

Nitric Oxide Levels and Sustained Virological Response to Pegylated-Interferon alpha2a plus Ribavirin in Chronic HCV Genotype 4 Hepatitis: a Prospective Study

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Abstract

Background: The role of nitric oxide (NO) in infectious diseases is gaining attention because of its antiviral effects. **Aim:** To evaluate whether serum and hepatic NO levels are predictors of the outcome of treatment in patients with chronic hepatitis C genotype 4. **Methods:** Fifty six patients with chronic HCV genotype 4 treated with pegylated interferon (IFN) alpha-2a plus ribavirin underwent blood tests, assessment of serum level of NO and hepatic tissue expression of NO synthase (iNOS) before and during treatment. **Results:** The pre-treatment serum NO level was significantly higher in sustained responders (SR) [39.583 (35-43.8)] compared to relapsers [36.25 (26-43.8)], and non responders (NR) [35.417 (25.0-43.8)]. During treatment, the serum NO level was significantly higher in SR [58.125 (47.9-60.6)] compared to relapsers [53.854 (47.9-59.4)] and NR [50 (42.9-59.4)]. The pre-treatment iNOS expression was significantly higher in SR [37.5 (15-75)] compared with either relapsers [25 (15-45)] and or NR [20 (2-45)]. In multivariate logistic regression analysis, the serum NO was correlated with the virological response to pegylated interferon alpha-2a plus ribavirin therapy. **Conclusion:** In patients with chronic hepatitis C, nitric oxide levels may be associated with the outcome of pegylated-IFN- α 2a plus ribavirin treatment.

Key words

HCV – nitric oxide – pegylated IFN- α – ribavirin – iNOS – immunohistochemistry – HCV genotype 4 – virological response.

Introduction

Hepatitis C virus (HCV) infection affects about 3% of the world population [1]. Globally, 3-4 million people are newly infected each year, with the predominant prevalence of infection with genotype 1, followed by genotypes 2 and 3. Genotypes 4, 5, and 6 have specific geographical distribution. Less than 20% of the acutely infected persons clear the virus, the rest (>80%) become chronically infected for decades, a significant fraction of whom die of HCV-related illnesses [2]. Egypt has a high prevalence of HCV especially genotype 4a. Hepatitis C virus is a leading cause of hepatocellular carcinoma (HCC) and chronic liver disease in Egypt [3, 4].

The current standard therapy for HCV infection is a combination of pegylated interferon (IFN)-alpha parenterally administered once weekly and daily oral ribavirin, with an overall response rate of about 40–60% [5, 6]. The response to combination therapy depends on several factors including the genotype of the virus, the serum level of HCV RNA before treatment, fibrosis stage, and the host immune response [5, 7].

The earliest host responses to viral infections are non-specific and involve the induction of cytokines, among them, IFNs. Interferons have been found to induce the production of nitric oxide (NO) [8]. There is an increasing evidence that NO is one of the most versatile mediators in the control of viral infections as well as in the pathogenesis of many human infectious and inflammatory diseases. This makes it reasonable to consider this multifunctional molecule as a potential player in HCV pathogenesis [8].

Nitric oxide is produced from L-arginine by one of three NO synthases (NOS) enzymes: two constitutive; neuronal type (nNOS: type 1 [NOS-1]) and or endothelial type (eNOS; type 3 [NOS-3]) and one inducible (iNOS: type 2 [NOS-2]). There is a controversy regarding the production of NO in chronic HCV patients with studies reporting an increase [9, 10], a decrease [11], or no change [12, 13] in its level. The effect of either IFN- α or ribavirin on NO production and the possible role of NO in the efficacy of both drugs are not clear [14, 15].

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In this study, we investigated the possible relation between NO markers (serum NO level and hepatic tissue expression of iNOS) and the virological response to pegylated INF- α 2a plus ribavirin therapy in patients with chronic HCV genotype 4.

