Monitoring oxidative damage in patients with liver cirrhosis and different daily alcohol intake

P Clot, M Tabone, S Aricò, E Albano

Abstract
This study looked at the possible association between alcohol abuse and free radical mediated oxidative injury by examining the presence of oxidative damage, as monitored by erythrocyte malondialdehyde and plasma lipid hydroperoxides, in patients with liver cirrhosis and different lifetime daily alcohol intake. All patients with an alcohol intake above 100 g/day (ALC) showed concentrations of malondialdehyde and lipid hydroperoxide on average four to fivefold higher than cirrhotic patients with alcohol intake below 100 g/day (NAC) or healthy controls. Further subgrouping of ALC patients showed that those with alcohol intake ranging between 100 and 200 g/day (ALC1) had malondialdehyde and lipid hydroperoxide concentrations significantly lower than those with an intake higher than 200 g/day (ALC2). These differences were not related to the extent of liver injury or to the liver derangement as assessed by Child's classification. The increase in lipid peroxidation markers in ALC cirrhotic patients was associated with a decrease in, respectively, plasma α-tocopherol and erythrocyte glutathione concentrations. Significant differences were also seen between ALC1 and ALC2 groups in plasma α-tocopherol, but not in erythrocyte glutathione concentrations. The concentrations of α-tocopherol and glutathione in the blood of NAC patients were in contrast not substantially different from those of healthy controls. The close association between oxidative damage and alcohol abuse suggested that free radical intermediates produced during ethanol metabolism might be responsible for causing oxidative damage.

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In recent years the finding that reactive oxygen species and hydroxethyl radicals1–3 are produced during the hepatic metabolism of ethanol has given new emphasis to the possible participation of free radical mediated damage in the pathogenesis of tissue injury resulting from alcohol abuse. Consistently, several authors have reported that either acute or chronic alcohol intoxication of rats is associated with the appearance of lipid peroxidation, as measured by conjugated diene absorbance and malondialdehyde accumulation in the liver as well as by the breath exhalation of pentane and ethane.4–8 The occurrence of oxidative injury during ethanol intoxication is further supported by several reports concerning the decrease in the hepatic content of antioxidants, such as glutathione and α-tocopherol.7–8 These findings are not limited to rodents, as stimulation of lipid peroxidation and glutathione depletion have also been seen in baboons treated with alcohol.9

The presence of oxidative damage in relation to alcoholic intoxication has also been investigated in clinical studies performed on alcoholic patients. These studies, however, have given contradictory results. For instance, lipid peroxide concentrations, measured by the thiobarbituric method in liver and in serum were found to be higher in heavy drinkers than in non-drinkers.10 A significant increase in the conjugated diene content has also been detected in the lipids extracted from hepatic biopsy specimens of alcoholic patients at different stages of liver disease. These patients also presented a decrease in the hepatic concentrations of glutathione and α-tocopherol that was unrelated with their nutritional state.11–13 Other authors have raised doubts, however, about the specificity of the association between oxidative damage and ethanol intoxication, as an increase in liver or serum thiobarbituric acid reactive substances has been seen in patients suffering from alcoholic as well as non-alcoholic liver disease.14–15

A further problem in the interpretation of these results is that subsequent studies have shown that the analytical methods most often used for the measurement of lipid peroxidation in alcoholic patients were unspecific or unsuitable for the detection of oxidative damage in humans.16

The above criticisms have prompted us to reinvestigate the problem by using different and more appropriate analytical procedures. Moreover, in the light of the recent experimental findings concerning the formation of free radicals during ethanol metabolism, we have investigated whether the detection of oxidative damage in patients with alcoholic cirrhosis might be specifically related to alcohol consumption.

Methods
We enrolled 72 consecutive patients with liver cirrhosis admitted to the Division of Gastroenterology of a general district hospital in Turin, Italy, during a six month period. All admissions occurred either from the emergency ward of our hospital, where all liver patients undergo gastroenterological consultation, or from our outpatient clinic. We excluded from the study 25 patients because of...
the presence of malignancies (n=8), biliary tract disease (n=5), primary biliary cirrhosis (n=4), autoimmune, metabolic or drug induced liver cirrhosis (n=8). In all the remaining 47 patients, liver cirrhosis was diagnosed according to laparoscopy, with or without liver biopsy; all of them had evidence of abnormal liver function tests lasting for at least six months.

