Oxidative stress in chronic hepatitis C: The effect of interferon therapy and correlation with pathological features

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AIMS: To evaluate the oxidative stress parameters before, during and after interferon treatment.
PATIENTS / METHODS: Twenty patients were treated with interferon α2b 5 MU, three times a week, subcutaneously, for 12 months. Liver biopsy was performed six months before treatment and at the six month follow-up. Chromosomal breakage studies were evaluated by the adjusted clastogenic score (ACS, normal value [nv] 1.1±2.4%). Plasma malondialdehyde (MDA) was measured according to the Yagi method (nv 6.6±1.4 nmol/mL) and total thiols using the Ellman's reagent (DTNB) (nv 9.8±1.3 µmol/g protein). A serum marker of fibrogenesis, the amino-terminal propeptide of Procollagen type III (PIIIP), was quantified by radioimmunoassay (nv 0.37±0.18 U/L).
RESULTS: Compared with reference samples, the plasma of patients before treatment showed an increase of ACS (9.2±3.2%, P<0.001); higher MDA values (12.6±2.7nmol/mL, P<0.001) and total plasma sulfhydryl groups (t-SH) were decreased (6.3±1.1 µmol/g protein, P<0.001). During treatment and at the follow-up, a decrease in ACS was noticed in all patients (P<0.001), but without normalization; a decrease in MDA was seen, with progressive normalization until the end of the follow up only in sustained responders (P<0.003), while an increase of t-SH was seen, with progressive normalization until the end of follow up in all patients (P<0.005). A positive correlation of ACS with grading of inflammation was found (r=0.52, P<0.03) but not with fibrosis staging. In contrast, plasma MDA correlates with fibrosis staging (r=0.51, P<0.03) and with PIIIP (r=0.57, P<0.03) but without grading of inflammation.
CONCLUSIONS: The present study confirmed the presence of oxidative stress in chronic hepatitis C patients. Interferon promotes a long term inhibition of oxidative stress with concomitant improvement of activity and fibrosis. In the management of chronic hepatitis C, adjuvant therapy with antioxidants could be useful.

Key Words: *Hepatitis C; Histology; Interferon treatment; Oxidative stress*

Stress oxydatif et hépatite C chronique : effet du traitement à l'interféron et corrélation avec des paramètres biologiques pathologiques

BUT : Évaluer les paramètres du stress oxydatif avant, pendant et après le traitement à l'interféron.
PATIENTS ET MÉTHODE : Vingt patients ont été traités à l'interféron alpha-2b à raison de 5 millions d'unités, par voie sous-cutanée, trois fois par semaine, pendant 12 mois. Des biopsies du foie ont été pratiquées six mois avant le traitement et après six mois de suivi. Les paramètres en question ont été évalués comme suit : les cassures chromosomiques, à l'aide du facteur clastogène redressé (FCR; valeurs normales [v.n.] : 1,1±2,4 %); le malondialdéhyde (MDA), selon la méthode Yagi (v.n. : 6,6±1,4 nmol/ml) et les thiols totaux, à l'aide du réactif d'Ellman (v.n. : 9,8±1,3 µmol/g protéine). Un marqueur sérique de la fibrogenèse, le propeptide amino-terminal du procollagène de type III (PPIII), a été quantifié par dosage radioimmunologique (v.n. : 0,37±0,18 U/l).
RÉSULTATS : Comparativement aux échantillons de référence, les valeurs plasmatiques des paramètres précédents, avant le traitement, s'établissaient comme suit : augmentation de la concentration de FCR(9,2±3,2 %; p<0,001) et de MDA (12,6±2,7 nmol/ml; p<0,001) et diminution de la concentration totale de thiols (6,3±1,1 µmol/g de protéine; p<0,001). Pendant le traitement et le suivi, nous avons enregistré les variations suivantes : diminution du FCR tendant vers la normalisation sans toutefois l'atteindre chez tous les patients (p<0,001); diminution du MDA, accompagnée d'une normalisation progressive jusqu'à la fin du suivi chez les sujets répondants seulement (p<0,003); augmentation des thiols, accompagnée d'une normalisation progressive jusqu'à la fin du suivi chez tous les patients (p<0,005). Une corrélation positive a été relevée entre le FCR et le degré d'inflammation (r=0,52; p<0,03) mais pas avec le stade de la fibrose. Par contre, la concentration plasmatique de MDA était en corrélation avec le stade de la fibrose (r=0,51; p<0,03) et le PIIIP (r=0,57; p<0,03) mais pas avec le degré d'inflammation.
CONCLUSION : La présente étude a confirmé la présence de stress oxydatif chez les patients atteints d'hépatite C chronique. Le traitement à l'interféron favorise l'inhibition prolongée de ce stress, qui se traduit par une amélioration de l'activité hépatique et une diminution de la fibrose. L'adjonction d'antioxydants pourrait s'avérer utile dans le traitement de l'hépatite C chronique.

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Hepatitis C virus is the most common cause of chronic viral hepatitis in the western world. The hepatic damage is due both to the cytopathic effect of the virus and the inflammatory changes secondary to immune activation (1). It has been suggested that oxidative stress plays a role in viral hepatitis, demonstrated by the occurrence of lipid peroxidation with increased levels of malondialdehyde (MDA) in the plasma (2,3). MDA-adducts can be localized in the livers of patients with the use of specific antibodies (4). Other studies indicated oxidative changes in protein molecules (5) and DNA (6). Total plasma sulfhydryl groups (t-SH), reflecting the antioxidant capacity of the plasma, were found to be decreased in patients (7). Discordant results have been reported for extra and intracellular levels of reduced glutathione (GSH) (8,9). Increased oxyradical production in hepatitis C may be detected by direct measurements using electroparamagnetic resonance and spin trapping techniques on liver biopsies (10).

