BASIC RESEARCH

Multiple pathogenic factor-induced complications of cirrhosis in rats: A new model of hepatopulmonary syndrome with intestinal endotoxemia

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cirrhosis, various pathological parameters got worse and pulmonary edema formed. The predisposition of glycine antagonized the effects of LPS and significantly alleviated various pathological alterations.

CONCLUSION: The results suggest that: (1) a characteristic rat model of HPS can be non-invasively induced by multiple pathogenic factors including high fat diet, alcohol, cholesterol and CCl₄; (2) this model can be used for study of hepatopulmonary syndrome and is clinically relevant; and (3) intestinal endotoxemia (IETM) and its accompanying cytokines, such as TNF- α , exert a crucial role in the pathogenesis of HPS in this model.

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Key words: Alcohol; Carbon tetrachloride; Endotoxin; High fat diet

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Abstract

AIM: To develop and characterize a practical model of Hepatopulmonary syndrome (HPS) in rats.

METHODS: The experimental animals were randomized into five feeding groups: (1) control (fed standard diet), (2) control plus intraperitoneal injection with lipopolysaccharide (LPS), (3) cirrhosis (fed a diet of maize flour, lard, cholesterol, and alcohol plus subcutaneously injection with carbon tetrachloride (CCl₄) oil solution), (4) cirrhosis plus LPS, and (5) cirrhosis plus glycine and LPS. The blood, liver and lung tissues of rats were sampled for analysis and characterization. Technetium 99m-labeled macroaggregated albumin (Tc99m-MAA) was used to test the dilatation of pulmonary microvasculature.

RESULTS: Typical cirrhosis and subsequent hepatopulmonary syndrome was observed in the cirrhosis groups after an 8 wk feeding period. In rats with cirrhosis, there were a decreased PaO₂ and PaCO₂ in arterial blood, markedly decreased arterial O₂ content, a significantly increased alveolar to arterial oxygen gradient, an increased number of bacterial translocated within mesenteric lymph node, a significant higher level of LPS and tumor necrosis factor- α (TNF- α) in plasma, and a significant greater ratio of Tc99m-MAA brain-over-lung radioactivity. After LPS administration in rats with

INTRODUCTION

In the physiological situation, endotoxin, derived from gram-negative bacteria in the intestinal lumen, is continuously released. But no distinct pathogenic effect is exhibited, because only a small portion of endotoxin from the intestine is absorbed into the portal circulation and delivered to the liver, where the endotoxin is quickly cleared by intrahepatic Kupffer cells. On the contrary, in liver disease, particularly in liver failure, a large amount of endotoxin from the intestine reaches the portal blood or flows into systemic circulation directly through shunting of portal blood away from the liver due to bacterial overgrowth in the intestinal lumen and enhanced permeability of enteric mucosa, impaired phagocytic capacity of Kupffer cells that fail to uptake and clear endotoxin, increased levels of bile acid and bilirubin in plasma which inhibit the phagocytosis of Kupffer cells, and absence of bile salt in the intestinal lumen which favor gram-negative bacterial overgrowth and endotoxin

production. As a result, there is an abnormal increased level of endotoxin in systemic blood in the end in patients with liver disease, which is named intestinal endotoxemia (IETM) and can lead to various pathological complications.

In the liver, endotoxin acts upon Kupffer cells by way of specific receptors and nonspecific membrane-conjoint fashion. Of these, a principal fashion is to activate intracellular signaling pathways, thereby releasing a variety of cytokines and other bioactive mediators. The latter will produce a massive biologic effect and participate in hepatic multiple pathophysiologic courses inducing and aggravating liver injury and further promoting liver failure. In the last 20 years, Han *et al*^[1,2] have conducted sequential experimental studies and clinical investigations and documented that IETM exists in various types of cirrhosis and is pathophysiologically associated with multiple features of liver failure, for example, hepatorenal syndrome and hepatic encephalopathy^[3-10]. Hepatopulmonary syndrome (HPS) is also associated with endotoxin^[11]. Clinically, HPS is recognized in as many as 15%-20% of patients undergoing evaluation for orthotopic liver transplantation^[12] and is found most commonly in the setting of cirrhosis and appears to occur across the spectrum of etiologies of liver disease^[13-15]. The presence of HPS increases mortality in the setting of cirrhosis and may influence the frequency and severity of complications of portal hypertension^[16]. But it remains to be determined whether IETM plays a direct role in the pathogenesis of HPS which is the triad of basic liver disease, dilatation of pulmonary vasculature, and hypoxemia^[17-22]. Few data are available on the pathogenesis of HPS in experimental animals and patients with liver diseases. Moreover, models for HPS that are developed non-invasively and reflect real clinical causes of liver disease such as high fat diets, toxins, and alcohol are missing. So, the goal of the present study is to establish a practical model of hepatopulmonary syndrome (HPS) in rats and to characterize its fundamental pathogenesis. Our results suggest that rats with cirrhosis induced by multiple pathogenic factors for 8 wk spontaneously develop hepatopulmonary syndrome which is associated with IETM.

