

SERUM LEVEL OF IL-6, TNF- α , IL-4, IL-10 IN PATIENTS WITH CIRRHOSIS DUE TO CHRONIC HEPATITIS B

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Summary

Objectives: This study was conducted to evaluate the serum level of interleukin - 6 (IL - 6), tumor necrosis factor α (TNF - α), interleukin - 4 (IL - 4) and interleukin - 10 (IL - 10) in a sample of patients affected by chronic HBV infection in various stage of liver cirrhosis to evaluate differences of the aforementioned cytokines between Child - Pugh classes, as well as between chronic hepatitis B (CHB) patients and healthy controls.

Patients and methods: 78 chronic hepatitis B patients who were divided into three groups according to the Child - Pugh scoring system: group A comprised 24 patients with Child Pugh class A; group B comprised 39 patients with Child Pugh class B; and group C comprised 15 patients with Child Pugh class C. The study also included 10 healthy controls for comparing. Biochemical markers of liver disease were measured by clinical routine methods approved by IFCC. Serum concentrations of IL - 6, TNF - α , IL - 4, and IL - 10 were measured with the Human Cytokine/Chemokine ELISA Kit, Arigo Biolaboratories Corporation, Taiwan.

Results: CHB patients showed statistically significant difference in TNF - α ($p < 0.001$), IL - 4 ($p < 0.01$) and IL - 10 ($p < 0.01$) levels versus healthy controls. Non-cirrhosis patients (Group A) showed statistically significant difference in TNF - α ($p < 0.001$), IL - 10 ($p < 0.05$) levels versus cirrhosis patients (Group B and C). Serum IL - 4 levels were significantly different between group B and group C patients ($p < 0.05$). There was not any correlation between cytokines and biochemical markers of liver cirrhosis.

Conclusions: This study evaluated the serum cytokine levels (IL - 6, TNF - α , IL - 10, and IL - 4) of chronic hepatitis B patients, as well as the differences in such levels between patients and healthy controls. Although no correlations of cytokine levels with biochemical markers of liver disease were observed, serum levels of TNF - α , IL - 4, IL - 10 increase significantly in chronic hepatitis B patient, especially elevating in cirrhosis group (TNF - α , IL - 10); IL - 4 only increased in severe cirrhosis group (group C). TNF - α , IL - 4, IL - 10 but not IL - 6 may be a possible mediator in the pathogenesis of liver fibrosis.

Key words: Fibrosis, cirrhosis, hepatocellular injury, inflammation, cytokine, IL - 4, IL - 6, IL - 10, TNF anpha.

INTRODUCTION

Hepatitis B virus (HBV) infections is a serious global health problems because of their high

morbidity and mortality. It is estimated that 2 billion people worldwide have been infected with HBV, among which 296 million people in the world had chronic hepatitis B infection (CHB) in 2019 (WHO 2021). Chronic HBV infections can have severe consequences including complications, reducing quality of life, disability, costly health care and even death. WHO estimated about 1.5 million new HBV infections and approximate 820 000 related deaths annually, mostly from cirrhosis and hepatocellular carcinoma (WHO 2021). However, the pathogenesis of liver damage during chronic HBV infections is not fully understood. The reasons

