Ischemic Preconditioning Attenuates Morphological and Biochemical Changes in Hepatic Ischemia/Reperfusion in Rats

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Abstract

Objective: Ischemic preconditioning (IPC) has been gradually introduced into clinical liver surgery and transplantation in recent years. However, the protective effects of IPC on hepatic warm ischemia/reperfusion (I/R) injury and the potential mechanisms involved are not fully understood. We aimed to evaluate whether the reduction of apoptotic sinusoidal endothelial cells (SECs), induced by IPC, contributes to its protective effect. Methods: Male Wistar rats were randomized into three experimental groups: the continuous clamping group underwent 60 min of 70% hepatic ischemia; the IPC group received 10 min ischemia followed by 10 min reperfusion prior to ischemia, and the sham control (sham) underwent a sham operation without ischemia. Hepatocyte and SEC apoptosis, liver necrotic areas and the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), hyaluronic acid, tumor necrosis factor, myeloperoxidase (MPO) and malondialdehyde were determined. Expression of cysteine-aspartic acid protease-3 (caspase-3) in hepatocytes and SECs was also investigated. Furthermore, the hepatic leukocyte infiltration was assessed by intravital fluorescence microscopy. Results: IPC exhibited a significant alleviation of their postischemic liver function. Serum AST, ALT and tissue MPO were significantly decreased by IPC, and the degree of hepatocyte and SEC apoptosis was significantly inhibited, as shown by the decreased numbers of adherent leukocytes. Conclusions: IPC attenuates hepatic I/R injury by the reduction of leukocyte infiltration, the reduction hepatic enzymatic leakage and the depression of apoptotic cells. SECs are more sensitive to apoptosis induced by warm I/R injury compared to hepatocytes.

Introduction

Hepatic ischemia/reperfusion (I/R) injury occurs in trauma, hemorrhagic and cardiac shock, vascular diseases and hepatic surgery, including liver tumor resection and liver transplantation [1–5]. In the transplantation setting, I/R injury plays an essential role in the initial poor function and consequent failure of many liver grafts [6–8]. To date, most investigations have always focused on cold ischemia (cold preservation). However, warm ischemia injury also plays a significant role before and after liver transplantation, especially living liver transplantation. The reduction of warm ischemia may contribute to improving graft function.

As a powerful strategy of experimentally and clinically reducing damage after I/R [3, 9–15], ischemic preconditioning (IPC), a well-established phenomenon induced
by brief periods of I/R, can render an organ resistant to subsequent episodes of more prolonged ischemia. IPC was first described by Murry et al. [16]. They used several brief episodes of myocardial ischemia prior to pre-treated ischemia in an animal heart model. Since then IPC has shown great promise for organ-protective strategies and is considered the gold standard [17]. Subsequent-ly, a vast number of investigations have shown the protective effect of IPC on hepatic I/R injury [1, 3, 18–21]. Furthermore, previous studies have focused on sinusoidal endothelial cells (SECs) and their vulnerability in cold I/R injury. Hence SECs are believed to be the primary target cells during this injury [22, 23]. However, whether or not SECs will also be more susceptible in warm I/R injury compared to other nonparenchymal cells and hepaticocytes has not been investigated thoroughly [24], and the potential mechanisms are not completely understood. Furthermore, whether the reduction of apoptotic SECs induced by IPC contributes to its protective effect in warm ischemia has not yet been proven. Based on these hypotheses, we assessed and compared the extent of the injury and the type of cells in the liver that undergo apoptosis and/or necrosis at various time points following warm I/R injury in a rat model randomized or not random-ized to IPC. We also touched upon the possible protec-tive mechanism. Our study will help to further under-stand the mechanisms of warm injury to the graft before liver transplantation, especially in living donor liver transplantation.

**Materials and Methods**

**Animals**

Male Wistar rats aged between 8 and 10 weeks, weighing between 220 and 260 g (n = 75), were purchased from Shanghai Animal Center (Chinese Academy of Science, Shanghai, China). The animals were fed a standard laboratory diet with water and food ad libitum. They were kept under constant environmental conditions with a 12-hour light-dark cycle. All animal experiments were approved by the Animal Care Committee of Zhejiang University in accordance with the Principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1985).

