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Oxidative Status in Chronic Hepatitis C: The Influence of Antiviral Therapy and Prognostic Value of Serum Hydroperoxide Assay

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The effect of α-interferon (α-IFN) and ribavirin (RBV) treatment on oxidative status in chronic hepatitis C (CHC) is unknown.

Aim: To study the time course of oxidative status in patients with CHC during α-IFN and RBV administration, and to evaluate the role of oxidative status in order to predict the therapeutic response.

Patients and methods: Fifty one patients with CHC were studied. All received a combination of α-IFN and RBV for 6 or 12 months in relation to the type of response. The hydroperoxides concentration in serum test samples by D-ROM test was measured in all of the patients before therapy. In 27 patients, hydroperoxides were also measured during the treatment and during the 12 subsequent months.

Results: Cross-sectional analysis demonstrates that patients with a successive long-term response had a lower basal serum hydroperoxide concentration than non-responders (280 ± 40.8 vs 337 ± 83 CARR Units, $p < 0.05$). This resulted to be an independent factor predictive of long-term response in the multi-varied analysis. Longitudinal observation on 27 patients showed that the mean hydroperoxide concentration decreased significantly during treatment (T_0 329 ± 79.2 vs T_{12} 272 ± 34.5 CARR Units) and that the decrease in the mean values was mainly due to variations in the relapsers group.

Conclusions: Normal basal hydroperoxide concentration helps to predict long-term response to combination therapy. The D-ROM test may be used for screening patients before treatment.

Keywords: Oxidative status; Serum hydroperoxides; Radical cation; Hepatitis C; Interferon and ribavirin

INTRODUCTION

Several well documented findings indicate that various factors contribute to liver injury in chronic hepatitis C (CHC). Different combinations of virus-, host- and environmental-related factors are responsible for the wide spectrum of severity and progression of the disease.[1] Nevertheless, the pathological mechanism determining liver damage in the hepatitis C virus (HCV) infection is still poorly defined.[2]

The "oxidative stress," which is the imbalance between oxidants, such as free radicals, and antioxidants in favour of the oxidants, has been associated with several diseases in humans.[3]

High number of clinical and experimental studies[1–3] highlighted the involvement of oxidative damage in CHC. The following experimental observations have been reported on this topic: (1) an increase of concentration of radical species: protein carbonyl groups,[4] 8-hydroxi-deossiguanosine,[5] lipoperoxides[6,7] and malondialdehyde (MDA),[4,8] in plasma and liver tissue; (2) a decrease of antioxidant compounds (glutathione (GSH),[9] vitamin A and E[7,8,10] and some flavonoids[11]) in plasma and liver tissue; (3) an induction of cell enzymatic antioxidant systems such as superoxide dismutase (SOD), glutathione peroxidase (GSPx), α-glutathione-*S*-transferase (α-GST) and catalase (CAT).[12,13]

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In a recent report, Okuda and co-workers[14] suggest that oxidative injury may occur as a direct result of HCV core protein expression, both *in vivo* and *in vitro*, and may involve a direct effect of this protein on liver mitochondria. However, it is widely demonstrated that the virus causes inflammation, which in turn yields an abnormal production of radical molecules playing a crucial role in generating oxidative stress. The leading mechanism of toxicity of free radical molecules is the peroxidation of membrane phospholipids, an event that provokes overt cytotoxicity (hepatocyte necrosis) and able to trigger hepatic fibrogenesis.[2]

Treatment with recombinant α-interferon (α-IFN), alone or in combination with ribavirin (RBV), became the standard therapy for CHC.[15,16] However, the effects of these drugs on the oxidative balance are unknown. Preliminary data suggest that α-IFN enhances the production of several radical species and increases SOD plasma levels, GSH turnover,[17] as well as erythrocyte levels of GSPx.[18] On the other hand, there is some evidence that haemolytic anaemia, induced by RBV administration, is the result of an oxidative stress leading to the lipid peroxidation of the red cell membrane.[19]

No information about the influence of combination therapy (IFN and RBV) on oxidative status of patients with CHC is present in literature; therefore, this study was aimed at investigating the time course of oxidative status in patients with CHC during the combined α-IFN and RBV administration, and to evaluate the potential role of the pre-treatment oxidative state in predicting long-term virological response.