In all patients, lifetime mean daily alcohol intake was assessed at the time of admission, according to a previously described standardised questionnaire, presented as part of a survey of life habits. In brief, alcohol intake was investigated for a 10 year period, starting from the age of 16, and for each period the average daily consumption of wine, beer, and spirits was separately measured as multiples of 25 g ethanol, which were considered to be equivalent to 0.25 litre of wine, or 0.5 litre of beer, or two measures of spirits. The mean lifetime daily alcohol intake, expressed as a discrete scale of multiples of 25 g, was calculated accordingly.

The 47 patients (34 men, 13 women; mean age 56 years, ranging from 31 to 80) were divided into three groups on the basis of different mean lifetime daily alcohol intakes. The first group (ALC1) consisted of 10 subjects whose mean lifetime daily alcohol intake was between 100 and 200 g, the second group (ALC2) consisted of 20 subjects whose mean lifetime daily alcohol intake was higher than 200 g, and the third group (NAC) consisted of 17 patients whose mean lifetime daily alcohol intake was lower than 100 g. The first two groups of patients (ALC1 and ALC2) were considered together for some findings and termed as group ALC. Child’s classification was used to assess the degree of liver function derangement.

Healthy controls (17 men, 7 women; mean age 57 years, ranging from 35 to 75), all drinking less than 100 g ethanol/day, were also studied.

At the time of the blood testing, all subjects had abstained from alcohol for at least 48 hours. All subjects gave informed consent to the analysis and the study was planned according to the guidelines of the university ethical committee.

Blood samples were taken using EDTA as the anticoagulating agent and immediately refrigerated in ice. The plasma was separated by centrifugation at 4°C and the leucocyte layer was immediately removed by aspiration. The remaining erythrocytes were washed in 5 ml ice cold phosphate buffered saline pH 7.4 and sedimented for five minutes at 1500 rpm. Aliquots (0.5 ml) of the packed erythrocyte were added to 1 ml 50% trichloroacetic acid and the supernatant was used for measurement of free malondialdehyde and of reduced glutathione.

Free malondialdehyde was routinely estimated by reacting for 10 minutes in a boiling bath of 0-8 ml of protein free supernatant with equal volume of 0-67% thio-barbituric acid solution in water as previously described.19

The effective measurement of malondialdehyde in the erythrocyte extracts was confirmed by high performance liquid chromatography (HPLC) analysis according to Esterbauer et al.20 Briefly 0-2 ml of packed erythrocytes were thoroughly mixed with the same volume of ice cold acetonitrile in 1.5 ml Eppendorf tubes. After 20 minutes of extraction in ice the samples were centrifuged for one minute at 13 000 rpm and 20 μl of supernatant analysed by HPLC using an Lichrospher 100-NH2 column (Merck, Darmstadt, Germany) and acetonitrile/30 mM TRIS buffer, pH 7-4 (1:9 vol/vol) as eluent. The effluent was monitored at 270 nm wavelength and the malondialdehyde peak in the chromatogram was identified by comparison with that of free malondialdehyde standard freshly prepared by the hydrolysis of malondialdehyde-bisdiacetate in 1% sulphuric acid solution.

Plasma lipid hydroperoxides, mostly phospholipid hydroperoxides, were estimated by the method of Kamiya and Udenfriend.21 This method involves the oxidation of 15-methylcarbamoyl-3,7-dimethylamino-10-H-phenothiazine after treatment with phospholipase D according to Ohishi et al21 using a kit supplied by Kamiya Biomedical Co (Thousand Oaks, CA, USA) and cumene hydroperoxide as standard. The phospholipid content of plasma was determined by an enzymatic kit (Boehringer Biochemia, Mannheim, Germany).

The content of glutathione in red cells was determined spectrophotometrically at 412 nm by the reaction with dithionitro-benzoic acid according to Owens and Belcher.22

The plasma content of α-tocopherol was measured by HPLC analysis according to Burton et al.23 Plasma aliquots (1 ml) were mixed with 1 ml 100 mM sodium dodecylphosphate solution in water, 2 ml absolute ethanol, and 1 ml n-heptane and shaken vigorously for one minute. After 15 minutes of extraction in the dark the heptane phase was separated by centrifugation and 50 μl aliquots were used for the HPLC determination using Lichrosorb CN250 RT column (Merck, Darmstadt, Germany) and a fluorescence detector operating at 296 nm excitation and 325 nm emission wavelengths.


**Results**

Previous studies in our laboratory have shown that malondialdehyde can be measured in the erythrocyte by the reaction with thiobarbituric acid.\(^{18}\) Thus, this parameter has been used to monitor 47 patients with liver cirrhosis and different mean lifetime daily alcohol intake.