**Experimental Design**

The animals were randomized into three experimental groups: the continuous clamping group (CC) underwent 60 min of 70% hepatic ischemia; the IPC group received 10 min of ischemia followed by 10 min reperfusion prior to ischemia, and the sham control underwent a sham operation without ischemia.

**Surgical Procedure**

Animals were acclimatized for at least 3 days before surgery. Food was stopped 12 h before surgery. All surgical procedures were performed under general anesthesia using sodium pentobarbital 160 mg/kg via an intraperitoneal injection. After a midline laparotomy had been performed, partial hepatic ischemia was induced by clamping the portal vein, hepatic artery and bile duct supplying the median and left lobes of the liver with a microvascu-lar clip, which accounts for 70% of the liver mass [2]. Heparin 100 IU was administered via the caudal vein. As the blood supply to the omental and right lobes remained uninterrupted, intestinal congestion was avoided during clamping by the bypass portal flow through the nonischemic lobes. The duration of the partial hepatic ischemia was selected based on a pilot experiment, in which 60 min of ischemia caused pronounced liver injury without any fatal accidents, such as severe liver failure or death of the animals. After 60 min of partial hepatic ischemia, the clip was removed to initiate hepatic reperfusion and the abdominal cavity was closed with a 4-0 silk suture. The animals were allowed to recover from the anesthesia and given free access to food and water. The sham-operated group underwent the same procedures without clamping the pedicle of the liver lobes. The animals were sacrificed after I/R injury at five sampling time points (0.5, 2, 6, 12, 24 h, n = 5/time point). A blood sample, collected from the inferior vena cava of each rat at kill, was centrifuged, and the serum was stored at −80°C until analysis.

**IPC Protocol**

IPC included a 10-min period of ischemia followed by 10 min reperfusion before 60 min ischemic insult. Briefly, a microvascular clamp was placed on the left lateral lobe for the ischemic period. The clamp was then removed to allow reperfusion for the required period before replacing it for the second ischemic insult. The animal was kept anesthetized for the entire period of preconditioning. After the clamp had been replaced for the second insult, the incision was closed and the animal was allowed to recover.

**Biochemical Determinations**

TNF-α, Hyaluronic Acid, Aspartate Aminotransferase and Alanine Aminotransferase Assay

To assess liver damage, hepatic injury was evaluated by determination of the alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels using an Automated Chemical Analyzer (7600; Hitachi, Tokyo, Japan). Serum hyaluronic acid (HA) levels were measured using the radioimmunoassay method (SHN Medical Research Center, Shanghai, China). Serum TNF-α levels were assayed with enzyme-linked immunosorbent assay (ELISA). The TNF-α ELISA kit was purchased from R&D Co., USA.

Myeloperoxidase and Malondialdehyde Assay

For assessment of neutrophil sequestration and activation in the ischemic liver, the myeloperoxidase (MPO) contents were measured. Lipid peroxidation, an indicator of oxidative injury, was determined by measuring the formation of malondialdehyde (MDA). The liver tissue MPO and MDA levels were assayed using commercial kits (NJJC Bio Inc., Nanjing, China) according to the manufacturer’s instructions.

**Morphometric Assessment of I/R Injury**

Hematoxylin-Eosin Staining

Partial specimens of the left lateral and median lobes were fixed in 10% formaldehyde and embedded in paraffin. Hematoxylin and eosin (HE)-stained sections (4 μm) were evaluated at
**Results**

**Biochemical Assessment**

**Assessment of Serum ALT Levels**

A significant ALT increase was observed in the CC group at 0.5 h after reperfusion, whereas a minor increase was seen in the IPC group. The maximal effect was observed at 2 and 6 h after reperfusion with a 2-fold reduction in the IPC group. The serum ALT levels of IPC and CC then gradually decreased to normal levels after 6–24 h reperfusion (fig. 1a).