Reactive oxygen species released from sequestered phagocytes and activated macrophages probably represent the major source of superoxide production in hepatitis C. Nonstructural proteins, encoded by the hepatitis C virus genome, are implicated via the activation of NADPH oxidase (11). In addition, tumour necrosis factor alpha (TNFα), a cytokine known to stimulate superoxide production by competent cells, is produced in excess (12). Superoxide may also arise from the xanthine oxidase reaction. This enzyme is increased in virus-infected tissues, coupled with enhanced catalysis of ATP that provides the substrates xanthine and hypoxanthine (13).

Iron-mediated, hydroxyl-radical formation was considered to be implicated in the hepatocellular injury and subsequent fibrosis by Pietrangelo (14), but questioned by others (15,16). Aldehydic breakdown products such as MDA and 4-hydroxynonenal were thought to be responsible for the observed correlation between lipid peroxidation, activation of stellate cells (17) and the stimulation of collagen alpha 1 gene expression (18,19).

The increased free radical formation in chronic hepatitis C may be one of the mechanisms underlying the increased risk of hepatocellular carcinoma. Genomic damage is documented not only by 8-hydroxyguanosine formation in DNA (6,20,21), but also by increased chromosomal breakage in blood cultures of hepatitis patients. The clastogenic effects in patients' lymphocytes are induced by chromosome damaging agents in the plasma. The term 'chromosome breakage factor' or 'clastogenic factor' (CF) is used for the presence of clastogenic activity in the plasma. CF contains lipid peroxidation products, cytokines such as TNFα and unusual nucleotides of inosine, shown to be clastogenic at micro molar concentrations. The generation of CFs as well as their clastogenic effects are regularly inhibited by superoxide dismutase. This was also the case for CFs from patients with hepatitis C (22).

In the present study, three oxidative stress markers (MDA, t-SH, CF) were studied before, during and after interferon therapy to evaluate the effects of the treatment on these parameters, and to look for correlations with histology and the N-terminal propeptide of procollagen type III (PIIIP), a useful marker of fibrogenesis in the serum of patients (23).

PATIENTS

Twenty interferon α2b naive patients with chronic hepatitis C, 12 men and eight women (mean age 43.5±11 years, range: 27 to 65) who fulfilled the entry criteria, were included in the study. Inclusion criteria were as follows: elevation of serum aminotransferases 1.5 times the normal value for at least six months, and the presence of serum HCV RNA. Serum hepatitis B surface antigen, alcohol abuse or other metabolic and autoimmune causes of chronic liver disease were excluded. Liver biopsy showed histological features of chronic hepatitis. The details concerning clinical, biochemical, virological and histological data are summarized in Table 1.

All patients were treated with α2b interferon 5 MU, three times a week, subcutaneously for 12 months and completed the six months of follow-up. The patients were prospectively studied before initiating the treatment, during therapy at three, six and 12 months and at the six month follow-up. Twenty healthy blood donors of similar age served as the control group. The healthy subjects were selected on the basis of physical examination and normal laboratory findings, including liver function tests. Individuals with alcohol ingestion were excluded. Informed consent was obtained from each patient and all procedures in the study were in accordance with the 1975 Helsinki Declaration. The Ethics Committee of the University of Lisbon approved the protocol.

METHODS

HCV-RNA and HCV genotype

Serum HCV was detected by using the Amplicor HCV polymerase chain reaction (Roche Molecular Systems, Switzerland). Viral genotype was assessed by enzyme linked immunosorbent assay (Gen-eti-K; Deia-Sorim, Italy).

Liver histology

A specimen was obtained six months before starting the treatment and at the end of the follow-up using a Menghini needle. The specimens were formalin fixed and embedded in paraffin. Two to 3 µm sections were taken and stained with hematoxylin-eosin and the Sweets and Gordon and chromotrope aniline blue methods for collagen. Histopathological findings were assessed and scored by two experienced pathologists (see Acknowledgements) blinded to the clinical data, using the histological activity index (HAI) of Knodell modified by Scheuer (24). A separate score was given to the grading of inflammation (score 0 to 4), with the individual evaluation of portal/periportal activity, lobular activity and fibrosis staging (0=none; 1=enlarged fibrotic portal tracts; 2=periportal or portal-portal septa but intact architecture; 3=fibrosis with architectural distortion but no obvious cirrhosis; 4=probable or definite cirrhosis).

