1 Once delayed non-invasive remote ischemic preconditioning protects against 2 early stroke by modulating neuroinflammatory responses in rats

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20**Running title:** Once delayed RIPC protects early stroke

21**Abstract**

Once delayed non-invasive remote ischemic preconditioning (RIPC) has been proven to provide endogenous protection against injury induced by ischemia–reperfusion in the central nervous system. However, for this ischemic preconditioning method, it is still unclear how long this protection can maintain and what the underlying mechanism is. In this study, we tested the hypothesis that once delayed non-invasive RIPC protects brain injury at short reperfusion time. The rat was stimulated by transient middle cerebral artery occlusion (MCAo) for 90 min, and subsequent reperfusion was performed at 6 h, 72 h and 7 days after MCAo. RIPC was conducted in both hind limbs 24 h before MCAo for 3 cycles (10 min ischemia/ 10 min reperfusion). The infarct size was measured by 2, 3, 5-triphenyl-2H-tetrazolium chloride (TTC) staining and Cresyl violet (CV) staining. The mRNA and protein levels of inflammatory cytokines in the brain were measured by real-time RT-PCR and ELISA. The results showed that once delayed non-invasive RIPC reduced the infarct size, improved neurological functions and behavioral performance at 6 and 72 h post-stroke. There was no change by reperfusion at 7 d after MCAo. RIPC reduced the levels of TNF α , IL-1 β and IL-6 in the brain at 72 h post stroke. It also reduced the levels of TNF α and IL-1 β when reperfusion at 6 h after MCAo. Our results strongly supported that once delayed non-invasive RIPC protects against stroke as a non-invasive neuroprotective strategy, which maintained for both short and middle term ischemic reperfusion time. The protective effect is mediated by the modulation of inflammatory response in the ischemic brain.

Key words: cerebral ischemia, reperfusion, RIPC, time window, pro-inflammation

44Introduction

45 Ischemic stroke is considered to be the third most fatal and disabling disease in
46the world. At present, the most effective treatment for stroke is intravenous
47thrombolysis or intravascular interventional treatment within several hours after the
48onset of stroke. Unfortunately, the proportion of patients who can be treated within a
49few hours is less than 5%, and even if the infarction is lifted and blood reperfusion is
50established, the ischemia-reperfusion injury of the brain tissue cannot be ignored,
51which causes the current unsatisfactory results. Neuroprotective drugs developed over
52the years have been proven effective in animal models of stroke, but have poor
53clinical efficacy [1]. Therefore, it is urgent to find auxiliary or alternative treatment to
54further improve the treatment effect of stroke. In recent years, a variety of remote
55ischemic preconditioning (RIPC) methods have been tested as feasible treatment
56strategies for stroke. Our previous studies and other researches have proved that RIPC
57has a protective effect on stroke in both basic research [2-4] and clinical experiments
58[5-7]. RIPC is easy to handle and relatively resistant to reperfusion injury, so it has
59great clinical advantages. However, for the once delayed non-invasive RIPC method,
60the duration of protection and mechanisms are still unclear.

61 Preconditioning is a phenomenon in which the brain protects itself against future
62injury by adapting to low doses of noxious insults [8]. The concept of cerebral
63ischemic tolerance was first introduced in the early 1990s. Kitagawa et al. reported
64the neuroprotective effects against neuronal cell death when adding 2 minutes of
65transient ischemia 24 hours before global cerebral ischemia in rats [9]. As ischemic

conditioning is difficult to realize in the *in situ* organ, the concept of RIPC is proposed. RIPC is an endogenous protective mechanism through which the short-term sub-lethal ischemia of remote organs can protect the main organs from further severe ischemia. Acute and delayed preconditioning in both heart and brain has different mechanisms. Acute/early preconditioning performed 1 to 3 hours before stroke onset is related to a rapid response such as changes in ion channel permeability and post-translational modifications of proteins and the protection lasted only several hours. While delayed preconditioning induced 1 to 7 days before stroke onset induced gene activation and protein synthesis and the protection lasted several days [10-13]. In most experiments, the protective effects on the brain need hours and sometimes days to fully manifest; thus, delayed preconditioning has been studied as a more effective strategy. To date, studies on the mechanisms of both cardiac and cerebral preconditioning at the molecular, cellular and tissue levels span nearly 30 years [14]. Many studies have shown that the neuroprotective mechanisms of RIPC by a complex cellular regulatory process that involves multiple cellular signaling pathways and leads to enhanced tolerance to ischemia/hypoxia.