**Assessment of Serum AST Levels**

As shown in figure 1b, significant increases in the serum AST activity was observed after I/R from 0.5 to 12 h in the CC group compared with the sham group, indicating impaired liver function after I/R injury. In contrast, the serum AST activity at 2 and 6 h was significantly lower in the IPC group (fig. 1b).

**Assessment of Serum HA Levels**

In the CC group, a significant increase was observed after 2–12 h of reperfusion compared to the sham group, indicating impaired SEC function after I/R injury. No significant differences were evaluated between the two groups (fig. 1c). The HA changes of the CC group and the IPC group were statistically nonsignificant followed by stably increasing levels for the rest of the study period. In the CC group, a major increase was observed after reperfusion from 0.5 to 24 h. HA levels in IPC tended to be lower than those in the CC group.

**Assessment of Serum TNF-α Levels**

The TNF-α level remarkably increased immediately after reperfusion and remained at high levels in the CC group compared to the IPC group, indicating that IPC can decrease serum TNF-α levels. In contrast, an exchange of the TNF-α level in the CC and the IPC groups after 6 h reperfusion was identified, and at 24 h, the difference reached statistical significance (fig. 1d).

Statistical Analysis

All values were expressed as mean ± SD. Data were analyzed using the SPSS 16.0 software package. Statistical significance between two groups of parametric data was evaluated by one-way ANOVA, and a Student-Newman-Keuls posttest was used for estimation of the stochastic probability in intergroup comparisons. Differences at p < 0.05 were considered statistically significant.
Assessment of Liver MPO and MDA Levels

After 0.5–24 h of reperfusion, the livers were harvested and the tissue levels of MPO and MDA were assessed. Hepatic I/R injury led to an elevation of the tissue MPO levels at 2–24 h after reperfusion, and the increases in the MPO levels were significantly inhibited by IPC (p < 0.05). The MDA was significantly decreased at 0.5–24 h after reperfusion in the IPC group compared to the CC group (p < 0.05) (tables 1, 2).

Histological Evaluation

HE Staining

No pathologic changes were observed in liver samples at 6 h after the sham operation. Only few nuclear condensations and fragmentations of the type associated with apoptotic cell death were focally identified in SECs and hepatocytes. Liver necrotic areas can be calculated on HE-stained tissue section. Other evidence of liver injury was absent or minimal at 6 h after perfusion (fig. 2).
TUNEL Staining
As shown in figure 3, apoptosis appeared in the early phase (0.5–2 h) of reperfusion in both SECs and hepatocytes. Its peak was reached at 6–12 h after reperfusion. Hepatocytes showed changes in apoptosis levels later than SECs. A smaller number of TUNEL-positive SECs were detected in the IPC group, whereas a greater number of positive cells were detected in the CC group. These histological changes were associated with serum ALT and AST levels. However, it was noteworthy that the magnitude of apoptosis in SECs (3.5 positive SECs/high field) was approximately 50 times greater than that observed in hepatocytes (1.5 positive hepatocytes/high field) (fig. 4).

Table 1. Tissue MPO contents at various reperfusion times (U/g wet tissue)

<table>
<thead>
<tr>
<th></th>
<th>0.5 h</th>
<th>2 h</th>
<th>6 h</th>
<th>12 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>0.62 ± 0.04</td>
<td>0.61 ± 0.05</td>
<td>0.56 ± 0.03</td>
<td>0.90 ± 0.30</td>
<td>0.73 ± 0.13</td>
</tr>
<tr>
<td>IPC</td>
<td>0.94 ± 0.05*</td>
<td>0.65 ± 0.14</td>
<td>0.87 ± 0.16</td>
<td>1.02 ± 0.04</td>
<td>1.45 ± 0.25</td>
</tr>
<tr>
<td>CC</td>
<td>0.95 ± 0.06</td>
<td>1.05 ± 0.15*</td>
<td>1.64 ± 0.32*</td>
<td>1.95 ± 0.08*</td>
<td>2.11 ± 0.45*</td>
</tr>
</tbody>
</table>

Data represent mean ± SD of n = 5 rats/group. * p < 0.05 vs. sham group; † p < 0.05 vs. IPC group.

