RANTES, TNF-α, Oxidative Stress and Hematological Abnormalities in Hepatitis C Virus Infection.

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ABSTRACT

Chronic infection with hepatitis C virus (HCV) is associated with failures of T-cell–mediated immune clearance and with abnormal B-cell growth and activation.HCV infection is characterized by a systemic oxidative stress that is most likely caused by a combination of chronic inflammation, iron overload, liver damage, and proteins encoded by HCV. Following viral infection, multiple pro-inflammatory mediators contribute to recruitment of immune cells to the liver and to the generation of an anti-viral immune response. Recent publications mark chemokines and their receptors as key players in leukocyte recirculation through the inflamed liver. The present study involved 75 male subjects, included into two groups: Group1 (n=30) control group; group II (n=45) patients with chronic HCV. For all subjects the following investigations were performed: estimation of the levels of bilirubin, albumin, prothrombin concentration, glycosylated hemoglobin (HbA_{1C}), creatinine, α-fetoprotein (AFP), HCV RNA, and activities of alanine and aspartate transaminases (ALT& AST) as well as alkaline phosphatase. Also, Regulated on Activation Normal T Cell Expressed and Secreted (RANTES), tumor necrosis factor alpha (TNF-α), malondialdehyde (MDA) and nitric oxide (NO**)** were assessed**.** Plasma HCV-RNA concentration (viral load) was determined by real time PCR step one using Applied Biosystem. Complete blood picture was assayed using Abbott cell dyn 3700 hematology analyzer. There were significant increase of the levels of RANTES,TNF-α, MDA and NO in HCV infected patients compared with control group ($P < 0.05$) and in these patients, these levels showed significant positive correlation with the HCV RNA viral load. Also, mild leucopenia, thrombocytopenia, neutropenia, lymphocytosis, with consequent significant increase in the lymphocytes / neutrophils (L/N) ratio were detected in these patients.

Conclusion: The data support the concept of chemokines (RANTES) as mediators of liver cell injury in hepatitis C infection. In addition, MDA and NO levels might be used as monitoring markers for oxidative stress in hepatitis C infection.

Key words: *chronic liver disease; RANTES, TNF-α, nitric oxide, MDA, Real time PCR, and complete blood picture.*

INTRODUCTION

The World Health Organization estimates reported that about 3% of the world's population has been infected with hepatitis C virus (HCV), **(WHO)[(1)].**

HCV is a major human pathogen, infecting more than 170 million individuals; approximately 70% of those infected become chronic carriers and are at severe risk of developing liver fibrosis and cirrhosis [2].

The majority of persons with HCV infection progress to chronic infection, which can lead to hepatic fibrosis and the subsequent occurrence of cirrhosis, liver failure, and hepatocellular carcinoma [3].

During many chronic infections, virus spreads rapidly from the site of initial infection to distal tissues. T cells must first become activated in the lymph nodes and spleen and then gain the ability to migrate to infected organs. Chemokines orchestrate all stages of that T cell response from recruitment of naive T cells to inflamed lymphoid tissue, migration of T cells within lymphoid organs, movement of activated T cells from lymphoid tissues to effector sites, and the movement of effector T cells within non-lymphoid tissues [4].

RANTES (regulated on activation normal T cell expressed and secreted) is a chemokine that play a role in immune responses to viral infections. It was originally considered a T cell-specific chemokine, but now it is known to be expressed by other cell types including epithelial cells and platelets[5]. It is a member of the chemokine family with potent chemotactic properties for T lymphocytes, natural killer (NK), memory T cells, esinophils, dendritic cells and monocytes [5-9].

Production of chemokines in the liver is likely to play a role in HCV infection, as infiltration of lymphocytes has been observed in concomitance with chronic disease[10].

Increased levels of circulating TNF-α and TNF receptors have been reported in patients with hepatitis C, although the exact role of that cytokine in the pathogenesis of HCV infection is still unclear [11].

