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Oxidative Stress EPR Measurement in Human Liver by Radical-probe Technique. Correlation with Etiology, Histology and Cell Proliferation

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The role of reactive oxygen species (ROS) in liver disease is controversial. This mostly reflects the difficulties to quantify ROS *in vivo*, particularly in humans. We aimed to measure the presence of ROS in diseased human liver and identify possible relations between ROS levels and etiology, histology and hepatocyte proliferation. Liver biopsy specimens from 102 individuals: 18 healthy controls and 84 patients (42 HCV chronic hepatitis (CHC), 19 HBV chronic hepatitis (CHB), 7 PBC, 4 PSC, 4 HCV relapsing hepatitis after liver transplantation, 3 autoimmune hepatitis, 3 hepatocellular carcinoma, 2 alcoholic hepatitis) underwent analysis by radical-probe electron paramagnetic resonance (EPR). ROS in patients (median $= 5 \times 10^{-6}$ mmol/mg) were higher than in controls (median $= 3 \times 10^{-11}$ mmol/mg) ($p < 0.001$). Progressively increasing levels of ROS were recorded passing from control values to CHB (median $= 4 \times 10^{-7}$ mmol/mg), CHC (median $= 3 \times 10^{-6}$ mmol/mg) and PBC (median $= 2 \times 10^{-5}$ mmol/mg), the differences being significant ($p < 0.001$). ROS in CHC positively correlated with histological disease activity ($r = 0.92$; $p < 0.001$). No correlation was found between ROS and hepatocyte proliferation rate, presence/degree of steatosis, serum ferritin levels and aminotransferases. ROS overproduction in liver appears to be a common thread linking different pathologic conditions and seems to be influenced by diseases' etiologies.

Keywords: EPR; Chronic hepatitis; Free radicals; Inflammation; Immune response; Human liver

Abbreviations: AgNOR proteins, silver-stained nucleolar organizer-region-associated proteins; AH, alcoholic hepatitis; AIH, autoimmune hepatitis; AULD, PBC and PSC; ALT, Alanine aminotransferase; AP, Alkaline phosphatase; AST, aspartate aminotransferase; CHB, HBV related chronic hepatitis; CHC, HCV related chronic hepatitis; EPR, electron paramagnetic resonance; GSH, reduced glutathione; HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; HNE, 4-hydroxynonenal; HSC, hepatic stellate cells; MDA, malondialdehyde; Mn-SOD, Mn-superoxide dismutase; ROS, reactive oxygen species or oxygen free radicals; PCC, protein carbonyl content; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis

INTRODUCTION

Oxygen centered free-radicals, often referred to as reactive oxygen species (ROS), have been recognized as widespread mediators both of cell injury and of either inter- or intra-cellular signaling processes. In particular, in the last few years the role of oxidative stress in regulating fibrogenesis during liver disease has attracted much interest.[1] ROS are thought to be involved in the activation of hepatic quiescent stellate cells (HSC), and their subsequent deposition of extracellular matrix components in liver tissue.[2,3] Interestingly, ROS are supposed to take part in HSCs activation both in liver diseases with a prominent

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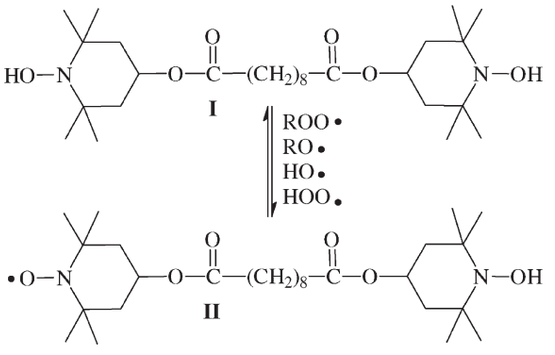
inflammatory component such as viral and autoimmune hepatic injury,[4] and in conditions characterized by hepatic fibrosis, such as genetic hemochromatosis, with little or no inflammation.[5]

Chronic viral hepatitis, autoimmune hepatitis (AIH) and primary chronic cholestathic liver diseases are among the prototypes of long-standing hepatic injury in which immune system activation towards a known or unknown pathogen is considered among the main factors leading to disease progression through hepatic fibrosis, cirrhosis and eventually to malignant transformation.[6–8] In these settings, the role of ROS in mediating liver injury is almost always inferred from animal or *in vitro* studies. Due to the difficulties to quantify or simply detect ROS in humans, works studying the role of oxygen centered radicals during liver disease have focused mainly on the measurement of indirect markers of oxidative stress, such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE),[9–11] hepatic or serum glutathione (H-GSH, S-GSH),[12,13] protein carbonyl content (PCC),[14] formation of 8-hydroxydeoxyguanosine in liver,[7] overexpression of Mn-superoxide dismutase (Mn-SOD),[15] plasma ubiquinol–ubiqunone ratio,[16] serum vitamin E content[17] and the production of ROS by polymorphonuclear cells isolated from blood.[18]

