P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BASIC RESEARCH •

Protective effect of nitric oxide induced by ischemic preconditioning on reperfusion injury of rat liver graft

Jian-Ping Gong, Bing Tu, Wei Wang, Yong Peng, Shou-Bai Li, Lu-Nan Yan

Jian-Ping Gong, Wei Wang, Lu-Nan Yan, Department of General Surgery, Huaxi Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China
Jian-Ping Gong, Bing Tu, Yong Peng, Shou-Bai Li, Department of General Surgery, the Second Affiliated Hospital of Chongqing Medical University, Chongqing 400010, China
Supported by the National Natural Science Foundation of China, No. 30200278, and China Postdoctoral Science Foundation, No. 2001-5
Correspondence to: Dr. Jian-Ping Gong, Department of General Surgery, the Second Affiliated Hospital of Chongqing Medical University, 74 Linjiang Road, Chongqing 400010, China. gongjianping11@hotmail.com
Telephone: +86-23-63766701 **Fax:** +86-23-63829191
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Abstract

AIM: Ischemic preconditioning (IP) is a brief ischemic episode, which confers a state of protection against the subsequent long-term ischemia-reperfusion injuries. However, little is known regarding the use of IP before the sustained cold storage and liver transplantation. The present study was designed to evaluate the protective effect of IP on the long-term preservation of liver graft and the prolonged anhepatic-phase injury.

METHODS: Male Sprague-Dawley rats were used as donors and recipients of orthotopic liver transplantation. All livers underwent 10 min of ischemia followed by 10 min of reperfusion before harvest. Rat liver transplantation was performed with the portal vein clamped for 25 min. Tolerance of transplanted liver to the reperfusion injury and liver damage were investigated. The changes in adenosine concentration in hepatic tissue and those of nitric oxide (NO) and tumor necrosis factor (TNF) in serum were also assessed.

RESULTS: Recipients with IP significantly improved their one-week survival rate and liver function, they had increased levels of circulating NO and hepatic adenosine, and a reduced level of serum TNF, as compared to controls. Histological changes indicating hepatic injuries appeared improved in the IP group compared with those in control group. The protective effect of IP was also obtained by administration of adenosine, while blockage of the NO pathway using Nω-nitro-L-arginine methyl ester abolished the protective effect of IP.

CONCLUSION: IP appears to have a protective effect on the long-term preservation of liver graft and the prolonged anhepatic-phase injuries. NO may be involved in this process.

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INTRODUCTION

Liver transplantation is an accepted therapy for patients with end-stage liver diseases[1]. Hepatic ischemia-reperfusion (I/R) injury associated with liver transplantation is an unresolved problem in the clinical practice. Primary non-function of liver graft remains one of the most severe complications of liver transplantation, and poor initial graft function also occurs in one third of cases after liver transplantation. To deal with these complications, no effective treatment can be used but retransplantation[2,3]. Evidently, approaches need to be established to handle or even to avoid these complications.

The underlying mechanisms of cold I/R injuries are still poorly understood. Long-term ischemia of liver graft leads to impairment of liver function and reduction in survival rate of the recipients[4]. On the other hand, anhepatic phase is the most important parameter for orthotopic rat liver transplantation (ORLT), because prolonged anhepatic phase can be associated with an endotoxin-like syndrome caused by the warm intestinal ischemia and is not related to the cold ischemia injuries of the liver[5]. The hepatic reticulo-endothelial system plays a key role in the elimination of endotoxins. The detoxification system is usually deficient in recipients due to liver failure and is completely absent during the anhepatic phase of transplantation. The endotoxin content has been proven to increase markedly in the portal circulation of cirrhotic patients compared to that in the peripheral blood[6]. The hypothermia may affect the ability of Kupffer cells to eliminate endotoxins and to release cytokines, such as tumor-necrosis factor (TNF). It has been shown that TNF is partly responsible for initiation of the lethal toxicity of endotoxins[7].