So far, there are many ways of RIPC, and their effects are not completely consistent. Previous study showed that non-invasive RIPC 5 min ischemia/ 5 min reperfusion for 3 cycles contributed neuroprotection by activating adenosine A1 receptor [15]. Another study showed that 4 cycles of RIPC (5 min/cycle, 40 min total) decreased the expression of neuroinflammation by improving the peripheral immune cell response [2]. Our previous research showed that 3 cycles of RIPC (10 min

88ischemia/ 10 min reperfusion) significantly reduced infarct size and
 89neuroinflammation by modulating the expression of HIF-1 α [4]. Moreover, studies
 90showed that acute (15 min before ischemia), delayed (24 h before ischemia) and
 91chronic (9 d repeated ischemic conditioning) ischemic preconditioning all reduced
 92infarct size in heart following myocardial ischemia [16]. For the 3 cycles of once
 93delayed non-invasive RIPC (10 min ischemia/ 10 min reperfusion), it has been proven
 94effective in our previous study, whereas, the duration of this protection lasting is still
 95unclear.

96 In this study, we aimed to provide insights into demonstrating how long the
 97protective effect of once delayed non-invasive RIPC can be maintained. First, we
 98tested the infarct size, neurological and behavioral deficiencies at different reperfusion
 99time compared with MCAo and RIPC+MCAo group. Then, we tested the mRNA and
 100protein levels of pro-inflammatory cytokines in the ischemic brain by real-time RT-
 101PCR and ELISA respectively.

102Materials and methods

103 Male Sprague-Dawley (SD) rats were purchased from Vital River Laboratory
 104Animal Technology Co., Ltd (Beijing, China). The weights were 280-320g. They are
 105placed in a control room at a temperature of 24 ° C and a standard 12-hour light-dark
 106cycle. They can freely obtain food and water and are randomly divided into different
 107groups. The number of animals per group is 12 to 14. All procedures in this study
 108were conducted in accordance with ethical standards, with the Helsinki Declaration,

with national and international guidelines, and have been approved by the Authors Institutional Review Board.

Middle cerebral artery occlusion (MCAo)

In our experiments, rats were anesthetized with 3-5 % isoflurane in 70 % nitrous oxide and 30 % oxygen, and maintained with 1-3 % isoflurane. For the MCAo model, ischemia and reperfusion was established as previously described [4]. Briefly, the common carotid artery (CCA), right internal carotid artery (ICA), and external carotid artery (ECA) were exposed during the procedure. A silicone-coated nylon suture with a diameter of 0.38 ± 0.02 mm was inserted into the ICA; the silicone-coated nylon suture was within 18-20 mm from the ECA bifurcation to block the MCA, and withdrawn after 90 minutes of occlusion to allow MCA to re-open. Rat rectal temperature was maintained at $37 \pm 0.5^{\circ}$ C during the entire procedure. The rats in the sham group underwent surgery without MCA occlusion. The cerebral blood flow (CBF) during the surgery before and after occlusion were measured by laser Doppler perfusion monitoring with a laser Doppler probe (PeriFlux System 5000, Perimed AB, Sweden) interfaced to a laptop equipped with the PeriSoft data acquisition software (PeriSoft Systems, Inc., Sweden). The blood gas (PaCO_2 , PaO_2 [mmHg] and pH) and blood sugar (mmol/L) were also examined during the surgery as previously described [17].

Remote ischemic preconditioning (RIPC)

In our experiments, delayed non-invasive RIPC was used, in contrast to the

130invasive, direct femoral artery occlusion. Briefly, twenty-four hours before MCAo,
131RIPC was conducted in both hind limbs of rats anesthetized with 1-3 % isoflurane.
132Two strip gauze bandages were tied on the two hind limbs simultaneously to occlude
133blood circulation for 10 minutes, and then released for 10 minutes to allow
134reperfusion. The occlusion/reperfusion cycle was repeated for 3 times.

135Behavioral testing

136 Behavioral tests were conducted as described previously [4, 17]. Behavioral tests
137were performed to assess rats' neurological function after stroke, including tail hang
138tests, home cages test and postural reflexes test. All behavioral tests are performed by
139a person who does not understand the experimental conditions. We trained rats three
140days before surgery and tested their baseline one day before surgery. All behavioral
141tests were evaluated before the animals were sacrificed.