Oxidative stress occurs due to discordance in balance between pro-oxidants and antioxidants. Normally, a rise in oxidative stress concomitantly enhances the anti oxidative activity to protect the cell from damage. Chronic exposure to increased levels of oxidative stress may results in excess of reactive oxygen species (ROS) within the hepatocytes [12].

HCV up-regulates hepatic inducible nitric oxide synthase (iNOS) gene expression leading to liver inflammation, hepatocellular damage & fibrosis[13]. Liver tissue from HCV-infected patients was shown to express elevated levels of iNOS transcripts compared with non–HCV-infected patients [14].

Recently, **Streiff et al.** [15] have reported abnormal peripheral blood count in patients with HCV infection.

The present study aimed to assess the serum levels of RANTES, TNF-α, complete blood count and oxidative stress markers in patients with hepatitis C and to find if they have a relation to the virus load in these patients.

SUBJECTS & METHODS

The present study included 45 male patients with hepatitis C virus (HCV). They were recruited from the Internal Medicine Department of October 6 University Hospital, beside thirty healthy male subjects, matched for age and body mass index, were recruited as a control group. The control subjects were among the

attendants to the Outpatient Clinic for minor surgery i.e. hernia, pilonidal sinus …etc. All patients underwent a thorough clinical examination including history taking, complete medical and laboratory evaluation including a liver ultrasound scan.

HCV infection was diagnosed by clinical as well as biochemical data and by detection of anti-HCV antibodies by **Abbot Axsym system.** Plasma HCV-RNA concentration (viral load) was determined by real time PCR step one using Applied Biosystem with detection limits up to 50 IU/ml of plasma.

Inclusion criteria included patients with positive HCV infection. Exclusion criteria included patients with positive hepatitis B surface antigen, HIV concomitant infection, patients with history of Schistosomasis (rectal snip), prior anti-HCV treatment, recent use of steatogenic or antiviral agents: hepatic decompensation (ascites, jaundice, bleeding varicocele, or hepatic encephalopathy) or history of alcohol intake were also excluded.

None showed clinical or biochemical signs of advanced liver or kidney diseases (their plasma prothrombin time, serum albumin and creatinine were within normal ranges).

Twelve ml. blood were withdrawn from each patient and control subject after 10 hours fasting. Blood was withdrawn in sterile syringe, 1.8-ml from the blood sample was taken on 200 µl. sodium citrate, centrifuged, and plasma was separated and used for estimation of prothrombin concentration by clotting method on ♀Stago STA-Compact analyzer–France. Two ml. of the blood sample was placed in vacuum tube containing K_2 EDTA, one ml. was used as such for estimation of HbA_{1c} [16] using automated Hitachi 911, and complete blood counts which were performed in duplicate within 20 minutes of collection by Abbott cell-Dyn 3700 hematology analyzer. The remaining ml. of the K_2 EDTA sample was centrifuged and the separated plasma was used for estimation of the viral load of HCV in patients using the real time PCR (Figure 1). The remaining part of the blood sample was placed in sterile plain tubes, centrifuged and the serum was stored at −70°C until used for estimation of the levels of total bilirubin, albumin, creatinine, nitric oxide**,** malondialdehyde**,** and the activities of serum alanine and aspartate transaminases (ALT, AST), and alkaline phosphatase using automated Hitachi 911multichannel analyzer (Boehringer Manheim Diagnostics, Indianapolis).

Hepatitis markers (HBsAg [17], anti-HCV- Ab by 3rd generation ELISA) [18] were searched in the same serum sample which was also used for assay of α-Fetoprotein by MEIA method on Abbott Axsym– USA, and RANTES as well as TNF-α which were measured by sandwich ELISA technique using RANTES, TNF-α antigens ELISA kits purchased from Raytech ELISA kits (Helena laboratories, Beaumont, Texas) [19,20].

