Remote ischemic preconditioning promotes early liver cell proliferation in a rat model of small-for-size liver transplantation

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A B S T R A C T

Background: The size of the liver donor graft is a major concern in living donor liver transplantation. Rapid regeneration is essential for the survival of these grafts. The purpose of this study was to investigate the effect of remote ischemic preconditioning (RIPC) on liver regeneration in a rat small-for-size liver transplantation model.

Methods: We established rat models of small-for-size liver transplantation (30%) in the presence or absence (control) of remote ischemic preconditioning. We observed liver mass regeneration, serum alanine aminotransferase, hepatic pathologic alterations, flow cytometry, and Ki-67 antigen immunohistochemistry. In addition, using Western blotting and reverse-transcriptase–polymerase chain reaction, we assessed the activation of cell cycle progression as well as tumor necrosis factor-α and interleukin-6 expression.

Results: Compared with the control group, serum alanine aminotransferase activity was significantly lower and histopathology changes were significantly attenuated in the RIPC group. Remote ischemic preconditioning induced a high level of interleukin-6 mRNA in small grafts, but suppressed the expression of tumor necrosis factor-α. The proliferation index, indicated by the S-phase and G2/M-phase ratio [(S+G2/M)/(G0/G1+S+G2/M)], was significantly increased in the RIPC group at 24 h (58.25% ± 0.506% versus 53.405% ± 1.25%; P = .007). Meanwhile, cell cycle progression and regeneration (Ki-67) were initiated early in liver grafts treated with RIPC.

Conclusions: These results suggest that RIPC can protect liver cells against ischemia reperfusion injury in the small grafts and enhance liver regeneration. Interleukin-6 may be a critical mediator in the stimulatory effect on liver cell regeneration, which may make RIPC valuable as a hepatoprotective modality.
1. Introduction
Living-related liver transplantation was developed to significantly improve clinical outcomes owing to the scarcity of cadaveric grafts [1–3]. The size of the graft is a major risk factor in adult-to-adult living donor liver transplantation. Graft failure may be characterized by coagulopathy, ascites, encephalopathy, cholestasis, and histological features of ischemia after implantation. Small graft size has been considered a dominant factor contributing to impaired post-operative graft function [4,5]. The mechanism leading to injury in a small-for-size graft has been studied in clinical and animal models [6–11]. Partial grafts must rapidly regenerate to enable hepatic mass recovery and normal function.

The ability of the liver to restore major tissue loss within a few weeks [12] involves numerous interacting cells and a complex network of mediators. Experiments using partial hepatectomy model have proved that early activation of tumor necrosis factor (TNF)-α and interleukin (IL)-6 responsive transcription factors is a critical process in the initiation of the regenerative response [8–10]. Recent studies have highlighted the IL-6/Stat3 pathway as a major signaling pathway involved in liver regeneration after hepatectomy. Studies have also demonstrated the importance of the IL-6/Stat3 activation pathway in the recovery of liver grafts from ischemia-reperfusion [13]. Ischemia-reperfusion injury is often seen in organ transplants, major organ resections, and shock. Ischemic-reperfusion injury significantly contributes to morbidity and mortality after liver surgery, especially in patients with liver disease.

Ischemic preconditioning (IPC), a brief period of ischemia followed by a short reperfusion before ischemia insult, serves to protect diverse organs against subsequent prolonged ischemic insults and reperfusion injury [14]. It has been considered to be beneficial not only for reducing hepatic ischemia-reperfusion injury in hepatectomy and liver transplantation [15–17], but also for enhancing liver regeneration [18,19]. However, its main disadvantage is trauma to major vessels and stress to the target organ. Remote preconditioning is a novel method in which brief ischemia of one organ has been shown to confer protection on distant organs without direct stress to the organ or blood vessels. Remote IPC (RIPC) of the limb reduces ischemia-reperfusion injury to the heart, lungs, and other organs in humans [20] and animals [21,22]. Studies have investigated the protective effect of RIPC of the hind limb in reducing the adverse effects of liver ischemia-reperfusion injury and underlying mechanism involved in increased hepatic blood flow, the role of heme oxygenase, modulation of the hepatic microcirculation, the role of High Mobility Group-Box1 and, and others [23–26]. None of these studies addressed the hepatic proliferation in vivo seen in the effect of RIPC on the small-for-size liver graft. In the present study, we aimed to investigate the possible effect of RIPC on hepatocyte proliferation, and the underlying mechanism.

