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Oxidative Damage to Plasma Proteins in Patients with Chronic Alcohol Dependence: The Effect of Smoking

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Abstract. *Background: Accumulating evidence implicates oxidative stress in ethanol-induced toxicity. Ethanol has been reported to be involved in oxidative damage, mostly in vitro, or in post mortem tissues, while biochemical abnormalities in the blood or serum are scanty or lacking. The aim of the present study was to examine the oxidative status of plasma proteins as markers of oxidative stress in subjects with chronic alcohol dependence (CAD). Since smoking has also been associated with oxidative stress this factor was also considered. Patients and Methods: A total of 71 patients with CAD and 61 healthy volunteers of comparable age were included in the study. The protein carbonyl assay was carried out in plasma, as a reliable measure of general oxidative protein damage, in these two groups. Results: Increased plasma protein carbonyls (PCs) were found in patients with CAD as compared with the control group [mean values (nmol/mg protein): 4.73±1.46 and 3.62±0.91 respectively, $p<0.000001$]. Within the control group, smokers had higher PCs than the non-smokers, however this difference was of marginal significance [mean values (nmol/mg protein): 3.93±1.32 and 3.47±0.63, respectively]. The CAD group had significantly increased PCs compared with both the smoker and the non-smoker subgroups of the controls ($p<0.001$ and $p<0.0001$, respectively). Duration of alcohol consumption, daily alcohol intake, smoke load, folic acid and vitamin B12 levels did not correlate significantly with PC levels. Conclusion: The above results support the evidence for systemic oxidative stress in CAD, which must be attributed mainly to alcohol consumption, while smoking may act synergistically.*

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Alcohol dependence is a chronic disorder characterized by the habitual consumption of alcohol, often resulting in interference with physical or mental health, as well as with social and/or occupational behaviour. Alcohol produces both physical and psychological addiction when consumed in excess for a long period. Chronic alcohol dependence (CAD) may result in multiple-organ damage derived from the detrimental effect of ethanol and its metabolites. Much of the cell damage that occurs over time has been increasingly attributed to oxidative stress, which is considered to play an important role in the pathogenesis of ethanol toxicity (1-3).

Cell biomolecules such as lipids, DNA and proteins are targets of reactive species formed during oxidative stress (4-7). Lipid peroxidation in chronic alcoholism has been studied extensively (8-15). Ethanol or acetaldehyde may also induce cell toxicity via protein oxidation. Ethanol-fed rats were found to have decreased thiol concentrations and reduced glutamine synthetase activity, together with increased protein carbonyls in the liver (16). Oxidative modification of proteins *in vivo* may potentially be more important than damage to lipids, since a variety of cellular functions is affected, including signal transduction mechanisms, transport and enzymatic systems, and receptor functioning (17). Secondary damage to other biomolecules such as inactivation of DNA repair enzymes, malfunction of DNA polymerases during DNA replication and the development of new antigens provoking autoimmune responses may also occur (18). The chemical reactions resulting from attack of reactive oxygen species (ROS) on proteins are complex (7, 19) and can lead to a variety of products. Oxidation can result in cleavage of the polypeptide backbone, cross-linking and modifications of the side-chains of virtually every amino acid (20-22).

Although there is a large body of evidence implicating ethanol oxidative damage *in vitro* or in post mortem tissue(s) -mainly liver- biochemical data in the periphery of humans is limited. Plasma proteins may be targets of ROS

and other free radicals, produced by ethanol metabolism and/or smoking, the oxidative alterations of which could be detected as an increase in their carbonyl content. Since there is no complete understanding of the molecular mechanisms by which alcohol exerts its toxic effects, we conducted the present study in order to explore the possibility that alcohol may trigger, at least in part, damage to plasma proteins. Due to the fact that smoking is strongly related with alcoholism we also evaluated this parameter. We used the protein carbonyl (PC) assay, as a reliable measure of general oxidative protein damage (23-25).

Patients and Methods

There were a total of 132 participants included in the study. Informed consent was obtained from each subject and the study protocol had the approval of the scientific committee of our hospital. The study was in accordance with the Declaration of Helsinki ethical guidelines.

