Noninvasive delayed limb ischemic preconditioning attenuates myocardial ischemia-reperfusion injury in rats by a mitochondrial K_{ATP} channel-dependent mechanism

Yan-Na Wu, Hui Yu, Xue-Hui Zhu, Heng-Jie Yuan, Yi Kang, Jian-Jie Jiao, Wei-Zhen Gao, Yan-Xia Liu, and Jian-Shi Lou*

Department of Pharmacology, College of Basic Medical Sciences, Tianjin Medical University, Tianjin, 300070, China

* Correspondence author

Jian-Shi Lou

Department of Pharmacology,

Tianjin Medical University,

Tianjin 300070, P.R. China

Tel: +86- 22-23542686,

Fax: +86-22-23542524

E-mail: jianshilou@163.com

Running head: limb preconditioning and mitochondrial K_{ATP} channels

ABSTRACT

We previously demonstrated in rat that noninvasive delayed limb ischemic preconditioning (LIPC) induced by three cycles of 5-minute occlusion and 5-minute reperfusion of the left hind limb per day for three days confers the same cardioprotective effect as local IPC of the heart, but the mechanism has not been in depth-studied. The aim of this project was to tested the hypotheses that delayed LIPC enhances myocardial antioxidative ability during ischemia-reperfusion by a mitochondrial K_{ATP} channel(mito K_{ATP})-dependent mechanism. Rats were randomized to five groups: the ischemia-reperfusion(IR)-control group, the myocardial ischemic preconditioning(MIPC) group, the LIPC group, the IR-5HD group and the LIPC-5HD group. The MIPC group underwent local ischemic preconditioning induced by three cycles of 5-minute occlusion and 5-minute reperfusion of the left anterior descending coronary arteries. The LIPC and LIPC-5HD groups underwent LIPC induced by three cycles of 5-minute occlusion and 5-minute reperfusion of the left hind limb using a modified blood pressure aerocyst per day for three days. All rats were subjected to myocardial ischemia-reperfusion injury. The IR-5HD and LIPC-5HD groups received the mito K_{ATP} channel blocker 5-Hydroxydecanoate Na (5-HD) before and during the myocardial ischemia-reperfusion injury. Compared with the IR-control group, both the LIPC and MIPC groups showed an amelioration of ventricular arrhythmia, reduced myocardial infarct size, increased activities of total superoxide dismutase, manganese-superoxide dismutase (Mn-SOD) and glutathione peroxidase, increased expression of Mn-SOD mRNA and decreased xanthine oxidase activity and

malondialdehyde concentration. These beneficial effects of LIPC were prevented by 5-HD. In conclusion**,** delayed LIPC offered similar cardioprotection as local IPC. These results support the hypothesis that the activation of mito K_{ATP} channels enhances myocardial antioxidative ability during ischemia-reperfusion, thereby contributing, at least in part, to the anti-arrhythmic and anti-infarct effects of delayed LIPC.

Key words: limb ischemic preconditioning; mitochondrial ATP sensitive potassium channel; myocardial ischemia-reperfusion; antioxidant enzymes

INTRODUCTION

Ischemic preconditioning (IPC) is an innate protective strategy that markedly reduces ischemia-reperfusion (IR) injury. Local IPC is induced by exposing tissues to brief periods of sublethal ischemia before a potentially lethal ischemia in the same tissue. Despite the effectiveness of local IPC in reducing myocardial IR injury, it has not been universally accepted as a clinical tool. This may be related to the practical difficulties associated with the local induction of cardiac ischemia, as well as to the ethical reasons. Fortunately, the IPC stimulus has systemic protective effects, and a brief ischemia in one tissue confers resistance to subsequent sustained ischemic insults in another tissue; this phenomenon is called remote ischemic preconditioning (RIPC). Skeletal muscle is relatively resistant to damage from ischemia, and its blood supply is easily accessible. This suggests that RIPC of the heart can be achieved clinically by transient limb ischemia, i.e., by limb ischemic preconditioning (LIPC). However, the molecular and cellular mechanisms underlying this endogenous adaptive process are not well understood and must be clarified before LIPC can be clinically applied.

