# Ischemic preconditioning improves hepatic regeneration with reduced injury following reduced-size rat liver transplantation★

Liu Xian-zhong[1], Yao Ai-hua[2], Wang Xuan[1], Zhong Ji-wei[2], Li Xiang-cheng[2]

**Abstract**

**BACKGROUND:** Recently, liver transplantation technique has been developed rapidly, and prevention of ischemia/reperfusion injury and protection of liver regeneration have become a research focus. Ischemic preconditioning (IPC) is an effective method for protecting liver ischemic injury. However, the mechanism remains controversial.
**OBJECTIVE:** To investigate the mechanism of IPC on hepatic injury and regeneration after reduced-size rat liver transplantation.
**METHODS:** Animals were randomly divided into 3 groups. Rat reduced-size liver transplantation model was established in liver transplantation group. IPC +liver transplantation group underwent first porta hepatis blocking for 10 minutes before liver graft reperfusion, followed by reperfusion for 15 minutes. The ligament around the liver was dissociated in the sham-surgery group. The samples were collected 0.5, 2, 6 and 24 hours post-operation. The hepatic injury was examined by the serum alanine aminotransferase (ALT) and hepatic tissue histopathology analysis of grafts. Semi-quantitative immunohistochemistry and westernblotting were used to examine the redox factor-1 (Ref-1) protein expression. The hepatic regeneration of the grafts was examined by the expression of proliferating cell nuclear antigen (PCNA) in hepatic cells.
**RESULTS AND CONCLUSION:** Compared with liver transplantation group, the ALT values at 6 and 24 hours after operation in IPC group decreased significantly ($P < 0.05$; $P < 0.01$). Pathological analysis indicated that there were lots of inflammation cells around the portal veins, the serious sinus hepaticus dilation and damage of hepatic tissue in liver transplantation group. However, the tissue injury observed in IPC group was comparatively slight. Semi-quantitative immunohistochemistry revealed that Ref-1 protein was more abundant in IPC grafts tissue compared to liver transplantation group. These observations were supported by westernblotting studies where Ref-1 protein was shown to be over-expressed in IPC specimens at 24 hours after reduced-size liver transplantation ($P < 0.05$). In addition, the number of PCNA-positive cells in IPC group was more than liver transplantation group at 2, 6 and 24 hours after operation ($P < 0.05$). IPC improves hepatic regeneration and relieves grafts injury in earlier period after reduced-size rat liver transplantation, which is associated with the over-expression of Ref-1 protein.

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## INTRODUCTION

The liver posses powerful regenerated function. The living donor liver transplantation relieves the donor deficiency. Success of relative living donor liver transplantation and small-for-size liver transplantation in clinical practice depend mostly on the liver regeneration and self-repairing capability[1]. Ischemic preconditioning (IPC) has been considered to be an effective strategy to protect graft from ischemia/reperfusion (IR) injury after liver transplantation[1-4]. However the exact mechanism of IPC remains unclear. Oxidative stress is recognized as a critical cause of injury in reperfused tissue [5-7]. The production of active oxygen, concerned with Rac-1 in early period of reperfusion, could promote intracellular oxidative stress[8], and activate redox-dependent signal, e.g. nuclear factor kappa B. However, Redox factor-1 (Ref-1) has been shown to defend the oxidative stress injury in a redox-dependent manner in the cell[9]. The present study focused on relation among IPC, oxidative stress and Ref-1, investigated the mechanism of IPC on hepatic injury and regeneration after reduced-size rat liver transplantation.

## MATERIALS AND METHODS

### Design

A randomized, controlled, animal trial.

### Time and setting

The experiment was performed at Laboratory Animal Center of Nanjing Medical University from May 2009 to December 2009.

### Materials

A total of 100 adult male Lewis rats weighing 230–250 g were used as donors and recipients. The rats were fed with standard rodent diet and water, and cared according to the guidelines approved by the China Association of Laboratory Animal Care and the Institutional Animal Care Committee. The rats were randomly divided into three groups: partial liver transplantation (PLT), PLT+ IPC, and sham-surgery groups.

