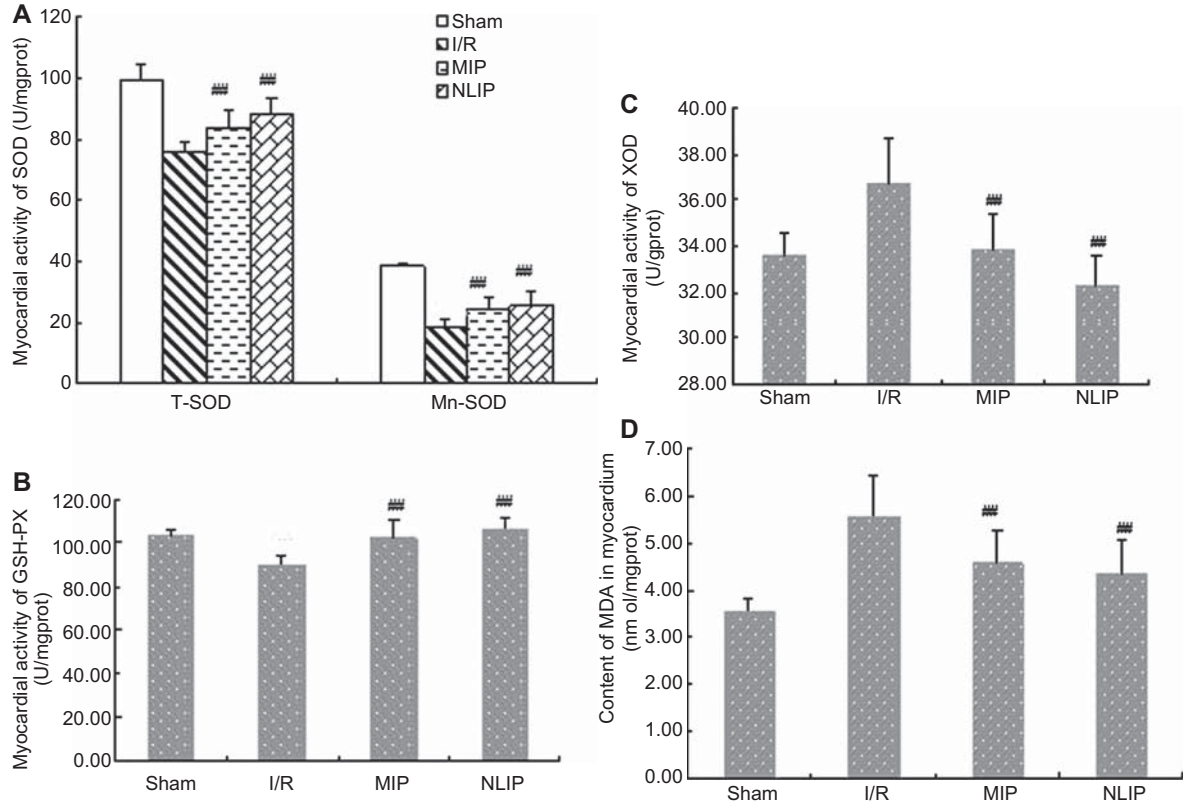

Figure 4. The effects of MIP and NLIP on activity of myocardial T-SOD, Mn-SOD (A), GSH-PX (B), XOD (C) and content of MDA (D) in diabetic rats (n = 6, 8). $^{\#\#}p < 0.01$ vs I/R group;$^{\Delta}$ $p < 0.05$, $^{\Delta\Delta}p < 0.01$ vs Sham group. Values are group means ± standard deviation.