Table 2. Tissue MDA contents at various reperfusion times (nmol/mg protein)

<table>
<thead>
<tr>
<th></th>
<th>0.5 h</th>
<th>2 h</th>
<th>6 h</th>
<th>12 h</th>
<th>24 h</th>
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<tbody>
<tr>
<td>Sham</td>
<td>1.96 ± 0.09</td>
<td>1.99 ± 0.22</td>
<td>1.94 ± 0.08</td>
<td>1.98 ± 0.03</td>
<td>2.07 ± 0.16</td>
</tr>
<tr>
<td>IPC</td>
<td>2.21 ± 0.10</td>
<td>2.46 ± 0.10</td>
<td>2.99 ± 0.24</td>
<td>2.82 ± 0.06*</td>
<td>2.88 ± 0.41</td>
</tr>
<tr>
<td>CC</td>
<td>3.58 ± 0.27*</td>
<td>3.89 ± 0.19*</td>
<td>4.55 ± 0.09*</td>
<td>3.58 ± 0.22*</td>
<td>3.96 ± 1.55*</td>
</tr>
</tbody>
</table>

Data represent mean ± SD of n = 5 rats/group. * p < 0.05 vs. sham group; † p < 0.05 vs. IPC group.
Immunohistochemical Staining of Caspase-3
In these groups, few positive caspase-3 cells were observed immediately after reperfusion. After 2–12 h of reperfusion, positive expression of caspase-3 was observed in massive SECs, but only occasionally in hepatocytes. Peak expression occurred in the 6th hour. Major apoptotic areas were located around central veins. At 24 h, caspase-3 activation on SECs disappeared, and small numbers of hepatocytes were positive for caspase-3 immunostaining (fig. 5).

Assessment of Leukocyte Infiltration in Living Liver
As shown in figure 6, leukocyte infiltration was blocked in the IPC group compared with the CC group at 2–12 h after reperfusion (p < 0.05), indicating that leukocyte infiltration may participate in the induction of hepatic injury in the early phase (fig. 6).

Discussion
IPC, introduced by Murry et al. [16] in 1986, has shown great promise in organ-protective strategies [3, 14, 26]. A short period of I/R protects various tissues against subsequent sustained ischemic insults. Previous studies [4, 14, 27] show the apoptosis of hepatocytes and SECs to be a critical mechanism of injury in the ischemic liver. The apoptosis of SECs has played a pivotal role in cold preser-
In this study, we presumed that SECs may be the initial cells in warm I/R injury as has been described in cold ischemia [22, 23, 29, 30]. To our knowledge, few investigations have focused on SECs after warm ischemia [24, 31].

In liver surgery, most surgeons clamp hepatic blood supply segmentally rather than totally [3, 32]. In the present study, as an objective analysis of IPC in normothermic ischemia, we applied the partial hepatic ischemia model, allowing us to evaluate a series of changes of related parameters.

HA is a biochemical marker of liver cell function and represents the function of SECs [33]. An increase in serum HA means damage to SECs. To further understand the degree of SECs injury, we not only evaluated histological changes, but assayed serum HA concentrations. In the present study, the difference of HA levels after reperfusion was significant in the CC and the IPC groups, indicating that IPC effectively ameliorated the impairment of SEC function in the early phase after reperfusion. On the other hand, the serum HA levels were consistent with ALT, AST leakage and histological changes of SECs. In the present experiment, IPC significantly decreased the serum ALT and AST concentrations at 2 and 6 h after I/R, indicating that it effectively ameliorated the impairment of hepatic function in the early stage after reperfusion. When the number of SEC apoptosis reached its peak after reperfusion at 6 h, at the same time, the serum HA levels also reached the peak. Similarly, when the amount of apoptosis of SECs was close to the baseline, HA levels continued to decrease. In the present study, we combined caspase-3 staining with TUNEL assay in order to objectively assess the occurrence of apoptotic cell death in our liver specimens. The conventional DNA fragmentation detection by TUNEL may fail as a specific marker of apoptosis [24]. Caspase-3 is an established member of the caspase family known to be involved in the final execution phase of apoptosis. In the present study, experimental data showed that

