A 7-day profile of oxidative stress and antioxidant status in patients with acute liver failure

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Received: 28 December 2007 / Accepted: 12 August 2008 / Published online: 16 September 2008
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Abstract

Purpose Acute liver failure (ALF) is characterized by a rapid and massive destruction of hepatocytes. The role of oxidative stress in perpetuating the injury is undefined and may be a potential therapeutic target. Our aim was to study serial variation in oxidative stress and antioxidant status in patients with ALF.

Methods The study involved a prospective case–control study set in a tertiary care referral center. Thirty-two consecutive patients admitted with ALF were included with 23 healthy controls for comparison. Level of systemic oxidative stress as defined by superoxide dismutase (SOD), lipid peroxidation products (thiobarbituric acid reactive derivatives [TBARS]), and the total antioxidant capacity as the ferric reducing ability of plasma (FRAP) was measured at baseline on days 3 and 7.

Results The patients were aged 24 years (range 13–60 years) and included 20 females. Thirteen (40.6%) patients died. Patients with ALF had significantly increased systemic oxidative stress at presentation, as reflected by higher levels of SOD ($P < 0.001$) and TBARS ($P < 0.001$) than controls. Both TBARS levels and FRAP decreased progressively from admission to the end of first week among the survivors ($P = 0.004$ and 0.015 , respectively). The antioxidant status reflected by FRAP ($P = 0.001$) was significantly lower in ALF patients than controls. No relation was found between the level of oxidative stress and the mortality or complications.

Conclusion A high level of systemic oxidative stress exists in ALF, with depletion of antioxidant reserves.

Further studies are needed to define the clinical correlation of the large pro-oxidant burden.

Keywords Acute liver failure · Oxidative stress · Antioxidant status profile

Introduction

Acute liver failure (ALF) is characterized by a sudden and massive necrosis and apoptosis of hepatocytes. The main causes of ALF are viral hepatitis [1–3] or drug-induced liver toxicity [4–6]. Indirect evidence from animal studies suggests the role of reactive oxygen species and oxidative stress in the pathophysiology of ALF [7–10]. Oxidative stress has been implicated in a variety of liver diseases such as viral hepatitis [11, 12] and drug-induced hepatotoxicity [13, 14]. Furthermore, the dying hepatocytes can be a source of massive oxidative stress along with a depletion of antioxidant defenses. The oxidative stress can contribute to further loss of hepatocytes and impede regeneration, culminating in a vicious cycle. To the best of our knowledge, no human report is available, which has investigated this issue systematically. The aim of this study was to evaluate the role of oxidative stress when the liver fails acutely and to see the variation in oxidative stress and antioxidant levels as the liver injury progresses or resolves. We also sought to study the relation, if any, of the level of oxidative stress with the patient outcomes and complications.

Patients and methods

Thirty-two consecutive patients with ALF admitted to the intensive care unit, Department of Gastroenterology and

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Human Nutrition, All India Institute of Medical Sciences (AIIMS), were prospectively included in this study. Also 23 age- and sex-matched healthy controls among hospital staff and patients' relatives were enrolled for comparison of the oxidative stress and antioxidant levels. The controls were all nonsmoking and nonalcoholic healthy subjects free of any chronic illness. The median age of the controls was 20 years (range 20–58 years) and included ten females. According to the criteria laid down by the International Association for the Study of Liver, ALF was defined by the occurrence of encephalopathy within 4 weeks of onset of symptoms in the absence of any pre-existing liver disease [15]. The diagnosis was confirmed by the presence of submassive or massive necrosis in the postmortem liver biopsy specimen of patients who died. Pre-encephalopathy period, icterus-encephalopathy period, grading of encephalopathy, clinical cerebral edema, and criteria for infection have been defined earlier [16–18].

Management protocol

All patients were managed in the Department of Gastroenterology ICU, with standard organ support systems. A uniform management protocol was followed. Prophylactic antibiotics (piperacillin-tazobactam, vancomycin, and fluconazole) were instituted in each case. Cerebral edema was managed conservatively by bolus mannitol. Liver transplantation and liver replacement therapies were unavailable, and all patients were followed up until recovery or demise.