The reactive oxygen species (ROS) generated during reperfusion have been implicated as one of the major causes of IR injury. The attenuation of oxidative stress during IR injury should be an important element of cardioprotection. Recent studies suggest that the opening of mito K_{ATP} channels prevents oxidative stress during reperfusion and may therefore play an important role in affording cardioprotection in ischemic heart (Ferranti *et al.* 2003). It has been well known that mito K_{ATP} channels

play a key role in the signaling pathway of local IPC of the heart (Carreira *et al.* 2005; Wei *et al.* 2004). Moses *et al*. (2005) first reported that mito K_{ATP} channels play a central role in RIPC mechanisms (Moses *et al.* 2005). Several studies have pointed to the important role played by ATP-sensitive potassium channels in the acute cardioprotection induced by LIPC (Konstantinov *et al.* 2005b; Schmidt *et al.* 2007; Shahid *et al.* 2008). However, there is very little information regarding the involvement of the channels in the delayed cardioprotection induced by LIPC. And it is not clear whether LIPC diminishes oxidative damage in delayed IR injury or whether the opening of mito K_{ATP} channels is related to this effect.

We previously demonstrated in rat that delayed LIPC induced by three cycles of 5-minute occlusion and 5-minute reperfusion of the left hind limb per day for three days confers the same cardioprotective effect as local IPC of the heart, and promotes the synthesis and increase the activity of superoxide dismutase (SOD) in myocardium, hinted the involvement of anti-oxidation mechanism in the cardioprotection (Li *et al.* 2009). The present study was designed to test the hypotheses that delayed LIPC enhances myocardial antioxidative ability during ischemia-reperfusion, by a mito K_{ATP} channel-dependent mechanism.

METHODS

A total of 86 male Wistar rats (240 to 270 g) were provided by Beijing Weitonglihua Laboratory Animal Technique Co., Ltd. All animal experiments were conducted in accordance with the “Guide for the Care and Use of Laboratory

Animals" published by the National Institutes of Health (NIH publication No. 86-23, revised 1985). The study protocol was approved by the Laboratory Animal Care and Use Committee of Tianjin Medical University, Tianjin, People's Republic of China.

General surgical preparation

General surgical preparation was performed as previously described (Li *et al.* 2009). Briefly, rats were anesthetized with 1 g/kg IP of urethane (Tairuida Pharmaceutical and Chemical Co., Ltd., Jinan, China). The lead II ECG, mean arterial pressure (MAP) and heart rate (HR) of each rat were continuously recorded on a BL-420 data acquisition and analysis system (Taimeng Scientific and Technologic Co., Ltd., Chengdu, China). Each animal's trachea was intubated and an HX-300 animal respirator (Chengdu Technology and Market Co., Ltd., China) was used for ventilation at a rate of 55-60 cycles/min and a tidal volume of 8-10 ml/kg. In each case, the left carotid artery was cannulated with a fluid-filled catheter connected to a pressure transducer for arterial pressure monitoring. The left femoral vein was cannulated to allow for the delivery of normal saline or a drug infusion. The chest was opened via a left parasternal chest incision. After the heart was exposed, a 3-0 suture was passed around the left anterior descending coronary artery (LAD). The ends of the thread were passed through a piece of polyethylene tube to form a snare. The LAD was occluded with the snare clamped against the surface of the heart; this caused an area of epicardial cyanosis with regional hypokinesis and ST segment elevation. Reperfusion was achieved by releasing the snare and was confirmed by the conspicuous hyperemic flushing of the previously ischemic myocardium and a

gradual resolution of the changes in the ECG signal. All rats underwent 30 minutes of LAD occlusion and 120 minutes of reperfusion.

Induction of LIPC

LIPC was induced as previously described with a slight modification (Li *et al.* 2009). Rats were anesthetized with sodium pentobarbital (30 mg/kg, IP). A modified blood pressure aerocyst was placed around the left thigh and was inflated until the pulse of the dorsal pedal artery just disappeared; the pulse was monitored by a noninvasive blood pressure measuring system (Taimeng Scientific and Technologic Co., Ltd., Chengdu, China). After 5 minutes, the aerocyst was deflated and the left hind limb was reperfused for 5 minutes. This inflation/deflation cycle was performed three times each day for three days.