PATIENTS AND METHODS

Patients

Our study group included 51 patients with serological (elevated aminotransferase activity, presence of HCV antibodies and HCV-RNA positivity) and histological diagnosis of CHC. The group underwent treatment with α-IFN (5 million units three times weekly) and RBV (1.0–1.2 g/day in relation to body weight) for 6 or 12 month period.

Definition of response: (1) Long-term responders (LTR) were patients with persistent absence of serum HCV-RNA 6 months or more after completion of therapy; (2) Relapsers (RR) were patients who had undetectable serum HCV-RNA at the end of therapy but who subsequently redeveloped viremia; (3) Non-responders (NR) were patients who failed to clear HCV-RNA from serum during therapy.

In NR, therapy was stopped at the sixth month of treatment. Ten patients received α-IFN and RBV as first line therapy, whereas among the other 41 patients, 19 were NR and 22 RR to a previous treatment with α-IFN alone. In the latter group of patients, the wash-out period between the two treatments ranged from 6 to 24 months (mean 11 months).

The dietary regime was free and monitored by using a food frequency-questionnaire and a food-preference list to rule out the effects of specific diet and to evaluate the fruit and vegetable intake. During the experimental period patients followed precise instruction about to avoid anti-oxidant supplementation.

All patients gave written informed consent to treatment with α-IFN plus RBV and the study protocol is conform to the ethical guidelines of the 1975 Declaration of Helsinki. The study design consisted of two parts: (1) cross-sectional study performed over all 51 patients (whose characteristics are illustrated in Table I(a); (2) longitudinal study performed in a subgroup of 27 patients (Table I(b)).

A blood sample was taken from each individual before starting therapy (T_0). For longitudinal observation, additional blood samples were taken from a subgroup of 27 patients, at the first (T_1), third (T_3), sixth (T_6), ninth (T_9) and twelfth (T_{12}) month of therapy and at the third (F_3), sixth (F_6),

TABLE I Characteristics of the populations investigated

Study group	(a) Cross-sectional study	(b) Longitudinal study
No. of patients	51	27
M/F	36/15	19/8
Age in years (range)	45 (24–63)	44 (24–61)
Weight in kg (range)	73.1 (45–104)	74 (49–93)
HAI*		
Grading, mean (range)	5.6 (2–10)	6.9 (3–10)
Staging, mean (range)	1.8 (0–4)	2.5 (1–4)
ALT, mean (range)	106 (18–326)	103 (18–195)
Genotype 1b	42	21
Genotype non-'1b	9	6
Viremia levels (Eq/ml)		
$< 3 \times 10^6$ copies/ml	28	15
$\geq 3 \times 10^6$ copies/ml	23	12

*Histologic activity index according to Knodell *et al.*.[24]

ninth (F_9) and twelfth (F_{12}) month of follow-up. Blood samples were left at room temperature for about 30 min and then centrifuged at 4000 rpm for 10 min. The serum obtained was distributed in 0.5 ml aliquots, then stored and frozen at −80°C until use. The measurement of serum hydroperoxide concentration was performed at the end of the study on stored aliquots.

Methods

Aminotransferase (ALT) levels were measured by Roche-Diagnostic Boeringer Mannaheim test (Germany) in agreement with the routine procedure recommended by the manufacturer.

The oxidative status was studied by measuring the serum hydroperoxide concentration according to a method automated by the Diacron (D-ROM test, Diacron, Italy).[20,21]

Serum hydroperoxides are the products of the result of dehydrogenation and peroxidation of several cellular components including proteins, peptides, amino acids, lipids and fatty acids.