As Figure 1 shows, in all 30 patients with cirrhosis associated with high alcohol intake (ALC) (mean lifetime daily alcohol intake above 100 g) we have found a relevant increase in erythrocyte malondialdehyde, which was on average about fourfold higher than healthy controls. Cirrhotic patients (n = 17) with mean lifetime daily alcohol intake below 100 g (NAC) also showed a moderate, but significant, rise compared with the controls in erythrocyte malondialdehyde (Fig 1). The mean value of malondialdehyde in NAC was, however, about 2.5-fold lower compared with ALC patients.

As thiobarbituric acid reactive substances other than malondialdehyde can be present in biological samples we have verified whether the values obtained by the thiobarbituric acid assay resulted from the presence of malondialdehyde in the erythrocytes. The simultaneous analysis of 20 different samples using the thiobarbituric acid reaction and a direct HPLC assay for malondialdehyde showed a close correlation between the values measured by the two tests (correlation coefficient \(r=0.97\)) (Fig 2).

Recently, a new assay for measuring lipid hydroperoxides in the phospholipid fraction of the plasma has been proposed.\(^{20}\) The use of this assay confirmed that in ALC patients not only erythrocyte malondialdehyde, but also plasma lipid hydroperoxides were dramatically increased, being on average fivefold higher than in the controls (Fig 1). Conversely, no significant difference in plasma lipid hydroperoxides was seen between NAC and healthy controls (Fig 1). These differences were not related to the changes in plasma lipids induced by alcohol because in the ALC cirrhotic patients triglyceride and cholesterol concentrations were only moderately increased (data not shown). Furthermore, when corrected for plasma phospholipids, lipid hydroperoxides were not related to those of ALC1 (daily alcohol intake above 200 g) than ALC2 (daily alcohol intake above 200 g) (Table I). Moreover, the indices of lipid peroxidation in the two alcoholic groups were significantly different not only from the control values, but also from those in NAC patients (Table I).

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**Table 1**  
*Erythrocyte malondialdehyde and plasma lipid hydroperoxide content in cirrhotic patients with different daily alcohol intake*

<table>
<thead>
<tr>
<th></th>
<th>Malondialdehyde (nmol/ml packed cells)</th>
<th>Lipid hydroperoxide (nmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ALC1 (n=10)</strong></td>
<td>2.3 (0.2)*†</td>
<td>4.8 (0.9)*†</td>
</tr>
<tr>
<td><strong>ALC2 (n=20)</strong></td>
<td>5.4 (0.6)*‡</td>
<td>13.8 (1.9)*‡</td>
</tr>
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</table>

Patients with cirrhosis (ALC) were grouped according to the daily alcohol consumption estimated according to Corrao et al.\(^{14}\) as ALC1 (consuming on average 100–200 g ethanol/day) and ALC2 (consuming more than 200 g ethanol/day). Controls subjects were all drinking less than 100 g ethanol/day. The results are mean (SEM). *p<0.001 compared with controls; †p<0.02 compared with NAC; ‡p<0.05 compared with ALC1.
The association between the increase in malonaldehyde and lipid hydroperoxides and alcohol intake did not result from differences in the extent of liver injury, because serum AST and GGT activities were similar in the two groups (Table II). Moreover, when the ALC patients were grouped according to the criteria suggested by Child,23 no significant differences were evident between the mean values of malonaldehyde and lipid hydroperoxides (Table III). These results show that the markers of lipid peroxidation detected in the blood of ALC patients were related to alcohol consumption rather than to the derangement of liver functions.

As a decrease in the antioxidant content of both liver and blood values has often been reported in alcoholic patients, we have investigated whether the changes in blood antioxidants are specifically related to alcohol intake and to the presence of oxidative damage. In all the ALC patients the content of reduced glutathione of the erythrocyte was decreased by about 50% of the normal values, while it was practically unchanged in NAC subjects (Fig 3). Similarly, the measurement of plasma α-tocopherol concentration showed an appreciable decrease in cirrhotic patients with alcohol abuse (ALC groups), but not in the patients with moderate alcohol intake (NAC group) (Fig 3). The decrease in α-tocopherol among drinkers was more evident in the ALC2 group, where it accounted for about 43% of the control values, compared with a 28% loss in ALC1 group. Such a difference among ALC1 and ALC2 groups was statistically significant (Fig 3). Conversely, erythrocyte glutathione did not show appreciable variations among ALC1 and ALC2 groups (Fig 3).