Fig. 4. Evidence of hepatocyte and SEC apoptosis was assessed by the TUNEL assay. In situ TUNEL staining of livers subjected to 60 min of ischemia and 6–24 h of reperfusion showed no apoptotic cells in the sham group (a) and only rare cells stained positive in the IPC group (b). In contrast, massive staining of positive cells is visible in the CC group (c). Among the positive cells, massive SECs and few hepatocytes appeared at different time points (d, e). Values are mean ± SD. * p < 0.05 vs. sham group; # p < 0.05 vs. IPC group.
IPC reduced hepatic enzymatic leakage, decreased TNF-α levels after early reperfusion and decreased the number of apoptotic SECs compared with the CC group. The reduction of SEC apoptosis was remarkable, and the appearance of apoptotic SECs occurred earlier than that of hepatocyte apoptosis. However, Tejima et al. [34] found that IPC decreased injury in hepatocytes via direct protection of hepatocytes, which is mediated by the suppression of Kupffer cell activation, not via suppression of changes in SECs. We do not explain the exact reasons for this disparity but provide the following possibilities. These conflicting findings may vary depending on the length and type of ischemia, the scope of the ischemic area, and whether or not the organ was excised. Cell death is the key form of hepatic ischemia injury; the ultimate fate of the postischemic liver relies on the balance between cell necrosis or apoptosis and hepatocyte proliferation. Previous observations show that 50–70% of endothelial cells and 40–60% of hepatocytes appear to undergo apoptosis during I/R rather than cell necrosis [24, 28, 35]. In the present study, the occurrence of apoptotic cells is associated with inflammatory reactions and reactive oxygen species (ROS) stress, which result in the activation of the apoptotic pathway mediated by mitochondria. Most recently, Arab et al. [36] demonstrated that hepatic parenchyma is able to resist against some types of I/R injury and parenchymal cells are not susceptible to I/R injury, indicating the cell death by apoptosis is the most important change in the early phase after hepatic reperfusion injury.

In the present study, a number of apoptotic cells did not increase remarkably after reperfusion in the CC group, partly because 60 min of 70% hepatic warm ischemia is insufficient to induce heavy liver injury. Additionally, we also observed that most of the distribution of apoptosis cells and necrotic areas were located around zone 3 of the liver lobule, indicating that the cells of this site are more susceptible to reperfusion. Although IPC significantly decreased cell apoptosis, IPC failed to abolish the occurrence of cell necrosis after reperfusion. In our study, we observed that liver necrosis was partially inhibited by IPC, although the differences of the necrotic areas failed to reach statistical significance when compared to the CC group. Furthermore, IPC significantly reduced tissue MPO concentrations, whereas CC caused a remarkable increase in MPO levels after reperfusion from 2 to 24 h (p < 0.05). MPO is a hemoprotein that is
abundantly expressed in neutrophils and secreted during their activation. It possesses potent proinflammatory properties and contributes directly to tissue injury. Liver MPO activity represents a degree of leukocyte infiltration [2]. Beauchamp et al. [37] suggest that IPC protects SECs partly through a decreased adhesion of leukocytes, secondary to a decreased production of endothelial adhesion molecules. Several studies have shown that adhesion molecules are essential for leukocyte attachment and penetration through SECs and postsinusoidal venules of the postischemic liver [38, 39]. Yuan et al. [40] observed that IPC exerted protective effects on both SECs and hepatocytes during liver I/R injury and explained a mechanism involving a reduction in leukocyte infiltration and modulation of the imbalance of the endogenous oxidant-antioxidant system. For further observation of adherent leukocytes in the liver, we applied the IFM technique [2]. In the present study, we found that the inhibitory effects