All these studies give evidence of an imbalance between oxidative stress and antioxidant status during liver disease. Whether this discrepancy in humans depends on a real overproduction of reactive oxygen species appears still speculative. In particular, the absence of quantitative ROS measurements in different liver conditions has limited the understanding of their clinical value in human tissue and their role as tissue-damaging mediators in relation to different disease etiologies is still unclear.

To our knowledge, there is no report in literature, directly assessing ROS in human liver tissue. A promising method of measuring ROS in real biological samples of growing complexity from sub-cellular fractions to whole animals was presented by one of us a few years ago.[19] It was founded on the great sensitivity and specificity of electron paramagnetic resonance (EPR) in detecting free radicals. The technique is based on the use of a suitable hydroxylamine probe, which reacts with ROS produced in the tissue under examination, generating a stable and long-lived nitroxide radical which is eventually measured by EPR.[20,21] The probe employed is the bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate (**I**) which gives fast reaction with conceivably all the oxygen centered radicals of biological interest, including superoxide ($HOO^{\cdot}$), to form the nitroxide **II**. Furthermore, it is also able to detect nitrogen reactive species, such as peroxynitrite, involved in the inflammatory process.



The lack of selectivity of the probe toward a given radical species is certainly the major limitation of the technique, as compared to other more established techniques such as *spin-trapping*, since it does not allow the identification of the actual species being detected. On the other hand, this limitation may turn into an advantage if, like the present case, what one wants to know is the overall oxidative stress status of the biological environment in which the probe is distributed. In this conjunction it is interesting to point out that, thanks to its peculiar physicochemical properties, the probe is able to diffuse in any compartment of a biological environment, thus reaching any site of generation, of radical species without any need to destroy compartimentation, e.g. by homogenation of the tissue. Therefore, although the present technique relies on the *ex vivo* measurement of radical species, these are generated in truly pseudo-physiological conditions and are therefore representative of the *in vivo* status.

The main advantage of this method is certainly its simplicity, as no particular sample treatment is required other than a short (5 min) incubation of the tissue with a solution of the hydroxylamine probe. This, together with the good sensitivity, makes the technique suitable for applications in real clinical settings. Indeed, the method has lately been implemented by setting a procedure suited to quantify ROS in human hepatic tissue which has been described and discussed in these pages[22] and has been recently reviewed.[23] Unfortunately, at that stage the limited number of human samples employed in the investigation prevented any assessment of the diagnostic relevance of the technique and allowed only tentative correlations between the measured level of ROS and other relevant clinical or biochemical parameters associated with the liver disease. This study represents the necessary extension of that preliminary investigation on a significant number of subjects (102 individuals) and aims to assess whether there are relationships between

hepatic ROS levels and etiology (CHB, CHC and PBC), disease activity, hepatocyte proliferation rate, ferritin content, and other clinical parameters related to human liver disease.

MATERIALS AND METHODS

Patients

Ninety patients, who referred to a liver unit as outpatients, with the indication of fine needle liver biopsy for diagnostic or staging purposes, were consecutively enrolled. Six of them were subsequently excluded because liver biopsy specimens were judged too short, (i.e. less than 4 cm in length) so that 84 patients were finally considered in the study. Each patient underwent an ultrasound-oriented liver biopsy using a 17–19 gauge modified Menghini needle. Forty-two patients (22 males and 20 females), mean age 45 ± 13 years (SD), had diagnosis of HCV chronic infection. All were repeatedly positive for antibodies against HCV (anti-HCV, EIA 2nd generation), RIBA II 2nd generation positive, and HCV–RNA positive. Serum HCV–RNA was detected by RT-PCR, and genotypes of HCV were identified by PCR with typed-specific primers from the core regions and then classified according to Simmonds.[24]

Nineteen patients (11 males and 8 females), mean age 42 ± 14 years (SD), were affected by HBV chronic hepatitis, presenting transaminases elevation for more than six months, presence of hepatitis B surface antigen in serum, hepatitis B e antigen in serum and/or detectable HBV–DNA in serum by a commercially available fluid phase hybridization assay (Abbott, hepatitis B viral DNA). Six patients were HBeAg negative, while all 19 patients had HBV–DNA detectable in serum. None of the HBV infected patients had detectable anti-hepatitis D antibodies. Serum markers for HBV and anti-hepatitis D virus were tested using commercial radioimmunoassay kits (Abbott, Chicago, IL, USA).

