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Evaluation of nitric oxide (NO) levels in hepatitis C virus (HCV) infection: Relationship to schistosomiasis and liver cirrhosis among Egyptian patients

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Abstract. Nitric oxide (NO), a recently discovered free radical, is overproduced in liver cirrhosis. Hepatitis C virus (HCV) might increase NO levels via increased inducible NO synthase (iNOS). This work was carried out to study the effect of HCV-induced liver cirrhosis on NO levels among Egyptian patients. The study included 46 patients with liver cirrhosis, and 30 healthy individuals of matched age and sex. NO levels determined as the stable endproduct nitrate, showed a statistically significant increase among patients compared to the control group ($P < 0.001$). Furthermore, NO levels increased proportionally with the severity of liver cirrhosis as assessed by Child's classification ($P < 0.05$). Moreover, schistosomial infection enhanced NO levels in cirrhotic patients with HCV infection compared to non-bilharzial patients ($P < 0.001$). Polymerase chain reaction (PCR) and branched DNA assays were used for detection of HCV RNA positivity, and measurement of the virus load, respectively. Both showed a positive correlation with the NO levels ($P < 0.001$). At a nitrate cutoff value of 70 μmol/L, the sensitivity and specificity were 83.0% and 73.0%, respectively. Chi square analysis showed a significant correlation between ALT levels and both HCV RNA positivity by polymerase chain reaction (PCR) ($P < 0.02$), and virus load ($P < 0.05$). Interestingly enough, there was a significant positive correlation between HCV RNA and schistosomal antibody titer as measured by hemaglutination inhibition assay (HAI) ($P < 0.05$). The data presented in this report indicated an association between NO levels and the development and progression of liver cirrhosis. Furthermore, the findings obtained from this study demonstrated that schistomiasis is an important risk factor involved in enhancement of NO levels and virus replication. The latter may aggravate liver cell injury and hence the development of cirrhosis.

Keywords: Nitric oxide, HCV RNA, liver cirrhosis, schistomiasis

1. Introduction

Patients with liver cirrhosis have several systemic hemodynamic disturbances including hypotention and low systemic vascular resistance. As cirrhosis progresses, vascular resistance continues to decrease and the low arterial pressure may lead to secondary disturbances in renal and hepatic blood flow and development of ascitis [18]. The exact mechanism of these hemodynamic disorders have not yet been clearly elucidated. It has been reported that nitric oxide, was originally discovered as an endothelium-derived relaxing factor [16] may be a causative factor of the hemodynamic disorders in patients with liver cirrhosis. Nitric oxide (NO) is an inorganic free radical synthesized

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from L-arginine by a family of isoenzymes called NO synthases. Two of these are constitutively expressed and a third is inducible by immunological stimuli. NO released by the constitutive enzymes acts as an important signaling molecule in the cardiovascular and nervous systems [24]. NO released by the inducible NO synthase (iNOS) is generated for long periods by the cells of the immune system among others, and has been shown to be cytostatic/cytotoxic for a variety of microorganisms as well as tumor cells [9]. Study of the function of NO in the liver in vitro has shown that NO inhibits total protein synthesis [5], and mitochondrial aconitase activity [23], and stimulates cyclic guanosine monophosphate (cGMP) synthesis and release [2]. NO most likely acts as vasodilator and inhibits platelet aggregation in a slow flow sinusoidal vascular bed, thereby, preventing vessel thrombosis and organ infarction [8]. The endotoxin found in patients with cirrhotic liver may induce nitric oxide synthase directly in blood vessels or indirectly through cytokines leading to an increased synthesis and release of nitric oxide that may account for the hemodynamic abnormalities [13].