142 For the tail suspension test, hung the tail of the rat about 10 cm from the ground.
143Stroke rats will turn to the opposite side (left) of the ischemic hemisphere, and the
144head will rotate more than 90 °. Each rat was hung for no more than 5 seconds, and
145each rat was hung a total of 20 times. The percentage of head turns was calculated.

146 Rats usually used their forelimbs to explore the cage margin. For the home cage
147limb test, we calculated the number of times when rats' ipsilateral, contralateral, or
148both forelimbs contacted the cage wall. Instruct the rat to touch the cage wall 20
149times. Use the following formula to calculate the percentage of ipsilateral forelimbs
150used: $[\text{ipsilateral} + (\text{both} / 2)] \times 100\%$.

151 In a postural reflex test, rat was placed on a table. We held its tail in one hand,
152 and pushed its shoulder nearly 20 cm for 3 times with the other hand. Non-ischemic
153 rats grasped the table vigorously during the push and scored zero. Rats that had less
154 resistance and became stiff during the referral process received 1 point. If the rat was
155 not resistant, the score was 2.

156 We also used the Longa scoring system to measure neurological deficits at
157 different times after reperfusion to assess stroke outcomes. The scores were based on
158 the following criteria: 0 = no defect, 1 = inability to stretch the left front foot, 2 =
159 circle left 3 = Fall to the left, 4 = Unable to walk away and lose consciousness, 5 =
160 Death.

161 **Infarct size measurement—TTC staining**

162 For 6 and 72 hours reperfusion animals, the infarct area was measured using 2,
163 5-triphenyl-2H-tetrazolium chloride (TTC) staining. Measure non-ischemic
164 hemispheres and non-ischemic region and calculate infarct area according to the
165 following formula: $[(\text{area of the non-ischemic hemisphere} - \text{area of the non-ischemic}$
166 $\text{region in the ischemic hemisphere}) / \text{area of the non-ischemic hemisphere}] \times 100\%$.
167 Detailed protocols have been described previously.

168 **Infarct size measurement—Cresyl violet staining**

169 For the long-term reperfusion induced by stroke, the infarct volume was
170 measured by Cresyl violet (CV) staining as the TTC staining method does not reflect
171 the infarct size clearly. Animals were anesthetized and transcardially perfused with

172N.S., followed by 4% paraformaldehyde in 0.1 M PBS. Brains were post fixed for 12
173h in 4% paraformaldehyde and dehydrated in 20% and 30% sucrose in PBS,
174respectively. Brains were frozen and sectioned coronally (30 μ m) and pasted on the
175slides. Slides were rehydrated with 0.1M PBS for 5 min. Then the slides were stained
176in 0.1% CV solution at 37°C for 10 min and differentiation in 1% glacial acetic acid.
177The slides were washed twice with ddH₂O and immersed in 95% ethanol for 2
178minutes. Then, they were cleared twice for 5 min with xylene, sealed with neutral
179gum, and finally observed under microscope.

180Quantitative RT-PCR analysis

181 To measure TNF α , IL-1 β and IL-6 mRNA expression, total RNA was isolated
182from the ischemic brain, which was collected on ice and stored at -80°C immediately
183after the animals were euthanized. RNA was extracted with TRIzol reagent (Cat#
18415596-026, Life Technologies, California, USA) according to the manufacturer's
185instructions. The purified RNA was then reverse-transcribed into cDNA using the
186Reverse Transcription System (Cat# E6300S, New England BioLabs® Inc., Ipswich,
187MA, USA). Quantitative RT-PCR analysis of the mRNA level of TNF α (TNF α , F,
188TGAAGTTCGGGGTGATCGGT, TNF α , R, GGCTACGGGCTTGTCACCTCG; IL-1 β
189F, CCCAACTGGTACATCAGCACCTCTC, IL-1 β R,
190CTATGTCCCGACCATTGCTG; IL-6, F, GATTGTATGAACAGCGATGATGC, IL-
1916, R, AGAAACGGAAGTCCAGAAGACC) was performed using the SYBR Green
192Prime Script kit (RR420A, TAKARA). GAPDH (GAPDH F,
193TTCCTACCCCAATGTATCCG; GAPDH R, CCACCCTGTTGCTGTAGCCATA)

194 was chosen as the housekeeping gene. The real-time PCR program steps were: 95 °C
195 for 5 min, 45 cycles at 95 °C for 5 s, 60 °C for 5 s, and 72 °C for 10 s, followed by 72 °C
196 for 1 min.

