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Oxidative stress and a thrombophilic condition in alcoholics without severe liver disease

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Background and Objectives. The degree of oxidative stress and its association with a thrombophilic condition, if any, were investigated in alcoholics before the onset of severe liver disease.

Design and Methods. Reactive oxygen species and total antioxidant capacity were evaluated using two new kinetic spectrophotometric methods in a selected group of 45 consecutive chronic alcohol abusers and 42 apparently healthy moderate drinkers, used as controls. The hemostatic system was explored by detecting the plasma levels of prothrombin fragment 1+2 (F1+2) and thrombin-antithrombin complexes (TAT) with enzyme-linked immunosorbent assays, while D-dimer plasma levels were measured with a turbidimetric immunoassay.

Results. Reactive oxygen species were significantly higher (p<0.001) in heavy drinkers than in controls: 328.1 (143.4-847.2) U.CARR vs 250 (200.7-366.8) U.CARR, respectively. The total antioxidant capacity was similar in chronic alcohol abusers and in moderate drinkers: 360.2 (336.8-374.4) µMol HClO/mL vs 369 (362-378.4) µMol HClO/mL, respectively. All molecular markers of hemostatic system activation were significantly increased in chronic alcohol abusers in comparison with those in moderate drinkers, as follows: TAT: 2.5 (1.4-13) µg/L vs 1.5 (1-4.1) µg/L, respectively (p<0.001); F1+2: 1.7 (0.5-5.2) nMol/L vs 0.9 (0.4-1.1) nMol/L, respectively (p<0.01); D-dimer: 235.5 (208-462) ng/mL vs 163.5 (71-233) ng/mL, respectively (p<0.001).

Interpretation and Conclusions. Our results suggest that oxidative stress and a thrombophilic condition can be observed in heavy drinkers without severe liver disease. The new test available for measuring reactive oxygen species in serum proved to be reliable and useful as an early marker of tissue damage.
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Key words: alcoholism, oxidative stress, thrombin generation

There is increasing evidence of an association between oxidative stress, resulting from abnormal free radical generation and/or decreased antioxidant defences, and tissue damage.[1] Endothelial cell dysfunction and hemostatic disorders due to a perturbation of the redox balance have recently been reported in type 2 diabetes and human immunodeficiency virus (HIV) infection.[2,3] Analogously, the role of oxidative stress in the development of alcohol-related toxicity and injury in various tissues has been the focus of a great deal of research.[4-10] Alcohol can act as an antioxidant and an oxidant, and its intake seems to exert both beneficial and untoward effects on vessel walls and the hemostatic system, depending on drinking habits.[5,10-15] In particular, there is increasing consensus in the literature that light-to-moderate regular alcohol intake may protect against atherosclerosis and its sequelae, according to the so-called *French paradox*,[13] characterized by a relatively low mortality from coronary heart disease despite a high intake of saturated fat. More recently, an association has been reported between regular light-to-moderate drinking and a lower mortality rate than that in non-drinkers.[16-18] This health-promoting effect has been mainly related to the presence in alcoholic beverages, but especially in wine, of antioxidant substances, such as polyphenols.[5,10,13-15,18] Recent observations indicated that such compounds counteracted ethanol in protecting vessel walls against the oxidation of low density lipoproteins (LDL)[5,10,19] and decreasing both platelet activity and thromboxane generation.[10,13,15] On the other hand, heavy drinking has been associated with increased risk of atherosclerosis and stroke.[10,20,21] In a recent study,[21] we reported that chronic alcohol abuse had a strong and independent effect of promoting carotid atherosclerosis, but the mediating mechanisms are not fully understood so far. Many findings are con-

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sistent with the hypothesis that, depending on the amount drunk, a predominant pro-oxidant effect of alcohol itself might outweigh any antioxidant effect of a beverage's polyphenols and cellular antioxidant mechanisms.[5] Nevertheless, data from literature concerning the relationship between alcohol-related variations of blood redox balance and atherosclerosis are rather contradictory,[22] a fact which is not surprising given the lack of trials comparable as regards the size of the populations studied, the extent of liver injuries and the alcohol intake. Since there has been no definite conclusion in previous reports, further clinical and laboratory evaluations are needed. The present study was undertaken to investigate: a) the degree of oxidative stress in chronic alcohol abusers, in the absence of severe liver disease; and b) whether a perturbation of the redox balance, if any, is associated with an ongoing prothrombotic risk in such patients, as recently reported in other pathologies.[2,3]