### Establishment of reduced-size rat liver transplantation

#### *PLT group*

Liver transplantation was performed according to previously described method[10]. In initial experiment, the ratio of liver to body mass was estimated in 15 rats. The mean value of total body mass was (241.5±5.6) g,

and the mean value of liver weights was (10.3±1.9) g. Partial hepatectomy was performed according to previously described method[11]. Briefly, the rats were subjected to midventral laparotomy and approximately 50% liver resection (left, triangle lobe and caudae lobe), and the weight of the remaining liver lobes was recorded (mean (5.5±0.6) g, approximately 50% of whole liver weight). The donor liver was perfused in situ via the abdominal aorta with ice-cold isotonic saline solution containing heparin (10 U/mL). In addition, inferior vena cava was cut open. Following cuff preparation, the grafts were stored in a beaker containing Ringer's solution (4 °C). At the end of storage, the liver was slowly flushed with 20 mL of cold (4 °C) Ringer's lactate and transplanted orthotopically into recipient animal. The suprahepatic vena cava was anastomosed by continuous sutures. The portal vein and infrahepatic vena cava were anastomosed by the cuff technique. Then, cystic duct cannula was placed into recipient bile duct. The anhepatic phase was (18±3) minutes. The recipient operation time was (55±12) minutes. The hepatic artery was not reconstructed.

### *IPC and sham-surgery groups*

Before donor liver harvest, the portal vein and hepatic artery were interrupted by placing a bulldog clamp for 10 minutes. Reflow was initiated by removing of the clamp for 15 minutes[1]. In the sham-surgery group, the perihepatic ligament was ligated and cut, followed by operation. In the preliminary experiment, the recipient animal survival rate for one week was 70%.

### Study design

In PLT group ($n$=40), there were 20 donors and 20 recipients; in IPC group ($n$=40), there were 20 donors and 20 recipients. To obtain blood and tissue samples, recipients in PLT and IPC groups were sacrificed at 0.5, 2, 6, and 24 hours after portal vein reperfusion as well as five animals at corresponding time point in the sham-surgery group. Plasma samples were collected from the inferior vena cava, and separated by centrifugation. The liver tissue was carefully excised and stored at −80 °C for analysis.

### Measurements

#### *Serum levels of alanine aminotransferase (ALT)*

Plasma samples were collected from the inferior vena cava and centrifuged. Serum levels of ALT were measured using clinical automated analysis (Hitachi 7600-10, Hitachi High-Technologies Corporation, Japan).

#### *Histopathological examination*

Rat liver tissue was carefully excised, fixated by 10% formaldehyde solution, imbedded with paraffin, stained with hematoxylin and eosin and observed by light microscopy.

#### *Expression of Ref-1 protein by western blot analysis*

The tissue protein was extracted from liver tissue samples using a tissue protein extract kit (Bio-Pierce, USA), and transferred to a microcentrifuge. The supernatant concentration was measured with the BCR protein assay kit (Shanghai Shenergy Biocolor Bioscience & Technology Company, China). Equal amounts of 40-μg protein were transferred to electrophoresis on sodium dodecyl sulfate polyacrylamide gel electropheresis and stained with Coomassie blue to document equal protein loading. Following spotting on nitrocellulose membranes (Roche, USA), the membrane was blocked with 10% skimmed dry milk in PBS for 1 hour at room temperature and incubated with a primary multi-antibody against Ref-1 (1: 1 000; rabbit anti-mouse, Sant Cruz Biotechnology, CA, USA) and tublin (1: 500, Sant Cruz Biotechnology). After washing, the filters were incubated with a horseradish peroxidase-conjugated secondary antibody (1: 2 000, rabbit-anti-goat Amersham, Arlington Heights, Ill, USA) and scanned using DNR Bio-Imaging systems (P.O.BOX 300, Belgium). The relative amount of the protein in a ratio to tublin was determined.

