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Original Article

Turk J Med Sci
2012; 42 (5): 918-929
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E-mail: medsci@tubitak.gov.tr
doi:10.3906/sag-1106-2

# Limb remote ischemic postconditioning is effective but also time-course-limited in protecting the brain from I/R injury

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**Aim:** To determine the effect of limb remote ischemic postconditioning (LIPoC) against cerebral ischemia reperfusion in rats and the relationship between cycle number or remote postconditioning episode duration and the protective function of LIPoC.

**Materials and methods:** Transient focal ischemia/reperfusion was induced by 90 min of middle cerebral artery occlusion (MCAO) with nylon monofilament and 22 h of reperfusion in male Sprague-Dawley rats. The animals underwent LIPoC consisting of 1, 2, or 3 cycles, with each occlusion or release lasting for 5 min (5/5), 10 min (10/10), or 15 min (15/15) of bilateral femoral artery occlusion/release. Neurological score, infarct volume, water content, blood-brain barrier permeability (BBB), neuronal analysis, immunohistochemical detection, and mitochondrial permeability transition pore opening were measured.

**Results:** Infarct volume was significantly reduced in the groups with 2 cycles of 15/15, 3 cycles of 10/10, and 2 cycles of 10/10. These 3 groups also had attenuated brain edema, BBB disruption, and apoptosis compared to both the MCAO and sham groups.

**Conclusion:** This study affirmed the protective effect of LIPoC on cerebral reperfusion injury. Furthermore, it indicated that the accumulative time of limb occlusion/reperfusion might be crucial in remote postconditioning and that LIPoC exerted its maximum protective effect if the accumulative time of limb occlusion/reperfusion lasted from 40 to 60 min.

**Key words:** Stroke, ischemia/reperfusion injury, middle cerebral artery occlusion, remote ischemic postconditioning, limb, rat

## Introduction

Stroke accounts for 9% of all the deaths in the world and is the second most common cause of death. Since the majority (about 80%) of strokes are ischemic (1), resulting from occlusion of a major cerebral artery, commonly the middle cerebral artery by a thrombus or embolism, restoration of the blood supply within the recommended therapy window can reduce more extensive brain tissue injury by salvaging the reversibly damaged penumbra of tissue. However, unmodified blood is not an ideal perfusate for reflow to a brain subjected to prior ischemia and may produce the so-called cerebral reperfusion injury.

Innovative strategies have been developed to limit or prevent further brain damage during reperfusion. Ischemic preconditioning was the first endogenous mechanism-dependent method. It was introduced by Murry et al. (2) for myocardial infarction. However, it failed to be translated into clinical practice because the timing of preconditioning necessitates knowledge of stroke or other ischemic events before it happens. Postconditioning, which produces no greater but

Received: 08.06.2011 – Accepted: 18.10.2011

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at least similar protection as preconditioning in stroke, has no such requirement. However, the methodological hurdles for postconditioning protection may greatly hinder its use in clinical practice. More recently, Kerendi et al. (3) introduced brief renal artery occlusion and reperfusion, which they named "remote postconditioning," to reduce myocardial infarct size in rats. This development might make the widespread use of postconditioning in the clinic possible. This approach was subsequently applied to limbs in a less invasive way (4,5) or noninvasive way (6-9).

In this study, we investigated whether limb remote ischemic postconditioning (LIPoC) could protect the brain from ischemia/reperfusion (I/R) injury in a middle cerebral artery occlusion (MCAO) rat model. Although the number of cycles and the duration of an occlusion/reperfusion episode are critical in ischemic conditioning, there have been no reports concerning the relationship between cycle number or remote postconditioning episode duration and the protective function of LIPoC. In this study, we used different postconditioning protocols to test whether the protective effect of LIPoC on the brain against I/R was dependent on cycle number and episode duration.

## Materials and methods

### Experimental animals

All procedures in this study were in compliance with the Guide for Care and Use of Laboratory Animals. The number of animals, experimental study design, and experimental treatment of animals were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC). Adult male Sprague-Dawley rats weighing from 250 to 280 g from Shanghai Laboratory Animal Center were used.

### MCAO with reperfusion

The rats were weighed and intraperitoneally (i.p.) anesthetized with 10% chloral hydrate (300 mg/kg). A heating pad and heating lamp were used to maintain core body (rectal) temperature at 36.5 ± 0.5 °C. Rats were subjected to MCAO as described previously, with minor modifications (10,11). In brief, the right common carotid artery, internal carotid artery, and external carotid artery were surgically exposed. The external carotid artery was then isolated and coagulated. A poly-L-lysine precoated nylon monofilament with a diameter of 0.26 mm and a rounded tip was inserted into the internal carotid artery through the external carotid artery stump and then gently advanced to occlude the middle cerebral artery (MCA). After 90 min of MCAO, the filament was removed to restore blood flow (reperfusion). The rats were allowed to recover after incision closure and were housed individually until euthanization. All animals had free access to food and water.

Cerebral blood flow (CBF) was monitored from the beginning of surgery until 30 min after reperfusion with a probe attached to the skull above the supply territory of the MCA (2 mm caudal to the bregma and 6 mm lateral to the midline) by laser Doppler flowmetry (PeriFlux System 5000, PERIMED, Stockholm, Sweden). The procedure was considered successful if more than a 75% decline in CBF was observed after MCAO.

### CBF measurement

Only rats having a reduction in CBF to less than 25% of baseline were considered to have successful occlusion of the MCA in this model and were included in the study. Rats with subarachnoid hemorrhage were excluded from the protocol.