Design and Methods

Patients and controls

Redox balance and activation of the hemostatic system were investigated in a selected group of 45 consecutive heavy drinkers (33 males, 12 females), hospitalized for detoxification, who gave their informed consent to participation in the study, according to the principles of the Declaration of Helsinki. The daily ethanol intake was >120 to <400 g. The mean daily alcohol intake was 233 g ± 86, mean ± SD. The patients' mean age was 47 ± 6 years. In order to limit the previously reported age-dependent increase of thrombophilic molecular markers, [23-25] patients over 55 years of age were excluded from the study. All patients consumed excessive quantities of alcohol until the day of hospitalization and were dependent on alcohol according to DSM-IV (*Diagnostic and Statistical Manual of Mental Disorders, 4th ed., revised criteria*[26]). The mean duration of alcohol abuse was 16±9 years, mean ± SD. Most of the patients (88.9%) had cerebral and cerebellar atrophy, while 58.3% were affected by motor-sensory polyneuropathy. None showed electromyographic signs of alcoholic myopathy. None had laboratory or clinical evidence of renal failure. None had a history of thrombosis or had previously received anticoagulant therapy. Patients with prolonged clotting tests (prothrombin time, activated partial thromboplastin time), clinical or biochemical features of acute infection or inflammation were not enrolled in the study. Severe liver disease was excluded by means of clinical, biochemical and ultrasound examinations. Patients affected by severe liver disease (acute or chronic hepatitis, parasitic diseases, malignancies, cirrhosis) or with evidence of abnormal liver function tests lasting for at least six months were excluded from the study. A condition of liver steatosis was documented in all patients. For ethical reasons, liver biopsies were not performed unless clinically necessary. No patient was affected by wasting syndrome (body mass index

Table 1. Indices of nutritional status, liver function and hemostatic activity of 45 chronic heavy drinkers and 42 controls. Results are given as medians with their ranges. The asterisks show the significance level of differences between the two groups.

	Heavy drinkers (n=45)	*Controls (n=42)*
BMI	24.4 (20.9-28.0)	22 (19-25)
γ-GT (U/L)	84.5 (11-2139)°	32 (8-50)
Bilirubin (mg/dL)	0.55 (0.25-1.01)	0.52 (0.30-0.96)
Alkaline phosphatase (U/L)	62 (31-108)	56 (35-76)
Prothrombin activity (%)	88 (68-100)	89 (72-100)
Activated partial thromboplastin time (sec)	34 (25-42)	31 (25-35)
Fibrinogen (mg/dL)	309 (185-403)	300 (180-380)
Folate (ng/mL)	3.9 (0.3-7.9) *	9.8 (4.2-19)
B_{12} (pg/mL)	457 (192-1133)	657 (243-894)
MCV (fL)	100.2 (71-117) *	89 (80-94)

** p<0.01. °p<0.0001.*

was between 20.9 and 28.0). Decreased folate plasma levels, but normal B_{12} plasma levels were recorded in most patients (Table 1). In line with the most recent evidence from literature,[27] alcohol abuse was confirmed by elevated levels of γ-glutamylaminotransferase (γ-GT) and mean corpuscolar volume (MCV) (Table 1). The control group consisted of 42 apparently healthy moderate drinkers (23 males, 19 females, aged 38±5, mean±SD), sampled in the same way as the patients, who consumed less than 30 g pure alcohol per day. Neither patients nor controls were given vitamin-mineral supplements. The main characteristics of the patients and controls are reported in Table 1.