#### *Measurement of proliferating cell nuclear antigen (PCNA) and Ref-1 by immunohistochemical analysis*

The paraffin sections of the liver tissues were immunochemically stained for PCNA and Ref-1 using immunohistochemistry techniques (SP method). Briefly, after de-paraffinization, the sections were quenched for endogenous peroxidase in absolute methanol containing 0.3% $H_2O_2$ for 20 minutes. The sections were processed to retrieve the antigens by conventional microwave oven heating in 10 mol/L citric acid buffer (pH. 8.0) for 10 minutes. Sections were blocked with goat serum (ZLI-9022, BeiJing Zhongshan Goldenbridge Biotecnology), followed by treatment of the primary antibodies (Ref-1, Santa Cruz Biotechnology, CA, USA; PCNA, Dako Cytomation, Denmark) with 1: 100, dilution at 4 °C overnight. After washing, rabbit anti-rat and rabbit anti-mouse IgG-HRP (SANTA CRUZ BIOTECHONOLOGY, CA, USA), 1: 1 000 dilution, were applied (60 minutes at room temperature). The sections were washed and the signal was developed with the DAB Substrate kit (ZLI-9032, BeiJing Zhongshan Goldenbridge Biotecnology). The sections were counterstained with Hematoxylin (ZYMED Laboratories, Germany). The number of PCNA-positive nuclei was counted in at least three rats per group, and 3 ×100 hepatocytes were quantified for each rat, by two investigators. The label results were analyzed by Image Pro Plus software. The mean for each time point was plotted with indicated standard deviations.

Histological assessment of the liver was also performed by hematoxylin and eosin staining with interpretation by a single-blinded pathologist.

### Main outcome measures

Inflammation cells infiltration, hepatic sinusoid, hepatic tissue damage, haemorrhage and necrosis in grafts.

### Statistical analysis

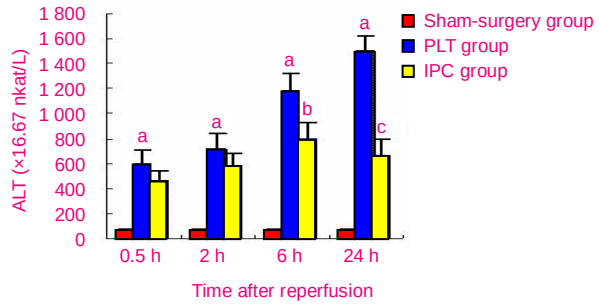
Results are expressed as Means±SD. Statistical data were analyzed using SPSS 10.0 software. Significant differences between two groups were identified by the unpaired Student $t$-test or Fisher's test, respectively. $P$-value < 0.05 was considered statistically significant.

## RESULTS

### Analysis for liver injury and regeneration

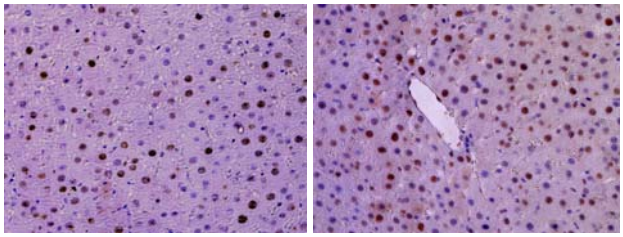
Compared with the sham-surgery group, the ALT values were significantly elevated at each point in PLT group. When the liver grafts were pretreated with IPC, the increased ALT values reduced significantly compared with PLT group at 6 and 24

hours after reperfusion ($P < 0.01$, Figure 1). After labeled with PCNA antibody, the hepatic PCNA-positive cells in IPC group were more than PLT group at 2, 6 and 24 hours after reperfusion ($P < 0.05$, Figure 2, Table 1).