Ischemic preconditioning (IP) is a process by which a brief ischemic episode confers a state of protection against the subsequent more sustained ischemic insult[8]. Recent studies have demonstrated that a brief ischemia treatment, followed by an episode of reperfusion, can reduce the sustained I/R injury[9,10]. These observations suggest a potential application of IP in liver transplantation. In this study, the protective effect of IP on the long-term preservation of liver grafts and the prolonged anhepatic phase was observed.

MATERIALS AND METHODS

Materials

Male *Sprague-Dawley* rats weighing 200 to 230 g supplied by the Center of Experimental Animal in Sichuan University, were used as donors and recipients. Animals were bred in a controlled environment with a 12 hour light/dark cycle. Donor rats were fasted for 12 hours with free access to water before surgery, while the recipients had free access to normal rat chow and water before surgery. This study was approved by Sichuan Bioethics Committee, and the procedures were carried out according to the routine animal-care guidelines.

ORLT

Liver transplantation was performed according to Kamada' s cuff-technique[11] under anesthesia with ether inhalation. Briefly, the abdomen was opened through a midline incision and the liver was freed from its ligaments with minimal manipulation. The donor bile duct was transected, and a 0.4 cm

length of tube was insert into the lumen of the bile duct and secured with a circumferential 5-0 silk suture. The animal was injected intravenously with 50 U of heparin. The liver was perfused through portal vein with an intravenous cannula connected with a syringe with 36 U of heparin in 6 ml of cold saline. The donor liver was placed in the saline bath at 4 ℃ for 100 min. The cuff preparation of portal vein and infrahepatic vena cava was performed also in the saline-ice bath. After the recipient liver was removed, the donor liver was implanted in the orthotopic position by connecting the suprahepatic vena cava with a running suture, inserting cuffs into the infrahepatic vena cava and the portal vein, and splint tube into the bile duct. The anhepatic phase was estimated to be 25 min for all recipients. ORLT was performed without artery reconstruction.

Experimental design

A total number of 128 rats were randomly divided into 4 groups, 32 for each. 1) Control group, the donor livers were flushed through the portal veins with physiological saline containing heparin only before harvested. 2) IP group, before the donor livers were harvested, the portal vein and hepatic artery were interrupted for 10 min, and the blood flow was restored for 10 min, then the liver was treated as control group. 3) Adenosine group, the donor livers were flushed through the portal vein with physiological saline containing heparin and adenosine (10 mmol/L) only before harvested. 4) Nω-nitro-L-arginine methyl ester (NAME) group, the donor livers were treated as IP group, but NAME (10 mmol/L), an NO synthesis inhibitor, was included in flushing solution. For each group, half of animals were used to investigate the one-week survival rate of recipients, and the remaining animals were for sample collection of blood from infrahepatic vena cava and hepatic tissue after 2 hours of reperfusion.

Determination of liver function and survival

Serum alanine transaminase (ALT) was measured using an automated analyzer (BECKMAN CX7, Beckman Instruments, Fullerton, CA). Survival of recipients was observed for 7 days after operation.

Determination of serum NO

Values of circulating nitrate and nitrite were determined to reflect serum NO level. Serum was separated by centrifugation and stored at -70 ℃ before use. Nitrite was measured after enzymatic conversion by nitrate reductase using the Griess reaction, as described by Schmidt[12]. Values obtained represented the sum of serum nitrite and nitrate.

Determination of serum TNF

Serum was separated by centrifugation, and concentration of serum TNF was measured by radioimmunoassay. The TNF standard (100 μl, Sigma, ST. Louis, MO. USA) and the serum samples (100 μl) were added separately to appropriate tubes. Two hundred μl of the 0 ng/ml TNF standard was added to each non-specific binding tube. All tubes were added 100 μl of ^{125}I-TNF reagent (Sigma, ST. Louis, MO. USA). TNF antiserum (100 μl) was also added to each tube, except the nonspecific binding tubes. Incubation was done at 4 ℃ for 24 hours following gentle agitation for 2-3 seconds. With 500 μl of precipitating reagent added, the tubes were vortexed immediately, and incubated for 20 minutes at room temperature (-25 ℃). All the tubes were centrifuged at 1500×g for 25 min, the suspension was dropped out. Radioactivity was read using a gamma counter (262 Factory, Xi' an, Shaanxi, China).