As seen with the markers of lipid peroxidation the differences in the concentrations of α-tocopherol were not any more evident when the patients with cirrhosis of grade A or B were compared with those with cirrhosis of grade C (Table III). Furthermore, an inverse linear correlation (r=0.68) was evident between plasma lipid hydroperoxide and α-tocopherol concentrations (Fig 4). These findings show that the decrease in plasma α-tocopherol was related to the intake of alcohol and was strictly associated with the development of lipid peroxidation.

**Discussion**

Previous studies concerning the presence of lipid peroxidation in blood and liver specimens from alcoholic patients10–15 were performed using analytical tests that have been subsequently proved to be prone to artefacts or unsuitable for use in humans.16 For instance, much evidence has been obtained using the estimation of thiobarbituric acid reactive substances that are generated by the decomposition of lipid hydroperoxides present in the plasma. Human plasma contains many substances, however, including sugars, amino acids, and bile pigments, which by reacting with thiobarbituric acid form chromogens, which can lead to an overestimation of the effective content of peroxidation products.16–25 Indeed the malonaldehyde values in healthy controls reported by Suematsu et al10 and Tanner et al12 are at least twice those detected using a more specific HPLC analysis of the malonaldehyde-thiobarbituric acid adducts.26 A further problem in the interpretation of previous studies relies on the fact that most of the malonaldehyde measured is generated by decomposition of lipid hydroperoxides during the acid heating stage of the test. This process might be greatly modified by the antioxidant content of the samples. Thus, a decrease in the concentration of antioxidants in the tissues can substantially increase the formation of thiobarbituric acid reactive substances during the assay.16 This should be taken into account because the concentrations of α-tocopherol, the main lipid soluble chain breaking antioxidant, are reduced in the liver as well as in the plasma of alcoholic patients.12,27

Caution should be also applied to the interpretation of the results concerning an increase.
Monitoring oxidative damage in patients with liver cirrhosis and different daily alcohol intake

in conjugated dienes among drinkers.11,13 Recent researches show that in human tissues the main compound containing conjugated diene bonds is represented by octadeca-9(cis)-11-(trans)-dienoic acid, an isomer of linoleic acid which, despite having the diene configuration, does not originate from lipid peroxidation.29 The lack of specificity of this index is suggested by the increase in the plasma concentrations of octadeca-9(cis)-11-(trans)-dienoic acid in alcoholic patients as well as in patients suffering from a number of diseases unrelated to alcohol consumption.28

Researches performed in our laboratory have shown that a number of carbonyl products derived from the peroxidative degradation of unsaturated fatty acids can be readily detected in human erythrocytes.19 Thus, the thiobarbituric acid test performed in the acid extract of erythrocytes can be regarded as a reliable measure for lipid peroxidation in vivo, providing care is taken to perform the reaction in the protein free supernatant.

The determination in vivo of lipid peroxidation has also been attempted by using an assay for plasma lipid hydroperoxides based on the reaction of organic hydroperoxides with a leuco-derivative of methylene blue (10-methylcarbamoyl-3,7-dimethylamino-10-phenothiazine).20 With this method we have found that lipid hydroperoxides concentrations in the plasma of healthy controls are on average 2 nmol/ml (range 0-4-3-2 nmol/ml). These values are lower than those detected by the same method by Taheishi et al31 in Japanese controls (7-65 (2-77)), but are consistent with the concentrations of plasma lipid hydroperoxide ranging from 0-5 to 4 nmol/ml found by other authors using different analytical procedures.32,33

The results obtained by the use of the above assays of lipid peroxidation have shown that peroxidation products are actually increased in both the erythrocytes and the plasma of patients with cirrhosis associated to alcohol abuse. It is interesting to note that there is a good linear correlation (r=0.71) between the values of erythrocyte malonildialdehyde and those of plasma lipid hydroperoxides in the same subjects, showing that the two tests are actually measuring the same phenomenon. The presence of lipid peroxidation seems specific for the alcohol associated disease, as patients with cirrhosis and mean lifetime daily alcohol intake below 100 g show only a small increase in erythrocyte malonildialdehyde, without changes in plasma lipid hydroperoxide content. The relation between the stimulation of lipid peroxidation and the amount of alcohol consumed is further strengthened by the finding that among alcohol abusers there are significant differences in malonildialdehyde and lipid hydroperoxides in relation to the daily ethanol intake. These findings are consistent with a recent report by Lettérón et al34 who have found that the exhalation of ethane, another end product of lipid peroxidation, is increased fivefold compared with controls in 89 alcohol abusers, but not in 52 patients with non-alcoholic liver diseases. Furthermore, in the above study ethane exhalation is weakly, but significantly, correlated with the daily ethanol intake before hospital admission.34

It has been proposed that the presence of lipid peroxidation products in the blood might reflect the extent of liver damage rather than the presence of a pro-oxidant stimulus.15 In this study, however, the indices of liver injury are essentially the same in all groups of patients irrespective of the alcohol intake and the differences among alcoholic patients are no more appreciable when the same patients are grouped according to the Child’s criteria for the estimation of liver derangement.53 Thus, ethanol consumption is seen as a main factor associated with the detection of lipid peroxidation.

