Protective Effects of Remote Limb Preconditioning on Ischemia Reperfusion Injury in Rats Liver

Bing Zhang, Yan Zhao, Lei Bao, Lan-Hui Wang, Huai-Bin Guo, Wan-Xing Zhang, Hong-Fang Tuo, Chun-Cheng Wang

ABSTRACT

AIM: Direct ischemic preconditioning of liver can reduce ischemia-reperfusion injury (IRI). Remote limb preconditioning has been shown to attenuate IRI to the heart. This study determined to investigate the protective effects of remote limb ischemic preconditioning in reducing ischemia reperfusion injury in rats liver.

METHODS: Fifty male SD (Sprague-Dawley) rats were randomly allocated to five groups (n=10 per group): control group (C), sham-operated group (SO), ischemia-reperfusion group (IR), ischemic preconditioning group (HIPC), and limb ischemic preconditioning group (LIPC). The hepatic tissue sample and the blood of all groups were examined after experiments.

RESULTS: The levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in IR, HIPC and LIPC groups increased significantly than that in C and SO group (P<0.05), and there were significantly difference between IR group, HIPC and LIPC groups (P<0.05). There was no significant difference between HIPC and LIPC groups, C and SO groups (P>0.05). TUNEL-positive cells could be seen rarely in SO group. The apoptosis index (AI) in IR, HIPC and LIPC groups increased significantly than those in SO group (P<0.05). In IR group, AI were significantly higher than those in HIPC and LIPC groups (P<0.05). There was no statistical significance between HIPC and LIPC groups (P>0.05).

CONCLUSION: Remote limb ischemic preconditioning has similar protective effect on the IR injured liver as the hepatic ischemic preconditioning and it can effectively attenuate the apoptosis.
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METHODS

Animals and reagents
Fifty male healthy SD (Sprague-Dawley) rats (weighing 227±12.6 g) were obtained from the Experimental Animal Center, Hebei Medical University, Shijiazhuang, China. All animals received human care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals.” The rats were kept in standard conditions and fed with water and rodent chow ad libitum. The animals were randomly divided into five groups: control group (C), sham-operated (SO), ischemia-reperfusion (IR), hepatic ischemic preconditioning (HIPC) and limb ischemic preconditioning (LIPC).

Techniques of experiment
Animals were maintained in a temperature-controlled environment and fasted (with water allowed) for twelve hours before the study. The rat liver ischemia-reperfusion model was established according to Nauta’s method by anesthetizing with an intraperitoneal injection of 10% pentobarbital 3.5 mL/kg. In C group, only blood samples taken from the heart; in SO group, only dissected out hepatoduodenal ligaments; in IR group, ischemia was initiated by clamping the portal venous and hepatic arterial branches to the left and median hepatic lobes for 30 min, after warm ischemia, the clip was removed initiating hepatic reperfusion; in HIPC group, the portal venous and hepatic arterial branches to the left and median lobes were subjected to three cycles of 10 min ischemia followed by 10 min reperfusion before IR; in LIPC group, both the left and right hind limb were subjected to three cycles of 10 min ischemia and followed by 10 min reperfusion by clamping the femoral artery prior to IR.

Detection of the levels of ALT and AST in serum
Blood samples taken from the heart were conserved in the freezer at -70 ℃ after 15 minute centrifugation with the speed of 2500 round/minute. Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured by a spectrophotometer (type-756, Shanghai Precision Instrument Factory, Shanghai, China).

Histological examination
Liver tissue was obtained after 6 h reperfusion and fixed in 4% formaldehyde. Tissues were embedded in paraffin, and 5-μm sections were cut and stained with haematoxylin-eosin and then sections were observed under a light microscopy.

Detection of apoptosis
TUNEL (terminal-deoxynucleotidyl transferase mediated d-UTP nick end labeling) staining for apoptotic nuclei was detected in dewaxed sections using the TUNEL assay kit (Roche Co., Mannheim, Germany). Dewaxed and rehydrated tissue section by heating at 60 ℃, followed by washing in xylene twice and rehydration through a graded series of ethanol. Incubated tissue section for 15-30 min at 21-37 ℃ with Proteinase K working solution, and then added TUNEL reaction mixture on sample. After signal conversion, apoptotic nuclei were analyzed under light microscopy by a blinded reviewer. The nuclei of positive cell showed brown or dark brown. 10 views of representative fields were observed to calculate the apoptosis positive index (AI): AI=(A1+A2+...+An)/n (n=10).

Statistical analysis
Data were expressed as mean±SD. All analyses were conducted with SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was performed when comparing four groups, and SNK q post-test for two-group comparison. A P value less than 0.05 was considered statistically significant.

RESULTS

Serum aminotransferase
Serum ALT and AST levels were higher 6 h after operation in the IR group, HIPC group and LIPC group compared with C and SO groups (P<0.05). It was lower in HIPC and LIPC relatively (P<0.05), but there was no significant difference between these two groups (P>0.05). There was no significant difference between C and SO groups (Table 1, figure 1).

