Nitric Oxide Levels in Sera of HCV-Infected Subjects

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Abstract: Hepatitis C virus infection is the leading cause of advanced liver disease. The prevalence rates of infection range from 0.5-2% in the developed world, through 6.5% in parts of equatorial Africa, to as high as 20% in Egypt. Nitric Oxide is a short-living biological mediator generated from L-arginine by NO synthase (NOS) and it possesses a wide range of physiological functions. The present study aimed at the determination of NO levels in sera of HCV-infected patients compared with its levels in sera of non-infected individuals. 250 serum samples from patients with different liver affections were screened for HCV infection including anti-HCV antibodies (HCV-Ab) and HCV antigen (HCV-Ag) using ELISA technique in addition to 30 serum samples from healthy individuals as negative controls. The Detection of anti- HCV-Ab revealed that 150 serum samples are positive. In addition, a total of 30 healthy individuals were negative for anti–HCV Ab. The detection of HCV-Ag revealed that 129 serum sample were HCV-Ag positive while all controls were negative for HCV-Ag. All 129 individuals showing positive anti-HCV and HCV-Ag were classified histopathologicaly into 3 groups: non-cirrhotic liver (n=70), cirrhotic liver (n=23) and hepatocellular carcinoma (HCC) (n=36). HCV-Ag was detected in non-cirrhotic patients, cirrhotic and HCC patients with detection rate 96%, 74% and 94% respectively. In non-cirrhotic patients serum NO was detected in a rate of 91.4%, in cirrhotic patients the rate was 91.3% and in HCC patients it was detected in a rate of 63.8% .There were statistically significant differences between the mean NO concentrations of the different pathological classifications of the liver disorders compared to that of the control group. A significant correlation was shown between the NO levels and the HCV-Ag levels in sera of HCV patients with different pathological classification of liver disorders. It is concluded that the elevated serum NO level accompanies HCV-infection and correlates well with active viral infection indicated by high HCV-Ag levels.
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Introduction

In 1989 the virus responsible for most transfusion-associated non-A non-B hepatitis was identified and cloned, and named hepatitis C virus (Houghton, 1996). Hepatitis C is caused by infection with the hepatitis C virus (HCV), an enveloped, single stranded, positive sense RNA virus (Purcell, 1994; Houghton, 1996). The virus infects liver cells and can cause severe inflammation of the liver with long-term complications (Viral Hepatitis Prevention Board, 1995; WHO, 2002). HCV induces several complex mechanisms that lead to inflammation, insulin resistance, steatosis, fibrosis, apoptosis, altered gene expression, and hepatocellular carcinoma (HCC) (Fartoux *et al.,* 2005; Pekow *et al.,* 2007; Sheikh *et al.,* 2008).

Hepatitis C Virus (HCV) is recognized as a major threat to global public health. An estimated 170 million people worldwide are infected, most of them chronically infected and at risk for liver cirrhosis and HCC (Pybus *et al.,* 2003). Egypt has possibly the highest HCV prevalence in the world; 10%–20% of the general population is infected and HCV is the leading cause of HCC and chronic liver disease in the country (Angelico *et al.,* 1997; Ray *et al.,* 2000; Hassan *et al.*, 2001; Pybus *et al.,* 2003).

Serologic assays for HCV are based on detecting HCV antibodies or HCV RNA. The most common serologic test for detecting antibodies to HCV is the enzyme-linked immunosorbent assay (ELISA) (sensitivity 95%, specificity 95, Attallah *et al.,* 2008). Some laboratories automatically confirm a positive ELISA test by a supplemental recombinant radio-immunoblot assay (RIBA) to increase the specificity of the test (e.g., to decrease the number of false positives). Furthermore, a very small percentage of patients infected with HCV are unable to mount an immune response to the viral protein and do not produce antibody. These false negatives occur in persons with HIV infections, renal failure, and HCV-associated mixed cryoglobulinemia. Confirmation of ongoing infection therefore requires the detection of HCV RNA in blood. HCV RNA is detected by polymerase chain reaction (PCR) using either a qualitative or quantitative assay. These assays may detect a viral count as low as 9.6 IU/L. A negative qualitative test supports the absence of viremia (Strader *et al*., 2004).

