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Measurement of Oxidative Stress in Human Liver by EPR Spin-Probe Technique

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A method for the measurement of reactive oxygen species (ROS) in human hepatic tissue has been developed. The method is based on the EPR detection of the nitroxide radical produced by reaction of the hydroxylamine spin-probe bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate with ROS generated under pseudo-physiologic conditions in fine needle biopsies of healthy (10 controls) and diseased (22 patients) human liver. Measures of malonaldehyde in 9 liver biopsies (3 controls and 6 patients) have also been obtained by high pressure liquid chromatography and values parallel those obtained by the spin-probe technique. The amount of ROS found in healthy human liver (median $= 1.8 \times 10^{-11}$ mol/mg) was significantly lower than values found in liver affected by hepatitis B (median $= 5.8 \times 10^{-10}$ mol/mg; $p < 0.02$) or by hepatitis C (median $= 2.7 \times 10^{-9}$ mol/mg; $p < 0.003$) as well as compared to some other non-viral liver diseases (NVLD): autoimmune hepatitis, primary biliary cirrhosis, primary schlerosing cholangitis (median $= 9.8 \times 10^{-9}$ mol/mg; $p < 0.005$). NVLD also showed significantly higher ROS levels compared to hepatitis B ($p < 0.04$) and hepatitis C ($p < 0.04$).

The mechanism, potentiality and limitations of our method are discussed.

Keywords: Free radicals, ROS, EPR, hepatitis, human liver

Abbreviations: ROS, Reactive oxygen species; AIH, Autoimmune hepatitis; PBC, Primary biliary cirrhosis; PSC, Primary schlerosing cholangitis; NVLD, Non-viral liver disease; CAH, Chronic autoimmune hepatitis; FNH, Focal nodular hyperplasia; HCC, Hepatocellular carcinoma; HBV, Hepatitis B virus; HCV, Hepatitis C virus; HDV, Hepatitis D virus; EDTA, Ethylenediaminetetraacetic acid; TEMPO, 2,2,6,6-Tetramethyl-1-piperidinyloxyl; TEMPOH, 1-Hydroxyl-2,2,6,6-tetramethylpiperidine; TEMPO-choline, 2-[4-(2,2,6,6-tetramethyl-1-piperidinyloxy)-oxyethyl]trimethylammonium chloride; Trolox®, 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; SOD, Superoxide dismutase; EPR, Electron paramagnetic resonance; TBA, Thiobarbituric acid; MDA, Malonaldehyde

INTRODUCTION

The role of oxidative stress in the pathophysiology of several diseases, among which vascular disorders,[1] cancer[2] and degenerative

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diseases,[3] as well as its involvement in the process of aging,[4] has been assessed or suggested by many scientists during last decades. More recently the peculiar role of free radicals even under physiological conditions has been recognized, particularly their involvement in both inter- and intracellular signaling processes have been hypothesized.[5,6] All these reflect in an increasing need of techniques able to measure radicals and their reactions in biologic systems, particularly *in vivo*.[7]

The superoxide radical anion, $O_2^{\bullet -}$, and in general oxygen centered radicals, sometimes referred to as *reactive oxygen species* (ROS), are among the most important radical species involved in biologic processes and are mostly generated by cellular respiration and oxidation during the metabolism of arachidonic acid or xenobiotics. Oxygen centered radicals are also produced in phagocytic cells, mainly through the activation of NADPH oxidase, where they conceivably serve the scope of damaging the structure of the pathogen agents as well as representing a chemiotactic factor.[8] This partly constitutes the rational for their involvement in acute and chronic inflammation.

Indeed the role of ROS in the inflammation associated to infectious disease is highly controversial, especially during viral infections where they appear to be among the main mediators of tissue damage.[9]

Due to their high reactivity and, consequently, limited life-time the detection of free radicals *in vivo* is still troublesome and techniques able to directly assess ROS in human tissue are still scarcely available. Thus the vast majority of methods developed so far is based on the detection of the end-products arising from the reaction of free radicals with the biomolecules adjacent (or close) to their source of formation.[10] Given the multitude of structures which can be potentially attacked by free radicals in biologic systems, the monitoring of only one (or very few) reaction product can in some cases bring to misleading quantitative measurement of free radicals' activity.

Recently some of us has suggested the use of the highly lypophilic spin scavenger bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate which will quantitatively react with oxygen centered radicals generated in biologic systems to give a stable nitroxide measurable by electron paramagnetic resonance (EPR).[11] This technique has been successfully applied in mice as a whole-body harvest approach, as well as in microsomes and cell cultures.

The aim of this work was to develop a spin-probe technique, based on the same radical scavenger, to quantitatively measure ROS in human tissues, and which could be easily applied in clinical settings. We chose infected human liver biopsies because hepatitis constitutes the prototype of a chronic viral infection in which the long-standing host response to the pathogen seems to be more detrimental than the pathogen itself.