2. Materials and methods

2.1. Experimental design and surgical procedure
We used male Sprague–Dawley rats (250–300 g; Vital River Experimental Animal Co., Beijing, China) as donors and recipients. The animals were given free access to tap water and a standard rat diet. We housed the rats in accordance with institutional animal care policies. Research procedures complied with the Ethics Committee for animal experiments.

We randomly divided the animals into the RIPC group and the control group. We executed non-arterIALIZED orthotopic liver transplantation as previously described [27]. In RIPC groups, the animal liver donators were subjected to RIPC treatment before the liver donor experienced laparotomy supplying the left and caudate lobes of the liver (approximately 30%). Control group animals underwent an identical experimental protocol without RIPC. Body temperature was maintained at 37°C and the graft was stored in Ringer’s solution at 0°C to 4°C, with a mean time of 60 min (range, 51–67 min). The technique of RIPC involved a limb tourniquet, which we applied around the hind limb at the inguinal level without skin insult. We monitored the method of hind limb ischemia and perfusion using a pulse oximeter (Kangtai Medical-Technology Co., Tianjin, China) modified for application in rats, and by the change in foot color. The procedure involved 5 min of ischemia followed by 5 min of reperfusion; this was repeated for four cycles. We based this on Tapuria and colleagues’ study [28], which used a similar protocol of hind limb preconditioning before liver ischemia-reperfusion injury in a rat model. At each time point, we killed animals, collected liver tissue and serum samples, and froze them at −80°C. We also fixed liver tissues in 10% formalin for histology.

2.2. Serum alanine aminotransferase (ALT)
We obtained blood serum when we killed the animals after centrifugation. We measured the plasma level of ALT in a clinical laboratory to assess the extent of hepatocellular damage.

2.3. Histological investigation
We obtained liver tissue at each time point and fixed it in formalin. We cut a paraffin-embedded sample of the liver tissue into 4-μm sections and stained it with hematoxylin and eosin. The mitotic index is expressed as the percentage of mitotic hepatocytes per total number of hepatocytes in 20 high-power fields.

2.4. Detection of cell cycle
We performed hepatocyte isolation using an enzymatic technique previously described (Howard et al., 1967). We washed single cell suspensions of hepatocytes, fixed them
with 75% ethanol, and stained them with 50 μg/mL propidium iodide (Sigma-Aldrich Trading Co., Shanghai, China) for 30 min at room temperature. We detected samples with a FACSort flow cytometer (Becton Dickinson & Company, NJ, USA), and cell cycle was analyzed by Modfit software (Verity Software House, ME).

2.5. Ki-67 Immunohistology

We used Ki-67 immunostaining to assess the percentage of proliferating hepatocytes. We deparaffinized liver paraffin sections with xylene and ethanol and washed the tissue sections with H2O (distilled). We demasked epitopes by heat treatment in a pressure cooker. After blockage of endogenous peroxidase with 3% H2O2 for 30 min, we incubated sections with a mouse anti-rat Ki-67 antibody (Cell Signaling Technology, MA) overnight at 4°C. After they were washed, we incubated the slides with a horseradish-peroxidase conjugate secondary anti-mouse antibody. When color developed by incubation with diaminobenzidine (Zhongshan Golden Bridge, Beijing, China), we counterstained the sections with hematoxylin. A positive cell was considered to be one with brown nuclear staining; we randomly selected 10 high-power fields to calculate the relative number of positive cells.