Experimental protocols

The rats were randomly assigned to one of five treatment groups (Fig. 1). (1) In the IR-control group (n=16), the rats underwent 30-minute occlusion and 120-minute reperfusion of LAD. (2) The MIPC group (n=16) was used as a positive control. The rats underwent three cycles of 5-minute occlusion and 5-minute reperfusion of LAD, followed by 30 minutes of myocardial ischemia and 120 minutes of reperfusion. (3) In the LIPC group (n=16), the rats underwent LIPC for three days, and then 30 minutes of myocardial ischemia and 120 minutes of reperfusion on the fourth day. (4) In the IR-5HD group (n=16), the rats received intravenous bolus injections of 9 mg/kg of the mito K_{ATP} channel blocker 5-Hydroxydecanoate Na (5-HD) (Sigma, USA) 10 minutes before a 30-minute LAD occlusion; they received a 1 mg/kg intravenous

infusion of 5-HD during a 30-minute occlusion and a 120-minute reperfusion. (5) Finally, in the LIPC-5HD group (n=16), the rats underwent LIPC for three days. On the fourth day, the experiment was continued as for the 5-HD group.

ECG monitoring

Ventricular arrhythmias during LAD occlusion and reperfusion were evaluated according to the criteria of the Lambeth Conventions (Walker *et al.* 1988). The onset time and the incidence of ventricular premature contraction (VPC), ventricular tachycardia (VT) and ventricular fibrillation (VF) were assessed.

Infarct size assessment

At the end of 120 minutes of reperfusion, the LAD was ligated and 2 ml of trypan blue dye (0.6%) was injected via the right femoral vein; this allowed the normally perfused myocardium to be stained blue. The heart was then excised, rinsed off excess dye and blood, and frozen at -20°C for 20 minutes. The frozen ventricles were sliced transversely from apex to base into 1 mm sections and the risk region (the non-stained area) was isolated. The sections were incubated with 1% triphenyltetrazolium chloride (TTC) at 37°C for 20 minutes; the TTC stained the non-infarcted regions a brick red, while the infarcted myocardium remained pale. The tissue sections were then fixed in a 10% formalin solution for 24 hours and were weighed. The size of myocardial infarction was defined as the ratio of the weight of the infarct region (infarct size, IS) to the risk region (area at risk, AAR); it was expressed as a percentage (IS/AAR %).

Activity of antioxidant enzymes and content of malondialdehyde assays

After 120 minutes of reperfusion, the heart was excised and washed in saline at 4°C. The atria, right ventricle and interventricular septum were trimmed away. Two-thirds of the myocardial tissue close to the atria was stored at -80°C to measure the activities of total superoxide dismutase (SOD), manganese-superoxide dismutase (Mn-SOD), glutathione peroxidase (GSH-PX) and xanthine oxidase (XOD), as well as the content of malondialdehyde (MDA) with commercial kits (Nanjing JianCheng Bioengineering Institute, Nanjing, China). SOD and Mn-SOD were assayed by the xanthine/xanthine oxidase method (McCord and Fridovich 1969). GSH-PX was estimated by the method of Sazuke *et al*.(1989) (Sazuka *et al.* 1989). XOD was measured by the modified method described by Sugawara *et al*.(1999) (Sugawara *et al.* 1999). MDA was determined by the thiobarbituric acid method (Ohkawa *et al.* 1979)). The remainder of the myocardial sample was immediately frozen in liquid nitrogen and the expression of Mn-SOD mRNA was determined by RT-PCR.

Reverse transcription polymerase chain reaction amplification (RT-PCR)

The total Mn-SOD RNA was extracted from each group of rats with Trizol Reagent kits (Invitrogen Life Technologies, USA) according to the manufacturer's instructions. The quantification and purity of the RNA were assured by the ratio of OD260 to OD280 as determined by a 751-GW ultraviolet spectrophotometer (Bio-Rad Laboratories, Milan, Italy); RNA samples with an OD260 to OD280 ratio between 1.8 and 2.0 were used for RT-PCR with the Mastercycler Gradient Authorized Thermal Cycler PCR System (Eppendorf, Hamburg, Germany). The first-strand cDNA was generated from the total RNA using avian myeloblastosis virus