Blood sample collection and handling

Blood samples from patients were collected on the first day of hospital admission, before withdrawal, after a 12-h fast. Since pre-analytic conditions may affect the assays of molecular markers of hemostasis, blood collection and handling were carried out under strictly standardized conditions[28,29] and in line with manufacturers' recommendations. In order to evaluate the degree of thrombin generation and fibrinolytic activity, venous blood was taken from the two groups of subjects, heavy drinkers and controls. The first 4 mL were discarded and then the blood was taken into evacuated tubes containing 0.129 M trisodium citrate, with a ratio of anticoagulant to blood of 1:9 (vol/vol) (Venoject VT-050SCBS, Terumo Europe, Leuven, Belgium). Plasma was obtained by centrifugation (2,000 x g for 20 min), immediately transferred into coded plastic tubes, snap-frozen and stored at –80°C until analysis. The assay was carried out after thawing the frozen aliquots for 5 min. in a water-bath at 37°C and gently mixing. Additional

samples were taken from both the patients and the controls to detect the serum levels of reactive oxygen species, such as hydroperoxides, and the degree of the antioxidant activity. Serum was obtained by centrifugation (1,500 x g for 15 min), transferred into coded plastic tubes, rapidly frozen and stored at –80°C until analysis. Since hydroperoxides have shown to be sufficiently stable over time (Franzini C. *et al.*, unpublished data), no antioxidation treatment was required before analysis. On the other hand, rapid collection and storage of serum was the only procedure recommended for the evaluation of the antioxidant capacity. In order to avoid falsely elevated results, no preservative (cyanide, sodium azide, etc.) was indicated by the manufacturer.

Laboratory procedures and statistical analysis

Reactive oxygen species. Reactive oxygen species were evaluated in serum by a new kinetic spectrophotometric assay (d-ROMs test, Diacron Italy), kindly provided by Diacron S.r.l. and used in line with the manufacturer's recommendations. The test measures the serum levels of reactive oxygen species, such as hydroperoxides, generated in cells as a consequence of the respiratory burst. The test takes advantage of the capacity of hydroperoxides to generate free radicals in the presence of transition metals (Fe, Cu, etc.) which act as catalyzers. When free radicals react with a correctly buffered chromogenic substance, they develop a colored complex that can be measured photometrically presenting a maximum peak absorbance at 505 nm. The concentration of the colored complex is directly proportional to the concentration of hydroperoxides. Results are expressed in arbitrary units, called "U. CARR." One U.CARR. corresponds to 0.08 mg/100 mL H_2O_2. The test can be performed according to either a kinetic or an end-point analysis. According to the kinetic method, 10 µL of chromogenic substrate (R1) and 1 mL of buffer (R2) should be mixed with 10 µL of specimen (serum) for 1 min at 37°C. A blank reagent, obtained by replacing serum with distilled water, and a standard with assigned value should be included for each series of assays. The 505 nm absorbance must be recorded immediately and after 1, 2 and 3 min. The absorbance value of the reagent blank must be subtracted from those of the standard and samples. The concentrations of d-ROMs may be calculated from the following formula:

$$\Delta \text{ Abs./min.} \times F = \text{U.CARR.}$$

where Δ Abs./min. are the mean differences of the absorbances recorded at 1, 2 and 3 min., and F is a factor with an assigned value. According to the *end-point-analysis*, the absorbances must be measured after incubating a solution composed of 5 µL of serum, 1 mL of R2 and 10 µL of R1 at 37° C for 75 min. A blank reagent, obtained by replacing serum with distilled water, and a standard should be included for each series of assays. The absorbance value of the reagent blank is subtracted from those of the standard and the samples. In this case, the concentrations of d-ROMs are given as follows: (Abs. sample/Abs. standard) x [standard], where Abs. are the absorbances and [ ] is the standard concentration. An example of assay linearity, referred to the kinetic analysis, is reported in Figure 1. Intra-assay coefficient of variation calculated on 20 aliquots of fresh serum was 2.1%, while inter-assay evaluated on 20 aliquots of frozen serum was 3.1%. Accuracy was evaluated using a control serum with assigned value, provided by the manufacturer. The method was shown to be linear up to 500 U.CARR. Samples giving results above 500 U.CARR. should be diluted with distilled water and reassayed after dilution. The results should then be multiplied by the dilution factor. We automated the test on Arco (Biogamma, Rome), according to the kinetic analysis, using 500 µL of R2, 5 µL of R1 and 5 µL of serum. Calibration was automatically performed by the instrument in triplicate using a calibrator with an assigned value. Calibration was performed at the beginning and at the end of the analytic run to verify the stability of reagents. The concentrations of d-ROMs in U.CARR. were calculated automatically by the analyzer based on the reference curve. Reference values, calculated by the manufacturer on about 4,000 apparently healthy subjects, have been suggested between 250 and 300 U.CARR. Increased levels of d-ROMs indicate a condition of oxidative stress. Decreased concentrations of d-ROMs have been reported during antioxidant supplementation or in patients under treatment with corticosteroids (unpublished data from the manufacturer).

