

Oxidative stress-related parameters in the liver of non-alcoholic fatty liver disease patients

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A B S T R A C T

Oxidative stress is implicated in the pathogenesis of non-alcoholic fatty liver disease (NAFLD). In the present study, hepatic and plasma oxidative stress-related parameters were measured and correlated with clinical and histological findings in 31 NAFLD patients showing increased body mass index. Liver protein carbonyl content was enhanced by 403 % in patients with steatosis ($n = 15$) compared with control values ($n = 12$), whereas glutathione content, superoxide dismutase (SOD) activity and the ferric reducing ability of plasma (FRAP) were decreased by 57 %, 48 % and 21 % ($P < 0.05$) respectively. No changes in microsomal *p*-nitrophenol hydroxylation and the total content of cytochrome P450 (CYP) or CYP2E1 were observed. Patients with steatohepatitis ($n = 16$) exhibited protein carbonyl content comparable with that of controls, whereas glutathione content, SOD and catalase activities were decreased by 27 %, 64 % and 48 % ($P < 0.05$). In addition, FRAP values in patients with steatohepatitis were reduced by 33 % and 15 % ($P < 0.05$) when compared with controls and patients with steatosis respectively, whereas *p*-nitrophenol hydroxylation (52 %) and CYP2E1 content (142 %) were significantly increased ($P < 0.05$) compared with controls. It is concluded that oxidative stress is developed in the liver of NAFLD patients with steatosis and is exacerbated further in patients with steatohepatitis, which is associated with CYP2E1 induction. Substantial protein oxidation is followed by proteolysis of the modified proteins, which may explain the co-existence of a diminished antioxidant capacity and protein oxidation in the liver of patients with steatohepatitis.

INTRODUCTION

Non-alcoholic steatohepatitis is a distinct hepatic disorder observed in patients without a history of significant alcohol consumption which histologically resembles alcohol-induced liver damage [1]. Recently referred to

as non-alcoholic fatty liver disease (NAFLD) [2], this entity is primarily associated with obesity, Type II diabetes and/or hyperlipidaemia [2–4]. Fatty liver, the earliest and most prevalent stage of NAFLD [5,6], is thought to sensitize the liver to additional necroinflammatory insults [7], thus promoting disease progression to

Key words: non-alcoholic fatty liver disease, oxidative stress, steatohepatitis, steatosis.

Abbreviations: BMI, body mass index; CAT, catalase; CYP, cytochrome P450; FRAP, ferric reducing ability of plasma; GSH, reduced glutathione; GSH-Px, glutathione peroxidase; NAFLD, non-alcoholic fatty liver disease; *p*-NPH, *p*-nitrophenol hydroxylation; ROS, reactive oxygen species; RNS, reactive nitrogen species; SOD, superoxide dismutase.

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steatohepatitis and cirrhosis [1,2,8]. A number of factors point to the multifactorial nature of this disease, including derangement in metabolic parameters, endotoxin-induced cytokine release and oxidative stress [7,9]. The metabolic parameters include mitochondrial dysfunction, amino acid imbalance, hyperglycaemia and imbalances in antiketogenic and ketogenic hormones in portal blood [7–9].

Under normal conditions, hepatic aerobic metabolism involves a steady-state production of pro-oxidants such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are balanced by a similar rate of their consumption by antioxidants [10]. Imbalance in the pro-oxidant/antioxidant equilibrium in favour of pro-oxidants constitutes the oxidative stress phenomenon, a condition that may induce a number of pathophysiological events in the liver [10–12]. Hepatotoxicity by oxidative stress may be achieved through a direct attack of ROS and RNS on essential biomolecules with loss of their biological functions and cell viability [10–12]. Alternatively, ROS may indirectly activate redox sensitive transcription factors such as nuclear factor κ B (NF- κ B) [13] or activator protein-1 (AP-1) [14], thus triggering the production of cytotoxic, proinflammatory and/or fibrogenic mediators by Kupffer cells and other non-parenchymal cells [15]. Several lines of evidence suggest that chronic oxidative stress may be important in the progression of NAFLD. Studies in animal models of NAFLD indicate a higher free radical activity in the liver as shown by (i) the increase in mitochondrial superoxide radical and H_2O_2 generation [16], (ii) the induction of the microsomal cytochrome P450 (CYP) isoforms CYP2E1 [17] and CYP4A10/4A14 [18], characterized by their high pro-oxidant activity [18,19], and (iii) the associated lipid peroxidation response [20]. In agreement with these animal studies, patients with NAFLD exhibit increased levels of hepatic CYP2E1 [21] and thiobarbituric acid reactants, which are a marker of lipid peroxidation [22]. In a recent study [23], NAFLD was associated with an increase in serological parameters of oxidative stress. The disorder in redox status was detected in more than 90% of the patients and evidenced by significant enhancement in malondialdehyde (92%) and 4-hydroxynonenal (98%) levels in erythrocytes from NAFLD patients and a reduction in antioxidant capacity of plasma, in comparison with values in healthy subjects of similar age or in patients with mild steatosis [23]. In view of these considerations, the aim of our present study was to investigate the molecular mechanism underlying liver oxidative stress in NAFLD. For this purpose, parameters affording antioxidant protection, namely superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) activities and total glutathione (GSH) content, were determined in the liver of NAFLD patients. Results obtained were correlated with the content and activity of CYP2E1 and the total