Material and Methods

Patients

This study was carried out in the Department of Internal Medicine, Minia University. The study included 56 out-patients who were positive for: anti-HCV antibody by third generation-ELISA (BIOELISA HCV kit, BIOkit, S.A Barcelona); HCV-RNA [quantitative PCR by Roche Amplicor HCV monitor version 2.0 (Roche Diagnostics, Branchburg, NJ)]; and HCV genotype 4 [second-generation line probe assay (Inno-LiPA HCV II; Innogenetics, Ghent, Belgium)]. Patients with other causes of chronic liver disease (e.g. hepatitis B, biliary cirrhosis), patients with acute hepatitis, patients with known contraindication to either INF- α or ribavirin, and patients subjected to any condition that has been reported to affect NO production (e.g. diabetes, nitrate drugs) were excluded from the study. The elected patients were classified as having low viral load (< 800,000 IU/ml) or high viral load (> 800,000 IU/ml) [16]. This work was completed according to the ethical rules of the Faculty of Medicine, Minia University, Egypt and conforms to the ethical guidelines of the 1975 Declaration of Helsinki. Informed consent was obtained from all patients.

Liver histology

Liver biopsy was performed in all patients before the initiation of therapy. Biopsy specimens were fixed in formalin, embedded in paraffin sections and stained with haematoxylin and eosin. Biopsy specimens were examined for grading and staging according to the Metavir System [17]. Liver biopsy samples were also used for immunohistochemical detection of iNOS.

Treatment protocol and definition of response

All patients received pegylated IFN- α 2a (Pegasys, Roche Ltd, Basel, Switzerland) 180 μ g/week subcutaneously together with ribavirin (Virin, Sigma) 1,000 to 1,200 mg daily (1,000 mg for those with weight \leq 75 kg and 1,200 mg for those with weight \geq 75 kg), for 48 weeks [7]. The patients were followed for 72 weeks and their virological response (HCV-RNA by PCR) was recorded. Early virological response (ER) was defined by undetectable or at least \geq 2 log reduction of HCV-RNA at 3 months after initiation of therapy, while non-responders (NR) were defined by < 2 log drop compared to baseline HCV-RNA at 3 months after initiation of therapy. Sustained responders (SR) were defined when the HCV-RNA was undetectable at 6 months after end of therapy while relapsers were defined when HCV-RNA was detectable at 6 months after stop of therapy.

Measurement of serum NO

The sera of patients were sampled at each visit and stored for assessment of total nitrite before the initiation of

therapy and 3 months after. Blood samples were collected, left to clot for 20 minutes, and centrifuged (5 minutes at 2000 rpm). Serum was isolated and stored at -80 °C till the time of the assay. Nitric oxide was assayed colorimetrically by measuring the accumulation of its stable degradation product, total NO using Griess reagent [18].

Immunohistochemistry of iNOS expression

Immunohistochemistry was performed as previously described [19]. Sections from liver biopsies were deparaffinised in xylene and rehydrated in graded alcohol. H₂O₂ 0.3% was applied to block endogenous peroxidase activity. Slides were pre-treated in a microwave oven in citrate buffer (pH 6). Subsequently, slides were incubated with the primary antibody for iNOS (Thermo scientific). The ultra-Vision detection system (Thermo scientific) was used as follows: sections were incubated with biotinylated goat anti-polyvalent, then with streptavidin peroxidase and finally with DAB Plus chromogen. Slides were counterstained and mounted. Negative control and positive control slides were included. For the evaluation of iNOS, positively stained hepatic cells were counted at 200x in each case. The mean values were calculated and expressed as percentage in relation to total hepatic cells [20, 21].

Statistical analysis

The statistical analysis was made with SPSS® Inc for windows release 16 and the ROC curve analysis was done to determine cutoff values. Differences between two related groups of paired continuous variables were tested by using Wilcoxon signed rank test. The difference between the two independent groups of continuous variables was tested by using Mann-Whitney test. Multivariate logistic regression analysis was made to study the independent variables, Multivariate regression analysis was used to predict response in relation to serum nitrite and iNOS. Odds ratio with 95% confidence interval (CI) was denoted for each analysis. Statistical significance was determined at a p value of \leq 0.05 and was 2-sided.