D-ROM test utilises a spectrophotometric procedure, which is based on the capability of an aromatic diamine to develop a purple colour when it is transformed into its cation-radical form by the reactive oxygen species (ROS).[22,23] In the experimental acidic conditions of the test (acetate buffer, pH 4.8), serum hydroperoxides react through the Fenton reaction with the iron released by serum transport proteins and catalyses the formation of ROS.

$$ROOH + Fe^{2+} \longrightarrow RO^{\cdot} + OH^{-} + Fe^{3+}$$

$$ROOH + Fe^{3+} \longrightarrow ROO^{\cdot} + OH^{-} + Fe^{2+} + H^{+}$$

The peroxyl ($ROO^{\cdot}$) and alcoxyl ($RO^{\cdot}$) radicals produced, whose amount is directly proportional to plasma peroxides concentration, are chemically trapped by the chromogen (*N*,*N*-diethyl-*p*-phenyldiamine, (C)) in an electronic-transfer process. This leads to the formation of cation radical of this chromogen (C^{+}).

$$ROO^{\cdot} + C \text{ (colourless)} \longrightarrow ROO^{-} + C^{\cdot +} \text{ (coloured)}$$

$$RO^{\cdot} + C \text{ (colourless)} \longrightarrow RO^{-} + C^{\cdot +} \text{ (coloured)}$$

The purple colour resulting from these reactions was monitored at 505 nm in an ECOM F 6124 Eppendorf photometer (purchased from Diacron s.r.l., Italy). The kinetic of the colour development gives a measure of the amount of hydroperoxides present in 5 μl of serum sample tested in the assay.

The results are expressed in arbitrary units (CARR Units); the normal range of this method, which is tested over a large population of healthy controls (4547 clinically asymptomatic subjects), is 250–300 CARR Units.[23] Values outside this range indicate a modification of the prooxidant/antioxidant equilibrium.

Between and within-assay coefficient of variation was less than 2.9 and 0.5%, respectively. Measurement performed on samples stored at different timelengths demonstrated that the oxidative status of the sample did not change at −20°C up to 6 months.

The D-ROM assay is handless, reliable, reproducible, and cost effective. In addition, it is the only one method presently capable of measuring hydroperoxides in biological samples.

Liver morphological impairments were evaluated through the histological activity index (HAI), scoring system by Knodell *et al*..[24]

Statistical analysis was performed with a SPSS/PC + 8 statistical package (SPSS Inc. Chicago, IL).

Average differences amongst the groups of patients were analysed by one-way ANOVA test (repeated measure or one way) as appropriate. χ^{-2} test was used to compare proportions, while the non-parametric test was used to compare nominal variables. A logistic regression model was used in the statistical analysis of predictive factors for the subsequent development of long term response. A p-value <0.05 was considered significant.

RESULTS

Cross-sectional Study

At study entry, 25/51 (49%) patients showed serum hydroperoxides levels higher than the normal value. The mean of the basal values of serum hydroperoxide in the total population was 307.0 ± 68.1 CARR Units. No significant difference was observed between patients with and without elevated hydroperoxides in relation to the age, initial histological diagnosis (grading and staging), ALT, genotype and baseline viral load. At the end of the study period, 24 patients resulted as LTR, 13 NR and 14 RR.

The baseline characteristics of the 51 subjects, according to the type of response, are summarised in Table II. The three groups of patients were homogeneous by sex, age, histological severity of the disease (grading and staging), ALT and viremia levels (Fig. 1). The only statistically significant difference was observed for the body weight between NR and RR groups.

The cross-sectional analysis demonstrates that at baseline, patients with subsequent long-term response had serum hydroperoxides concentration lower than NR. The two groups (LTR vs NR) showed T_0 mean values of 280.0 ± 40.8 vs 337.0 ± 83 CARR Units, $p < 0.05$ (Fig. 2).