MATERIALS AND METHODS

Animal and reagents

Male Wister rats, weighing 230-280 g obtained from the Animal Center of Shanxi Medical University, were employed. All animals received care during the study, under a protocol that was in accordance with institutional guidelines for animal research and was approved by the Ethical and Research Committee of the Shanxi Medical University. Lipopolysaccharide (LPS, from *Escherichia coli* serotype 0111:B4) was purchased from Sigma (St Louis, Missouri, USA). TNF- α radioimmunoassay kits were provided by the Radioimmunity Institute of PLA General Hospital. *Limulus amoebocyte* lysate (LAL) reagent, for determination of LPS in plasma, was obtained from Shanghai Yi Hua Scientific, Inc. CCl₄ oil solution was from TIANJIN KE SAI OU Science and Technology Company, LTD (Tianjin, China). Cholesterol was from TIANJIN Chemical Reagent Company (Tianjin, China). All reagents

used in the present study were at analytical grade.

Induction of cirrhosis

The experimental animals were randomized into five groups (6 animals per group): (1) control (fed standard diet from Animal Center of Shanxi Medical University, China), (2) control plus lipopolysaccharide (LPS), (3) cirrhosis, (4) cirrhosis plus LPS, and (5) cirrhosis plus glycine and LPS. Animals in the cirrhosis group were fed a mixture of maize flour, lard, cholesterol, and alcohol plus subcutaneously injection with carbon tetrachloride (CCl₄) oil solution for 8 wk^[23]. The CCl₄ oil solution (400 g/L) was injected at 0.5 mL/100 g body weight at the first day of experiment and at 0.3 mL/100 g body weight from the third day on at an interval of two days until the experimental end. Lard was used only in the first two weeks accounting for 20% of the feed. Cholesterol was appended at 0.5% of feed for the whole experiment. Alcohol was used in the drinking water exclusively (300 mL/L) during the whole experiment. LPS (3 μ g/g body weight) was administrated with an intra-peritoneal injection at the end of the 8th wk. Glycine (1 g/rat) was administrated by gavage 4 h before LPS administration in the cirrhotic rats. Glycine is an antagonist towards LPS which has been demonstrated. The purpose of additional LPS and glycine is to consolidate the effect of IETM in the pathogenesis of HPS. The normal control animals had free access to the standard food and water. The blood, liver and lung tissues of rats were sampled 3 h after LPS treatments.

Analysis of blood gas

The rats were fasted overnight before sacrificing for samples. The blood from the coeliac artery of various animals was aseptically collected for gas analysis.

Measurements of LPS, TNF- α , ALT, AST, TB, Hyp levels and PVP

The contents of LPS, TNF- α , alanine transferase (ALT), aspartate transaminase (AST) and total bilirubin (TB) in plasma were measured according to the manufacture's instructions. Hydroxyproline (or Hyp) in liver tissue was determined employing chloramines T method^[24]. The technique of simple water column was used for examination of portal vein pressure (PVP).

Histology and pulmonary coefficient

Samples from lung and liver were collected into 100 g/L phosphate buffered formaldehyde and fixed overnight. Serial 4 μ m thick sections were prepared after the samples were dehydrated in graded ethanol solutions, cleared in chloroform, and embedded in Paraplast. Staining was performed with hematoxylin and eosin (HE) and studied under light microscopy in a blind fashion.

For transmission electron microscopy (TEM), a pulmonary sample was prepared as described in the product literature (Technical Bulletins 405 and 406, Electron Microscopy Sciences, Hatfield, PA).

