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# FAILURE OF ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY STUDIES TO DETECT ELEVATED FREE RADICAL SIGNALS IN LIVER BIOPSY SPECIMENS FROM PATIENTS WITH ALCOHOLIC LIVER DISEASE

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Electron paramagnetic resonance spectroscopy (EPR) was used to study free radicals and transition metal complexes in liver tissue taken from patients with liver disease. Samples were frozen to 77K directly following biopsy to prevent deterioration. Our major aim was to compare signals from patients suffering from alcohol abuse with those from patients having liver damage not induced by alcohol. Samples were obtained from 19 chronic alcohol abusers and 7 non-alcoholic liver disease patients. Of the 19 alcoholic patients, 18 had an increased fat content, 6 had Mallory's hyaline, 12 had an acute inflammatory response, 9 had increased stainable iron and 4 had evidence of fibrosis. A signal derived from free radicals with a spectroscopic splitting factor of g = 2.0045 was found in all samples. This signal in the alcoholic patients had a mean amplitude of 2.96 cm (±1.42 SD), and in patients with non-alcoholic liver disease 2.12 cm (±0.82) (p = 0.10 NS), measured under identical instrument settings.

The molar proportion of diene conjugated linoleic acid (DCLA), a free radical marker, in the sera of alcoholic patients was 2.68% (±1.93), but did not correlate with the free radical signals obtained by EPR spectroscopy. Also, there was no correlation between the free radical derived EPR signal and fat content, Mallory's hyaline, inflammatory infiltrate, iron or fibrosis in the liver biopsy specimens. Similarly the concentrations of aspartate transaminase, albumin, and gamma-glutamyl transferase in serum samples showed no correlations with free radical concentrations.

The absence of any significant increase in the stable free radical signal in the presence of alcohol induced liver disease and the lack of correlation between the signal and either histological or serological evidence of liver damage, suggests that alcohol derived free radicals may not be involved in the pathogenesis of alcoholic liver disease.

Unusually large sextet features characteristic of MN(II) complexes were observed for all liver samples. Such signals are very rare in human tissue, showing that there is a strong accumulation of Mn (II) in the liver. However, no systematic trends were observed. In some samples signals characteristic of iron-sulphur cluster units were detected, but again no correlations could be discovered.

KEY WORDS: Free radicals. Alcoholic liver disease. Ethanol. EPR.

## INTRODUCTION

The biochemical mechanism by which alcohol causes liver disease is unclear. Acetaldehyde,[1] immunoreactive products such as Mallory's hyaline[2] and altered

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intracellular redox changes[3] have been postulated as playing a part. Free radicals generated during either the oxidation of alcohol by the microsomal ethanol oxidising system[4] or by xanthine oxidase[5] can cause cellular disruption by initiating lipid peroxidation,[6] denaturating proteins[7] or causing strand breakage of DNA.[8] It has therefore been postulated that free radicals may play an important role in hepatocyte damage in alcoholic liver disease.[4-9]

Techniques available to study free radical reactions in biological systems have relied upon markers such as conjugated dienes of fatty acids.[10] The measurement of diene conjugated linoleic acid, an 18 carbon fatty acid has been widely used to study free radical reactions and has been shown to be the major conjugated diene in biological systems.[10] Its concentration in the serum of alcoholics has been shown to be increased whilst drinking alcohol, but rapidly falls to normal levels upon abstinence. This finding has been taken to imply increased levels of free radical activity in alcoholics.[11]

Direct techniques for quantification of free radicals are available but are less readily performed. Chemiluminescence, the emission of light following the stimulation of lipid peroxidation, is an *in vivo* technique, but due to its lack of specificity it does not necessarily confirm free radical reactions.[12] Electron paramagnetic resonance spectroscopy (EPR) allows the direct study in tissue samples of stable free radicals and paramagnetic transition-metals.[13,14] The source of EPR signals can sometimes be identified by the g-value or by hyperfine splittings. Signal amplitudes are proportional to free radical concentrations for a given type of radical.[15]

The aims of this study were (i) to compare concentrations of stable free radicals directly by EPR studies of liver specimens from patients with alcoholic and non-alcoholic liver disease, in order to assess whether any extra stable free radical signal was induced by ethanol. (ii) to assess possible relationships between free radical concentrations in alcoholics as quantified by EPR spectroscopy in liver specimens and indirectly by measuring diene conjugated linoleic acid concentrations in serum. (iii) to seek a correlation between free radical activity and histological liver damage in alcoholics. (iv) To compare the accumulation of Mn (II) with the extent of damage and with alcohol abuse.