197 ELISA for quantifying pro-inflammatory cytokines

198 We measured 3 pro-inflammatory cytokines: TNF α , IL-1 β and IL-6 using ELISA
199 kit (Expandbio, Beijing, China) at 6, 72 h and 7 d after MCAo. The procedure was
200 conducted according to the manufacturer's instructions. Briefly, dilute the standard to
201 five gradients according to the instructions, and keep the sample volume of each
202 gradient in 50 μ l. After adding samples, incubate the mixture for 30 minutes at 37 °C.
203 Washed 5 times, then added 50 microliters of enzyme-labeled reagent, and incubate
204 again at 37 °C for 30 minutes. After 5 times of washing, add chromogenic reagents A
205 and B solution for 15 min. Finally, add stop solution and read the OD value at 450
206 nm.

207 Statistical Analysis

208 Statistical analysis was performed using Prism 5 (GraphPad software, Inc., La
209 Jolla, USA). Results are presented as the means $\pm$ SEM. The difference between
210 means was assessed by the Student's t test (single comparisons) or by one-way
211 ANOVA with Newman-Keuls Multiple Comparison test as a post hoc test (for
212 multiple comparisons). A value of $P < 0.05$ was considered statistically significant.
213 The number of rats in each group was 12-14.

214 Results

2151. **RIPC reduced infarct size at short reperfusion time following stroke**

216 To test whether once non-invasive delayed RIPC was neuroprotective in different
217 reperfusion time including 6 h, 72 h and 7 d. We measured the weight and infarct size
218 by TTC and CV staining. Firstly, we detected the CBF levels, blood sugar and blood
219 gas between groups which were the requirement of stroke model. CBF levels were
220 monitored during MCAo surgery, and there was no difference between groups. It was
221 reduced nearly 78% of baseline during ischemia and reestablished to 80% of baseline
222 following reperfusion (Fig. 1B). At the same time, there were no differences of blood
223 sugar and blood gas between groups, either (Fig. 1C and D). Results showed that
224 RIPC significantly attenuated the weight loss at 72 h post-stroke (Fig. 2D), while
225 RIPC had no effect of the weight loss at other reperfusion time (Fig. 2A and G). TTC
226 staining results showed that RIPC significantly reduced infarct size from 39.26 ± 1.51
227 to 31.31 ± 1.68 after reperfusion 6 h (Fig. 2B and C). Similarly, RIPC significantly
228 reduced infarct size from 47.11 ± 1.14 to 36.44 ± 1.82 at 72 h post-stroke (Fig. 2E and
229 F). Whereas, CV staining results showed that there was no significant difference of
230 the infarct size receiving RIPC compared with MCAo group at 7 d post-stroke (Fig.
231 H and I).

2322. **RIPC improved neurological and behavioral function at early stroke**

233 After validating that once non-invasive delayed RIPC reduced infarct size at
234 acute and middle term ischemic reperfusion time following stroke, we further
235 examined the neurological score and behavioral performance receiving RIPC. The

236 results showed that RIPC significantly attenuated neurological dysfunction at 6 and
237 72 h post-stroke, while there's no significant change at reperfusion 7 d after MCAo
238 (Fig. 3A). Such protection of RIPC in these reperfusion time-points were also
239 observed in the behavioral performance test especially in the tail hang test and home
240 cage test, in which RIPC significantly improved the behavioral performance at 6 and
241 72 h post-stroke (Fig. 3B and C). There was no significant difference in the postural
242 reflex test at any of the reperfusion time-point, but we also observed a decrease trend
243 in RIPC group (Fig. 3D).

244 **3. RIPC down-regulated pro-inflammatory factors in the ischemic brain at** 245 **short-term ischemic reperfusion time following stroke**

246 To test how the inflammatory status was regulated by RIPC at different
247 reperfusion time after MCAo, we measured the effect of RIPC on the mRNA and
248 protein levels of the pro-inflammatory cytokines, including TNF α , IL-1 β and IL-6 in
249 the ischemic brain. The results showed that MCAo increased the mRNA and protein
250 levels of TNF α in the ischemic brain at any reperfusion time we tested (Fig. 4A and
251 5A). However, RIPC significantly decreased TNF α expression at 6 and 72 h post-
252 stroke compared with MCAo group at both mRNA and protein levels, while no
253 significant change was observed at 7 d post-stroke (Fig. 4A and 5A). Similar results
254 were observed in IL-1 β and IL-6 expression. It is showed that MCAo up-regulated IL-
255 1 β mRNA and protein expression at 6 and 72 h after reperfusion and only mRNA
256 level at 7 d after reperfusion, while RIPC decreased the IL-1 β expression at mRNA
257 and protein 72 h post-stroke (Fig. 4B and 5B) and mRNA level 6 h post-stroke (Fig.