Seven patients (all females), mean age 52 ± 9 years (SD), were affected by primary biliary cirrhosis (PBC) at the histological examination. In all seven patients antimitochondrial antibodies in a titer of 1:80 or greater were found in serum.

Four patients (3 males), mean age 39 ± 9 years (SD), were found to be affected by primary sclerosing cholangitis (PSC). They presented typical cholangiographic abnormalities involving the biliary tree associated with an elevation in serum alkaline phosphatase (AP) for more than six months, in the absence of any other possible pathologic conditions. Four patients (2 males), mean age 53 ± 6 years (SD), had HCV relapsing hepatitis after orthotopic liver transplantation. Three patients (all females; 28, 34 and 42 years old), satisfied the criteria for the diagnosis of AIH according to the recently formulated scoring system.[25] Three liver biopsies (3 males; 58, 62 and 65 years old) came from three unifocal hepatocellular carcinomas (HCC) on HCV related cirrhosis. Two patients (2 males; 24 and 28 years old) were affected by alcoholic hepatitis (AH) as suggested by history of alcohol abuse for more than 6 months, a characteristic pattern of mild transaminases elevation with aspartate aminotransferase (AST), alanine aminotransferase (ALT) and a steatohepatitis pattern at histology examination. Eighteen hepatic tissue specimens (used as healthy controls), were obtained at surgery from people (10 males, mean age 49 ± 6 years (SD)) with normal liver function, submitted to cholecistectomy. The study design was in accordance with the declaration of Helsinki principles, and gained the approval of the local ethical committee in December 98. Written informed consent was obtained from all patients and controls.

Morphological Evaluation

Each liver biopsy (fixed in 10% buffered formaldehyde, embedded in paraffin, cut and routinely stained with hemotoxylin–eosin and periodic acid-Schiff) was evaluated by two expert pathologists, who were unaware of both EPR and silver-stained nucleolar organizer-region-associated (AgNOR) protein measurements. Viral hepatitis B and C have been scored according to Knodell's histological activity index.[26] A semiquantitative score (0–3) was used to evaluate the presence and extent of steatosis. Histological staging of PBC has been performed in accordance to Scheuer's classification.[27]

Electron Paramagnetic Resonance Spectroscopy

The detailed description of the technique is given elsewhere.[22] Briefly, before biopsy fixing, a small weighted portion of each biopsy specimen from patients (mean weight = 2.3 ± 1.9 mg (SD)) and controls (mean weight = 2.5 ± 1.5 mg (SD)) was cut and immediately treated with 1 ml of 1 mM hydroxylamine probe in physiologic solution containing 1 mM EDTA for 5 min at 37°C. Optimal time of incubation had been determined in preliminary experiments.[22] After that time, the sample was quickly frozen in liquid nitrogen to block any enzymatic reaction and by the time of measurement had reached room temperature. About 50 μl of the solution were transferred and sealed in a calibrated capillary glass tube, which was placed inside the cavity of a Bruker ESP 300 EPR spectrometer equipped with an NMR gaussmeter for field calibration, a Bruker ER 033M FF-lock and a Hewlett-Packard 5350B microwave frequency

counter. The spectra of the nitroxide radicals generated by reaction of the probe with the radicals present in the tissue were then recorded using the following instrumental settings: modulation amplitude = 1.0 G; conversion time = 163.84 ms; time constant = 163.84 ms; receiver gain 1.0 e5; microwave power = 6.3 mW. The intensity of the first spectral line of the nitroxide was used to obtain the absolute amount of radicals per tissue milligram after calibration of the spectrometer response with known solutions of TEMPO-coline in water, using an artificial ruby crystal as internal standard.

The hydroxylamine spin-probe employed in the present investigation was bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate (**I**) which is not commercially available but can be easily prepared from the parent piperidine as previously described.[23]