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why viral persistence and transformation from acute to chronic infection are not fully understood, but both factors including viral and host characteristics can influence the outcome of the infection^[1]. The host response to hepatitis viruses involves components of the immune system, including cytokines that regulate T-lymphocyte activity. Cytokines are a group of protein molecules, consists of different types of molecules, such as the interleukins, the tumor necrosis factor family, the interferons..., involved in almost biological processes including growth, differentiation, cell survival, hematopoiesis, immunological functions, inflammation, apoptosis, necrosis and fibrosis^[2]. The effects of cytokines are widespread throughout multiple regulatory molecule networks. Cytokines are synthesized by a wide variety of cells, mainly the Th1 and Th2 cells, in which, Th1 cells produce pro - inflammatory cytokines, whereas the Th2 cells produce anti - inflammatory cytokines. Th1 cytokines are involved in cell - mediated immunity, are associated with recovery and play a important role in protection from intracellular pathogens. Th2 cytokines regulate humoral immune responses, are often associated with the development of persistent infections by their high levels^[2]. The liver is a major position for production of cytokines and they are involved in physiologic and pathologic processes in the liver including liver growth and regeneration, inflammatory processes including viral liver disease, liver fibrosis and cirrhosis. They can recognize virus - infected cells and can regulate the immunological and inflammatory responses, viral clearance and tissue damage progression. Alterations of cytokine activities have been observed during HBV infections, while an imbalance of pro - inflammatory and anti - inflammatory cytokine production influences their immune pathogenesis^[1,3]. In particular, alterations in serum and intrahepatic Th1 and Th2 cytokine patterns relates to viral persistence, host immune response, liver damage and liver disease progression from chronic hepatitis to liver fibrosis, cirrhosis or hepatocellular carcinoma^[3,4].

Fibrosis is consequence of cytokines mediator and liver injury, which is defined as an excess deposition of the components of the extracellular matrix (i.e. collagens, glycoproteins, and

proteoglycans) within the liver. In addition to fibrosis, the complications of cirrhosis include portal hypertension, ascites, hepatorenal syndrome, and hepatic encephalopathy (HE)^[5]. Given that cytokines are involved in many pathological processes in the liver which effect on the development of fibrosis, it is of interest to study their levels in chronic hepatitis patients and their alteration between fibrosis Child - Pugh stages.

Our study was conducted to evaluate serum levels of interleukin - 6 (IL - 6), tumor necrosis factor α (TNF - α), interleukin - 4 (IL - 4), and interleukin - 10 (IL - 10) of patients affected by chronic HBV infection compared with healthy controls; and to access differences of the aforementioned cytokines between fibrosis stages of CHB patients base on Child - Pugh score classification.

SUBJECTS AND METHODS

Subjects: A total of 78 hepatitis B patients were selected from patients attending the Outpatient Clinic and/or Inpatient Hepatitis Department at Vietnam National Hospital for Tropical diseases during the period August - December 2020. The study population comprised 17 women and 61 men and their ages ranged from 30 to 72 years (Table 1). Ten healthy individuals of matched age and sex were also selected as a control group. Patients were diagnosed with liver cirrhosis on the basis of clinical examination, ultrasound, and laboratory investigations. Severity of liver failure was assessed according to the Child - Pugh score classification. All patients were counselled to fulfill informed consent to participate in the study as required by Vietnam Medical Ethics Council, and was approved by the Ethical Committee of our hospital.

Clinical and laboratory data: Clinical and laboratory data were collected at time of stable state. Clinical data included age, gender, Child - Pugh score. Laboratory data included blood albumin, bilirubin, enzyme AST, ALT, GGT and INR.

Measurement of TNF - α , IL - 6, IL - 4, IL - 10 concentration: Samples of peripheral venous blood taken at the same time collecting laboratory data were centrifuged at 2500rpm, at 4°C

centrifuge temperature for 10 minutes. The serum was stored immediately at -20°C until cytokines assay. Cytokines concentrations were measured using commercially quantitative and wick enzyme immunoassay (ELISA) according to the manufacturer's recommendations (Human IL - 6/TNF - α / IL - 10/ IL - 4 ELISA Kit, Arigo Biolaboratories Corporation, Taiwan). A monoclonal antibody specific for IL - 6, TNF - α , IL - 10, IL - 4 has been precoated onto a microplate corresponding. Standards or samples are pipetted into the wells and any cytokines present is bound by the immobilized antibody. After washing away any unbound substances, a biotin - conjugated antibody specific for corresponding cytokines is added to each well and incubate. Following a washing to remove unbound substances, streptavidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After washing away any unbound antibody - enzyme reagent, a substrate

solution (TMB) is added to the wells and color develops in proportion to the amount of each cytokines bound in the initial step. The color development is stopped by the addition of acid and the intensity of the color is measured at a wavelength of $450\text{nm} \pm 2\text{nm}$. The concentration of each cytokines in the sample is then determined by comparing the O.D of samples to the standard curve. All samples were coded so that investigator running the assay was blinded as to their source. Results were expressed by mean \pm SD (pg/mL) for all cytokines.