2.6. Reverse-transcriptase–polymerase chain reaction (RT-PCR)

We extracted total RNA from liver tissue by TRIzol Reagent (Invitrogen, Shanghai, China) and converted it to cDNA. We performed DNA amplification by PCR. The primers used were as follows: IL-6 sense: 5'-TGTGTGACACCCAAGACCTCCCTCCC-3' antisense: 5'-ACGGAAAACCAGACCGAGCA-3' (product length = 308 bp); TNF-α sense: 5'-CATCTGCTGGGTGCGGGG-3' antisense: 5'-GCTGCTCCCGCTCCGTCGG-3' (product length = 204 bp); glyceraldehyde 3-phosphate dehydrogenase sense (GAPDH): 5'-TGCTGAGTTAGCTGCTGGAG-3' antisense: 5'-GTCTGTGAGTGGCTGTGGCAGG-3' (product length = 288 bp). We electrophoresed 10 μL of PCR products on 1.5% agarose gel (Sigma) containing 5 μg/mL ethidium bromide (Sigma). We calculated the molecular weight with DL-500 DNA markers (TaKaRa, Dalian, China). To compare the relative levels of mRNA, each sample was normalized against its respective GAPDH.

2.7. Western blotting

For Western blot analysis, we separated the total extracts prepared from liver tissues onto sodium dodecyl sulfate–polyacrylamide gels and electrophoretically transferred them onto polyvinylidene difluoride (PVDF) membrane (Roche Diagnostics Co., IN). We incubated the membranes with the primary antibody cyclin D1 (Cell Signaling Technology) and then incubated them with corresponding peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). We visualized the results via a chemiluminescence detection System (Pierce ECL Western Blotting Substrate Detection System; Thermo Scientific, Rockford, IL) and exposure to autoradiography film (Biomax XAR film; Kodak, Shenzhen, China).

2.8. Statistical analysis

The results are presented as means ± standard deviations. We examined differences between groups for statistical significance using the t-test. \( P < .05 \) was considered statistically significant.

3. Results

3.1. Liver mass recovery

We used the liver–body weight ratio to assess restitution of the residual of liver lobes. The remnant liver mass–body weight ratio at 24 h after surgery in the control groups was 1.78% ± 0.17%; RIPC in treatment slightly increased the relative liver weight after 24 h, to 1.92% ± 0.23%, but the difference was not statistically significant (Fig. 1).

3.2. Hepatocellular proliferation

Immunohistochemical staining of Ki-67 confirmed that the mitotic activity of hepatocytes enhanced and increased in the RIPC group. Compared with controls, the Ki-67 labeling index of the RIPC group was significantly higher at 24 h (57.64% ± 3.37% versus 67.35% ± 3.44%; \( P < .05 \)) (Fig. 2A and B). It resulted in stimulation of DNA replication after orthotopic transplantation, compared with the control group. The proliferation index increased significantly at 24 h in the RIPC group compared with the control group. There was a statistically significant difference (58.25% ± 0.50% versus 53.40±1.25%; \( P < .01 \)) (Fig. 3).

Fig. 1 – Recovery of graft weight after small-for-size liver transplantation. The liver mass of two groups at 24 h increased significantly compared with that at 2 h (1.78% ± 0.17% versus 1.18% ±0.02%, ** \( P < .05 \); 1.92% ± 0.23% versus 1.19% ± 0.09%, * \( P < .05 \)), but there was no significant difference between the RIPC and control groups (\( n = 3 \), each sample repeated three times).
3.3. Histologic analysis and serum levels of ALT

We observed an apparent dilation of the sinusoid, hepatocellular edema, degeneration, and hepatic lobule distortion in both groups. The severity of sinusoidal congestion, cell edema, hepatocellular necrosis, and neutrophil infiltration was less evident in the RIPC group compared with the control group (Fig. 4). The serum ALT levels increased rapidly after transplantation in both groups and reached the peak at 6 h. The ALT levels in the RIPC group were significantly lower than in the controls at 2, 6, and 24 h after reperfusion (2 h: 506.80 ± 36.74 versus 1153.80 ± 157.57 U/L; 6 h: 1140.44 ± 98.57 versus 1606.43 ± 49.25; 24 h: 860.56 ± 74.05 versus 1193.80 ± 38.07; \(P < .05\)) (Fig. 5A and B).