Assay for adenosine from hepatic tissue

Adenosine standard was purchased from Sigma (St. Louis, MO, USA). High-performance liquid chromatography (HPLC) was performed using a Beckman Gold Nouveau system equipped with a 168 photo-diode-array detector (210 nm; 262 Factory, Xi' an, Shaanxi, China). Satisfactory separation of the marker substances was obtained with a reversed-phase column and eluted at a flow rate of 1 ml/min. Adenosine separation was allowed to precede in a phosphate-buffer solution (331 mmol/L KH_2PO_4, pH 6.24) containing 3.5% CH_3CN and 2.3 mmol/L *t*-butylamine (TBA). Lyophilized liver tissue was homogenized in 0.5 ml of 0.42 mol/L perchloric acid and incubated for 20 min at 4 ℃. The supernatant was separated by centrifugation at 3 000 rpm for 10 min at 0.5 ℃, and neutralized with 85 μl/200 μl NaOH. After 5 min of centrifugation at 3 000 rpm, 20 μl of the supernatant was subjected to HPLC.

Histopathologic examination

Liver samples were fixed in 10% neutral buffered formalin, embedded in paraffin. Sections of 5 μm in thickness were prepared, stained with hematoxylin and eosin, and observed under a light microscope.

Statistics

All statistical computations were performed using SPSS software (version 10.0 for Windows 98; SPSS, Inc., Chicago, Illinois). The *P* values less than 0.05 were considered statistically significant.

RESULTS

Animal survival

The survival rates of these four groups are shown in Figure 1. Most of the recipients died within 3 days after liver transplantation. The survival rate at day 7 was higher in IP group (87.5%, 7 of 8) and adenosine group (87.5%, 7 of 8) than that in control group (37.5%, 3 of 8) and NAME group (25%, 2 of 8, $P<0.05$, Figure1).

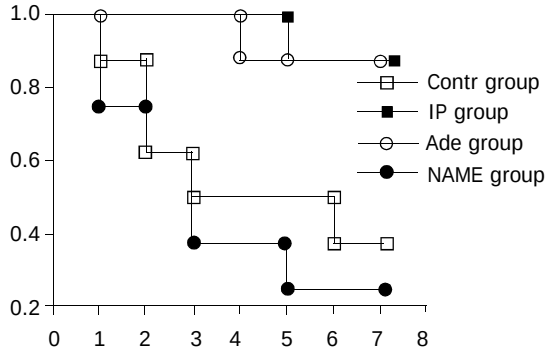

Figure 1 Survival of recipients in different groups. The donor livers were treated respectively as described in Materials and Methods, and the recipients were divided into control (Contr group), IP-(IP group), adenosine-(Ade group), and NAME-treated groups. The 7-day survival rate was 37.5% (3/8) and 25% (2/8), respectively, in the control group and NAME group, compared with 87.5% (7/8) in both the IP group and adenosine group ($P<0.05$). The survival curves were calculated using Kaplan-meier's methods.

Liver function

The serum ALT values in control group (588±58 U/L) were significantly higher as compared to those in IP group (287±82 U/L) ($P<0.001$). Meanwhile, administration of adenosine also reduced the level of serum ALT (357±93 U/L) as compared to that in control group ($P<0.001$). However, with administration of NAME, the response in ALT level to IP was abrogated (634±65 U/L, $P>0.05$, Figure 2).

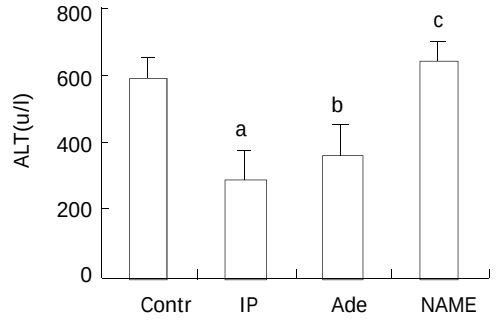

Figure 2 Values of serum ALT in different groups of recipients at 2 hours after ORLT. The ALT levels in control group and NAME group increased significantly compared with IP group and adenosine group. [a]*P* and [b]*P*<0.001, [c]*P*>0.05 *vs*. control group. The values were expressed as means ± SD.

