

# Protective effects of tumor necrosis factor $\alpha$ antibody and ulinastatin on liver ischemic reperfusion in rats

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Supported by the National Natural Science Foundation of China, No. 30070741

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**Received:** 2003-08-26 **Accepted:** 2003-10-27

## Abstract

**AIM:** To study the protective effects of tumor necrosis factor  $\alpha$  (TNF  $\alpha$ ) antibody and ulinastatin on liver ischemic reperfusion in rats.

**METHODS:** One hundred and twenty male SD rats were randomly divided into four groups: normal control group, ischemic group, TNF $\alpha$  antibody group and TNF $\alpha$  antibody + ulinastatin group. The animals were killed at 0, 3, 6, 9, 12 h after ischemia for 60 min and followed by reperfusion. Serum alanine aminotransferase (ALT), malondialdehyde (MDA) and liver histopathology were observed.

**RESULTS:** After ischemic reperfusion, the serum ALT and MDA were remarkably increased, and the hepatic congestion was obvious. Treatment of TNF $\alpha$  antibody and ulinastatin could significantly decrease serum ALT and MDA levels, and relieve hepatic congestion.

**CONCLUSION:** Ulinastatin and TNF $\alpha$  antibody can suppress the inflammatory reaction induced by hepatic ischemic reperfusion, and have protective effects on rat hepatic ischemic reperfusion injury.

Yang YL, Li JP, Xu XP, Dou KF, Yue SQ, Li KZ. Protective effects of tumor necrosis factor  $\alpha$  antibody and ulinastatin on liver ischemic reperfusion in rats. *World J Gastroenterol* 2004; 10(21): 3161-3164

<http://www.wjgnet.com/1007-9327/10/3161.asp>

## INTRODUCTION

Liver ischemic reperfusion injury is induced when liver gets the retrieval of its blood perfusion or oxygen supply, and hepatic injury would aggravate due to ischemia and hypoxia injury<sup>[1-4]</sup>. Hepatic insufficiency or primary liver graft non-function can be caused by liver ischemic reperfusion injury after portal blockage, hemorrhagic shock or liver transplantation. As liver ischemic reperfusion is hard to be avoided in hepatic surgical practice and the existing prevention and cure methods are not satisfactory, research of the mechanisms and therapy on liver ischemic reperfusion becomes one of the hotspots in hepatic surgery<sup>[5,6]</sup>.

Cytokines are polypeptides with extensive biological activities, and play important roles in the immunoregulation. They prevent body from diseases and accelerate tissue rehabilitation. But on the other hand, too many cytokines can also lead to or aggravate tissue damages<sup>[7,8]</sup>. Recent researches have demonstrated that TNF  $\alpha$  plays an important role in ischemic reperfusion injury of liver<sup>[9-12]</sup>. At the same time, ulinastatin has been applied in the clinical treatment of pancreatitis, shock and extracorporeal circulation because of its significant inhibitory effect on inflammation<sup>[13-15]</sup>. In the present study, we attempted to relieve ischemic reperfusion injury of liver by using TNF  $\alpha$  antibody and ulinastatin, so as to provide experimental and theoretic bases for prevention and treatment of liver ischemic reperfusion injury.

## MATERIALS AND METHODS

### Animals

A total of 120 male Spargue-Dawfey (SD) rats weighing 230 $\pm$ 20 g, were obtained from Animal Research Center of Shaanxi Chinese Medical Institute, and fed with standard rat chow.

### Drugs

Ulinastatin (Tianpu Co. Ltd., Guangdong, China) was diluted to 50 U/L by saline prior to use. TNF  $\alpha$  monoclonal antibody (Jingmei Co. Ltd., Guangdong, China) was diluted 100 times by saline prior to use.