The weight of wet lungs (gram, g) and body (kilogram, kg) of animals were recorded and pulmonary filtration coefficient was calculated as a ratio of the whole wet lungs weight (g) to body weight (kg).

Table 1 Changes of hepatic function in rats (mean $\pm$ SD, $n = 6$)

Group	ALT (U/L)	AST (U/L)	TB (mmol/L)	Hyp (mg/g)	PVP (H ₂ O cm)
Control	37.24 $\pm$ 6.96	68.68 $\pm$ 13.36	8.15 $\pm$ 1.70	0.60 $\pm$ 0.07	10.31 $\pm$ 1.16
Cirrhosis	84.12 $\pm$ 13.14 ^a	186.23 $\pm$ 31.83 ^a	40.25 $\pm$ 3.21 ^a	2.08 $\pm$ 0.09 ^a	18.38 $\pm$ 2.53 ^a

^a $P < 0.01$ vs normal control.

Bacterial translocation

The rats were anesthetized, and the abdominal skins were shaved and sterilized with an iodine solution. Mesenteric lymph nodes were dissected aseptically and crushed and plated onto chocolate agar plates containing blood, and were incubated at 37°C for 24 h. The number of colony forming units was counted (less than 20 colonies for minor colonies, 20-50 for middle colonies, more than 50 for major colonies) and Gram-dyeing at this time in cirrhosis was compared with normal controls^[25].

Intrapulmonary vascular dilatations

The technetium 99m-labeled macroaggregated albumin (Tc99m-MAA) was used to prove the dilatation of pulmonary microvasculature^[26]. Animals in four groups except the cirrhosis + glycine + LPS group were tested. Thirty minutes after the injection of 200 μ Ci of 99mTc-labeled albumin macroaggregates into the tail vein of experimental rats, the brain and lung radioactivity was scanned and ratio of brain-over-lung radioactivity was calculated.

Statistical analysis

Data were evaluated using analysis of variance and multiple comparisons and correlation between groups with SPSS software. All values are reported as means $\pm$ SD. P values of less than 0.05 were considered statistically significant.

RESULTS

Animal behavior

During the whole experiment, a soft, sparse, ruffled, lack-luster fur covering the bodies of animals was found and a low spirit, fatigue, unappetizing, gradually decreased body weight were displayed in rats with cirrhosis. The mortality of cirrhotic animals accounts for approximately 20 per cent (14/70).

Abnormal hepatic function and histology

At the end of the 8th wk, the levels of AST, ALT, TB in plasma, hydroxyproline in hepatic tissues, and portal vein pressure (PVP) were significantly greater in rats with cirrhosis than in those of controls (Table 1).

Characteristically, the hepatic destructive architecture by fibrous septa that encompass regenerative nodules of hepatocytes and the formation of false lobules were histopathologically observed (Figure 1).

Disturbance of blood gas

In the rats with cirrhosis, pH was normal, partial pressures of O₂ and CO₂ in arterial blood (PaO₂, PaCO₂) were lower, arterial oxygen content (O₂ cont) was markedly fallen; the alveolar to arterial oxygen gradient was sharply widened.

Figure 1 Histology of rat liver. Panel A: normal liver structure showing normal sinusoids and cord located around central vein (HE, $\times 400$); Panel B: typical liver cirrhosis with regenerative nodules of hepatocytes separated by fibrous septa (HE, $\times 40$).

After administrating an extra LPS to the cirrhotic rats, respiratory defects above got worse. The predisposition of glycine alleviated various pathological features (Table 2). There was a close correlation between decreased O₂ content and increased plasma LPS (Table 3).

Abnormal pulmonary morphology

Histopathologic studies showed massive accumulation of giant macrophages in the alveolar spaces and its wall, and widened alveolar wall architecture in which enlarged blood vessel cavity and increased numbers of blood capillaries (normal control 5.25 ± 1.22 , cirrhosis 8.28 ± 1.24 , $P < 0.05$) were observed in cirrhotic rats. After administrating an extra LPS, alveoli wall further widened with decreased density of cells and narrowed alveoli space, integrity of type I cell membrane destructed with discontinued zigzag lesion, taken together with infiltration of polymorphs and fibrinous exudates, indicating interstitial pulmonary edema and inflammatory reaction. There was severe stasis of blood in alveoli walls and in numerous red cells extravasated airspace, indicating the widespread dilatation of alveolar capillaries and the augmentation of permeability of microvasculature. However, when normal rats were administered an extra LPS, only a mild pathological change was observed. The predisposition of glycine could alleviate above-mentioned various pathological features (Figure 2).