Studies in vitro have shown that both rat and human liver microsomes can produce hydroxyethyl radicals during ethanol metabolism and that the formation of these radical species increases with the concentration of ethanol added as well as the induction of cytochrome P-450E1 by chronic alcohol exposure.35 Furthermore, reactive oxygen species are generated in the liver during chronic ethanol consumption as a result of the modification of several enzymatic functions, thus contributing to the stimulation of lipid peroxidation.12,27 Because the presence of hydroxyethyl free radicals and lipid derived radicals has also been detected in vivo in ethanol fed animals,4 it is possible that the association between the extent of lipid peroxidation and that of alcohol consumption might reflect an increased formation of hydroxyethyl radicals by the liver.

It is known that a decrease in the antioxidant defences of the liver might also influence the development of free radical mediated oxidative damage. Experimental and clinical studies have shown that chronic alcohol consumption is associated with a decrease in the hepatic content of glutathione and α-tocopherol,8,12,13,26 which represent the main antioxidants in cytosol and cell membranes, respectively. Recent reports show that in alcoholic patients the changes in liver
glutathione and α-tocopherol are independent from the nutritional conditions and are reflected by a parallel decrease in the blood concentrations in the two antioxidants.\textsuperscript{27} 37 38 The reduction in erythrocyte glutathione and plasma α-tocopherol seen in cirrhotic patients with high alcohol intake is consistent with the results published by Bell et al.\textsuperscript{29} and Loguerocio et al.\textsuperscript{30} Our results show, however, that the decrease in blood antioxidants is independent from the severity of liver damage. Preliminary experiments in our laboratory have shown that the incubation of human erythrocytes and plasma with ethanol or acetaldehyde does not result in the formation of malondialdehyde or lipid hydroperoxides, or affect the content of glutathione and α-tocopherol. On the other hand, after stimulation of peroxidative reactions in isolated hepatocytes about 50% of the total amounts of carboxyl products formed is released in the extracellular space.\textsuperscript{39} This suggests the possibility that peroxidative damage of hepatocytes consequent to ethanol metabolism might lead to the leakage of fatty acid oxidation products such as lipid hydroperoxides, malondialdehyde, and ethane, which can be detected in the blood or in the expired air. The appearance of blood markers of lipid peroxidation in relation to hepatic injury by ethanol is supported by a study using rats fed a high fat ethanol containing diet by intragastric infusion. In these animals the development of liver injury and fibrosis is, in fact, associated with an increase in the fluorescent adducts between plasma proteins and aldehydes derived from lipid peroxidation, including malondialdehyde.\textsuperscript{40} Consistently, an increase of lipid peroxidation associated with the development of hepatic fibrosis has also been seen by Kamimura et al.\textsuperscript{41} in the liver of rats fed intragastrically with ethanol and a high fat diet.

The liver is known as the main storage tissue of α-tocopherol\textsuperscript{42} and oxidative hepatic injury causes a decrease in the secretion of this antioxidant into the plasma well before the liver content of α-tocopherol is affected.\textsuperscript{43} Interestingly, the concentration of α-tocopherol in the plasma of heavy drinkers decreases with the amount of ethanol consumed and an inverse linear correlation with lipid hydroperoxide concentrations is evident. Thus, the lowering of α-tocopherol in the plasma might also reflect the pro-oxidant action of alcohol in the liver.

In conclusion, the results obtained show that the occurrence of stimulation of lipid peroxidation in patient with liver cirrhosis associated with alcohol abuse is related to the estimate of alcohol consumed. This finding suggests the possibility that free radical species produced during liver metabolism of ethanol might be responsible for causing peroxidative damage. Furthermore, the specific association between oxidative injuries and high alcohol intake gives new emphasis to their possible participation in the pathogenesis of alcoholic cirrhosis.\textsuperscript{40} This work has been supported by the Italian Ministry of University and Scientific and Technological Research (Projects Carroco E and Pastore E, and the Consiglio Nazionale delle Ricerche (Projects pr-1978-027 and pr-1982-041).
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