2584B). Whereas, no any significant changes of IL-1 β expression were observed at 7 d
259following MCAo (Fig. 4B and 5B). Finally, it was showed that RIPC down-regulated
260the expression of IL-6 on the middle-term of reperfusion time (72 h post-stroke) (Fig.
2614C and 5C).

262Discussion

263 In the present study, we investigated the neuroprotection of once delayed non-
264invasive RIPC against stroke at different reperfusion time. An important finding of
265this study was that once delayed non-invasive RIPC reduced infarct size, attenuated
266the loss of neurological function and behavioral performance only at acute reperfusion
267time – 6 h post-stroke and middle-term reperfusion time – 72 h. However, for the
268long-term ischemic reperfusion injury, such as 7 d, there was no protection by RIPC.
269Moreover, RIPC significantly reduced the mRNA and protein levels of pro-
270inflammatory cytokines including TNF α , IL-1 β and IL-6 in the ischemic brain
271reperfusion 6 and/or 72 h post-stroke. However, there was no difference between
272MCAo and RIPC+MCAo group on ischemic reperfusion 7 d. Collectively, these
273findings showed that this ischemic preconditioning method - once delayed non-
274invasive RIPC protected against stroke as a non-invasive neuroprotective strategy just
275at short term reperfusion time. The protective effect was mediated by the modulation
276of inflammatory response in the ischemic brain.

277 As early as 1986, Murry et al. proposed the concept of ischemic preconditioning
278with the findings that 4 cycles of a 5 min ischemia/ 5 min reperfusion had a protection

279in myocardial ischemia [12]. They indicated that ischemic preconditioning was a short
 280period of ischemia, which did not affect the ischemic tissue, but had a protective
 281effect on subsequent, prolonged ischemia. While Kitagawa et al. first introduced
 282cerebral ischemic tolerance in the early 1990s. They discovered 2 min of transient
 283ischemia 24 h before global cerebral ischemia had a neuroprotective effect against
 284neuronal cell death [9, 18]. Researchers regarded ischemic preconditioning as a
 285powerful tool in understanding the endogenous mechanisms by which the ischemic
 286organs are protected [19]. In terms of clinical applicability for myocardial infarction
 287and stroke treatment, RIPC had advantages over conventional ischemic
 288preconditioning by reducing the higher risk directly to the ischemic organ [20, 21].
 289RIPC refers to a repeated transient ischemia/ reperfusion in a remote organ to prevent
 290prolonged ischemia of other vital organ, which now was widely used in the protection
 291of heart and brain ischemia. In contract to invasive RIPC, we mainly focus on non-
 292invasive method which is often established by tourniquet or strip gauze bandages. The
 293important findings of our study were that once delayed non-invasive RIPC (3 cycles
 294of 10 min ischemia/ 10 min reperfusion) reduced infarct size, attenuated the loss of
 295neurological function and behavioral performance only at acute (6 h) and middle-term
 296(72 h) reperfusion time, but not long-term (7 d) (Figs 2 and 3). The results were
 297consistent with our previous study, in which we have demonstrated that RIPC reduced
 298ischemic/ reperfusion injury at 48 h post-stroke [4]. Moreover, the results were
 299consistent with the data which published by Perez-Pinzon MA et al. They found that
 300ischemic preconditioning *in situ* protected rats against ischemic neuronal damage after

3013 but not 7 d of reperfusion following global ischemia [22]. Although different
302ischemic preconditioning positions were used, these results supported that transient
303ischemic preconditioning treatment could not maintain long-term protection.
304However, other studies showed that once rapid non-invasive RIPC (3 cycles of 15 min
305ischemia/ 15 min reperfusion) had chronic protective effect against distal MCAo even
306after 60 d [3]. And these contradictory conclusions can be explained by the different
307model chosen for the two experiments. Moreover, these results supported that RIPC
308was more effective to improve the infarct in the cortex rather than basal ganglia injury