## MATERIALS AND METHODS

### *Subjects*

Patients had been admitted for detoxification of alcohol and had been drinking in excess of 80 gm of alcohol per day for the preceding year and had not been abstinent immediately prior to admission. 19 patients with alcoholic liver disease were biopsied 9 male, 10 female, mean age 51 years (range 33–70). All had an elevated aspartate transaminase (AST) concentration (>50 IU/1) (Table 1). 13 were on no medication, 5 were prescribed chlormethiazole and one was taking a L-dopa preparation. None were clinically malnourished and only two had serum albumin concentrations below the normal range.

Samples from patients with non-alcohol related liver disease were obtained at the time of biopsy which was clinically indicated. Seven patients with non-alcoholic liver disease were biopsied, 2 male, 5 female, mean age 56 years (range 52–64) – three had chronic active hepatitis (hepatitis B surface antigen negative), three had rheu-

TABLE 1
Summary of serological and histological results for patients with alcoholic liver disease (1–19), and mean value for non-alcoholic subjects (Non-ALD), with free radical signal amplitude and serum DCLA molar ratio

| Pt. No. | Bil | Alb | AST | GGT | FR | DCLA (MR) | Fat | NH | Fib | Inflammation |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 36 | 44 | 82 | 1183 | 1.0 | 1.6 | 3 | 1 | 0 | 0 |
| 2 | 5 | 44 | 96 | 225 | 1.5 | 1.7 | 2 | 0 | 0 | 1 |
| 3 | 10 | 47 | 175 | 189 | 4.2 | 1.0 | 3 | 0 | 0 | 3 |
| 4 | 12 | 42 | 52 | 203 | 5.9 | 4.5 | 2 | 0 | 0 | 0 |
| 5 | 7 | 44 | 200 | 366 | 3.0 | 1.4 | 0 | 1 | 0 | 1 |
| 6 | 13 | 44 | 220 | 1271 | 3.0 | • | 3 | 2 | 0 | 1 |
| 7 | 150 | 28 | 277 | 988 | 5.0 | 3.1 | 1 | 0 | 0 | 1 |
| 8 | 4 | 36 | 140 | 88 | 2.3 | 1.5 | 3 | 0 | 0 | 0 |
| 9 | 10 | 35 | 54 | 134 | 0.8 | 4.5 | 2 | 2 | 3 | 1 |
| 10 | 17 | 42 | 172 | 310 | 1.8 | 2.0 | 1 | 0 | 0 | 1 |
| 11 | 36 | 33 | 675 | 429 | 4.3 | 1.7 | 3 | 0 | 0 | 1 |
| 12 | 80 | 48 | 80 | 90 | 3.3 | 1.9 | 1 | 0 | 0 | 0 |
| 13 | 11 | 43 | 90 | 121 | 3.5 | 3.2 | 1 | 0 | 0 | 1 |
| 14 | 9 | 40 | 58 | 119 | 2.0 | 3.1 | 2 | 0 | 0 | 0 |
| 15 | 51 | 41 | 300 | 359 | 2.2 | 1.5 | 2 | 1 | 0 | 2 |
| 16 | 78 | 31 | 539 | 2870 | 4.3 | 3.7 | 2 | 0 | 0 | 0 |
| 17 | 13 | 32 | 52 | 60 | 2.9 | 3.5 | 1 | 1 | 1 | 1 |
| 18 | 26 | 42 | 140 | 268 | 1.2 | 4.5 | 1 | 0 | 3 | 1 |
| 19 | 148 | 28 | 275 | 710 | 4.0 | 1.6 | 3 | 0 | 1 | 0 |
| Non-ALD | 57 | 32 | 108 | 215 | 2.1 | 1.8 | 0 | 0 | 1 | 0 |