Viral markers

Serum sample from each patient was tested for hepatitis B surface antigen (HBsAg), IgM antibody against hepatitis B core antigen (IgM anti-HBc), and IgM antibody against hepatitis A virus (IgM anti-HAV) using commercial immunosorbent assay (ELISA) test kits (Organon, Teknika, Netherlands). IgM antibody to ORF-1, -2, and -3 of hepatitis E virus (HEV) and HEV RNA were tested by methods developed at our institute [19, 20]. Antihepatitis C (HCV) antibody was tested by using a third-generation commercial ELISA (Xcyton, Bangalore, India) test system.

Measurement of oxidative stress and antioxidant levels

An aliquot of 10 ml of blood was collected in plain and EDTA vials on days 1, 3, and 7 after admission among surviving patients. The serum and plasma samples were stored at -80°C for subsequent analysis. Ferric reducing ability of plasma (FRAP) was used as a marker of total

antioxidant capacity (TAC)/antioxidant reserves, whereas thiobarbituric acid reactive substances (TBARS) and superoxide dismutase (SOD) were used as markers of oxidative stress.

Estimation of lipid peroxidation

TBARS was estimated by the method of Belch et al. [21]. Briefly 0.5 ml of serum was added to 40% trichloroacetic acid and centrifuged for 10 min at 2,500 rpm to precipitate proteins. From this, 0.5 ml of supernatant was taken and 0.5 ml of 0.67% thiobarbituric acid was added. Similar steps were taken for the standard (1,1,3,3-tetraethoxypropane). The tubes were covered and boiled for 1 h in boiling water bath. The tubes were then cooled immediately in chilled water bath and optical density (OD) was recorded at 530 nm. The results were calculated according to the standard graph.

Estimation of serum SOD

SOD assay was done following the auto-oxidation of pyrogallol [22]. Tris-EDTA (50 mM) buffer was prepared and its pH was adjusted at 8.5. Pyrogallol solution was prepared (20 mM) in 40 mM of hydrochloric acid. SOD enzyme was procured from Sigma, India. The change in OD (ΔOD) for pyrogallol was set at 0.02–0.03 according to the sample. An aliquot of 50 μl of samples was diluted with 1,400 μl of buffer. Samples were then incubated at 37°C for 10 min, and 50 μl of adjusted pyrogallol (ΔOD per minute = 0.03) working solution was added to the samples. The change in OD was recorded both at 1 min 30 s and at 3 min 30 s at 420 nm and was calibrated according to the standard graph.

Estimation of TAC

FRAP was estimated by the method of Benzie and Strain [23]. For the working FRAP reagent, we mixed 10 vol of acetate buffer, pH 3.6, 300 mmol/l; 1 vol of TPTZ (Sigma), 10 mmol/l in 40 mmol/l HCl; and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (Sigma), 20 mmol/l. For the standard graph, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (Sigma) was used. To 1 mL of the working FRAP reagent, 33 μl of sample was added and the change in OD was recorded at 30 s and 4 min. The results were calculated using the graph generated by the standard.

Data analysis and statistics

All continuous variables are expressed as median (range). Kruskal–Wallis test was used to compare measures of oxidative stress and antioxidant reserves between groups (controls, cases at days 1, 3, and 7). The Friedman test was

used for nonparametric comparison of multiple-related samples within groups. The level of significance was set at $P = 0.05$. Statistical software SPSS (Version 10, SPSS Inc., Chicago, IL) was used for the statistical analysis.

Results

The median age of the study patients was 24 years (range 13–60 years) and included 20 females. All patients were admitted within 3 days of onset of encephalopathy. Eleven of the females were pregnant. The baseline features of the study patients are detailed in Table 1. The complications of infection, renal failure, gastrointestinal tract bleeding, and seizures were seen in 20 (62.5%), 10 (31.3%), 3 (9.4%), and 7 (21.9%) patients, respectively. Thirteen (40.6%) patients died.

Oxidative stress

Patients with ALF had significantly increased level of TBARS (nmol/ml) at baseline in comparison with that of healthy controls ($P < 0.001$). The TBARS level showed a significant decline from baseline to days 3 and 7 ($P = 0.003$) among surviving ALF patients (Figs. 1 and 2). The SOD (U/ml) level was significantly higher at baseline among ALF patients than healthy controls ($P = 0.001$). The level also gradually decreased toward day 7 among

Table 1 Baseline characteristics of the patients ($n = 32$)

