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Stepwise Deterioration of Prothrombin, Factors I, IV but Not Factor XIII in Progressive Stages of Liver Cirrhosis and HCC

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Abstract: This study was designated to investigate the haemostatic changes during the gradual progression of liver diseases. The study included forty patients; thirty of them had liver cirrhosis with different stages (classified according to Childs-Pugh classification) and 10 with hepatocellular carcinoma (HCC). Haemostatic variables including fibrinogen (FI), calcium (FIV), transglutaminase (FXIII), prothrombin time (PT) and platelet count were estimated and compared with the baseline levels of healthy subjects (n=10). The results obtained demonstrated that fibrinogen level progressively decreased and PT prolonged progressively in Child A, Child B, Child C and reached the maxima in HCC patients. Calcium significantly increased in mild (Child A) and moderate (Child B) but not in Child C cirrhosis and HCC patients, whereas FXIII level did not show any change. Some haemostatic variables were correlated with the hepatic markers albumin and bilirubin but not with aminotransferases. The results of this study indicated that the haemostatic abnormalities in fibrinogen, calcium and PT (but not FXIII) go in parallel to the gradual dysfunction of liver.

Key words: Child-Pugh classification, coagulation factors, fibrinogen, FIV, FXIII, HCC, liver cirrhosis and PT

INTRODUCTION

Liver performs a number of functions that are closely related to various blood components, where it manufactures almost all proteins involved in blood coagulation and fibrinolysis. The hepatic reticuloendothelial system also plays an important role in disposing activated coagulation and fibrinolysis-related factors and inhibitors. Mild fibrotic changes in liver tissue may progress into liver cirrhosis. Over years the normal liver architecture is gradually deteriorated and this interferes with blood flow and functions (Clark and Kumar, 1998). The severity of liver cirrhosis is classified according to Child-Pugh score (Pugh *et al*., 1973), depending upon the level of bilirubin, albumin, prothrombin time (PT), presence or severity of ascites and encephalopathy. In some cases, cirrhosis progresses into Hepatocellular Carcinoma (HCC).

A wide spectrum of hematological disturbances is observed in patients with chronic liver disease. The most commonly encountered abnormalities are anemia and bleeding (Solomon, 1994). Acute and chronic liver diseases are invariably associated with coagulation disorders due to multiple causes such as decreased synthesis of clotting and inhibitor factors, decreased clearance of activated factors, quantitative and qualitative platelet defects, hyperfibrinolysis, and accelerated intravascular coagulation. Previous reports have shown that hepatocellular diseases may display decreases in the vitamin K-dependent factors (FII, FVII, FIX, FX), whereas other parameters remain normal. Except for FVIII and vWF, all procoagulant and inhibitory factors are decreased, which is a reflection of impaired protein synthesis. Vitamin K deficiency leads to the production of abnormal vitamin K-dependent factors. The factors lack gamma-carboxy glutamic acid residues in the N-terminal part of their molecules (Mammen, 1992). In addition to vitamin K-dependent coagulation factors, fibrinogen (FI) and (FV) are variably decreased in patients with liver disease (Tripodi, 2006). Calcium (FIV), on the other hand, decreases with the progression of cirrhosis from compensative (Child A and B) to uncompensative stage (Wang *et al*., 2004). Also, some of the components of the fibrinolytic system are altered in the direction of hyperfibrinolysis (high plasma level of tissue plasminogen activator and low level of α_2-plasmin inhibitor), but others are altered in the direction of hypofibrinolysis (low plasminogen and high plasminogen activation inhibitor type 1) (Lisman *et al.,* 2001). FXIII deficiency, however, was found to be rare in patients with liver cirrhosis, however it is associated with a clinical bleeding tendency and an unfavorable prognosis for future hemorrhages and survival (Tacke *et al*., 2006).

These studies and others did not step wisely monitor the haemostatic changes during the gradual deterioration of liver disease. This triggers our interest to follow the level of some coagulation factors in patients with different cirrhotic stages and HCC, and to test the correlation between some haemostatic and the commonly used hepatic markers.