[a]$P < 0.05$, PLT group *vs*. sham-surgery group; [b]$P < 0.05$ and [c]$P < 0.01$, IPC group *vs*. PLT group

Figure 1 Serum alanine aminotransferase (ALT) in the sham-surgery group, partial liver transplantation (PLT) group and ischemic preconditioning (IPC) group after reperfusion



A: PCNA-positive liver cells increased in partial liver transplantation group

B: Enhanced expression PCNA-positive liver cells in ischemic preconditioning group

Figure 2 Proliferating cell nuclear antigen (PCNA)-positive cells in livers harvested 24 h after reperfusion (Immunohistochemistry staining, SP method, ×400)

Table 1 Comparison of the number of proliferating cell nuclear antigen (PCNA)- positive cells at each time point after reperfusion between partial liver transplantation (PLT) group and ischemic preconditioning (IPC) group ($\bar{x}\pm s$, $n$=10)

| Group | Number of PCNA-positive cells at each time point | | |
|---|---|---|---|
| | 2 h | 6 h | 24 h |
| PLT | 78.65±21.79 | 97.59±30.06 | 127.03±35.6 |
| IPC | 127.50±32.44[a] | 151.75±37.89[a] | 193.84±49.60[a] |

[a]$P < 0.05$, IPC group *vs*. PLT group

### Activation of Ref-1 during reperfusion after operation

Compared with sham-surgery group, the activation of Ref-1 was increased at 0.5 hours after reperfusion and decreased by 24 hours in grafts. The expression of Ref-1 was slight in hepatic tissue of sham-surgery group. However, the activation of Ref-1 protein promoted obviously at 0.5 and 2 hours after reperfusion in IPC group (Figure 3). The expression of Ref-1 protein in IPC group was increased compared with PLT group at 24 hours after reperfusion by immunohistochemical analysis (Figure 4).

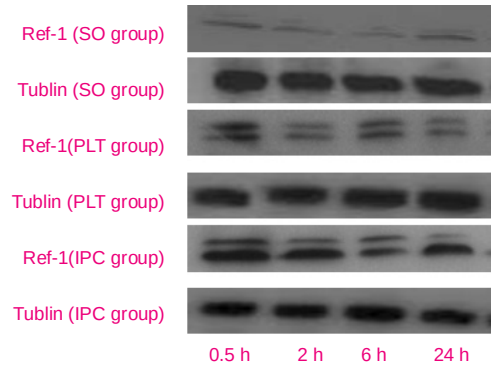
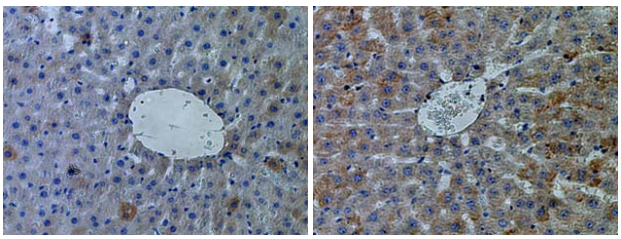
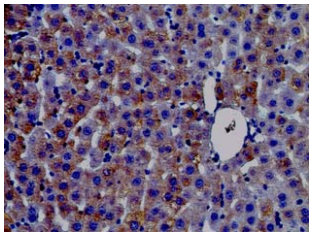



Figure 3 Expression of Redox factor-1 (Ref-1) protein during first 24 h after reperfusion by Western blot: compared to the sham-surgery group, the expression of Ref-1 in partial liver transplantation group increased at each time point after reperfusion. In ischemic preconditioning group, the Ref-1 expression was stimulated at 0.5 and 2 h after reperfusion



A: A few Ref-1 protein positive expression in liver cytoplasm of sham-surgery group, buffy staining

B: Ref-1 protein expression increased in partial liver transplantation group, buffy staining



C: Enhanced expression of Ref-1 in ischemic preconditioning group, buffy staining

Figure 4 Expression of Redox factor-1 (Ref-1) protein in liver tissue harvested 2 h after reperfusion (immunohistochemistry staining, SP method, ×400)

### Histopathological analysis

There was a large amount of inflammation cells infiltration around the portal veins, the serious sinus hepaticus dilation and damage of hepatic tissue in PLT group. However, the damage of hepatic tissue in IPC group was comparatively slight. The hemorrhage and necrosis in grafts were not observed in all groups.

## DISCUSSION

During liver transplantation, there is an unavoidable ischemic stage, which results in grafts injury after reperfusion. Living-related liver transplantation is developed to alleviate the mortality resulting from the scarcity of suitable cadaveric grafts[12-13]. The main problem in using living-related liver

transplantation for adults is graft size disparity[14-15]. In addition, I/R, which is inevitable in liver transplantation, reduces liver regeneration after hepatectomy[15].