antioxidant capacity of plasma, and were analysed in terms of the clinical, histological and pathophysiological aspects of NAFLD.

METHODS

Patients and laboratory investigations

Thirty-one patients [average body mass index (BMI) of 42.4 ± 1.8 kg/m², age range of 18–55 years], who underwent therapeutic gastroplasty or gastrectomy with a gastro-jejunal anastomosis, were included in this study. Twelve patients (BMI = 22.0 ± 1.0 kg/m²) who underwent anti-reflux surgery or cholecystectomy were included as a control group. A complete clinical history, including data on nutrition and alcohol consumption together with anthropometric measurements, were obtained. Laboratory tests included liver enzymes, bilirubin, albumin, hepatitis B and C serology and auto-antibodies (anti-nuclear, anti-mitochondrial and anti-smooth muscle antibodies). In addition, serum levels of ferritin, transferrin saturation, ceruloplasmin, cholesterol, triacylglycerols (triglycerides), low-density lipoprotein, high-density lipoprotein and glucose were also determined. Subjects whom consumed two or more alcohol drinks per week or who had any blood tests suggesting other specific liver diseases were excluded. Selected patients were subjected to a diet of 25 kcal/kg (where 1 kcal \equiv 4.184 kJ), with 30% of the calories given as lipids and 15% as proteins, for at least 2 days prior to surgery.

Liver biopsies of approx. 2 cm³ were taken for histological diagnosis, immunohistochemistry and parameters related to oxidative stress. Liver samples were fixed in 10% formaldehyde, paraffin-embedded and sections were stained with either haematoxylin/eosin or Van Gieson's stain. Sections of each liver biopsy were observed in a blinded manner and evaluated for histological abnormalities by means of a previously defined code [24] to give three groups of individuals: (a) control group (normal liver histology), (b) patients with steatosis (macrovesicular steatosis), and (c) patients with steatohepatitis (steatosis and lobular inflammation with hepatocyte ballooning, without the presence of fibrosis). Steatosis was graded as absent (0), mild (1), moderate (2) and severe (3).

The Ethics Committee of the University of Chile Clinical Hospital approved this study protocol, and the study was performed according to Helsinki criteria. Informed consent was obtained from all patients.

Liver parameters related to oxidative stress and total antioxidant capacity of plasma

Liver samples were blotted on to filter paper, weighed and minced in ice-cold 0.25 M sucrose. SOD activity was

Table 1 Clinical and biochemical parameters in control subjects and in patients with non-alcoholic fatty liver disease as a function of the morphological characteristics of the liver

Values represent means \pm S.E.M. for the number of subjects indicated. The significant differences between mean values ($P < 0.05$), assessed by one-way ANOVA and Bonferroni's test, are shown by the letters identifying each group. Abbreviations: LDL, low-density lipoprotein; HDL, high-density lipoprotein; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ -GT, γ -glutamyltransferase; IU, international units. Parentheses show the percentage of subjects exhibiting altered values compared with the normal values.