The patient group constituted of 71 patients with chronic alcohol dependence according to the DSM IV (Diagnostic and Statistical Manual, 4th Edition) criteria of the American Psychiatric Association (26). They were all out-patients of the "Athena" Drug and Alcohol Addiction clinic, attached to the Department of Psychiatry of Athens National University. Patients were included in the study after abstaining from drinking for 24 h. Evaluation of alcohol abuse was made using the Pattern of Abuse tool (a tool of the World Health Organization, for alcoholism that examines past and present history; 27), the section on alcoholism of Composite International Diagnostic Interview (28) and the Diagnostic Interview Schedule (29). All alcoholic patients were smokers. The average alcohol consumption, the mean duration of alcohol abuse and the smoke load are shown in Table I. No patient exhibited any significant nutritional deficits.

The control group consisted of 61 healthy volunteers of comparable age. Control participants either did not drink at all or did so only occasionally (< once/week). According to their smoking habits they were divided into smokers and non-smokers. They were free of cardiopulmonary, gastrointestinal or hepato-biliary-pancreatic disorders. Demographic characteristics of both patients and controls are shown in Table I.

Blood sampling was performed at the time of admission to the detoxification program, at which point they were in a drug-free state. Venus blood samples were taken after overnight fasting at 8.00-10.00 hours into heparinized tubes. The aliquots, for the PC analysis were immediately centrifuged at 3000 rpm x 15 min and the plasma was stored at –80˚C until analysis.

Protein carbonyl assay. The determination of PCs was made spectrophotometrically according to the method of Reznick and Packer (25) after modification (23, 24). Analytically, the method used is described below.

All plasma samples were diluted 1:20, then 400 μl of each sample was taken and divided equally into two 2 ml eppendorff tubes. For each 0.1 ml of sample that was present, 0.4 ml of 10 mM 2,4-dinitrophenylhydrazine (DNPH) (in 2.5 M HCl) was added to one tube and 0.4 ml of 2.5 M HCl to the other. The tubes were then incubated for 1 h on a rotator (Blood cell mixer) in the dark. The protein was then precipitated out by adding an equal volume (1000 μl) of 20% (w/v) trichloroacetic acid (TCA) and the samples were left on ice for 10 min in order to maximize the precipitation effect. The protein was spun down at 6000 x g for 5 min (Centrifuge: Heraus, Biofuge-stratos), the supernatant was discarded and a further 1000 μl of 10% (w/v) of TCA was added to the pellet which was broken up with a glass rod. The protein was spun down again to 6000 x g for 5 min and the supernatant removed. The pellet was subsequently washed with 1 ml of an ethyl acetate:ethanol mixture (1:1) to remove excess DNPH. This was repeated three times. The final protein pellet was dissolved in 1 ml of 6 M guanidine HCl (pH 2.3) and the samples left for 10 min at 37oC. The samples were then centrifuged at 6000 x g for 5 min to remove any particles that could interfere with the absorbance readings. Finally the absorbance (A) of both (DNPH and HCl) solutions were measured at 280 nm and 370 nm, respectively. Standard curves for the measurement of protein concentrations were carried out at A280 using bovine serum alboumin (BSA) (range of concentrations 0.05 mg/ml-10 mg/ml) in guanidine hydrochloride solution (6 M). Results were expressed as nmoles of carbonyls per milligram of protein based on the following equations:

Protein concentration (mg/ml) = A_{280} of HCl solution x 1.868 mg/ml (from standard curve using guanidine hydrochloride)

Carbonyl concentration (mol/ml) = (A_{370} of DNPH solution - A_{370} of HCl solution) x 45.45 nmol/ml (molar extinction coefficient)

Carbonyl content (nmol/mg of protein) = Protein concentration/carbonyl concentration

Activities of γ-glutamyl transferase (γ-GT_L), alanine aminotransferase (ALT_L), aspartate aminotransferase (AST_{S2}) and alkaline phosphatase (ALP_{L6}) were measured by using an autoanalyzer (Cobas Integra 800, Roche) (30-33).