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Table 1: Hepatic serum markers in normal, cirrhotic and HCC groups

Group (Stage)		Bilirubin (mg/dl)	Albumin (mg/dl)	Ascites	Encephalopathy grade
I (Normal)		0.73±0.20	4.42±0.38	No	None
	IIa (Child A)	0.68±0.22	4.1± 0.48	No	none
Cirrhosis	IIb (Child B)	2.13±0.63[a]	3.84±0.26	No to mild	0-1
	IIc (Child C)	4.25±1.17[a]	2.6±0.35[a]	Moderate	1-2
III (HCC)		3.74±0.4[a]	2.72±0.24[a]	Severe	2-4

(a): significant difference of the corresponding group versus the normal group.
(b): significant difference of the corresponding group versus Child A group.
(c): significant difference of the corresponding group versus Child B group.
(d): significant difference of the corresponding group versus Child C group.

MATERIALS AND METHODS

Patients and grouping: The study included 40 patients (30 males and 10 females aged 35-70 years) admitted to the National Institute of Liver, Monofia University in Egypt. The initial presentation proved post-hepatitis cirrhosis in 30 patients and the development of HCC (in 10 patients). According to Child's classification (Pugh *et al.,* 1973), cirrhotic patients were divided into three grades (10 patients each): mild (Child A), moderate (Child B) and advanced (Child C) cirrhosis. Another 10 patients were diagnosed with HCC. During the study period, patients did not receive anticoagulant treatment, and those with active bleeding were excluded. In addition, 10 healthy subjects were voluntarily taken as a normal control. After an ethical committee approved the study protocol, patients were informed and blood samples were collected with anticoagulant (3.8% sodium citrate (1:10 ratio) for fibrinogen or with heparin for the determination of calcium, where plasma was immediately collected by centrifugation and used for haemostatic parameters. Another part of blood was left to clot and serum was recovered by centrifugation and used in other biochemical investigations.

Investigations

Haemostatic variables: Plasma fibrinogen level (FI) was performed based on Clauss (1957) method using the commercially available kit (Technoclone, GmbH, Austria) and following the manufacturer instructions. PT was estimated by thromboblastin with calcium (ThromboMax, DiaMed, Schweiz). FXIII was measured according to Flckensher and Stüber (1991) using Berichrom FXIII reagents. Heparinzed plasma was used to determine calcium concentration according to the method of Faulkner and Meites (1982) using the reagents of Diamond Diagnostics. Platelets were counted (in cells/cm^3) by coulter counter (S-plus STKR, counter electronic Co., Florida, USA).

Hepatic variables: Liver transaminases (ALT, AST), albumin and bilirubin were determined in serum using the commercially available kits following the manufacturers instructions designated for each variable.

Statistical Analysis: Data are presented as mean (±Standard deviation), comparison between groups was performed by ANOVA and Pearson correlation coefficients between variables were assessed by Graphpad software (Graphpad, USA). P Value of less than 0.05 was considered significant.

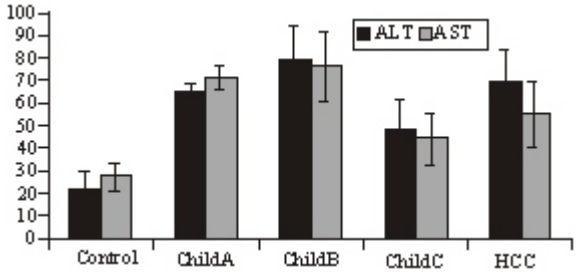

Fig. 1: Mean values of serum aminotransaminases in normal, cirrhotic and HCC patients

RESULTS

Hepatic variables: To assess the integrity of liver architecture and function, serum levels of transaminases, albumin and bilirubin were investigated. Also, abdominal ultrasonography was performed to detect cirrhosis, presence or absence of ascites and/or tumors. Accordingly, patients were categorized into 3 cirrhotic stages (according to the calculated score of Child's system) or HCC. Patients with Child A (group IIa) were encephalopathy none and have no ascites. Serum bilirubin and albumin were normal (0.68±0.22 and 4.1± 0.48 mg/dl, respectively) (Table 1). Higher levels (less than 2-fold increase) of transaminases (ALT and AST) were observed (Fig. 1). Patients in subgroup IIb were encephalopathy none, or with grade 1 with or without ascites. Serum bilirubin (2.13±0.63 mg/dl) was higher and albumin (3.84±0.26mg/dl) was lower than both normal control and Child A groups. Also, these patients had an elevated (about 2-fold increase) ALT and AST. Cirrhotic patients with Child C had advanced grade of encephalopathy (grades 2, 3 or 4) and ascites. Serum bilirubin was 4-fold the normal level (4.25±1.17 mg/dl) and a marked decrease in serum albumin (2.6±0.35 mg/dl) was observed. The average Child's scores of cirrhotic patients in subgroups IIa, IIb and IIc were 5, 7 and 10, respectively. Patients with HCC (gp III) had advanced grade of encephalopathy, ascites, and the levels of both

serum bilirubin and albumin were dramatically deteriorated (3.74±0.4, 2.72±0.24, respectively) similar to the pattern of patients with cirrhosis Child C.