Pulmonary coefficient

Pulmonary filtration coefficient is a reliable index reflecting whether pulmonary edema exists. A normal pulmonary

Table 2 Analysis of blood gas in rats (mean $\pm$ SD)

Groups	<i>n</i>	pH	PaCO ₂ (mmHg)	PaO ₂ (mmHg)	O ₂ cont (mL/dL)	AaDO ₂ (mmHg)
Normal control	8	7.38 $\pm$ 0.03	33.94 $\pm$ 4.21	94.71 $\pm$ 13.72	20.40 $\pm$ 0.32	3.44 $\pm$ 3.26
Normal + LPS	7	7.31 $\pm$ 0.02	28.13 $\pm$ 2.56	117.51 $\pm$ 22.30	20.63 $\pm$ 0.34	4.60 $\pm$ 2.24
Cirrhosis	7	7.34 $\pm$ 0.14	28.76 $\pm$ 5.57	88.87 $\pm$ 28.46	19.80 $\pm$ 1.1 ^a	9.90 $\pm$ 2.08 ^c
Cirrhosis + LPS	6	7.32 $\pm$ 0.04	24.98 $\pm$ 8.69	112.55 $\pm$ 24.02	20.58 $\pm$ 0.27	18.48 $\pm$ 7.86 ^{c,c}
Cirrhosis + Glycine + LPS	6	7.31 $\pm$ 0.10	33.25 $\pm$ 5.10	110.40 $\pm$ 12.71	20.60 $\pm$ 0.14	-

^a*P* < 0.05 *vs* other groups; ^c*P* < 0.05 *vs* normal control; ^c*P* < 0.05 *vs* cirrhosis group.

Table 3 Analysis of correlation

PAIRS	<i>n</i>	<i>r</i>	<i>P</i> value
Plasma LPS-Plasma TNF- α	30	0.443	< 0.01
Plasma LPS-O ₂ content	30	0.47	< 0.05
Plasma LPS-Tc99MAA	20	0.505	< 0.05
Plasma TNF- α -Tc99MAA	20	0.475	< 0.05

Table 4 Changes of pulmonary coefficient in rats (mean $\pm$ SD, *n* = 6)

Groups	Pulmonary coefficient
Normal control	7.3489 $\pm$ 0.8225
Normal + LPS	7.9728 $\pm$ 0.9203
Cirrhosis	7.5992 $\pm$ 0.7960
Cirrhosis + LPS	10.8895 $\pm$ 4.6496 ^a
Cirrhosis + Glycine + LPS	8.5914 $\pm$ 0.1875

^a*P* < 0.01 *vs* normal control.

Table 5 Bacterial translocation of mesenteric lymph node in rats (*n* = 5)

Groups	Minor clony	Major clony
Normal control	5	0
Cirrhosis	2	3

Gram reaction: gram-negative predominantly.

Figure 2 Electron microscopy of lung from cirrhotic liver. Panel A: enlarged alveolar capillaries with narrowed alveolar space filled with exudates ($\times$ 6000); Panel B: destruction of the type I alveolar epithelium, showing a discontinued zigzag lesion ($\times$ 6000).

filtration coefficient was seen in cirrhotic rats, indicating there was no edema in the lungs. Whereas cirrhotic animals administered an extra LPS showed a significant increased pulmonary filtration coefficient, obvious pulmonary edema due to lung injury with increased vascular permeability. Glycine could alleviate the above-mentioned pathological features (Table 4).

Bacterial translocation

The goal of the present study was to identify whether

bacterial translocation occurs by calculation of colony numbers of bacteria in the mesenteric lymph node so as to further judge the presence of blood bacteremia. The results showed that the colony number of bacteria was markedly higher in rats with cirrhosis than those in normal controls and Gram-negative bacteria accounted for the majority (Table 5).