Patients frequently come to medical attention based on elevated alanine aminotransferase (ALT) levels, which are indirect markers of liver cell necrosis (Hui *et al*., 2003). Although these measurements have been used to monitor HCV infection and the efficacy of therapy, the recognition that many infected patients may have normal ALT levels has limited their utility. Furthermore, the normalization of alanine aminotransferase levels with antiviral therapy is not proof of successful virus eradication. Viral quantification has, therefore, replaced ALT levels in monitoring treatment response.

Nitric oxide (NO) is a short-living biological mediator generated from L-arginine by NO synthase (NOS). The NOS family of enzymes identified to date includes constitutively expressed endothelial eNOS and neuronal nNOS, as well as inducible iNOS. NO exerts a broad spectrum of physiological functions, including regulation of vascular reactivity, platelet and leukocyte activation, neurotransmission, regulation of cellular proliferation, and nonspecific immunity reactions (de Belder *et al*., 1994). In the liver, NO is generated by eNOS and iNOS, and this generation can mediate a number of physiological and disease reactions involving this organ (Li *et* Billiar 1999). Therefore, the present work was planed to estimate NO levels in sera of HCV-infected patients and non-infected subjects for comparison.

2. Materials and Methods:

Subjects:

250 hepatitis subjects (165 males,85 females) with age range 20-79 years were included in the present study.They attended to the Internal Medicine University Hospital, Mansoura University and blood sample was withdrown from each subject for the preparation of serum. 30 apparently healthy subjects (14 males, 16 females) in the same age range were also included as negative controls for comparison.

Detection of anti- HCV antibodies in sera:

The HCV–Ab ELISA is an immuno-enzymatic method in which the wells of a microtiter plate are coated with HCV-specific synthetic antigens derived from core and NS regions (NS3, NS4 and NS5) representing epitopes of HCV. Diluted serum samples were added to these wells of antibodies specific for HCV present in the sample; they would be captured by the HCV antigen in the wells. After washing out all the other components of the sample, a rabbit anti-human IgG/IgM labeled with horseradish peroxidase was added and, if the antigen/antibody complex was present, the conjugate would bind to the complex. The enzyme captured on the solid phase, acting on the substrate / chromogen mixture, generates an optical signal that is proportional to the amount of anti- HCV antibodies present in the sample**.**

Detection of HCV-antigen in sera:

As a sensitive and specific assay, the ELISA technique of Attallah *et al.* (2008) was used to detect HCV antigen in serum. Diluted serum samples (1: 400) in coating buffer (pH 9.6), were tested (50 µl per well) for hepatitis C virus antigen. In brief, coating ELISA plate was sealed by adding 100 ml/well of blocking buffer and incubated overnight at 2-8 C°. After blocking of free binding sites with 0.5% bovine serum albumin (BSA), the plate was washed 5 times using tap water then a specific polyclonal antibody for HCV diluted in PBS-T20 was added (50 µl per well) and incubated at 37 C° for 2hrs. After washing, 50 µl / well of anti-rabbit IgG alkaline phosphate conjugate diluted in 0.2% BSA in PBS-T20, were added and the plate was incubated at 37 C° for 1hr. The amount of coupled conjugate was determined by incubation with substrate for 30 min. at 37 C°. The reaction was stopped and the absorbance was read at 490 nm using ELISA reader (∑960 Metretech, Germany). The cut-off value was = 0.21.

Measurment of total nitric oxide in sera:

NO under aerobic conditions oxidizes to its inactive and a stable end products namely**,** nitrites (NO^{2-}) and nitrates (NO^{3-}). Firstly nitrates should be converted to nitrites using metallic cadmium then total NO concentration is measured by Griess reagent reaction. Nitric oxide product forms a violet colored complex. 50 µl of serum samples were diluted with 200 µl dist. water then 50 µl of deproteinizing solution were added to complete the volume 300 µl, (dilution 1:6). The diluted samples were vortex mixed and centrifuged at 4000 r.p.m for 5 minutes. The resulting supernatant (230 µl) was then added into Micro-centrifuge tubes containing tensfer reagent. The micro-centrifuge tube was incubated for 2 hours at 37 C°, and re-centrifuged at 4000 r.p.m. for 5 minutes. The supernatant was added into Micro-titer plate using 200 µl/well of the supernatant for each sample. 200 µl of each of dist. H_2O (Blank), 7 serial concentrations of standards and diluted samples were dispensed into the wells of the microtiter plate. 50 µl of color reagent were added to all wells. The contents of the wells were gently mixed by lateral thrumming on the edges of the microtiter plate for 60 seconds. The absorbance values were read at 490 nm in microtiter plate reader. The color intensity is proportional to the amount of NO present in the sample. To calculate the concentration of an unknown sample the absorbance

value of the blank well was subtracted from the absorbances of all wells. A standard curve was plotted using the absorbance value for each standard versus its concentration. The concentration of the unknown samples was determined by interpolation from the standard curve.

3. Results:

Serum samples from 250 hepatitis subjects were screened for HCV infection including anti-HCV-Abs and HCV-Ag using ELISA. 150 serum samples out of 250 were positive for anti-HCV-Abs. In addition, all the healthy individuals included as controls were negative for anti-HCV-Abs. Also 129 out of the 150 (86%) serum samples positive for anti-HCV-Abs were positive for HCV-Ag (Table 1).

The cut-off level of ELISA above or below which the tested serum sample is considered HCV positive or negative was calculated as the mean ELISA optical densities of 8 serum samples from healthy volunteers ±3 standard deviations. The value equals 0.21 and serum samples from 8 HCV infected individuals showed ODs above the cut-off level (Fig. 1).

The 129 individuals showing positive anti-HCV-Ab and HCV-Ag were classified histopathologicaly into 3 groups. Group I includes 70 patients (54.3) with non-cirrhotic liver, group II includes 23 patients (17.8%) with cirrhotic liver and group III includes 36 patients (27.9%) with HCC. HCV-Ag was detected in non-cirrhotic, cirrhotic and HCC patients with detection rates 96%, 74% and 94% respectively.

NO was detected in 108 out of total 129 HCV patients with detection rate 83.7%. All healthy individuals were negative for NO. NO was evaluated in cirrhotic patients with detection rate 91.3%, while in HCC patients it was detected in 23 out of 36 with detection rate 63.9% (Table 2).

Quantitatively non-cirrhotic patients had significantly higher (P=0.048) serum level of NO (36.46±21.15 μM) compared with those subjects considered negative for NO (10.99 ± 1.31 μM). The level of NO in cirrhotic patients (40.17 ± 16.73 μM) was significantly higher (P = 0.027) than that in patients negative for NO (8.11± 0.919 μM). HCC patients had higher significant (P = 0.006) level of NO (38.16 ± 39.63 μM) compared with the corresponding patients negative for NO (7.74 ± 3.84 μM). In addition all healthy controls showed very low serum levels of NO (Table 3).

Highly significant differences (P<0.0001) were found between the mean concentration (μM) of NO of the different pathological classifications of the liver disorders compared to that of the control group. But there are no significant differences among the means of the concentration of NO of the different pathological classifications of liver disorders. Apositive correlation (r = 0.34, P < 0.0001) was shown between the NO levels and the HCV-Ag levels in sera of HCV patients with different pathological classification of liver disorders (Fig. 2).

4. Discussion

The data presented in the present study illustrated that, the differences between mean concentrations of NO, of each class of the different pathological classifications of the liver disorders (non-cirrhotic, cirrhotic and HCC) and the control group, were statistically highly significant. Also no significant differences between mean concentrations of NO among the different pathological classifications of liver disorders were found. This means that the estimation of serum NO can be used to differentiate these liver disorders from controls but it can not differentiate them from each other.

In that respect, Hassan *et al*. (2002) found significant elevation of serum nitrate level in cirrhotic patients compared to controls. These investigators found also that increased serum nitrate levels in cirrhotic patients were more evident in patients with mixed viral infection compared to those with viral etiology.