Parameter (normal range)	(a) Controls ($n = 12$)	(b) Steatosis ($n = 15$)	(c) Steatohepatitis ($n = 16$)
Age (years)	42 \pm 3	43 \pm 4	38 \pm 3
Male/female ratio	6/6	3/12	3/13
BMI (< 25 kg/m ²)	22 \pm 1	35 \pm 2 (100) ^{a,c}	47 \pm 2 (100) ^{a,b}
Cholesterol (< 200 mg/dl)	156 \pm 11	194 \pm 13 (33)	180 \pm 12 (31)
Triacylglycerols (< 165 mg/dl)	109 \pm 15	135 \pm 27 (15)	186 \pm 31 (40)
LDL (< 140 mg/dl)	113 \pm 9	128 \pm 10 (30)	99 \pm 8 (14)
HDL (> 40 mg/dl)	51 \pm 4	44 \pm 3 (38)	42 \pm 3 (43)
Fasting glucose (< 110 mg/dl)	93 \pm 4	104 \pm 5 (20)	96 \pm 8 (8)
AST (< 40 IU/l)	25 \pm 3	34 \pm 7 (20)	28 \pm 3 (19)
ALT (< 50 IU/l)	30 \pm 3	53 \pm 11 (28)	47 \pm 5 (28)
AST/ALT ratio	0.78 \pm 0.08	0.66 \pm 0.13	0.63 \pm 0.08
γ -GT (< 78 units/l)	34 \pm 6	41 \pm 8 (8)	36 \pm 3 (0)
Bilirubin (< 1.1 mg/dl)	0.62 \pm 0.06	0.55 \pm 0.07 (0)	0.63 \pm 0.07 (6)
Albumin (> 3.5 g/dl)	4.1 \pm 0.1	4.1 \pm 0.1 (0)	3.9 \pm 0.1 (0)

determined in liver homogenates (10%, w/v) prepared in 0.25 M sucrose by using an assay based on the SOD-mediated increase in the rate of autoxidation of 5,6,6a, 11b-tetrahydro-3, 9,10-trihydroxybenzo[c]fluorene in aqueous alkaline solution to yield a chromophore with maximum absorbance at 525 nm [25]. The results were expressed as units/mg of protein (where 1 unit is defined as the enzyme activity that doubles the autoxidation background). The activity of CAT and GSH-Px were assayed in liver homogenates (10%, w/v) prepared in 1.15% (w/v) KCl/0.01 M Tris/HCl (pH 7.40) buffer. CAT activity was assayed in 2400-g supernatants by following the kinetics of H₂O₂ breakdown at 240 nm, and results were expressed on the basis of the rate constant of the first order reaction (k)/mg of protein [26]. Soluble GSH-Px activity was measured in the cytosolic fraction (100 000-g supernatant) by using a spectrophotometric method based on the reduction of glutathione disulphide coupled to NADPH oxidation by glutathione reductase [27]. GSH-Px activity was expressed as units/mg of protein (where 1 unit is defined as the enzyme activity that oxidizes 1 μ mol NADPH/min).

Liver protein oxidation was assayed by the reaction of 2,4-dinitrophenylhydrazine with protein carbonyls [28], and results were expressed as nmol carbonyl groups/mg of protein. Total GSH equivalents were assayed by the method of Tietze [29], and results were expressed as nmol/mg of protein. Proteins were measured according to Lowry et al. [30].

Microsomes were prepared by ultracentrifugation and used to measure the total content of CYP [31], the hydroxylation of *p*-nitrophenol to 4-nitrocatechol

catalysed by CYP2E1 [32] and CYP2E1 levels. The latter parameter was determined by Western immunoblotting using SDS/10% (w/v) polyacrylamide gels with 20 μ g of protein per lane [33]. After being transferred on to nitrocellulose sheets, blots were developed with a polyclonal antibody to human hepatic cytochrome P450E1 (Daiichi Pure Chemicals Co., Tokyo, Japan). Alkaline phosphatase was used as second antibody, and bands were stained with Nitro Blue Tetrazolium/5-bromo-4-chloro-3-indolylphosphate solution. Results were expressed as the patient/control ratio of arbitrary densitometric units.

Total antioxidant capacity of plasma was assessed as the ferric reducing ability of plasma (FRAP) samples [34].

All reagents used were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.), Merck (Darmstadt, Germany) and Riedel-de Haen (Germany), and were of the highest commercial grade available.

Statistical analysis

Results are expressed as means \pm S.E.M. for the number of patients indicated. The sources of variation for multiple comparisons were assessed by ANOVA, followed by Bonferroni's multiple comparison test. The differences were considered statistically significant at $P < 0.05$.