Cytogenetic methods

The CF-test was performed using previously described methodology. Endogenous clastogens released from cells as a consequence of increased free radical production were detected (25). After preparation of an ultra filtrate (cut-off 30,000 DA) from the patients' plasma, the clastogenic activity of the samples was studied on cultures set up with whole blood (0.4 mL) from healthy donors. The culture medium (4 mL RPMI, Gibco, France) was supplemented with 1 mL of fetal calf serum. Lymphocyte proliferation was stimulated by addition of phytohemagglutinin (Welcome Diagnostics, United Kingdom). The ultra filtrates, which had been maintained at –70°C until handling, were added to the test cultures at the start of the 48 h incubation period. If cytotoxic effects were observed with the usual dose of 250 µL, the assay was repeated with 100 µL. Slides were prepared according to classical cytogenetic procedures after the addition of colchicine to the cultures for

the last 2 h. The presence of structural chromosomal aberrations was studied on coded slides. The total number of aberrations observed in 50 mitoses from five different slides were multiplied by two to give a numerical score expressed as a percentage. The spontaneous chromosomal aberration rate of the test cultures was determined for each experimental set by two simultaneous untreated cultures of the same donor's blood. This aberration rate was deduced from that of the ultra-filtrate treated cultures. The difference between the two values was called the adjusted clastogenic score (ACS). According to previous studies in 100 healthy persons, the addition of plasma ultra filtrates does not significantly increase the chromosomal aberration rate in the test cultures (0% for 52 samples, +2% and +4% additional aberrations for another 43 samples). Only five of the normal samples yielded increases of +6% aberrations (26). The mean ACS of the 100 samples from healthy adults was 0.8±1.0.

Biochemical assays

Whole blood specimens were placed in test tubes containing heparin. The mixed sample was centrifuged for 5 min at 3000 rpm and the plasma was separated. Samples were stored at –70°C until use.

Lipid peroxidation was evaluated by measuring the formation of thiobarbituric acid-reactive substances in the serum of patients and the controls expressed as total malondialdehyde (MDA), according to the Yagi method (27).

Thiobarbituric acid-reactive substances were determined as follows: 4.0 mL of N/12 sulphuric acid (H_2SO_4) was added to 0.5 mL of plasma and mixed gently. Then, 0.5 mL of 10% phosphotungstic acid was added and mixed. After allowing it to stand at room temperature for 5 min, the mixture was centrifuged at 3000 rpm for 10 min. The supernatant was discarded and the sediment was mixed with 2.0 mL of N/12 H_2SO_4 and 0.3 mL of 10% phosphotungstic acid. The mixture was centrifuged at 3000 rpm for 10 min. The sediment was suspended in 4.0 mL of distilled water and 1.0 mL of thiobarbituric acid (TBA) reagent was added. TBA reagent was prepared by mixing equal volumes of 0.67% TBA aqueous solution and glacial acetic acid. The reaction mixture was heated for 60 min at 95°C in an oil bath. After cooling with tap water, 5.0 mL of n-butanol were added and the mixture was shaken vigorously. After centrifugation at 3000 rpm for 15 min, the mixture was taken for fluorimetric measurement at 553 nm with 515 nm excitation. The results are expressed in nmol/mL of MDA.

t-SH groups were measured using Ellman's reagent 5, 5′-dithiobis-2-nitrobenzoic acid (DTNB). The thiol-disulfide interchange reaction between DTNB and thiols was the basis of this spectrophotometric assay (28). An aliquot of plasma (0.20 mL) was mixed in a 10 mL test tube with 0.6 mL of Tris (0.25 M)-EDTA (20 mM) buffer, followed by addition of 40 µL of 10 mM DTNB and 3.16 mL of absolute methanol. The test tube was capped, and the colour was developed for 15 min to 20 min, followed by centrifugation at 3000 g for 10 min at ambient temperature. The absorbance of the supernatant at 412 nm (A) was subtracted from a DTNB blank (B) and a blank containing the sample without DTNB. A value of 0.03 at 412 nm for the sample blank was consistently obtained. Total SH groups were calculated as followed: (A-B-0.03) × 1.47 nM. The results are expressed as micromoles t-SH per gram of protein, after determination of the protein content of the sample with the Lowry assay (29).

Serum amino-terminal propeptide of procollagen type III

Serum was stored at –70°C until quantification with a commercial radioimmunoassay (RIA-gnost PIIIP c.t., CIS bio international, France). The assay was performed according to the instructions of the manufacturer. The normal level of 29 healthy individuals ranged from 0.14 U/mL to 0.65 U/mL (mean ± SD 0.37±0.18 U/mL) (23).

TABLE 1
Clinical and histological characteristics of the patients

	Interferon α2b
Patients (n)	20
Male/Female	12/8
Mean age (range)	43.5 (27–65)
Route of infection, n (%)	
Transfusion	6 (30)
Sporadic	3 (15)
IVDU	11 (55)
Serum HCV-RNA, n (%)	
>2 millions copies/mL (%)	5 (25)
≤2 millions copies/mL (%)	15 (75)
Genotype 1	13 (65)
Liver histology, n (%)	
F0–F1	7 (35)
F2	8 (40)
F3–F4	5 (25)

F Fibrosis score; HCV-RNA Hepatitis C virus RNA; IVDU Intravenous drug users

Serum aminotransferases

Alanine aminotransferase (ALT) range 0 U/L to 25 U/L) and aspartate aminotransferase (AST) range 0 U/L to 29 U/L) were determined by standard automated techniques.

Alfa interferon treatment and definition of response

All the patients were treated with 5 MU of recombinant α2b interferon (IFN), three times a week, subcutaneously for 12 months and divided into three groups: sustained responders (SR) with normalization of aminotransferases and undetectable serum HCV RNA observed at the end of the 12 months treatment and during the six month follow-up; relapsed patients (RR) with normalization of aminotransferases and undetectable serum HCV RNA observed at the end of interferon therapy, but with reappearance of viremia and abnormal AST and ALT after stopping IFN; and nonresponders (NR) with elevated liver enzymes and HCV RNA positivity seen at any moment, during and after cessation of the treatment.

