Ischemic preconditioning decreases C-X-C chemokine expression and neutrophil accumulation early after liver transplantation in rats

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INTRODUCTION
Liver transplantation as an effective therapy for end-stage liver diseases has been accepted. Though preservation techniques have been greatly improved, ischemia/reperfusion injury resulting in primary liver nonfunction still poses significant clinical problems and contributes to mortality[1-2]. Jaeschke et al.[3-6] established that there were two distinct phases of liver injury after warm ischemia and reperfusion. The initial phase of injury which is far less than that observed at later time points is characterized by Kupffer cell-induced oxidant stress. Events occurred during the initial phase including activation of Kupffer cells, initiate a complex inflammatory pathway that culminates in hepatic accumulation of neutrophils[7]. Recruited neutrophils directly damage hepatocytes by releasing oxidants and proteases and are responsible for the later phase of liver injury induced by ischemia/reperfusion. Activated PMN has also been implicated as a vital factor in the development of ischemia/reperfusion injury in both experimental and clinical liver transplantsations[8-11].

C-X-C chemokines are a group of molecules that have both inflammatory and reparatory properties and are best known for their neutrophil chemotactic properties[12,13]. MIP-2, belonging to C-X-C chemokines has been shown not only to regulate PMN recruitment from vascular compartment to the tissues[14] but also to cause PMN activation[15]. Kataoka et al. demonstrated that MIP-2 played a crucial role in PMN recruitment and activation after liver transplantation[16]. IP is a process of a short period of ischemia and reperfusion, which leads to an unexpected resistance to a long-term ischemia/reperfusion injury. It has been documented in several organs, including the liver[17-19]. In experimental liver transplantation, IP has been confirmed as an effective strategy for protecting the grafts from ischemia/reperfusion injury

MATERIALS AND METHODS
Animals
Male Sprague Dawley rats weighing 200 to 250 g were used as donors and recipients of orthotopic liver transplantation (OLT). They were housed in pathogen-free water and fasted for 14 hours before operation. All experiments were performed in compliance with the standards for animal use and care set by Institutional Animal Care Committee.

Surgical procedures
OLT. Liver transplantation was performed according to Kamada’s cuff-technique with minor modifications. Before liver harvesting, 1 mL saline containing 50 units of heparin was given intravenously, and the donor liver was perfused in situ via the portal vein with 20 mL of cold physiological saline
solution to which 50 units of heparin was added. Following cuff preparation, the liver was stored in a beaker containing University of Wisconsin solution at 4 °C for 24 hours. At the end of storage, the liver was slowly flushed with 20 mL of cold (4 °C) Ringer’s lactate and transplanted orthotopically into a recipient animal. The hepatic artery was not reconstructed.

IP. Before harvesting donor liver, the portal vein and hepatic artery were interrupted by placing a bulldog clamp for 10 min. Reflow was initiated by removing of the clamp for another 10 min.

In sham group, the left phrenic vein and the right supraparenal vein were ligated and the hepatic artery was freed by ligating and dividing.

Experimental design
All rats were randomly divided into: sham groups, non-IP group and IP group. To obtain blood and tissue samples, six animals were killed in non-IP and IP group after 1, 2, 4 and 6 hr of reperfusion and four animals at each time point in sham group. Plasma samples were collected from inferior vena cava, and separated by centrifugation, and median lobe of the liver was carefully excised and stored at -80 °C for analysis. Serum levels of alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) were measured using standard clinical automated analysis. Serum levels of MIP-2 (IBL Immuno-Biological Laboratories, Hamburg) and TNF-α (R & D Systems, Inc) were measured using commercial enzyme-linked immunosorbent assay kits, respectively.

MPO assay
MPO was used as a marker of pulmonary and hepatic neutrophil infiltration[22]. MPO activity was measured photometrically employing 3, 3’, 5, 5’-tetrathiomethylbenzidine as a substrate. Frozen liver tissues were macerated, homogenized, sonicated, and centrifuged at 4 000 g for 12 minutes at 4 °C as described previously[22]. MPO activity was measured in the supernatant, with calculations based on the absorbance change at 460 nm. All values were normalized to tissue weight.