After withdrawal of the occluding filament, ipsilateral blood flow was restored to approximately 90% of baseline level. Reductions in CBF during MCAO and during reperfusion were not significantly different among the treated groups. There was no reduction in CBF in all groups that underwent sham operations.

### Limb remote ischemic postconditioning

Rats were randomly assigned to 11 groups (Figure 1). All groups were subjected to 90 min of MCAO. MCAO group rats were subjected to 90 min of ischemia only, without any further interruption of reperfusion. Bilateral femoral arteries were separated below the groin ligament for later induction of femoral artery occlusion or sham limb surgery. Rats in the sham group received surgery with ischemia and reperfusion, but only bilateral femoral arteries separation without postconditioning. LIPoC was carried out by occluding and releasing the bilateral femoral arteries for 1, 2, or 3 cycles, with each

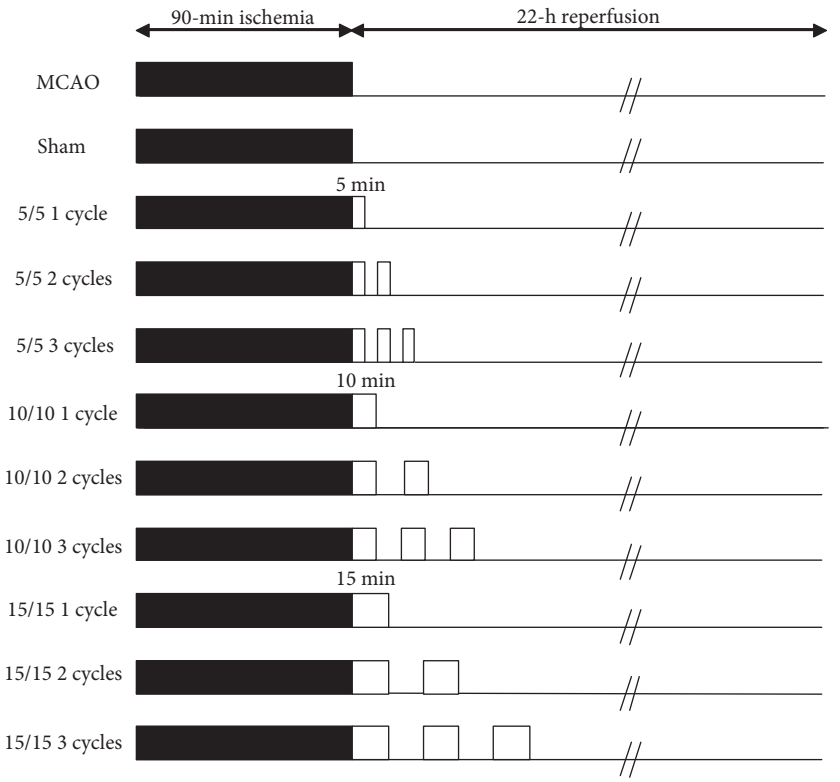


Figure 1. Experimental protocols for cerebral ischemia and reperfusion in rats. 15/15, 10/10 and 5/5 represent 15 min/15 min bilateral femoral arteries occlusion/release, 10 min/10 min bilateral femoral arteries occlusion/release, and 5 min/5 min bilateral femoral arteries occlusion/release, respectively.

occlusion or release lasting for 5 min (5/5), 10 min (10/10), or 15 min (15/15). LIPoC was initiated at the beginning of reperfusion.

**Neurological evaluation**

After the suture was removed, the rats were returned to their separate cages. After 24 h, the rats were assessed neurologically by an observer who was blind to the animal groups. The neurobehavioral scoring was performed using a 6-point scale as was previously described by Longa et al. (11): normal motor function = 0; flexion of contralateral forelimb upon suspension vertically by tail or failure to extend forepaw = 1; circling to the contralateral side but having normal posture at rest = 2; loss of righting reflex = 3; no spontaneous motor activity = 4. Death was considered as a score of 5 only when a large infarct volume was present in the absence of subarachnoid hemorrhage. If rats died due to subarachnoid hemorrhage or pulmonary insufficiency and asphyxia, they were eliminated from the study.

**Measurement of infarct volume**

After being sacrificed with chloral hydrate (800 mg/kg), the animals were decapitated and the brains were rapidly removed and cooled in –20 °C saline for 10 min. Brains (n = 5 for each group) were sliced into 2-mm-thick coronal sections and stained with standard 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich, Vienna, Austria) for 30 min at 37 °C for photographing and calculations (12). The infarcted zone was demarcated and analyzed by ImageJ software (National Institutes of Health, version 1.43). Infarct volumes of all sections were added together to derive the total infarct area, which was multiplied by the thickness of the brain sections to obtain the infarct volume, and the ischemic area was expressed as a percentage of the whole brain area.

**Water content**

Brain edema was analyzed by water content determination. Rats were decapitated with chloral hydrate (800 mg/kg) 22 h after reperfusion and the wet weight of the brain samples was determined. The brain samples were then dried in an oven at 110 °C for 24 h and reweighed. The water content of the samples was then measured by the wet and dry method as follows: water content (%) = (wet weight – dry weight) × 100/wet weight (13).