Ratio of Tc99m-MAA brain-over-lung radioactivity

The test rests upon the principle that Tc99m-MAA has a larger diameter, about 20-60 μ m than the normal capillary, about 8-15 μ m, and can be trapped within normal pulmonary precapillary when injected *via* the tail vein but can pass through dilated pulmonary capillaries into the capillary bed of the systemic circulation and can be detected in the brain and other organs scanned. The results demonstrated that the ratio of Tc99m-MAA brain-over-lung radioactivity was significantly raised in cirrhotic rats, indicating intrapulmonary vasodilatation. The manifestations became more characteristic after an extra injection of LPS. The pulmonary microcirculation tended to slight dilatation after administration of LPS in rats fed standard diet (Figures 3 and 4). The ratio of brain-over-lung radioactivity was significantly correlated with the increased plasma LPS and TNF- α in cirrhotic rats (Table 3).

Levels of endotoxin and TNF- α in plasma

The change trend of endotoxin and TNF- α in plasma

Figure 3 Tc99m-MAA scanning images of the rat brain and lung. Panel A: representative rat from control group fed standard diet (see details in Method), showing a full distribution of Tc99m-MAA in the lung; Panel B: representative rat from the group fed standard diet plus intraperitoneal injection with lipopolysaccharide (LPS), showing slight radioactivity in the brain; Panel C: representative rat from the cirrhotic group fed a mixture of maize flour, lard, cholesterol, and alcohol plus intraperitoneal injection with carbon tetrachloride (CCl₄) oil solution for 8 wk, showing significant increased radioactivity in the brain; Panel D: representative rat from cirrhotic animals plus LPS injection, showing much increased radioactivity in the brain.

was similar. They rose obviously in rats with cirrhosis and further increased after adding a given dosage of LPS. The precondition of glycine could alleviate various pathological alterations (Table 6). There was a close correlation between contents of both endotoxin and TNF- α (Table 3).

Analysis of correlation

It was demonstrated in the present study that there are close correlations between plasma LPS and plasma TNF- α , arterial O₂ content, Tc99m-MAA brain-over-lung radioactivity and between plasma TNF- α and Tc99m-MAA brain-over-lung radioactivity, which strongly suggest that intestinal endotoxemia (IETM) and its accompanying cytokine, TNF- α , are important in pathogenesis of HPS (Table 3).

DISCUSSION

In the present study, a new model of hepatopulmonary syndrome (HPS) was achieved after the induction of cirrhosis in rats with multiple pathogenic factors including high fat, alcohol, carbon tetrachloride, cholesterol and maize flour, which was close to the spectrum of etiologies of liver disease. It was also demonstrated that intestinal endotoxemia (IETM) plays an important role in the development of HPS.

Several protocols have been proposed for clinical diagnosis of HPS^[27]. However, the following criteria set by Roisin *et al*^[20] are better for experimental models of HPS: (1) presence of chronic hepatic disease (alcoholic,

Figure 4 Ratio of brain-over-lung radioactivity in the rats. Control, fed standard diet; LPS, fed standard diet plus LPS injection; Cirrhosis, fed a mixture of maize flour, lard, cholesterol, and alcohol plus intraperitoneal injection with carbon tetrachloride (CCl₄) oil solution for 8 wk. ^a*P* < 0.05 vs normal control group; ^c*P* < 0.05 vs cirrhosis group.

Table 6 Levels of LPS and TNF- α in plasma in rats (mean $\pm$ SD, *n* = 6)

Groups	LPS (EU/mL)	TNF- α (ng/mL)
Normal control	0.0571 $\pm$ 0.0104	0.6629 $\pm$ 0.1620
Normal + LPS	0.5550 $\pm$ 0.0699 ^a	0.9344 $\pm$ 0.2819 ^a
Cirrhosis	0.1655 $\pm$ 0.0410 ^a	1.0588 $\pm$ 0.1337 ^a
Cirrhosis + LPS	0.5269 $\pm$ 0.0659 ^a	1.5571 $\pm$ 0.1785 ^a
Cirrhosis + Glycine + LPS	0.3144 $\pm$ 0.0649 ^{a,c}	1.2560 $\pm$ 0.1356 ^{a,b}

LPS: ^a*P* < 0.05 vs normal control group; ^c*P* < 0.05 vs cirrhosis + LPS group; TNF- α : ^a*P* < 0.05 vs normal control group; ^b*P* < 0.05 vs cirrhosis + LPS group.