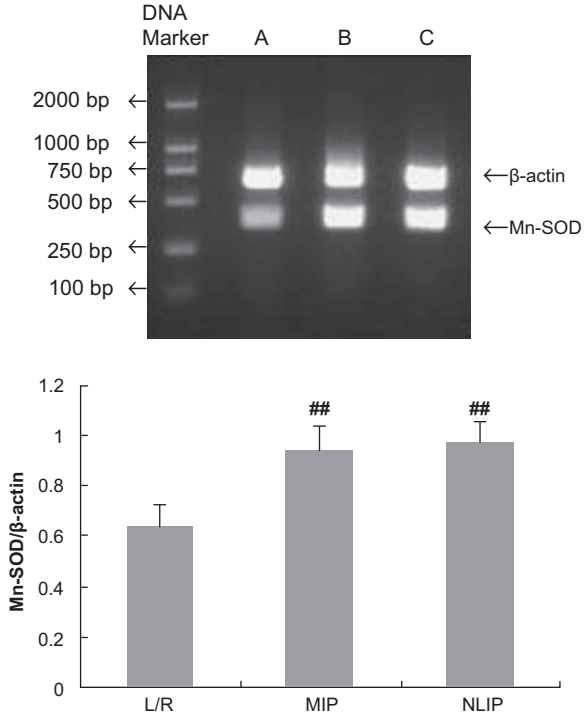

Figure 5. RT-PCR analysis of Mn-SOD transcription in diabetic myocardium after 30 min LAD occlusion and 2 h of reperfusion (n = 8). (A) I/R group; (B) MIP group; (C) NLIP group. β-actin served as a control gene. $^{\#\#}p < 0.01$ vs I/R group. Values are group means ± standard deviation.

far has explored the effects of NLIP in this aspect. The present study showed for the first time that repeated NLIP elevated activities of T-SOD, Mn-SOD and GSH-PX and up-regulated the gene transcription of Mn-SOD, while attenuated activity of XOD and content of MDA in diabetic myocardium subjected to ischemia-reperfusion, resulting in a reduced oxidative damage. Thereby, a delayed cardioprotection of repeated NLIP against I/R injury was displayed by reducing the incidence of arrhythmia and the release of CK-MB and cTnI and limiting infarct size of myocardium. These observations support the hypothesis that the attenuation of oxidative stress during I/R contributes, at least in part, to the cardioprotective effects of repeated NLIP.

The actual mechanism through which remote ischemic pre-conditioning confers cardioprotection is currently unknown, although several hypotheses have been proposed: (1) The neural hypothesis was demonstrated by Gho et al. [30] and further developed with the proposition that endogenous substances such as adenosine, bradykinin, calcitonin gene-related peptide (CGRP), released by the remotely pre-conditioned organ, stimulated afferent nerve fibres, which then relay to efferent nerve fibres terminating on the myocardium to confer cardioprotection. (2) The humoral hypothesis proposes that the endogenous substance (such as adenosine,

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bradykinin, opioids, CGRP) or some other as yet unidentified humoral factor generated in the remote organ or tissue enters the blood stream and activates its respective receptor in the myocardium, thereby recruiting the various intracellular pathways of cardioprotection implicated in ischemic pre-conditioning [31]. (3) The third hypothesis proposes that transient ischemia and reperfusion of an organ or tissue provokes a systemic protective response, which suppresses inflammation and apoptosis [32–34]. Whether RIP also activates pro-survival kinases of the reperfusion injury salvage kinase (RISK) pathway and results in the inhibition of the mitochondrial permeability transition pore (mPTP), as in IP, remains to be determined [35]. A recent study suggested that the activation of the mitogen-activated protein kinases (MAPKs) JNK, p38 and Erk1/2 within the remote organ might contribute to RIP-induced cardioprotection [36]. However, it remains to be determined whether Akt and these MAPKs would have been activated within the myocardium at the onset of myocardial reperfusion as part of the RISK pathway. Some studies had shown that the threshold of pre-conditioning stimulus raised in diabetic myocardium [37,38] and that there might be a defect of protein kinase signalling such as the PI3K-AKT [37,39] and the JAK/STAT [40] pathway in the diabetic heart, which may contribute to the lack of protection in these settings. Thus, whether repeated NLIP can increase the intensity of stimulus, thereby improve the signal transduction of IP, still needs further research. In the present study, repeated NLIP for 3 days maintained a cardioprotectively pre-conditioned state in diabetic rats. Consequently, it showed that NLIP conferred anti-arrhythmic and anti-infarct effects to the extent of classical MIP. In addition, the results of the current study suggested that anti-oxidation may represent a major mechanism whereby repeated NLIP conferred its cardioprotection. The underlying molecular mechanism of NLIP merits further in-depth studies.

In summary, results from the current study demonstrated for the first time that non-invasive limb ischemic pre-conditioning reduces oxidative stress and attenuates myocardium ischemia-reperfusion injury in diabetic rats. The ability to use transient limb ischemia as the remote pre-conditioning stimulus has facilitated its translation from the 'bench-side' to the 'bedside' for the benefit of children and adults undergoing elective cardiac surgery and adults undergoing surgery for repair of an abdominal aortic aneurysm (AAA) [41–43]. Our study may foster further clinical evaluation to determine whether the repeated NLIP, as a prophylactically cardioprotective intervention, can improve clinical outcomes in diabetic patient groups.

Declaration of interest

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