Hepatitis C virus (HCV) causes acute and often chronic liver disease. Hepatocellular injury might result from both a host response to inhibit viral spread and from processes initiated by the virus itself [15]. As a chronic inflammatory disease, it frequently leads to the increased production of NO via inducible NOS [11]. The latter effects of released NO might play an important role in the pathogenesis of HCV-induced liver cirrhosis. In this work, the effects of HCV infection on NO release have been investigated. Furthermore, the relationship and potential role of NO release in HCV-induced liver cirrhosis have been addressed.

2. Materials and methods

Forty six patients (group I) presented to the department of Medicine at Ain Shams University hospitals in the period from June 1998 – August 1999 were included in the present study. Thirty healthy individuals (group II) of matched age and sex were selected as a control group. Both groups were subjected to full history-taking and clinical examinaton. Laboratory work-up included liver function testing (alanine amino transferase ALT & aspartate amino transferase AST), renal function testing (BUN and serum creatinine), and measurement of HCV antibodies by 2nd generation enzyme immunoassay (EIA). Hemaglutination inhibition assay (HAI) was used for measurement of schistosomal antibody titer. Abdominal ultrasound, and liver biopsy were performed for all patients to confirm the presence of liver cirrhosis. A written consent was obtained from each patient, and the study protocol was conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

2.1. Measurement of nitric oxide levels

Serum nitrate concentration as a stable endproduct of NO was measured by an endpoint one-step enzymatic assay using nitrate reductase as described by Bories and Bories [3]. The concomitant reduction of nitrate to nitrite by NADPH was monitored by the oxidation of the coenzyme and the decrease in absorbance at 340 nm. All blood samples were withdrawn after overnight fasting (12 hours) to omit nitrates from external sources.

2.2. HCV antibody assay

Patients' sera were analyzed for hepatitis C (HCV) antibodies using second generation microparticle enzyme immunoassay kits (Abbott Co., Chicago, IL, USA).

2.3. Polymerase chain reaction (*PCR*)

Sera were tested qualitatively for HCV RNA using a nested RT-PCR as described by Ravaggi et al. [21]. Positive, negative, and amplification controls were used in each assay. All precautions were taken to avoid contamination [12]. All PCR reagents were purchased from Promega Chemical Company (Madison, WI, USA), and primers were synthesized as described [20,19] using applied Biosynthesis DNA synthesizer and were kindly donated by M. Kotb, Ph.D. at Veterans Administration Medical Center (Memphis, TN, USA). The amplification procedure was carried out in a Hybaid Omnigene thermal cycler (UK). Detection of PCR products was carried out by using 2% agarose gel electrophoresis [22].

2.4. HCV-RNA quantification by branched DNA (*bDNA*) *technology*

DNA QUANTIPLEX^{TM} HCV RNA 2.0 assay is a signal amplification hybridization assay to quantify HCV RNA in patients' sera and plasma [6]. QUANTIPLEX^{TM} HCV RNA 2.0 kits were purchased form CHIRON (Emeryville, CA, USA). After release of the virus genomic RNA, its capture to the microplate

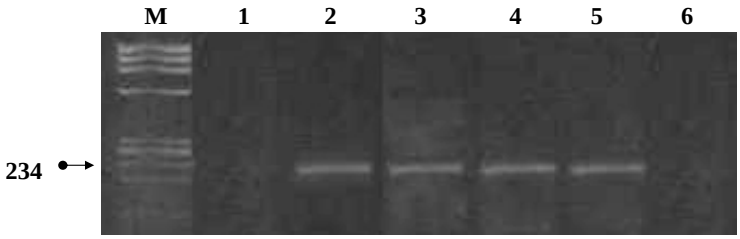

Fig. 1. Agarose gel electrophoresis showing the 2nd PCR product (234 bp) of HCV RNA. M represents $\phi\times$ 174 DNA/ Hae III marker. Lane 1: negative control; lane 2: positive control; lane 3: amplification control; lanes 4 and 5: positive HCV samples. Lane 6 was negative sample for HCV-RNA. The arrow points to the 234 bp band.