TABLE II Characteristics of the population investigated, in relation to the type of response

	LTR	RR	NR	*p*
No. of patients (%)	24 (47.1)	14 (27.5)	13 (25.5)	
M/F	19/5	8/6	9/4	NS
Age in years (range)	42 (24–63)	49 (27–63)	45 (25–59)	NS
Weight in kg (range)	73.1 (45–104)	66 (51–78)	80.8 (67–93)	$p<0.05$*
HAI†				
Grading, mean (range)	5.43 (3–9)	5.50 (2–9)	6.14 (3–10)	NS
Staging, mean (range)	1.43 (0–4)	1.70 (0–4)	2.71 (0–4)	NS
ALT (range)	108.2 (46–280)	95.9 (43–169)	113.9 (18–326)	NS
Viremia level				
Mean copies Eq/ml $\times 10^6$ (range)	7.98 (0.96–27.1)	11.90 (0.74–5.9)	7.03 (0.53–20.32)	NS

* RR vs NR. † Histologic activity index according to Knodell *et al.*[24]

Test sensitivity and specificity of the assay in identifying LTR patients, was equal to 66.7% (CL 0.44–0.84) and 63% (CL 0.42–0.81), respectively. Positive predictive value for LTR was 61.5% (CL 0.40–0.80). A receiver operating curve (ROC) was used to determine the cut-off hydroperoxide concentration able to discriminate between the patients LTR and those non-LTR with the maximal diagnostic accuracy. The best value was 301 CARR Units with a diagnostic accuracy of 66.6 (CL 51.6–79.6).

Logistic regression analysis showed that baseline low values of hydroperoxide concentration before starting therapy was associated with the highest probability to obtain subsequent long-term response ($p = 0.037$).

Longitudinal Study

All 27 patients enrolled in the longitudinal study have been submitted to a previous therapeutic treatment with IFN mono-therapy. As expected, most LTR patients (7/10) to the second (combined) therapeutic treatment were RR to the first therapy with IFN alone.[16] The three groups of patients in this population (LTR, RR, NR) were homogeneous by sex, age, histological severity of disease and cytological activity, while the only statistical significant difference was related to the body weight between NR and RR groups.

Monitoring of hydroperoxides in our population shows that basal concentration became significantly lower during the treatment period ($T_0 = 329.4 \pm 79.2$ vs $T_{12} = 272.0 \pm 34.5$ CARR Units, $p < 0.05$) and that the reduction also remained at the end of the follow-up period ($F_{12} = 295.5 \pm 67.7$ CARR Units). The decrease of the mean values was mainly due to variations in the RR group ($T_0 = 340.4 \pm 81.0$ vs $T_{12} = 276.6 \pm 46.7$, $p < 0.05$). In fact, both the LTR groups with mean baseline values within the normal range ($T_0 = 284.6 \pm 51.8$ CARR Units) and the NR group with higher mean basal values ($T_0 = 373.3 \pm 84.5$ CARR Units), hydroperoxide concentration did not change significantly during the observation period. On the contrary, the parameter of the RR group gradually improved and reached normal range during therapy, while returning to the values similar to the basal levels at the end of the follow-up (Fig. 3).