### Experimental grouping

The rats were randomly divided into four groups. Group I: the control group, sham operation was performed, hepatic lobes of the rats were exposed without any treatment. Group II: ischemic reperfusion injury group, in which blood stream of the rats' liver lobes were blocked and then recovered after 60 min. Group III: TNF  $\alpha$  antibody treatment group, in which TNF  $\alpha$  antibody (2.0 mg/kg) was injected into the rats through dorsum veins of penis 5 min prior to reperfusion. Group IV: TNF  $\alpha$  antibody and ulinastatin treatment group, in which both TNF  $\alpha$  antibody (2.0 mg/kg) and ulinastatin (500 000 U/L, 0.5 mL) were simultaneously injected into the rats through dorsum veins of penis 5 min prior to reperfusion. Blood samples (2 mL) of all animals in each group were taken from hepatic superior and inferior vena cava at 0, 3, 6, 9 and 12 h after reperfusion. Then the rats were killed and liver samples were obtained.

### Operation

The animals were intraabdominally anesthetized by pentobarbital sodium (30 mg/kg, 0.1 mL/10 g), and incised through median incision of the abdomen. After the liver pedicel between left and middle lobes of liver was exposed, ligaments between liver and septum transversum and abdominal wall were cut. The scatheless vascular clamp was used to block blood stream of portal veins and hepatic arteries of left and middle lobes of liver. After 60 min, the vascular clamp was released and blood stream recovered. So approximately seventy percent of liver was hypoxia, thus severe congestion of the mesentery vein was prevented.

**Table 1** Levels of serum ALT in rats (U/L)

Group	n	Time of after reperfusion (h)				
		0	3	6	9	12
I <sup>d</sup>	6	40.52±8.33	42.36±3.71	43.19±7.64	42.92±5.18	42.66±9.27
II	6	263.92±16.90	315.61±21.02	374.26±19.56	289.11±16.32	257.94±27.41
III	6	238.73±10.62	254.06±13.78	273.17±18.29	213.26±26.54	172.53±36.46
IV <sup>b</sup>	6	173.42±15.33	189.08±24.52	203.17±23.19	175.36±38.66	163.13±32.27

<sup>b</sup> $P < 0.01$  vs other groups, <sup>d</sup> $P < 0.01$  vs group II and III.

**Table 2** Levels of serum MDA of rats (mmol/L)

Group	n	Time of after reperfusion (h)				
		0	3	6	9	12
I <sup>d</sup>	6	7.82±0.17	7.91±0.08	8.11±0.13	8.04±0.67	7.93±0.41
II	6	13.81±4.19	38.65±4.73	46.36±2.54	35.09±5.12	24.09±3.20
III	6	10.71±2.52	18.23±3.58	23.31±4.49	16.83±2.29	11.26±3.16
IV <sup>b</sup>	6	9.12±3.41	12.29±6.83	15.18±3.22	11.72±4.15	9.42±5.28

<sup>b</sup> $P < 0.01$  vs group II and III, <sup>d</sup> $P < 0.01$  vs other groups.

### Determination of ALT and MDA in serum

The blood samples in each group were poured into centrifuge tubes and the placement lasted for 20 min without shaking. After centrifugation at 2 000 r/min for 10 min, the sample serum was extracted and stored at -80 °C for determination.

ALT levels of sample serum were determined by an automatic biochemistry analyzer. MDA levels of sample serum were determined by the method introduced by Mourek *et al.*<sup>[16]</sup>, and the kit was purchased from Juli Biomedical Engineering Institute of Nanjing, China.

### Pathological changes of liver

Fresh tissues of liver in each group were sampled. Haematoxylin-Eosin (HE) staining was performed on 100 g/L formaldehyde-fixed tissue sections. The histological patterns of the liver samples were observed under light microscope.

### Statistical analysis

All the data were analyzed by Student's *t* test and expressed as mean±SD.  $P < 0.05$  was considered statistically significant and  $P < 0.01$  as very statistically significant.