Statistical Analysis:

Statistical analysis was done using SPSS (Statistical package for social science) program version 17.The Kolmogorov-Smirnov test was used to verify the normal or non-normal distribution of values. The quantitative data were presented as mean ± standard deviation. Unpaired student t test was used for comparison of two groups. The qualitative data

were presented in the form of number and percentage. The Pearson correlation coefficient was used for correlations. Significance was considered with P value <0.05; insignificance was considered when P value was >0.05.

RESULTS

The present study showed significant increase of serum levels of RANTES, TNF-α, MDA and NO in patients with chronic HCV infection compared with control group (Table 1 and figure 2). Further, these parameters showed significant positive correlations with the HCV load and with the activities of ALT (Table 2).Also, total leucocytic, platelets and neutrophils mean counts were significantly lower, while lymphocytes mean count was significantly higher in HCV patients compared to controls (Table 1).

Table (1): Clinical data, liver function tests, RANTES, TNF-α, MDA, NO, and blood picture. In HCV patients versus control group (mean ±SD)

	Control n=30	Chronic HCV n=45	P
Age(years)	41.43±3.2	42.8±5.8	>0.05
Body mass Index (kg/m^2)	24.27± 0.97	24.35±1.30	>0.7
Total bilirubin (mg/dl)	0.84 ± 0.08	1.09 ± 0.16	<0.001*
AST (IU/l)	21.13 ± 3.40	50.0 ±13.6	<0.001*
ALT (IU/l)	22.13 ± 3.06	63.3± 18.2	<0.001*
Alkaline phosphatase (U/l)	90.73± 11.9	95.76 ±17.7	>0.1
Albumin (g./dl)	4.16 ± 0.19	4.03± 0.305	<0.05*
Prothrombin concentration (%)	84.7 ± 4.9	74.4 ±6.29	<0.001*
α-Fetoprotein (ng/ml)	3.96 ±0.54	4.35 ± 0.9	<0.05*
HbA_{1c} %	4.73±0.47	4.88±0.55	>0.2
Hb, g. /dl.	13.77±0.56	13.45±0.81	>0.05
RBCs, 1X10^{6}cell/mm^3	4.7 ±0.49	4.6± 0.99	>0.05
TLC, 1X10^{3}cell/mm^3	5.97 ± 1.16	5.21 ± 1.07	<0.01*
Neutrophils,%	55.30 ± 8.85	51.13 ± 8.53	<0.05*
Lymphocytes,%	32.77 ± 5.41	40.78 ± 7.51	<0.001*
L/N ratio	0.591 ± 0.016	0.795 ± 0.054	<0.001*
Monocytes , %	5.1 ±1.33	5.18 ± 2.40	.>0.05
Esinophils, %	2.92 ±2.3	2.94 ± 2.03	.>0.05
Basophils, %	0.57 ± 0.31	0.59 ±0.41	.>0.05
Platelets, 10^{3}cells / mm^3.	242.33±48.83	169.67±28.11	<0.001*
Creatinine, mg/dl	0.82 ±0.11	0.86±.14	>0.1
RANTES, (ng/ml)	24.97± 12.17	77.27 ±25.45	<0.001*
TNF-α,(pg/ml)	17.17 ± 7.01	49.84 ± 13.07	<0.001*
MDA,(nmol/ml)	4.81 ± 0.87	13.63 ± 1.53	<0.001*
NO, µmol/L)	17.29 ± 2.63	20.49 ± 3.98	<0.01*
HCV-RNA (IU/ml)	undetected	1212860 ± 373662.8	<0.001*

P< 0.05 is considered significant.

Table (2): Correlation between biochemical parameters and HCV RNA viral load among patients with HCV infection.