MATERIALS AND METHODS

Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), was purchased from Aldrich and stored at −20°C. Superoxide dismutase (SOD) from bovine eritrocites (Fluka) and catalase from bovine liver (Aldrich) were stored at −18°C and +5°C, respectively and a solution with the desired activity was prepared immediately before use. Bis(1-hydroxy-2,2,6,6-tetramethyl-4-piperidinyl)decandioate was synthesized by following a previously described procedure[12] and used as hydrochloride salt. All the other materials were commercially available and were used as received.

EPR Measurements

Human liver (0.9–6.2 mg) was obtained by fine needle biopsies from 22 patients with liver disease

(10 patients affected by hepatitis C virus (HCV) chronic hepatitis, 2 with hepatitis B virus (HBV) chronic hepatitis, 1 HBV–hepatitis D virus (HBV–HDV) chronic hepatitis, 2 primary biliary cirrhosis (PBC), 1 chronic autoimmune hepatitis (CAH), 1 primary schlerosing cholangitis (PSC), 2 alcoholic chronic hepatitis, 1 focal nodular hyperplasia (FNH), 1 hepatocellular carcinoma (HCC) and 1 HCV relapsing hepatitis after liver transplantation) were treated with 1 mL of hydroxylamine probe **I** 1 mM in physiologic solution containing 1 mM EDTA (or deferoxamine) for 5 min at 37°C. After that time the sample was quickly frozen in liquid nitrogen to stop any enzymatic reaction and subsequently allowed to reach room temperature. About 50 μL of the solution were transferred and sealed (to prevent dispersion of infected material) in a calibrated capillary glass tube which was in turn placed inside an EPR quartz tube. The actual volume of sample was chosen such to cover the entire sensitive area of the EPR cavity. Ten controls were obtained by the same technique from 50 to 100 mg of surgical healthy hepatic tissue and were similarly treated. All patients gave informed consent to participate in the study.

The nitroxide radical **II** generated by reaction of the probe with the radicals present in the tissue were then measured by EPR on a Bruker ESP 300 spectrometer equipped with an NMR gaussmeter for field calibration, a Bruker ER 033M FF-lock and a Hewlett-Packard 5350B microwave frequency counter for the determination of the *g*-factor which was corrected with respect to that of perylene radical cation in concentrated H_2SO_4 ($g = 2.00258$). Spectra were recorded using the following instrumental settings: modulation amplitude = 1.0 G; conversion time = 163.84 ms; time constant = 163.84 ms; receiver gain 1.0e5; 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 milligram tissue after calibration of the spectrometer response with a standard solution of 2-[4-(2,2,6,6-tetramethyl-1-piperidinyloxy)-oxyethyl]trimethylammonium chloride (TEMPO-choline) in water, using an artificial ruby crystal as internal standard.

In order to evaluate the extent of oxidation of the hydroxylamine by atmospheric oxygen under our experimental conditions, a reference sample containing only the solution of hydroxylamine in physiologic solution was prepared for each bioptic sample and treated in the same way.

To investigate the role of the time from tissue extraction to hydroxylamine addition (t_0), of the time of incubation (t_1) and of the time from sample preparation to measurement (t_2) on the overall EPR signal intensity, 5–7 tissue samples were obtained from the same biopsy and similarly treated except for the parameter under investigation. In order to assess the nature of the chemical species responsible for the oxidation of the hydroxylamine to nitroxide, three biopsies were split each into two samples, one of which was treated as reported above, while the other was additioned of 0.5 mL of 0.2 M Trolox® in physiologic solution immediately prior to incubation with 0.5 mL of 2 mmol hydroxylamine solution. In a similarly matched set of experiments, SOD (final concentration 1000 U/mg of tissue) or SOD and catalase (for each final concentration 1000 U/mg of tissue) were used instead of Trolox® to treat only one sample from each biopsy. Control experiments were performed using thermally denatured SOD. The same experiments in the absence and in the presence of SOD or SOD and catalase were repeated after homogenation of the tissue.

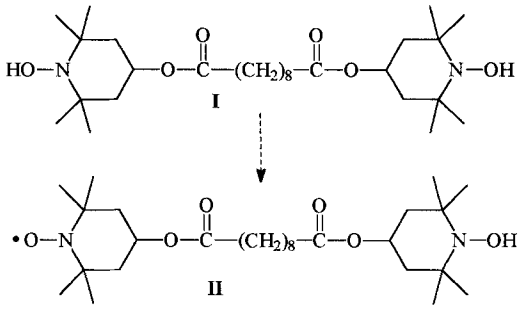
To test the role of tissue homogenation in our measures, three biopsies were divided into two samples each, one of which was homogenized by treatment with ultraturrax (IKA, T25 set at 6000 rpm for 10–30 s) after the addition of the hydroxylamine solution and then frozen in liquid nitrogen after 30 s incubation at 37°C, while the other was treated according to the standard protocols and results were compared.

