Clinical significance of fibrotic, haemostatic and endotoxic changes in patients with liver cirrhosis

C.J. Li[1], Z.H. Yang[2], F.G. Lu[1], X.L. Shi[1], D.L. Liu[1]

(1) Department of Gastroenterology, the Second Xiangya Hospital of Central South University, Changsha 410011, Hunan Province, China ; (2) Department of Medicine, the Hospital of National University of Defence Technology, Changsha 410073, Hunan Province, China.

Abstract

Background and study aims : **To investigate the relationship among fibrotic, haemostatic and endotoxic changes in patients with different degrees of liver cirrhosis.**

Patients and methods : **Liver fibrotic markers, including hyaluronic acid (HA), Ceollagen IV (Col-IV), laminin (LN), and N-terminal pro-peptide of collagen type III (PIIINP), were determined by radioimmunoassay. A series of haemostatic tests, including prothrombin time (PT), international normalized ratio, activated partial thromboplastin time, antithrombin-III, thrombin time, fibrinogen, fibrin(ogen) degradation product and D-dimer were determined using an automatic coagulation analyszer. Plasma levels of endotoxin were detected quantitatively using an endotoxin detection kit. Correlation analysis of the data was performed.**

Results : **Based on Child-Pugh classification, statistically significant differences in fibrotic markers and haemostatic parameters were found in 249 patients with liver cirrhosis, while no significant differences in endotoxin levels were observed. Based on ascites classification, statistically significant differences in fibrotic markers (such as HA,−Col-IV and PIIINP, except for LN) and haemostatic parameters were found. As for endotoxin levels, there were significant differences between the ascites, spontaneous bacterial peritonitis (SBP) and no-ascites groups, while no significant differences were observed between the ascites and SBP groups. Correlation analysis demonstrated some correlation among fibrotic markers, haemostatic parameters and endotoxin.**

Conclusions : **A close relationship exists between the severity of cirrhosis and fibrotic changes, as well as haemostatic changes. Endotoxin may be an important contributing factor to the development of ascites in cirrhosis. Some correlation may exist between fibrosis, haemostatic and endotoxin.** (Acta gastroenterol. belg., **2018**, 81, **404-409**).

Keywords : Liver cirrhosis, Liver fibrotic markers, Haemostatic parameters, Endotoxin, Child-Pugh grade.

Introduction

Liver cirrhosis is common in China. Traditionally, liver biopsy has been the "gold standard" for diagnosing cirrhosis. However, liver biopsy is limited in clinical practice. The development of non-invasive and easy serological tests for early diagnosis and dynamic monitoring of liver cirrhosis is of great clinical significance.

Prognosis of cirrhosis differs significantly among patients for various reasons. Abnormal coagulation is an important factor. In patients with decompensated cirrhosis, liver function is seriously damaged, with increased susceptibility to bacterial infection and severe endotoxemia. Intestinal endotoxins can directly damage liver cells and induce them to release thromboplastin-like substances, which enter the blood circulation and activate the exogenous coagulation system (1). Endotoxins can also directly activate factor XII and activate the endogenous coagulation system. Endotoxins can damage the vascular endothelium and expose collagen, leading to disorders of coagulation and the anticoagulation mechanism, resulting in hyperfibrinolysis (2,3).

In the present study, to investigate the relationship among fibrotic, haemostatic and endotoxic changes in patients with different degrees of liver cirrhosis, we measured the levels of liver fibrotic markers, haemostatic parameters and endotoxin levels, and compared them with the degree of liver failure.

Patients and methods

Patient selection criteria

We included patients who were admitted to the Second Xiangya Hospital of Central South University, China, from March 2013 to September 2015, and given a diagnosis of liver cirrhosis. We excluded patients with severe diseases (such as cardiovascular, cerebrovascular, haematologic, respiratory, and urinary disease, psychosis and diabetes), malignancies (such as hepatocellular carcinoma), with liver transplant, portal hypertension shunting or devascularisz̈ation surgery, endoscopic therapy, haemorrhagic events, thromboembolic disease, those taking drugs that affect coagulation, or blood products. The Ethics Committee of the Second Xiangya Hospital of Central South University, Changsha, China, approved this study.