Pre-encephalopathy interval (days) ^a	5 (1–27)
Icterus-encephalopathy interval (days) ^b	3 (1–20)
Grade 3,4 encephalopathy	24 (75%)
Cerebral edema	10 (31.3%)
Prothrombin time (seconds prolonged)	
3–25	15 (46.9%)
25–60	8 (25.0%)
>60	9 (28.1%)
Bilirubin (mg/dl)	14.7 (4.4–31.0)
Alanine-aminotransferase (IU/l)	996.5 (40.0–4570.0)
Sodium (meq/l)	141.0 (116.0–158.0)
Arterial ammonia (μ mol/l)	72.6 (17.0–477.2)
Etiology	
HAV	0
HBV	2
HEV	20
Dual acute	3
Etiology unknown	7

All quantitative data is depicted as median (range)

^a Interval from symptom onset to encephalopathy onset

^b Interval from onset of jaundice to encephalopathy onset

Fig. 1 TBARS significantly higher in cases on all days than controls (Kruskal–Wallis test, $P < 0.001$). TBARS significantly different on all days among cases (Friedman test, $P = 0.003$)

Fig. 2 Serum SOD significantly higher in cases at baseline than controls (Kruskal–Wallis test, $P = 0.001$). Serum SOD not different among cases during 7 days (Friedman test, $P = \text{NS}$)

surviving patients, but was not statistically significant (Table 2).

Antioxidant capacity

The FRAP level was significantly lower among ALF patients at baseline and on days 3 and 7 among survivors than healthy controls ($P < 0.001$). The FRAP levels

Table 2 Oxidative stress and antioxidant levels over the initial week among the survivors ($n = 11$)

	Day			<i>P</i> -value
	1	3	7	
FRAP (μmolFe^{2+} liberated)	196.6 (115.6–477.9)	182.8 (84.1–305.6)	173.3 (94.2–231.6)	0.015
TBARS (nmol/ml)	12.04 (2.5–23.4)	8.44 (4.8–18.9)	6.15 (3.5–17.8)	0.004
SOD (U/ml)	4.22 (0.1–10.1)	1.5 (0.1–8.3)	1.88 (0.1–6.2)	0.178

All quantitative data are depicted as median (range), compared using Friedman test

Fig. 3 FRAP significantly lower in cases on all days than controls (Kruskal–Wallis test, $P < 0.001$). FRAP significantly different on all days among cases (Friedman test, $P < 0.015$)

showed a significant increase from baseline to day 7 (Fig. 3) among the survivors ($P = 0.015$).

Among ALF patients, a significant negative correlation existed between the level of oxidative stress, as reflected by the TBARS levels, and the antioxidant capacity of plasma,

as reflected by the FRAP levels at baseline (Spearman $\rho = -0.452$; $P = 0.009$). At days 3 and 7, this correlation between TBARS and FRAP levels was lost among the survivors (Spearman $\rho = -0.266$ and -0.358 , respectively; $P = 0.209$ and 0.310 , respectively). No correlation was, however, found between the SOD and the FRAP levels at baseline (Spearman $\rho = 0.254$; $P = 0.160$) and subsequently among the survivors. Among controls, the SOD and TBARS levels were unrelated to the FRAP levels (Spearman $\rho = 0.208$ and 0.248 , respectively; $P = 0.342$ and 0.254 , respectively).

Clinical correlation

No relation was found between the level of systemic oxidative stress or antioxidant levels, and either the patient outcome, or complications like cerebral edema, deeper encephalopathy grade, renal failure, or seizures (Table 3).

Discussion

Oxidative stress has been implicated in pathophysiology of various liver diseases. However, the role of oxidative stress has not been investigated in ALF in humans. The level of systemic oxidative stress was significantly higher among ALF patients as indicated by higher TBARS and SOD

Table 3 Systemic antioxidant levels and oxidant stress related to complications and outcome among ALF patients ($n = 32$)