**Blood-brain barrier permeability**

The integrity of the blood-brain barrier (BBB) was evaluated by using Evans blue (EB; Sigma-Aldrich, St. Louis, MO, USA) dye extravasations, as described by Kaya et al. (14) Briefly, the rats received 2 mL/kg of 2% EB solution in saline by tail vein injection 30 min after MCAO. The thoracic cavity was opened under anesthesia 22 h after reperfusion. The rats were perfused with 250 mL of saline transcardially to wash out intravascular EB until colorless perfusion fluid was obtained from the atrium. After decapitation, the brains were removed and the hemispheres were separated and weighed. The right and left hemispheres were separately homogenized in 2.5 mL of phosphate-buffered saline to extract the EB, and to precipitate protein 2.5 mL of 60% trichloroacetic acid was added and mixed by vortex for 3 min. The samples were kept at 4 °C for 30 min and then centrifuged for 30 min at 1000 × *g*. The amount of EB in the supernatants was measured at 630 nm using a spectrophotometer (ST-360, KHB, Shanghai, China). EB levels were expressed as μg/g of brain tissue against a standard curve.

**Neuronal analysis and immunohistochemical detection**

The brains were immersed overnight in 10% phosphate-buffered formalin. The tissues from an area 4 mm anterior and 8 mm posterior to the bregma were then cut into 6 serial 2-mm coronal sections. The third section was used to analyze the effect of LIPoC. The sections were embedded in paraffin and cut at 5 μm.

The slices were stained with TUNEL as described in the manufacturer's protocol (Roche, Mannheim, Germany). The selected regions for TUNEL-positive neuron counting were sampled at the ischemic core and ischemic penumbra of the cortex (Figure 2). The TUNEL-positive neurons were counted in the penumbra of the cortex.

**Preparation of brain mitochondria**

Mitochondria were isolated according to the procedure described by Clark and Nicklas (15). Rats were decapitated and the brains were immediately removed and homogenized in an ice-cold isolation buffer (0.25 mol/L sucrose, 1 mmol/L K-EDTA, 10 mmol/L Tris-HCl, pH 7.4) with a Teflon pestle. The homogenate was immediately centrifuged at 2000 × *g* for 3 min, the supernatant was centrifuged again at 2000 × *g* for 3 min, and the second supernatant was decanted and centrifuged at 12,000 × *g* for 8 min. The supernatant was discarded and the pellet was resuspended in isolation buffer without K-EDTA. The suspension was then centrifuged at 12,000 × *g* for 10 min. The resulting brown mitochondrial pellet was resuspended in the same buffer. All of the above procedures were carried out at 0-4 °C.

Mitochondrial protein concentration was quantified according to the method of Bradford using 1 mg/mL BSA as standard.

**Measurement of mitochondrial permeability transition pore opening in isolated brain mitochondria**

Opening of the mitochondrial permeability transition pore (mPTP) causes mitochondrial swelling, which results in a reduction of absorbance at 520 nm ($A_{520}$). Mitochondrial swelling was determined by the method reported earlier (16). Mitochondria (approximately 0.2 mg of protein) were preincubated in a medium containing KCl at 110 mmol/L, MOPS at 20 mmol/L, Tris-HCl at 10 mmol/L, rotenone at 0.5 mmol/L, and antimycin at 0.5 mmol/L (pH 7.4) for 10 min. After a 10-min equilibration period, 200 μmol/L $CaCl_2$ was added to induce mPTP opening. The absorbance at 520 nm was measured spectrophotometrically for 6 min.

**Statistical analysis**

Values are shown as mean ± SD. Unless otherwise stated, differences were assessed using Student's t-test and considered statistically significant at $P < 0.05$. One-way ANOVA and Student-Newman-Keuls tests were used for post hoc analysis.