wells was mediated by a set of specific, synthetic oligonucleotide target probes. A second set of target probes hybridize to the viral RNA and branched DNA (bDNA) amplifiers. The two sets of target probes bind to the 5′ untranslated and core regions of the HCV genome. Signal amplification was achieved by the alkaline phosphatase-conjugated label molecules which are hybridized to the immobilized complexes. Detection was accomplished by incubating the complexes with a chemiluminescent substrate and measuring the light emission generated by the bound alkaline phosphatase reacting with the chemiluminescent substrate. Results were recorded as luminescent counts by a plate luminometer where light emission was directly proportional to the amount of HCV RNA present in each sample. A standard curve was defined by light emission from a set of four different calibrators with known copy numbers of recombinant bacteriophage. The results were expressed as mEq/ml of duplicate samples with a percent coefficient of variation (%CV) not exceeding 25%. One HCV mEq/ml in the QUANTIPLEX$^{\mathrm{TM}}$ HCV RNA 2.0 assay (bDNA) is equal to the luminescence generated by 10^6 molecules of an HCV RNA transcript. Viral concentration from 0.2–1.2 mEq/ml was considered as low viremia, 1.2–12.0 mEq/ml represented moderate viremia, and 12.0–120.0 mEq/ml was considered as high viremia.

2.5. Statistical analysis

Univariate analyses were performed using a Chi square test of association or Fisher's exact model to test the association of categorial variables, whereas a t test was used for continuous variables. Correlation coeffecient analysis was done to study the correlation of different parameters. Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) software. A cutoff value for serum nitrates was determined using receiver operating characteristic (ROC) curve, area under the curve was calculated according to Zweig and Campbell [25].

3. Results

The patients' group included 33 males, and 13 females. The mean value of their ages was 47 $\pm$ 7.8 years. They were classified according to Child's classification [4] into Child A (15/46, 32.6%), B (15/46, 32.6%), and Child C (16/46, 34.7%). Fifteen patients (32.6%) were negative for schistosomal Ab, and 31 (67.4%) were positive. Detection of HCV Ab positivity by ELISA showed that 11 patients (23.9%) were positive, and that all of them were also positive for HCV RNA (Fig. 1). Ten patients were positive for both schistosomal Ab and HCV RNA. Moreover, using QUANTIPLEX$^{\mathrm{TM}}$ HCV RNA 2.0 assay (bDNA), 2 patients showed mild viremia, 7 patients showed moderate viremia, and 2 patients had high viremia. On the other hand, the control group included, 16 males and 14 females, the mean value of their ages was 43.5 $\pm$ 10.7 years. All individuals showed normal laboratory findings and were negative for HCV antibodies.

Using nonparametric Mann-Whitney test, the mean ranks of ALT, and AST in patients' group (49.99 range: 9–207 IU/L, and 51.23 range: 15–220 IU/L, respectively) were significantly higher than those of the control group (20.88 range: 8–41 IU/L, and 18.98 range: 8–40 IU/L, respectively).

Measurement of NO levels showed a highly significant increase in the patients' group (123.4 $\pm$ 72.4 μmol/L) compared to the control group (46.5 $\pm$ 38.7 μmol/L) ($t = 5.98$, $P < 0.001$)(table 1). When nitrate levels were correlated with the clinical and pathological parameters of the patients, a statistically significant increase in nitrate levels in relation to the severity of cirrhosis as assessed by Child's classification was found (Table 1). The levels of nitrates showed significant differences between schistosomal and non schistosomal patients. However, no significant difference was shown between patients with mixed infection (schistosomiasis and HCV) than in cirrhotic patients with schis-

Table 1
Serum nitrate levels in relation to the different clinicopathological factors in patients with liver cirrhosis

