Article

Expression of Redox Factor-1 in Early Injury Period after Liver Transplantation in Rat Model

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The aims of this study were to observe the relationship between injury of graft and expression of redox factor-1 (Ref-1) in early period (24 h) after liver transplantation in rat model. One hundred and fifty adult male Wister rats were randomly divided into three groups including liver transplant group, sham surgery group and untreated control group. After liver transplantation, animals were sacrificed at different time points, and the changes and significance of the expression of Ref-1 were then explored by immunohistochemistry, serology and histopathology. As compared with sham surgery group and untreated control group, the expression of Ref-1 protein in transplant group was stronger in early period after liver transplantation. With pathology analysis, lots of infiltrating inflammation cells were found around the portal veins. Hepatic tissues were injury. However, the injury in sham surgery and untreated control group were comparatively slight. The serum ALT and AST levels reached the peak at 6-12 h, and decreased significantly after 12 h. These data suggested that the degree of liver injury in earlier period after transplantation peaked at 6 h and then decreased. And Ref-1 protein induced by hepatic ischemic reperfusion injury might play a critical role in repairing the injury. *Cellular & Molecular Immunology*. 2009;6(4): 309-313.

Key Words: liver transplantation, Ref-1, hepatic lesion

Introduction

The major factors affecting early period survival after liver transplatation focus on the function of liver, kidney, intestine and lung. After transplantation of liver, the probability of many complications increase including complicating liver failure, acute renal failure and intestine lesion. These complications lead to endotoxemia and ARDS which increase the rate of postoperative death. But the factors causing organs dysfunction is a great deal. Recently, a research indicates that oxidation stress plays a major role in tissue ischemic damage inside or outside the cells (1, 2). Redox factor-1 (Ref-1), also called HAP1, APE or APEX, is paid attention by scholars on cell accommodation redox (3). Oxidation stress inside the cell in early period after reperfusion can activate oxidoreduction dependent signals such as NF-κB and Ref-1 which can keep cells from injury of oxidoreduction (4). Our research is to investigate the mechanism of hepatic failure after graft which leads to the endotoxemia and ARDS through ischemic reperfusion injury. At the same time we investigate the rule of Ref-1 variance and approach the relationship between Ref-1 and organ dysfunction.

Materials and Methods

Animals

One hundred and fifty 6-8 weeks male Wistar rats weighting 200-250 g were bought from Laboratory Animal Centre of Jilin University. All experiments were performed according to Animal Experimental Ethics Committee guidelines.

Experimental design and surgical procedure

All the animals were randomly divided into three groups: transplant group, fake surgery group and untreated control group. Hepatic transplantation model in transplant group was performed as described previously (5). Fake surgery model was performed as follows: firstly we disconnected the connective between the upper part of the liver and the diaphragm, then mutilated and ligated the hepatic proper artery. We ligated and sheared the right suprarenal vein. The vessels between the left liver and esophagus were also ligated.

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Then a hepatic ischemic reperfusion model was set up in rat. In untreated control group there were no surgery procedures. Animals were sacrificed and detected at different time points after liver transplantation.

Assay of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels

Serum levels of ALT and AST at each time point after surgery were measured using an automated chemical analyzer to assess the extent of hepatocellular damage.

Histopathology

Hepatic tissues were harvested and formalin-fixed at each time point after surgery. Paraffin-embedded rat liver specimens were sectioned and stained with hematoxylin and eosin. Sections were observed under light microscope.

Immunohistochemistry

Liver samples were fixed by 10% formalin and sliced at 3 μm to deparaffinage and hydrate. Then the samples were incubated at room temperature for 30 min. After blocking with serum, the sections were dropwised by 50 μl 1:100 diluted first antibody to Ref-1 (Jimpy mice anti rat Ref-1 monoclonal antibody, Santa cruz Biotechnology, USA), and stayed over night at 4°C. After washing, they were incubated with 50 μl biotinylated secondary antibody and then HRP linked streptavidin (rabbit anti Jimpy mice, Maixin Bioengineering Company, Fuzhou, China) at room temperature for 20 min. Samples were colorated with 3, 3-diaminobenzidine (DAB substate kit, Boster, Wuhan) and counterstained with hematoxylin.