General demographics

A total of 249 patients (195 males and 54 females) fulfilled the selection criteria. Their ages ranged from 16-87 years, with a median age of 49 years. Patient data were collected and analyszed retrospectively. According

Correspondence to : Chen-Jie Li, Department of Gastroenterology, the Second Xiangya Hospital of Central South University, Changsha 410011, Hunan Province, China.
E-mail: chenjielimao@126.com

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to etiology, 151 patients had cirrhosis caused by hepatitis B virus, 18 by hepatitis C virus, 12 by alcohol, 28 by hepatitis B or C virus combined with alcohol, and 40 by other factors (including primary biliary cirrhosis, Wilson's disease and cryptogenesis). Based on the Child-Pugh classification system, 37 patients with cirrhosis were class A, 76 were class B and 136 were class C. Clinical (ascites and encephalopathy) and laboratory (albumin, bilirubin, and prothrombin time) parameters were determined to assess the degree of liver failure. Seventy-nine patients were included in the no-ascites group, 102 in the ascites group and 68 in the spontaneous bacterial peritonitis (SBP) group based on ascites (whether with ascites or infection). The patients in each group were similar in terms of age, sex and etiology of cirrhosis.

Blood collection and testing

Blood was drawn by venipuncture in the morning after an overnight fast. Liver function tests were determined using an automatic biochemical analysis. Liver fibrotic markers, including hyaluronic acid (HA), Collagen IV (Ceol-IV), laminin (LN) and N-terminal pro-peptide of collagen type III (PIIINP) were determined using a semi-automatic luminescence immunoassay analysis (JETLIA-962). A series of haemostatic tests, including prothrombin time (PT), international normalised ratio (INR), activated partial thromboplastin time (APTT), antithrombin-III (AT-III), thrombin time (TT), fibrinogen (FIB), fibrin(ogen) degradation product (FDP) and D-dimer, were conducted using an automatic coagulation analyszer. Plasma levels of endotoxin were detected quantitatively using an endotoxin detection kit.

Statistical analysis

Data are described by medians and quartiles because normal distribution of the parameters was improbable. Differences in the results were tested for significance using the Kruskal Wallis test. Correlations among the liver fibrotic markers, haemostatic parameters and endotoxin levels were assessed using the Spearman's rank order method. Statistical analysis was performed using SPSS version 18.0. A P value < 0.05 was considered statistically significant.

Results

Liver fibrotic markers, haemostatic parameters and endotoxin levels in relation to Child-Pugh classification of cirrhosis

The markers of liver fibrosis HA, Ceol-IV, LN and PIIINP gradually increased significantly from Child-Pugh class A to C. The haemostatic parameters PT, INR, APTT, TT, FDP, and D-dimer gradually increased from Child-Pugh class A to C, while AT-III and FIB gradually decreased. These differences were significant. Plasma levels of endotoxin in patients with cirrhosis were 0.071, 0.109 and 0.108 EU/mL in Child-Pugh class A, B, and C, respectively. The levels in patients with Child-Pugh class B and C were higher than in patients with class A cirrhosis, but the differences were not significant (Table 1) .

Liver fibrotic markers, haemostatic parameters and endotoxin levels in relation to ascites classification of cirrhosis

The markers of liver fibrosis HA, Col-IV, and PIIINP gradually increased from no-ascites to ascites, and then to SBP, with significant differences. However, the differences in LN in each group were not significant. The haemostatic parameters PT, INR, APTT, TT, FDP and D-dimer gradually increased from no-ascites to ascites, and then to SBP, while AT-III and FIB gradually decreased. The differences were significant. There were significant differences in the plasma levels of endotoxin

Table 1. — **Liver fibrotic markers, haemostatic parameters and endotoxin levels in the Child-Pugh classification of cirrhosis**