	FRAP (μMFe^{2+} lib)	<i>P</i> -value	TBARS (nmol/ml)	<i>P</i> -value	SOD (U/ml)	<i>P</i> -value
Died ($n = 13$)	264.7 (105–529.9)	0.323	7.53 (0.98–23.9)	0.099	5.9 (0.1–10.6)	0.677
Survived ($n = 19$)	196.6 (111–505.5)		10.96 (2.5–23.4)		3.95 (0.1–6.8)	
Cerebral edema ($n = 10$)	267.35 (117–529.9)	0.070	7.34 (0.9–23.9)	0.952	3.97 (0.1–9.2)	0.562
No cerebral edema ($n = 22$)	170.41 (104.6–526)		11.13 (2.02–23.4)		4.13 (0.1–10.6)	
HE Grade 3/4 ($n = 8$)	159.11 (111–526)	0.334	11.17 (3.7–23.4)	0.593	4.16 (0.910.6)	0.623
HE Grade 1/2 ($n = 24$)	213.00 (105–529.9)		8.71 (0.9–23.9)		3.81 (0.05–9.2)	
Renal failure ($n = 10$)	407.45 (105–529.9)	0.008	7.75 (0.9–23.8)	0.535	2.31 (0.1–9.2)	0.920
No renal failure ($n = 22$)	170.41 (111–445.2)		10.71 (2.0–23.4)		4.18 (0.1–10.6)	
Seizures ($n = 7$)	349.04 (111–526.4)	0.281	7.14 (3.9–23.9)	0.175	5.94 (1.5–9.2)	0.721
No seizures ($n = 25$)	196.60 (105–529.9)		10.96 (0.9–23.4)		3.95 (0.1–10.6)	

All quantitative data are depicted as median (range). Mann–Whitney *U*-test has been used for comparisons

levels. The source of markers of oxidative stress is the ongoing hepatocyte injury and cell death. The lipid peroxidation products are liberated in the circulation as the membrane lipids are oxidized during cell lysis. These products are also a source of cytotoxicity and are suggested to invoke the inflammatory response in the body [24, 25]. Hence, an increased TBARS level may reflect the large-scale destruction of hepatocytes that may further aggravate the inflammation and cell death. The high SOD level may be a result of the overexpression of SOD mRNA, which is a normal physiologic response against the acute stress [26–29]. Thus, the increased SOD levels may be a result of body's adaptive response against the prevalent oxidative stress during ALF, which needs to be studied further by measuring expression of *SOD* gene in patients with ALF.

Acetaminophen toxicity, which is the most common etiology of ALF in the West, has oxidative stress as a key pathogenic factor for liver cell injury and antioxidant therapy as treatment. Acetaminophen toxicity leading to liver failure is mediated by hepatic glutathione depletion and mitochondrial oxidant stress [30]. No data are available on the intrahepatic or systemic oxidative stress among postnecrotic ALF. Among patients with nonalcoholic fatty liver disease (NAFLD) and alcoholic liver disease, both increased oxidative stress [31] and an immune response to lipid peroxidation products have been shown to aggravate liver injury [32].

Further support for the physiologic significance of the high oxidative stress is reflected by the depleted systemic antioxidant reserves. We found a significant negative correlation between the TBARS and the FRAP levels. Therefore, the levels of antioxidant reserves may be reduced by the increased utilization for quenching of the oxidant stress. Poor oral intake during the prodromal and hepatitis phase preceding the liver failure may also contribute to the depleted antioxidant reserves in these patients. Interestingly, among the survivors, the TBARS level decreased through the first week. This would reflect decreasing oxidative stress as the liver recovers. However, the oxidative stress at the end of first week still continued to be significantly higher than control levels. Also the antioxidant levels remained significantly lower even after 1 week among recovering patients, which indicates that there was a hyperdeficit of antioxidants during the acute phase of the disease.

We did not find a higher level of oxidative stress among the nonsurvivors. Also, we did not find any relation of oxidative stress with the complications of ALF. This is because oxidative stress may be a reflection of hepatocyte injury rather than having a causal role. The limitations of the present study include the small number of patients. A type-2 error might have underpowered the study to detect the clinical correlations of the level of oxidative stress.

Another shortcoming was that normal levels for the assays among pregnant women were not known. Because a large number (11/20) of the females were pregnant, this might have influenced the comparisons. Finally, local milieu in the remaining hepatocytes might be different from the circulatory compartment. Perlemuter et al. [33] have recently reported that erythrocyte and plasma antioxidant defenses did not reflect intrahepatic peroxidation in patients with NAFLD. This discrepancy between the systemic and intrahepatic oxidative stress and the antioxidant defenses in the setting of ALF cannot be discounted. Future studies should compare the level of oxidative stress between ALF and severe hepatitis without liver failure.

In conclusion, this study suggested that there is a high level of systemic oxidative stress when the patient presents with ALF along with a deficit in antioxidant defenses. The correlation of oxidative stress with disease severity and clinical outcomes and the influence of antioxidants as a therapeutic modality in ALF need to be studied further.