	Number of patients	Nitrate levels[a] (mean ± SD)	P
Control group	30	46.5 ± 38.7	
Patients' group	46	123.4 ± 72.4	$< 0.001^*$
Cirrhosis			
Child A and B	30	112.3 ± 46.3	
Child C	16	159.8 ± 65.8	$< 0.05^*$
Schistosomiasis			
Non schistosomal	15	71.88 ± 30.7	
Schistosomal Ab +ve	31	152.77 ± 69.12	$< 0.001^*$
Virus load			
Low and moderate viremia[b]	9	99.22 ± 40.31	
High viremia[c]	2	214.5 ± 10.6	$< 0.001^*$
Mixed infection			
Schistosomal Ab +ve & HCV RNA −ve	21	149.62 ± 77.7	
Schistosomal Ab +ve & HCV RNA +ve	10	159.4 ± 49.4	0.719

[a]Nitrate levels were measured in triplicate (mean ± SD), and were expressed in μmol/L.
[b]Virus load (0.2–12 mEq/ml).
[c]Virus load (12–120 mEq/ml).
$^*P$ value was significant at levels < 0.05.

tosomal infection only (Table 1). Nitrate levels were significantly higher in cirrhotic patients with mixed infection than in cirrhotic patients due to other etiologies (159.4 ± 49.4, and 71.2 ± 31.7, respectively, $P < 0.001$). Furthermore, correlating nitrate levels with the virus load in HCV RNA +ve patients using quantitative bDNA, a significant correlation was obtained (Table 1).

The determined cutoff value for nitrates that maximizes the sum of sensitivity, specificity, positive and negative predictive values was 70 μmol/L (Fig. 2). At this level, the sensitivity was 82.6% and specificity was 73.3%. Using this value, chi square analysis showed no significant correlation between nitrate levels and all other parameters studied. However, a significant correlation was found between HCV RNA concentrations and ALT levels ($X^2 = 5.03,\ P = 0.02$), virus load and schistosomal infestation ($X^2 = 3.67$ and $P < 0.05$) (Table 2).

When we classified HCV RNA +ve patients according to their virus load, schistosomal infestation and Child's classification, no correlation was found. On the other hand, a positive correlation was found between the levels of viremia and ALT concentraions ($X^2 = 6.5,\ P = 0.04$) when the cutoff values for ALT was 45 IU/L. Furthermore, linear regression analysis showed no significant correlation between the nitrate levels and any of the other parameters. However, a positive correlation was observed between the levels of ALT and AST ($r = 0.89,\ P < 0.001$).

4. Discussion

The data presentented in this study demonstrated a significant increase in the mean nitrate levels in cirrhotic patients compared to normal controls. Moreover, a significant increase in nitrate mean levels was encountered with advanced cirrhosis as determined by Child's grading [4]. This indicated that the production of endogenous NO was enhanced in patients with liver cirrhosis, particularly, in the decompensated subgroup and confirmed previous data by Hori et al. [10] who reported that increased production of endogenous NO corresponds to the progress of liver cirrhosis. Others reported that plasma NO levels were significantly higher in patients with cirrhosis and hepatocellular carcinoma (HCC) than those with chronic HCV infection without cirrhosis [17]. No statistically significant difference was observed between nitrate levels among schistosomal and non schistosomal patients suffering from liver cirrhosis. However, a significant increase in nitrate levels was encountered in cirrhotic patients with mixed infection compared to those who had a nonviral etiology. These findings match those reported by others [1]. In their studies, the authors relied on measurement of HCV Ab seropositivity for diagnosis of HCV infection. These assays are less specific and, as well, cannot discreminate whether the patient has an active disease or asymptomatically viremic [14]. However, in this work, HCV infection was confirmed by HCV RNA positivity and a significant positive correlation was found between nitrate levels, virus load and the extent of cirrho-