Statistical analysis

Statistical analysis at the different times of the treatment was performed by analysis of variance, associated with the Student-Newman-Keuls q-test.

Coefficient correlations were evaluated with linear regression analysis or the Spearman rank-order test. We used the Mann-Whitney test for nonparametric analysis. Data were expressed as mean ± SD or, in the case of ACS, as percentage. $P<0.05$ was considered statistically significant.

RESULTS

The clinical, biochemical and histological characteristics of the patients were presented in Table 1. The majority of the patients were male (60%), with low viral loads in 75% of patients and with a prevalence of genotype 1 in 65% of patients. The severity of histology was moderate, with a score for staging of two or lower in 75% of the cases.

TABLE 2
Cytogenetic, biochemical and histological data of the 20 patients before, during and after interferon therapy. Comparison with controls

	Controls (n=20)	Patients (n=20) 0 months	P*	3 months	P†	6 months	P†	12 months	P†	Follow-up 6 months	P†
MDA *(nmoL/mL)*	6.6±1.4	11.7±3.3	<0.01	9.3±2.7	<0.01	9.1±2.6	<0.005	9.4±2.1	<0.01	9.2±3.1	<0.01
ACS *(%)*	1.1±2.4	9.2±3.2	<0.01	4.5±3.3	<0.001	4.4±3.4	<0.001	5.1±2.7	<0.001	3.5±2.3	<0.001
t-SH *(µmol/g protein)*	9.8±1.3	6.1±1.5	<0.01	7.2±2.0	<0.05	7.4±1.9	0.02	7.3±1.7	0.02	7.2±1.8	0.04
AST *(U/L)*	27±2.2	46.3±18.5	0.001	43.9±42.3	ns	51.7±45.1	ns	44.9±28.2	ns	43.3±27.9	ns
ALT *(U/L)*	20±4.6	81.8±38.8	0.001	58.8±50.2	ns	61.7±49.8	ns	69.3±42.9	ns	70.2±42.4	ns

**Comparing patients with controls; †Comparing the beginning with the different months along the treatment and at the end of follow up. ACS Adjusted clastogenic score; ALT Alanine aminotransferase; AST Aspartate aminotransferase; MDA Malondialdehyde; ns Not significant; t-SH Total sulfhydryl group.*

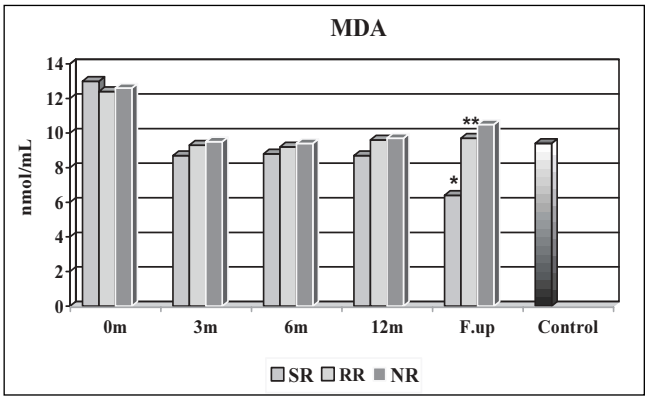

Figure 1) *Prospective evaluation of malondialdehyde (MDA) according to the type of response before, during and after alpha interferon treatment. Sustained response (SR) – five patients (before 13±0.4 nmol/mL and after *6.4±0.8 nmol/mL, P<0.005); Nonresponse (NR) – 11 patients (before 12.6±0.9 nmol/mL and after 10.5±2.4 nmol/mL, **P<0.01); Relapse (RR) – four patients (before 12.4±2.8 nmol/mL and after 10.2±2.1 nmol/mL, P= NS). Before treatment: SR vs RR vs NR P=NS. During the treatment: SR vs NR vs RR, P=NS in all the periods studied. At six month follow-up (F.up): *SR vs NR P= 0.003; **SR vs RR P=0.007; NR vs RR P=NS. Control group of 20 healthy individuals (6.6±1.4 nmoL/mL)*

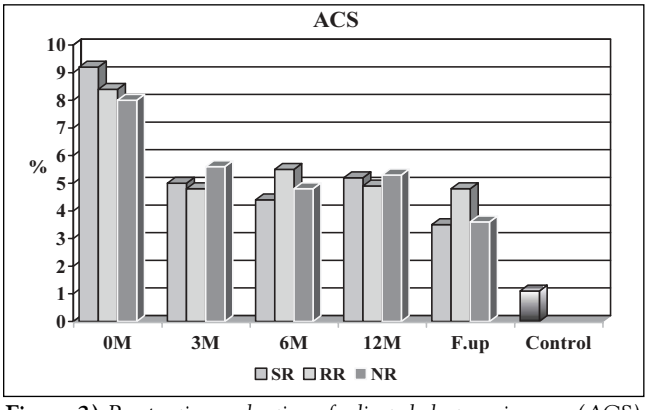

Figure 2) *Prospective evaluation of adjusted clastogenic score (ACS) according to the type of response before, during and after alpha interferon treatment. Sustained response (SR) – five patients (before 9.2±2.4% and after 3.5±1.0%, P<0.05); Nonresponse (NR) – 11 patients (before 8.0±3.4% and after 3.6±3.4%, P<0.007); Relapse (RR) – four patients (before 8.4±5.1% and after 4.8±4.1%, P<0.01). SR vs NR+RR P=NS in all the periods studied). Before treatment: SR vs NR vs RR P=NS. During the treatment: SR vs RR vs NR, P=NS in all the periods studied. At six month follow-up (F.up): SR vs NR vs RR P=NS. Control group of 20 healthy individuals (1.1±2.4%)*