The present results assured that HCV infection (manifested by detection of either HCV -Ag or HCV-Ab) is usually associated with elevated serum NO level. This is evident from the highly significant positive correlation between levels of HCV -Ag and serum NO concentrations (Fig. 2). It can be, therefore, speculated that HCV infection may induce nitric oxide synthase (NOS) leading to elevated serum NO level in HCV infected hepatitis patients. Previously, Rhman *et al.* (2001) found that the immunologic (type 2) isoform of NOS (i.e. inducible NOS, iNOS) was elevated in HCV-associated HCC. It is well known that iNOS generates NO from L-arginine in inflamed tissues. Since NO is involved in the body's defense mechanism towards HCV infection (Sharara *et al.,* 1997; Weiss *et al.,* 1999), its generation in some organs especially the liver by both eNOS and iNOS was studied by sevsral investigators (Li and Billar,1999; McNaughton *et al.,*2002). It is thought that iNOS expression can be accelerated in HCV infection both directly and indirectly by IFN-γ secretion from immune cells (Hassan *et al.,* 2002).

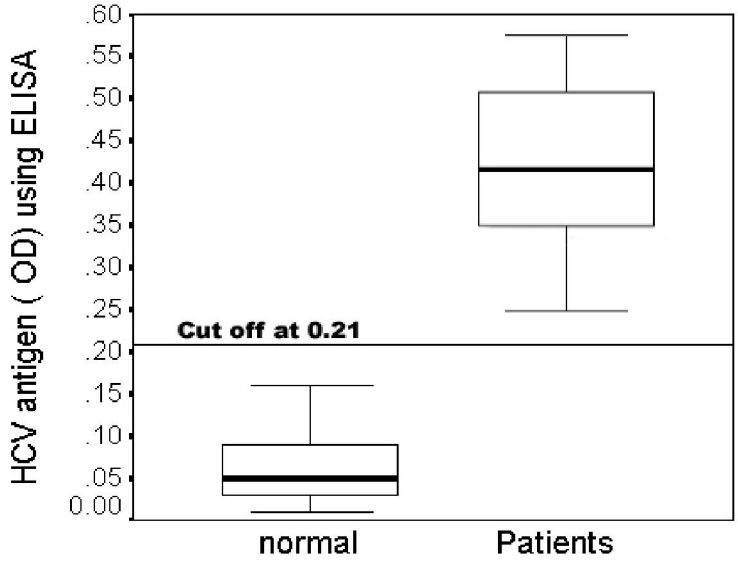

Figure 1: Cut-off level of ELISA HCV antigen (the mean optical density at 490nm + 3 SD).

Table 1: Incidence of HCV-Ab and HCV-Ag in hepatitis patients and control subjects

	30 Controls subjects		250 Hepatitis patients	
	Negative	Positive	Negative	Positive
HCV-Ab	30	0	100	150
HCV-Ag	30	0	21	129

Table 2: Detection of NO in patients with different pathological lassifications of liver disorders:

Groups	No. of case	Serum NO		Detection rate %
		Positive	Negative	
Normal controls	30	0	30	0 %
Group I: Non cirrhotic patients	70	64	6	91.4 %
Group II: Cirrhotic patients	23	21	2	91.3 %
Group III: HCC patients	36	23	13	63.9 %

Table 3: Levels of NO in sera of patients with different pathological states of liver diseases:

Groups	Serum NO				P value
	Negative		Positive		
	No / Total	M ± SD*	No / Total	M ± SD*	
Normal controls	30/30	8.10±3.88	0/30	-	-
Non-cirrhotic	6/70	10.99±1.31	64/70	36.46±21.15	0.048
Cirrhotic	2/23	8.11±0.919	21/23	40.17±16.73	0.027
HCC **	13/36	7.74±3.84	23/36	38.16±39.63	0.006

* : Mean ± standard deviation.
**: Hepatocellular carcinoma. (µM): Micromolar.