reverse transcriptase and oligo-(dT)-primers (Dalian Bioengineering Ltd., Dalian, China). The cDNA products were amplified by PCR in a total volume of 40 μl with 1.25 U TaKaRa Ex Taq HS (TaKaRa, Japan) and 20 pmol each of the upstream and downstream primers. After pre-denaturation at 94°C for 5 minutes, 31 cycles were allowed to run for 45 seconds at 94°C; this was followed by 45 seconds at 64°C, 1 minute at 72°C and a final extension at 72°C for 10 minutes. The primers for Mn-SOD were sense 5'- GAC CTG CCT TAC GAC TAT GG -3' and antisense 3'- GAC CTT GCT CCT TAT TGA AGC -5'. The primers for β-actin were sense 5'- TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA -3' and antisense 5'- CTA GAA GCA TTG CGG TGG ACG ATG GAG GG -3' (Dalian Bioengineering Ltd., Dalian, China). The predicted sizes of the amplified Mn-SOD and β-actin DNA products were 666 bps and 358 bps, respectively. The amplified products (6 μl) were loaded onto 2% agarose gels that had been previously stained with 1 μg ethidium bromide; they were electrophoresed at 80 V for 20 minutes and then examined under a universal hood II gel imaging system (Bio-Rad Laboratories, Milan, Italy). The images were analyzed with Quality One software, and the semi-quantitative measure of mRNA expression was expressed as the ratio of integrated optical density (IOD) with Mn-SOD/β-actin.

Statistical analyses

All data were expressed as means ± standard deviation. Comparisons were carried out with paired or unpaired *t*-tests or one-way ANOVA procedures as appropriate. Incidences of ventricular arrhythmia were compared using a chi-squared

test or Fisher's test. Differences were considered to be significant when $P<0.05$. All data summaries and statistical analyses were performed with SPSS 11.5.

RESULTS

Eighty-six rats were randomly assigned to one of five groups. Six rats were excluded because of sustained VF and/or hypotension during the LAD occlusion and reperfusion. Data are reported on the remaining 80 rats; 16 rats were in each group.

Hemodynamics

Baseline MAP and HR (before the 30-minute occlusion) were comparable among the five groups. The LAD occlusion and reperfusion produced similar decreases in MAP and HR in all groups ($P<0.05$). No significant differences in MAP and HR were found among these groups at any point (Tab. 1).

Arrhythmias

Compared to the IR-control group, the LIPC and MIPC groups showed delayed VPC and VT onset times, reduced total number of VPC and fewer incidences of VT and VF during 30-minute occlusion. These anti-arrhythmia effects of LIPC were prevented by 5-HD. There were no significant differences between the IR-5HD group and the IR-control group (Tab. 2 and Fig. 2).

Infarct size

The AAR was comparable among all groups(IR-control: 223.6±40.3, MIPC: 203.4±27.4, LIPC: 203.8±39.3, IR-5HD: 221.2±43.3, LIPC-5HD: 203.8±30.8 mg, respectively). However, the infarct sizes after ischemia and reperfusion were

significantly smaller in the LIPC and MIPC groups than in the IR-control, IR-5HD and LIPC-5HD groups ($P<0.05$) (Fig. 3).

Activity of antioxidant enzymes and content of malondialdehyde assays

Compared to the IR-control group, the total SOD, Mn-SOD and GSH-PX activities were significantly preserved, the Mn-SOD mRNA expression was markedly increased and the MDA content and XOD activity were significantly decreased in the LIPC and MIPC groups. These effects of LIPC were prevented by 5-HD in the LIPC-5HD group. There were no significant differences between the IR-5HD and IR-control groups (Tab. 3 and Fig. 4).

DISCUSSION

In the present study, LIPC consisted from three cycles of 5-minute occlusion and 5-minute reperfusion of the left hind limb per day for three days maintained the rats in a preconditioned state that was cardioprotective. And it offered a similar level of anti-arrhythmic and anti-infarct effects as acute local IPC of the heart, which is consistent with the previous study (Li *et al.* 2009) The major findings are as follows: (1) the protection offered by delayed LIPC is associated with the increase in myocardial antioxidative ability and the opening of mito K_{ATP} channels; (2) the increase in myocardial antioxidative ability of delayed LIPC is related to the activation of mito K_{ATP} channels. These observations support the hypothesis that the activation of mito K_{ATP} channels enhances myocardial antioxidative ability during IR and therefore contributes, at least in part, to the anti-arrhythmic and anti-infarct

effects of delayed LIPC.