Results

Basic data of the studied groups

This study included 56 patients (50 male and 6 female) and their age ranged from 37 to 53 years. There were 28 patients categorised as having low viral load and 28 as high viral load. There were 31 (55.4%) patients with histological activity A1, 21 (37.5%) with A2, and 4 (7.1%) with A3. Stages of fibrosis were as follows: F1: in 23 patients (41.1%), F2: in 18 patients (32.1%), and F3: in 15 patients (26.8%).

The virological response to interferon plus ribavirin therapy

The virological responses are shown in Table I: 9 patients (16%) were NR; 47 patients were ER, 32 SR and 15 patients were relapsers. Twenty-four patients (43%) were total NR. There was no significant difference between the SR and total NR with regard to basic data (Table II).

ROC curve analysis was performed to determine the cut-off values of NO and iNOS.

Table I. Response to interferon and ribavirin therapy in the study group

Type of response	N (%)
Early responders	32 (57.15%)
Sustained responders	32 (57.15%)
Relapsers	15 (26.8%)
Total	47 (84%)
Early non-responders	9 (16%)
Total non-responders (Early non responders + Relapsers)	24 (42.85%)

Table II. Basic data of sustained responder and non-sustained responder groups

Demographic, laboratory, and histopathological data		Study group (n= 56)		p value
		SR (n= 32)	Total NR (n= 24)	
Age (years)	Median (range)	44 (37-53)	42 (37-53)	0.589
Sex	Male	28 (87.5%)	22 (91.7%)	0.87
	Female	4 (12.5%)	2 (8.3%)	
Viral load	High	16 (50%)	12 (50%)	1.0
	Low	16 (50%)	12 (50%)	
AST (IU/L)	Median (range)	36 (25 - 57)	36 (25 - 121)	0.973
ALT (IU/L)	Median (range)	33 (18 - 109)	29 (19 - 115)	0.590
Bilirubin (mg/dl)	Median (range)	0.9 (0.7 - 2.3)	0.9 (0.7 - 2.3)	0.705
Albumin (g/dl)	Median (range)	3.9 (3.5 - 4.8)	3.8 (3.5 - 4.8)	0.485
INR	Median (range)	1.03 (1 -1.3)	1.1 (1 -1.3)	0.215
Histological activity score (Metavir system)	A1	19 (59.4%)	12 (50%)	0.645
	A2	10 (31.2%)	11 (45.8%)	
	A3	3 (3.4%)	1 (4.2%)	
Stage of fibrosis (Metavir system)	F1	16 (50%)	7 (29.2%)	0.363
	F2	7 (21.9%)	11 (45.8%)	
	F3	9 (28.1%)	6 (25%)	

SR: sustained responders, Total NR: non responders and relapsers.

Serum levels of NO (μM) during treatment

Three months after starting therapy, the median serum NO level [55.833 (42.9-60.6)] was significantly higher than before treatment [37.6042 (25 - 43.75)] ($p < 0.001$).

Relation between serum NO (μM) levels and the virological response

The pre-treatment serum NO level was significantly lower in NR (Table III) compared to ER ($P=0.01$). The pre-treatment serum NO level was significantly higher in SR compared to relapsers and total NR ($P=0.01$, $P=0.01$ respectively) with the cut-off point being 36.25. During treatment, the serum NO level was significantly lower in early NR [45 (42.9-51)] compared to ER [56.25 (47.9-60.6)] ($P=0.05$), and significantly higher in SR [58.125 (47.9-60.6)]