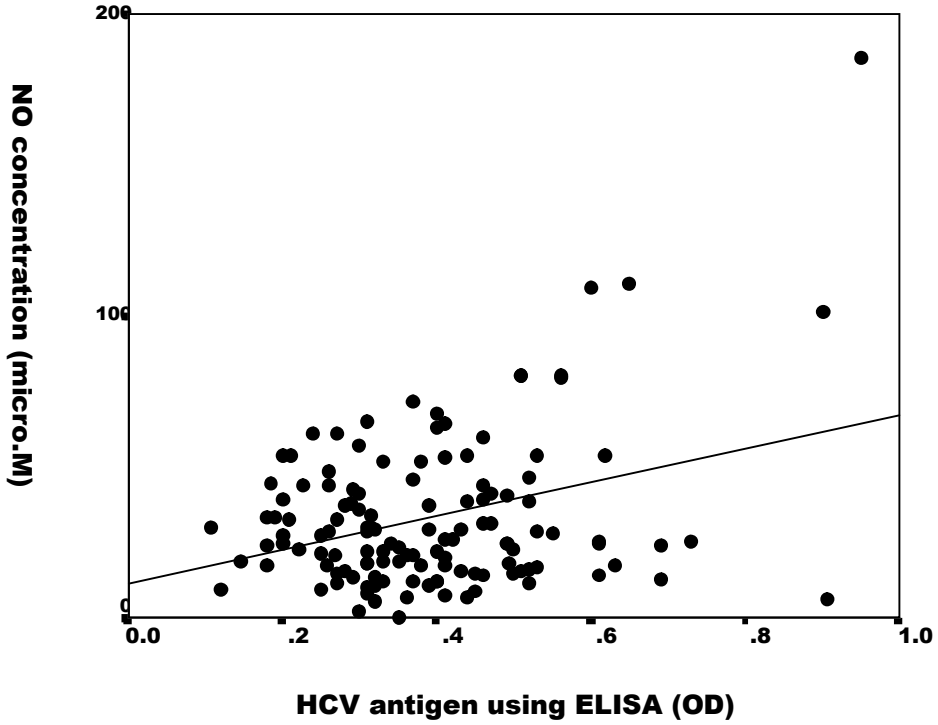

Figure 2: Correlation between HCV antigen and levels of nitric oxide in chronic hepatitis C (n = 129, r = 0.34, P = 0.0001).

Machida *et al*. (2004) determined the amount of nitrite and nitrate in the culture supernatant of HCV-infected cells to confirm the role of iNOS induction and found that this amount was elevated in HCV-infected cells compared to non-infected or uv-inactivated cells. These authors found that treatment of HCV-infected cells with iNOS specific inhibitors or the general NOS inhibitor L-NMMA (L-N-monomethyl arginine) suppressed the elevation of nitrite and nitrate levels in the supernatant of HCV-infected cell culture. Also, they found that cells treated with the stimulatory cytokines or the NO donor SNAP(S-nitroso-N-acetylpenicillamine) produced high levels of nitrite and nitrate. There was also an association between total NO (both nitrite and nitrate) and viral load in serum of HCV-infected patients (Mihm *et al*., 1997; Machida *et al*., 2004). Furthermore, Machida *et al*., (2004) have demonstrated that HCV induces the production of NO in hepatocytes and B cells by activating iNOS promotor which is mediated by two HCV proteins (core and NS3 proteins).

In HCV cirrhosis, there is a significant increase in the serum nitrate (de Lucas *et al.,* 2002; El-Sherif *et al.,* 2008). Serum NOx level in patients with chronic HCV (n = 47), or liver cirrhosis (n = 18) showed a higher concentration (92.5±7.4 and 78.0±7.8 μmol L^{-1}, respectively) than controls. (Higashino *et al.,* 2010).

Several authors found significant correlation between serum NO levels and degree of viremia in HCV RNA positive patients (Mihm *et al.,* 1997). Hassan *et al.* (2002) confirmed the positive correlation between serum nitrate levels and HCV virus load and extent of cirrhosis. The present results confirm also this correlation (Fig. 2) since HCV -Ag load represents well the degree of viremia.

In conclusion, serum NO level is elevated in non-cirrhotic, cirrhotic and HCC patients with HCV infection and its levels correlates well with serum HCV-Ag levels but it can not differentiate between the three liver affections.

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