3.4. Expression of TNF-\(\alpha\), IL-6, and cell cycle regulators in regenerating liver

The RT-PCR results showed that TNF-\(\alpha\) and IL-6 mRNA were expressed in the control and RIPC groups. The levels of pro-inflammatory cytokines TNF-\(\alpha\) and IL-6 were rapidly up-regulated after transplantation in the control and RIPC-treated groups (Fig. 6). The messenger RNA expression of TNF-\(\alpha\) was significantly suppressed (Fig. 6B and D), but IL-6 mRNA levels in the RIPC groups were markedly higher than those in the control group at 6 and 12 h after operation (\(P < .01\)) (Fig. 6C). We assessed progression of cell cycle by cyclinD1 expression. We evaluated the expression of cyclin D1 protein in the regenerating livers by Western blotting after surgery. Figure 7 shows a significantly high expression of cyclin D1 in the RIPC group compared with the control group. The successful initiation of the cell cycle pathway of RIPC may be the reason for the mild liver histologic injury.

4. Discussion

The capability of liver regeneration is considered a crucial factor in the survival and functional recovery of liver grafts after partial liver transplantation. However, liver regeneration
is suppressed in small-for-size liver grafts [29,30], which prevents or delays the recovery of liver mass and function after partial liver transplantation. A study has shown that ischemia-reperfusion impairs the regenerative capacity of the liver in both rat and mouse models [31]. A number of therapeutic methods have been investigated to alleviate liver ischemia-reperfusion injury and improve hepatic function. Remote ischemic preconditioning before hepatic ischemia-reperfusion injury reduced the biochemical markers of hepatic injury and improved hepatic perfusion, systemic hemodynamics, and peripheral oxygen saturation in the experimental liver warm ischemia-reperfusion model [23-25]. In the present study using small-for-size liver grafts, we found a similar result demonstrating that RIPC led to a lower ALT level and reduced the extent of liver histological injury in the early stage. However, the recovery of ALT levels in the RIPC group was delayed compared with the control group. The mechanisms of the hepatoprotective effect of RIPC might be related to its ability to inhibit the production of pro-inflammatory cytokine TNF-α and activate cell proliferation in the liver.

Many studies have investigated the mechanism of small-for-size graft injury after liver transplantation in both animal experiments and clinical research [6,8-11]. Graft size is inversely related to the degree of graft damage in liver transplantation. The underlying mechanisms of small graft dysfunction and failure remain unclear. Previous studies have indicated that liver regeneration is markedly suppressed in small-for-size transplantation by interfering with the production of IL-6 and TNF-α or defective progression from the IL-6 signaling pathway to cellular proliferation [29,30]. To avoid this graft dysfunction and failure, rapid regeneration of the graft is essential to ensure hepatic mass recovery, and subsequent protective measures are required to maintain liver function. In this study, flow cytometry demonstrated that RIPC could enhance cell cycle progress. Grafts treated with RIPC had significantly increased Ki-67 expression at 24 h after reperfusion compared with the controls. Zhang et al [32] showed that proliferating liver cells can resist ischemia-reperfusion injury. This result implies that hepatocyte proliferation was promoted in the RIPC group during the early phase after surgery, which is possibly related to RIPC protection.

Fig. 3 — We stained DNA with propidium iodide and (A) detected cell cycle progress by flow cytometry. (B) The proliferation index increased in the RIPC group compared with the control group; there was a statistically significant difference (n = 3, each sample was repeated three times; **P < .01).
Liver regeneration is a complex, multi-step process involving numerous cellular components and a complex network of mediators. Among them, TNF-α and IL-6 have been shown to be crucial effectors in liver regeneration [19,30,33]. Studies have suggested that IL-6 and TNF-α are important in the initiation of hepatic regeneration using specific gene knockout mice [33,34]. These major regenerative cytokines and growth factors are higher in quarter-size liver grafts that

![Histopathology examination of small-for-size liver grafts in control and RIPC-treated rats. Hematoxylin and eosin-stained sections of RIPC-treated grafts demonstrated normal architecture, with mild to moderate sinusoidal dilatation and disintegration. Control grafts exhibited more severe cell swelling, infiltrating inflammation, and focal necrosis and hepatocyte collapse. The result is representative of the independent experiments (n = 3; original magnification, ×200).](image)