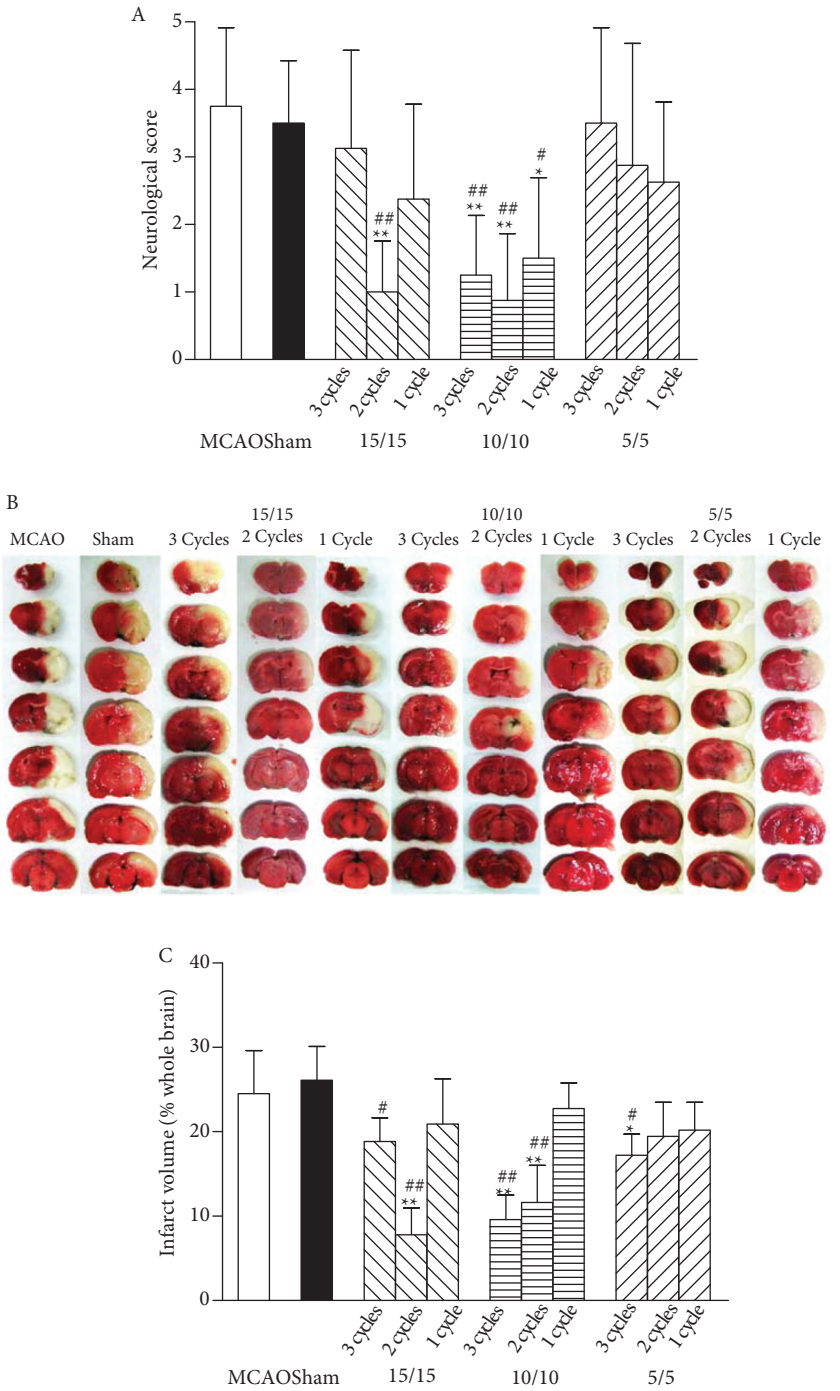


Figure 2. Diagram indicates regions for H&E analysis of TUNEL. A coronal brain section near the bregma was selected. The ischemic core (A) and the ischemic penumbra (B) are the 2 regions indicated.

## Results

### Neurological score

Rats in groups for 2 cycles of 15/15, 3 cycles of 10/10, and 2 cycles of 10/10 showed a significantly improved neurological outcome compared with both the MCAO and sham groups ($P < 0.01$). The group with 1 cycle of 10/10 also showed an improved neurological score ($P < 0.05$). However, the other groups failed to show any obvious improvement in their scores (Figure 3A).

### TTC staining

Figure 3B shows representative photographs derived from postmortem TTC-stained sections at 22 h after MCAO. The infarct volume in groups with 2 cycles of 15/15, 3 cycles of 10/10, and 2 cycles of 10/10 decreased significantly (7.79 ± 3.19%, 9.60 ± 1.89%, and 11.60 ± 4.42%, respectively, versus sham group at 26.10 ± 4.02%; $P < 0.01$). The infarct volume for 3 cycles of 15/15 and 3 cycles of 5/5 was reduced significantly compared to the sham group (18.85 ± 2.81% and 17.22 ± 2.50%, respectively, versus 26.10 ± 4.02%; $P < 0.05$) (Figure 3C).

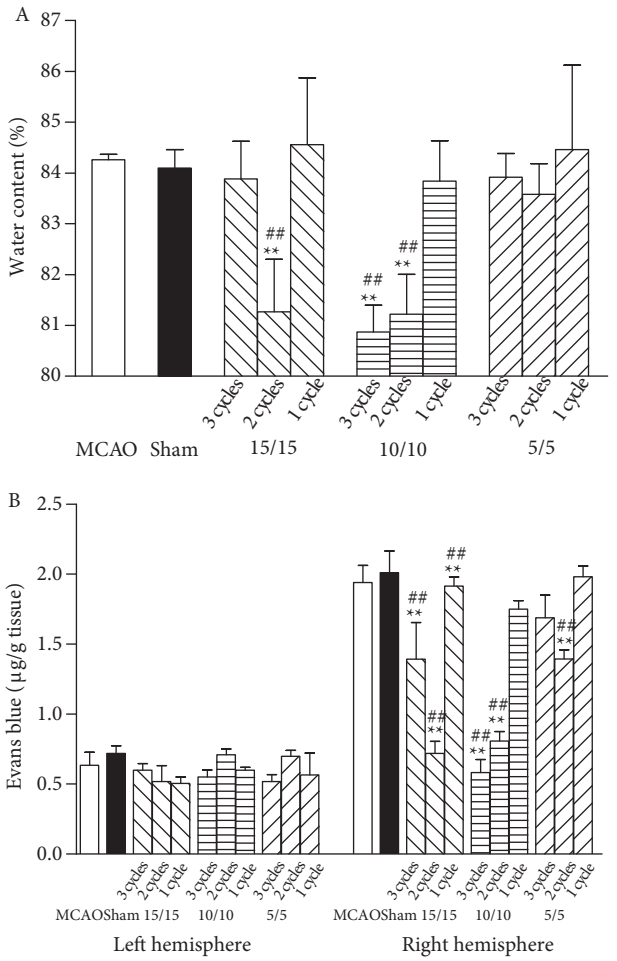


Figure 3. The effect of LIPoC on neurological score (A) and infarction (B and C) after cerebral ischemia and reperfusion in rats. Values expressed as mean ± SD; n = 8 in neurological score evaluation and n = 5 in infarct volume measurement. *$P < 0.05$, **$P < 0.01$ vs. MCAO; #$P < 0.05$, ##$P < 0.01$ vs. sham.