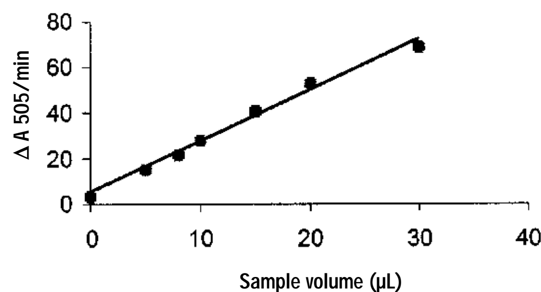

Figure 1. Linearity of the d-ROMS test according to the kinetic analysis.

Antioxidant capacity. The antioxidant capacity in serum was evaluated manually by a new spectrophotometric test (OXY-ADSORBENT test, Diacron, Italy), according to a *fixed-time analysis*. This is based on the capacity of a massive dose of HClO to oxidate the physiologic antioxidant reef (uric acid, GSH, thiol groups, vitamins, GSH-Px, SOD, catalase, etc.) The efficacy of the antioxidant system can be monitored indirectly by measuring the excess of HClO in serum. As HClO reacts with a correctly buffered chromogenic substrate, a colored complex develops that can be measured photometrically, presenting a maximum peak of absorbance at 505 or

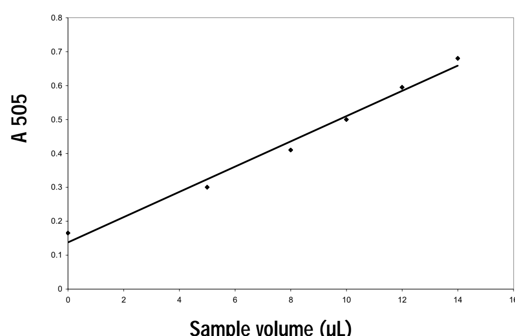

Figure 2. Linearity of the OXY-ADSORBENT test according to the *fixed-time* analysis.

Table 2. Total antioxidant capacity referred to serum concentration of HClO.

Normal	>350 μmol HClO/mL
Mild reduction	350-320 μmol HClO/mL
Moderate reduction	320-280 μmol HClO/mL
Severe reduction	280-250 μmol HClO/mL
Maximal reduction	<250 μmol HClO/mL

546 nm. The concentration of the colored complex is directly proportional to the concentration of HClO and indirectly to the antioxidant capacity. According to the manufacturer's recommendations, 10 μL of sample (serum), previously diluted 1:100 with distilled water, must be added and mixed with 1 mL of oxidant solution (R1) and 10 μL of chromogenic substance (R2). For each series of assays, a standard with an assigned value, previously diluted 1:100 with distilled water as for the samples, and a blank reagent, obtained by replacing serum with distilled water, were included. The absorbances were measured immediately at 505 nm or 546 nm. The absorbance value of the reagent blank was subtracted from those of the standard and the samples. The antioxidant capacity, expressed as μMol HClO/mL of serum, was calculated by the following formula: (Abs.blank - Abs. sample) / (Abs. blank - Abs. standard) x [standard], where Abs. are the absorbances and [ ] is the standard concentration. An example of assay linearity is reported in Figure 2. The intra-assay coefficient of variation tested on 20 aliquots of fresh serum was 2.2%, while the inter-assay evaluated on 20 aliquots of frozen serum was 6.3%. Expected values are reported in Table 2.