Statistical analysis. All variables were checked for normality using the Shapiro-Wilk's W test and for homogeneity of variances with the Leven's test. Statistical analysis was done by analysis of covariance (ANCOVA) with diagnostic group and sex as factors

Table I. *Clinical and demographic data of studied groups.*

	Controls	CAD	*p*
No.	61	71	
Gender (males/females)	27/34	58/13	<0.001†
Age (years)*	44.8±17.9	45±11	NS
Smokers	18 (30%)	(100%)	<0.001†
Smoke load§	25 (13-31)	37 (15-42)	0.06
Duration of alcohol use (years)*	–	16.7±7.6	
Average alcohol consumption* (g/day)	–	360±258	
Average age of onset of alcohol use (years)		29	

*Mean±SD, §Cigarettes/day (min-max), †χ^2-test, N.S.: non significant.

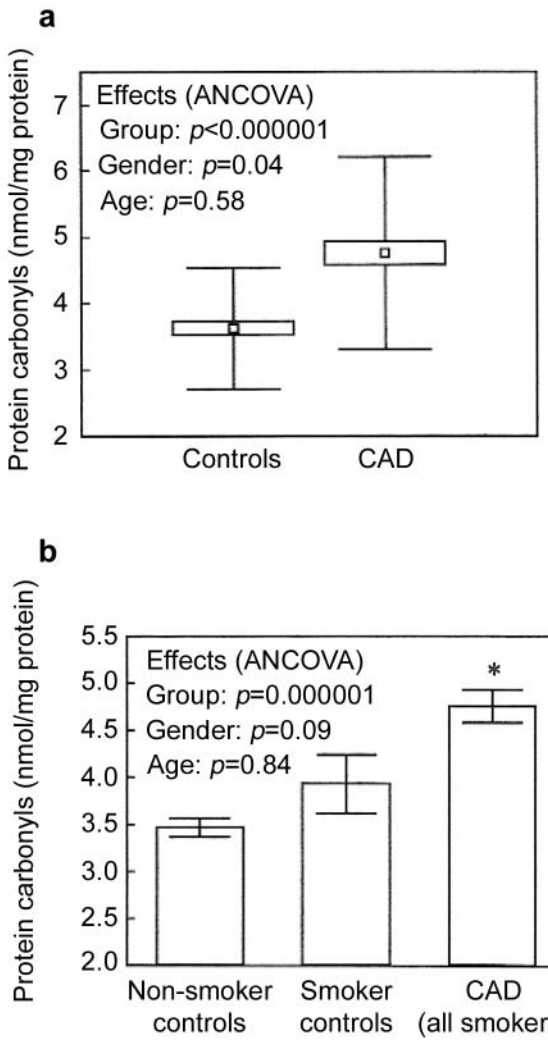

Figure 1. *Box and whiskers plots (mean value, standard error and standard deviation) of (a) plasma protein carbonyls in CAD patients and the controls; (b) in CAD patients and two subgroups of the controls (smokers and non-smokers). *Newman-Keuls post-hoc tests: CAD vs. smoker controls p=0.00089, CAD vs. non-smoker controls p=0.000022, Smoker vs. non-smoker controls p=0.07.*