ABBREVIATIONS: (normal values)
Bil - Bilirubin (3–22 μmol/1)
Alb - Albumin (30–50 g/1)
AST - Aspartate transaminase (10–40 U/1)
GGT - Gamma glutamyl transferase (10–55 U/1)
FR - Amplitude of free radical derived signal (cm)
DCLA - Molar ratio percent of diene conjugated linoleic acid
Fat - Hepatic fat content (0–3)
MH - Hepatic mallory's hyaline (0–3)
Fib - Hepatic fibrosis (0–3)
Inflammation - Hepatic inflammation (0–3).

matoid arthritis with elevated serum AST concentrations, and one had mitochondrial antibody positive primary binary cirrhosis.

All patients gave written informed consent and the study was authorised by the South Sefton District ethical committee.

## SAMPLE PREPARATION

Needle biopsy specimens were obtained percutaneously using a modified Menghini technique (1.6 mm), under local anaesthesia. Samples were divided into two. One portion was sent for routine histological assessment, and the other was immediately plunged into liquid nitrogen, and stored at 77K for later analysis by EPR spectroscopy. Liver biopsies were performed within four days of hospital admission. Blood samples were collected at the time of biopsy and serum was stored at -20°C, for later analysis by HPLC for the diene conjugate of linoleic acid.

*EPR Spectroscory*

A Bruker ER 200D SRC Q band spectrometer was used with an ER 4111 variable temperature unit, and an ER 053 QRD microwave bridge. A continuous flow of nitrogen gas from boiling liquid nitrogen was used to maintain a cavity temperature of approximately 100K. Incident power was optimised at 1.0 mW, with a microwave frequency of *ca* 33900 GHz. The magnetic field was scanned between *ca* 11500 and 12500 G.

Samples (4 × 1 mm) were placed in a signal free quartz tube, sealed at one end, which was then attached to a long aluminium arm securely clamped to the EPR spectrometer. Spectra were recorded at 100K using carefully controlled conditions for signal comparisons.

## HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR DCLA

Serum was prepared and analysed according to the method of Iversen & Dormandy.[16] Following cleavage of fatty acid residues from phospholipids with phospholipase A2, protein was removed by precipitation and the samples purified on a *Bond Elut* (Technicol UK) column. Simultaneous analyses using high performance liquid chromatography (HPLC) with optical density detection at 234 nm for the conjugated dienes and 205 nm for total fatty acids was performed. Results are expressed as the percentage proportion of the fatty acid that is diene conjugated.

## LIVER HISTOPATHOLOGY

Formalin – fixed liver biopsy (5 $\mu$m) sections were stained with haematoxylin and eosin, a reticulin stain and modified Perl's Prussian blue stain. Each specimen was analysed by the same pathologist, and a grading was established for inflammatory infiltrate, Mallory's hyaline, fibrosis, fat content and stainable iron. The grading was between 0 and 3.

## STATISTICAL ANALYSIS

Was by Spearman Rank correlation with a p value of less than 0.05 considered significant.

## RESULTS

All but one of the alcoholic patients had an increased fat content, 6 had Mallory's hyaline, 12 had an acute inflammatory response, 9 had increased stainable iron, and 4 had evidence of fibrosis (Table 1). None exhibited cirrhosis.

Reproducible signals (coefficient of variation of 6.9% for the free radical signal) were obtained with g values equal to 2.0045 ± 0.0001 (Figures 1 & 2). Signal amplitude in the alcoholic group was 2.96 cm (+/−1.42 SD), compared to 2.12 (+/−0.82) in the non-alcoholic group (p = 0.10 NS).