	RANTES,(ng/ml)		TNF-α(pg/ml)		MDA, (nmol/ml)		NO, (µmol/L)		ALT (IU/ml)	
	r	P	r	P	r	P	r	P	r	P
HCV RNA with	0.608	<0.001*	0.728	<0.001*	0.570	<0.001*	0.625	<0.001*	0.563	<0.001*
ALT with	0.786	<0.001*	0.720	<0.001*	0.666	<0.001*	0.791	<0.001*		

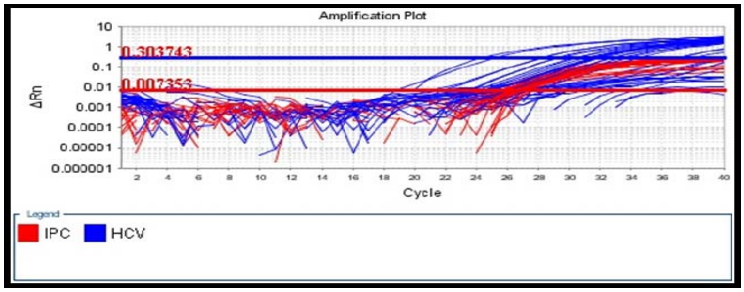

Figure 1: Amplification plot of HCV by real time PCR

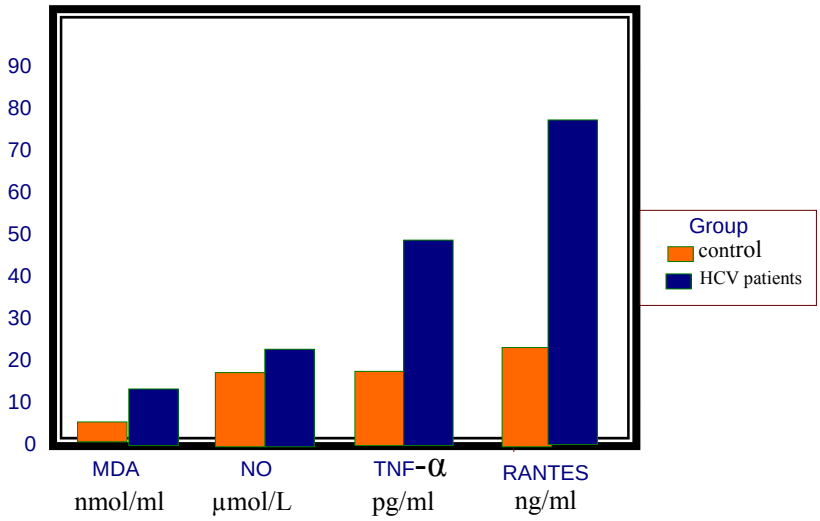

Figure (2): mean serum levels of RANTES, TNF-α, NO and MDA in Chronic HCV patients compared with control group

DISCUSSION

During HCV infection, chronic inflammation, regeneration and fibrosis are the key elements leading to liver dysfunction. Cytokines and chemokines are major regulators of these processes. Therefore, the outcome of HCV infection depends in part on a complex network of cytokine

and chemokine interactions that orchestrate innate and adaptive immune responses to HCV infection[21].

The role of chemokines in chronic hepatitis C virus (HCV) infection is not fully understood[22].**Li et al.** [23] find that Toll like receptor-3 (TLR3) senses HCV infection in cultured hepatoma cells, leading to nuclear factor kappa B (NF-κB) activation and the production of numerous chemokines and inflammatory cytokines, such as RANTES, macrophage inflammatory protein 1α and β (MIP-1α, MIP-1β), inducible protein-10(IP-10) and IL-6.

Although the precise role of TNF-α in the immunopathogenesis of HCV infection is far from clear, it represents a key mediator in determining HCV clearance and hepatitis progression[24].

In the present study, there were significant increase of serum levels of RANTES and TNF-α in patients with chronic HCV infection compared with control group ($P<0.05$).

Elsammak et al.[25] have demonstrated increased levels of serum TNF-α in 27 HCV infected Egyptian patients and these levels showed significant positive correlation with the HCV RNA viral load. Also, **Talaat**[26] has reported elevation of TNF- α levels in 82 HCV-infected patients (20 mild, 20 moderate, and 20 severe cirrhosis patients and 22 patients with HCC)at different stages of disease. A significantly positive correlation between serum levels of TNF- α and grade of disease was recorded.

