Remote limb ischemic post-conditioning attenuates ischemia-reperfusion injury in rat skin flap by limiting oxidative stress

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DOI: http://dx.doi.org/10.1590/S0102-86502016001000003

ABSTRACT

PURPOSE: To investigate the effect of remote ischemic post-conditioning (RIPoC) against ischemia-reperfusion (I/R) injury on flaps of rats.

METHODS: Sprague-Dawley rats were randomized into the Sham, Control, RIPoC1 and RIPoC2 groups. All the animals were submitted to a 5×4 cm superficial inferior epigastric artery flap. Eight hours of flap ischemia was induced and two protocols of limb RIPoC were applied. Tissue MDA level and SOD activity in 24-h reperfusion were assessed. Flap survival was assessed 7 days postoperatively.

RESULTS: Compared to the Control group, the RIPoC1 group showed statistically decreased MDA level at 6-, 12-, and 24-h reperfusion (P = 0.01, P < 0.01 and P < 0.01, respectively), and statistically increased SOD activity at 12- and 24-h reperfusion (P < 0.05 and P < 0.01, respectively). Flap survival rate on the 7th day was significantly higher in the RIPoC1 group than the control group (47.9 ± 6.4 vs. 29.4 ± 7.1 %, P < 0.01).

CONCLUSION: Three cycles of 5-min Limb remote ischemic post-conditioning rather than a single cycle of 15-min limb RIPoC has protective effect on flaps against ischemia-reperfusion injury by attenuating oxidative stress.

Key words: Ischemic Postconditioning. Ischemia. Reperfusion Injury. Oxidative Stress. Rats.
Introduction

Free flap transplantation is frequently used in plastic and reconstructive surgery. In spite of the high success rate, reperfusion injury by abrupt restoration of circulation after prolonged ischemia has been remained an unsolved problem associated with partial to total flap loss.

Excessive production of free radicals and/or reactive oxygen species during reperfusion triggers lipid peroxidation and initiates ischemia reperfusion (I/R) damage. Malondialdehyde (MDA) is one of intermediate products of lipid peroxidation, and its level is commonly used as a biomarker of oxidative stress. On the contrary, endogenous antioxidant enzymes, such as superoxide dismutase (SOD) and catalase, act as “free radical scavengers” which can transform superoxide anion to the less reactive species $\text{H}_2\text{O}_2$.

In recent years, numerous labs have demonstrated that ischemic post-conditioning (IPostC), defined as brief intermittent episodes of ischemia at the onset of reperfusion, is an endogenous protective strategy against I/R injury for multiple organs. The underlying mechanism of IPostC involves limiting the oxidative stress by reducing reactive oxygen metabolites and increasing antioxidant enzymes’ activity in tissues after prolonged ischemic insult. However, repetitive ischemic insult and hypoxia added directly on the primary ischemic tissue are still unacceptable concept and impracticable. Remote ischemic post-conditioning (RIPoC) refers to ischemic tolerance induced by transient ischemia at a site distant to the ischemic target organs immediately at the time of its reperfusion. In contrast to the clinical limitation of IPostC, RIPoC could be a non-invasive clinical intervention. Recent preclinical papers show that RIPoC performed in the limbs can generate effective cardioprotection and neuroprotection against I/R injury similar to IPostC, yet its protective effect on skin flap has not yet been well investigated.

In the present study, we aimed to evaluate the anti-ischemic properties of two protocols of limb RIPoC in a rat model of epigastric island flaps subjected to I/R injury, and the possible effect of RIPoC on reduction of I/R-induced oxidative stress.

Methods

All procedures were conducted in compliance with NIH Guiding Principles for Research Involving Animals and were approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University School of Medicine.

Male Sprague-Dawley (SD) rats, weighing between 290g to 350g, were randomized into four groups (n=16 in each group): the Sham, Control, RIPoC1 and RIPoC2 groups. The animals were kept in a room with a controlled environment with a 12-h day/night lighting cycle, and allowed access to standard rodent chow and tap water. General anesthesia was induced with intraperitoneal injections of ketamine 80 mg/kg and maintained with a booster injection of one third of initial dose.