### Brain water content

A significant decrease in brain water content was observed for 2 cycles of 15/15, 3 cycles of 10/10, and 2 cycles of 10/10 compared to both the MCAO and sham groups (81.27 ± 1.03%, 80.87 ± 0.53%, and 81.22 ± 0.78%, respectively, versus sham group at 84.10 ± 0.36%; $P < 0.01$). There were no changes in the other groups (Figure 4A).

### Blood-brain barrier permeability

There were no significant differences of EB content on the contralateral side (left hemisphere). On the ipsilateral side (right hemisphere), EB content decreased significantly with 3 cycles of 15/15, 2 cycles of 15/15, 3 cycles of 10/10, 2 cycles of 10/10, and 2 cycles of 5/5 ($P < 0.01$), and especially in the groups treated with 2 cycles of 15/15, 3 cycles of 10/10, and 2 cycles of 10/10 (0.72 ± 0.08 μg/g tissue, 0.58 ± 0.09 μg/g tissue, and 0.81 ± 0.07 μg/g tissue, respectively, versus sham group at 2.01 ± 0.15 μg/g tissue ) (Figure 4B).

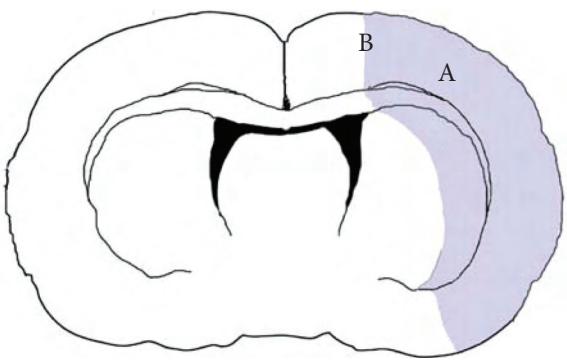


Figure 4. The effect of LIPoC on brain water content (A) and blood-brain barrier permeability (B) after cerebral ischemia and reperfusion in rats. Values expressed as mean ± SD; n = 5. *$P < 0.05$, **$P < 0.01$ vs. MCAO; #$P < 0.05$, ##$P < 0.01$ vs. sham.

### Histological examination

Sample micrographs of coronal brain sections stained with hematoxylin and eosin (H&E) are given for each group. Ischemic damage involving both the cortex and striatum of the ipsilateral cerebral hemisphere characterized by typical necrosis, neuronal loss, tissue edema, and gliotic cavity was observed in both the MCAO and sham groups (Figure 5A). After LIPoC, the infarct area was obviously decreased in the groups treated with 2 cycles of 15/15, 3 cycles of

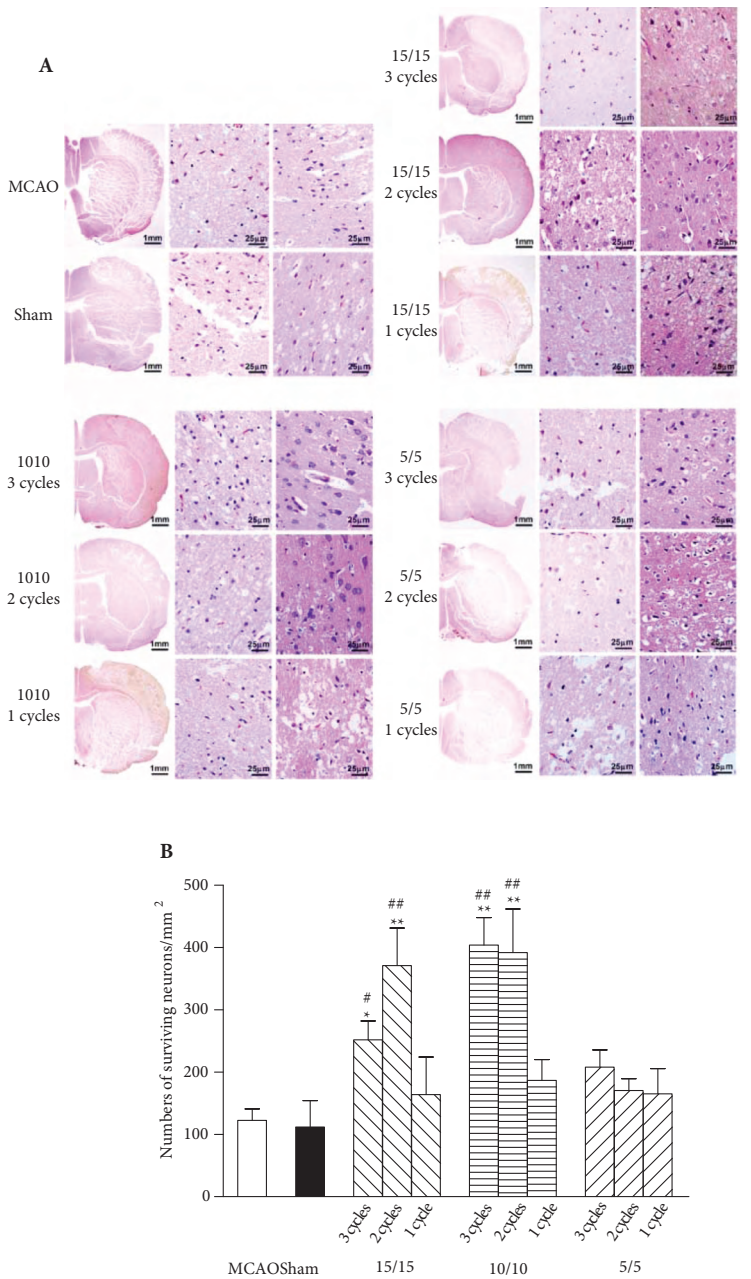


Figure 5. The effect of LIPoC on H&E staining of the core (left) and penumbra (right) of the cortex (A); and the number of surviving neurons in the penumbra of the cortex (B). Effect of LIPoC on values expressed as mean ± SD; n = 5. *P < 0.05, **P < 0.01 vs. MCAO; #P < 0.05, ##P < 0.01 vs. sham.