Serum TNF and NO level

TNF concentration in serum was measured 2 hours after ORLT as described previously[5,6]. In IP group (1.15±0.23 ng/ml) and adenosine group (1.14±0.27 ng/ml), it was significantly lower compared to that in control group (1.59±0.35 ng/ml, *P*<0.01). The level in NAME group (1.71±0.23 ng/ml) was as high as that in control group (*P*>0.05, Figure 3).

Concentrations of NO were shown to be 32.96±6.10 μmol/L, 29.14±6.49 μmol/L in IP and adenosine groups, respectively, which were significantly higher than that in control group (15.44±2.99 μmol/L, *P*<0.001). The value of the recipients in NAME group was 13.74±3.11 μmol/L, which was similar to that in control group (*P*>0.05, Figure 4).

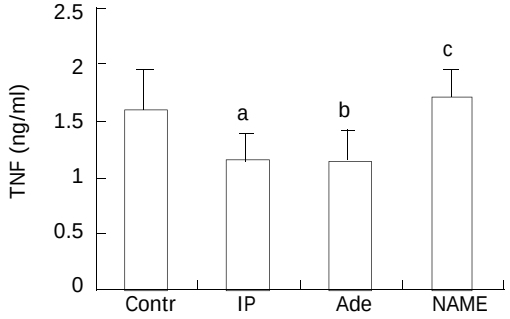

Figure 3 Serum TNF levels measured at 2 hours after ORLT in different recipient groups. In control and NAME groups, the concentrations of serum TNF were elevated significantly as compared to those in IP and adenosine groups. [a]*P* and [b]*P*<0.01, [c]*P*>0.05 *vs*. control group. Values were expressed as means ± SD.

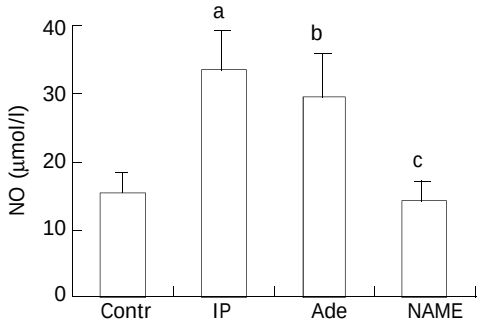

Figure 4 NO levels in serum at 2 hours after ORLT. When the donor livers were pretreated with IP or adenosine, the serum NO levels were elevated significantly. When IP-treatment followed by administration with NAME, the serum NO concentration was as low as that in control group. [a]*P* and [b]*P*<0.001, [c]*P*>0.05 *vs*. control group. Values were expressed as means ± SD.

Adenosine in hepatic tissue

Figure 5 shows the levels of hepatic adenosine at 2 hours after ORLT in liver grafts. Concentrations of tissue adenosine were 7.22±1.83 mol/g, 5.68±1.32 mol/g, and 5.56±1.19 mol/g in liver grafts pretreated with IP, adenosine and IP+NAME, respectively, which were higher than that in the reference liver grafts (3.69±0.54 μmol per gram of dry liver tissue, *P*<0.05). Animal survival and liver function were improved after adenosine administration. The protective effect of IP was abrogated in NAME group, though the content of adenosine in the tissue also increased.

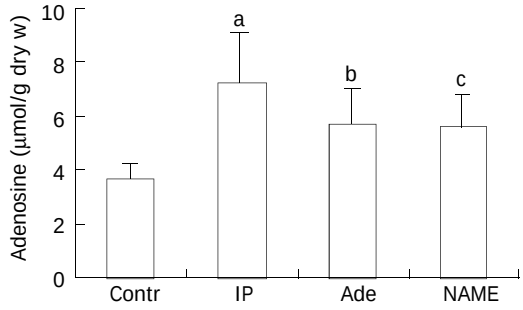

Figure 5 Adenosine concentrations in liver tissues at 2 hours after ORLT. When the donor livers were pretreated with IP or adenosine, or even the IP-treatment followed NAME, the adenosine levels in dry hepatic tissue were elevated as compared to that in control group. [a]*P*, [b]*P* and [c]*P*<0.05 *vs*. control group. Results were expressed as means ± SD.