It has been suggested that various endogenous free radical scavenging enzymes prevent an ROS surge and mediate acute cardioprotection by local IPC (Das *et al.* 1993). In the present study, the level of MDA, a product of ROS interaction with cellular constituents and an indicator of oxidative stress, was lower in the MIPC and LIPC groups than in the IR group; this indicates that either ROS generation was decreased and/or the degradation of oxidative metabolites was accelerated by MIPC and LIPC. The decreased activity of XOD in the MIPC and LIPC groups suggests that ROS generation was reduced by MIPC and LIPC. SOD converts superoxide anion to hydrogen peroxide, which is then reduced to water by GSH-PX and catalase. In the present study, the activities of total SOD, Mn-SOD and GSH-PX and the expression of Mn-SOD mRNA were higher in the MIPC and LIPC groups than in the IR group; this indicates that the products of oxidative stress were degraded more rapidly by MIPC and LIPC. These findings lead us to believe that the observed cardioprotection induced by MIPC or LIPC may be related to its enhanced antioxidative ability. This notion is supported by several other studies. For example, the expression of Mn-SOD and GSH-PX in the AAR were consistently elevated after a repeated four-cycle, 10-minute IR of the femoral artery (Chen *et al.* 2005). In addition, genes involved in protection against oxidative stress (e.g., Hadhsc, Prdx4 and Fabp4) were up-regulated after RIPC induced by six cycles of 4 minutes of occlusion and 4 minutes of reperfusion of the femoral artery (Konstantinov *et al.* 2005a).

To date, mito K_{ATP} channels appears to be both a trigger and a mediator/effector

of cardioprotection (Ichinose *et al.* 2003). Since 5-HD was administered just before lethal ischemia and during 30-min occlusion and 120-min reperfusion, the present study demonstrates that mito K_{ATP} channels play a mediator/effector role in the late phase of LIPC. Since it has also been reported to be involved in MIPC (Carreira *et al.* 2005; Wei *et al.* 2004), the opening of mito K_{ATP} channels may represent an important common feature of LIPC and MIPC, and it is likely to be a critical cellular process in cardioprotection against IR injury. The mechanism by which the opening of mito K_{ATP} channels produces cardioprotection against IR injury still remains to be determined. Several possible mechanisms have been proposed. The opening of mito K_{ATP} channels may: (1) enhance mitochondrial respiration, thus preventing mitochondrial ROS release (Ferranti *et al.* 2003); (2) improve energy metabolism (Carreira *et al.* 2005) or (3) decrease mitochondrial calcium uptake and prevent mitochondrial permeability transition (Carreira *et al.* 2005).

Moreover, the results point to a mechanistic link between the mito K_{ATP} channel activation and the enhanced antioxidative ability in the delayed cardioprotection induced by LIPC; that is, the opening of the mito K_{ATP} channels increases myocardial antioxidative ability during IR. A study by Facundo *et al*. (2007) suggested that mito K_{ATP} channels act as reactive oxygen sensors that decrease mitochondrial free radical generation in response to enhanced local levels of oxidants (Facundo *et al.* 2007). As a result, these channels regulate the mitochondrial redox state under physiological conditions and prevent oxidative stress under pathological conditions like IR. It is thought that the opening of mito K_{ATP} channels results in an increase in the protective