Surgical procedure

The abdomen was shaved with an electric hair clipper, and asepsis was maintained by providing a local sterile. A 5×4 cm island pedicle flap was elevated in all animals with the base at left superficial inferior epigastric (SIE) vessels (Figure 1). The flap consisted of skin, subcutaneous tissue, and intimately attached panniculus carnosus. The SIE pedicle was carefully isolated down to the left femoral vessels, and de-nervated under a microscope to accurately mimic a free tissue transfer.

In the Control group, an 8-h flap ischemia was induced using an S&T microvascular clip with 15g compression on the pedicle. During the period of ischemia, the rats were kept...
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anesthetized and the flap was placed in gauze moistened with warm saline solution. After the clip was removed, the vascular patency of the pedicles was confirmed under an operating microscope before the flap was repositioned and sutured. The Sham group underwent the same flap elevation and vessel dissection as the Control group, but flap I/R was not induced (Figure 2).

The RIPoC groups underwent the same procedure as the Control group, and 3 cycles of 5-min limb ischemia followed by 5-min reperfusion (RIPoC1), or 1 cycle of 15-min limb ischemia followed by limb reperfusion (RIPoC2), at the onset of flap reperfusion. Limb ischemia was induced by placing a tourniquet around the base of the right hind limb to stop the arterial blood supply. Circulatory arrest in the limbs was confirmed by observing the empurpled limb skin and via vascular Doppler (Philips). The rationale for the timing used in this experimental design was based on our experience and the literatures. All the animals were sacrificed by a lethal dose (100 mg/kg) of intracardiac Nembutal, after the observations were completed (Figure 3).

SOD activity and MDA content

A full-thickness, 2-mm punch biopsy of the flap skin was collected at the distal end of the flap, after 3h, 6h, 12h, 24h after reperfusion, respectively. The samples were rinsed, weighed, and homogenized in 9 volumes of ice-cold buffers. Supernatant homogenate was collected after centrifugation at 5000 rpm/min for 15 min at 4 C. The SOD activity of homogenates was determined by the xanthine oxidase (hydroxylamine) method, using a commercial kit (A001; Nanjing jiancheng Bioengineering Institute, China), and the results are expressed as nmol/mg wet weight. The MDA content of homogenates was determined spectrophotometrically by thiobarbituric acid colorimetric method, using a commercial kit (A003; Nanjing jiancheng Bioengineering Institute, China), and the results are expressed as µmol/mg wet weight.
Flap survival analysis

At the 7th day after reperfusion, the animal was re-anesthetized and the appearance of the skin flap was recorded and photographed in a standardized fashion. The flap viable area was determined based on color, capillary refill, and the pin-prick test. Ratios of viable area to original flap area were calculated by digital planimetry software (Image-Pro Plus Version 7.0), expressed as a percentage (percent survival).

Statistical analysis

The results were expressed as mean ± standard deviation (SD). Student t-test was used for comparisons between two means.

Results

MDA content and SOD activity

Ischemia and the subsequent reperfusion resulted in significantly higher level of MDA content and lower level of SOD activity in flap tissue from both the Control and RIPoC groups than that in tissue from the Sham group, throughout the 24-h reperfusion period (Figure 4).

Compared with the controls, induction of RIPoC1 significantly attenuated I/R-induced elevation in MDA at 6-, 12-, and 24-h reperfusion timepoints (P = 0.01, P < 0.01 and P < 0.01, respectively). In contrast, differences in MDA level between the Control and RIPoC2 groups at each timepoint were not significant (P > 0.05). Compared with the controls, RIPoC1 significantly attenuated the decrease in SOD activity at 12- and 24-h reperfusion timepoints (P < 0.05 and P < 0.01, respectively) while RIPoC2 only attenuated the decrease in SOD activity at 24-h reperfusion timepoints (P < 0.05). Detailed statistical evaluation of the MDA levels and SOD activity (Tables 1 and 2).