Data of patients at different stages of the treatment
Comparison with controls

At the start of treatment, all patients showed abnormal values for the three biomarkers of oxidative stress (Table 2). Plasma ultra filtrates of the patients were highly clastogenic (ACS 9.2±3.2%), while the values for controls (1.1±2.4%) corresponded to our laboratory standard for samples from healthy blood donors (P<0.001). The MDA values were higher in patients (11.7±3.3 nmol/equivalents/mL) than in controls (6.6±1.4 nmol/equivalents/ml, P<0.001). Plasma thiols were decreased in patients (6.1±1.5 µmol/g protein) compared with controls (9.8±1.3 µmol/g protein, P<0.001). If increases or decreases of 2 SD from the mean observed in controls were considered as the limit of normal, all results higher than 9.4 nmol/equivalents/mL plasma for MDA, lower than 7.2 µmol/g protein for t-SH, as well as ACS of +5.9% or higher, were in the abnormal range. According to these criteria, there was a concordance between these three biomarkers in 13 patients (65%). Only one patient had the three biomarkers in the normal range. All others had abnormal values in two or more parameters. Using linear regression analysis, no significant correlation was found between AST, ALT and the biomarkers of oxidative stress.

The significant decreases were noticed along the treatment in ACS and MDA (P<0.001). At the same time, an increase of t-SH levels was seen (P=0.02) (Table 2). The most prominent increases and decreases of all biomarkers were seen at the third month of therapy, maintaining a statistically significant plateau six months after stopping the treatment.

Also the levels of aminotransferases decreased, but the decrease was not as striking as that of the oxidative stress markers. AST remained practically unchanged (46 U/L at the start of treatment and 43 U/L at the end). The decrease for ALT was most important after three months, comparable to the oxidative stress markers.

Comparison of patients according to their response to IFN treatment

Among the total of 20 patients, five (25%) were SR, while 11 were NR and four were RR.

Before treatment, MDA and ACS values did not differ among the three types of response (Figures 1 and 2); t-SH values were higher in the SR group compared with NR (P<0.005), but they were not different from RR (Figure 3).

During the treatment, a significant decrease of MDA (Figure 1) was noticed in the three groups of response (P<0.01).

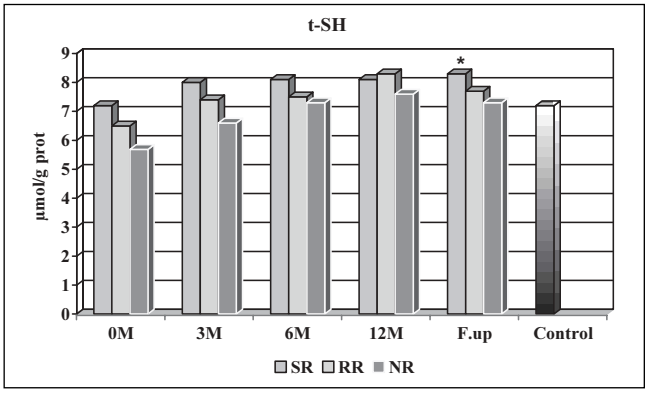

Figure 3) *Prospective evaluation of t-SH groups (total sulfhydryl groups) according to the type of response before, during and after alpha interferon treatment. Sustained response (SR) – five patients (before 7.2±0.7 µmol/g of protein (prot) and after 8.3±0.6 µmol/g of protein, P<0.03); Nonresponse (NR) – 11 patients (before 5.7±0.4 µmol/g of protein and after 7.3±0.4 µmol/g of protein, P<0.005); Relapse (RR) – four patients (before 6.5±0.7 µmol/g of protein and after 7.7±1.1 µmol/g of protein, P=0.09). Before treatment: *SR vs NR P<0.005; SR vs RR P=NS; RR vs NR P=NS. During the treatment: SR vs RR vs NR P =NS in all the periods studied. At six month follow-up (F.up) *SR vs NR P<0.001; SR vs RR P=NS; RR vs NR P=NS. Control group of 20 healthy individuals (9.8±1.3 µmoL/g of protein)*