Table 1 Levels of ALT and AST in serum (mean±SD).

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>ALT(U/L)</th>
<th>AST(U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>10</td>
<td>49.32±6.22</td>
<td>75.48±8.02</td>
</tr>
<tr>
<td>SO</td>
<td>10</td>
<td>52.95±6.95</td>
<td>79.66±10.63</td>
</tr>
<tr>
<td>IR</td>
<td>10</td>
<td>654.06±104.45</td>
<td>782.49±112.40</td>
</tr>
<tr>
<td>HIPC</td>
<td>10</td>
<td>395.52±98.18</td>
<td>431.75±40.89</td>
</tr>
<tr>
<td>LIPC</td>
<td>10</td>
<td>437.25±48.41</td>
<td>481.53±48.17</td>
</tr>
<tr>
<td>F</td>
<td>10</td>
<td>167.58±8.43</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Figure 1 Levels of ALT and AST in serum.

IR-Induced Apoptosis
The TUNEL-positive apoptotic cell nucleus was brown observed under light microscope. Positive cell was rare in the SO group, it was more common to be observed in IR group. Both HIPC and LIPC attenuated the apoptosis (Figure 2). Apoptosis index was calculated by
vessels could cause neuromuscular paralysis. Moreover, some researchers used to occlude blood flow to the limb, but this method was much more invasive.

In some previous animal experiments, external tourniquet was totally occluded we used small size bulldog clamps to clip the bilateral femoral artery to achieve the preconditioning, and from the experiment results we assumed this method was practicable.

In conclusion, remote preconditioning has greater potential for clinical application than conventional preconditioning, since it can be performed in a non-vital organ, avoiding the high risk of inducing ischemia as preconditioning in the vital organ.

### DISCUSSION

Recent studies have demonstrated that remote ischemic preconditioning, a process for the use of a non-vital distant organ temporarily rendered to short sublethal ischemic preconditioning, is able to protect other organs from the subsequent ischemia reperfusion injury. Transient remote vascular occlusion has been shown to be protective of IR injury in the heart, kidney[9,10], and brain[11], the most commonly used preconditioning vascular is femoral artery. The mechanisms accounting for remote preconditioning have been investigated extensively in the field of myocardial ischemia, such as neural and humoral pathway[12,13], inflammatory mediator[14,15], and etc. However, they have received much less attention in the field of liver.

In this study, we hypothesized that it may modulate hepatocellular apoptosis to attenuate the hepatocyte injury.

The results of this study showed that serum alanine transaminase (ALT) and aspartate transaminase (AST) increased significantly after 30 min of ischemia which indicated hepatocyte function was impaired, and much lower degree of these parameters in both HIPC and LIPC groups showed protective effects of these two interventions.

On the basis of previous studies, we knew that during reperfusion large numbers of oxygen free radicals were generated and NF-κB was activated afterwards which could increase the gene transcription of many pro-inflammatory cytokines (such as TNF-α and IL-1)[14,15], TNF-α and other mediators activated many of the proteins involved in apoptosis, such as the proteases caspase-3 and caspase-8, along with mitochondria cytochrome-C released to the cytoplasm[16,17]. The cascade of events that started with these substances led to DNA destruction and cell death. With this in mind, we observed the liver tissue apoptosis by means of TUNEL assay to confirm the DNA injury. The results affirmed our hypothesis, but the protective pathway of remote preconditioning needed investigating further.

In our studies, hindlimb preconditioning was induced by three cycles of 10 min occlusion of the bilateral femoral arteries followed with 10 min reperfusion; this was consistent with all previous studies[18,19]. This was based on evidence that three cycles of 10 min LIPC was more effective than a single cycle and a study indicated a single cycle IPC did not reduce liver damage in patients undergoing major liver resection[20].

In previous animal experiments, external tourniquet was used to occlude blood flow to the limb, but this method was much commonly applied in big animals. Moreover, some researchers found that the pressure of tourniquet needed to occlude the femoral vessels could cause neuromuscular paralysis[21]. In this study, because the limb artery of rat was very thin and the femoral pulse was not palpable, and it was not feasible to perform the ultrasonic Doppler to exam the blood flow. In order to ensure the hindlimb artery was totally occluded we used small size bulldog clamps to clip the bilateral femoral artery to achieve the preconditioning, and from the experiment results we assumed this method was practicable.

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**Peer reviewers:** Yin Zhengfeng, Professor, Molecular Oncology Laboratory, Eastern Hepatobiliary Surgery Hospital, 225 Changhui Rd., Shanghai, 200438, China; Quanda Liu, MD, PhD, associate professor, Institute of Hepatobiliary Gastrointestinal Diseases, General Hospital of Chinese PLA Second Artillery, XinWai Street 16th, Beijing, 100088, China.