TABLE 1 - MDA levels at each timepoint in each group.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Sham</th>
<th>Control</th>
<th>RIPoC1</th>
<th>RIPoC2</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
</tr>
</thead>
<tbody>
<tr>
<td>3. h</td>
<td>52.4 ± 8.6</td>
<td>86.1 ± 11.2</td>
<td>80.3 ± 6.9</td>
<td>83.1 ± 7.5</td>
<td>&lt; 0.01</td>
<td>0.23</td>
<td>0.54</td>
</tr>
<tr>
<td>6. h</td>
<td>50.4 ± 10.7</td>
<td>126.6 ± 9.0</td>
<td>111.5 ± 11.2</td>
<td>119.1 ± 9.6</td>
<td>&lt; 0.01</td>
<td>0.01</td>
<td>0.17</td>
</tr>
<tr>
<td>12. h</td>
<td>56.3 ± 9.5</td>
<td>147.2 ± 10.0</td>
<td>129.7 ± 11.5</td>
<td>139.8 ± 6.6</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
<td>0.1</td>
</tr>
<tr>
<td>24. h</td>
<td>57.0 ± 7.2</td>
<td>188.7 ± 15.6</td>
<td>162.8 ± 17.2</td>
<td>179.0 ± 15.8</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
<td>0.1</td>
</tr>
</tbody>
</table>

P1: Control group vs. Sham group; P2: Control group vs. RIPoC1 group; P3: Control group vs. RIPoC2 group; p value in bold when less than 0.05.

All data were analyzed using SPSS software 19.0. Values <0.05 were considered to be statistically significant.
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Flap survival rate

The viable region of the flap was measured 7 days after reperfusion (Figure 5). Compared to the Sham group, significant decrease in ratios of the survival area to original flap area was observed in the Control group (29.4 ± 7.1% vs. 92.0 ± 4.4%, P < 0.01), RIPoC1 group (47.9 ± 6.4% vs. 92.0 ± 4.4%, P < 0.01), and RIPoC2 group (34.4 ± 4.2% vs. 92.0 ± 4.4%, P < 0.01). Compared to the controls, induction of limb RIPoC1 significantly increased the flap survival rate (P < 0.01). In contrast, difference in flap survival rate between the Control and RIPoC2 groups was not significant (P > 0.05) (Table 3).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Sham</th>
<th>Control</th>
<th>RIPoC1</th>
<th>RIPoC2</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avr. ± SD (nmol/mg wet weight)</td>
<td>3. h 87.0 ± 10.1</td>
<td>62.3 ± 6.9</td>
<td>66.7 ± 7.5</td>
<td>64.6 ± 6.2</td>
<td>&lt; 0.01</td>
<td>0.24</td>
<td>0.48</td>
</tr>
<tr>
<td>6. h</td>
<td>85.8 ± 7.7</td>
<td>48.5 ± 8.3</td>
<td>56.3 ± 6.9</td>
<td>53.3 ± 7.1</td>
<td>&lt; 0.01</td>
<td>0.06</td>
<td>0.23</td>
</tr>
<tr>
<td>12. h</td>
<td>84.8 ± 9.9</td>
<td>33.2 ± 7.6</td>
<td>41.0 ± 6.5</td>
<td>35.2 ± 4.2</td>
<td>&lt; 0.01</td>
<td>0.05</td>
<td>0.54</td>
</tr>
<tr>
<td>24. h</td>
<td>88.6 ± 6.8</td>
<td>20.0 ± 5.8</td>
<td>30.2 ± 4.9</td>
<td>26.1 ± 5.3</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Table 2 - SOD levels at each timepoint in each group.

P1: Control group vs. Sham group; P2: Control group vs. RIPoC1 group; P3: Control group vs. RIPoC2 group; p < 0.01

Discussion

IPostC has been proven to confer protection against flap I/R injury. Moon et al.11 reported that IPostC could decrease flap necrosis by reducing MPO activity and attenuating acute inflammatory reaction. Experimental data from Coskunfirat et al.12 showed that IPostC applied by means of 6 cycles of 30 seconds yields an optimal anti-ischemia protection in a rat skin flap model. In the present study, we expand further on this concept by applying post-conditioning at a distance (hind limbs) in a rat skin flap model, and showed that RIPoC was an effective therapeutic intervention against flap I/R injury.