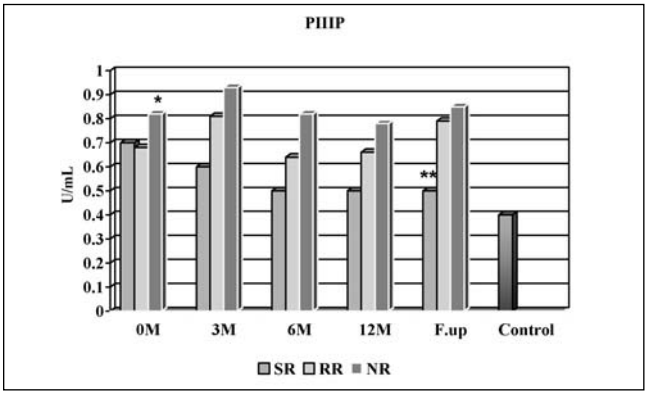

Figure 4) *Prospective evaluation of PIIIP (amino-terminal propeptide of procollagen type III [upper limit of normal value, 0.65 U/mL]) according to the type of response before, during and after alpha interferon treatment. SR Sustained response – five patients (before 0.7±0.1 U/mL and after 0.5±0.16 U/mL* P<0.01); NR Nonresponse – 11 patients (before 0.82±0.2 U/mL and after 0.85±0.24 U/mL, P=NS); RR Relapse – four patients (before 0.68±0.2 U/mL and after 0.79±1.0 U/mL, P=NS). Before treatment: *NR vs RR and SR P<0.01; SR vs NR and RR significant at six months, 12 months and the six month follow-up (**P<0.002). Control group of 20 healthy individuals (0.4±0.2 U/mL).*

However, at the six month follow-up, the normalization of MDA was only seen in the group of SR (P=0.003). The ACS (Figure 2) decreased in SR (P<0.05), NR (P=0.007) and RR (P<0.01). The clastogenic activity remained slightly increased in all groups compared with the mean values observed in healthy blood donors (P<0.01). Clastogenic scores higher than 6% were only observed in three NR patients before starting therapy. An increase of t-SH (Figure 3) was seen until the end of therapy and at the six month follow-up, reaching normalization in all types of responses. At the six month follow-up the values were higher in SR than in NR (P<0.001) but they were not different from RR.

AST and ALT values, which were higher in the SR group at the beginning IFN therapy, decreased to normal (104 U/L to 20 U/L) with concomitant negativation of HCV-RNA. The NR and RR groups maintained the elevation of aminotransferases without disappearance of HCV-RNA.

PIIIP decreased significantly from 0.7 U/mL to 0.5 U/mL in the SR group (P<0.01), while it remained unchanged in the NR and RR groups (Figure 4).

Control biopsies were available for three SR patients, four RR patients and eight NR patients (Table 3). The grading of inflammation and the fibrosis staging had decreased in two SR patients. Fibrosis and necroinflammatory scores remained unchanged or increased in three RR patients and in six NR patients.

Correlation of oxidative stress parameters with PIIIP and histological severity

Liver biopsies were performed in all patients before the treatment and in 15 patients at the end of the follow-up. The statistical evaluation with Spearman rank order test showed significant correlations between ACS and HAI (n=23, r=0.47, P<0.05). However, the significance was only observed with the grading of inflammation (r=0.52, P<0.03) but not with the staging of fibrosis (Table 4).

Linear regression analysis was used for the study of MDA and t-SH. MDA was also significantly correlated with HAI (n=23, r=0.48, P<0.05), but in contrast to ACS, this was true with fibrosis staging (r=0.51, P<0.03) but not with the grading of inflammation. Neither grading nor staging were correlated with plasma thiols and ALT. The correlation of PIIIP with MDA was significant (r=0.57, P<0.03) but none was found with ACS, t-SH and ALT (Table 4).

The number of HCV-RNA copies/mL varied considerably from patient to patient, and there was no correlation with the levels of ACS, MDA, t-SH and ALT. Genotype 1 was associated with a more severe HAI score (P<0.01), with a positive correlation with the grading of inflammation (r=0.47, P<0.05), but not with fibrosis staging.

DISCUSSION

The results of the present study confirm that patients with chronic hepatitis C are in oxidative stress (ie, their prooxidant-antioxidant balance has shifted towards the prooxidant side). When the antioxidant defences are overwhelmed because of increased free radical production, oxidative damage occurs in lipids, proteins and nucleic acids. Among the three biomarkers of oxidative stress chosen for this study, the quantification of lipid peroxides by the measurement of thiobarbituric acid reactive materials is a convenient and frequently used assay (27). The results are given as equivalents of MDA, but one has to keep in mind that this method measures only the aldehydes derived from lipid hydroperoxides. MDA is also formed during the enzymatic degradation of the arachidonic acid of membranes, and detection of MDA by HPLC would probably have yielded higher values. Nevertheless, the values observed with the Yagi assay were significantly increased in all patients compared to controls. Except for the patients with a sustained response to IFN, they were reduced by the treatment, but not completely normalized.

TABLE 3
Liver biopsy before and after treatment, according to the type of response

Sustained response Necroinflammation – fibrosis (grading – staging)		Relapse Necroinflammation – fibrosis (grading – staging)		Nonresponse Necroinflammation – fibrosis (grading – staging)	
Before	After	Before	After	Before	After
3–2	4–1	3–1	4–2	3–1	0–1
4–2	1–1	2–1	1–1	3–1	4–2
6–1	1–1	2–1	2–1	6–3	4–2
		6–2	6–2	2–1	2–1
				5–1	4–2
				4–2	4–2
				5–3	4–3
				2–1	2–1

TABLE 4
Correlation between histological severity and PIIP with ALT, genotype 1 and oxidative stress parameters

	Necroinflammation	Fibrosis	HAI	PIIIP (U/mL)
MDA (nmol/mL)	r=0.44; NS	r=0.51; P<0.03	r=0.48; P<0.05	r=0.57; P<0.03
ACS (%)	r=0.53; P<0.03	r=0.34; NS	r=0.48; P<0.05	r=0.31; NS
t-SH (mmol/g prot)	r=0.13; NS	r=0.04; NS	r=0.07; NS	r=0.23; NS
ALT (IU/L)	r=0.19; NS	r=0.17; NS	r=0.02; NS	r=0.28; NS
Genotype 1	P<0.05	P= 0.32	P<0.01	P<0.34