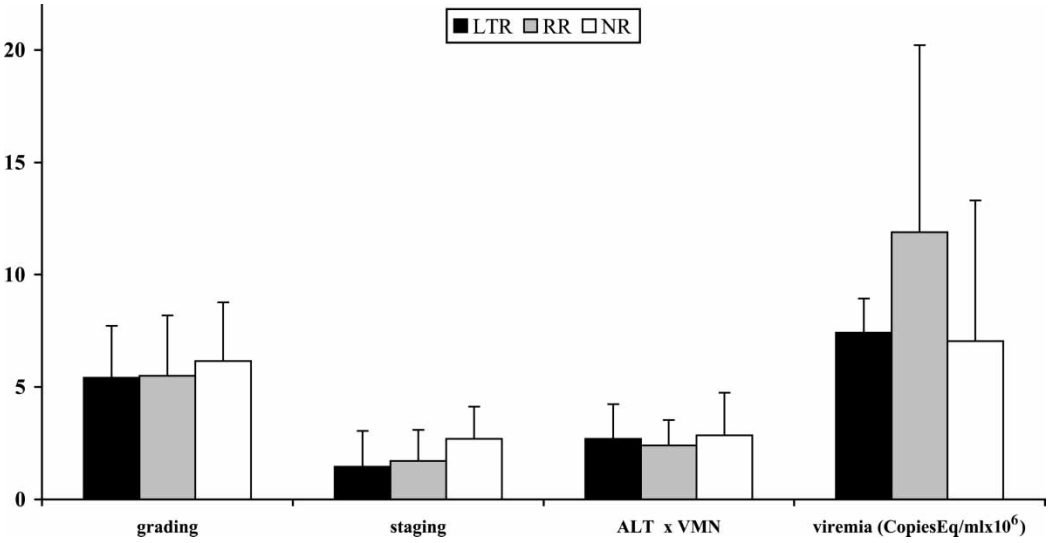

FIGURE 1 Mean ± SD of grading, staging, ALT and viremia levels in total population (51 patients) according to type of response.