Apoptosis cells assay

Paraffin sections of samples were deparaffinaged and dehydrated, and then incubated in 20 μg/ml protease K at room temperature for 3 min. After washing twice, samples were put in natrium citricum buffer (pH = 6.0) at 37°C for 5 min. After washed with PBS for 5 min twice, 50 μl TUNEL (TdT-mediated dUTP nick end labeling) reaction mixture were added to the samples, and incubated at 37°C for 60 min. After rinsing, the sections were incubated with Converter-POD for 30 min at 37°C and developed with DAB Substate Kit (Boster, Wuhan). The slides were lightly counterstained with hematoxylin and then dehydrated and mounted.

Western blotting analysis

Hepatic tissues were homogenated, and total proteins were extracted by tissue protein extraction kit (Pierce Biotechnology, USA). Then the samples were quantitated with BCA kit. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were processed. Briefly, the whole cell extracts (30 μg/lane) were separated by SDS-PAGE and transferred to nitrocellulose membrane. After blocked in Tris-buffered saline with 5% (w/v) nonfat dry milk, membranes were incubated with primary antibodies according to the manufacturer's instructions and then incubated with horseradish peroxidase conjugated secondary antibody. The proteins were detected by the enhanced chemiluminescence (ECL) system (Pierce, Rockford, IL) using X-ray film.

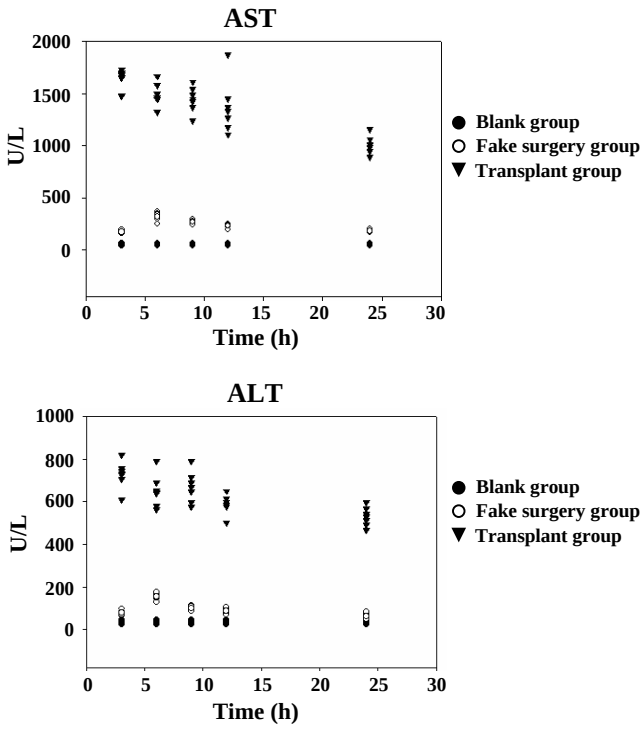

Figure 1. Serum AST and ALT levels in the transplant group, fake surgery group and untreated control group at different times after graft. Serum levels of ALT and AST at each time point after surgery were measured using an automated chemical analyzer.

Statistical analysis

All the data were analyzed by SPSS11.5. Group comparison was tested by variance analysis.

Results

The levels of AST and ALT significantly increased after liver transplantation

Serum levels of AST and ALT were measured to demonstrate the degree of the hepatic lesion. The values of AST and ALT in transplant group were markedly higher than that of fake surgery group and untreated control group at each time point ($p < 0.05$). As shown in Figure 1, we deduced that the peak of hepatic injury in transplant group was at 3 h after graft while fake surgery group peaked at 6 h after ischemic reperfusion. The outcome indicated that the hepatic injury of graft was caused not only by ischemic reperfusion but also by other mechanisms.