Haemostatic variables: Platelet count was normal in Child A cirrhotic patients (195.5±31.2 cells/cc). Cirrhotic patients with child B and C (gp IIb and IIc) and HCC patients, however revealed a marked thrmpocytopenia (Table 2). The range of PT was 12.40 to 29.10 sec in all patients investigated. The mean levels of Child A, Child B, Child C, and HCC patients were 14.48±2.36, 17.92±3.30, 21.84±6.70, and 20.60±3.85 sec, respectively. Compared with normal group, PT was significantly prolonged in patients with Child C and HCC. Compared to patients with Child A, PT was significantly increased in cirrhotic patients with Child C.

Coagulation factors: Plasma fibrinogen concentration ranged from 0.36 to 3.46 g/l. The variation in fibrinogen level in the patients with liver cirrhosis and HCC is shown in Table 3. Compared to normal control (2.74±0.44 g/l), fibrinogen significantly and gradually decreased, in parallel to the severity of liver disease. In cirrhotic patients (Childs A, B and C) the fibrinogen concentrations were 1.91±0.43, 1.76±0.55 and 0.94±0.30, respectively. Also, HCC patients had lower plasma fibrinogen compared to all stages of cirrhosis (0.99±0.28 g/l). Plasma calcium (FIV) was above the baseline of normal subjects in cirrhotic patients with Childs A and B (14.48±3.1 and 14.88±2.84). Child C and HCC patients, however, showed a normal calcium level (9.41±1.83 and 8.32±1.78, respectively) (Table 3). The range of FXIII was 70 to 140 %, and the average values of normal, Child A, Child B, Child C and HCC patients were: 100.8±16.6, 107.6±13.8, 110.4±17.2, 98.9± 18.08, and 86.80±13.5, respectively.

Also, hepatic variables (albumin and bilirubin) were correlated well with fibrinogen, while PT and platelet count were weakly correlated with calcium (Table 4). Transaminases, however were not correlated with all haemostatic variables investigated.

Table 2: Prothrombin time, INR and platelets

Group (Stage)		PT (sec)	INR	Platelets
I (Normal)		12.83±0.36	1.32± 0.06	232.4±28.5
Cirrhosis	IIa (Child A)	14.48±2.36	1.43± 0.15	195.5±31.2 [a]
	IIb (Child B)	17.92±3.30[a]	2.2± 0.80	86.0±23.1 [a,b]
	IIc (Child C)	21.84±6.70 [a,b]	3.38± 2.05	82.5±14.9 [a,b]
V (HCC)		20.60±3.85[a,b]	3.29±1.30	81.2±12.3 [a,b]

(a): significant difference of the corresponding group versus the normal group.
(b): significant difference of the corresponding group versus Child A group.

Table 3: Fibrinogen, calcium and FXIII levels in cirrhotic and HCC patients

Group (Stage)		Fibrinogen	Calcium	FXIII
I (Normal)		2.74±0.44	9.33±0.73	100.8±16.6
Cirrhosis	IIa (Child A)	1.91±0.43[a]	14.48±3.1[a]	107.6±13.8
	IIb (Child B)	1.76 ±0.55[a]	14.88±2.84[a]	110.4±17.2
	IIc (Child C)	0.94±0.3[a,b,c]	9.41±1.83[b,c]	98.90±18.1
III (HCC)		0.99±0.28[a,b,c]	8.32±1.78[b,c]	86.80±13.5

(a): significant difference of the corresponding group versus the normal group.
(b): significant difference of the corresponding group versus Child A group.
(c): significant difference of the corresponding group versus Child B group.