Hung et al.[27] have shown that HCV infection interferes with the insulin signaling pathway in hepatocytes, increasing the inflammatory response with production of cytokines such as TNF-α and IL-6, and increasing oxidative stress. Also, **Parvaiz et al.** [28] have reported that HCV infection up-regulates the inflammatory cytokine TNF-α.

Yoneda et al. [29] have reported significantly higher levels of serum RANTES levels in 79 Japanese chronic HCV patients before therapy compared to controls.

It was postulated that RANTES was transcriptionally induced in human hepatoma cells by treatment with TNF-α via activation of NF-kappa B and p38 mitogen activated protein (MAP) kinase, presumably suggesting that TNF-α induced expression of RANTES plays important roles in cell-mediated liver injury in liver diseases[30]. Hepatitis C virus was shown to be capable of RANTES gene expression in both non-hepatic and hepatic cell lines[9].

HCV infection was reported to be associated by increased markers of oxidative stress[31]. Under conditions of oxidative stress, as seen in certain chronic inflammatory disorders including hepatitis C, reactive NO Species (RNS), such as peroxynitrite and nitrogen oxides, are currently considered as the main mediators of the deleterious effects to the host of NO, including cytotoxicity and DNA damage [32].

Malondialdehyde (MDA) is the end-product of lipid peroxidation and forms by degradation of the polyunsaturated lipids by ROS.

In the current study, HCV infected patients showed significantly

increased serum levels of stress markers (MDA and NO) and these levels showed significant positive correlation with the HCV RNA viral load.

Ali et al.[33] have shown that serum MDA and NO levels and myeloperoxidase (MPO) activity were significantly higher and the activities of paraoxonase and arylesterase were significantly lower in 23 chronic HCV hepatitis Egyptian patients compared to 21 healthy subjects. **Nakhjavani et al.**[34] have reported that oxidative stress and lipid-peroxidation play a major role in liver injury in chronic HCV infection and that viral load correlated with the serum level of oxidized LDL.

Moreover, **Ozenirler et al.**[35] have reported that oxidative stress increases in chronic hepatitis patients manifested by increases in MDA. However, antioxidant defense mechanisms may not increase sufficiently and accordingly, therefore, the balance destabilizes in favor of oxidative stress

In the present study, the means count of platelets, total leucocytes, as well as neutrophils were significantly lower, and that of lymphocytes was significantly higher, while those of Hb and erythrocytes were not significantly different in patients with HCV infection compared with controls. **Streiff et al.** [15] have reported significant neutropenia as well as thrombocytopenia (TCP), but with no significant difference in the means of Hb and erythrocytes between HCV infected patients and controls. **Louie et al.** [36] have reported that the prevalence of TCP ranged from 0.16% to 45.4% and more than half of the studies reported a TCP prevalence of 24% or more. In the current study, considering TCP as a platelet count $\leq 150 \times 10^3$ /mm^3 [37], 13 of the 45 (28.9%) Egyptian patients had TCP. Such TCP could be attributed to multiple mechanisms including platelets splenic sequestration, bone marrow suppression, and reductions of the level or activity of the hematopoietic growth factor thrombopoietin [38]. Recently, **Martinez-Camacho et al.**[39] have calculated the lymphocytes/neutrophils (L/N) ratio and found that ratio was significantly higher in HCV infection (0.86) than that of HBV infection (0.56) and of African American controls. Lymphocytosis was defined as ratio of lymphocytes to neutrophils (L/N) above 0.6. L/N ratios were calculated to avoid the impact of hypersplenism and constitutional leucopenia seen in African Americans (AA).

In the present study, the calculated L/N ratio was significantly higher in HCV infected patients (0.795) than controls (0.591), $P<0.001$ among Egyptians.