For all samples a strong sextet of lines, having g = 2.002 and A ($^{55}$Mn) = 94.5 G

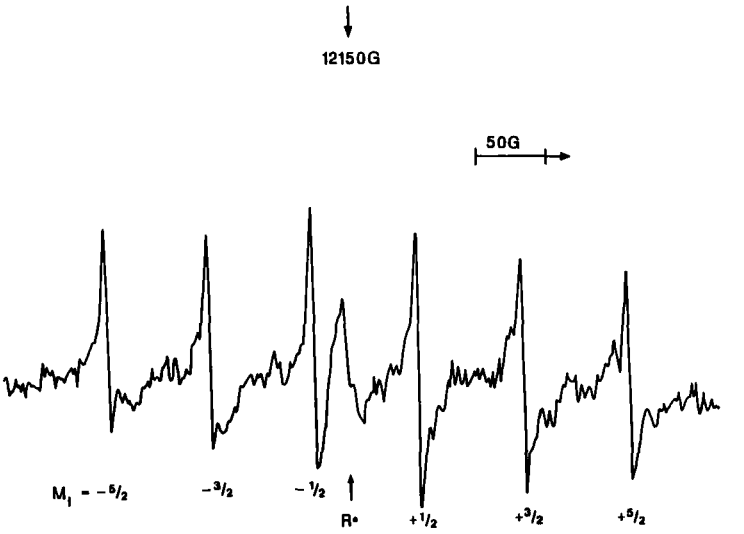


FIGURE 1 First derivative Q-Band EPR spectrum from a patient with alcoholic liver disease. Six equally spaced signals characteristic of manganese (II) are seen together with a free radical-derived signal (R) at g = 2.0045. Mid range 12092 G, scan range 1000 G.

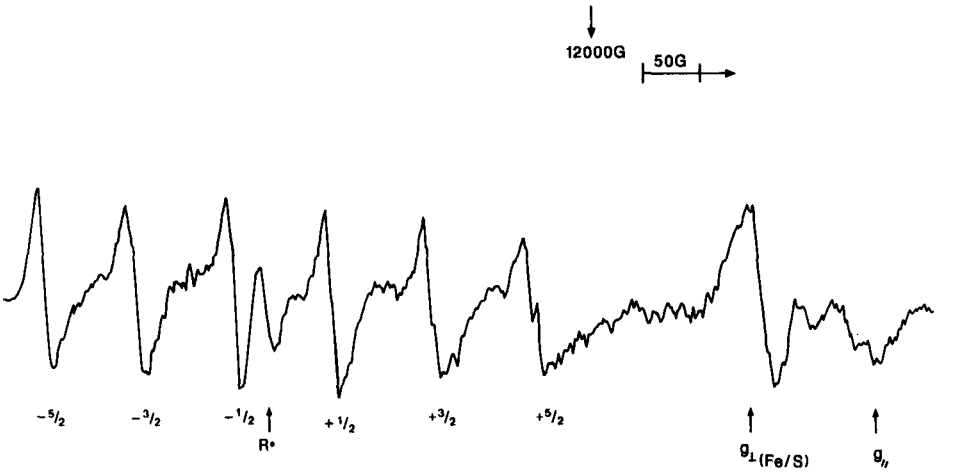


FIGURE 2 Q-Band EPR spectrum from a patient with alcoholic liver disease. The six manganese signals are shown (Mn), the free radical-derived signal (R) and a prominent iron-sulphur cluster (FeS).

were detected. These are characteristic of high-spin divalent manganese in symmetrical sites showing very small g-value variation and small zero-field splittings (Figures 1 & 2). In some cases (Figure 2) features having $g_i = 1.930$ and $g_{ii} = 1.911$ were also detected by EPR spectroscopy. These are characteristic of iron-sulphur cluster centres. These features were obtained from samples taken from both

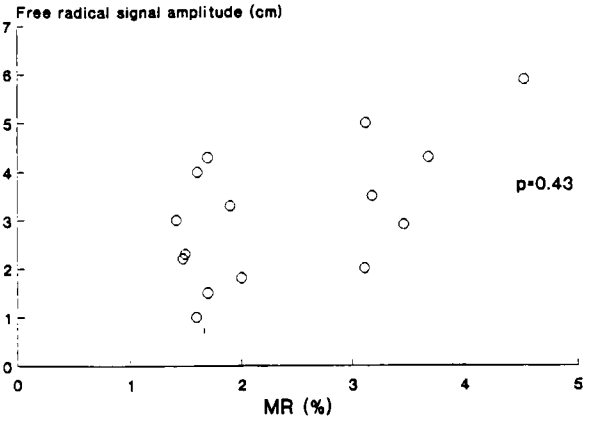


FIGURE 3 Relationship between free radical activity as measured by EPR compared to serum marker of free radical activity - diene conjugated linoleic acid (expressed as molar ratio, MR).