Histopathologic examination

Hepatocyte swelling and ballooning were observed in tissues from the control and NAME groups. No identifiable morphological alterations or only minor changes were found in IP and adenosine groups (data not shown).

DISCUSSION

The ORLT model, established in 1973 by Lee *et al*.[13], has received wide acceptance in the study of liver transplantation. IP is a procedure originally described during heart transplantation. A brief ischemia treatment followed by reperfusion has been shown to be able to decrease infarct size after subsequent prolonged I/R[8,14,15]. IP has been found to be protective during transplantation of several organs including brain, intestine and skeletal muscle[16-18]. In liver, IP was found to reduce tissue damages and mortality after warm ischemia and cold ischemic storage[5, 19].

In the present study, The harvested livers were preserved in saline for 100 min at 4 ℃ and the anhepatic phase was selected for 25 minutes, poor outcomes of recipients in control group were observed, and the survival rate 7 days after operation was 37.5% (3/8). In IP group, ALT level was reduced markedly and the survival rate was significantly elevated (87.5%, 7 of 8). The protective effect of IP was further indicated by the morphologic parameters presented.

The effects of a long anhepatic phase on the graft were associated with an endotoxin-like syndrome induced by the prolonged congestion of internal organs. Endotoxemia has been shown to be one of the processes causative for early graft dysfunction[20,21]. This was most probably associated with Kupffer cell activation by splanchnic endotoxin accumulation and release during intestinal congestion/reperfusion[22,23]. During the ORLT with a long anhepatic phase, Kupffer cells were shown to be directly responsible for overproduction of TNF, causing endotoxicosis-like syndrome[24]. The cytotoxic effects of TNF are associated with activation of phospholipase

A_2, release of ceramide, formation of reactive oxygen intermediate (ROI) and promotion of cell apoptosis. However, it is not known whether the protective effect of IP in this study was associated with TNF.

The definite mechanism underlying IP is not clear, and there are several explanations about its protective effects. According to some authors, the protective effects were not attributed to blood flow alterations[25,26]. However, the IP protective effect was associated to certain substances produced by ischemic tissue against injury[4]. On the contrary, another study indicated that IP could improve blood flow, and decrease hepatic vascular resistance of liver grafts preserved in cold storage[10], and this was probably related to some potential mediators such as NO and adenosine.

NO was reported to exert a protective effect through inhibiting endothelin synthesis[27], and the upregulating effect of adenosine NO release in endothelial cells was also documented[28]. During ischemia, adenosine is rapidly formed from adenosine triphosphate and reaches high concentrations. Enhanced adenosine level might in turn induce NO synthesis through the activation of adenosine A_2 receptors[27]. The functions of the two mediators might lead to an improvement in graft blood flow and accordingly improve liver function. Since the ROIs are known to cause cell injury by promoting the peroxidation of lipids and proteins in cell membranes, it is possible that the antioxidant action of NO might also be involved in the protective effect of IP.

In the present study, the concentrations of NO in serum and adenosine in hepatic tissues of IP group were significantly higher than those in control group. In contrast, the level of serum TNF was greatly reduced after IP treatment. Furthermore, the protective effect of IP was also achieved by administration of adenosine before the donor livers were harvested. Our results showed NAME had negative effects on the protective effect of IP and suppressed NO synthesis, indicating that, in the absence of NO, adenosine was unable to develop protective effects. IP might exert its protective role by inducing the production of NO.

In summary, the data presented suggest that an elevated adenosine level can induce the generation of NO under IP, conferring protection to liver grafts and recipients. IP may be an important approach to reduce the transplantation risk resulted from preservation/reperfusion injuries. Further studies are needed for further understanding of the underlying mechanisms.

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