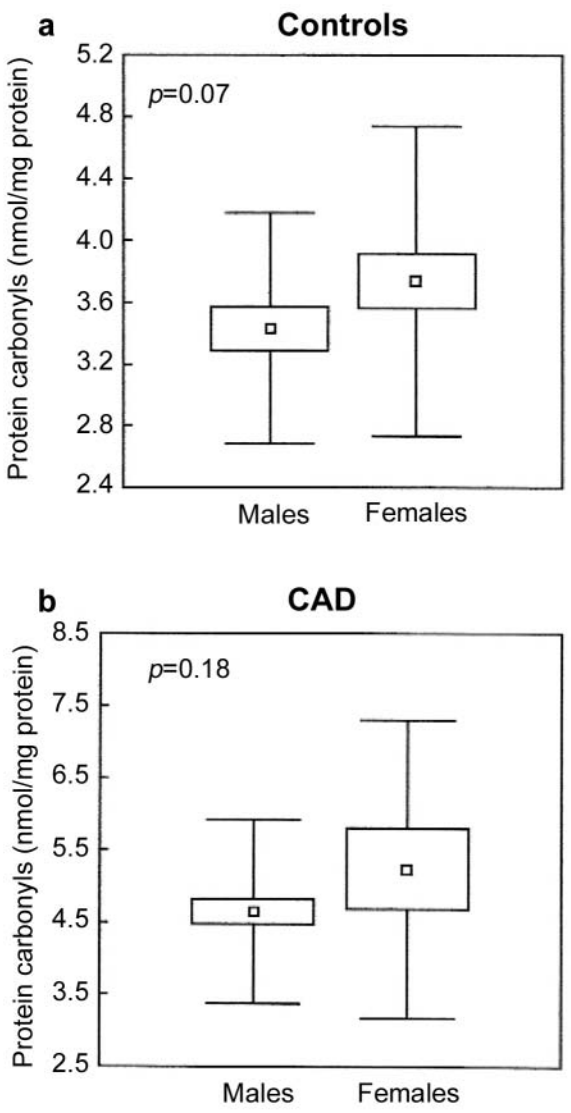

Figure 2. *Box and whiskers plots (mean value, standard error and standard deviation) of plasma protein carbonyls in males and females, in (a) the control group and (b) the CAD group.*

and age as covariate. Pearson's or Spearman's correlation coefficients and χ^2-test were also used when appropriate. The level of statistical significance was set at 0.05.

Results

The CAD group showed significantly higher levels of PCs compared with the entire control group (Figure 1a). Since the alcoholic patients were all smokers, the control group was subdivided into smoker and non-smoker subgroups. Comparisons of the CAD group with both smoker and non-smoker subgroups of the controls revealed statistically significant differences (Table II, Figure 1b). Within the

Table II. *Biochemical data of studied groups*.*

	Controls	CAD	*p*
PCs (nmol/mg of protein)			
All subjects	3.62±0.91	4.73±1.46	<0.000001‡
Smokers	3.93±1.32		0.001‡
Non-smokers	3.47±0.63		<0.0001‡
ASTL	36.7±27.1	20±6	<0.001‡
ALTL	22.1±20.2	22±8	N.S.
γ-GTS2	124.2±137.3	20±9	<0.0001‡
ALPL6	65.2±22.5	66.8±28	N.S.

*Mean±SD, ‡ANCOVA with Newmann-Keuls post-hoc tests. N.S.: non significant.

control group, smokers presented with higher protein carbonyl levels compared with the non-smokers, however the difference was marginally significant ($p=0.07$).

Age did not affect PCs in patients or the control group. Females presented with a tendency for higher levels of PC only in the control group (Figure 2). Age of onset, average alcohol consumption (g per day), age of onset of smoking, number of cigarettes per day, did not correlate with PCs in the CAD group. Additionally, no correlation was observed between PC levels and vitamin B12, folate levels or liver enzyme activities.

Consequently, the CAD group was stratified according to the duration of alcohol consumption (<10, 10-20, >20 years). One-way ANOVA did not reveal any statistically significant difference among the subgroups.

Discussion

It has been well established that ethanol and its metabolites, namely, acetaldehyde and hydroxy-ethyl free radicals result in oxidative stress in experimental animals and alcoholic patients, which is expressed as oxidative modification of cellular constituents, mainly lipids. Oxidation of lipids has been reported in plasma, erythrocytes and liver biopsy samples (34-36). However, data regarding oxidative protein damage in the plasma of CAD patients is limited (37, 38).