Group	Child-Pugh A (*n*=37)	Child-Pugh B (*n*=76)	Child-Pugh C (*n*=136)	*P* value^{+}	*P* value^{++}	*P* value^{+++}	*P* value*
HA	166.00(115.25,208.00)	338.50(232.75,496.00)	517.50(258.50,990.00)	0.000	0.010	0.000	0.000
Col-IV	130.00(81.00,206.63)	259.20(143.30,347.68)	370.00(269.98,543.68)	0.000	0.000	0.000	0.000
LN	46.30(24.25,194.75)	293.50(40.15,679.00)	210.50(57.50,592.08)	0.003	0.678	0.004	0.007
PIIINP	8.75(5.25,15.00)	16.90(11.10,30.50)	21.75(13.75,34.00)	0.000	0.036	0.000	0.000
PT(s)	14.00(13.25,14.90)	16.10(14.73,17.3)	21.20(19.10,26.08)	0.006	0.000	0.000	0.000
INR	1.14(1.07,1.24)	1.35(1.22,1.46)	1.86(1.63,2.50)	0.005	0.000	0.000	0.000
APTT(s)	45.20(40.95,48.40)	47.90(43.00,52.10)	56.95(50.30,69.60)	0.068	0.000	0.000	0.000
AT3(%)	61.00(52.25,70.73)	42.00(33.00,52.75)	26.10(17.00,37.00)	0.001	0.000	0.000	0.000
TT(s)	18.45(16.93,20.08)	18.30(17.10,19.98)	20.20(18.53,21.88)	0.881	0.000	0.000	0.000
FIB(g/L)	1.95(1.78,2.32)	1.85(1.51,2.27)	1.40(1.12,1.76)	0.180	0.000	0.000	0.000
FDP(ug/mL)	1.38(1.10,1.84)	2.70(1.56,5.42)	7.30(4.10,13.30)	0.000	0.000	0.000	0.000
D-dimer(ug/mL)	0.37(0.28,0.56)	1.26(0.52,2.81)	2.86(1.73,4.67)	0.000	0.000	0.000	0.000
Endotoxin(EU/mL)	0.071(0.041,0.191)	0.109(0.588,0.253)	0.108(0.663,0.194)				0.174

Note : + Child-Pugh class A cases *vs* class B cases. ++ Child-Pugh class B cases *vs* class C cases. +++ Child-Pugh class A cases *vs* class C cases. * Compares Child-Pugh class A, B and C cases. All scores expressed as median with interquartile range.

Table 2. — **Liver fibrotic markers, haemostatic parameters and endotoxin levels in the ascites classification of cirrhosis**

Group	No-ascites group (*n*=79)	Ascites group (*n*=102)	SBP group (*n*=68)	*P* value^{+}	*P* value^{++}	*P* value^{+++}	*P* value*
HA	276.00(150.00,649.00)	380.50(206.75,715.00)	453.00(262.00,733.50)	0.026	0.523	0.009	0.019
Col-IV	236.10(113.00,380.00)	310.00(197.18,413.75)	383.00(288.25,551.70)	0.005	0.000	0.000	0.000
LN	256.50(38.48,639.65)	197.50(52.25,585.00)	259.00(52.50,700.00)				0.941
PIIINP	14.60(8.15,22.25)	21.00(12.23,32.83)	20.90(14.50,31.35)	0.001	0.430	0.000	0.000
PT(s)	15.70(13.70,17.80)	18.90(16.50,21.33)	22.25(17.90,27.78)	0.000	0.005	0.000	0.000
INR	1.26(1.10,1.48)	1.61(1.38,1.87)	2.06(1.50,2.63)	0.000	0.005	0.000	0.000
APTT(s)	47.90(43.90,52.10)	53.10(46.88,62.35)	55.90(48.60,69.60)	0.000	0.197	0.000	0.000
AT3(%)	46.15(33.00,62.50)	34.00(23.20,42.00)	22.10(16.43,37.10)	0.000	0.007	0.000	0.000
TT(s)	18.60(17.10,20.25)	19.40(18.20,21.08)	20.50(18.75,21.93)	0.010	0.018	0.000	0.000
FIB(g/L)	2.01(1.60,2.33)	1.53(1.26,1.80)	1.39(1.11,1.85)	0.000	0.413	0.000	0.000
FDP(ug/mL)	1.60(1.29,2.58)	5.80(3.20,11.40)	9.20(4.80,19.30)	0.000	0.011	0.000	0.000
D-dimer(ug/mL)	0.52(0.31,1.13)	2.63(1.32,3.61)	3.43(2.02,5.41)	0.000	0.009	0.000	0.000
Endotoxin(EU/mL)	0.073(0.041,0.135)	0.118(0.072,0.207)	0.117(0.068,0.333)	0.000	0.716	0.000	0.003