RESULTS

The main clinical and biochemical characteristics of NAFLD patients and the percentage of subjects exhibiting altered values in relation to normal values are shown in Table 1. Patients were divided into three groups

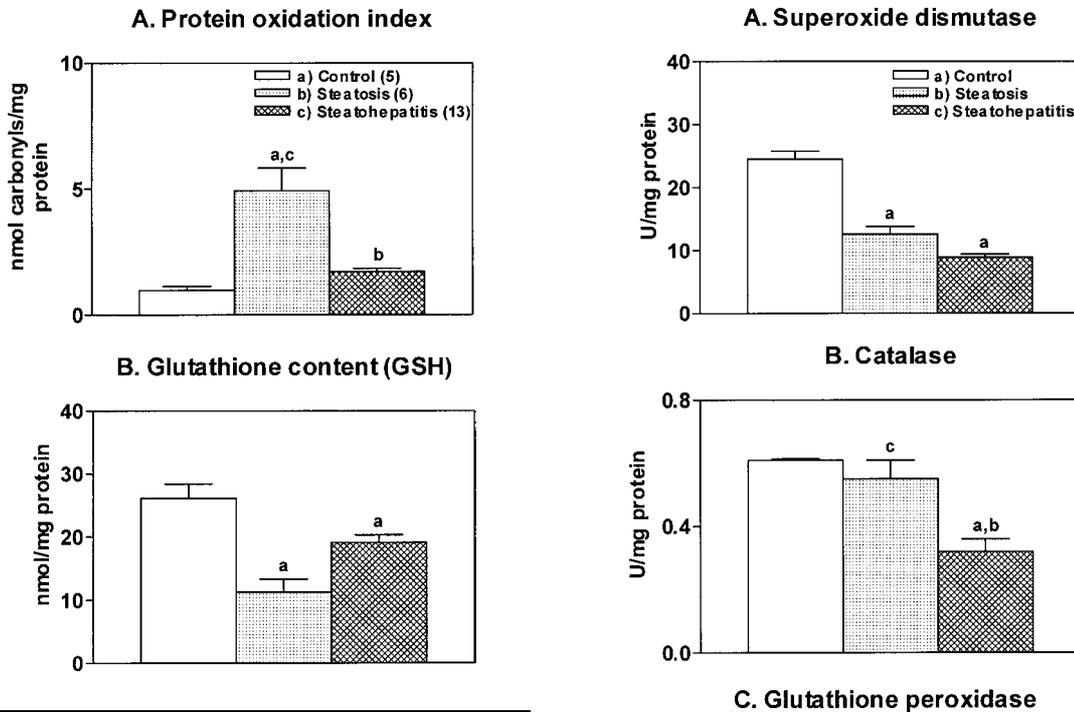


Figure 1 Protein oxidation index (A) and GSH content (B) in the liver of control subjects and in patients with non-alcoholic fatty liver disease with different degrees of liver injury

Values represent means \pm S.E.M. for the number of subjects per group indicated in parentheses. The significance between mean values was assessed by one-way ANOVA and Bonferroni's multiple comparison test, and is shown by the letters identifying each group of patients ($P < 0.05$).

according to their hepatic histology: (a) normal histology (controls, 12/43 = 28%), (b) steatosis (15/43 = 35%) and (c) steatohepatitis (16/43 = 37%). The steatosis score of control, steatosis and steatohepatitis groups were 0 ± 0 ($n = 12$), 1.22 ± 0.12 ($n = 11$) and 2.06 ± 0.23 ($n = 10$) respectively ($P < 0.05$ when control compared with steatosis or steatohepatitis; $P < 0.05$ when steatosis compared with steatohepatitis). The three groups revealed no significant differences in the parameters studied, except for BMI values that were increased in steatosis and steatohepatitis groups compared with controls ($P < 0.05$). Subjects were predominantly female and were negative for hepatitis B and C virus infection and for genetic or autoimmune liver diseases. The majority of patients were asymptomatic, with normal or mild alterations of liver function tests and lipid profile.