NOR Silver Staining and Quantitative Analysis of AgNOR Proteins

Liver biopsies were fixed in 10% buffered formalin and paraffin wax embedded. Histological sections were stained with silver for AgNOR proteins according to Ploton *et al.*[28] Sections were mounted on poly-L-lysine pre-treated slides, dewaxed in xylene and ethanol, and progressively rehydrated. Slides were then removed from water to heat-resistant plastic slide holders, fully immersed in 10 mM sodium citrate buffer (pH 6.0), and boiled in a commercially available pressure cooker at 120°C for 20 min.[29] After cooling to room temperature, sections were stained with silver according to the "one-step" method originally described by Ploton *et al.*[28] Silver-staining was carried out using a solution of one volume 2% gelatin in 1% aqueous formic acid and two volumes of 50% silver nitrate. The staining reaction was performed for 10 min at 37°C. After washing, the sections were dehydrated and mounted in a synthetic medium. Quantitative analysis of AgNOR proteins was performed by measuring the area of silver-stained structures within the nuclei of hepatocytes present in the whole section of the biopsy specimen at high magnification (40 ×), using a specific image analysis system (Image-Pro Plus 4.0, Media Cybernetics, Maryland, USA). The AgNOR proliferation index (AgNOR-PI), expressed as the percentage of hepatocytes with AgNOR protein area $\geq 7\,\mu m^2$, was automatically obtained at the end of each case. The same procedure had been previously used to determine hepatocyte proliferation in human liver biopsies.[30]

Statistics

Since the distribution of both ROS and AgNOR-PI variables could not be approximated by a gaussian curve (Kolmogorov–Smirnov test: $z = 2.08$ and 1.66, respectively; $p < 0.001$), non-parametrical tests were used for statistical analysis. For comparison of two groups of individuals, the Mann–Whitney *U*-test (two tailed) was employed. When more than two groups were compared, the Kruskal–Wallis test was used. Correlation coefficients (r) were calculated by the Spearman rank test. Data are given as medians and upper and lower values if not otherwise stated. Probability is two-sided and considered significant when < 0.05.

RESULTS

Patient and control characteristics are summarized in Table I. Since both PBC and AIH are unanimously considered prototypes of organ-specific immune-mediated diseases,[31] a single group named autoimmune liver disease (AULD), encompassing both entities has also been statistically analyzed. We decided not to include PSC in this category, since its etiology is still debated.

EPR spectra obtained from healthy and diseased liver samples showed significantly different signal intensities attributed to nitroxide **II** ($a_N = 15.52$ G, $g = 2.0062$) (Fig. 1). Preliminary investigations revealed that the life-time of radical **II** in samples prepared from healthy and diseased liver tissue was not significantly different, therefore, the absolute

TABLE I Patient and control characteristics

Variable	Controls ($N = 18$)	Patients ($N = 84$)	p
Age (yrs)	49 ± 6	46 ± 17	NS
Sex (M/F)	10/8	43/41	NS*
ALT (U/l)	19 ± 11	126 ± 86	< 0.05
GGT (U/l)	34 ± 14	79 ± 65	< 0.05
AF (U/l)	217 ± 39	266 ± 76	NS
Ferritin (ugr/ml)	41 ± 39	254 ± 127	< 0.05
Albumin (g/dl)	4.1 ± 0.51	3.9 ± 0.64	NS
Prothrombin time (s)	12.02 ± 0.9	11.97 ± 0.54	NS

Variables are expressed as mean ± SD except for sex, where absolute numbers are shown. p-Values were obtained by Student unpaired two tailed t-test where not otherwise stated. *Fisher's exact test.

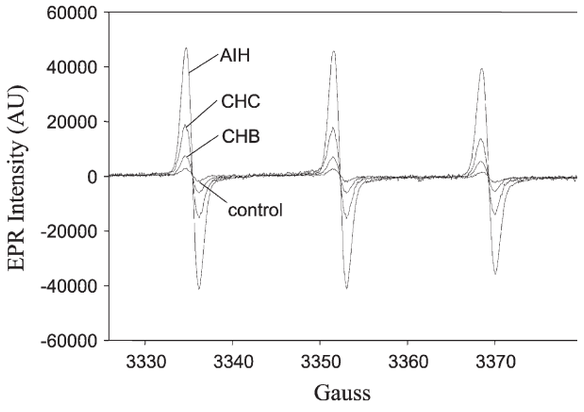


FIGURE 1 EPR spectra of the nitroxide (**II**) generated by incubation of the hydroxylamine spin-probe (1 mM) in physiologic solution with 1 mg of wet liver tissue from an healthy control or with the same amount of liver biopsy from patients affected by chronic hepatitis B (CHB), chronic hepatitis C (CHC) or autoimmune hepatitis (AIH). Signals are superimposed in the same scale for comparison.

concentration of nitroxide **II** in the sample was used to measure ROS production, after calibration of the spectrometer response.[22]

ROS production in liver tissue from patients affected by liver disease (median $= 5 \times 10^{-6}$ mmol/mg; range $= 7 \times 10^{-8}$, 9×10^{-5}) was found to be significantly higher when compared to free radicals activity in control tissue (median $= 3 \times 10^{-11}$ mmol/mg; range $= 5 \times 10^{-13}$, 6×10^{-10}) ($p < 0.001$) (Fig. 2).