alcoholic patients and those with non-alcoholic liver disease and may be derived from succinate dehydrogenase.[14]

There was no correlation between the amplitude of the liver free radical signal in the alcoholic group and the level of either fat ($p = 0.34$), fibrosis ($p = 0.5$), iron ($p = 0.16$), Mallory's hyaline ($p = 0.71$) or inflammation ($p = 0.48$), in liver biopsies. Neither was there a correlation between signal amplitude derived from free radicals and either a serum marker of liver disease (AST) (mean 177.6 U/1 ± 171.5 SD) ($p = 0.23$), a marker of hepatic enzyme induction - gamma glutamyl transferase (mean 543 U/1 ± 693.9) ($p = 0.18$), or synthetic liver function (serum albumin) (mean 39 g/l ± 6.4) ($p = 0.77$). The molar ratio of the diene conjugated linoleic acid in the alcoholic group was 2.55% (± 1.20) and there was no correlation with the free radical - derived EPR signal ($p = 0.43$) (Figure 3).

## DISCUSSION

The technique of EPR spectroscopy allowed the reliable identification of a free radical signal in human liver biopsy samples both from alcoholics and patients with non-alcoholic liver disease.

There was no significant increase in hepatic stable free radical concentration measured by EPR spectroscopy in the alcoholic group compared with the non-alcoholic patients. Correlations were not found between the EPR free radical signal amplitude and histological and biochemical evidence of liver damage, nor with the serum concentration of the free radical marker diene conjugated linoleic acid.

The origin of the stable free radicals that were detected by EPR spectroscopy is not clear, and it is impossible to assign any specific structure in the absence of more information. The signal seen may have been derived from a number of stable radicals, including ascorbyl free radicals[17] age pigments[18] and melanin.[19]

The radicals detected by EPR are unable to react with oxygen to give $RO_2^{\cdot}$ radicals, since these were not detected. Thus they are either so strongly stabilized

that this reaction is unfavourable, or they are so closely occluded within, say, a protein, that they are inaccessible for further reaction. The small but significant shift in the g-value from that of the free spin (2.0023) suggests the presence of significant spin-density on oxygen, and semiquinone radicals seem likely candidates.

These EPR-visible radical signals seem to be characteristic of liver tissue rather than of alcohol abuse. In on-going studies of a wide range of human tumours, tissue samples were directly frozen to 77K, as in the present studies. The EPR spectra run at 77K generally failed to show any significant free radical signals. (Deighton N, Rowland I, Symons MCR, unpublished data). However tissue from both normal and tumour samples taken from human livers showed signals similar to those recorded herein and with comparible intensities. Again, no precise identification can be offered.

Recent studies employing spin traps (which convert unstable free radicals into stable nitroxide radicals) administered to hepatocyte preparations during active alcohol metabolism, have demonstrated the formation of the hydroxyethyl radical ($CH_3CHOH$),[20,21] hydroxyl radical[22] and superoxide radical.[23] Also, one of us (MCRS) has shown that $CH_3CHOH$ radicals are formed from excess ethanol in blood by the action of oxy haemoglobin.[24] Thus it is probable that ethanol is a source of $CH_3CHOH$ radicals. These are active and will react with many substrates in cells or in plasma and may sometimes lead to serious damage. However they clearly do not enhance the intensity of the EPR-visible stable trapped radicals in liver, nor do they seem to give rise to stable free radicals of other types.

In a related study,[25] we were also unable to find any increase in stable free radical concentrations in livers from rats fed with ethanol, iron, or both, over a period of a year, despite extensive cellular damage. We are forced to conclude that the formation of small active radicals such as $CH_3CHOH$ do not lead to an increase in the concentration of stable free radicals.