Parameters related to oxidative stress in the liver of patients with NAFLD are shown in Figures 1–4. Total protein oxidation, assessed by the content of hepatic protein carbonyls, was significantly increased ($P < 0.05$) in the liver of patients with steatosis when compared with that of patients with steatohepatitis (188%) or control subjects (403%; Figure 1A). The content of total hepatic GSH equivalents was significantly decreased

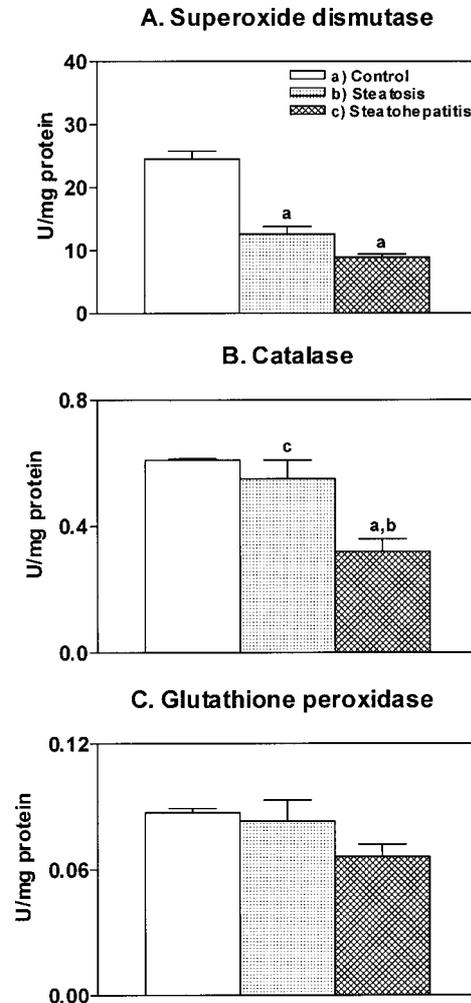


Figure 2 Specific activity of SOD (A), CAT (B) and GSH-Px (C) in the liver of control subjects and in patients with non-alcoholic fatty liver disease with different degrees of liver injury

Values represent means \pm S.E.M. for 6–13 subjects per group. The significance between mean values was assessed by one-way ANOVA and Bonferroni's multiple comparison test, and is shown by the letters identifying each group of patients ($P < 0.05$).

($P < 0.05$) compared with control values in patients with steatosis (57%) and steatohepatitis (27%; Figure 1B). Accordingly, patients with steatosis exhibited a 1280% increase in the ratio of hepatic protein carbonyl/GSH content (0.48 ± 0.10 nmol carbonyls/nmol GSH, $n = 6$; $P < 0.05$ compared with control) compared with control values (0.0375 ± 0.007 , $n = 5$), whereas no significant changes in this parameter were observed in patients with steatohepatitis (0.090 ± 0.009 nmol carbonyls/nmol GSH, $n = 13$). The specific activity of SOD exhibited 48% and 64% decreases in the liver of patients with steatosis and steatohepatitis respectively, compared with control values (Figure 2A), whereas that of CAT was significantly reduced in patients with steatohepatitis

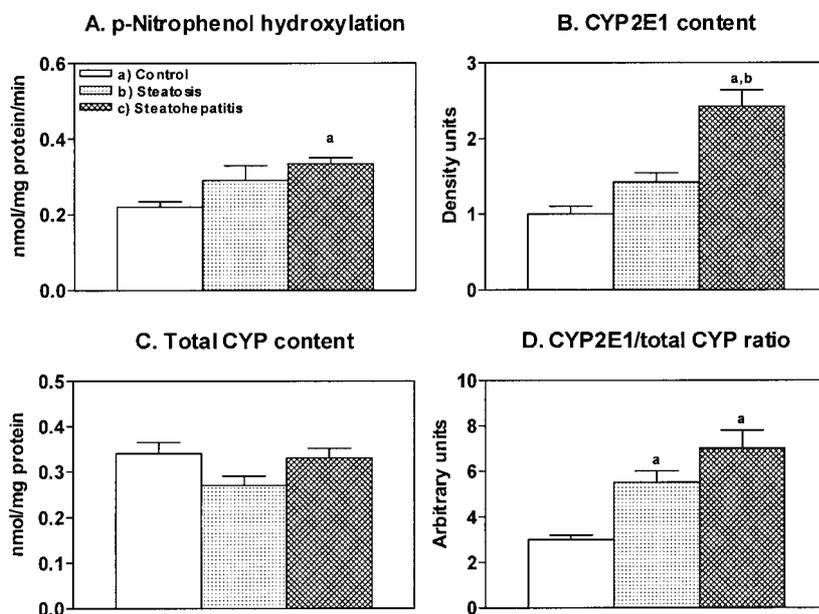


Figure 3 Microsomal *p*-NPH (A), CYP2E1 content (B), total CYP content (C) and CYP2E1/total CYP content ratio (D) in the liver of control subjects and in patients with non-alcoholic fatty liver disease with different degrees of liver injury. Values represent means \pm S.E.M. for 6–10 subjects per group. The significance between mean values was assessed by one-way ANOVA and Bonferroni's multiple comparison test, and is shown by the letters identifying each group of patients ($P < 0.05$).

compared with patients with steatosis (42 %) and controls (48 %; Figure 2B). However, no significant changes were observed in the activity of hepatic GSH-Px between the two subgroups of NAFLD patients and controls studied (Figure 2C).