Note : + No-ascites cases *vs* ascites cases. ++ Ascites cases *vs* SBP cases. +++ No-ascites cases *vs* SBP cases. * Compares no-ascites, ascites and SBP cases. All scores expressed as median with interquartile range.

Table 3. — **Relationship among liver fibrotic markers, haemostatic parameters and endotoxin levels in cirrhosis**

	Levels of endotoxin	
	r_s	*P* value
HA	-0.026	0.699
Col-IV	0.145	0.029
LN	0.111	0.095
PIIINP	-0.002	0.970
PT	0.100	0.116
INR	0.104	0.103
APTT	0.048	0.450
AT-III	-0.106	0.119
TT	0.065	0.315
FDP	0.088	0.185
FIB	-0.047	0.474
D-dimer	0.162	0.012

$P < 0.05$ was considered to be significantly different.

in patients with cirrhosis in the no-ascites, ascites and SBP groups. However, further analysis showed significant differences between the ascites, SBP and no-ascites groups, while no significant differences were observed between the ascites and SBP groups (Table 2).

Relationship among liver fibrotic markers, haemostatic parameters and endotoxin levels in cirrhosis

Correlation analysis of the entire cirrhosis group showed a significant positive relationship between plasma levels of endotoxin and haemostatic parameters D-dimer (rs=0.162, *P*=0.012), as well as plasma levels of endotoxin and liver fibrotic marker Col-IV (rs=0.145, *P*=0.029). HA was significantly correlated with haemostatic parameters except FDP, and PIIINP was significantly correlated with haemostatic parameters except FIB. LN was significantly correlated with haemostatic parameters AT-III and FDP. Col-IV was significantly correlated with all the haemostatic parameters(Tables 3 and 4).

Discussion

Liver cirrhosis is a chronic, progressive and diffuse liver disease that develops from liver fibrosis. Serum HA, LN, PIIINP and Col-IV are four common markers of liver fibrosis (4). Hyaluronic acid (HA) is a glucosamine polysaccharide that is widely present in the extracellular

Table 4. — **Relationship between liver fibrotic markers and haemostatic parameters in cirrhosis**

	HA		Col-IV		LN		PIIINP	
	r_s	P value	r_s	P value	r_s	P value	r_s	P value
PT	0.391	0.000	0.524	0.000	0.123	0.063	0.314	0.000
INR	0.388	0.000	0.526	0.000	0.123	0.064	0.327	0.000
APTT	0.304	0.000	0.453	0.000	0.104	0.120	0.323	0.000
AT-III	-0.321	0.000	-0.507	0.000	-0.242	0.001	-0.310	0.000
TT	0.246	0.000	0.344	0.000	0.115	0.088	0.296	0.000
FDP	0.112	0.106	0.274	0.000	-0.190	0.006	0.139	0.045
FIB	-0.148	0.029	-0.207	0.002	-0.019	0.777	-0.120	0.077
D-dimer	0.189	0.005	0.355	0.000	-0.120	0.076	0.210	0.002

$P < 0.05$ was considered to be significantly different.