Hybridization
Chunks (1 cm³) from fresh rat liver were immediately fixed in 4 % paraformaldehyde at 4 °C for 8 h and dehydrated through graded ethanol, then embedded in paraffin and sectioned. The sections were layered onto glass slides by standard procedures. Five µm thick sections were cut for analysis. They were deparaffinized with xylene and quickly rehydrated through graded ethanol. The deparaffinized sections were quenched from endogenous peroxidase activity in 3 % H₂O₂ diluted in methanol. Then, the expression of MIP-2 mRNA was detected in rat liver tissue sections using a commercial ISH kit (TBD biotech Co.). All solutions of it were RNase free. The sections were digested using solution I (0.4 % pepsin, 0.1 M Hcl, TBD biotech Co.), then washed in 0.5 M PBS twice for 5 minutes each. The non-specific IgG binding sites were blocked with blocking solution I (1.5 % normal blocking serum, TBD biotech Co.) at RT and excess serum was blotted from the sections. Then, the sections covered with coverslips were prehybridized with prehybridized solution (formamide, standard saline citrate, 100xdenhardt’s, SDS, sperm DNA, TBD biotech Co.) at 37 °C for 4 h in a sealed humidity chamber. The sections were washed twice in 0.5 M PBS and hybridized with digitoxin-labeled oligonucleotide probe synthesized by TBD cooperation Lab, (5’-CCACCTCGCCAGCTCCTAATGCTGTACTGGT CCTGCTCC-3’). Hybridization was performed at 38 °C overnight in a humidity chamber, and washed in 2xSSC, 0.5xSSC, 0.2xSSC for 5 minutes each. In the following step, the sections were blocked with blocking solution II (25 µg/ml avidin, TBD biotech Co.) for 15 minutes at RT, then excess solution was blotted. The sections covered by coverslips were incubated with biotinylated mouse anti-digitoxin antibody (TBD Biotech Co.) for 1 h at 37 °C in a sealed humidity chamber and washed three times in 0.01 M PBS for 5 minutes each. The sections were incubated with streptavidin-biotin-peroxidase complex (TBD Biotech Co.) at 37 °C for 1 h and washed three times in 0.01 M PBS for 5 minutes each. The complex was detected with DAB (0.1 % diaminonbenzidine, 0.02 % hydrogen peroxide, TBD Biotech Co.) in sections and counterstained with hematoxylin (Sigma). The first negative control group was performed in which the mouse anti-digitoxin antibody was substituted with normal mouse serum and the second was performed with RNase (20 µg/ml, Sigma) before hybridization. The last one was hybridized without any oligonucleotide probe.

Histology study
Liver samples were fixed in 4 % neutral buffered formalin, paraplast-embedded and cut into 4 µm thick sections, and stained with hematoxylin-eosin according to the standard procedures.

Statistical analysis
The data were expressed as mean ± SEM. Means of different groups were compared using a one-way ANOVA. The Student t test was performed to evaluate the significant differences between groups. Significance was determined at P<0.05.

RESULTS
Serum levels of ALT, LDH
Both ALT (A) and LDH (B) were significantly elevated after OLT without IP, compared with sham-operated group. When the liver grafts were pretreated with IP, the increases in ALT and LDH were relatively reduced to the non-IP group in each time point respectively (Figure 1).

![Figure 1](image-url) Changes in serum ALT (A) and LDH (B) levels. The serum ALT and LDH levels were significantly elevated in non-IP groups compared with sham-operated group (p<0.01, non-IP group vs sham-operated group). After IP, the increase was significantly reduced at 4 and 6 hour points after reperfusion (p<0.05, IP group vs non-IP group).
**Hepatic PMNs accumulation**

PMNs accumulation in the grafted livers was assessed by investigating the levels of MPO. Figure 2 shows changes in PMNs accumulation in three groups. The increases in non-IP groups were significantly reduced in IP group, especially at 4 and 6 hour points after reperfusion.

**Intragraft MIP-2 mRNA expression**

*In situ* hybridization was performed at 4 hour point after reperfusion in all three groups (Figure 5). MIP-2 mRNA was not detectable in sham operated group (A). MIP-2 mRNA in the non-IP group (B) was remarkably up-regulated at 4 hour point and after IP (C), the expression was detectable at a lower level. It was emphasized that the MIP-2 mRNA was mostly expressed in hepatocytes in grafted livers.

**Histological changes of grafts**

Similar to serum transferase indicating liver function, histological change of the graft showed that 24 hours cold storage in UW solution and reperfusion resulted in grafted liver damage. After IP and OLT, the damage was ameliated.

**DISCUSSION**

In this study, we demonstrated that 24 hours cold storage in
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