Hemoglobin A_{1c} levels were estimated in the present study to asses the glycemic state of the controls and chronic HCV patients since it is now widely recognized that chronic hepatitis C is a metabolic disease, strongly associated with type 2 diabetes mellitus and insulin resistance [29]

CONCLUSION

High linear correlation of RANTES, MDA, NO and TNF-α with HCV RNA viral load has been

demonstrated and this makes the measurement of these peptides a reliable marker. Oxidative stress was documented in HCV cases manifested by high levels of MDA and NO. HCV testing should be considered for persons with unexplained neutropenia, and thrombocytopenia .L/N ratio was significantly higher in HCV infection, a finding that requires to be confirmed by repeating the assay on large number of cases, and to be performed during and after therapy to ascertain its ability to serve as an inexpensive pre-treatment tool to predict poor virological response to HCV therapy.

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دراسة المنشط الكيميائى رانتيس، معامل تنخر الورم الفا، الأجهاد التأكسدي والتغيرات غير الطبيعية في صورة الدم بين مرضي الإلتهاب الكبدي الوبائي الفيروسي سى

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واقسام الكيمياء الحيوية[٣] والباطنة العامة [٤]- كلية الطب – *جامعة* ٦ اكتوبر

يهدف هذا البحث إلى قياس المنشط الكيميائى رانتيس ، معامل تنحرالورم الفا فى مرضى الالتهاب الكبدى الفيروسى سى ومدى تأثيره علي الجهد التأكسدي عند هؤلاء المرضي

اشتملت هذه الدراسة على ٧٥ رجلا، منهم ٣٠ رجلا أصحاء من المترددين علي العيادة الخارجيه لأجراء جراحات بسيطه مثل الفتاق ولا يعانون من أي أمراض ويمثلون المجموعه الضابطه للبحث . اشتملت مجموعة المرضى على٤٥ رجلا يعانون من الالتهاب الكبدى الفيروسى سى من المرضى المترددين على العيادة الخارجية للأمراض المتوطنة فى مستشفي جامعة ٦ اكتوبر. تم أخذ ١٢سم^3 دم بعد فترة صيام ١٠ ساعات وزعت علي ثلاث انابيب معقمه: الاولى بها ٠.٢ سم^3 سترات الصوديوم ووضع بها ١.٨ سم^3 دم لقياس زمن ونشاط البروثرومبين والثانية تحتوى على K_2EDTA لقياس الهيموجلوبين المسكر وصورة دم كاملة شاملة عد الصفائح الدمويه ، ثم فصل البلازما لقياس HCV-RNA concentration (viral load) باستخدامReal Time PCR.والثالثة لاتحتوى على اى مانع للتجلط لفصل مصل الدم الذي استخدم لقياس المنشط الكيميائى رانتيس ، معامل تنحرالورم الفا، ثنائي ألدهيد المالون ، اكسيدالنتريك وظائف الكبد، الكرياتنين، والفافيتوبروتين وقد تم تشخيص فيروس التهاب الكبد الوبائي من خلال الكشف عن الأجسام المضادة لفيروس الكبد سي ، وتم تاكيد التشخيص باستخدام جهاز Real Time PCR. لقياس تركيزHCV RNA . (viral load)

أظهرت نتائج البحث زيادة كبيرة وذات دلالة احصائية في مستويات المنشط الكيميائى رانتيس ، معامل تنحرالورم الفا ،أكسدة الدهون متمثلة في ثنائي ألدهيد المالون واكسيد النيتريك في المرضى الذين يعانون من فيروس التهاب الكبد الوبائي سي مقارنة بالمجموعة الضابطة للبحث. كذلك اظهرت نتائج البحث علاقة ارتباط إيجابية وذات دلالة احصائية بينهم وبين شدة المرض متمثلة فيHCV RNA(viral load) في مرضى الالتهاب الكبدى الفيروسى.

كذلك أظهرت النتائج تغيرات ملحوظة هامة وذات دلالة احصائية في صورة الدم متمثلة في نقصا في العدد الكلي لخلايا الدم البيضاء ، الخلايا متعادلة الصبغه (Neutrophils)والصفائح الدمويه ، مع زيادة الخلايا الليمفاوية في مجموعة المرضي مقارنة بالمجموعة الضابطة.