References

1. Trey C, Davidson CS. The management of fulminant hepatic failure. In Popper H, Schaffner F, editors. *Progress in Liver Diseases*, vol. III. New York: Grune & Stratton; 1970. 282–298.
2. Fagan EA, Williams R. Fulminant viral hepatitis. *Br Med Bull* 1990;46:462–480.
3. Fagan E, Yousef G, Brahm J, Garelick H, Mann G, Wolstenholme A, et al. Persistence of hepatitis A virus in fulminant hepatitis and after liver transplantation. *J Med Virol* 1990;30:131–136. doi:10.1002/jmv.1890300210.
4. Ruprah M, Mant TGK, Flanagan RJ. Acute carbon tetrachloride poisoning in 19 patients: implications for diagnosis and treatment. *Lancet* 1985;1:1027–1029. doi:10.1016/S0140-6736(85)91624-1.
5. Smilkstein MJ, Knapp GL, Kulig KW, Rumack BH. Efficacy of oral *N*-acetylcysteine in the treatment of acetaminophen overdose: analysis of the National Multicenter Study (1976 to 1985). *N Engl J Med* 1988;319:1557–1562.
6. Seeff LB, Cuccherini BA, Zimmerman HJ, Adler E, Benjamin SB. Acetaminophen hepatotoxicity in alcoholics: a therapeutic misadventure. *Ann Intern Med* 1986;104:399–404.
7. Pawa S, Ali S. Boron ameliorates fulminant hepatic failure by counteracting the changes associated with the oxidative stress. *Chem Biol Interact* 2006;160:89–98. doi:10.1016/j.cbi.2005.12.002.
8. Detry O, Gaspar Y, Cheramy-Bien JP, De Roover A, Honoré P, Meurisse M, et al. Oxidative stress in the liver and the brain of rats in fulminant hepatic failure. *Transplant Proc* 2005;37:2883–2885. doi:10.1016/j.transproceed.2005.05.012.
9. Shapiro H, Ashkenazi M, Weizman N, Shahmurov M, Aeed H, Bruck R. Curcumin ameliorates acute thioacetamide-induced hepatotoxicity. *J Gastroenterol Hepatol* 2006;21:358–366. doi:10.1111/j.1440-1746.2005.03984.x.
10. Ritter C, Reinke A, Andrades M, Martins MR, Rocha J, Menna-Barreto S, et al. Protective effect of *N*-acetylcysteine and deferrioxamine on carbon tetrachloride-induced acute hepatic failure in rats. *Crit Care Med* 2004;32:2079–2083. doi:10.1097/01.CCM.0000142699.54266.D9.

