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Effect of matrine on cold ischemia and reperfusion injury of sinusoidal endothelial cells in rat orthotopic liver transplantation[1]

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KEY WORDS liver; transplantation; matrine; reperfusion injury; vascular endothelium; rats

ABSTRACT

AIM: To study the mechanism and prevention of matrine (Mat) on cold ischemia/reperfusion injury of sinusoidal endothelial cells (SEC) in rat orthotopic liver transplantation (OLT). **METHODS**: One hundred and twenty-six syngeneic SD rats were randomly divided into four groups (n=18): untreated group, 40 mg/kg treated group, 80 mg/kg treated group, and pseudo-treated group. After 5 h of preservation in Ringer's (LR) solution, orthotopic implantation of the donor liver was performed. At 1, 2, and 4 h after reperfusion of the portal vein, 6 rats were killed in each group to collect the serum and the median lobe of liver for assay. **RESULTS:** The level of hylluronic acid (HA) and intercellular adhesion molecule-1 (ICAM-1) decreased significantly in both treated groups at different times post-transplantation, and their pathological changes of SEC were ameliorated, too. **CONCLUSION:** Matrine can prevent SEC from ischemia and reperfusion injury in rat orthotopic liver transplantation.

INTRODUCTION

Preservation injury continues to be a major clinical problem in orthotopic liver transplantation (OLT) with a 10 % incidence of primary nonfunction[1-3]. The underlying mechanism of cold ischemia/reperfusion injury that leads to primary nonfunction is still unknown. The cold ischemia and reperfusion injury typically causes rounding and detachment of the sinusoidal endothelial cells (SEC)[4,5], and the degree of SEC injury has been shown to correlate with graft viability after transplantation in animal[6-8] and clinical[9] studies. It is currently hypothesized that SEC impairment[10] and/or liver microcirculatory disturbances[11,12], mediated by oxygen radicals[13], activation of Kupffer cells[14,15], or sinusoidal accumulation of leukocytes[16], are the primary causes of storage-related graft failure.

Matrine (Mat), an alkaloid found in kinds of *Sophora* plants in *Leguminosae*, shows pharmacological effects such as anti-inflammation[17], immuno-inhibition[18], anti-liver fibrosis[19,20], and anti-arrhythmia[21], and has been widely used in treatment of chronic liver disease[22]. Pharmacological studies revealed no obvious side-effect of Mat[23,24]. Mat treatment markedly inhibited the activation of Kupffer cells and decreased the serum level of TNF and IL-6, it suggests that matrine may have a protective effect on liver injury[17]. The present study was designed to evaluate the effects of

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Mat on SEC injury caused by preservation and reperfusion using a rat orthotopic liver transplantation model. Hylluronic acid (HA) elimination and intercellular adhesion molecule-1 (ICAM-1) expression were used to establish sinusoidal endothelial cells function.

MATERIALS AND METHODS

Reagents Mat, a parenteral solution (10 g/L), was purchased from Mingxing Pharmaceutical Factory, Guangzhou. HA RIA kit was from Shanghai Ocean Research Biomedical Technology Center, Shanghai. Mouse-anti-rat CD54 (ICAM-1) monoclonal antibody, MCA773, was purchased from Serotec Co in England.

Animals SD rats, ♂, weighing 200 g±10 g, from the Medical Center of Animal Experimental Nanjing Unit PLA (Grade II, Certification No 97001). Prior to being used in the study, rats were fasted for 12 h and were allowed free access to water.

Experimental design Rat liver transplantation was performed by the technique of Kamada and Calne [25] under ether anesthesia, and the hepatic artery was not reconstructed. Ringer's (LR) solution was used for the perfusion. The graft liver was preserved in Ringer's solution at 4 °C for 5 h, then transplanted orthotopically. The explantation of the recipient liver required <10 min and the rewarming time of the graft (ie, clamping of the portal vein and the subhepatic vena cava during implantation) did not exceed 20 min.

The animals were randomly assigned into 4 experimental groups (n=18 in each group) as follows: 1) a control group in which the recipients were injected ip normal saline (NS, 1 mL) 1.5 h before laparotomy. 2) a low-dose treated group in which the recipients were injected ip matrine (40 mg/kg) 1.5 h before surgery. 3) a high-dose treated group, in which the transplantation was performed following the injection of Matrine (80 mg/kg) as above. 4) a sham-operated control group, in which the liver was mobilized as the others without hepatectomy to exclude the influence of surgery.

Assesment of HA plasma level Six samples of blood were collected for assay via the subhepatic vena cava at 1, 2, and 4 h after reperfusion of the portal vein in each group. The serum was separated immediately and stored at -70 °C until analysis. HA plasma levels were determined in duplicate using a HA RIA kit according to the manufacturer's protocols.