Measurements of Malonaldehyde (MDA)

MDA was measured by reaction of thiobarbituric acid (TBA) with the biological sample according to standard procedures.[13] Specifically, approximately 3 mg of liver biopsy samples were weighted and homogenized in 250 μL of physiologic solution then treated with 500 μL of 0.6% TBA and 250 μL of 1 N HCl. The solution was heated at 95°C for 20 min and the MDA–TBA complex was measured by high pressure liquid chromatography (HPLC, Hewlett-Packard HP 1050) monitoring at 532 nm. A calibration curve was obtained with authentic MDA generated by acid hydrolysis of MDA-bis-dimethylacetal (1,1,3,3-tetramethoxypropane).

Statistic Treatment of Data

Since the distribution of ROS concentration in liver biopsies could not be approximated to a Gaussian curve thus requiring non-parametrical tests, Mann–Withney *U* test was employed to assess the difference of absolute amount of radicals per milligram tissue between two different groups of patients. When comparison was made among more than two groups Kruskal Wallis test was employed. For both tests $p < 0.05$ was required for statistic significance.



SCHEME 1

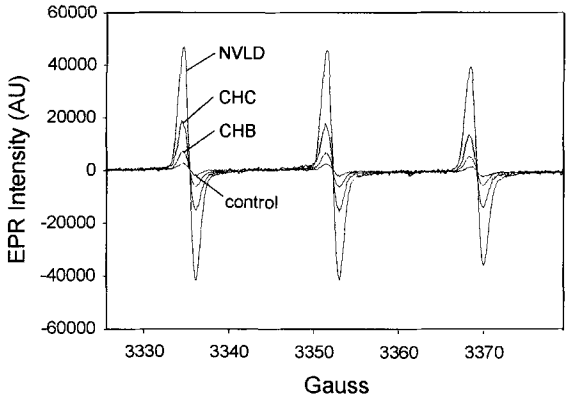


FIGURE 1 EPR spectra of the nitroxide generated by incubation of the hydroxylamine spin-probe (1 mM) in physiologic solution with 1 mg of healthy liver (control) or with the same amount of liver biopsy from patients affected by CHB, CHC or immuno-mediated liver disease. Signals are superimposed in the same scale for comparison.

RESULTS

When the samples prepared by incubating *ca.* 1 mg of human liver biopsy for 1–10 min at 37°C with 1 mL of physiologic solution containing ethylenediamine tetraacetic acid (EDTA) (1 mM) and the hydroxylamine **I** (1 mM) were analyzed by EPR spectroscopy, it was possible to observe an intense three lines EPR signal which was attributed to the nitroxide **II** on the basis of its spectral parameters ($a_N = 15.52$ G, $g = 2.0062$) (Scheme 1).

Analogous results were obtained when using deferoxamine as transition metals chelating agent. In all cases signal intensities were sensibly higher than those obtained from blank samples which were prepared by leaving the same solution exposed to atmospheric oxygen, in the absence of liver tissue, under the same experimental conditions employed for the bioptic samples (see Figure 1). This indicates that the nitroxide radical detected is due to the interaction of the hydroxylamine probe with the human tissue and is therefore related to the oxidizing activity of the tissue itself. In order to assess the nature of the chemical species and processes responsible for the oxidation of the hydroxylamine, three biopsies were split each into two samples which were incubated for 5 min at 37°C

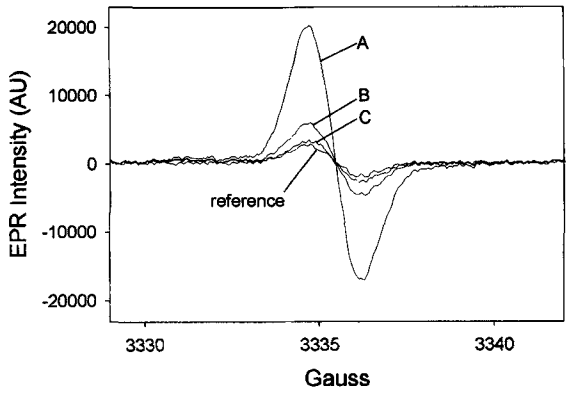
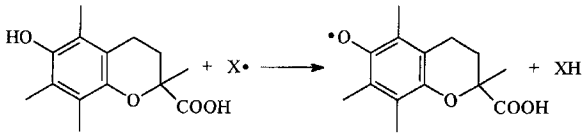


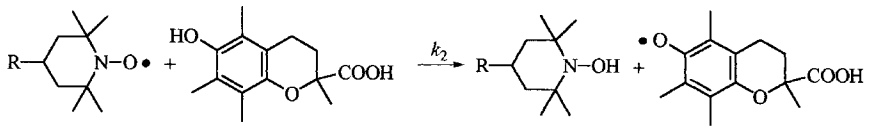
FIGURE 2 Low field expansion of the EPR spectra obtained by incubation at 37°C for 5 min of the *ca.* 1 mg of homogenated liver biopsy from a patient affected by CHC with: 1 mM probe + 1 mM EDTA (A); 1 mM probe + 1 mM EDTA + 1000 UI SOD + 1000 UI catalase (B); 1 mM probe + 1 mM EDTA + 0.1 M Trolox® (C). The reference was obtained by exposing a 1 mM solution of the probe to atmospheric oxygen for the same time required for the above measurements.