The protective effect of RIPoC was initially described in the heart13, and applied to other organs14-16. RIPoC conducted in the limbs is most commonly used in the previous studies because of its convenience and safety for clinical application17. However, varying protocols of limb RIPoC were selected in different studies,
while the optimal one remains to be determined. In the present study, two experimental protocols of limb RIPoC were evaluated. Limb RIPoC1 applied as 3 cycles of 5-minute ischemia/5-minute reperfusion has been proven as a valid protection against I/R injury in brain8,19, while Limb RIPoC2 with 1 cycle of 15-minute ischemia has been proven to confer a strong degree of cardioprotection against I/R injury20. Our findings indicated that limb RIPoC1 significantly reduced the flap necrosis area at the 7th days after reperfusion compared to the controls. On the contrary, limb Rchemiadid not provide equiemia protection for the flap.

The optimal number of stimuli cycles applied in RIPoC remains a controversial subject. Some researchers concluded hat protection by remote conditioning requires the completion of a sufficient stimulus during index ischemia. Study by Kanoria et al21 demonstrated that remote conditioning with multiple cycles of I/R stimuli was more effective than with a single cycle of stimulus in cardioprotection. Loukogeorgakis et al22 reported that I/R injury was significantly prevented by an arm RIPoC with 3 cycles of 5-minute ischemia/5-minute reperfusion, but shortening the stimulus to 2 cycles was much less protective, indicating that RIPoC is a dose-response protection. In the present study, in spite of the same total duration of limb ischemia, RIPoC1 rather than RIPoC2 was found effective in limiting flap I/R damage, which supports the hypothesis that multiple cycles of short limb RIPoC was more effective than a single cycle of long limb RIPoC.

Understanding the mechanisms responsible for the ischemic conditioning therapy is important for its clinical practice which aims at mimicking the protective mechanisms. In agreement with previous studies23,24, high level of oxidative stress was induced in the rat flap subjected to 8-h ischemia, demonstrated by increased MDA contents and decreased SOD activities. The level of MDA indirectly reflects the severity of cellular injury from free radical attack, while SOD activity indirectly reflects the level of scavenging capacity. When the oxidative stress occurred, the rapid overproduction of free radicals overwhelms the cellular detoxification and scavenging capacity within the body25. In the present study, however, these changes were attenuated by limb RIPoC1 conducted immediately after reperfusion in the flap. Compared to the Control group, limb RIPoC1 produced an increased MDA contents appearing at 6 h after reperfusion, and a decreased SOD activities appearing at 12 h after reperfusion, while the differences in these parameters between the Control and RIPoC2 groups were insignificant. Consistent with the previous studies that demonstrated the antioxidative effect of RIPoC on liver and kidney26, our results provide promising evidence for its effect on decreasing the production of peroxidation products and increasing free radical scavenging capacity in the early stage of flap reperfusion injury.

There’s limitation that should be mentioned. Since flap necrosis was the primary endpoint, based on our experience and the previous literature21, 8-hour ischemia was conducted to establish a stable model of I/R injury with partial flap loss. Therefore, our findings might focus on flaps with severe ischemia induced by vaso-occlusive crisis, when RIPoC could be applied as an add-on therapy to re-explorations. Its effect on routine free flap transplantation, in which warm ischemia time usually does not exceed 2 hours, was nevertheless not well discussed in the current study. Thus, further studies based on a model with different ischemia time are needed, and assessments of acute biomarkers of I/R such as neutrophil infiltration are recoened.

Conclusions

The remote ischemic post-co that RIPoC applied as 3 cycles of 5-min limb ischemia followed by 5-min reperfusion limits I/R injury inepprotective and iPoC may be mediated by limiting oxidatap tissues in the early stage of reon. Thus, intervention with RIPoC, which targets the first few minutes of reperfusion, may be a valuable clinical “after-injury strategy” for free flap research.

References

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Received: Sept 22, 2015
Review: Nov 17, 2015
Accepted: Dec 19, 2015
Conflict of interest: none
Financial source: none

*Research performed at Shanghai Ninth People’s Hospital, School of Medicine, Jiao Tong University, China.