## RESULTS

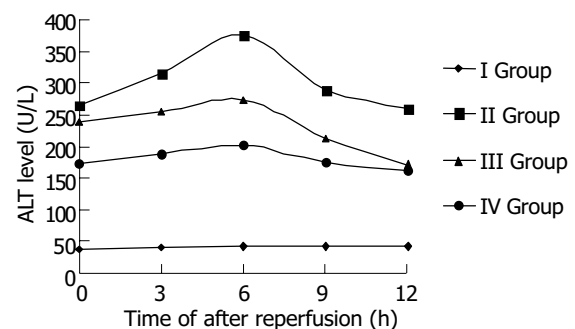
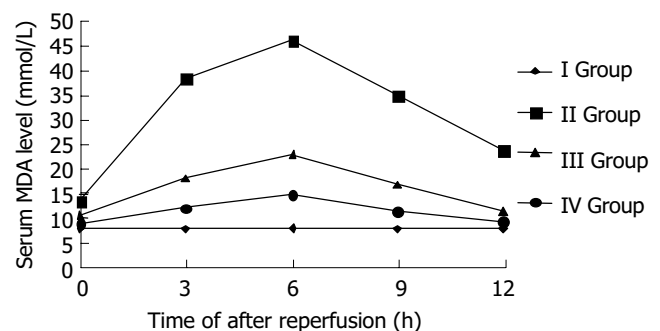
### ALT levels of sample serum

ALT levels of sample serum in each group were determined to assess the liver function of rats. The levels of ALT in ischemic reperfusion injury group at different time points were significantly higher than those in control ( $P < 0.01$ ). The levels of ALT increased gradually after reperfusion, reached the peak 6 h after reperfusion, and then decreased mildly. The levels of ALT in TNF $\alpha$  antibody treatment group were remarkably lower than those in ischemic reperfusion injury group ( $P < 0.01$ ). The levels of ALT in TNF $\alpha$  antibody and ulinastatin treatment group were lower than those in ischemic reperfusion injury group and TNF $\alpha$  antibody treatment group ( $P < 0.01$ ).

### MDA levels of sample serum

The oxygen-derived free radicals induced lipid peroxidation reaction of polyvalent unsaturated fatty acid at plasmalemma, which developed lipid peroxidation products, such as MDA. So the MDA levels of sample serum showed the degree of lipid peroxidation injury of liver. The levels of MDA in ischemic reperfusion injury group at different time points were significantly

higher than those in control ( $P < 0.01$ ). The levels of MDA increased gradually after reperfusion, reached the peak 6 h after reperfusion, and then decreased. The levels of MDA in TNF $\alpha$  antibody treatment group were remarkably lower than those in ischemic reperfusion injury group ( $P < 0.01$ ). The levels of MDA in TNF $\alpha$  antibody and ulinastatin treatment group were lower than those in ischemic reperfusion injury group and TNF $\alpha$  antibody treatment group ( $P < 0.01$ ).

**Figure 1** Serum ALT level after reperfusion in rats.**Figure 2** Serum MDA level after reperfusion in rats.

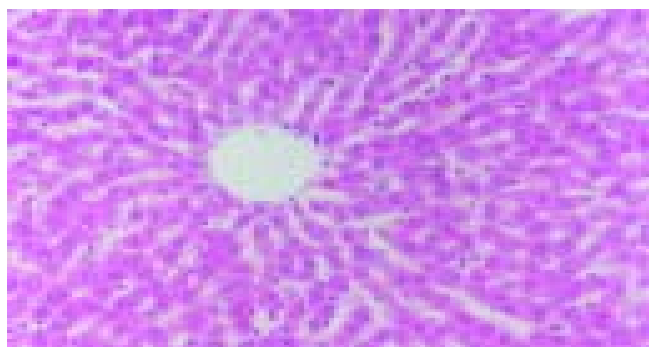
### Histopathological observation

The blood stream in middle and left lobes of livers of rats was reperused 1 h after blocked for 60 min. In ischemic reperfusion injury group, the middle and left lobes of livers were found to be swollen and faint. HE staining showed disorganized hepatic

lobules and extensive hepatocytic edema with various degrees of vacuolation and lamellar necrosis. The liver tissues in TNF $\alpha$  antibody treatment group and in TNF $\alpha$  antibody + ulinastatin treatment group were found to insignificantly swollen and faint in appearance. HE staining showed a sprinkle of hepatocytic edema without vacuolation and lamellar necrosis.



**Figure 3** Disorganized hepatic lobules and extensive hepatocytic edema with various degrees of vacuolation and lamellar necrosis in ischemic reperfusion injury group.



**Figure 4** A sprinkle of hepatocytic edema without vacuolation and lamellar necrosis in TNF $\alpha$  antibody + ulinastatin treatment group.