with the hydroxylamine **I** (1 mM) and EDTA (1 mM) in physiologic solution in the presence and absence, respectively of 0.1 M Trolox®, a water soluble mimic of α-tocopherol[14] which is known to behave as extremely effective radical scavenger giving a very fast hydrogen abstraction reaction with oxygen and carbon centered radicals.[15] While in the absence of the phenolic radical trap very intense EPR signals due to the nitroxide **II** were observed, when the liver tissue was incubated with Trolox® no nitroxide could be detected other than the amount formed by direct oxidation of the probe by atmospheric oxygen (see Figure 2). This clearly indicates that some radical species, conceivably oxygen centered radicals, generated in the liver tissue under pseudo-physiologic conditions is responsible for the oxidation of the hydroxylamine probe, thus when the phenolic trap is present in the system it competes with the probe in trapping the radical species (Equation (1)), preventing the formation of the nitroxide **II**:[19]



(1)

In principle, the inhibition of the signal formation in the presence of Trolox® could be due to a fast regeneration of the hydroxylamine from the nitroxide formed (Equation (2)), nevertheless the occurrence of this reaction under our experimental conditions can be excluded on the basis of EPR experiments. Thus, when 1 mM nitroxide **II** was mixed with 1–100 mM α-tocopherol in benzene inside the cavity of the EPR spectrometer, no reaction affecting the concentration of the nitroxide could be observed which allowed to estimate the rate constant for reaction (2) as $k_2 < 1\,M^{-1}\,s^{-1}$:



(2)

Experiments with SOD and Catalase

Since our measurements give little or no information about which is the actual radical species (X•) trapped by the probe under our usual experimental conditions, in a similar set of experiments the liver biopsies were incubated with the hydroxylamine and EDTA dissolved in 1 mL physiologic solution containing or not SOD (1000 U/mg of tissue) or SOD and catalase (for each 1000 U/mg of tissue). In the presence of these enzymes who are able to remove the superoxide radical anion from the reaction medium according to

Equations (3) and (4) the formation of the nitroxide **II** was strongly impaired:

$$H^+ + O_2^{-\bullet} + H_2O \xrightarrow{SOD} 3/2HOOH \quad (3)$$

$$HOOH \xrightarrow{catalase} 1/2O_2 + H_2O \quad (4)$$

Thus in all the experiments the amount of nitroxide produced by interaction with the hepatic tissue in the presence of SOD ranged from 30% to 70% of the amount detected in the absence of the enzyme and similar results were obtained when also catalase was present in the system (32–64% of the value registered in the same biopsy when only the probe and EDTA were present). When the same experiments were repeated using homogenated hepatic tissue from the biopsy instead of the whole biopsy sample, while the signal obtained in the absence of SOD and catalase was similar to that obtained without homogenation (*vide infra*), the presence of SOD with or without catalase reduced the amount of nitroxide detected to about 10% of the original value.

However, when the homogenated tissue was reacted with the hydroxylamine probe in the presence of SOD which had been previously denatured by thermal shock, the EPR signal obtained ranged from 19% to 28% of that obtained in the absence of the enzyme under the same conditions.

Role of Time and Other Experimental Conditions

With the aim to define the most convenient experimental settings, the role of the time from biopsy extraction to incubation with the hydroxylamine solution t_0, the time-length of incubation of the reaction mixture at 37°C, t_1, and of the time from sample preparation to the EPR measurement, t_2, on the result of the measurements were investigated. Results collected in Figure 3, which refers to the dependence of the amount of nitroxide detected as a function of t_0 and t_1, indicates that the signal intensity decreases very slowly on prolonging the time from tissue extraction from the human body to incubation with the solution of hydroxylamine, at least as long as the tissue sample is kept at 37°C for all the time. Clearly it would be advisable to keep this lap of time as short as possible but for practical reasons