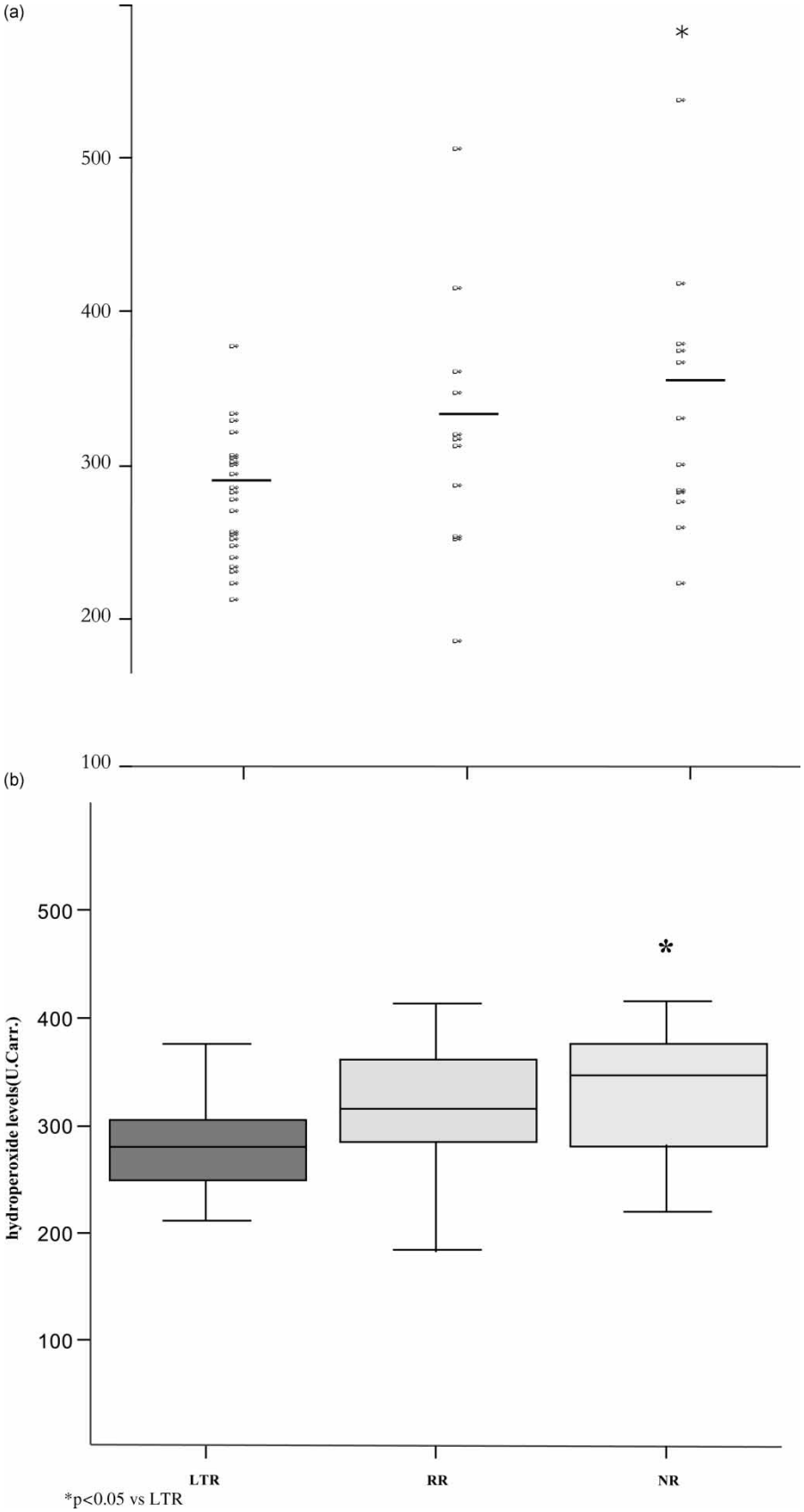

FIGURE 2 Individual (a) and mean ± SD (b) value of the baseline hydroperoxides levels in the three groups of patients.

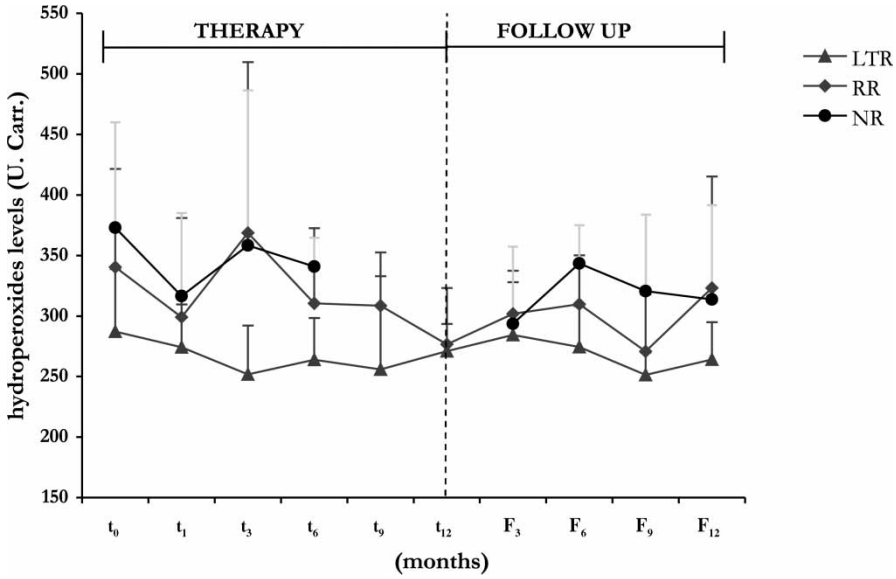

FIGURE 3 Mean ± SD of hydroperoxides levels in the three groups of patients during therapy and follow-up.

DISCUSSION

It is widely reported that the oxidative stress-related mechanism contributes to the pathogenesis of the liver damage in CHC.[25] However, until now no information regarding the status and the modifications of the oxidative stress profile in CHC during antiviral treatment (α-IFN and RBV) has been described.

The aim of our study was to evaluate the time course of oxidative status during antiviral therapy, up to 12 months after. Previous clinical observations were limited to the first 4–12 weeks of treatment with α-IFN alone.[18]

The results of our retrospective analysis showed that the trend of oxidative status parallels the therapeutic response, demonstrating that this parameter can be used as a predictive marker of prognosis.

The amount of hydroperoxides in serum represents a reliable index of the free radical attack, being a marker of the oxidative degradation of lipids, peptides and aminoacids. For this reason, an assay able to measure serum hydroperoxide concentration (the D-ROM test) was used. This assay was also selected because hydroperoxides are produced in the first stages of the oxidative process, therefore they could be considered an early and sensitive marker of peroxidation, useful even in patients with less severe liver disease.[26] Moreover, the assay is simple, rapid, cost effective and particularly suitable for large-scale studies.