Molecular markers of thrombophilia. The degree of thrombin formation *in vivo* was investigated by measuring plasma concentrations of prothrombin fragment 1+2 (F1+2) and thrombin-antithrombin complexes (TAT),[30,31] using a commercial enzyme-linked immunosorbent assay (ELISA) (Enzygnost F 1+2 micro, and Enzygnost TAT micro, respectively, BehringWerke AG, Marburg, Germany), based on the sandwich principle. The intra-assay coefficient of variation (C.V.) for F1+2 was between 5 and 7.5%, while the inter-assay C.V. was between 6 and 13%. According to the manufacturer, F1+2 reference values were between 0.4-1.1 nmol/L. The intra-assay coefficient of variation for TAT was between 4 and 6%, while the inter-assay C.V. was between 6 and 9%. According to the manufacturer, TAT reference values are between 1.0-4.1 μg/L.

Fibrinolytic activity was explored by measuring plasma levels of D-dimer, a specific product derived from plasmin-induced degradation of stabilized fibrin,[32] by means of IL Test™ D-Dimer, a new and reliable turbidimetric immunoassay, automated on IL Coagulation System ACL 7000 (Instrumentation Laboratory).[33] The reagent is a suspension of polystyrene latex particles coated with a monoclonal antibody specific for the D-dimer domain included in fibrin soluble derivatives. When the sample is mixed with the reagent and the reaction buffer, the coated latex particles agglutinate. D-dimer levels are directly proportional to the degree of agglutination and can be calculated photometrically by measuring the decrease in transmitted light at 405 nm caused by the aggregates. The intra-assay coefficient of variation, assessed over multiple runs of low D-dimer control (IL Test™ D-dimer control), was 4.3%. The inter-assay coefficient of variation, assessed on 20 aliquots of frozen low D-dimer control was 6.5%. The manufacturer suggests reference values below 250 ng/mL.

Statistical analysis. Results are given as medians with their ranges. Data from patients and controls were compared using the Mann-Whitney U-test. Spearman's rank test was used to evaluate the correlation between parameters. A p value <0.05 was considered to be statistically significant.

Results

Results are reported in Table 3. Serum levels of d-ROMs were significantly higher ($p<0.001$) in heavy drinkers than in controls: 328.1 (143.4-847.2) U.CARR vs 250 (200.7-366.8) U.CARR, respectively. The total antioxidant capacity was similar in chronic alcohol abusers and in moderate drinkers: 360.2 (336.8-374.4) μMol HClO/mL vs 369 (362-378.4) μMol HClO/mL, respectively. Plasma levels of TAT were higher ($p<0.001$) in heavy drinkers than in controls: 2.5 (1.4-13) μg/L vs 1.5 (1-4.1) μg/L, respectively. Similarly, plasma levels of F1+2 were higher ($p<0.01$) in alcoholics than in controls: 1.7 (0.5-5.2) nMol/L vs 0.9 (0.4-1.1) nMol/L, respectively. Plasma levels of D-dimer were significantly higher ($p<0.001$) in alcoholics than in moderate drinkers: 235.5 (208-462) ng/mL vs 163.5 (71-233) ng/mL, respectively. No correlation between oxidative stress parameters and hemostatic factors was found.

Table 3. Plasma levels of thrombin-antithrombin complexes (TAT), prothrombin fragment 1+2 (F1+2) and D-dimer, serum levels of reactive oxygen species (d-ROMs) and antioxidant capacity (OXY-ADS test) in 45 heavy drinkers and in 42 controls. Results are given as medians with their ranges. A *p* value <0.05 was considered to be statistically significant.

	Heavy drinkers (n=50)	*Controls (n=42)*
TAT (mg/L)	2.5 (1.4-13)°	1.5 (1-4.1)
F1+2 (nMol/L)	1.7 (0.5-5.2)*	0.9 (0.4-1.1)
D-Dimer (ng/mL)	235.5 (208-462)°	163.5 (71-233)
d-ROMs (U.CARR)	328.1 (143.4-847.2)°	250 (200.7-366.8)
OXY-ADS.Test (mMol HClO/mL)	360.2 (336.8-374.4) n.s	369 (362-378.4)