ROS produced during the MIPC phase and a decrease in the levels of ROS generated during reperfusion (Colantuono *et al.* 2008). As Matejíková *et al.*(2009) indicated that potential mechanisms of protection conferred by mito K_{ATP} opening in the rat heart might involve a temporal increase in ROS production in the preconditioning phase triggering changes in the pro/antioxidant balance in the myocardium and attenuating ROS production during subsequent prolonged ischemia (Matejikova *et al.* 2009). There are also conflicting reports that demonstrated that MIPC increases ROS release independently of mito K_{ATP} channels and suggested that the activity of this channel prevents oxidative reperfusion damage by decreasing ROS production (Facundo *et al.* 2006). Since 5-HD was administered just before lethal ischemia and during 30-min occlusion and 120-min reperfusion, the present study did not detect the relationship between the mito K_{ATP} channel activation and the ROS production during LIPC procedure. But Shahid *et al.*(2008) demonstrated that brief bilateral femoral artery ischemia resulted in acute preconditioning against myocardial IR injury (Shahid *et al.* 2008). The cardioprotection was mediated by a combination of mito K_{ATP} channels opening and increased ROS production. Moreover, activation of mito K_{ATP} channels was working upstream, which subsequently increased the production of ROS.

Currently, the relationship between mito K_{ATP} channels and SOD remains unknown. The present study showed that 5-HD blocked the increase of total SOD and Mn-SOD activity, as well as the increase in Mn-SOD mRNA expression induced by LIPC. Therefore, it appears that mito K_{ATP} channels work upstream of SOD. However, further study is required to more fully understand the mechanism responsible for the

opening of mito K_{ATP} channels and the increased activity and expression of SOD.

There is another type of K_{ATP} channel, i.e., sarcolemmal ATP-sensitive potassium channel (sarc K_{ATP}). But the role of it in delayed myocardial protection is not as well studied as that of the mito K_{ATP} channel. Patel *et al*. (2005) reported that the sarc K_{ATP} channel is required as a trigger but not a mediator for delayed MIPC-induced infarct size reduction in rat hearts, whereas the mito K_{ATP} channel is an end effector of delayed MIPC in rats (Patel *et al.* 2005). A similar conclusion was obtained in a study on the delayed cardioprotection induced by kappa-opioid receptor agonist U50488H (Chen *et al.* 2003). It was indicated that administration of selective channel blocker HMR-1098, before preconditioning, but not before lethal ischaemia, abolished the cardioprotection of U50488H. Early cardioprotection of LIPC was abolished by the addition of 5-HD and glibenclamide but not by HMR-1098 administered before IR, indicating a mechanism that involves mito K_{ATP} channel, but not sarc K_{ATP} channel (Kristiansen *et al.* 2005). Although several studies indicated that 5-HD is not a specific mito K_{ATP} channel inhibitor since it suppressed ischemia-induced epicardial action potential duration shortening that is usually associated with the opening of sarc K_{ATP} channel (Bernardo *et al.* 1999), the concentrations of 5-HD used in the present study affect only mito K_{ATP} channel (Sato *et al.* 1998). The role of sarc K_{ATP} channel will be examined in a later study.

The signal pathway for LIPC is unclear. Information transfer in LIPC may be mediated by humoral mediators or through a neurogenic path or a combination (Kanoria *et al.* 2007). There is evidence that cardioprotection from LIPC is triggered

by the release of endogenous opioids, adenosine, nitric oxide, norepinephrine and/or calcitonin gene-related peptide from the preconditioned limb. These signals may work directly or through receptors to trigger intracellular signal pathways such as protein kinase C, mitogen-activated protein kinases and NFkappaB; these pathways in turn are amplified and influence effectors like mito K_{ATP} channels and neutrophils, resulting in protection (Kanoria *et al.* 2007)(Kanoria *et al*. 2007). This study did not detect the pathways upstream of the opening mito K_{ATP} channels; they will be examined in a later study.

In conclusion, LIPC consisted from three cycles of 5-minute occlusion and 5-minute reperfusion of the left hind limb per day for three days provides similar anti-arrhythmic and anti-infarct cardioprotection and works through similar mechanisms as acute local IPC of the heart. It protects the myocardium from IR injury by enhancing antioxidative ability through a process involving the activation of mito K_{ATP} channels.