10/10, and 2 cycles of 10/10, while the majority of the cells in the penumbra of these groups remained intact. The groups with 3 cycles of 15/15 and 3 cycles of 5/5 also showed improvement to some extent, whereas the other groups did not show any treatment effect (Figure 5B). Brain tissue structure was normal on the contralateral side.

At 2 cycles of 15/15, 3 cycles of 10/10, and 2 cycles of 10/10, there was significantly decreased cell death compare to the MCAO and sham groups (371 ± 60.3/mm$^2$, 404 ± 44.1/mm$^2$, and 392 ± 69.7/mm$^2$, respectively, versus sham group at 112 ± 42.3/mm$^2$; $P < 0.01$). The 15/15 cycles also showed a protective effect on neurons (252 ± 30.3/mm$^2$ versus sham group at 112 ± 42.3/mm$^2$; $P < 0.05$).

**TUNEL staining**

TUNEL-positive cells were distributed in the ischemic penumbra and ischemic core in the MCAO and sham groups and there was also much necrotic cellular debris in the core (Figure 6A). There were significantly fewer TUNEL-positive cells with 2 cycles of 15/15, 3 cycles of 10/10, and 2 cycles of 10/10 than in the MCAO and sham groups (237 ± 42.2/mm$^2$, 170 ± 80.5/mm$^2$, and 218 ± 66.6/mm$^2$, respectively, versus sham group at 709 ± 129.3/mm$^2$; $P < 0.01$; 66.6%, 76.0%, and 69.3% reduction, respectively). A 38.4% reduction in the number of TUNEL-positive cells was also observed in the group with 3 cycles of 5/5 (437 ± 64.7/mm$^2$ versus sham group at 709 ± 129.3/mm$^2$; $P < 0.05$) (Figure 6B). No TUNEL-positive cells were found on the contralateral side.

**mPTP opening**

In order to find out the possible mechanism of LIPoC on the MCAO rats, we measured the mPTP opening spectrophotometrically in brain mitochondria from MCAO rats (Figure 7A). After adding 200 μmol/L $CaCl_2$, the absorbance of isolated mitochondria suspension from groups treated with 2 cycles of 15/15, 3 cycles of 10/10, and 2 cycles of 10/10 decreased significantly ($P < 0.01$). However, the absorbance decrease of the mitochondria from the MCAO and sham groups was attenuated. Treatment with 3 cycles of 15/15 also increased the decline of the absorbance significantly. The other groups, except that treated with 1 cycle of 5/5, showed a slight decrease of absorbance, but not enough to be significant statistically (Figure 7B).

**Discussion**

The present study confirmed that LIPoC applied immediately at reperfusion reduced infarct size and attenuated reperfusion injury with regard to brain edema, integrity of the BBB, and neuron apoptosis. Moreover, we found that the protective effect of LIPoC on the brain against I/R might be related to the number of cycles and duration of the limb I/R episode. Multiple cycles of bilateral femoral artery occlusion/reperfusion might be needed for cerebral infarct reduction because single LIPoC failed to provide any benefit for the brain after transient focal ischemia.

Li et al. (4) first chose a limb as the remote organ to perform remote ischemic postconditioning before the onset of myocardial reperfusion, and it proved to be effective in protecting myocardium from I/R. Subsequently, Andreka et al. (6) extended LIPoC to the time of reperfusion during myocardial infarction in pigs, and Ren et al. (5) found that LIPoC carried out 3 h after bilateral common carotid artery reperfusion also significantly reduced infarct size in permanent focal cerebral ischemia. The present study also confirmed that LIPoC performed immediately at the time of reperfusion protects the brain from reperfusion injury. These observations suggest that LIPoC could potentially have an important clinical role in stroke because femoral artery occlusion can be carried out in a noninvasive way such as with a blood pressure cuff, which has been used in a previous human I/R injury study (9).

The mechanisms of LIPoC are still unknown. However, research from preconditioning or postconditioning against ischemia reperfusion in the brain or other organs may indicate the possible mechanism of the accumulative time-dependent effect of LIPoC. Kerendi et al. reported that adenosine might serve as a mediator of the remote postconditioning effect (3). There are 3 adenosine pathways that have been proposed to explain the remote postconditioning effect: 1) the humoral pathway suggests that adenosine released into the bloodstream by remote ischemic tissue binds to receptors in the I/R injured organ and then triggers protection; 2) the neural pathway suggests that adenosine released by remote ischemic tissue activates its afferent nerves, then activates