post necrotic, or primary biliary cirrhosis or active chronic hepatitis)-severe liver dysfunction may not be mandatory; (2) absence of primary cardiac and pulmonary diseases; (3) pulmonary gas exchange abnormalities-an increased alveolar to arterial oxygen gradient ($\geq$ 2.0 kPa) with or without hypoxemia; (4) the extrapulmonary appearance of intravenous radiolabelled microspheres or a positive contrast enhanced echocardiogram indicative of intrapulmonary vascular abnormalities. The abnormalities of arterial oxygenation are one of the characteristics of HPS. Clinically, oxygen partial pressure in arterial blood (PaO₂) exhibits a larger difference in patients with liver disease due to varying degree of impaired liver function^[28-32]. Thus, to qualify for this diagnosis, patients must be demonstrated to have an elevated alveolar to arterial oxygen gradient (AaDO₂) and an increased dilatation of the intrapulmonary microcirculation on the basis of primary liver disease.

In the present studies, rats challenged with the pathogenic factors displayed characteristics of HPS. A declined hepatic function, increased amounts of hydroxyproline in the liver architecture typical of cirrhosis and portal vein pressure, decreased PaO₂ and O₂ content, and significantly enlarged alveolar to arterial oxygen gradient were observed. The enlarged blood vessels in cirrhotic rats and further destruction of respiratory membrane in the presence of LPS indicate severe

hypoxemia resulting from the gas exchange abnormality in the lung; and the raised ratio of Tc99m-MAA brain-over-lung radioactivity indicates that a portion of technetium-99 macroaggregated albumin particles traversed the pulmonary capillary bed and deposited in systemic microvascular beds, which suggests that intrapulmonary vascular dilatations predominantly occurred in areas of alveoli. These characteristics were in accordance with manifestations of patients with HPS and Roisin's diagnostic criteria, suggesting that a new model of HPS has successfully been established in rats.

The inducing agents used in the present model reflect routine causes of cirrhosis and HPS. Of the ingredients we used, CCl₄ is a determinant and the mixture of maize flour, ethanol, cholesterol, and lard added in the first two weeks enhanced the effects of cirrhosis induction. Protein provided by pure corn flour accounts for about 9 per cent, which could merely meet half of the demands of normal rats. As far as the intrinsic quality is concerned, corn glue proteins lack lysine, tryptophan, and methionine, which are important amino acids for lipid metabolism. If they are deficient, hepatic fatty degeneration up to hepatic fibrosis will happen. Ethanol consumption could increase the requirement for choline. Choline is required for processing long-chain saturated fatty acids (C16-18) from an ordinary fatty meal. Choline deficiency impairs release of hepatic triglycerides and could raise the intrahepatic fatty content and directly damage hepatocytes. Prolonged hepatic fatty degeneration leads to formation of liver cirrhosis. After adding cholesterol to the feedstuff, it will further increase the requirement for choline due to damage caused by cholesterol to the cell membrane, which will consequently accelerate development of cirrhosis^[33]. Thus these complex pathogenic factors induce and intensify hepatic fatty degeneration, and formation of fatty cysts. The latter presses contiguous reticulated brackets accordingly forming fasciculi. Meanwhile, on account of hepatocytes in fatty degeneration being extraordinarily sensitive to CCl₄, a massive hepatocytic necrosis would occur, reticulated bracket would collapse due to contiguous regenerated nodular pushing, and ultimately develop fasciculi until hepatic cirrhosis^[23].

Induction of cirrhosis in rats through chronic common bile duct ligation (CBDL) has established a model that reproduces the physiologic features of human HPS^[34,35]. Compared with it, are the following advantages in the model of the present study: (1) with respect to the induction of cirrhosis and HPS, our method is non-invasive; (2) with respect to the clinical etiology of developing cirrhosis, hepatic pathologic changes resulted from complex pathogenic factors which might be more akin to what happens in patients; (3) with regard to pathogenesis of HPS, chronic poisoning of CCl₄, alcohol consumption, lack of choline, and high fat all together lead to a robust chronic dynamic course, i.e., hepatocytic necrosis - live fatty degeneration - liver fibrosis - liver cirrhosis - HPS successively. According to our experiment, in the course of HPS development, degeneration and necrosis of hepatocytes occur within the first two weeks of experimentation, fibrosis within 3 to 4 wk, early architectural disturbances typical of cirrhosis at the 6th wk