ACS Adjusted clastogenic score; ALT Alanine aminotransferase HAI Histological activity index; MDA Malondialdehyde; PIIIP Procollagen type III propeptide; t-SH Total sulfhydryl group

Interaction of IFN with the arachidonic acid cascade has been proposed as an explanation for the reduction of MDA values (30,31). Indeed, inhibition of virus replication by IFN involves modulation of the enzyme 2′,5′-oligoadenylate synthetase and an increase of this enzyme leads to decreased production of metabolites from the cyclo-oxygenase pathway, including MDA (32). However, the global decrease of oxyradical formation and consequently of nonenzymatic oxidation of lipids can explain the observed reduction in MDA equivalents. It is interesting that MDA was positively correlated with fibrosis scores and the serological marker of fibrogenesis, PIIIP. Other authors have reported that MDA is associated with activation of stellate cells and with the stimulation of collagen alpha 1 gene expression (18,19,33).

As previously reported, IFN prevents further development of fibrosis and improves the serological markers of fibrogenesis (23,34). In the NR and RR patients, fibrosis score and PIIIP values remained constant after treatment in the majority of the patients. However, in two of the three responders with control biopsies, the fibrosis score was reduced from two to one with concomitant normalization of PIIIP.

t-SH, the second biomarker of oxidative stress studied in our patients, is also a classical test and allows a global evaluation of a patient's antioxidant defences. This test was regularly abnormal before treatment. Values started to normalize from the third month on and were maintained during the follow-up period in all patients, despite the arrest of treatment.

The third biomarker of oxidative stress, clastogenic activity in patient's plasma, represents a global assay for increased superoxide production in the organism. A positive CF-test indicates an increase in circulating endogenous clastogens formed via superoxide-mediated pathways. Among the lipid peroxidation products, the aldehyde 4-hydroxynonenal is clastogenic at 0.1 μM concentration. MDA, on the other hand, is only a weak clastogen. This explains why no positive correlation was found between ACS and MDA. The two other identified clastogenic components of CFs, TNFα and inosine triphosphate are highly clastogenic and stimulate the superoxide production by competent cells. CFs are formed via superoxide and stimulates themselves the release of superoxide. This results in an auto-sustained process with permanent genotoxic effects. As also pointed out by other authors studying oxidative damage to DNA (6,20,21), persistence of unrepaired lesions in DNA might well be at the origin of the increased hepatoma risk in HCV-infected persons. CFs are also observed in other chronic inflammatory diseases, in which an increased incidence of cancer is also documented (35). In agreement with the link to inflammation, CF values were positively correlated with necroinflammatory scores. ACS was reduced to near normal values not only in responders, but also in nonresponders and relapsed patients. In the 15 patients whose control biopsies were available, inflammatory grading was reduced from 4.0±1.5 to 2.8±1.9. Given that in general inflammation precedes fibrogenesis, IFN treatment should be the most efficient, if given early in the disease process. Reduction of chronic oxyradical-related DNA damage might also reduce the risk of hepatocarcinoma.

One may assume that the effect of IFN on oxidative stress is not only due to the probable antioxidant properties of the drug, but also to its anti-inflammatory and antiviral action. However, given the importance of oxyradical induced damage, adjuvant therapy with antioxidants should be assayed. It is also noteworthy that the biomarkers of oxidative stress as well as aminotransferases levels were normalized after three months of treatment in the SR group. This early normalization of biomarkers may predict a future sustained response. Larger series of patients are necessary to evaluate this point.