Hepatic histopathology changes in the transplant group

After transplantation, part of the hepatic tissues structure was not very clear. Some hepatic cells began to degenerate. Hepatic sinus dilated and had local congestion. Leucocytes were also observed to infiltrate into hepatic tissues obviously

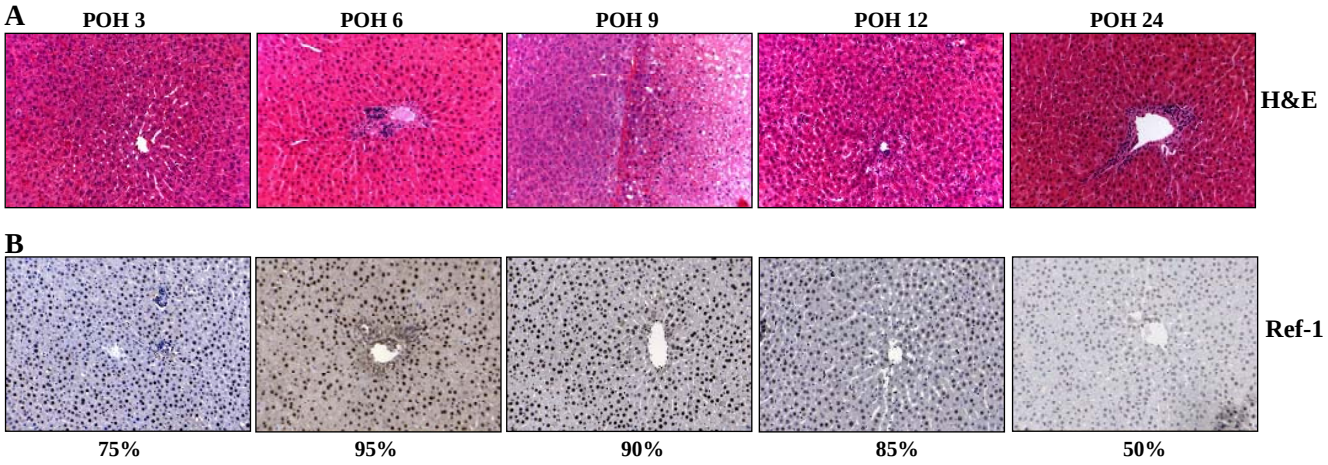

Figure 2. Histopathological and immunohistochemical analysis of livers at different time pionts in transplant group. (A) Liver samples in transplant group were stained by hematoxylin and eosin at 3-24 h after transplantation. (B) Immunohistochemical staining showed the expression of Ref-1 in hepatocytes at 3-24 h after transplantation. The percentage indicated the positive rate of Ref-1 in the hepatocytes (original magnification ×200).

(Figure 2 A). However, in fake surgery group the outlines of hepatic lobules were clear. Hepatic cells lined up in order except some degenerated lightly. A small part of leucocytes infiltrated into the hepatic tissues. But no changes were found in untreated control group.

Enhanced expression of Ref-1 in hepatocytes after liver transplantation

Positive granules of Ref-1 proteins were expressed in the nucleus of hepatic cells at 3 h after transplantation and peaked at 6 h after surgery with its positive rate at 95%. Then it fell down and reached its lowest point at 24 h after surgery with positive rate at 50% (Figure 2B). In the contrast, there were much less positive granules in fake surgery group while none was detected in untreated control group.

Apoptosis cells increased in liver after surgery

At each time point in transplant group, scattered buffy dyed cells (apoptosis cells) were counted. Positive granules expressed inside the nucleus of hepatic cells were also found at 3 h after transplation (data not shown). The number of positive dyed cells increased gradually at 6, 9 and 12 h, and reached its peak at 12 h after operation. At 24 h the number of apoptosis cells decreased a little. In fake surgery group, only a small quantity of positive granules could be observed, and in untreated control group, positive dyed cells were hardly detected (Figure 3).

Ref-1 protein expressed highly after transplantation

The Ref-1 protein in the transplant group expressed strongly, especially at 3 h and 6 h after transplant surgery. In the fake operation group the Ref-1 protein expressed weakly while in untreated control group Ref-1 protein was not detected.