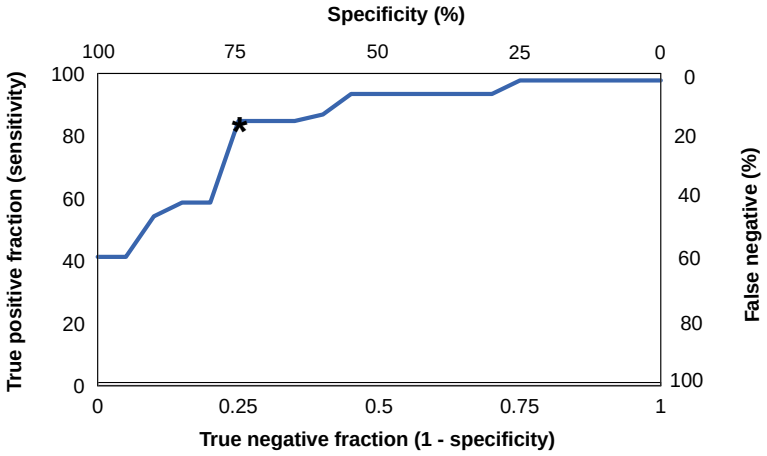

Fig. 2. Receiver Operating Characteristic (ROC) curve for nitrates in patients with liver cirrhosis. The best cutoff (astrisk) for nitrates was 70 umol/L. Area under the curve was 0.8164.

Table 2
The relationship between HCV RNA status and other parameters measured in patients with liver cirrhosis

Parameter	HCV RNA −ve ($n = 35$)	HCV RNA +ve ($n = 11$)	X^2	P
ALT				
< 45.0[a]	23	2		
> 45.0[a]	12	9	5.03	0.02*
AST				
< 41.0[a]	19	3		
> 41.0[a]	16	8	2.44	0.12
Nitrate				
< 70.0[b]	7	1		
> 70.0[b]	28	10	0.69	0.4
Schistosomal Ab				
−ve	14	1		
+ve	21	10	3.76	0.05*
Child grade				
A&B	23	7		
C	12	4	0.016	0.89

[a] ALT and AST were expressed in IU/L.
[b] The determined cutoff for serum nitrate was expressed in μmol/L.
* P value was significant at levels < 0.05.

sis. Furthermore, a cause-effect relationship between increased nitrate levels and the pathogenesis of liver cirrhosis needs to be established.

HCV infection as determined by HCV RNA virus load was significantly correlated with ALT levels. The latter finding illustrates the value of ALT as a significant biological marker in patients with HCV infection. The elevation of ALT levels might be due to virus-induced cytopathic effects on the hepatocytes with subsequent leakage of the enzyme in the blood. HCV RNA positivity was also positively correlating with previous schistosomal infection. This might indicate the impact of schistosomiasis as a major risk factor in HCV infection and furtherly supports similar findings by other investigators who claimed that schistosomiasis increased the prevalence of HCV infection in Egypt [7,14]. Moreover, a significant correlation was observed between the mean serum levels of nitrate and the degree of viremia in HCV RNA positive patients where a significant elevation in nitrate level was obtained in patients with high viremia (Table 2). According to these findings it is tempting to postulate that HCV infection might induce nitric oxide synthase (iNOS) expression both directly, and indirectly by stimulating IFN-γ secretion from immune cells [15].

Although liver cirrhosis due to only schistosomal infestation was not associated with increased nitrate lev-

els over non-schistosomal cirrhosis, we demonstrated, in this study, that nitrate levels were significantly higher in cirrhotic patients with mixed schistosomal and HCV infections, than in patients with non-schistosomal cirrhosis. This indicates that the inflammatory reaction in the cirrhotic liver increases in patients with mixed infection resulting in more iNOS production. Taken altogether, the data obtained from this work support the concept that NO is elevated in cirrhosis and that HCV infection might be responsible for this increase. Moreover, the severity of liver damage may be an important factor. Furthermore, the potential usefulness of NO as a valuable marker for assessment of cirrhotic patients needs to be investigated. The findings from this study may emphasize the need for effective programs for schistomiasis control and prevention of HCV infection and hence liver cirrhosis among Egyptian population.

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