**p<0.01; °p<0.001.*

Discussion

Our results show that both oxidative stress and a thrombophilic condition can be observed in heavy drinkers in the absence of severe liver disease. In particular, chronic alcohol abuse appears to be linked to increased serum levels of reactive oxygen species, such as hydroperoxides, with a normal antioxidant capacity. Previous observations[9,34] indicated that an adaptative enhancement of some antioxidant defences (superoxide dismutase, glutathione peroxidase, glutathione transferase, etc.) could be detected during chronic alcohol abuse when highly reactive oxidant species are generated in large amounts. Thus, even a normal antioxidant capacity, as we reported in the patients examined, could be inadequate, indicating the failure of any adaptative response. This might contribute to the development of alcohol-induced injury to liver[4,6,8] as well as to various extrahepatic tissues and systems, including vessels and the endothelium.[5,7,9,10] Just recently, endothelial cell dysfunction and hemostatic disorders due to a perturbation of the redox balance have been reported in some pathologies associated with a high thrombotic morbidity rate, such as type 2 diabetes and HIV infection.[2,3] Analogously, in chronic alcohol abusers we detected abnormal thrombin generation and fibrin degradation, as suggested by higher plasma levels of F1+2, TAT, and D-dimer, respectively, in the presence of oxidative stress. Since patients and controls were not completely matched for age, an age-related increase of such hemostatic markers of thrombophilia[23-25] could not be completely ruled out in the chronic alcohol abusers. Furthermore, differently from previous studies, we did not find a direct correlation between oxidative stress parameters and hemostatic indices in heavy drinkers. Nevertheless, different methodologic approaches make direct comparison between trials quite difficult and could partly explain some discrepancies. Other factors, besides those already investigated, probably play a role. A strong positive relationship between oxidative stress and homocysteine, considered to be an important risk factor for atherothrombosis, has been recently reported.[35-37] In particular, increased oxidative stress has been suggested to play a pathophysiologic role in the deleterious endothelial effects of homocysteine in humans.[36,37] Homocysteine has been supposed to rise as a consequence of consumption of wine and spirits[38] as well as of folate reduction,[39, 41] which is a common finding in chronic alcohol abusers.[40,41] In the present study, confirming data recently reported,[41] decreased plasma levels of folate and hyperhomocysteinemia were found in a subgroup of alcoholics who also exhibited elevated levels of reactive oxygen species (preliminary data).

In conclusion, the results obtained support the hypothesis that both alterations in the redox balance and a thrombophilic condition can be observed in heavy drinkers without severe liver diseases, such as cirrhosis and hepatitis C virus (HCV) hepatitis (a quarter of patients with alcoholic liver disease also have markers of HCV infection).[42] The new test for the measurement of reactive oxygen species in serum proved to be simple, accurate, reproducible and cheap. It could, therefore, be used in association with other simple routine laboratory tests (γ-GT, MCV), usually performed to screen for a condition of alcohol abuse,[23] as an early index of oxidative damage, which precedes and likely contributes to the evolution of alcohol-induced injury to various tissues and atherosclerosis.

Contributions and Acknowledgments

Each author gave a personal contribution to the conception and design, analysis and interpretation of data of the paper according to their own scientific background. RT; study conception and design (with Dr. PB), evaluation of redox balance and hemostatic parameters, interpretation of data, draft and revision of the article for clinically important intellectual content; MC; development and evaluation of the two new methods (dRoms and OXY-ADSORBENT TEST) for measuring reactive oxygen species and antioxidant capacity, respectively; MB; automization of the d-ROMs kit, dosage of reactive oxygen species and evaluation of results; GM and DB; patient recruitment and clinical assessment; MR; collection of samples, collection of data and statistical evaluation; PB (Chief of the Center for Alcohol Abuse and Alcoholism, Neurological Institute "C. Mondino", I.R.C.C.S., Department of Neurological Sciences, Universitry of Pavia); study conception and design (with RT), literature revision and final approval of the version to be submitted. The order of authorship was established on the basis of the role of each author in the context of the study.

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Disclosures

Conflict of interest: none.

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Manuscript processing

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Potential implications for clinical practice

The new simple, accurate and automated test, for measuring levels of reactive oxygen species could be used, in combination with other diagnostic procedures, in the assessment of chronic alcohol abuse.

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