Histochemical analysis of ICAM-1 At 1 h, 2 h, and 4 h after reperfusion of the portal vein, 6 rats were killed in each group to collect the median lobe of liver for assay. Embedded in OCT glue, they were stored in liquid nitrogen until analysis. Cryostat 6-μm frozen sections obtained from the cold-stored specimens were fixed in cold acetone for 10 min and the immunohistochemical staining was performed immediately according to manufacturer' protocols. In brief, primary antibodies, anti-rat-ICAM-1 monoclonal antibody, were incubated with RPMI-1640 medium including 10 % fetal bovine serum for 1 h at room temperature. Each primary antibody was diluted 1/50. Blocking for nonspecific protein binding was accomplished by applying normal goat serum. Diaminobenzidine hydrogen peroxide was used as a chromagen and the sections were counterstained with hematoxylin. Mouse IgG1 negative control was used to verify specific reactions of ICAM-1.

Light microscopy Six hepatic specimens were collected at 4 h after reperfusion of the portal vein in each group. Hepatic specimens for light microscopy were fixed with 10 % formalin and then embedded in paraffin. Sections were stained with hematoxylin and eosin for histologic examination.

Transmission electron microscopy For transmission electron microscopy, liver fragments of approximately 1 mm^3 were fixed in 2.5 % glutaraldehyde containing 0.1 mol/L phosphate buffer for 3 h. After washing in phosphate buffer, specimens were postfixed with osmium tetroxide, dehydrated in graded alcohols, and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and examined under an electron microscope (JEM-1200EX).

Statistical analysis Results are expressed as mean±SD. Data were analyzed with Statistical Analysis System (SAS). One-way analysis of variance (ANOVA) was used for multiple comparisons with Student-Newman-Keuls (snk) test. P value of <0.05 was considered statistically significant.

RESULTS

Changes in HA plasma levels Compared with the sham-operated control group, a significant elevation of serum HA was observed in the other 3 groups at different time points, with the peak of HA level at 2 h post-surgery. HA levels at different time points were ameliorated markedly by Mat treatment, and double-dose Mat treatment 80 mg/kg induced a significant elevation in HA at 1 h and 2 h *vs* Mat 40 mg/kg (Tab 1).

Changes in hepatic ICAM-1 expression In sham-operated control group, the histological findings indicated that the degree of liver cell injury and inflammatory cell infiltration in portal areas and sinusoids were mild, and there was almost no expression of ICAM-1. The obvious expression of ICAM-1 was recognized in sinusoidal endothelial cells in control group, and it could also be observed in some hepatocytes and sinusoidal space, with the strongest expression in almost all lobules at 4 h post transplantation (Fig 1A). In both treated groups, the expression of ICAM-1 was markedly decreased in the similar space as control, and no significant difference was noted between these two groups (Fig 1B).

Light microscopy In sham-operated control group, the histological findings indicated that the degree of liver cell injury, Kupffer cell hyperplasia, and inflammatory cell infiltration in portal areas and sinusoids were mild. In the control group, as shown in Fig 2A, histological examination revealed some focal necrosis of hepatocytes, extensive congestion, and inflammatory cells aggregating in hepatic sinusoid lumen, and the obvious Kupffer cell hyperplasia and rounding and detachment of SEC were observed, too. These were ameliorated markedly in both treated groups, and no significant difference was observed between the 2 treated groups (Fig 2B).

Transmission electron microscopy The hepatocytes and SEC of the sham-operated control livers showed normal appearance after harvest. Samples taken from the control group showed some typical injury to the endothelial cells, including rounding, swelling, detachment from the hepatocyte plate, loss of cytoplasmic processes, swollen mitochondria, and loss of fenestration in some areas (Fig 3A). The treated groups showed a reduction of the severity of the ultrastructural changes of SEC in the samples examined compared with the control. This was reflected by a reduction in the amount of cellular debris seen in the sinusoidal space, and the mitochondria were not swollen so severely (Fig 3B). No significant differences were noted between the two treated groups.