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REFERENCES

1. Gonzalez-Peralta R, Davis GL, Lau JYN. Pathogenetic mechanisms of hepatocellular damage in chronic hepatitis C virus infection. J Hepatol 1994;21:255-9.
2. Romero MJ, Bosch-Morell F, Romero B, Rodrigo JM, Serra MA, Romero FJ. Serum malondialdehyde: Possible use for the clinical management of chronic hepatitis C patients. Free Rad Biol Med 1998;25:993-7.
3. Higueras V, Raya A, Rodrigo JM, Serra MA, Romá J, Romero FJ. Interferon decreases serum lipid peroxidation products of hepatitis C patients. Free Radic Biol Med 1994;16:131-3.
4. Paradis V, Mathurin P, Kollinger MF, et al. In situ detection of lipid peroxidation in chronic hepatitis C: Correlation with pathological features. J Clin Pathol 1997;50:401-40.
5. De Maria N, Colantoni A, Fagiuoli S, et al. Association between reactive oxygen species and disease activity in chronic hepatitis C patients. Free Radic Biol Med 1996;21:291-5.
6. Kitada T, Seki S, Iwai S, Yamada T, Sakaguchi H, Wakasa K. In situ detection of oxidative DNA damage, 8-hydroxyguanosine, in chronic human liver disease. J Hepatol 2001;35:613-8.
7. Mutlu-Turkoglu U, Ademoglu E, Turkoglu S, Badur S, Uysal M, Toker G. The effects of interferon alpha on serum lipid peroxidation and total content in patients with chronic active hepatitis C. Res Commun Mol Pathol Pharmacol 1997;96:357-61.
8. Beloqui O, Prieto J, Suarez M, Gil B, Quian CH, Garcia N. N-acetil-cysteine enhances the response to interferon alpha in chronic hepatitis C: A pilot study. J Interferon Res 1993;13:279-82.
9. Lim HL, Myers BM, Hamilton BA, Davis GL, Lau JYN. Plasma glutathione concentration in patients with chronic hepatitis C virus infection. J Viral Hepat 1995;2:211-4.
10. Valgimigli M, Gaiani S, Pedulli S, Solendi F. Measurement of oxidative stress in human liver by EPR spin probe. Free Radic Res 2000;33:167-78.
11. Bureau C, Bernard J, Chaouche N, et al. Nonstrutural 3 protein of hepatitis C virus triggers an oxidative burst in human monocytes via activation of NADPH oxidase. J Biol Chem 2001;276:23077-83.
12. Boya P, de la Pena A, Beloqui O, et al. Tumor necrosis factor alpha gene expression and the response to interferon in chronic hepatitis C. Hepatology 1996;23:210-7.
13. Maeda H, Akaike T. Oxygen free radicals as pathogenic molecules in viral diseases. Proc Soc Exp Biol Med 1991;198:721-72.
14. Pietrangelo A. Iron, oxidative stress and liver fibrogenesis. J Hepatol 1998;28(Suppl 1):8-13.
15. Riggio O, Montagnese F, Fiore P, et al. Iron overload in patients with chronic viral hepatitis: How common is it? Am J Gastroenterol 1997;92:1298-301.
16. Di Bisceglie A, Bonkovsky HL, Chopra S, et al. Iron reduction as an adjuvant to interferon therapy in patients with chronic hepatitis C who have previously not responded to interferon: A multicenter, prospective, randomised, controlled trial. Hepatology 2000;32:135.
17. Parola M, Robino G. Oxidative stress-related molecules and liver fibrosis. J Hepatol 2001;35:297-306.
18. Poli G, Parola M. Oxidative damage and fibrogenesis. Free Radic Biol Med 1997;22:287-305.
19. Bedossa P, Houglum K, Trautwein C, Holstege A, Chojkier M. Stimulation of collagen α1 (I) gene expression is associated with lipid peroxidation in hepatocellular injury: A link to tissue fibrosis? Hepatology 1994;19:1262-71.
20. Farinati F, Cardin R, Degan P, et al. Oxidative DNA damage in circulating leukocytes occurs as an early event in chronic HCV infection. Free Radic Biol Med 1999;27:1284-91.
21. Cardin R, Saccoccio G, Masutti F, Bellentani S, Farinati F, Tiribelli C. DNA oxidative damage in leukocytes correlates with the severity of HCV-related liver disease: Validation in an open population study. J Hepatol 2001;34:587-92.
22. Emerit I, Serejo F, Filipe P, et al. Clastogenic factors as biomarkers of oxidative stress in chronic hepatitis C. Digestion 2000;62:200-7.
23. Serejo F, Costa A, Oliveira AG, Ramalho F, Batista A, Moura MC. Alpha interferon improves liver fibrosis in chronic hepatitis C: Clinical significance of the serum N-terminal propeptide of procollagen type III. Dig Dis Sci 2001;46:1684-9.
24. Sheuer PJ. Classification of chronic viral hepatitis. A need for reassessment. J Hepatol 1991;13:372-4.
25. Emerit I. Clastogenic factors. Detection and assay. Methods Enzymol 1990;186:555-64.
26. Emerit I, Organessian N, Sarkisian T, et al. Clastogenic factors in the plasma of Chernobyl accident recovery workers. Anticlastogenic effect of Ginkgo biloba extract. Radiat Res 1995;144:198-205.
27. Yagi K. Assay for serum lipid peroxide level and its clinical significance. In: Yagi K, ed. Lipid Peroxidation. New York: Academic Press, 1982:223-43.
28. Hu ML. Measurement of protein thiol groups and glutathione in plasma. Methods Enzymol 1994;233:380-5.
29. Lowry OH, Rosebrough NJ, Farr AL, Randall LJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:265-75.
30. Hanigan GE, William BRG. Interferon signalling through arachidonic acid-dependent pathway: A clue to adjuvant therapy for chronic viral hepatitis. Hepatology 1991;14:392-4.
31. Andreoni P, Cursaro C, Gasbarrini G. Interferonα increases prostaglandin E2 production by cultured liver biopsy in patients with chronic viral hepatitis: Can non-steroidal anti-inflammatory drugs improve the therapeutic response to interferon? J Hepatol 1993;19:228-31.
32. Merritt J, Ball A, Sielaff K, Meltzer D, Borden E. Modulation of 2', 5'-oligoadenylate Synthetase in patients treated with alpha-interferon: Effects of dose, schedule and route of administration. J Interferon Res 1992;May:7-16.
33. Lotte F, Piton A, Opolon P, Holstege A, Poynard T, Bedossa P. In situ detection of lipid peroxidation in chronic hepatitis C: Correlation with pathological features. J Clin Pathol 1997;50:401-6.
34. Bruno S, Battezzati PM, Bellati G, et al. Long term beneficial effects in sustained responders to interferon-alfa therapy for chronic hepatitis C. J Hepatol 2001;34:748-55.
35. Emerit I. Reactive oxygen species, chromosome mutations and cancer: Possible role of clastogenic factors in carcinogenesis. Free Rad Biol Med 1994;16:99-109.