As one strategy against I/R injury, recent studies have paid attention to IPC[16-18], a phenomenon in which brief periods of ischemia followed by reperfusion render tissues resistant to subsequent prolonged ischemia. IPC was originally characterized in dog hearts[19] and has since been recognized in a wide spectrum of animals and humans[20-21]. Studies have shown that IPC protected livers against cold I/R injury and improved recipient survival after liver transplantation[22-23].

The main mechanism of IPC protecting against I/R injury is to release active oxygen in reperfusion phase[24-25], and the burst of active oxygen is the triggering factor of Ref-1 protein expression[26], which imply IPC could activate the expression of Ref-1 protein by releasing active oxygen. At present, the studies with respect to the role of Ref-1 in IPC procedure are few. In the present study, we used Lewis rats to attempt to minimize the grafts injury from reject reaction. Model of 50% partial rat liver transplantation was established according to reduced liver grafts in clinical adult living donor liver transplantation.

The injury in liver transplantation is complicated. Oxidative stress is recognized as a critical cause of injury in reperfused tissue and remains a major concern in liver transplantation. Increasing studies suggest that intracellular as well as extracellular oxidative stress may play an important role in inducing postischemic tissue injury. At a very early phase of reperfusion, production of reactive oxygen species associated with Rac1 has recently been reported to mediate intracellular oxidative stress[8], which consequently activates redox-dependent pathologic signals including NF-κB. However, Ref-1 can defend the injury results from oxidative stress in cell[8,27] and protect hepatic I/R injury.

Ref-1, a 37-kDa protein, is initially described as a key endonuclease involved in base excision repair pathways of a variety of DNA lesions. In addition to this function, Ref-1 is known to act as a redox-dependent regulator of various transcription factors, such as AP-1 and NF-κB, thereby affecting transcriptional regulation of their target genes. Ref-1 protein contains two distinct domains. The N-terminal domain contains the nuclear localization sequence which is essential for redox activity while the endonuclease activity resides in the C-terminal region[26]. It is reported that adenoviral overexpression of Ref-1 in hepatic tissue results in significant suppression of reperfusion-induced oxidative stress, NF-κB activation, apoptosis, and acute hepatic injury[9]. Recently, it has been reported that constitutive activation of Stat3 protects against Fas-induced liver injury partly by up-regulation of Ref-1 in a redox-dependent manner[28-29].

In the present study, the expression of Ref-1 protein was increased in both IPC group and PLT group compared with the sham-surgery group. Significantly, the expression of Ref-1 protein in IPC group was higher than the PLT group in early period after reduced-size liver transplantation ($P < 0.05$), suggesting that the IPC promotes the expression of Ref-1 protein which relieves grafts injury.

Ape1/Ref-1 is located in the cytoplasm of highly metabolically active or proliferative cells, such as spermatocytes, hippocampal cells, and hepatocytes. Since highly metabolically active cells experience an increase in oxidative stress, the presence of Ape1/Ref-1 in the cytoplasm may reflect an involvement in cellular responses to oxygen tension. Usually, the factor that spurs Ref-1 expression will facilitate its intracellular activity. Stimulating factor changes the subcellular localization of Ref-1[30], almost shift from cytoplasm to nucleus. Results from the present study reveal that hepatic nucleus expression of Ref-1 in IPC group was less at 6 hours and increased at 24 hours after reperfusion, which may relate with the endonuclease function of Ref-1. However, the regulatory mechanism of Ref-1 distribution in cells remains unclear. Results from the present study provide the evidence that Ref-1 participates in the protecting mechanism of IPC for graft I/R injury and regeneration after partial liver transplantation.

Compared with PLT group, the serum ALT values at 6 and 24 hours after reperfusion were degraded obviously in IPC group ($P < 0.05$), and the ALT value at 5 days dropped to preoperative level, but in the PLT group its level remained high. These results reveal IPC relieve the grafts injury in early period after reduced-for-size liver transplantation. In order to investigate the effect of IPC on liver regeneration after transplantation, PCNA staining of hepatic tissue was performed. The expression of PCNA in hepatic tissue in IPC group was more obvious than that in PLT group ($P < 0.05$), which demonstrates IPC promotes the expression of PCNA and improves liver regeneration.