Discussion

The main complication of liver transplant surgery is donor liver rejection and primary graft liver dysfunction. Primary graft liver dysfunction is related to ischemic reperfusion injury. Recent years, some scholars presume that apoptosis plays an important role in ischemic reperfusion injury of hepatic graft (6). The research of Dimitrios E. confirmed that

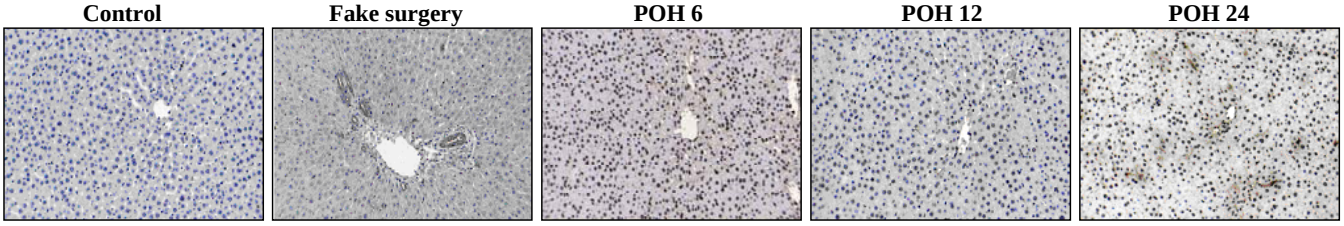

Figure 3. Apoptosis of hepatic cells increased after transplantation. The apoptosis cells in untreated control group, fake surgery group and transplant group were detected by using TUNEL method. (original magnification ×200).

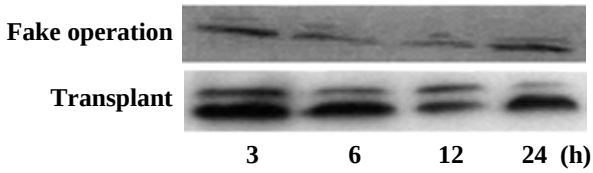

Figure 4. The expression of Ref-1 protein in the fake operation group and transplant group at different times after surgery. After operation at different time, the whole hepatic tissues protein were extracted and the expression of Ref-1 were detected by western blotting using Ref-1 antibody as described in materials and methods.

apoptosis participates in ischemic reperfusion injury (7). It produces a great quantity of oxygen free radicals in ischemic reperfusion process. The mechanism of these active oxygen inducing hepatic cells apoptosis may be related to active oxygen damaging the DNA and inducing poly-ADP ribose transferring enzyme activation and P53 acummulating and lipid peroxidation inducing Ca^{2+} level increasing (8). Active oxygen activate sensitive nuclear factor (NF) which induces apoptosis (9). The relationship between Ref-1 and apoptosis is testified in rat model (3, 10). The decrease of Ref-1 inducing DNA repair failure is the reason for cerebral apoptosis. Researches show that Ref-1 is an anti-apoptosis protein related to cell differentiation in neuron development process (11).

DNA repair mechanism is defense at the second line by organism antioxidant (The first defense mechanism is self anti-oxidant). It can repair the oxidation injury of DNA rapidly and efficiently. The expression of Ref-1 is related to ROS, Chun-ming Jiang thought active oxygen induce Ref-1 expression indirectly. It may be conducted by calcium dependent signal (12). Ref-1 can be cyclized by tyrosine kinase C at multiple Ser/Thr residue situs. Its cyclization at Thr-133 situs can be deactivation by incision enzyme (13). Debonera F. reported that hepatic cell apoptotic index is independent with cold inchemic time. But it is positive correlation to AST level after surgery (14). Some scholars indicate that Ref-1 up-regulates NF-κB binding with DNA and suppresses the apoptosis induced hypoxia (6). Otherwise some scholars think NF-κB is activated rapidly in early reperfusion period after transplant. It promotes the expression of mediators of inflammation, such as IL-1, TNF-α and ICAM-1, aggravating HIRI (Hepatic Ischemia Reperfusion Injury) (12, 15).

Generally DNA is damaged at N-glycon of basic radical by deoxyribose hydrolization under DNA glycosylase action which makes AP situs and basic radical sequence change. From the view of DNA injury repair, active oxygen may damage DNA. AP endonuclease is rate-limiting enzyme in ROS damage reparative process. So the induction of Ref-1 can be seen as double functional enzyme that can repair AP situs and basic radical sequence on DNA.

In conclusion, it is observed in this experiment that ischemical reperfusion injury is most severe at 6 h after transplantation of liver and then lessen gradually. This is because a great quantity of ROS generate after transplantation which activate the expression of Ref-1. Ref-1 repaired the apoptosis induced by ischemical reperfusion injury.

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