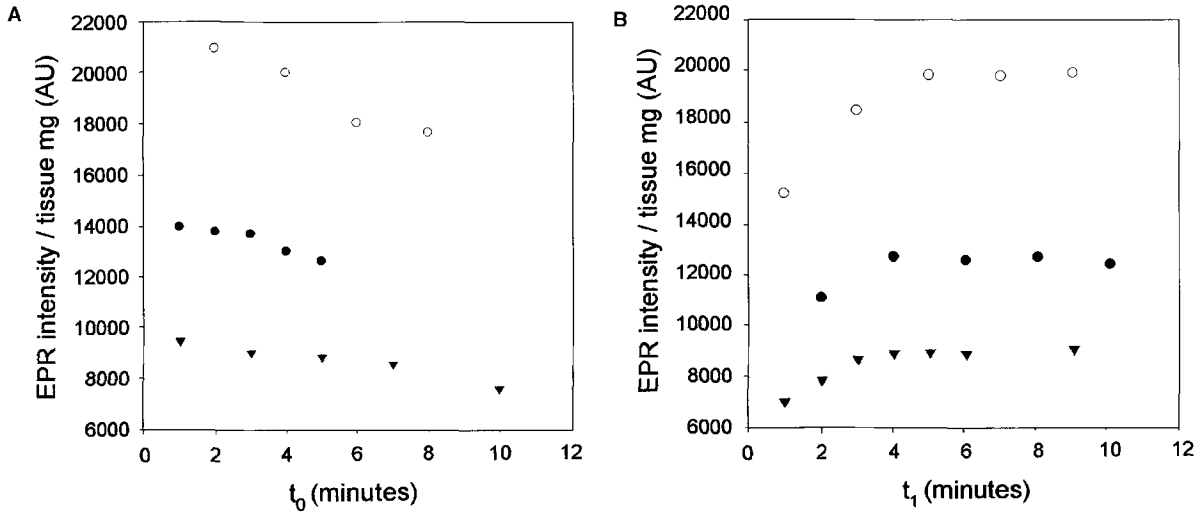


FIGURE 3 Dependence of the EPR signal, obtained by incubation of *ca.* 1 mg of diseased liver biopsy with 1 mM hydroxylamine probe, on the time (t_0) from tissue extraction to incubation (A), and on the time-length (t_1) of incubation (B). Each plot represents an individual biopsy from CHB (▼), CHC (●) and NVLD (○) diseased liver, respectively.

we chose to fix this time to 4 min in all our experiments, since this waiting causes the signal to decrease only by less than 10%. Figure 3 also reveals that the detected amount of nitroxide increases significantly with the incubation time until it reaches a plateau after about 3–4 min. This most probably represents the time required for the lypophilic probe to diffuse in both the extracellular and intracellular compartments where it can react with the superoxide produced *in situ*, (*vide infra*), therefore in all our measurements the incubation time was fixed to 5 min. The time elapsed from the recovery of the sample after freezing in liquid nitrogen to the EPR measurement (t_2), on the contrary, appeared to have no effect on the final result, thus the signal decreased only by about 10% on leaving the sample exposed to room light and temperature for 8 h. This is not surprizing considering the stability and persistence of the nitroxide radical generated from our probe.

Also homogenation of the hepatic tissue showed to have little or no effect on the measurement, thus incubation of the homogenated tissue with 1 mM hydroxylamine for 30 s to 5 min produced an EPR signal which was not significantly different from that obtained after 5 min incubation of the whole biopsy under the same experimental conditions (data not shown).

Quantitative Measurements

Quantitative measurements of the amount of nitroxide radical **II** produced in each sample were carried out after calibration of the spectrometer response with a standard solution of TEMPO-choline in water using an artificial ruby crystal as internal standard (see Materials and Methods). The absolute amount (in mmol) of nitroxide radical generated by reaction with ROS per milligram tissue could be calculated by the following expression:

$$[\mathrm{ROS}] = \frac{[\mathrm{NOX_S}] - [\mathrm{NOX_R}]}{W} V \qquad (5)$$

where $\mathrm{NOX_S}$ is the molar concentration of the nitroxide in the bioptic sample, $\mathrm{NOX_R}$ is the concentration of nitroxide found in the reference sample due to oxidation of **I** by air, V is the sample volume (usually 1.0×10^{-3} L) and W is the actual weight of the biopsy. Results are summarized in Table I.

The amount of oxygen free radicals in diseased liver (median $= 3.2 \times 10^{-9}$ mol/mg; range $= 1.9 \times 10^{-10}$ to 2.6×10^{-8}) was significantly higher than the control value (median $= 1.8 \times 10^{-11}$ mol/mg; range $= 3 \times 10^{-13}$ to 4.4×10^{-10}) ($p < 0.0002$) (Figure 4). In order to investigate whether different hepatocyte damaging mechanisms can

TABLE I Concentration of ROS (expressed as median value) measured in the liver tissue

Groups	ROS median (mol/mg)	Controls	LD	CHB	CHC
Controls	1.8×10^{-11} (3×10^{-13} to 4.4×10^{-10})	—	$p < 0.00001$	$p < 0.02$	$p < 0.0003$
LD	3.2×10^{-9} (1.9×10^{-10} to 2.6×10^{-8})	$p < 0.00001$	—	—	—
CHB	5.8×10^{-10} (4.7×10^{-10} to 1.8×10^{-9})	$p < 0.02$	—	—	n.s.
CHC	2.7×10^{-9} (1.9×10^{-10} to 7.7×10^{-9})	$p < 0.0003$	—	n.s.	—
NVLDs	9.8×10^{-9} (2.6×10^{-9} to 2.6×10^{-8})	$p < 0.005$	—	$p < 0.04$	$p < 0.04$

Data in parenthesis represent the range of concentration found in the samples belonging to each group. The values of p reported refer to the Mann–Withney test for the difference in ROS found between different groups. LD = All enrolled liver diseases considered as a single group; CHB = chronic hepatitis B; CHC = chronic hepatitis C; NVLDs = non-viral liver diseases (AIH, PSC, PBC); n.s. = not significant.