309 Inflammatory response plays an important role in the pathogenesis of ischemic
310stroke. A large number of studies have shown that neuroinflammatory response is
311involved in the prognosis of cerebral ischemia-reperfusion injury [23, 24]. Therefore,
312in theory, inhibiting the inflammatory response after stroke can reduce stroke injury
313and improve the clinical prognosis. Previous studies have shown that RIPC reduced
314systemic neuroinflammatory response [2-4, 15]. To examine whether reducing
315infarction was influenced by the elimination of inflammation in the ischemic brain,
316we then measured the levels of neuroinflammation in the ischemic brain. We found
317that RIPC reduced the mRNA and protein levels of pro-inflammatory cytokines,
318including TNF α , IL-1 β and IL-6 at acute and middle terms of reperfusion in the
319ischemic brain, but had no effect on long term reperfusion (Figs 4 and 5). The
320decrease of cytokines release is consistent with the reduction of the infarct size,
321suggesting that once delayed non-invasive RIPC improved the regional ischemia by
322reducing the expression of neuroinflammatory response in the ischemic brain after

323 short and middle term of reperfusion time.

324 In fact, RIPC has been moved into clinical trials for several years and it has been
325 proven to be effective in the prevention or treatment of cerebrovascular disease [5-7,
326 20]. The importance of our study was to confirm the limited therapeutic time window
327 of RIPC we used. For different ischemic preconditioning methods and stroke models,
328 RIPC may have different protective effect. Only known that how long it works, can
329 we explore the mechanism and complete further clinical transformation.

330 There are also several limitations in the present study. First of all, inhibitors of
331 inflammatory cytokines need to be used to demonstrate the interaction between brain
332 injury and inflammatory response in the future study. Secondly, the mechanism of
333 RIPC should be further explored, such as whether the hypothalamus-pituitary-adrenal
334 axis is involved in the protective effect of RIPC. Last but not the least, RIPC was used
335 only once in our experiment, and the parameters of RIPC worth studying to obtain a
336 chronic protective effects for further clinical application.

337 **Conclusion**

338 In this study, we provided strong evidence that once delayed non-invasive RIPC
339 protects against stroke as a non-invasive neuroprotective strategy which just at short
340 and middle term ischemic reperfusion time. The protective effect was mediated by the
341 modulation of inflammatory response in the ischemic brain.

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344animals.

345**Conflict of interest:** None declared.

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362**References**

- 363[1] O'Collins VE, Macleod MR, Donnan GA, Horky LL, van der Worp BH, Howells
364DW. 1,026 experimental treatments in acute stroke. *Ann Neurol*. 2006; 59:467-477.
- 365[2] Liu ZJ, Chen C, Li XR, Ran YY, Xu T, Zhang Y, et al. Remote ischemic
366preconditioning-mediated neuroprotection against stroke is associated with significant
367alterations in peripheral immune responses. *CNS Neuroscience & Therapeutics*. 2016;
36822:43-52.
- 369[3] Wei D, Ren C, Chen X, Zhao H. The chronic protective effects of limb remote
370preconditioning and the underlying mechanisms involved in inflammatory factors in
371rat stroke. *PLoS One*. 2012; 7:e30892.
- 372[4] Yang J, Liu CY, Du XN, Liu ML, Ji XM, Du HS, et al. Hypoxia inducible factor
373 α plays a key role in remote ischemic preconditioning against stroke by modulating
374inflammatory responses in rats. *J Am Heart Assoc*. 2018; 5.
- 375[5] Meng R, Asmaro K, Meng L, Liu Y, Ma C, Xi C, et al. Upper limb ischemic
376preconditioning prevents recurrent stroke in intracranial arterial stenosis. *Neurology*.
3772012; 79:1853-1861.
- 378[6] Mi T, Yu F, Ji XM, Sun YX, Q DM. The Interventional Effect of Remote Ischemic
379Preconditioning on Cerebral Small Vessel Disease: A Pilot Randomized Clinical Trial.
380*Eur Neurol*. 2016; 76:28-34.
- 381[7] Zhao WB, Meng R, Ma C, Hou BJ, Jiao LQ, Zhu FS, et al. Safety and efficacy of
382remote ischemic preconditioning in patients with severe carotid artery stenosis before
383carotid artery stenting: a proof-of-concept, randomized controlled trial. *Circulation*.
3842017; 135:1325-1335.