Measurement of hepatic CYP2E1 activity, through the assessment of microsomal *p*-nitrophenol hydroxylation (*p*-NPH) [32], showed no significant changes in patients with steatosis compared with control values, whereas it was enhanced by 52 % ($P < 0.05$) in those with steatohepatitis (Figure 3A). Content of CYP2E1 assessed by immunoblot analysis revealed 142 % and 70 % increases in patients with steatohepatitis compared with controls and patients with steatosis respectively, whereas, in control subjects and patients with steatosis, it was comparable (Figure 3B). Values of *p*-NPH and CYP2E1 content obtained from controls and NAFLD patients with steatosis or steatohepatitis were significantly correlated ($r = 0.96$, $P < 0.05$). In these conditions, the total content of CYP was found to be comparable in the liver of controls and NAFLD patients (Figure 3C), thus leading to a 83 % and 133 % enhancement in the CYP2E1/total CYP ratio in steatosis and steatohepatitis compared with control values respectively (Figure 3D).

The total antioxidant capacity of plasma, assessed by the FRAP index, exhibited 21 % and 33 % reduction in patients with steatosis and steatohepatitis compared with control values respectively (Figure 4). In these groups, the FRAP values negatively correlated with the steatosis score ($r = -0.997$; $P < 0.001$).

Antioxidant capacity of plasma (FRAP)

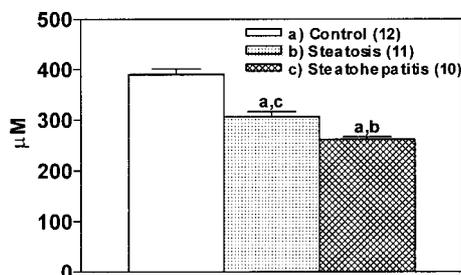


Figure 4 Total antioxidant capacity of plasma in control subjects and in patients with non-alcoholic fatty liver disease with different degrees of liver injury, measured as the FRAP.

Values represent means \pm S.E.M. for the number of subjects indicated in parentheses. The significance between mean values was assessed by one-way ANOVA and Bonferroni's multiple comparison test, and is shown by the letters identifying each group of patients ($P < 0.05$).

DISCUSSION

In agreement with earlier observations [35], patients in the present study show minimal changes in liver function tests and lipid profile but have elevated BMI over normal values, strengthening the association between obesity and the appearance of NAFLD [4,23,24]. According to the severity of the morphological alterations assessed

by liver histology, this condition was found to involve major changes in parameters related to oxidative stress in the liver and plasma samples. NAFLD patients with steatosis exhibit a substantial pro-oxidant condition in the liver, as evidenced by the higher content of protein carbonyls, GSH depletion and the consequent enhancement in the protein oxidation/GSH content ratio, when compared with values found in control subjects. This pro-oxidant condition occurs concomitantly with a significant decrease in hepatic SOD activity, changes involving an overall derangement in the antioxidant status of the liver, with the consequent diminution in the antioxidant capacity of plasma. The observation that NAFLD patients with steatosis exhibit a higher protein oxidation index in the liver may be of special pathological significance in those cases in which iron overload is evidenced [24,36,37]. Iron binding to proteins favours their interaction with ROS to produce carbonyl formation [38], a condition that may exacerbate oxidative damage in the liver [39].