of experimentation, namely, formation of false lobules and increased portal vein pressure; at about the 8th wk of the experiment, a typical HPS formed spontaneously with further increased portal vein pressure and overt ascites; (4) regarding to its applicable range, the model can be applied in studying pathogenesis of liver cirrhosis, HPS and other cirrhotic complications. Clinically while evidence is lacking it would be very interesting to know whether patients suffering from both cirrhosis and HPS have been exposed to complex pathogenic factors such as eating a high fat diet or using corn flour as their life time diet, abuse of alcohol, accidental contact of CCl₄ or other toxins. Such association could provide strategies for the prevention of cirrhosis and its complications. On the other hand, in terms of diagnosis, the possibility of IETM and liver injury should be examined in patients who suffer from dyspnea and hypoxia. In summary, the model that we have established by employing complex pathogenic factors is a non-invasive and practical model that reproduces the clinical context of HPS.

The Kupffer cells located in the liver exert a crucial role in clearing pathogenic organisms and their toxins from the portal circulation. During development of cirrhosis, on the one hand, the drained portal blood was obstructed that resulted in blood stasis and swelling of mucosa, weakened intestinal movements and decreased secretion of bile, which would lead to a bacterial overgrowth in the lumen of the bowel, particularly a profusion of Gram-negative enteric organisms, and overproduction of endotoxin; on the other hand, impaired mucosal barrier and decreased function of hepatocytes and Kupffer cells could cause an invasion of enteric organisms/endotoxin into blood and a formation of bacteremia and IETM. Endotoxin itself, in return, destroyed mitochondria and lysosome in enteric epithelial cells leading to cell autolysis. Ultimately, a vicious cycle could be formed between IETM and an increased permeability of enteric mucosa. In our present studies, there were significantly increased endotoxin and TNF- α in plasma and a rise in numbers of colonies of the organisms accompanied by a positive Gram-negative staining in the mesenteric lymph node in rats with HPS, suggesting that IETM is involved in the development of experimental HPS in rats. The observations in our present study were consistent with the fact that various infections can worsen the patient in the advanced stage of chronic liver disease.

Clinically, many patients in the advanced stage of chronic liver disease often develop a rapid deterioration of illness due to some inducing factors, in which various infections are one of the commonest inducing factors. In order to further explore the role of endotoxin in the pathogenesis of HPS, we designed an animal group for administering exogenous bacterial LPS so as to increase the levels of plasma endotoxin on the basis of cirrhosis, mimicking possible aggravated endotoxemia clinically in the advanced stage of chronic hepatic diseases. Also, we designed additional groups of cirrhotic rats with injection with bacterial LPS plus glycine, an agent for antagonizing endotoxin that can block the function of endotoxin^[36-42], to compare with. As a result, almost all pathologic alterations are further aggravated toward a vicious direction and pulmonary edema emerged after the administration of

bacterial LPS in cirrhotic animals, whereas the precondition of glycine strikingly antagonized the effect of bacterial LPS, which firmly demonstrated that IETM play a crucial role in the pathogenesis of HPS.

In addition, we observed increased production of TNF- α which might result from hypoxia. TNF- α could exert an important role in the injury of intestinal barrier function, which results in bacteremia and IETM. IETM could cause intrapulmonary vasodilatation and hypoxemia. In return, hypoxemia can exacerbate defective intestinal barrier by inducing some cytokine production (such as TNF- α)^[43]. As such, a vicious circle between hypoxemia and impaired enteric barrier function makes HPS further develop and deteriorate.

Finally, we also observed massive accumulation of giant macrophages in the alveolar spaces and an architecture of widened alveolar wall accompanying enlarged blood vessel cavity and increased numbers of blood capillaries. The angiogenesis and vasculogenesis could be due to LPS, TNF- α , or hypoxia-induced increased expression of inducible nitric oxide synthase (iNOS) and heme oxygenase 1 (HO-1) which produce NO and carbon monoxide (CO) respectively in the macrophages^[11,12]. In future studies, it would be interesting to know whether alterations of NO and/or CO occur and whether inhibition of iNOS or HO improves the experimental HPS in this model.

In conclusion, our data demonstrate that: (1) a new HPS model, which reproduces the human features of HPS, is established by employing noninvasive and multiple pathogenic factors in rats; (2) the model can reflect clinical causes to a great extent; and (3) IETM and its accompanying cytokine, such as TNF- α , may play a crucial role in the pathogenesis of HPS.

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