The most common method of quantifying oxidation of proteins is the carbonyl assay. It was developed as a "general assay" of oxidative protein damage and is based on the fact that several oxygen-derived species can attack amino acid residues (particularly histidine, arginine, lysine and proline) to produce carbonyl groups (>C=O), which can be measured after reaction with 2,4-dinitrophenylhydrazine (39-41). The carbonyl assay has become widely used since it is a reliable and relatively sensitive method, partly because it measures several different consequences of oxidative damage (42). Normal values in physiological fluids however have not been established and appear to vary widely. This can be attributed to: a) The sensitivity of the method used (spectrophotometric *vs.* ELISA *vs.* HPLC); b) Non-standardized procedures that may lead to variable losses of protein, depending on how the assay is performed and c) Interference caused by other compounds, such as products of unsaturated hydroxyaldehyde reaction with proteins (43). In the present study, strict precautions were taken to ensure reliability as has been previously criticized (44).

In the present study, a significant increase in plasma PCs was noted in subjects with CAD as compared with the entire control group and the results are compatible with previous observations in plasma (37) and serum (38) of smaller groups of patients. Furthermore, the CAD group had statistically significant increased protein carbonyls, compared with both the smoker and the non-smoker subgroup of the controls. Within the control group a marginally significant tendency towards increased PCs in smokers was noted, in accordance with the results of Reznick *et al.* reporting that cigarette smoke leads to the formation of protein carbonyls in human plasma, while antioxidant agents such as glutathione seem to protect both plasma proteins and isolated albumin against carbonyl formation (45). Protein carbonyls measured in the plasma of smokers have consistently been reported to be increased whether the difference reached statistical significance (46) or not (47). Thus, smoking might affect the levels of protein carbonyls measured *in vivo* and hence be a factor contributing to oxidative damage, especially in older age (48). Age of onset of smoking and the number of cigarettes per day did not affect PCs levels in the present study, indicating that smoke may affect PC concentration in plasma independently of smoke load. Thus, when estimating PCs as markers of oxidative stress in plasma, this parameter should be taken into account, especially in small PC elevations. Smoking is nearly always connected with alcohol abuse and this is the first time that it is being evaluated in regard to CAD. Since in the present study the CAD group (all smokers) differed significantly, not only from the non-smoker but also from the smoker subgroup of the controls, one can assume that this effect of ethanol is independent of smoking.

Our results support the notion that alcohol consumption may have a significant role in the whole oxidative stress process. In the present study, this role seemed independent of the duration and the amount of alcohol consumption. Elevated PC concentrations in plasma are suggestive of systemic oxidation with the potential of being subsequently detrimental to a number of vulnerable organs/tissues. Recently, chronic alcohol consumption has been reported to cause abnormal erythrocyte morphology and increased fragility as a result of oxidation of erythrocyte ghost proteins (49).

The principal mechanism by which ethanol is thought to generate tissue-damaging reactive oxygen species is by the induction of cytochrome P-450 2E1 (CYP2E1) (50). CYP2E1, is an abundant hepatic protein considered to result in hydroxyethyl radical formation in the liver after induction with ethanol. However, CYP2E1 is also present on the plasma membrane (51). In a rat total enteral nutrition model, CYP2E1 protein and activity was found to increase as blood alcohol concentration rose. At blood ethanol concentration >300 mg/dl, transcription of the CYP2E1 gene is turned on, followed by elevated mRNA levels and increases in CYP2E1 protein which can induce further oxidative damage (52). Oxidative modification of proteins can inactivate catalytic function, activate degradation by most common proteases and reduce life span (promoting premature aging) (20, 21). Furthermore, protein oxidation, under certain conditions shifts metabolism from anaerobic to aerobic and probably alters

nutritional state (17). Nutritional deficits which are common in chronic alcoholics result in additional deprivation of diet-derived antioxidants.

The slight PC increase observed in females *vs.* males may possibly reflect the greater vulnerability of women to the effects of ethanol, especially after chronic alcohol abuse. This has been attributed to body size with a subsequent smaller distribution space for alcohol, but more interestingly to the lower total gastric alcohol dehydrogenase activity in the stomach, a gender-related effect exacerbated by alcoholism (53, 54).

Conclusion

Our results indicate the occurrence of a systemic oxidative damage induced by chronic alcohol abuse. Additionally, smoking which is nearly always associated with alcohol abuse, may have an additive effect, thus overwhelming the plasma antioxidant defences, exacerbating the oxidative stress process (55).

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