Statistical analysis: The differences between groups of cirrhosis patients and the differences between patients and healthy controls were analyzed by means of hypothesis testing for the equality or not of the means. They were statistical evaluated by Student *t* - test, and *p* values lower than 0.05 were considered significant.

RESULTS

Characteristics of chronic hepatitis B patients and is heathy control in study

Table 1. Routine laboratory data regarding patients and healthy controls

	Heathy control (n = 10)	Group A (n = 24)	Group B (n = 39)	Group C (n = 15)
Age	43.4 \pm 9.60	51.25 \pm 9.71	49.51 \pm 12.53	51.13 \pm 11.07
Gender (M/F)	7/3	13/11	34/15	14/1
AST	21.2 \pm 4.13	61.74 \pm 103.27	451.33 \pm 712.42	139.67 \pm 125.62
ALT	20.1 \pm 8.99	54.29 \pm 76.85	414.80 \pm 687.50	98.73 \pm 175.07
GGT		112.11 \pm 256.07	191.90 \pm 295.68	120.31 \pm 181.87
INR		1.01 \pm 0.05	1.70 \pm 0.46	2.04 \pm 0.67
Bilirubin total		16.64 \pm 14.49	156.61 \pm 147.27	203.96 \pm 183.73
Bilirubin direct		6.73 \pm 11.27	88.57 \pm 97.91	123.10 \pm 128.23
Albumin		45.40 \pm 3.33	32.32 \pm 5.68	27.68 \pm 4.82

Serum Cytokine Levels (TNF - α , IL - 4 and IL - 10) in chronic hepatitis B is significantly higher than heathy control

The levels of TNF - α , IL - 4 and IL - 10 were significantly higher in serum of CHB patients than in healthy control (Table 2) but IL - 6 serum levels were not different between groups. In details, TNF - α (79.49 ± 106.97 vs. $47.85 \pm 26.60\text{pg/mL}$; $P < 0.05$), IL - 4 (6.13 ± 3.36 vs. $4.52 \pm 0.86\text{pg/mL}$; $P < 0.01$) and IL - 10 ($2.21 \pm 6.75\text{pg/mL}$ vs. under detection; $P < 0.01$) levels in CHB group (including group A, B, and C) were more elevated compared with controls.

Table 2. Levels of Cytokines in groups of patients

	TNF - α (mean \pm SD, pg/ml)	IL - 6 (mean \pm SD, pg/ml)	IL - 4 (mean \pm SD, pg/ml)	IL - 10 (mean \pm SD, pg/ml)
Healthy control	47.85 \pm 26.60	113.35 \pm 60.63	4.52 \pm 0.86	<i>Under detection</i>
Chronic hepatitis B (CHB)				
Group B	79.49 \pm 106.97*	112.78 \pm 120.48	6.13 \pm 3.36**	2.21 \pm 6.75**
Group A	35.16 \pm 24.02	105.55 \pm 112.551	5.79 \pm 2.12	0.56 \pm 2.22
Group B	95.68 \pm 106.31	109.8 \pm 120.77	5.41 \pm 3.02	2.89 \pm 7.47
Group C	65.43 \pm 75.35	136.29 \pm 132.59	8.54 \pm 4.54	3.06 \pm 8.82

*p < 0.05, **p < 0.01

Serum TNF - α , IL - 10 levels increased in cirrhosis patients (Group B and C), IL - 4 level increased in severe cirrhosis patients (Group C) comparing with non - cirrhosis patients (Group A).