Table III. Levels of NO markers in patients with different virological responses

	ER	NR	SR	Relapsers	Total NR
Pre-treatment serum nitrite (μM)	38.125** (26-43.8)	27.708 (25-36)	39.583# (35-43.8)	36.25 (26-43.8)	35.417 (25.0-43.8)
Hepatic iNOS scores (%)	30** (15-75)	10 (2-20)	37.5# (15-75)	25 (15-45)	20 (2-45)
Serum nitrite (μM) during treatment	56.25** (47.9-60.6)	45 (42.9-51)	58.125# (47.9-60.6)	53.854 (47.9-59.4)	50 (42.9-59.4)

ER: early responders, NR: non responders, SR: sustained responders. Values represent median (range), ** Significantly higher compared with ENR (Mann-Whitney test); # Significantly higher compared with relapsers and total NR (Mann-Whitney test)

compared to relapsers [53.854 (47.9-59.4)] and total NR [50 (42.9-59.4)] ($P=0.01$, $P=0.01$ respectively). Multivariate logistic regression analysis showed that pre-treatment serum NO and NO during treatment were independent predictors of virological response (Table IV).

Table IV. Multivariate logistic regression analysis for the virological response to INF- α plus Ribavirin therapy

Variant	β	Odds ratio	CI (95%)	P value
Pre-treatment serum nitrite	0.69	2.0128	1.17 - 3.45	0.012
Serum nitrite during treatment	0.50	1.6522	1.11 - 2.45	0.009
Pre-treatment liver iNOS	0.088	1.0924	0.99 - 1.19	0.057
Histological activity	-0.58	1.2920	0.08 - 4.04	0.56
Fibrosis	0.25	0.5547	0.28 - 5.95	0.75
Viral load	0.09	1.0964	0.15 - 7.9	0.93

Expression of iNOS immunostaining and virological response

The iNOS immunoreactivity was observed in the cytoplasm of hepatocytes in all patients, with overall expression percentage of 25% (2-70). Expression of iNOS was significantly lower in NR [10 (2-20)] compared to ER [30 (15-75)] ($P=0.001$). Also, iNOS expression was significantly higher in SR [37.5 (15-75)] compared to both relapsers [25 (15-45)] ($P=0.01$) and NR [20 (2-45)] ($P=0.01$) with the cut-off point being 20% (Table 3).

Discussion

Previous studies reported a normal, increased or decreased nitrite concentration in the serum of patients with chronic HCV hepatitis [15, 22-25]. The reason for these discrepancies remains to be explained. In this study, we found a significant positive correlation between serum NO and virological response, serum NO being higher in responders to pegylated IFN- α plus ribavirin therapy than

in NR. The presence of a natural variation in the immune response among patients is reflected on NO production. The patients who were able to produce NO in response to IFN- α plus ribavirin therapy represented the responders and vice versa. In line with this concept, Mihm et al reported a significant variation in the serum nitrite levels among non treated chronic HCV patients and patients treated with IFN-based therapy [26]. In our study, the serum NO level measured at three months after starting IFN- α plus ribavirin therapy showed a significant increase compared with NO level before treatment. However, when comparing the levels of responders with those of NR, although the former had significantly higher level of serum nitrite than the latter, the median values were similar between SR and non SR patients, with a considerable overlap in the reached values.

The main finding in our study was the pre-treatment serum NO levels with the impact on the early viral response. These findings triggered us to hypothesize the possible use of the NO markers as an independent predictor of virological response in HCV genotype 4 infected patients. The multivariate logistic regression analysis showed that the pre-treatment serum NO as well as the rise of serum nitrite during therapy are independent predictive factors for the efficacy of IFN- α plus ribavirin therapy. The mechanism of increased pre-treatment levels is unknown but may be related to IFN stimulated genes (ISGs). ISG-encoded proteins establish a general antiviral state within the cell [27]. Interferons achieve their potent antiviral effects through the regulation of hundreds of IFN-stimulated genes (ISGs). Interferons induce ISG transcription by activating the Jak-STAT pathway [28]. Induction of ISGs was also found in pretreatment liver biopsies of many patients with chronic viral C hepatitis, again demonstrating that HCV infection can lead to activation of the endogenous IFN system [29]. Notably, patients with preelevated expression of ISGs tended to respond poorly to therapy when compared with patients with low initial expression [29]. The cause of this differential response to therapy is not understood. Are patients with elevated initial expression refractory to further stimulation of ISGs by exogenous IFN? Does the administration of IFN to patients with low initial ISG values lead to ISG expression levels exceeding those found in the other group, possibly explaining a successful therapy in low-ISG patients? Are there specific ISGs important for viral clearance that are not activated in nonresponders? [30]. The elevated NO levels, on the contrary, are associated with better response to IFN therapy with the probability of the inverse relation between the ISGs and NO with regard to the antiviral response.