Role of Etiology in ROS Production

In order to investigate the role of etiology, ROS productions in control, CHB, CHC and PBC groups were compared (Fig. 3). ROS levels measured in the four groups were statistically different ($p < 0.001$). In particular, free radicals steady state in controls (median $= 3 \times 10^{-11}$ mmol/mg; range $= 5 \times 10^{-13}$, 6×10^{-10}) was lower than that measured in CHB group (median $= 4 \times 10^{-7}$ mmol/mg; range $= 9 \times 10^{-8}$, 8×10^{-6}) ($p < 0.001$), as well as lower compared to CHC group (median $= 3 \times 10^{-6}$ mmol/mg, range $= 4 \times 10^{-8}$; 7×10^{-5}) ($p < 0.001$), and to PBC (median $= 2 \times 10^{-5}$ mmol/mg, range $= 7 \times 10^{-6}$; 9×10^{-5}) ($p < 0.002$) (Fig. 3). Oxygen-free radicals in CHB (median $= 4 \times 10^{-7}$ mmol/mg; range $= 9 \times 10^{-8}$, 8×10^{-6}) were lower than those recorded in CHC (median $= 3 \times 10^{-6}$ mmol/mg, range $= 4 \times 10^{-8}$; 7×10^{-5}) ($p < 0.001$), as well as in PBC (median $= 2 \times 10^{-5}$ mmol/mg, range $= 7 \times 10^{-6}$; 9×10^{-5}) ($p < 0.001$). The amount of ROS production in CHC (median $= 3 \times 10^{-6}$ mmol/mg, range $= 4 \times 10^{-8}$; 7×10^{-5}) was lower than that detected in PBC (median $= 2 \times 10^{-5}$ mmol/mg, range $= 7 \times 10^{-6}$; 9×10^{-5}) ($p < 0.002$) (Fig. 2). When ROS values from AIH patients were added to PBC in the AULD group, the same statistically significant differences, as for PBC alone, were recorded.

Correlations Among ROS and Other Parameters

In patients affected by HBV chronic hepatitis, ROS levels in tissue neither showed any correlation with Knodell's total score, nor with histological staging and grading as assessed with score's single items. Patients with hepatitis B e antigen in serum showed similar ROS values with respect to e antigen negative patients. Oxygen-free radicals in CHC group were highly correlated to the degree of

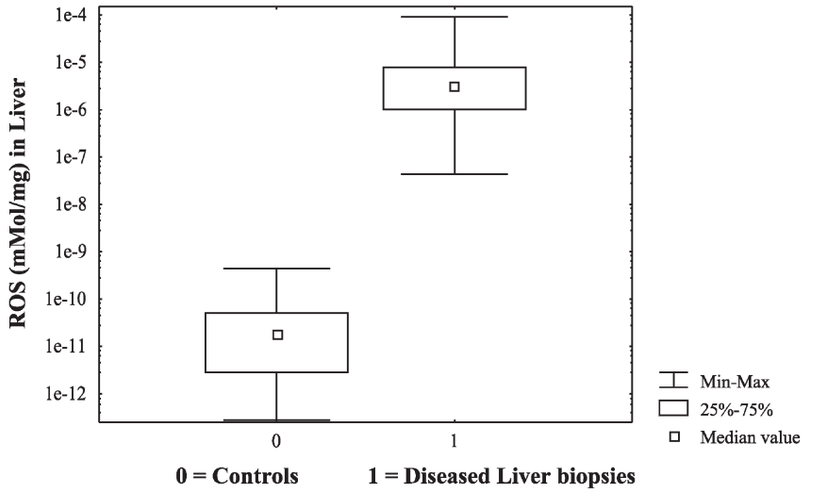


FIGURE 2 ROS in controls and patients. Oxygen free-radicals ([ROS] per mg wet-weight of liver tissue) in diseased liver biopsy specimens are significantly higher than those in controls ($p < 0.001$ at Mann–Whitney *U*-test). Worth noting, ROS in patients with liver disease have no overlapping values with control ones.