- 385[8] Dirnagl U, Simon RP, Hallenbeck JM. Ischemic tolerance and endogenous
386neuroprotection. Trends Neurosci. 2003; 26:248–254.
- 387[9] Kitagawa K, Matsumoto M, Tagaya M, Hata R, Ueda H, Niinobe M, et al.
388‘Ischemic tolerance’ phenomenon found in the brain. Brain Res. 1990; 528:21–24.
- 389[10] Murphy E, Steenbergen C. Mechanisms underlying acute protection from cardiac
390ischemiareperfusion injury. Physiol Rev. 2008; 88:581–609.
- 391[11] Kaneko T, Yokoyama K, Makita K. Late preconditioning with isoflurane in
392cultured rat cortical neurones. Br J Anaesth. 2005; 95:662–668.
- 393[12] Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: A delay of
394lethal cell injury in ischemic myocardium. Circulation. 1986; 74:1124–1136.
- 395[13] Kuzuya T, Hoshida S, Yamashita N, Fuji H, Oe H, Hori M, et al. Delayed effects
396of sublethal ischemia on the acquisition of tolerance to ischemia. Circ Res. 1993;
39772:1293–1299.
- 398[14] Cadet JL, Krasnova IN. Cellular and molecular neurobiology of brain
399preconditioning. Mol Neurobiol. 2009; 39:50–61.
- 400[15] Hu S, Dong HL, Zhang HP, Wang SQ, Hou LC, Chen SY, et al. Noninvasive
401limb remote ischemic preconditioning contributes neuroprotective effects via
402activation of adenosine A1 receptor and redox status after transient focal cerebral
403ischemia in rats. Brain Res. 2012; 1459:81-90.
- 404[16] Rohailla S, Clarizia N, Sourour M, Sourour W, Gelber N, Wei C, et al. Acute,
405Delayed and Chronic Remote Ischemic Conditioning Is Associated with
406Downregulation of mTOR and Enhanced Autophagy Signaling. Plos One. 2014; 9:

407e111291.

408[17] Du XN, Yang J, Liu CY, Wang SN, Zhang CC, Zhao H, et al. Hypoxia-inducible
409factor 1 α and 2 α have beneficial effects in remote ischemic preconditioning against
410stroke by modulating inflammatory responses in aged rats. *Front aging neurosci.*
4112020; 12:54.

412[18] Kitagawa K, Matsumoto M, Kuwabara K, Tagaya M, Ohtsuki T, Hata R, et al.
413‘Ischemic tolerance’ phenomenon detected in various brain regions. *Brain Res.* 1991;
414561:203–211.

415[19] Perez-Pinzon MA. Neuroprotective effects of ischemic preconditioning in brain
416mitochondria following cerebral ischemia. *J Bioenerg Biomembr.* 2004; 36:323–327.

417[20] Walsh SR, Nouraei SA, Tang TY, Sadat U, Carpenter RH, Gaunt ME. Remote
418ischemic preconditioning for cerebral and cardiac protection during carotid
419endarterectomy: results from a pilot randomized clinical trial. *Vasc Endovascular*
420Surg. 2010; 44: 434–439.

421[21] Gao X, Ren C, Zhao H. Protective effects of ischemic postconditioning
422compared with gradual reperfusion or preconditioning. *J Neurosci Res.* 2008; 86:
4232505–2511.

424[22] Perez-Pinzon MA, Xu GP, Dietrich WD, Rosenthal M, Sick TJ. Rapid
425preconditioning protects rats against ischemic neuronal damage after 3 but not 7 days
426of reperfusion following global cerebral ischemia. *J Cereb Blood Flow.* 1997; 17:
427175-182.

428[23] Shim R, Wong CHY. Ischemia, Immunosuppression and infection —tackling the
429predicaments of post-stroke complications. *Int J Mol Sci.* 2016; 17.

430[24] Nguyen TV, Frye JB, Zbesko JC, Stepanovic K, Hayes M, Urzua A, et al.
431Multiplex immunoassay characterization and species comparison of inflammation in
432acute and non-acute ischemic infarcts in human and mouse brain tissue. *Acta*
433*Neuropathol Commun.* 2016; 4:100.