The cut off points of the pre-treatment serum nitrite, hepatic iNOS, and serum nitrite during treatment were 36 μ M, 20%, and 54 μ M, respectively. A previous study reported a significant rise of serum nitrite at two weeks after initiating IFN in SR compared to NR indicating that the rise of serum nitrite was an independent predictive factor for the efficacy of IFN treatment [31]. Another study reported no role of NO in virological response to TNF- α in HCV patients because the levels of NO either in plasma or in extracts of

liver tissue were varied minimally between responders and NR to INF- α monotherapy [14].

In viral infections, the earliest host responses are non-specific and involve the induction of cytokines including IFNs. It has been found that iNOS is an IFN- γ -inducible protein in macrophages. Thus, NOS falls into the category of IFN-inducible proteins, activated during innate immune response and has a protective effect against viral infection [8, 31]. An indirect antimicrobial function of the iNOS pathway is thought to result from the NO-dependent induction of IFN- γ . Furthermore, iNOS-dependent host-protective effects during infectious diseases include inhibition of tissue fibrosis and termination of the immune response by apoptosis of activated CD4 T cells. During the resolution of infections, iNOS also participates in the regeneration of parenchymatous tissues, by protecting host cells from apoptosis and coordinating the synthesis of extracellular matrix [32]. At the molecular level, the antimicrobial activity of NO was originally thought to result from mutation of DNA, inhibition of DNA repair and synthesis, inhibition of protein synthesis, alteration of proteins by S-nitrosylation, ADP-ribosylation or tyrosine nitration, or inactivation of enzymes by disruption of Fe-S clusters, zinc fingers or haeme groups or by peroxidation of membrane lipids [31].

The effect of either IFN- α and/or ribavirin on NO production is not clear. It has been found that IFN- α 2b treatment for HCV patients increased the blood mononuclear cell NOS enzyme activity and iNOS antigen and mRNA expression speculating that induced NO production may be related to the antiviral action(s) of IFN- α [33]. Another study reported that ribavirin lowered NO production and concluded that the increasing numbers of SR to IFN- α plus ribavirin combination over IFN- α monotherapy may be attributable to ribavirin's prevention of IFN- α -mediated increase of NO in the milieu of cytotoxic lymphocytes [34]. There is a shortage of data addressing the effect of combined drugs on NO production. A significant increase in total urinary nitrite concentration was reported in SR compared to relapsers in chronic HCV patients treated with IFN- α plus ribavirin [35]. A recent study reported no change in serum NO (measured at the end of therapy) in SR compared with non treated patients [36].

Our study is the first that correlates the pretreatment serum nitrite to the virological response. Several factors influence the response to treatment including the virus genotype, viral load before treatment, liver histology, body weight and the host immune response [5, 7]. Patients with genotype 2 or 3 achieve higher SVR than patients with genotype 1 [37]. In Egypt, HCV genotype 4 constitutes up to 90% of HCV infections. HCV genotype 4 generally responds to treatment as genotype 1 does, and the current recommendation is to treat it in the same manner (48 weeks of therapy) [38, 39].

Conclusion

Our results allowed us to hypothesize a role of NO in the efficacy of INF- α plus ribavirin therapy and the possible

future consideration of researching an NO-based therapy to improve the therapeutic outcome of chronic HCV genotype 4 hepatitis.

Conflicts of interest

None of the authors have a conflict of interest regarding this study.

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