matrix(ECM). During liver tissue damage, the liver mesenchymal cells synthesiszze HA, and the level of HA increases. In addition, HA degradation in sinusoidal endothelial cells decreases, resulting in elevated serum HA levels. In a study of 486 patients with chronic hepatitis C, circulating HA levels were significantly higher in patients with liver cirrhosis than those without cirrhosis (5). LN is a non-collagen glycoprotein in the ECM. In liver fibrosis, a large amount of LN depositse in the interspace of sinusoidal endothelial cells, reducing the permeability of endothelial cells and forming capillaries. In a recent study of 87 patients with chronic hepatitis B, LN's sensitivity for evaluating significant fibrosis was 71.9%, and the specificity was 80% (6). Type III collagen is the main collagen component in ECM. PIIINP is formed by the extracellular type III procollagen, which is cleaved from the C-terminal globular peptide by endopeptidase. It was reported that the sensitivity of PIIINP for detecting cirrhosis was 94%, and the specificity was 81% (7). Col-IV is the main collagen component which constitutes the basement membrane. It proliferates in the early stage of liver fibrosis and depositesaround the sinusoidal space, which plays a key role in the formation of sinusoidal capillaries. Col-IV is an important indicator in early diagnosis of liver fibrosis and determines the severity of liver fibrosis. The results of this study showed that the markers of liver fibrosis, HA, LN, PIIINP and Col-IV, gradually increased from Child-Pugh class A to C, which was consistent with the literature (8,9). In addition, the levels of HA, PIIINP and Col-IV increased with the appearance of ascites. These results may be due to excess pathologic deposition of ECM, especially the sinusoidal capillary involved with Col-IV in liver injury. As the sinus cavity narrows intrahepatic blood flow resistance is increased and portal hypertension develops, which finally results in formation of ascites. The four markers of liver fibrosis have important clinical significance in judging the severity of liver cirrhosis.

A normal liver plays an important role in the regulation of dynamic equilibrium between the coagulation and anticoagulation system. The liver synthesizes most plasma pro- and anticoagulation factors and fibrinolysis factors. In addition, the liver has a function in the clearance and inactivation of activated coagulation factors (10). Patients with cirrhosis have complex haemostatic dysfunction related to reduced synthesis of pro- and anticoagulation factors, decreased clearance of activated coagulation factors, thrombocytopenia, platelet dysfunction, accelerated fibrinolysis and low-grade intravascular clotting (11-13). Our study showed a progressive increase in PT, INR, APTT, TT, FDP and D-dimer and a decrease in AT-III and FIB from Child-Pugh class A to C. This indicated that patients had abnormalities in haemostasis which were closely related to the degree of cirrhosis. Furthermore, in the no-ascites, ascites and SBP groups, PT, INR, APTT, TT, FDP and D-dimer gradually increased, and both AT-III and FIB gradually decreased. This indicated that the haemostatic abnormalities in patients with cirrhosis may be one of the mechanisms involved in the formation of ascites.

Patients with decompensated cirrhosis have increased susceptibility to bacterial infection and severe endotoxemia. Endotoxemia can cause microvascular inflammation in the liver, leading to microcirculation disorders and decreased Kupffer cell phagocytosis. In addition, endotoxins stimulate the release of Kupffer cells into the medium, resulting in a high metabolic state and increased oxygen consumption, ultimately resulting in hypoxia-induced hepatocellular injury (14). Animal models have confirmed that cirrhosis is accompanied by endotoxemia, which further aggravates the liver (15,16). Endotoxin can directly or indirectly cause hepatocyte necrosis or apoptosis, and is an important cofactor of other hepatotoxic substances that can cause liver damage and necrosis (17). By determining plasma levels of endotoxin and liver fibrosis indicators in patients with chronic hepatitis B, Yu GQ *et al.* (18) found that plasma levels of endotoxin gradually increased with Child-Pugh class, and a significant difference was noted among patients with Child-Pugh class A, B and C. Our study showed that plasma levels of endotoxin in patients with Child-Pugh class B and C cirrhosis were higher than in patients with Child-Pugh class A cirrhosis, but the difference was not significant. Cirrhotic endotoxemia is an important factor that causes disease progression and a series of complications (19). Cirrhotic endotoxemia induced by systemic and splanchnic vasodilation and by triggering of the hepatic inflammatory response via tumour necrosis factor(TNF)-α, may aggravate portal hypertension, which may promote bacterial translocation and increase serum endotoxin levels (20). He F *et al.* (21) showed that increased endotoxin in patients with cirrhosis with ascites may be associated with disease severity. Occult infection was present in patients with ascites who had no symptoms of infection. Our study showed that there were significant differences in endotoxin levels in patients with cirrhosis between the no-ascites and ascites groups, suggesting that infection may be an important contributing factor to the development of ascites in cirrhosis. However, there were no significant differences in endotoxin levels in patients with cirrhosis between the ascites and SBP groups, which was probably because some patients with ascites had occult infection.