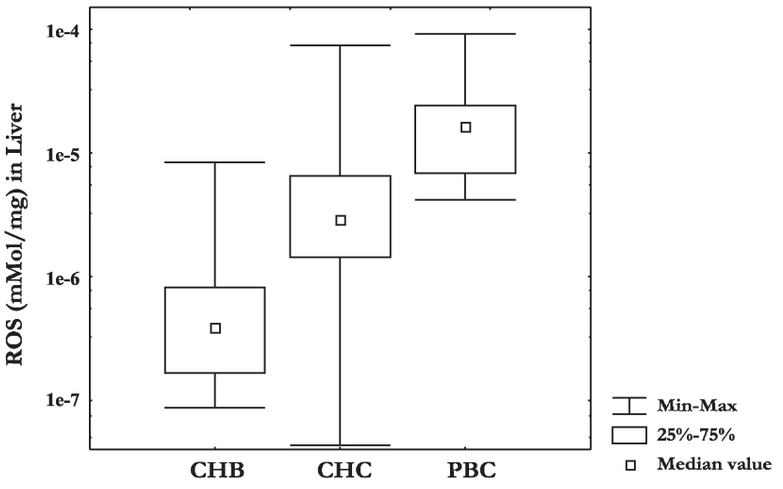


FIGURE 3 Role of etiology in ROS production. Oxygen free-radicals ([ROS] per mg wet-weight of liver tissue) in HCV chronic infected liver tissue (CHC) are significantly higher compared to levels recorded in patients with HBV chronic infection (CHB) ($p < 0.001$ at Mann–Whitney *U*-test), as well as lower in comparison to ROS measured in patients affected by primary biliary cirrhosis (PBC) ($p < 0.002$ at Mann–Whitney *U*-test).

histological inflammation according to Knodell's score ($r = 0.92$; $p < 0.001$) (Fig. 4), while no correlation was apparent in respect to Knodell's score remaining items. The two most common genotypes in CHC group were 1b and 2a, found in 49 and 27% of patients, respectively. ROS levels in these subgroups of patients were not significantly different. No correlation was found between ROS in tissue and disease activity as assessed by serum aminotransferases (AST, ALT). ALT showed a positive correlation only with Knodell's necrosis score ($r = 0.65$; $p < 0.001$). ROS measurements in CHB, CHC and PBC did not correlate with iron balance in serum, as assessed by ferritin, plasma total iron capacity and plasma iron concentration.

Oxygen-free radicals production in tissue showed no correlation with cell proliferation as assessed by AgNOR proteins analysis ($r = 0.146$; $p = 0.36$). Hepatocyte proliferation appeared partially influenced by etiology, though not in a significant manner, being AgNOR-PI 1.92% ± 2.01 in CHB patients, 0.56% ± 0.97 in CHC group and

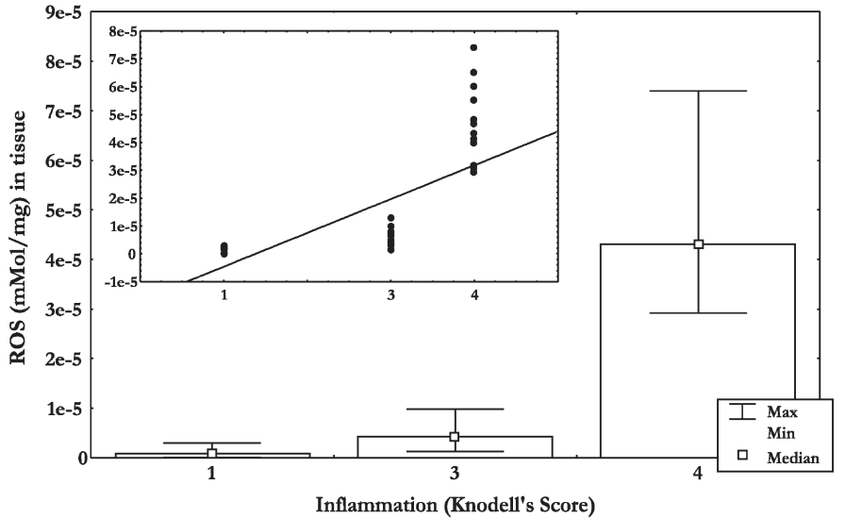


FIGURE 4 Correlation between ROS and inflammation in HCV subgroup. ROS levels (per mg wet-weight of liver tissue) in patients with HCV chronic hepatitis (CHC) are plotted in relation to hepatic histologic inflammation according to Knodell's score ($p < 0.001$ at Kruskal–Wallis test). The insert shows the positive correlation between ROS in liver and tissue inflammation in CHC ($r = 0.92$; $p < 0.001$ at Spearman-rank test).

1.38% ± 1.49 in patients belonging to PBC group ($p = 0.08$). A weak but significantly positive correlation between length of liver disease and AgNOR-PI was found ($r = 0.395$; $p = 0.0086$).

The role of fatty infiltration in ROS production was evaluated only in hepatitis C group since only four patients in hepatitis B group and none in PBC or AULD group showed steatosis at histological examination.