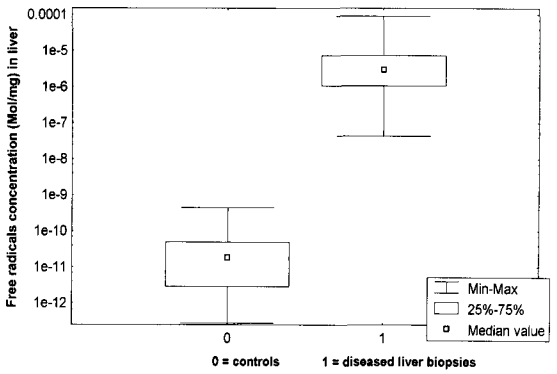


FIGURE 4 Levels of ROS in human hepatic tissue from healthy controls (0) and patients affected by liver diseases (1) ($p = 0.00001$ by Mann–Withney *U* test).

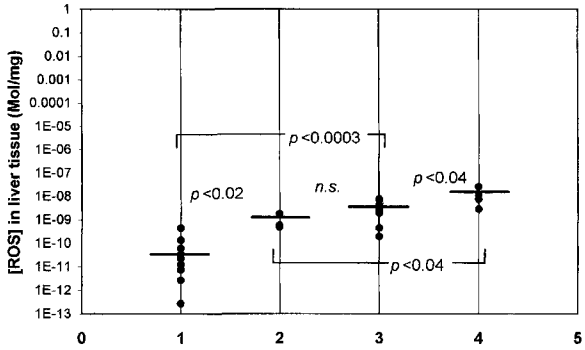


FIGURE 5 Levels of ROS found in liver biopsies from healthy controls (1), CHB patients (2), CHC patients (3) and NVLDs patients (4). Horizontal bars represent the median value for each group, while *p* refers to the Mann–Withney test for the difference between each pair of groups.

lead to different degree of hepatic ROS production, we clustered as non-viral liver diseases (NVLD) those liver diseases in which an intense immune system activation is present, i.e. autoimmune hepatitis (AIH), PBC, and PSC and compared this group with viral chronic hepatitis. Controls, chronic hepatitis B (CHB), chronic hepatitis C (CHC) and NVLDs differed significantly in relation to ROS values ($p < 0.0002$) (Figure 5). In particular ROS in control group (median $= 1.8 \times 10^{-11}$ mol/mg; range $= 3 \times 10^{-13}$ to 4.4×10^{-10}) was significantly lower than in hepatitis B group (median $= 5.8 \times 10^{-10}$ mol/mg; range $= 4.7 \times 10^{-10}$ to 1.8×10^{-9}) ($p < 0.02$), as well as compared to hepatitis C (median $= 2.7 \times 10^{-9}$ mol/mg; range $= 1.9 \times 10^{-10}$ to 7.7×10^{-9}) and NVLDs (median $= 9.8 \times 10^{-9}$ mol/mg; range $= 2.6 \times 10^{-9}$ to 2.6×10^{-8}) ($p < 0.003$, $p < 0.005$, respectively). ROS in NVLDs were significantly higher than in hepatitis B ($p < 0.04$) and hepatitis C ($p < 0.04$).

Comparison with Measurements of MDA

In order to validate our measurement and to establish a comparison with a more traditional technique employed for the evaluation of oxidative stress in biologic systems, 9 tissue samples (3 HCV, 3 NVLD and 3 healthy controls) were analyzed by the "thiobarbituric acid test"[26] measuring the TBA–MDA adduct.

TABLE II Level of MDA or MDA-like substances found in human hepatic biopsies and amount of ROS measured by the EPR-spin-probe method in the same biotic samples

Sample No.	Group	MDA (mol/mg)	ROS (mol/mg)
1	Control	6.0×10^{-11}	1.7×10^{-11}
2	Control	6.3×10^{-11}	3.0×10^{-11}
3	Control	5.5×10^{-11}	3.9×10^{-11}
4	HCV	8.5×10^{-11}	1.8×10^{-9}
5	HCV	8.9×10^{-11}	5.0×10^{-9}
6	HCV	7.0×10^{-11}	2.9×10^{-9}
7	NVLD	1.30×10^{-10}	1.0×10^{-8}
8	NVLD	1.24×10^{-10}	7.9×10^{-9}
9	NVLD	1.18×10^{-10}	1.8×10^{-8}

About 3 mg of homogenated tissue suspended in physiologic solution were heated with TBA under acidic conditions and analyzed by HPLC with 532 nm UV detection. Results expressed as MDA equivalents per milligram tissue are collected in Table II and satisfactorily parallel to those obtained in the same biopsies by the spin-probe method (Figure 6, $r = 0.83$, $p < 0.05$) considering the major difference between the two techniques and the complexity of the system under investigation.