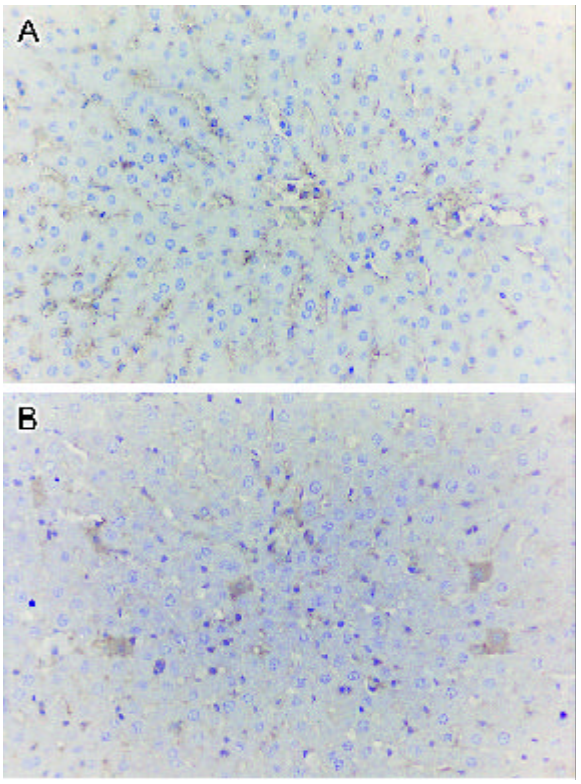

Fig 1. Immunohistochemical expression of ICAM-1 in rat liver 4 h after reperfusion. A: control group; B: a low-dose treated group. The obvious expression of ICAM-1 was recognized in sinusoidal endothelial cells in almost all lobules in A, and it could also be observed in some hepatocytes and sinusoidal space. Its expression was markedly decreased in the similar space as control in B. (Cryosections: 6 μm thick. Original magnifications: ×200).

DISCUSSION

With donor shortage becoming worse, preservation injury continues to be a major clinical problem with

Tab 1. Effect of matrine (Mat) on HA plasma level (μg/L). n=6. Mean±SD. ^{c}P<0.01 *vs* control. ^{d}P>0.05, ^{e}P<0.05, ^{f}P<0.01 *vs* Mat 40 mg/kg group.

Rats	1 h	2 h	4 h
Control	1 109±110	2 380±225	1 821±240
Mat 40 mg/kg	278±29^{c}	1 041±167^{c}	538±59^{c}
Mat 80 mg/kg	407±95^{ce}	1 419±96^{cf}	688±53^{cd}
Sham-operated	74±9	136±5	172±7

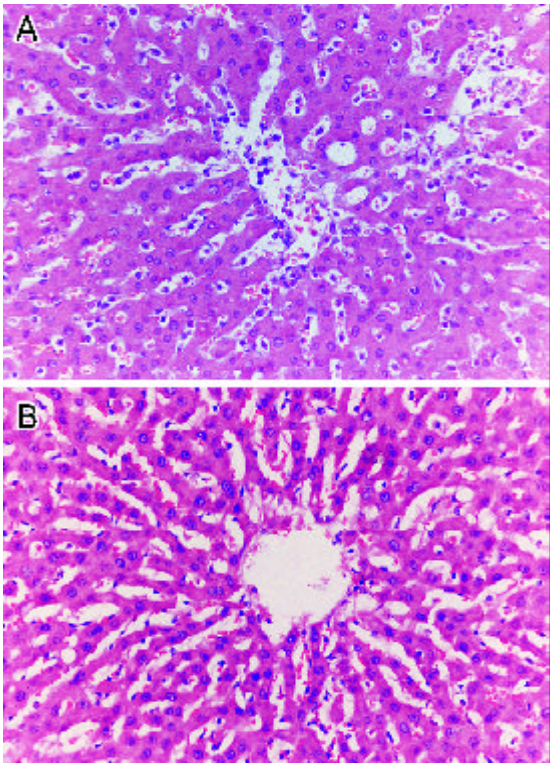

Fig 2. Histological appearance of the rat liver at 4 h after reperfusion. A: control group; B: Mat 40 mg/kg treated group. Some focal necrosis of hepatocytes, inflammatory cells aggregating in hepatic sinusoid lumen, and the obvious Kupffer cell hyperplasia and rounding and detachment of SEC were observed in A. These were ameliorated markedly in B. (HE staining paraffin-embedded 5-μm thick sections. ×200).