The presence of fatty infiltration was found in 20 patients out of 42 (48%) in CHC group, 14 of them (70%) showing a mild degree of fatty acid infiltration. The remaining patients presented a moderate degree of fatty acid infiltration. No patient was judged to present grade 3 fatty infiltrations. No correlation was apparent between the presence and degree of steatosis and levels of ROS production in hepatic tissue.

DISCUSSION

The nitroxide **II** detected and quantified by our EPR measurements is generated by oxidation of the hydroxylamine probe **I** in the biological sample under investigation, i.e. the hepatic tissue.[22] Since the probe **I** displays no particular selectivity it can be oxidized by hydroxyl, alkoxyl, peroxyl and alkyl radicals as well as by superoxide. Furthermore, cyclic hydroxylamines can also be oxidized to the corresponding nitroxides by peroxidase/H_2O_2 systems and peroxynitrite, and the nitroxide can be reduced back by ascorbate, GSH and by some radical reactions such as trapping of alkyl radicals.[22,23] Therefore the present radical-probe technique does not provide a mean to identify a particular radical species but should rather be regarded as a general way to measure the overall oxidative status of the system under investigation. However, since hydroxylamines display the highest reactivity toward oxygen-centered radicals the present technique provides a method to quantify ROS in a biological environment.[23]

Our results indicate that ROS production in liver disease is significantly increased in comparison to healthy liver tissue (Figs. 1 and 2). These data are in agreement with previous works showing a reduction of anti-oxidant status/increase of oxidative stress markers during liver diseases,[7–18] and for the first time provide evidence of a direct increase of ROS production in hepatic tissue during liver disease in humans. Whether the previously described reduction of scavenging products (i.e. vitamin E, hepatic- and serum-GSH) during liver disease is simply the consequence of ROS buffering, remains to be clarified. Since the liver is directly involved in metabolism of the majority of anti-oxidant molecules, a primary alteration in storage or synthesis of these radical scavengers appears to be a possible contribution to this oxidative imbalance.

Correlation with Etiologies

ROS production during liver disease seems to relate significantly to etiology. In particular, a statistically significant tendency of ROS levels to rise progressively from CHB through CHC, to PBC has been recorded. Studies comparing the degree of oxidative stress in liver disease are scarcely available. Hepatic malondialdehyde (MDA) and liver oxidized glutathione (GSSG) in chronic hepatitis C were reported to be higher as compared to chronic hepatitis B,[9,10] whereas glutathione concentrations in erythrocytes of patients with chronic hepatitis B and C have been reported not to differ significantly.[32] The *in situ* detection of lipid peroxidation by-products has shown that chronic cholestatic diseases, such as primary biliary cirrhosis, show an intense lipid peroxidation degree as compared to other chronic liver diseases.[11] Indeed, the detection of Mn-SOD in serum introduced the concept that PBC may be associated to higher levels of oxidative stress than in other liver diseases.[33] Because inflammatory cells are a recognized source of ROS,[34] the differential degree of immune system activation during different liver disease etiologies may be a key factor in determining different levels of ROS production. It has been observed that HBV-infected subjects who develop a self-limited *acute* hepatitis show a vigorous immune response towards HBV-proteins. On the contrary, peripheral blood T cells from patients with *chronic* HBV infection who do not clear the virus either appear to be completely unresponsive or show a weak responsiveness to HBV-antigens.[35] Therefore, our findings of intermediate levels of ROS production is not surprising, given the weak nature of immune response.

In hepatitis C, the vigor of the T cell response during the early stages of infection seems to be a critical determinant of disease resolution and control of infection.[36] During the acute phase of HCV infection, the immune response in subjects who subsequently develop a chronic hepatitis appears to be much weaker than that observed during chronic infection, while T-cell response to hepatitis C Virus proteins has shown little difference in persistent and self-limited hepatitis C virus infection.[37] It was speculated that patients able to mount an adequate immune response to HCV antigens in short times succeed in clearing the virus, whereas patients with persistent infection have a weaker immune response only during the acute infection, allowing virus replication during the chronic phase of the disease, despite the presence of significant immune system activation. These findings may imply that immune

system is hypo-activated in chronic hepatitis B, while it is hyper-activated in HCV chronic infection.[38] Furthermore, the positive correlation found between ROS production in HCV chronic infection and Knodell's score inflammation index supports this speculation.