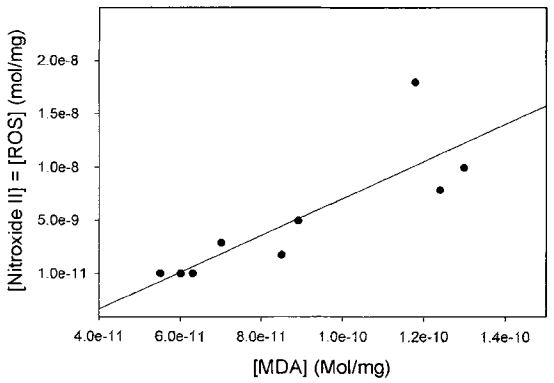


FIGURE 6 Plot of ROS levels found in healthy and diseased liver tissue by the EPR-spin-probe technique and the MDA levels measured in the same samples by the TBA-HPLC method (data from Table II). $p < 0.05$, $r = 0.83$.

DISCUSSION

The main problem arising when discussing the results of a measure of free radicals in a complex system like human liver is to realize what is actually being measured.

Aliphatic hydroxylamines are known to react with oxygen centered radicals to generate stabilized nitroxides. Thus the rate constant for hydrogen abstraction by peroxyl radicals has been reported as $k = 3 \times 10^5\,M^{-1}\,s^{-1}$ at 323 K from diethyl hydroxylamine[20] and as $k = 5 \times 10^5\,M^{-1}\,s^{-1}$ at 338 K from 1-hydroxyl-4-oxo-2,2,6,6-tetramethylpiperidine (TEMPOH).[21] TEMPOH is oxidized to the corresponding nitroxide by superoxide at pH 7.8 and room temperature with a rate constant of $1.7 \times 10^3\,M^{-1}\,s^{-1}$[22] and this value is expected to increase to $10^4\,M^{-1}\,s^{-1}$ under more acidic conditions where the superoxide exists predominantly in the more reactive $HOO^{\bullet}$ form. Alkoxyl radicals are known to give a very fast hydrogen abstraction from aliphatic hydroxylamines,[23] and hydroxyl radicals will react with rate constants of $10^9\,M^{-1}\,s^{-1}$ or higher.

Cyclic hydroxylamines can also be oxidized to the corresponding nitroxides by peroxidase/H_2O_2 systems[25] and the nitroxide can be reduced back by ascorbate. Furthermore hydroxylamines can undergo autoxidation in biologic systems due to the presence of metal ions such as Cu^{2+} and Fe^{3+}. Apart from autoxidation which can be neglected under our experimental conditions due to the presence of metal chelators (EDTA and deferoxamine) in any sample, all the other processes might potentially contribute to determine the total amount of nitroxide detected by EPR in our measurements. The same radical species or redox processes responsible for the generation of the nitroxide, however, are among the most important factors involved in the so called "oxidative stress" of a biologic system.[26]

Our experiments in the presence of Trolox® strongly indicate that nitroxide **II** is mostly generated from our probe by the scavenging of reactive radical species generated in the tissue, conceivably oxygen centered radicals, although no indication is provided about which radical species is actually involved.

Unfortunately no unambiguous answer could be obtained from the experiments in the presence of SOD or SOD and catalase. Indeed measurements in the presence of SOD with or without catalase, after homogenation of the bioptic sample, resulted in a roughly 90% suppression of the nitroxide formation. This apparently indicates that superoxide is the main responsible for the oxidation of the hydroxylamine probe. On the other hand the addition of the above enzymes to the whole biopsy resulted in only a partial suppression of the EPR signal. This can be explained by admitting that these large proteins cannot enter the cell and protect the intracellular and transmembrane oxidation of **I** unless the compartmentation is destroyed by homogenation.

Surprizingly also when thermally denatured SOD was added to the bioptic sample the EPR signal due to nitroxide **II** was markedly reduced (19–28% of the original value). Clearly the role of this enzyme under our experimental conditions consists mostly of a general non-specific radical scavenging activity, presumably due to the many –SH groups present in the protein. Although no definite conclusion can be drawn, these

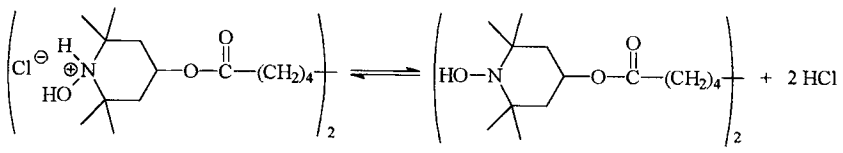
experiments however indicate that superoxide might provide only limited contribution to the formation of the nitroxide **II** (roughly 10%).

The present method can therefore be regarded as a general measure of oxidative stress in biologic systems rather than a detection of a specific radical species. Indeed due to the higher reactivity of oxygen centered radicals, compared to nitrogen and carbon centered radicals, toward hydroxylamines, we propose the present technique as a method for the evaluation of the steady-state levels of ROS in the system under investigation.