Progression from steatosis to steatohepatitis involves a further decrease in the antioxidant capacity of the liver, as shown by the significant decrease in the activity of CAT, in addition to that of SOD, two major enzymes affording antioxidant protection. These changes are accompanied by an increased CYP2E1 activity, which involves a high production of free radicals and reactive intermediates [18,19], thus rendering the liver more susceptible to oxidative stress. Enhancement in hepatic CYP2E1 activity could lead to SOD and CAT inactivation upon progression of the disease, considering that SOD [40–42] and CAT [40,43] are inactivated *in vitro* by superoxide radical, hydroxyl radical, H₂O₂ or singlet oxygen. Induction of CYP2E1 in the liver of NAFLD patients with steatohepatitis was evidenced by the significant increase in its activity (*p*-NPH) and content (immunoblot analysis) [44], in agreement with studies assessing liver CYP2E1 by immunohistochemistry [21], and occurs regardless of changes in total cytochrome P450 content. Although obesity increases the activity of CYP2E1 in human liver, as shown by studies measuring the pharmacokinetics of chlorzoxazone [44–46] or the plasma levels of inorganic fluoride after exposure to halogenated anaesthetics [47], no correlation with liver injury was carried out. Furthermore, progressive functional deficiency associated with the development of liver injury, mainly protein synthesis, could also contribute to the lower activity of antioxidant enzymes found in patients with more advanced stages of NAFLD. In addition to the reduction in liver antioxidant enzyme activity, a systemic reduction in the antioxidant capacity was observed in patients with steatohepatitis, as evidenced by the significant additional drop in their FRAP values compared with those in patients with steatosis. This may represent a consequence of the higher pro-oxidant status developed in patients with steatohepatitis, which

is likely to involve a high consumption of cellular and circulating antioxidants, partly due to the decrease in the activity of SOD and CAT otherwise lowering ROS levels. In agreement with data reported previously [35], immunohistochemical assessment of intrahepatic nitric oxide markers, namely, inducible nitric oxide synthase expression and nitrotyrosine reactivity, were significantly higher in the severe forms of NAFLD than in the mildest forms.

Despite the lower liver and plasma antioxidant capacities found in patients with steatohepatitis compared with patients with steatosis, the hepatic protein oxidation index was significantly lower in patients with the more severe condition. This discrepancy could be explained by the higher susceptibility to proteolytic attack exhibited by ROS-oxidized proteins [38]. Thus the levels of oxidized proteins may be linked to the pro-oxidant status in a biphasic manner, initially increasing with the establishment of a pro-oxidant activity, followed by a reduction in the levels of oxidized proteins as the proteolytic action is enhanced.

The development of a higher oxidative stress status in the liver of NAFLD patients with steatohepatitis may also lead to modulation of Kupffer cell function, through activation of transcription factors such as nuclear factor κ B and activator protein-1 [13,14]. These transcription factors up-regulate genes coding for proinflammatory and/or fibrogenic mediators that may induce progression of NAFLD. This mechanism may account for the increased serum levels of the Kupffer cell- and stellate cell-derived cytokines, such as tumour necrosis factor- α (TNF- α) [48] and transforming growth factor- β 1 (TGF- β 1) [49], observed in NAFLD patients with steatohepatitis. Tumour necrosis factor- α is also overexpressed in the adipose tissue of obese humans [50] and rodents [51]. Furthermore, a significant improvement in serum aspartate aminotransferase, γ -glutamyltransferase, alkaline phosphatase and transforming growth factor- β 1 levels and in the histological grade of hepatic inflammation and fibrosis were reported after long-term α -tocopherol treatment [49], supporting the role of oxidative stress as an indirect pathogenic mechanism in NAFLD, an effect not observed with shorter periods of α -tocopherol administration [52]. In agreement with these studies in NAFLD patients, the animal model of non-alcoholic steatohepatitis elicited by feeding rats a high-fat diet for 12 weeks showed an increased number and activity of Kupffer cells [53]. Furthermore, in the model of non-alcoholic steatohepatitis produced by feeding mice a diet deficient in methionine and choline, chronic oxidative stress development precedes stellate cell activation and the expression of pro-fibrogenic genes [54], features that are inhibited by α -tocopherol [55].

In conclusion, oxidative stress-related parameters are markedly altered in the liver of obese NAFLD patients exhibiting normal or mild alterations in liver function

tests. Changes in the redox balance of the liver include the development of a substantial pro-oxidant condition at the early stage of steatosis, with a derangement in some major hepatic enzymic antioxidant mechanisms, induction of CYP2E1 with pro-oxidant behaviour [18,19] and systemic reduction in the antioxidant capacity of plasma at the latter stages of steatohepatitis. These data and previous observations [49,55,56] suggest that nutritional support with antioxidants might be useful in preventing oxidative damage and/or progression of NAFLD.

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