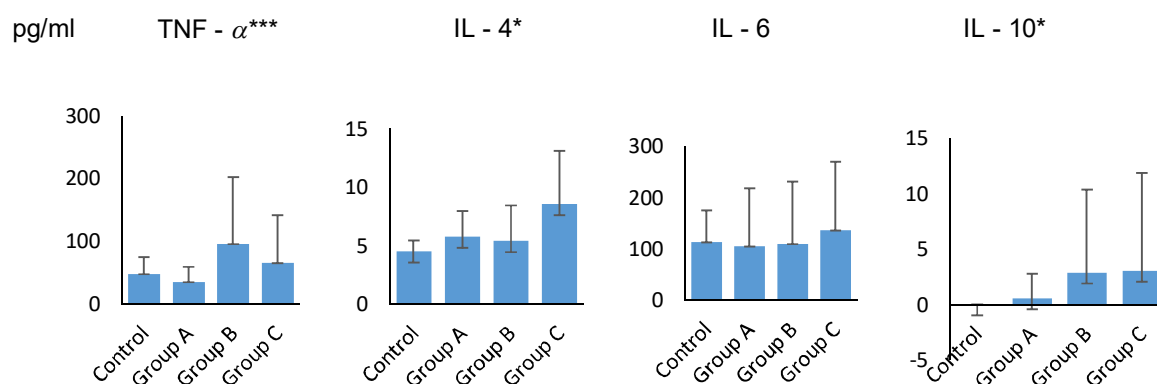


Figure 1. Serum cytokines level in cirrhosis and non - cirrhosis groups

Non - cirrhosis patients (Group A) showed statistically lower in TNF - α (**p < 0.001), IL - 10 (*p < 0.05) levels versus cirrhosis patients (Group B and C). Serum IL4 levels were significantly higher in severe cirrhosis group (C) than medium cirrhosis group B patients (*p < 0.05). IL - 6 was not different between groups.

DISCUSSIONS

Chronic hepatitis B are characterized by inflammatory liver disease of variable severity and increased risk of developing cirrhosis, liver failure and hepatocellular carcinoma. Hepatitis B virus (HBV) is hepatotropic, noncytopathic virus and HBV induced liver injury is mainly regulated by the host immune response against the virus infected cells and by the mediation of inflammatory cytokines^[5]. Changes in various cytokine activities that occur during the inflammatory response against these viruses are responsible for variable degree of liver damage^[5]. Many forms of liver injury are marked by fibrosis, that was an excess deposition of the components

of the extracellular matrix (i.e. collagens, glycoproteins, and proteoglycans) within the liver and finally become irreversible cirrhosis including complications of cirrhosis: portal hypertension, ascites, hepatorenal syndrome, and hepatic encephalopathy^[6]. In this study we examined the serum levels of pro - inflammatory cytokines (TNF - α , IL - 6) and anti - inflammatory cytokines (IL - 4, IL - 10) in chronic hepatitis B patients with different stage of Child - Pugh classification to evaluate the role of serum levels of these cytokines involving in pathological fibrosis development.

In liver physiology, pro - inflammation cytokines such as TNF - α , IL - 6 have critical role

in mechanism of hepatic injury, fibrosis and carcinoma. TNF - α has a dual effect that induce both hepatocyte cell death and hepatocyte proliferation. Circulating TNF - α levels increase during HBV infection and they are associated with severity of inflammation, fibrosis, and injury in liver. Moreover, they are also elevated in patients with HCC. IL - 6 is a pleiotropic cytokine that exerts its complex biological activities through different mechanisms^[7]. Elevated levels of IL - 6 have been associated with morbidity and disease activity in a variety of chronic diseases as well as in chronic hepatitis, liver cirrhosis and hepatocellular carcinoma^[6,7]. In our study, the concentration of TNF - α in the plasma of CHB patients was higher than in the control group ($p < 0.05$), and more elevation in plasma of cirrhosis patients compare with the non - cirrhosis ($p < 0.001$). Our results were in line with several studies that serum levels of TNF - α are significantly elevated in patients with acute and chronic liver diseases regardless of pathological etiologies. Serum levels of TNF - α are significantly higher in patients with cirrhosis than in the non - cirrhosis group, reaching the highest levels in decompensated cirrhosis^[6]. Serum TNF - α levels were not significantly correlated with serum ALT and AST activities. However, IL - 6 levels of CHB patients in this study were not different than in healthy controls, and between non - cirrhosis and cirrhosis groups. Such results have also been reported by other authors and it is concluded that measurement of TNF - α levels reflect liver injury despite normal levels of liver enzymes indicating that this cytokine could be used as a predictor of liver inflammation and involved in pathogenesis of liver fibrosis^[10].