PBC and AIH, clustered in the AULD group, show the highest ROS values. In patients affected by PSC, several immunological disorders have been described, which are typically associated with known autoimmune disorders.[39–41] Although the pathogenesis of PSC remains obscure, both genetic and environmental factors are involved, likely resulting in disturbances in the host immune system.[42] The presence of overlapping syndromes among PBC, AIH and PSC further underlies the possibility that immune system derangement is a common denominator in all three diseases.[43] Interestingly, ROS levels in patients affected by PSC were comparable to those measured in AULD group. Whether the present data support the hypothesis that auto-immune liver diseases have the strongest activation of immune system, as suggested by the highest levels of ROS production, remains to be confirmed.

The presence of cholestasis in PBC and PSC may also be suspected as a possible contribution to ROS generation. Hydrophobic bile acids hepatoxicity was reported to be significantly related to oxygen free radicals generation and reversed by preincubation with *d*-α-tocopherol.[44] Of note, among the seven PBC patients, the three who were receiving Ursodeoxycholic acid had lower ROS level than each of the other four patients.

Correlation with Hepatocyte Proliferation

In the present study, ROS production in liver diseases showed no correlation with hepatocyte proliferative activity, assessed by quantitative analysis of AgNOR proteins. Even when the role of etiology was excluded, proliferation did not appear to be influenced by ROS activity.

Both superoxide and hydrogen peroxide can each stimulate growth and growth responses in a considerable variety of cultured mammalian cell types when added exogenously.[45,46] Such observations led to suggestions that superoxide and hydrogen peroxide might function as mitogenic stimuli. However, a great amount of data, contrasting the positive role of oxidative stress in mediating cell proliferation, has also been reported.[47,48] It has been noted that, although low concentrations of hydrogen peroxide are generally growth stimulatory, the effects of higher concentrations of superoxide and hydrogen peroxide can have deleterious effect on mammalian cells and can lead to cell death.[49] In addition, the promoting effect of low levels of ROS in mediating proliferation may be highly cell specific,[50] and the critical balances in relation to proliferation and death may be very different for different cell types. Therefore, it is not completely surprising that ROS did not correlate with hepatocyte proliferation rate in our study. We found that the inflammation rather than the degree of ROS production in a precise moment as a possible determinant of hepatocyte proliferative activity.

Correlation with Ferritin Levels

Abundant evidence now exists from both *in vitro* and *in vivo* experiments that iron can catalyze the production of oxygen centered radicals.[51,52] Indeed, it has been reported that patients with hemochromatosis have increased mean plasma levels of TBA-reactants as well as decreased plasma concentrations of vitamin E and ascorbate compared to matched healthy controls.[53] However, in patients with milder iron overload, no effect of iron on oxidative balance status was reported.[54] It has, therefore, been suggested that iron overload can induce oxidative stress when body stores are substantially elevated, implying the existence of a threshold level under which the production of oxygen radicals may result unaffected.[55] Good correlation was reported between ferritin levels in serum and tissue iron measurement in different chronic liver diseases.[10,12] There is evidence supporting the idea that higher serum ferritin levels during active hepatitis may also reflect the degree of hepatocyte injury and/or the magnitude of the inflammatory response.[56] In our series, a positive correlation was found linking ferritin levels and transaminases in serum ($r = 0.54$, $p = 0.002$), while no correlation was found between ROS and transaminases and between ROS and ferritin levels. The lack of correlation in the latter case, may simply confirm that serum ferritin levels did not only reflect intracellular iron status, but also represented the inflammatory/necrotic undergoing process during chronic hepatitis.

Study Limitation

The different method used to obtain biopsies in controls with respect to the one employed in patients is probably the major limitation of our work. To avoid any methodological differences in surgical liver biopsy manipulation, the same Menghini-like fine needle used in patients were used in controls. Since anesthesia has well known effects on many hepatic enzymes, special care was taken to obtain liver specimens in controls shortly after liver isolation.

However, we cannot completely rule out that the different methods of tissue extraction could have partially affected final results.

The technique for oxidative stress assessment employed in the present work measures ROS production in liver biopsies *ex situ* and therefore may not completely represent the situation *in vivo*. A detailed discussion about advantages and drawbacks of the present technique for ROS measurement is given elsewhere.[22,23]

CONCLUSIONS

This work represents the first clinical application of an EPR radical probe technique, able to quantify ROS production in human hepatic tissue in real clinical settings. Our results indicate that ROS levels in liver tissue is remarkably increased during pathological conditions and significantly affected by disease etiology. Disease histological activity showed to positively affect ROS production in the HCV chronic hepatitis subgroup. Conversely, no link was found between a single assessment of ROS levels and hepatocytes proliferation activity. Interestingly, no correlation was found between ROS concentration in liver tissue and serum ferritin levels. The pathophysiological meaning and therapeutic implications of these findings are potentially worth investigating.

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