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445Figure legend

446**Figure 1. Experimental protocols and model of RIPC.** A. RIPC was conducted by

4473 cycles (60 min total) in both hind limbs under isoflurane. Non-RIPC rats were
 448exposed to the same anesthesia for 60 min. MCAo was induced by 90 min after RIPC
 44924 h. Neurological score, behavioral test and sample collection at 6 h, 72 h and 7 d
 450post-stroke. **B.** Cerebral blood flow during the MCAo surgery. Cerebral blood flow
 451was measured at five time points, baseline, 1, 5 and 10 minutes of ischemia and
 452reperfusion in the MCAo and RIPC+MCAo groups. Data were normalized to baseline
 453and expressed as percentages. **C.** Blood sugar value after surgery in each group. **D.**
 454Arterial blood gas parameter before and immediately after MCAo. MCAo, middle
 455cerebral artery occlusion; RIPC, remote ischemic preconditioning.

456**Figure 2. RIPC attenuated the weight loss of rats at 72 h reperfusion post-stroke**
 457**and reduced infarct size at 6 and 72 h after MCAo. A, D, G.** Weight of the rats in
 458the MCAo and RIPC+MCAo group. **B, E.** Representative images and infarct volume
 459of TTC staining in the MCAo and RIPC+MCAo group at 6 and 72 h post-stroke. **H.**
 460Representative images and infarct volume of CV staining. **C, F, I.** Statistical analysis
 461of infarct size. Statistical analysis was performed by ANOVA. **, *** $p < 0.01$,
 4620.001, vs MCAo, respectively. (N=12-14 per group).

463**Figure 3. RIPC improved neurological and behavioral function at 6 and 72 h**
 464**post-stroke. A.** Neurological score in the MCAo and RIPC+MCAo group at 6 h, 72 h
 465and 7 d post-stroke. **B, C, D.** Behavior tests, including tail hang test, home cage test
 466and postural reflex test. Statistical analysis was performed by ANOVA. *, ** $p < 0.05$,
 4670.01 vs MCAo, respectively. (N=12-14 per group).

468 Figure 4. RIPC down-regulated the mRNA levels of pro-inflammatory factors in
469 the ischemic brain. A, B, C. The mRNA levels of TNF α , IL-1 β and IL-6,
470 respectively. Statistical analysis was performed by ANOVA. *, **, *** p < 0.05, 0.01,
471 0.001 vs Sham, respectively. #, ## p < 0.05, 0.01 vs MCAo, respectively. (N=12-14
472 per group).

473 Figure 5. RIPC down-regulated the protein levels of pro-inflammatory factors in
474 the ischemic brain. A, B, C. The protein levels of TNF α , IL-1 β and IL-6,
475 respectively. Statistical analysis was performed by ANOVA. *, **, *** p < 0.05, 0.01,
476 0.001 vs Sham, respectively. #, ## p < 0.05, 0.01 vs MCAo, respectively. (N=12-14
477 per group).

A

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B

C

Blood sugar value after surgery in each group

Time point	Blood sugar value (mmol/L) (mean $\pm$ SEM) n=8		
	Sham	MCAo	RIPC+MCAo
0 min	5.6 $\pm$ 0.28	5.7 $\pm$ 0.22	5.6 $\pm$ 0.29
5 min	5.8 $\pm$ 0.12	5.9 $\pm$ 0.25	5.7 $\pm$ 0.34
10 min	5.8 $\pm$ 0.30	6.0 $\pm$ 0.32	5.9 $\pm$ 0.19

D

Arterial blood gas parameter before and immediately after MCAo

Time point	Group	Arterial blood gas parameter (mean $\pm$ SEM) n=8		
		pH	pCO ₂ (mmHg)	pO ₂ (mmHg)
Pre-MCAo	MCAo	7.40 $\pm$ 0.06	41.0 $\pm$ 2.8	103 $\pm$ 6.2
	RIPC+M	7.42 $\pm$ 0.09	41.8 $\pm$ 1.2	99 $\pm$ 7.2
Post-MCAo	MCAo	7.41 $\pm$ 0.02	40.9 $\pm$ 3.0	98 $\pm$ 6.5
	RIPC+M	7.41 $\pm$ 0.08	42.2 $\pm$ 1.1	101 $\pm$ 5.5

fig. 1

MCAo 6h

A

B

C

MCAo 72h

D

E

F

MCAo 7d

G

H

I

Fig. 2

Fig. 3

Fig. 4

Fig. 5