Anti - inflammatory cytokines including IL - 4 and IL - 10 were also evaluated in this study. IL - 4 can suppress HBV gene expression via regulating the expression of viral genes and the innate defense mechanism in liver^[2]. There are contradictory reports about IL - 4 levels in chronic hepatitis patients, some of them have reported lower levels, while others significantly elevated such levels IL - 4 in CHB patients. In our study the concentration of IL - 4 in the sera of CHB patients was higher significant than in the healthy controls ($p < 0.01$) and more elevation in plasma

of severe cirrhosis patients (group C) compared to the non - cirrhosis - group A ($p < 0.05$) or moderate group B ($p < 0.05$). It was not observed correlation of IL - 4 levels with any of the biochemical parameters. This results revealed that IL - 4 may involved in the protective role in inhibition of hepatocellular damage in chronic HBV infection. IL - 10 plays an anti - inflammatory role in the immune system because it inhibits the production of proinflammatory cytokines and limits T cell activation and differentiation^[11]. Due to its immune regulatory action, it has been assumed that inadequate levels of IL - 10 can determine long - term escape of pathogens from immune control and give rise to persistent infections^[11]. Song et al. studied in Vietnamese population have reported low IL - 10 levels in HBV patients and regardless of clinical progression of the infection^[8]. Our finding is in line with many other studies which have reported elevated IL - 10 levels in HBV patients^[10,11]. IL - 10 levels in CHB patients were statistically different from healthy controls ($p < 0.05$). Therefore, we can conclude that the elevated levels of IL - 10 in our CHB patients reflected one side inflammation were progressive, and on the other hand, the protection of hepatocellular from pro - inflammatory induced pathological process were stimulated.

In summary, our study revealed a significant elevation of both a pro - inflammatory (TNF - α), and anti - inflammatory (IL - 4 and IL - 10) cytokines levels in CHB patients. CHB patients showed statistically significant difference in TNF - α ($p < 0.001$), IL - 4 ($p < 0.01$) and IL - 10 ($p < 0.01$) levels versus healthy controls. Non - cirrhosis patients (Group A) showed statistically significant difference in TNF - α ($p < 0.001$), IL - 10 ($p < 0.05$) levels versus cirrhosis patients (Group B and C). Serum IL4 levels were significantly different between group B and group C patients ($p < 0.05$). No correlation between cytokines and biochemical markers of liver cirrhosis was observed. As a general remark we can argue that such alterations reflect the degree of activity of the inflammatory process in the liver, and also the effort to limitation of hepatic injury by anti - inflammatory cytokines.



CONCLUSIONS

Cytokines play an important role in defending the host against HBV, but they have also been implicated in the hepatocellular injury seen in the majority of chronically infected patients. Chronic virus infection with imbalance between different cytokines, leading to prolonged inflammation, hepatocellular injury, fibrosis, cirrhosis and hepatocarcinoma.

Our study evaluated both pro - and anti - inflammatory cytokine levels (IL - 6, TNF - α , and IL - 4, IL - 10) of chronic hepatitis B patients

with various cirrhosis stages. Although no correlations of cytokine levels with biochemical markers of liver disease were observed, serum levels of TNF - α , IL - 4, IL - 10 increased significantly in chronic hepatitis B patient, especially elevating in cirrhosis group (TNF - α , IL - 10); and IL - 4 only increased in severe cirrhosis group (group C). TNF - α , IL - 4, IL - 10 may be a possible mediator in the pathogenesis of liver fibrosis.

Additional studies on a large number of patients should be investigated to observe the changes of cytokine activities.

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