Furthermore, correlation analysis showed that plasma levels of endotoxin was associated with Col-IV in patients with cirrhosis. Animal experiments have shown that plasma endotoxin gradually increases in the process of liver fibrosis, and the markers of liver fibrosis are positively correlated with endotoxin level (22). Histologic–observation has also shown that plasma endotoxin level is positively correlated with liver collagen level. After injection of endotoxin, the degree of liver fibrosis increased (23). The mechanism of endotoxemia in liver fibrosis might be related to the release of cytokines such as TNF-α, TNF-β, interleukin(IL)-1,

endothelin-1, NO and free radicals by stimulate Kupffer cells. The proliferation of hepatic stellate cells (HSCs) is stimulated by TNF-α, TNF-β, IL-1, etc, which induces excess production of ECM and promotes transformation of resting HSCs into myofibroblasts or myofibroblast-like cells, thus triggering liver fibrosis (24). Endotoxin stimulates the proliferation of HSCs and expression of collagen genes by activating Kupffer cells, thus promoting the development of liver fibrosis.

Correlation analysis has shown that the plasma level of endotoxin is associated with D-dimer in cirrhosis. Dai LL *et al*. (25) proved that the incidence of endotoxemia and levels of endotoxin were closely related to coagulation abnormalities. Kinasewitz *et al.* (26) showed that almost all patients with sepsis had an abnormal coagulation reaction that was manifested as increased procoagulation, decreased anticoagulation and damaged endothelium. Violi *et al.* (2) found a strong association between endotoxemia and high plasma levels of D-dimer, which returned to normal following administration of non-absorbable antibiotics. Villa *et al.* (27) demonstrated that, in patients with cirrhosis, enoxaparin reduced bacterial translocation as improved intestinal microcirculation attenuated the intestinal damage caused by portal hypertension. Endotoxin can directly damage vascular endothelial cells, activate the coagulation system and stimulate the release of various cytokines, particularly TNF, which plays an important role in inducing disseminated intravascular coagulation). Endotoxin can also produce oxygen free radicals and release proteases, which further damage endothelial cells. Endotoxin may also promote platelet aggregation and increase procoagulant activity.

In our study, correlation analysis showed that HA was associated with haemostatic parameters except for FDP. Col-IV was associated with all the haemostatic parameters, LN was associated with AT-III and FDP and, PIIINP was associated with haemostatic parameters except for FIB in patients with cirrhosis. Serum liver fibrotic markers are non-traumatic indicators to determine the degree of liver fibrosis and evaluate the efficacy of anti-fibrosis treatment at present (28). At the same time, patients with chronic liver disease, especially those with cirrhosis, are often associated with intrahepatic hypercoagulability and microcirculation disorders, leading to decline of hepatocyte function, resulting in varying degrees of coagulation dysfunction, and aggravating coagulation disorders with liver function damage (29). Therefore, liver fibrotic markers in patients with cirrhosis have a good correlation with haemostatic parameters.

In summary, liver fibrotic markers have clinical importance for determining the severity of cirrhosis. Patients with cirrhosis have obvious abnormalities in haemostasis that are closely related to the degree of cirrhosis. Patients with decompensated cirrhosis have increased susceptibility to bacterial infection and severe endotoxemia. Endotoxemia is an important contributing factor to a series of complications, including ascites. In cirrhosis, plasma levels of endotoxin are associated with degree of liver fibrosis and coagulation abnormalities, while liver fibrosis is associated with coagulation abnormalities. Therefore, the assessment of liver fibrotic markers, haemostatic parameters and endotoxin levels in patients with cirrhosis has clinical importance. When liver fibrosis, haemostatic abnormalities and endotoxemia occur, timely interventions should be implemented to avoid progression to organ failure.

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