In order to validate our measurements we compared our results with the HPLC analysis of MDA (or MDA-like compounds) in a number of liver biopsies. We chose this reference because, despite its limitations, it is one of the most widely employed method for the evaluation of lipid peroxidation in biologic-like environments which, in turn, is a valuable indicator for the oxidative status of a biologic system.[26,27] A fairly good linear correlation (Figure 6, $r = 0.83$, $p < 0.05$) was obtained when comparing MDA levels with ROS measurements in the same biopsies which indicates that the two quantities being measured vary in the same way and conceivably are indicators for the same phenomenon.

Indeed hepatic[28] and serum[29] level of MDA in humans affected by HCV liver disease have been reported to increase compared to healthy controls or different liver diseases and a correlation between serum MDA levels and the severity of the HCV hepatitis has been observed.[29]

In a very complex biological system like the hepatic tissue, enzymatic and non-enzymatic processes capable of producing ROS under physiologic or pseudo-physiologic conditions are potentially located in any of the three main compartments into which the system can be ideally split, namely intracellular spaces, extracellular spaces and cell membranes (inside the phospholipid double layer). Due to their high reactivity, however, oxygen centered radicals would not escape the compartment into which have been generated and would, instead, react with structures or substrates present close to their site of production. As a consequence, radical probes or traps that do not possess the correct hydrophilicity/lypophilicity balance would be physically confined in only one of these compartments and would be able to specifically detect only the radicals produced in that compartment. The hydroxylamine probe **I**, due to the rapid protonation equilibrium between the free hydroxylamine form and the ammonium chloride form (Equation (6)), appears to have just the correct lypophylicity to cross the cell membrane and distribute on both intracellular and extracellular compartments, thus scavenging ROS wherever they are generated. This provides a somewhat global picture of the oxidative stress in a biological system which is desirable for quantitative measurements:



(6)

In this conjunction it is interesting to note that the distribution of the hydroxylamine in any compartment of the 1 mm thick biopsy is not an instantaneous process and follows a passive diffusion-like kinetic which appears to be complete after about 4 min (see Figure 3). Indeed after that time the amount of nitroxide produced is practically coincident with that obtained after homogenation of the hepatic tissue. The ability of this spin-probe technique to detect the averaged radical activity without any need to destroy compartmentation is of particular practical interest in those cases where homogenation of the

system cannot be easily performed such as when it is to be used *in vivo*.[10]

By means of our EPR-spin-probe technique we could quantify steady-state levels of oxygen free radicals in human liver tissue. Due to their limited life-time, the ROS measured are conceivably those produced in the bioptic tissue after its extraction. Nevertheless the short time of incubation needed and our peculiar experimental setting, allowed the tissue to be kept under pseudo-physiologic conditions and to preserve enzymatic and non-enzymatic activities practically coincident to those operating *in vivo*. This makes the present measurements highly representative of the oxidative stress in living human hepatic tissue.

Our results indicate that when liver tissue is exposed to a pathological process, the oxidative pressure is increased as compared to healthy liver tissue, see Figure 4. In addition, the degree of oxidative stress seems to be at least partly influenced by the etiology of liver disease. Although the present limited enumeration of cases prevents to draw definitive conclusions from our work, some preliminary speculations on the meaning of our data may be attempted.

We recorded an intriguing tendency of ROS to increase starting from CHB, through CHC, to NVLDs (Figure 5), thus possibly paralleling the degree of immune system activation in the different liver diseases.

NVLDs, such as AIH, PSC and PBC are characterized by unknown etiology and an intense activation of immune system, whose targets are still debated. Some recent insights into the pathophysiology of these diseases may induce to think all three entities being autoimmune in nature.[30,31] Indeed our results indicate that NVLDs are associated with significantly higher ROS production than HCV and HBV related hepatitis. On the contrary, the difference in oxyradicals recorded between CHB and CHC was not statistically significant. Among the very few investigations available in the literature contrasting data have been reported. Thus MDA in liver tissue was found to be increased in hepatitis C positive patients compared to patients affected by HBV chronic hepatitis,[28] while glutathione concentration in erythrocytes resulted not to differ in HBV and HCV positive patients.[32] Clearly more data are needed before any conclusion can be drawn.

Among the many questions raised from basic investigations in these last decades regarding the role of ROS in human disease, the majority are still open. Hopefully, the new possibilities offered by this EPR-spin-probe technique will provide some answer to these speculations.

CONCLUSIONS

We have described an EPR-spin-probe technique which allowed us to measure ROS in human hepatic tissue. This method appears to be easily reproducible and moderately invasive. The limited time required for both the sample preparation and measurement makes this technique of interest where a large number of samples needs to be screened, although no indication of the actual radical species being detected can be obtained.

The radical activity resulted to be increased during pathological conditions compared to healthy controls. This allowed some speculations on the role of etiology in different oxidative conditions. Further work is in progress to assess the clinical relevance of our technique and the possibility to extend its application to different human tissues other than liver.

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[19] As a control experiment the hydroxylamine **II** was reacted with photolytically generated *tert*-butyloxyl radicals in benzene inside the cavity of an EPR spectrometer, in the presence of 100 fold excess α-tocopherol: no formation of the nitroxide **I** could be observed.
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