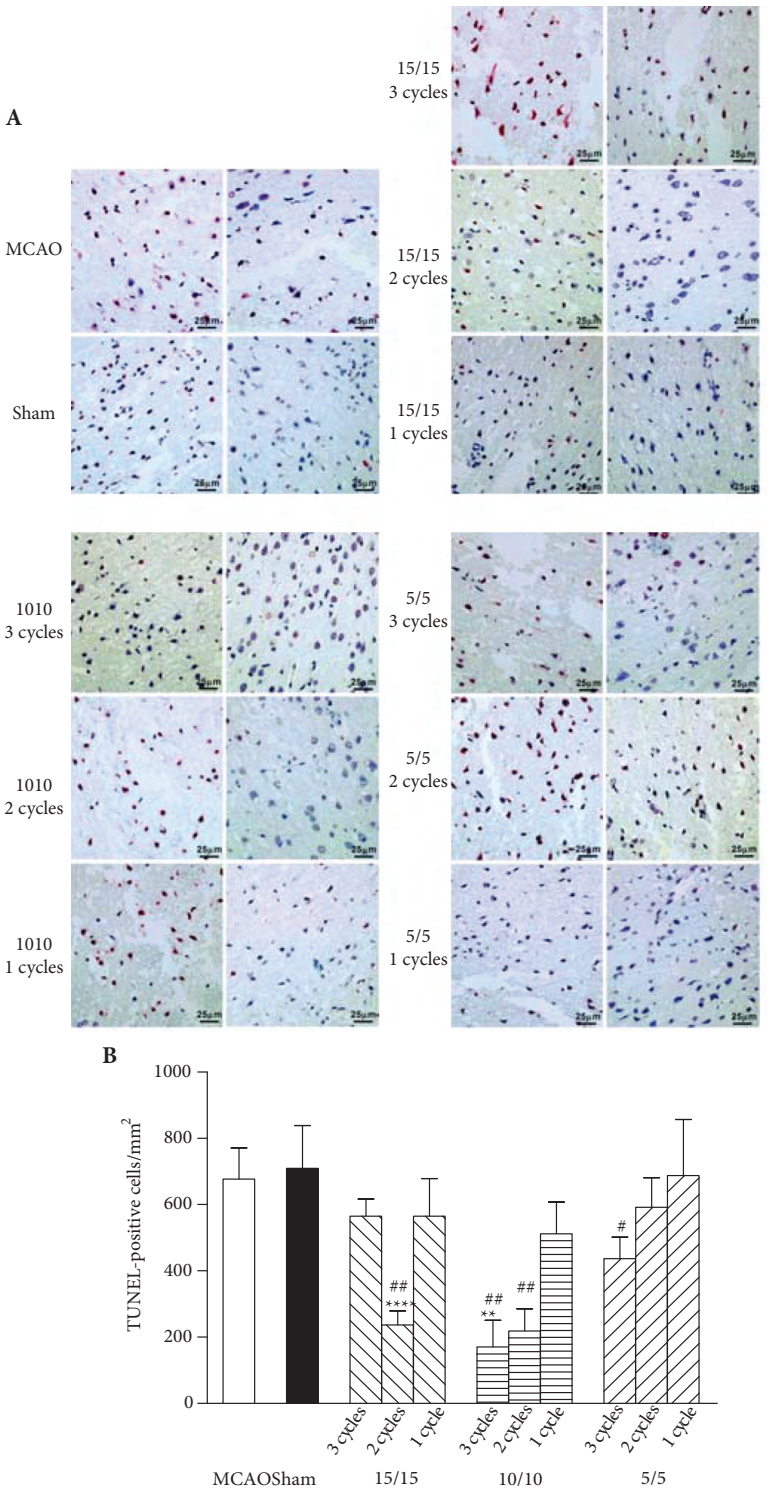


Figure 6. The effect of LIPoC on TUNEL staining of the core (left) and penumbra (right) of the cortex (A); and the number of cells immunopositive for TUNEL in the penumbra of the cortex (B). Effect of LIPoC on values expressed as mean ± SD; n = 5. **P < 0.01 vs. MCAO; #P < 0.05, ##P < 0.01 vs. sham.