**Fig. 6.** Alterations of hepatic microcirculation in the CC and IPC groups. Adherent leukocytes and sinusoidal sequestration of leukocytes at 2 h after reperfusion in the IPC (a) and CC groups (d). Adherent leukocytes and sinusoidal sequestration of leukocytes at 12 h after reperfusion in the IPC (b) and CC groups (e). Adherent leukocytes at 24 h after reperfusion in the IPC (c) and CC groups (f). The numbers of adherent leukocytes in postsinusoidal venules were 1.4 ± 0.55, 1.6 ± 0.55 and 1.6 ± 0.55 cells/mm venule length at 2, 12 and 24 h after reperfusion in the sham group. In contrast, the numbers of adherent leukocytes in postsinusoidal venules (22.4 ± 7.77 and 37 ± 6.55 cells/mm venule length, 39.0 ± 4.3 and 48.4 ± 6.19 cells/mm venule length, respectively) were more pronounced at 2 and 12 h after reperfusion in the IPC group than in the CC group (g). The data of 24 h in the CC and IPC groups did not reach significance. Values are mean ± SD. *p < 0.05 vs. sham group; **p < 0.05 vs. IPC group.
of IPC on hepatic function correlated very well with the reduction of adherent leukocytes in the liver. Analysis of leukocyte attachment by IFM revealed that the postischemic increases in the numbers of adherent leukocytes were significantly reduced in the IPC animals. Only a few sequestrated leukocytes were observed in the postsinusoidal venules of sham animals (fig. 6). The number of adherent leukocytes in the CC group was consistent with tissue MPO levels, indicating that leukocyte infiltration plays a significant role during hepatic I/R injury. Furthermore, these observations indicate that IPC has beneficial hemodynamic effects on hepatic I/R by alleviating postischemic leukocyte trapping. As a result, the suppression of leukocyte attachment plays an important role in improving hepatic I/R injury, where IPC has significantly decreased the number of adherent leukocytes.

Usually, oxidative stress is considered a mediator of programmed cell death. SECs are damaged by oxygen-free radicals produced on reperfusion following liver ischemia [41]. Actually, ROS and toxic free radicals have been implicated in the mediation of hepatic I/R injury [12]. In the present study, IPC significantly reduced tissue MDA and MPO levels, indicating that suppression of neutrophil sequestration and activation play a fundamental role in I/R injury by directly or indirectly inducing ROS production. Early hepatic injury after reperfusion is associated with the relevant triggers for apoptotic cell death. TNF-α has been shown to induce the necrosis and apoptosis of liver parenchymal and nonparenchymal cells [42] via stimulating hepatic infiltration of leukocytes [25]. Conversely, IPC can prevent systemic events caused by the release of TNF-α after liver reperfusion [42–44]. In the present study, we observed that there was a relatively lower, stable level of TNF-α in the IPC group in the early and subacute phases compared with the CC group, and other biological indexes and the degree of histological damage of the IPC group were mild. As a result, the pleiotropic effects of TNF-α may be the relation to the low or high of the serum concentration. Teoh et al. [45] mimicked IPC response by different-dose TNF-α (1 and 5 μg/kg body weight) injection to evaluate the injurious and proliferative index, showing that low-dose TNF-α is just as effective at reducing hepatic I/R injury as IPC. In contrast, they observed that higher doses of TNF-α (25 μg/kg body weight) augment hepatotoxicity rather than protect against I/R injury. Therefore, many investigators think that TNF-α has a dual role (apoptosis and proliferation) in both the early and late phases in case of hepatic I/R injury or major tissue loss [45–48].

In summary, the findings in the present study demonstrated that SECs were more sensitive to apoptosis induced by warm I/R injury than hepatocytes. IPC conferred protection to hepatic I/R through reducing hepatic enzymatic leakage, reducing cell apoptosis and improving neutrophil infiltration.

**Acknowledgments**

This study was supported by the National Basic Research Program of China (973 Program; No. 2009CB522403), the National Key R&D Program (No. 2008BAI60B02) and the National Science and Technology Major Projects (No. 2008ZX10002–026). We would like to thank Jennifer Meintjes from the University of Western Australia for revising the language.

**References**