Evidence that free radicals are involved in the production of alcoholic liver disease is based largely on indirect markers of free radical production such as thiobarbituric acid - reactive substances from rat liver homogenates incubated with alcohol,[26] increased exhaled hydrocarbon gases in man,[27,28] and increased circulating levels of diene conjugated linoleic acid.[11] As far as we are aware only one other study has compared indirect and direct EPR measurements of free radical activity in human samples and this also demonstrated a lack of correlation between EPR-detectable derived free radical activity and diene conjugated linoleic acid.[29] DCLA has been found in a wide variety of foodstuffs and alteration of diet can affect serum concentrations[30] and so its usefulness as a marker of free radical activity may be limited.

Finally, the amount of hepatic lipid peroxidation has been shown to be similar in patients with alcoholic and non-alcoholic liver disease. This similarity was attributed to leucocyte activity.[31] Had free radicals been important in the pathogenesis of alcoholic liver disease, and not just a product of leucocyte activity, a significant difference in activity would be expected between subjects with alcoholic and those with non-alcoholic liver disease. This was not found, and hence some further doubt has been cast on the importance of free radicals in this disorder.

It is therefore clear that free radicals are involved in the metabolism of ethanol but the absence of an increase in the hepatic stable free radical signal in alcoholic subjects, and the lack of correlation between the amplitude of the signal and histological or serological evidence of hepatocellular damage, suggests that free

radicals are probably not directly involved in the development of alcoholic liver disease.

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## *References*

1. R. Barry and J. Mcgivan (1985) Acetaldehyde alone may initiate hepatocellular damage in acute alcoholic liver disease. *Gut*, **26**, 1065–1069.
2. S. French, E. Burbige and G. Tarder (1979) Lymphocyte sequestration by the liver in alcoholic hepatitis. *Archives of Patholoy and Laboratory Medicine*, **103**, 146–152.
3. C. Lieber (1980) Alcohol, protein metabolism and liver injury. *Gastroenterology*, **79**, 373–390.
4. G. Winston and A. Cederbaum (1983) NADPH production of oxy radicals by purified components of the rat liver mixed-function oxidase system. Role in microsomal oxidation of ethanol. *Journal of Biological Chemistry*, **258**, 1514–1519.
5. K. Ohnishi and C. Lieber (1978) Respective role of superoxide and hydroxyl radical in the activity of the reconstituted microsomal ethanol oxidising system. *Archives of Biochemistry and Biophysics*, **91**, 798–803.
6. A. Cederbaum, E. Dicker and G. Cohen (1978) Effect of hydroxyl radical scavengers on microsomal oxidation of alcohols and on associated microsomal reactions. *Biochemistry*, **17**, 3058–3064.
7. S. Wolff, A. Garner and R. Dean (1986) Protein denaturation by hydrogen peroxid. *Trends in Biochemical Sciences*, **11**, 27–31.
8. J. Ward, J. Evans, C. Limoli and P. Calabro-Jones (1987) Radiation and hydrogen peroxide induced free radical damage to DNA. *British Journal of Cancer*, **55S8**, 105–112.
9. S. Klein and C. Lieber (1983) Increased microsomal oxidation of hydroxyl radical scavenging agents and ethanol after chronic consumption of ethanol. *Archives of Biochemistry and Biophysics*, **273**, 425–43.
10. S. Iversen and T. Dormandy (1984) Identification of a diene conjugated component of human lipid as octadeca-9,11 dienoic acid. *Federation of European Biochemical Societies Letters*, **171/2**, 320–324.
11. R. Fink, D. Marjot, P. Cawood, S. Iversen, M. Clemens, Patsalos, A. Norden and T. Dormandy (1985) Increased free radical activity in alcoholics. *Lancet*, **Aug**, 291–294.
12. I. Yamazaki (1987) *Free radicals in Biology* pp. 183–218 (Pryor, W. ed.), Academic Press. New York.
13. T. Slater (1972) *Free radical mechanisms in tissue injury*. Pion Ltd. London.
14. M.C.R. Symons (1978) *Chemical and biochemical aspects of ESR spectroscopy*. Van Nostrand Reinhold, Wokingham.
15. D. Borg (1976) *Free radicals in biology* (Pryor. W. ed.), Academic Press. New York.
16. S. Iversen, P. Cawood and T. Dormandy (1985) A method for the measurement of a diene-conjugated derivative of linoleic acid, 18:2(9, 11), in serum phospholipid, and possible origins. *Annals of Clinical Biochemistry*, **22**, 137–140.
17. N. Dodd (1973) Some EPR signals in tumour tissue. *British Journal of Cancer*, **28**, 257–262.
18. G. Elek, A. Rockenbauer and L. Jokay (1987) A study of free radicals in paraffin embedded and deparaffinized human heart muscle tissue using electron spin resonance. *Histochemistry*, **87**, 499–504.
19. L. Ambari, J. Hung, L. Edelstein and M. Van Woerst (1977) Quantification of melanin in cardiac and hepatic lipofuscin. *Experieenta*, **33**, 296–298.
20. K. Knecht, B. Bradford, R. Mason and R. Thurman (1990) *In vivo* formation of a free radical metabolite of ethanol. *Molecular Pharmacology*, **38**, 26–30.
21. L. Reinke, J. Rau and P. McCay (1990) Possible roles of free radicals in alcoholic tissue damage. *Free Radical Research Communications*, **9**, 205–211.
22. P.B. McCay, L.A. Reinke and J.M. Rau (1992) Hydroxyl radicals are genera ed by hepatic microsomes during NADPH oxidation: relationship to ethanol metabolism. *Free Radical Research Communications*, **15(6)**, 335–346.
23. K.T. Knecht, R.G. Thurman and R.P. Mason (1993) Role of superoxide and trace transition metals in the production of alpha-hydroxyethyl radical from ethanol by microsomes from alcohol dehydrogenase-deficient deermice. *Archives of Biochemistry and Biophysics*, **303**, 339–348.