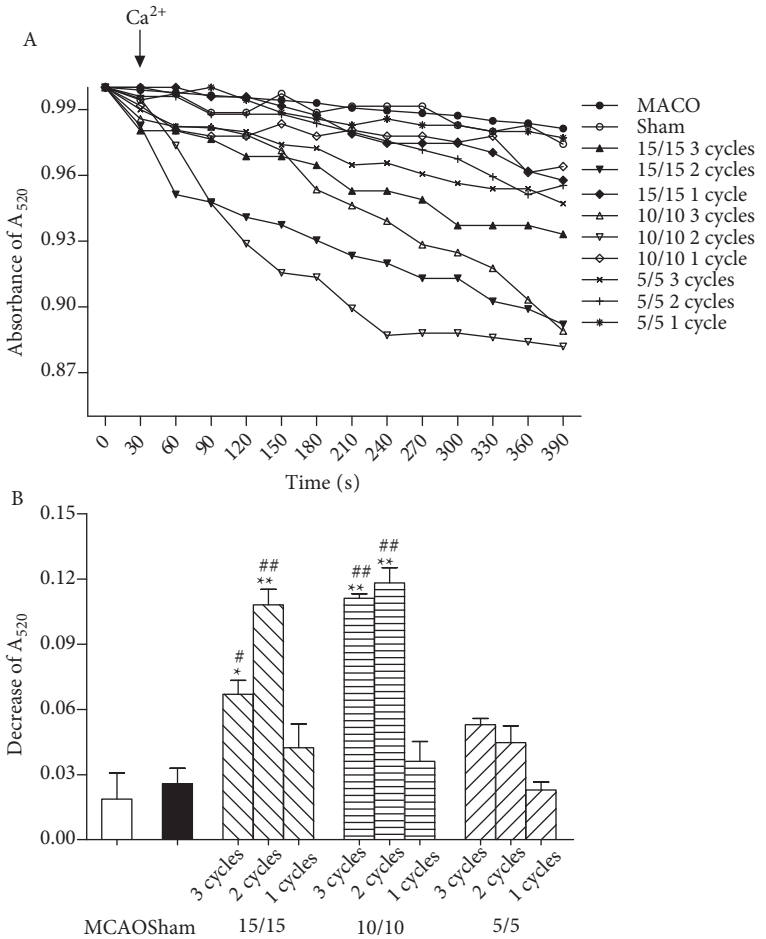


Figure 7. The effect of LIPoC on absorbance at 520 nm ($A_{520}$) in suspensions of rat brain mitochondria exposed to 200 μmol/L $CaCl_2$ with pooled curves of absorbance under different treatments (A) and absorbance data 15 min after addition of 200 μmol/L $CaCl_2$ (B). Values expressed as mean ± SD; n = 5. *P < 0.05, **P < 0.01 vs. MCAO; #P < 0.05, ##P < 0.01 vs. sham.

efferent nerves to protect the I/R injured organ; 3) adenosine released from remote ischemic tissue suppresses the systemic inflammatory response, such as inhibiting neutrophil accumulation in the area at risk of infarction (7). Ren et al. (5) reported that hexamethonium (ganglion blocker), capsacin (afferent nerve), or cycloheximide (protein synthesis inhibitor) could abolish the protective effect of remote postconditioning. Bradykinin and εPKC might contribute to the afferent nerve activation pathway, but why new protein is synthesized from neuroprotection is still unknown. Moreover, decrease in plasma CK and MDA activity also suggested that remote postconditioning could reduce the generation of reactive oxygen species (ROS) and attenuate the oxidant-mediated injury (4).

It is noteworthy that the number of cycles and duration of the I/R episode are critical in preconditioning (17) and postconditioning (18), and this principle may be also applicable in LIPoC. In our study, we attempted to clarify the relationship between cycle number or remote postconditioning episode duration and the protective function of LIPoC. In addition, we found that only in groups treated with 2 cycles of 15/15, 3 cycles of 10/10, and

2 cycles of 10/10 was there reduced infarct size and attenuation of reperfusion injury with regard to brain edema, integrity of the BBB, and neuron apoptosis, while the groups with 3 cycles of 15/15 and 3 cycles of 5/5 only had partial attenuation of these parameters. It is interesting that LIPoC exerted its maximum protective effect in rat brains against cerebral I/R injury when accumulative limb occlusion/reperfusion time lasted from 40 to 60 min. The results showing that 2 cycles of 5/5 and 1 cycle of 5/5 were not protective are not surprising. The requirement of postconditioning stimulus to cross a threshold may be determined by endogenous mediators, some of which (e.g., adenosine, bradykinin) are released during postconditioning.

However, the finding that 3 cycles of 15/15 were less protective than 2 cycles of 15/15, 3 cycles of 10/10, and 2 cycles of 10/10 was beyond our expectation. Cerebral I/R has been characterized by 4 major phenomena in the early stage: 1) rapid loss of ATP during ischemia followed by the first 15 min of reperfusion; 2) morphologic evidence of progressive damage to selected vulnerable tissues; 3) postischemic hypoperfusion representing "secondary ischemia"; and 4) inhibition of brain protein synthesis during reperfusion (19). All 4 characteristics are initiated at the beginning or early stage of reperfusion. Meanwhile, BBB disruption (20) and DNA fragmentation measured by TUNEL (21) begin at the late reperfusion stage (more than 4 and 3 h after reperfusion, respectively) and become most apparent by 12-24 h and 24 h after reperfusion, respectively. In the present study, the protocol with accumulative time of 40-60 min attenuated reperfusion injury with regard to brain edema, the integrity of the BBB, and neuron apoptosis, and these results indicated that the effect of LIPoC might last more than 4 h at the end of postconditioning. However, we did not observe any differences in the progress of the pathological changes after reperfusion between the MCAO and LIPoC groups, which might have revealed the time course of LIPoC or answered the question about whether LIPoC shows similar delayed protection as remote preconditioning.

Inhibition of mPTP opening is considered as the end effector of postconditioning. Adenosine, which exerts its effect on A2a and A3a receptors, triggered phosphoinositide-3-kinase (PI3K) pathways to affect mPTP(18). Postconditioning-mediated endogenous NO (low dose) also triggered NO-cGMP-PKG pathways to inhibit mPTP opening to protect I/R injured organs (22). In this study, 2 cycles of 15/15, 3 cycles of 10/10, and 2 cycles of 10/10 inhibited mPTP opening significantly. Adenosine is the product of ATP during the very first reperfusion and the concentration of adenosine in plasma may decrease when the accumulative limb occlusion/reperfusion time exceeds 60 min. An accumulative limb occlusion/reperfusion time longer than 60 min may also cause the overproduction of NO, damaging I/R injured organs.

The present study had several limitations. We did not select 5-min occlusion/reperfusion protocols or other protocols with 40-60 min of accumulative time to study. However, our study indicated that LIPoC might be potentially useful for attenuating cerebral reperfusion injury. Additionally, this is the first study on LIPoC to demonstrate that the effect of LIPoC is dependent on accumulative time and that the ideal accumulative time ranges from 40 to 60 min.

## Acknowledgment

This study was supported by the National High-tech R&D Program (863 Program) of China, No. 2006AA02A117.

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