In summary, results from the present study show that IPC relieved grafts I/R injury and improved liver regeneration in early period after reduced-for-size liver transplantation of rats. Moreover, IPC promoted Ref-1 protein activity and protected grafts injury by relieving cellular oxidative stress. The precise mechanism by which IPC promotes Ref-1 expression and the principal role of Ref-1 defending postischemic injury or/and promoting regeneration require further investigation.

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# 缺血预处理减轻大鼠减体积肝移植损伤并促进肝再生*

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**摘要**

**背景：**近年来，肝移植技术迅速发展，如何预防缺血再灌注损伤并有效保护肝再生成为研究的热点。缺血预处理是保护肝缺血损伤的有效方法，但其确切机制尚存争议。

**目的：**研究缺血预处理在大鼠减体积肝移植肝损伤和肝再生中的作用及机制。

**方法：**动物随机分为 3 组，肝移植组建立大鼠减体积肝移植模型。缺血预处理+肝移植组在供肝灌注前阻断第 1 肝门行缺血预处理 10 min，再灌注 15 min。假手术组在开腹后游离肝周韧带，然后关腹。分别于术后 0.5，2，6，24 h 取材。通过血清谷丙转氨酶水平和移植肝组织病理检查评估肝损伤。半定量免疫组织化学和 western blot 法测定氧化还原蛋白 1 表达水平，检测移植肝细胞增殖细胞核抗原评估肝再生情况。

**结果与结论：**与肝移植组相比，缺血预处理+肝移植组术后 6，24 h 受体血清谷丙转氨酶明显降低($P < 0.05$；$P < 0.01$)。病理学分析显示肝移植组术后 24 h 可见到门脉周围大量炎细胞浸润，肝窦扩张明显，肝组织损伤较重；而缺血预处理+肝移植组则损伤较轻。半定量免疫组织化学显示缺血预处理+肝移植组移植肝中 Ref-1 蛋白表达明显增加，这一结果同样在 westernblot 检测中得到验证；缺血预处理+肝移植组移植肝术后 24 h Ref-1 蛋白表达较肝移植组明显增强($P < 0.05$)。同时，术后 2，6 和 24 h 缺血预处理+肝移植组增殖细胞核抗原阳性细胞数较肝移植组明显增加($P < 0.05$)。结果提示缺血预处理可减轻大鼠减体积肝移植术后早期移植物肝损伤并促进肝再生，这与 Ref-1 蛋白高表达密切相关。

**关键词：**缺血预处理；肝再生；损伤；肝移植；大鼠

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**来自本文课题的更多信息—**

*课题创新点*：缺血再灌注损伤的研究报道较多，大部分侧重于细胞因子、转录因子和结构形态方面，肝移植损伤中活性氧作用的报道不多。大鼠减体积肝移植缺血预处理中 Ref-1 的作用报道更少。该研究结果显示，Ref-1 参与大鼠减体积肝移植中缺血预处理保护再灌注损伤并促进肝再生机制，同时，缺血预处理促进 Ref-1 蛋白表达从而减轻细胞氧化应激反应。

*课题评估的"金标准"*：文章采用了肝移植后肝脏损伤常用观察指标"血清转氨酶（ALT、AST）"，也应用了用的较多肝再生的观察指标 PCNA，细胞增殖核抗原指标。

*设计或课题的偏倚与不足*：文章以实验动物模型为基础，存在一定的影响因素，对于缺血预处理的具体分子机制应进一步证实。

*提供临床借鉴的价值*：本组结果提示缺血预处理可减轻大鼠减体积肝移植术后早期移植物肝损伤并促进肝再生，这与 Ref-1 蛋白高表达密切相关。文章对临床肝移植术后药物的应用，缺血再灌注损伤的预防及缺血预处理机制有指导意义，有助于减少肝移植后并发症，提高移植成活率。