24. F.A. Taiwo and M.C.R. Symons (1993) Interaction of ethanol with haemoglobin: implications for alcohol abuse. *Free Radical Research Communications*, **19(2)**, 121–4.
25. G.P. Butcher, N. Deighton, R.M. Batt, B. Ding, S. Haywood, J. Hoffman, M.J. Jackson, M.C.R. Symons and J.M. Rhodes (1993) Electron paramagnetic resonance spectroscopy of stable free radicals in the liver compared with ultrastructural and functional damage in a model of alcohol and iron overload. *Clinical Science*, **84**, 339–348.
26. M. Comporti, A. Hartman and N. Di Luzio (1967) Effect of *in vivo* and *in vitro* ethanol administration on liver lipid peroxidation. *Laboratory investigation*, **16**, 616–624.
27. S. Moscarella, G. Laffi, D. Coletta, U. Arena, A. Cappelini and P. Gentilini (1984) Volatile hydrocarbons in the breath of patients with chronic alcoholic liver disease: a possible marker of ethanol-induced lipoperoxidation. *Frontiers of gastrointestinal research Vol. 8* (P. Gentilini and M. Dianzini M. eds.), pp. 208–216. P. Rozen Tel Aviv.
28. P. Letteron, V. Duchatelle, A. Berson, B. Fromenty, C. Fisch, C. Degott, J.P. Benhamou and D. Pessayre (1993) Increased ethane exhalation, an *in vivo* index of lipid peroxidation, in alcohol abusers. *Gut*, **34**, 409–414.
29. D. Wickens, S. Davies, J. Fairbank, S. Tay, T. Slater and T. Dormandy (1990) Studies on cervical intraepithelial neoplasia: the level of octadeca-9,11-dienoic acid and measurement of free radical content by electron spin resonance spectroscopy. *American Journal of Obstetrics and Gynecology*, **162(3)**, 854–8.
30. M. Britton, C. Fong, D. Wickens and J. Yudkin (1992) Diet as a source of phopsholipid esterified 9,11-octadecadienoic acid in humans. *Clinical Science*, **83**, 97–101.
31. R. Mazzanti, S. Moscarella, G. Bensi, E. Altavilla, P. Gentiline (1989) Hepatic lipid peroxidation and aldehyde dehydrogenase activity in alcoholic and non-alcoholic liver disease. *Alcohol and Alcoholism*, **24**, 121–128.

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