Table 4: Pearson correlations between hepatic and haemostatic variables in cirrhotic and HCC patients

Variable	FI	FIV(Ca^{2+})	FXIII	PT	Platelet
Albumin	0.721	0.366	0.115	-0.643	0.731
Bilirubin	-0.747	-0.375	-0.112	0.636	-0.727
AST	-0.168	0.427	0.067	0.016	-0.271
ALT	-0.389	0.333	-0.114	0.236	-0.499

Correlation coefficient estimated by Pearson correlation

DISCUSSION

Fibrosis represents the initial stage of histological abnormalities of the liver tissue and occurs next to inflammation. This inflammation activates the hepatic stellate cells (HSC) and triggers the over production and deposition of the extracellular matrix (ECM) proteins, particularly collagen. This leads to the loss of the constitutional blood and oxygen infusion, and subsequently hepatic cells are converted into myofibroblasts (Friedman, 2001). This may represent the initial event that affects the synthetic capabilities of liver cells. There is a long list of factors triggering liver fibrogenesis including chronic infection with hepatitis viruses HBV and HCV (Poynard *et al.*, 2000; 2001). HCV is the most common cause of liver disease in Egypt. Untreated cases may progress into cirrhosis and few percentage of infected patients progress into HCC. This scenario takes years (15–20 years) during which many complications such as ascites, renal failure, hepatic encephalopathy and variceal bleeding may develop. Although the deterioration of the coagulation system in liver disease is well reported, gradual monitoring of haemostatic variables may help to follow the prognosis of liver disease.

Chronic infection with HCV was the underlying factor of the liver failure of the patients investigated. To ensure that, patients were screened for the absence of HBV and schistosomal infections and the involvement of HCV infection was confirmed by RT-PCR. The mechanism through which HCV leads to hepatic dysfunction was repeatedly reported, where the viral proteins usually transactivate many of the host cell genes (Shi *et al.*, 2008). Thus HCV-induced transformation of infected cells reflects the phenotypical changes (seen in chronically infected liver), which progress gradually over years.

Turcotte and Child (1964) and Pugh *et al.* (1973) published and modified a method to assess the operative risk in cirrhotic patients. According to this classification, patients are classified into three grades (Child A, Child B and Child C), which respectively reflect mild, moderate and severe conditions of cirrhosis. The classification based on both haemostatic (PT) and hepatic (albumin and bilirubin) markers, in addition to ascites and/or encephalopathy. Accordingly, the average scores of cirrhotic patients included in this work were 5, 7 and 10. The initial presentation depicted the progressive deterioration of the liver function, which was accompanied by haemostatic abnormalities. Platelets count progressively decreased from normal (gps IIa and

IIb) to marked thrombocytopenia in cirrhotic patients (gp IIc) and HCC patients. Thrombocytopenia is usually due to hyperslpnism in addition to other mechanisms that negatively alter platelet function (Ordinas *el al.*, 1996). Good correlations between platelet count and albumin (r = 0.7) and bilirubin (r = -0.73) were observed.

The formation of fibrin is a central event in the process of coagulation, initially involving the cleavage of four small peptides to produce fibrin monomers (Repke *el al.*, 1990), which spontaneously polymerize (Blombäck and Blombäck, 1972). This polymer is still susceptible to the fibrinolytic enzyme plasmin and requires the enzymatic action of FXIIIa to produce insoluble fibrin. This process reflects the integration between the coagulation factors investigated (FI, FIV and FXIII).

Also, fibrinogen (FI) is an important diagnostic index to follow the dynamics of the disease and may be helpful in diagnosing the haemorrhagic tendencies before they are clinically manifested. In consistence with previous studies (Arif *et al.*, 2002), the data obtained revealed a significant decrease in fibrinogen level in cirrhotic and HCC patients compared to normal subjects. The decrease was progressive, where the fibrinogen decreased by 30, 35.8, 65.7 and 63.9% (of the corresponding normal level) in patients with cirrhosis Childs A, B, C and HCC, respectively (Table 2). This decrease may occur due to the increase of the fibrinogen degradation products. Violi and his coworkers (Violi *et al.*, 1992) have demonstrated that patients with higher fibrinogen degradation products have higher levels of serum bilirubin indicating the association between the severity of liver disease and the low fibrinogen level. Similarly, the data revealed a negative correlation between fibrinogen and serum bilirubin (r = -0.75) and positive correlation with serum albumin (r = 0.72). No correlation, however, was noticed between fibrinogen and aminotransaminases (ATL and AST). The variable fibrinogen levels in cirrhotic patients encouraged some investigators to monitor the response of cirrhotic patients with hyperfibrinolytic activity to epsilon-aminocaproic acid treatment (Hu *et al.*, 2001). Other investigators have used plasma fibrinogen to differentiate liver failure with and without tumor (Miatto *et al.*, 1985). Herein, the levels of fibrinogen were quite similar in both cirrhosis (with Child C) and HCC patients, which minimize the chance of accurate discrimination among such cases. Liver diseases not only alter the concentration of circulating fibrinogen, but also make it functionally abnormal (Martinz *et al.*, 1978). The functional abnormality of the circulating fibrinogen molecule does not necessarily mean that the molecule secreted by the diseased liver is abnormal. It is conceivable that the abnormal liver secretes a normal fibrinogen and it undergoes rapid alteration in circulation due to abnormal plasma environment (Ratnoff and Forman, 1976).