11. Dikici I, Mehmetoglu I, Dikici N, Bitirgen M, Kurban S. Investigation of oxidative stress and some antioxidants in patients with acute and chronic viral hepatitis B and the effect of interferon-alpha treatment. *Clin Biochem* 2005;38:1141–1144. doi:10.1016/j.clinbiochem.2005.10.006.
12. Swietek K, Juszczak J. Reduced glutathione concentration in erythrocytes of patients with acute and chronic viral hepatitis. *J Viral Hepat* 1997;4:139–141.
13. Lu Y, Cederbaum AI. Cisplatin-induced hepatotoxicity is enhanced by elevated expression of cytochrome P450 2E1. *Toxicol Sci* 2006;89:515–523. doi:10.1093/toxsci/kfj031.
14. Liu LG, Yan H, Yao P, Zhang W, Zou LJ, Song FF, et al. CYP2E1-dependent hepatotoxicity and oxidative damage after ethanol administration in human primary hepatocytes. *World J Gastroenterol* 2005;11:4530–4535.
15. Tandon BN, Bernauau J, O'Grady J, Gupta SD, Krisch RE, Liaw YF, et al. Recommendations of the International Association for the Study of the Liver Subcommittee on nomenclature of acute and subacute liver failure. *J Gastroenterol Hepatol* 1999;14:403–404. doi:10.1046/j.1440-1746.1999.01905.x.
16. Bhatia V, Singh R, Acharya SK. Predictive value of arterial ammonia for complications and outcome in acute liver failure. *Gut* 2006;55:98–104. doi:10.1136/gut.2004.061754.
17. Riegler JL, Lake JR. Fulminant hepatic failure. *Med Clin North Am* 1993;77:1057–1083.
18. Bhatia V, Batra Y, Acharya SK. Prophylactic phenytoin does not improve cerebral edema or survival in ALF—a controlled clinical trial. *J Hepatol* 2004;41:89–96. doi:10.1016/j.jhep.2004.03.017.
19. Jameel S, Durgapal H, Habibullah CM, Khuroo MS, Panda SK. Enteric non-A non-B hepatitis: epidemics, animal transmission, and hepatitis E virus detection by the polymerase chain reaction. *J Med Virol* 1992;37:263–270. doi:10.1002/jmv.1890370405.
20. Nanda SK, Yalchinkaya K, Panigrahi AK, Acharya SK, Jameel S, Panda SK. Etiological role of hepatitis 'E' virus in sporadic fulminant hepatitis. *J Med Virol* 1994;42:133–137. doi:10.1002/jmv.1890420207.
21. Belch JFF, Bridges AB, Scott N, Chopra M. Oxygen free radicals and congestive heart failure. *Br Heart J* 1991;65:215–218. doi:10.1136/hrt.65.5.245.
22. Marklund S, Marklund G. Involvement of superoxide anion radical in the autooxidation of pyrogallol and a convenient assay for SOD. *Eur J Biochem* 1974;47:469–474. doi:10.1111/j.1432-1033.1974.tb03714.x.
23. Benzie IFF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal Biochem* 1996;239:70–76. doi:10.1006/abio.1996.0292.
24. Cipak A, Borovic S, Scukanec-Spoljar M, Kirac I, Zarkovic N. Possible involvement of 4-hydroxynonenal in splenocyte regulated liver regeneration. *Biofactors* 2005;24:217–226.
25. Park JE, Yang JH, Yoon SJ, Lee JH, Yang ES, Park JW. Lipid peroxidation mediated cytotoxicity and DNA damage in U937 cells. *Biochimie* 2002;84:1199–1205. doi:10.1016/S0300-9084(02)00039-1.
26. Akalin FA, Toklu E, Renda N. Analysis of superoxide dismutase activity levels in gingiva and gingival crevicular fluid in patients with chronic periodontitis and periodontally healthy controls. *J Clin Periodontol* 2005;32:238–243. doi:10.1111/j.1600-051X.2005.00669.x.
27. Raho G, Cassano N, D'Argento V, Vena GA, Zanotti F. Over-expression of Mn superoxide dismutase as a marker of oxidative stress in lesional skin of chronic idiopathic urticaria. *Clin Exp Dermatol* 2003;28:318–320. doi:10.1046/j.1365-2230.2003.01264.x.
28. Broide E, Klinowski E, Koukoulis G, Hadzic N, Portmann B, Baker A, et al. Superoxide dismutase activity in children with chronic liver diseases. *J Hepatol* 2000;32:188–192. doi:10.1016/S0168-8278(00)80062-8.
29. Choung BY, Byun SJ, Suh JG, Kim TY. Extracellular superoxide dismutase tissue distribution and the patterns of superoxide dismutase mRNA expression following ultraviolet irradiation on mouse skin. *Exp Dermatol* 2004;13:691–699. doi:10.1111/j.0906-6705.2004.00209.x.
30. Jaeschke H, Knight TR, Bajt ML. The role of oxidant stress and reactive nitrogen species in acetaminophen hepatotoxicity. *Toxicol Lett* 2003;144:279–288. doi:10.1016/S0378-4274(03)00239-X.
31. Madan K, Bhardwaj P, Thareja S, Gupta SD, Saraya A. Oxidant stress and antioxidant status among patients with nonalcoholic fatty liver disease (NAFLD). *J Clin Gastroenterol* 2006;40:930–935. doi:10.1097/01.mcg.0000212608.59090.08.
32. Albano E, Mottaran E, Vidali M, Reale E, Saksena S, Occhino G, et al. Immune response towards lipid peroxidation products as a predictor of progression of non-alcoholic fatty liver disease to advanced fibrosis. *Gut* 2005;54:987–993. doi:10.1136/gut.2004.057968.
33. Perlemuter G, Davit-Spraul A, Cosson C, Conti M, Bigorgne A, Paradis V, et al. Increase in liver antioxidant enzyme activities in non-alcoholic fatty liver disease. *Liver Int* 2005;25:946–953. doi:10.1111/j.1478-3231.2005.01126.x.