GRANTS

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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TABLES

Tab. 1. Systemic hemodynamics among the five experimental groups.

group	Pre-occlusion	Occlusion 30 min	Reperfusion 120 min
MAP (mmHg)			
IR-control	87 ± 6	67 ± 8**	76 ± 6**##
MIPC	87 ± 8	69 ± 14**	78 ± 8**##
LIPC	85 ± 9	68 ± 11**	74 ± 8**##
IR-5HD	85 ± 8	68 ± 9**	74 ± 8**##
LIPC-5HD	85 ± 9	65 ± 13**	77 ± 6**##
HR (bpm)			
IR-control	377 ± 34	332 ± 43**	336 ± 40**
MIPC	371 ± 18	343 ± 19**	350 ± 25*
LIPC	379 ± 24	345 ± 36**	356 ± 43*
IR-5HD	382 ± 30	336 ± 40**	348 ± 49**
LIPC-5HD	378 ± 22	341 ± 26**	349 ± 29**

MAP : mean arterial pressure; HR: heart rate; IR: ischemia-reperfusion; MIPC: myocardial ischemic preconditioning; LIPC: limb ischemic preconditioning; 5HD: 5-Hydroxydecanoate Na. Data are presented as means ± standard deviation from 16 rats per group. * P<0.05, ** P<0.01 vs pre-occlusion; ## P<0.01 vs occlusion 30 min.

Tab. 2. Ventricular arrhythmia during ischemia among the five experimental groups.

group	Onset time (min)		Incidence (%)	
	VPC	VT	VT	VF
IR-control	5.38±1.97	6.16±2.04	87.5	56.25
MIPC	11.85±1.79**	13.25±1.72**	25**	12.5**
LIPC	8.46±2.04**##	11.30±2.73**	43.75**	12.5**
IR-5HD	5.88±2.10	6.55±2.29	81.25	62.5
LIPC-5HD	5.96±2.35&&	6.81±2.97&&	81.25&	50&

VPC: ventricular premature contraction; VT: ventricular tachyarrhythmia; VF: ventricular fibrillation; IR: ischemia-reperfusion; MIPC: myocardial ischemic preconditioning; LIPC: limb ischemic preconditioning; 5HD: 5-Hydroxydecanoate Na. Data are presented as means ± standard deviation from 16 rats per group. **P<0.01 vs. IR-control group; ##P<0.01 vs. MIPC group; &P<0.05, &&P<0.01 vs. LIPC group.

Tab. 3. Activity of antioxidant enzymes and content of malondialdehyde in myocardium among the five experimental groups.

groups	Total SOD (U/mgprot)	Mn-SOD (U/mgprot)	GSH-PX (U/gprot)	XOD (U/gprot)	MDA (nmol/mgprot)
IR-control	144.78±16.47	46.37±12.18	79.38±7.95	91.20±10.69	2.01±0.17
MIPC	162.26± 6.41**	76.49±17.57*	90.62±9.80**	81.34±7.23*	1.79±0.18*
LIPC	162.67±14.21**	75.67± 3.36**	92.74±7.76**	76.29±8.77**	1.68±0.11**
IR-5HD	145.67±9.25	42.87±14.82	76.86±7.62	95.27±9.63	2.10±0.24
LIPC-5HD	145.07±14.02$^{\#\#}$	44.75±15.33$^{\#}$	79.00±7.51$^{\#\#}$	94.73±10.66$^{\#\#}$	2.10±0.23$^{\#\#}$

SOD: superoxide dismutase; Mn-SOD: manganese- superoxide dismutase; GSH-PX: glutathione peroxidase; XOD: xanthine oxidase; MDA: malondialdehyde; U/mg prot: unit per milligram protein; U/gprot: unit per gram protein; nmol/mgprot: nanomole per milligram protein; IR: ischemia-reperfusion; MIPC: myocardial ischemic preconditioning; LIPC: limb ischemic preconditioning; 5HD: 5-Hydroxydecanoate Na. Data are presented as means ± standard deviation from 8 hearts per group. $^{*}P<0.05$, $^{**}P<0.01$ vs. IR-control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. LIPC group.

FIGURE LEGENDS

Fig. 1. Experimental protocol.