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![The serum ALT levels after small-for-size liver transplantation. Values are expressed as mean ± standard deviation (n = 3) (*P < .05 compared with control groups).](image)
fail to regenerate than in half-size grafts that regenerate rapidly [35]. The liver underlying ischemic preconditioning could generate reactive oxygen species and TNF-α during early reperfusion [36,37]. Tumor necrosis factor-α is a known mitogen for hepatocytes and an important factor for liver cell proliferation. In several experimental studies, TNF-α and IL-6 have both been characterized as mediating the priming response of hepatocytes, whereby these resting cells gain proliferative capacity and undergo a transition from G0 to G1. In the present study, we demonstrated an early rise in the expression of TNF-α and IL-6 levels in the control group and RIPC group. Remote ischemic preconditioning significantly decreased the rise in the expression of TNF-α but not IL-6. The results suggest that IL-6 may have a key role in the induction of hepatocyte proliferation after partial liver transplantation under ischemia reperfusion.

Once they leave the resting state (G0/G1) and proceed through the phase of DNA synthesis (S) and mitosis (G2/M), hepatocytes achieve the capability of replication. There is a checkpoint between the G0/G1-phase and S-phase, regulated by cyclin D1. A high expression of cyclin D1 could make cells pass through the checkpoint and enter the S-phase and G2/M-phase, and promote cell proliferation in rat liver [38]. In the present study, we observed delayed IL-6 and TNF-α expression in the control group grafts; this suppressed liver regeneration agreed with results of previous research [29]. The mechanisms may be related to the activating TGF-β/Smad signaling pathway that up-regulates CDKI p21Cip1, leading to cell cycle arrest [39]. In the present study, we detected the expression of cyclin D1 protein in the RIPC group soon after reperfusion was begun. Compared with the control, RIPC could enhance cyclin D1 expression during the early

Fig. 6 – Effects of RIPC on levels of IL-6 and TNF-α expression. (A, B) We assessed intrahepatic IL-6 and TNF-α mRNA by reverse-transcriptase–polymerase chain reaction and normalized them to GAPDH. Each lane is a representative sample of one of three animals. (C, D) Quantitative analysis of IL-6 and TNF-α depicted graphically for control and RIPC-treated grafts (n = 3, each sample was repeated for three times; *P < .05 and **P < .01 versus control group).

Fig. 7 – Western blotting analysis for the expression of cyclin D1 at each time point. We calculated each lane’s relative gray value and normalized it to GAPDH. (A) We assessed the expression of cyclin D1 protein by Western blotting. (B) We used the relative ratio of cyclin D1/GAPDH to make a quantitative analysis of cyclin D1 protein. Each lane is a representative extract of one of three animals (**P < .01 compared with the control group.)
reperfusion in small-for-size liver transplantation. Expression of cyclin D1 enhances progression of cells through the G1 checkpoint and into the S-phase of the cell cycle [38,40]. Up-regulation of the cyclin D1 protein may be related to activation of the IL-6/Stat3 pathway. Gp130, which is a subunit of the IL-6 receptor, can activate the mitogen-activated protein kinase signaling cascade [41]. Stimulation of the mitogen-activated protein kinase pathway, which is important for cellular proliferation, makes p38 kinase transfer to cell nuclei and induces the production of cyclin D1 [42]. Interleukin-6 may be the crucial regulator in the small grafts treated with RIPC, but the intimate mechanism of the protection needs further study.

In conclusion, the present study shows an active effect of RIPC on liver cell proliferation during the early phase after small-for-size liver transplantation, which protected the liver from ischemia-reperfusion injury by reducing the inflammatory response. The mechanisms involved may be associated with the early up-regulation of IL-6, which was elated to the initiation of hepatocyte proliferation after injury. One limitation of the present model is that we studied only the early phase of liver regeneration. Differences later than 24 h cannot be excluded. The protective effects of RIPC in enhancing hepatocyte proliferation and preventing ischemic-reperfusion injury suggest that this process might become an alternate method to induce protection and promote regeneration in living donor liver transplantation.

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REFERENCES