Measurement of PT, on the other hand, reflects the integrity of other haemostatic factors such as FXa, FVa and FIV (Davie *el al.*, 1991). As anticipated, a prolonged PT was observed in parallel to the progression of liver failure. The longest PT was recorded in cirrhosis with Child C and HCC patients (21.8 and 20.6 sec, respectively) compared to 14.4 and 17.9 sec in patients of Child A and Child B, respectively. Since PT value is related to hepatic synthesis of proteins, it is widely used as a surrogate marker of liver function. Also, PT is one of the five items used to calculate Child's classes, the widely used system to assess the severity of liver cirrhosis (Butt *et al.*, 1998). Also, PT is crucially dependent on FVII, whose level in blood is influenced by the liver functional mass. This may explain the prolongation of PT in patients with progressive cirrhosis and HCC (Grimaudo *et al.*, 2005).

The role of trasnglutaminase (FXIII) in the coagulation process is limited to the covalent binding of specific glu residues in one fibrin molecule to lys residues in another. These isopeptide bonds stabilize the clot against proteolytic insult (Weisel, 2005). Although FXIII is generated in liver, in contrast to fibrinogen, it did not show significant variation among patients with mild and moderate cirrhosis, which agrees with previous reports (Klingemann *et al.*, 1978). Patients with Child C and HCC, however, had slightly (statistically insignificant) lower concentrations. The activity of FXIII and the level of its substrates, particularly collagen in the ECM are the limiting factors in the development of hepatic scar. The constitutive existence of FXIII in the ECM (Knittel *et al.*, 1996) fulfills its involvement in matrix assembly (Carmeliet *et al.*, 1998) and (Abdel-Aziz *et al.*, 1990). In our previous work (Mohamed *et al.*, 2005), FXIII was found with higher activity in fibrotic liver indicating the involvement of FXIII in cross-linking process during the early inflammatory stage of fibrosis. In liver the conditions favor the stability of ECM proteins and the development of fibrosis, where the high FXIII activity may be explained by the increased binding of the nuclear factor-kappa-B (NF-kB) to the NF-kB motif of the FXIII promoter (Mirza *et al.*, 1997). Nevertheless the association between FXIII activity and fibrosis may involve other factors such as the transforming growth factor-beta (TGF-b), a major fibrogenic growth factor, where FXIII is known to activate the latent TGF-b1, which in turn led to de novo synthesis of FXIII (Kojima *et al.*, 1993) and (Iredale *et al.*, 1998). Similar to fibrinogen, the activity of the enzyme in the circulation may differ from that in liver.

In addition to the abnormalities in calcium level due to other metabolic disorders, liver disease significantly affects the normal calcium level. Herein plasma calcium significantly increased in patients with mild and moderate cirrhosis. However, in Child C, its level was restored to the normal values and slightly decreased in HCC patients. Earlier studies have reported the deficiency of calcium with the progression of cirrhosis from compensative (Child A and B) to uncompensative

cirrhosis (Wang *et al*., 2004). The increase of calcium in early and moderate cirrhosis was explained by the probable decrease of vitamin D and the reduction of calcium absorption from the gut. This decrease induces the secretion of PTH (secondary hyperparathyroidism), which may increase bone resorption and the subsequent increase of blood calcium, which in turn switches off PTH secretion. This scenario is expected in healthy liver. In early stages of cirrhosis and due to the low clearance efficiency of the liver, PTH remains high and maintains bone resorption. In advanced stages (Child C and HCC), in contrast, the decrease of vitamin D3 leads to a decrease of calcium absorption from the gut. The secondary hyperparathyroidism and osteoporosis are increased due to low levels of serum osteocalcin and decrease bone formation (Duarte *et al*., 2001).

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