The first milestone of our study was the observation that 49% of our population of patients with CHC have a hydroperoxide concentration higher than the upper limit of normality (300 CARR Units). This finding confirms the existence of an imbalance in the oxidative homeostasis in patients with CHC. On the other hand, 51% (26/51) of the subjects investigated reveal serum hydroperoxide concentration within the normal range and 61.5% (16/26) of them belong to the LTR group. The high percentage of CHC patients not showing an alteration of hydroperoxide concentration is a surprising result. The possible explanation for this phenomenon is that the increase of hydroperoxides in these patients could be efficiently balanced by the endogenous antioxidant systems. In other words, the status of normal or abnormal serum hydroperoxide concentration in CHC could depend on specific host characteristics (efficient activation of antioxidant defences) and by dietary habits that will determine the intake of the endogenous antioxidant compounds. Indeed, the increase of ROS could be only detectable when a lack an efficient antioxidant defence occurs.

It is well known that the concentration of antioxidants in serum is the result of several variables related to the genetic pattern, nutrition state, lifestyle of the host and presence of the inflammatory reaction. When a genetic predisposition is inherited and appropriate environmental events take place, an imbalance in the oxidative status occurs.

Data collected with dietary questionnaire did not show significant differences between the three groups of patients with different therapeutic response and between patients with and without elevated levels of hydroperoxides. However, it is possible that the direct measurement of specific antioxidant compounds can reveal a correlation with hydroperoxides levels.

Our data demonstrates that patients with a basal serum hydroperoxide concentration inside the normal range, give a persistent positive biochemical and virological response to antiviral therapy in about two-thirds of the cases. Multivariate analysis confirms this statement showing that low baseline serum hydroperoxide concentration is an independent prognostic factor of favourable outcome. As a consequence, oxidative status can be considered a predictive parameter of good prognosis. Several viruses and host related parameters such as levels of serum HCV-RNA, viral genotype, race, iron liver content, etc.[27] have been suggested as predictive factors of response to IFN therapy in patients with CHC. Additionally, factors involved in oxidant/antioxidant balance such as GSH, MDA, α-GST and thioredoxin, a stress-inducible thiol-containing protein with a wide spectrum of biological activities, seem to be characterised by a predictive power in relation to IFN response and to the course of asymptomatic HCV carriers.[28] Furthermore, elevated and persistently altered levels of plasma α-GST activity and thioredoxin, predict relapse or non-response to antiviral therapy.[29,30] Results of our work are in agreement with this line of findings.

The pathogeneic mechanism linking an altered redox state to the resistance to antiviral treatment is unknown. We can assume that the lack of therapeutic response observed in patients with altered pre-treatment markers of oxidative stress, primarily depends on depletion of antioxidant defences (GSH, vitamin A, vitamin C, vitamin E) as frequently found in chronic HCV infection.[26,31] It is conceivable that the observed antioxidant depletion in immunocompetent cells[31] might interfere with the immunological mechanism involved in the viral clearance and in the suppression of virus related cytopathic effect.[28] This hypothesis supports the utilisation of antioxidants as complementary therapy in CHC, although the few therapeutical trials carried out up to now showed inconclusive results.[32–34]

Data obtained from the longitudinal study demonstrate that in most subjects with a sustained response, basal hydroperoxide concentration fall within the normal range during the treatment. This would indicate the ability to preserve the efficiency of the antioxidant defences during and after the therapy. In NR, whose basal hydroperoxide concentrations are frequently above the normal range, the oxidative stress parallels the absence of therapeutic efficacy. Additional studies are necessary to define whether oxidative stress directly contributes to the failure of HCV eradication during antiviral therapy or, in alternative, whether the factors determining oxidative stress are the most responsible for therapeutic failure.

In conclusion, our study confirms that CHC is characterised by an increased oxidative stress. The initial concentration of serum hydroperoxides is well related to type of response to antiviral treatment and could help in selecting patients more responsive to α-IFN and RBV administration. Further studies are also desirable to evaluate whether the restoration of normal oxidative balance, might improve the LTR ratio.

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