## DISCUSSION

Liver ischemic reperfusion injury can be frequently seen in surgical practice, and plays important roles in severe infection, trauma, shock, cardiorespiratory malfunction, organ transplantation, *etc.* Ischemic reperfusion can cause a series of injuries on metabolism, structure, and function in hepatic tissues and cells, and even liver function failure. So it is one of the major factors influencing the prognosis, operative success and survival of patients.

Cytokines, are the soluble polypeptides excreted by immunocytes, and play important roles in immunological activation and inflammatory reaction. The study of cytokine effects on ischemic reperfusion injury in liver has become highlights at present<sup>[17-22]</sup>. TNF $\alpha$  is the polypeptide excreted by activated macrophages, endothelial cells, neutrophilic granulocytes and B lymphocytes, and plays an important role in inflammatory reaction. Liver possesses tremendous Kupffer cells (KC) that have the great potency to produce TNF $\alpha$  in human body. At the time of liver ischemia, for the blockage of ATP production, calcium pump of liver is in dysfunction, which causes intracellular calcium overload. Aggravated calcium overload can activate Kupffer cells. On the other hand, Kupffer cells have complement receptors, intracellular protein was released during ischemic reperfusion, thus activating complements, which can also activate Kupffer cells. The activated Kupffer cells excrete TNF $\alpha$ . Liver has plenty of TNF $\alpha$  receptors, and also is the major target organ of TNF $\alpha$ <sup>[23-26]</sup>. Some mechanisms were used to explain

the liver damage caused by TNF $\alpha$ <sup>[27-30]</sup>. Firstly, TNF $\alpha$  could directly injure hepatocytes. Secondly, TNF $\alpha$  could activate neutrophilic granulocytes and mononuclear macrophages to express IL-1 and IL-6, and phosphatidase A2 that can decompose arachidonic acids. Inflammatory media were produced such as platelet active factor, leukotriene, and thromboxane A2. So the inflammatory reaction was aggravated. Thirdly, the toxic effect of TNF $\alpha$  on endothelial cells could induce the circulatory disorder of hepatic sinusoid, and TNF $\alpha$  could activate complement system that aggravates tissue damage by cytotoxicity. Finally, the oxygen-derived free radicals induced by TNF $\alpha$  could facilitate oxidation explosion of neutrophilic granulocytes, which could also result in liver damage. Therefore, the way to block the production of TNF $\alpha$  in liver can be used to prevent liver from injury by TNF $\alpha$ .

Ulinastatin is one kind of glycoproteins containing 143 amino acids with molecular weight of 67 ku. It is the typical urine protease inhibitor isolated and purified from human urine. Ulinastatin has two active function domains which have a wide restrained zymogram with no overlapped region each other. So ulinastatin can inhibit many hydrolytic enzymes in one time, including trypsin, phospholipase A<sub>2</sub>, aldase and elastase. Moreover, the components of low molecular mass decomposed from ulinastatin can also inhibit hydrolytic enzymes strongly. On the other hand, ulinastatin could ameliorate the shock situation by blocking the production of myocardial depressant factors and stabilizing the membrane of lysosome<sup>[31-33]</sup>. Recent researches found that ulinastatin could block the release of inflammatory factors, prevent cascade reaction of cytokines, inhibit excessive activation of leukocytes and block vicious circles among cytokines, inflammatory factors and leukocytes. So when pancreatitis, shock or other severe infection occurred, the action of multiple hydrolytic enzymes necessitated the use of ulinastatin, which can inhibit many hydrolytic enzymes at the same time and alleviate the vicious effects of multiple proteases on tissues and organs<sup>[34-36]</sup>.

The present study was to verify the protective effect of TNF $\alpha$  antibody and ulinastatin on ischemic reperfusion injury of liver. The results showed that the levels of ALT and MDA in TNF $\alpha$  antibody and ulinastatin treatment group were remarkably decreased as compared with those in ischemic reperfusion injury group. Pathological changes of liver also demonstrated the significant protective effect of TNF $\alpha$  antibody and ulinastatin on ischemic reperfusion injury of liver. The present results suggest TNF $\alpha$  antibody and ulinastatin can effectively prevent ischemic reperfusion injury of liver. But further work is needed to make clear of their best concentrations, best ratio and their effect on immune system of the body.

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