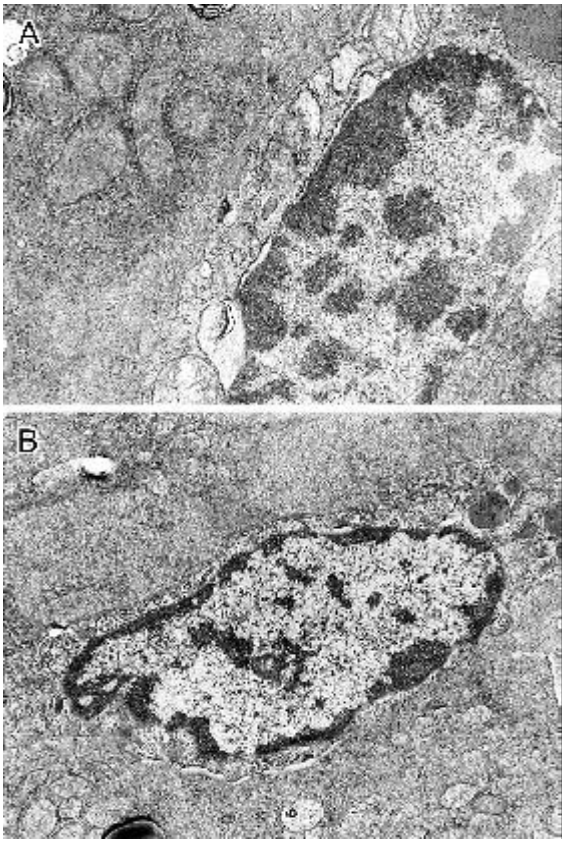

Fig 3. Electron microscopic pictures of rat liver 4 h after reperfusion. A: control group; B: Mat 40 mg/kg treated group. Some typical injuries to the endothelial cells were shown in A, and these were ameliorated markedly in B. (Original magnifications: A×12 000, B×6000).

a 10 % incidence of primary nonfunction[1-3]. The sinusoidal endothelial cells of the liver are susceptible to extended cold preservation[26]. It has been proposed that the preservation-reperfusion injury is mainly due to changes in the SEC surface[4,5]. SEC damage causes marked microcirculatory disturbances, leukocyte and platelet adhesion, diminished blood flow, and continuation of the ischemic process leading to massive hepatic necrosis[27,28]. It seems that SEC injury after preservation is linked to graft failure.

In the present investigation, rat livers were kept for 5 h in cold Ringer's solution (4 °C), exceeding the safety limit of 4 h[29]. Under these preservation and transplantation conditions (ie, a portal vein clamping time of less than 20 min), a postoperative survival rate of about 40 % was obtained. Thus, 5 h in cold Ringer's solution, although a severely compromising condition, should allow an adequate assessment of the mechanisms of cold ischemia/reperfusion injury that led to primary nonfunction. Usually, rats without Mat treatment recovered well from anesthesia; however, their clinical status began to deteriorate within 4 to 6 h, and nonsurvivors died within 24 h, mostly between 10 to 20 h.

Loss of SEC function and viability led to an increase of HA concentration. HA levels at different time points post transplantation were ameliorated markedly by Mat treatment, and double-dose Mat treatment resulted in a significant elevation in HA compared with low-dose treated group. It seems that increasing Mat dosage did not result in better therapeutic effect, which is in accordance with the result[17], and no obvious side effect was noted in our study. Zhang *et al* indicated that high dose of Mat treatment resulted in better antifibrotic effects[20], and it may be caused by a different pharmacological mechanism of Mat. Accordingly, the severity of the ultrastructural changes of SEC was reduced markedly by Mat treatment.

The precise protective mechanism of Mat is still

unknown. It has been recently stressed that a "no-reflow phenomenon", which is a microcirculatory injury caused by the formation of intracapillary thrombi due to the infiltration of inflammatory cells, may be an important factor in the pathogenesis of reperfusion injury[10-12]. Therefore, in this work, we focused on microcirculatory injury to investigate the protective effect of Mat. After hepatic ischemia and reperfusion, Kupffer cells release inflammatory cytokines (tumor necrotizing factor α, and others)[30,31]. Inflammatory cytokines activate the sinusoidal endothelial cells and hepatocytes to express ICAM-1 on their membrane surfaces and release leukocyte-activating factors such as interleukin-1 or platelet-activating factor in the blood[32], which induces lymphocyte-function-associated antigen-1 (LFA-1) on the surface of neutrophils, thus resulting in the adhesion of ICAM-1 and LFA-1. Thrombi of neutrophils may then be formed in hepatic sinusoids, causing peripheral microcirculatory failure at the inflammatory sites. On the other hand, increases in intracellular free calcium are associated with biochemical and histological evidence of cell death likely due to the activation of phospholipases and protein kinases[33]. During the preservation period, adenosine triphosphate (ATP) stores become depleted and intracellular acidosis increases, thereby preventing or reducing the amount of calcium that can be extruded[34]. In this study, our results suggest that Mat treatment decrease the expression of ICAM-1 and the adhesion of inflammatory cells to SEC, resulting in suppression of microcirculation injury caused by hepatic reperfusion. And no significant difference was noted between the two treated groups.

Mat has been shown to inhibit the activation of Kupffer cells and the release of TNF-α, IL-1, and IL-6 *in vitro* and *in vivo*[17,35,36]. And the results in this study indicated that Mat treatment inhibited the expression of ICAM-1 by SEC and the sinusoidal accumulation of inflammatory cells. These may provide a possible mechanism for Mat protective effect on SEC during the cold ischemia and reperfusion injury.

In conclusion, the result of this study demonstrated the protective effect of Mat against the cold ischemia and reperfusion injury of SEC in liver transplantation. It was mediated by inhibition of the activation of Kupffer cells, the release of inflammatory cytokines, and sinusoidal accumulation of leukocytes, *etc*.

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