IR: ischemia-reperfusion; MIPC: myocardial ischemic preconditioning; LIPC: limb ischemic preconditioning; 5HD: 5-Hydroxydecanoate Na; NS: normal saline

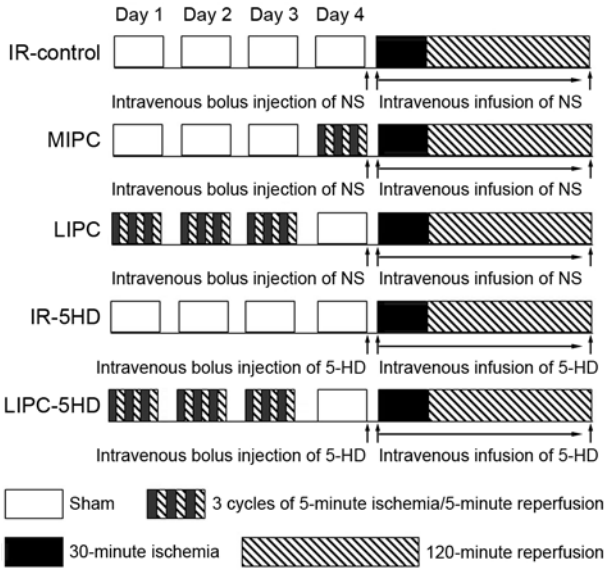

Fig. 2. Total number of VPC during ischemia among the five experimental groups. VPC: ventricular premature contraction; IR: ischemia-reperfusion; MIPC: myocardial ischemic preconditioning; LIPC: limb ischemic preconditioning; 5HD: 5-Hydroxydecanoate Na. Data are presented as means ± standard deviation from 16 rats per group. **P<0.01 vs. IR-control group.

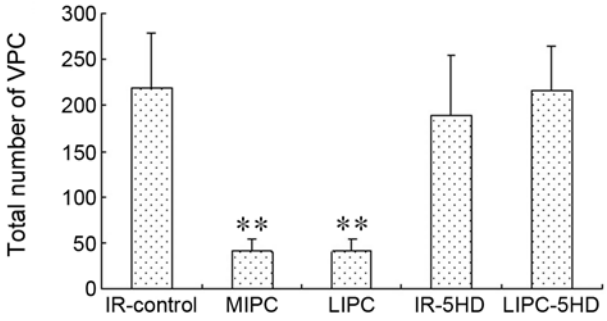

Fig. 3. Infarct size among the five experimental groups after ischemia and reperfusion.

IS: infarct size; AAR: area at risk; IR: ischemia-reperfusion; MIPC: myocardial ischemic preconditioning; LIPC: limb ischemic preconditioning; 5HD: 5-Hydroxydecanoate Na. Data are presented as means ± standard deviation from 8 hearts per group. *P<0.05, **P<0.01 vs. IR-control group; ##P<0.01 vs. LIPC group.

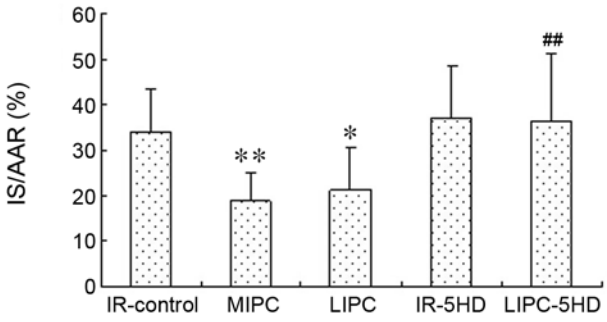

Fig. 4. RT-PCR analysis for Mn-SOD mRNA expression in myocardial tissue after ischemia and reperfusion.

A: Product expressed Mn-SODS mRNA. Lane M: DNA marker; Lane 1 and 2: IR-control group; Lane 3 and 4: MIPC group; Lane 5 and 6: LIPC group; Lane 7 and 8: IR-5HD group; Lane 9 and 10: LIPC-5HD group. B**:** Mn-SOD RT-PCR signals were quantified by densitometric absorbance units and expressed as ratios of IOD with Mn-SOD/β-actin.

IOD: integrated optical density; M: molecular weight marker; Mn-SOD: manganese-superoxide dismutase; RT-PCR: Reverse transcription polymerase chain reaction amplification; IR: ischemia-reperfusion; MIPC: myocardial ischemic preconditioning; LIPC: limb ischemic preconditioning; 5HD: 5-Hydroxydecanoate Na. Data are presented as means ± standard deviation from 8 hearts per group. *P<0.05, **P<0.01 vs. IR-control group; #P<0.05 vs. MIPC group; &&P<0.01 vs. LIPC group.