Increased LDL+VLDL Oxidizability and Plasma Homocysteine Levels in Chronic Alcoholic Patients

Jale Balkan, Pervin Vural, Serdar Öztçan, Hasan Mırsal, Mansur Beyazıyırek, Gülçin Aykaç-Toker and Müjdat Uysal

1Department of Biochemistry, Istanbul Faculty of Medicine, Istanbul University, 34093 Çapa-Istanbul, Turkey
2Anatolia Clinics, Balıklı Rum Hospital, Yedikule, İstanbul, Turkey

(Received August 3, 2004)

Summary The purpose of this study was to investigate the effect of heavy alcohol consumption on peroxidation status in apolipoprotein B-containing lipoproteins (LDL+VLDL) and plasma as well as plasma homocysteine (HC) levels in patients with chronic alcoholism who drank raki, a national Turkish beverage. For this reason, endogenous diene conjugate (DC) and lipid hydroperoxide (LOOH) levels and lag phase, maximum DC formation and propagation rate following copper induction were measured in apolipoprotein B-containing lipoproteins (LDL+VLDL) isolated by precipitation with dextrane sulfate and MgCl₂ from plasma. In addition, serum malondialdehyde (MDA), DC, HC, folate and vitamin B₁₂ levels as well as paraoxonase activity were determined. Serum MDA and DC levels were higher in heavy raki drinkers compared to control subjects. Significant increases in endogenous DC and LOOH levels in LDL+VLDL together with shortened lag phase were also observed in patients. In addition, HDL-cholesterol, HC and vitamin B₁₂ levels and HDL-associated paraoxonase activity were found to be higher, but folate levels to be lower in serum of heavy raki consumers. In conclusion, our results indicate that increases in LDL+VLDL oxidizability and plasma HC levels may enhance the susceptibility to vascular diseases in heavy raki drinkers.

Key Words LDL+VLDL oxidizability, homocysteine, alcoholism, raki drinkers

Alcohol intake has been reported to produce a double effect on blood vessels (1, 2). Light to moderate alcohol intake has a protective role in coronary heart disease (3, 4), but heavy alcohol ingestion has prooxidant effects (5, 6) and thus may increase the incidence of vascular diseases (1, 2). Although some controversial results have been presented (7, 8), a decrease in the susceptibility of LDL to oxidation (1–4) and increases in HDL-cholesterol levels (2, 3, 9) and HDL-associated paraoxonase activity (10) are found following moderate alcohol ingestion. However, there is relatively little information on the effect of heavy alcohol intake on in vivo LDL-oxidation. HDL-levels and paraoxonase activity in humans (1, 4, 11, 12).

On the other hand, chronic ethanol administration in rats (13, 14) and human beings (9, 15) has been shown to result in elevated homocysteine (HC) levels in the plasma. HC is found to have several potentially deleterious vascular actions such as increase of oxidative stress, impairment of endothelial function, and induction of thrombosis (16). Therefore, hyperhomocysteinemia may also increase the incidence of vascular disease in chronic alcoholics.

Raki, largely consumed by the Turkish population, is a national alcoholic beverage. Raki is an aniseed-flavored spirit obtained through the redistillation of wine grape distillate and contains 45% alcohol by volume. It can be drunk straight or by mixing with water half and half. There is no information on the toxic effects of raki consumption in the literature. Therefore, we wanted to investigate oxidative stress status in plasma and apo B-containing lipoproteins (LDL+VLDL) as well as plasma HC, folate and vitamin B₁₂ levels in Turkish heavy raki drinkers.

MATERIALS AND METHODS

Subjects. Thirty-two patients with chronic alcoholism (26 men and 6 women, 48.6 ± 9.86 y; mean ± SD, range 40 to 60 y) were studied after admission to the alcoholic detoxification unit at Anatolia Clinics in Balıklı Rum Hospital. Diagnosis of alcohol dependence was made according to the Diagnosis and Statistical Manual for Mental Disorders (DSM IV) of the American Psychiatric Association (17). Details of patients recorded at the time of admission included age, sex, height and weight, dietary and smoking habits, history of ischemic heart disease, diabetes mellitus, hypertension, malignancy and medication. The alcoholics had no signs or symptoms of any other diseases. They had negative hepatitis markers and exhibited no clinical or laboratory evidence of liver cirrhosis. Liver biopsies...
were not performed for ethical reasons. Alcoholics who consume only raki were included in this study. Their mean daily alcohol consumption was 225.9±88.2 g (range 90 to 450 g), and the mean duration of their alcoholism was 18.8±9.04 y (range 5 to 30 y). Current smokers (20-30 cigarettes per day) accounted for 65% of alcoholics. No patient was on lipid lowering drugs, vitamins or antioxidants at the time of investigation.

Twenty-four control subjects included 18 men and 6 women, mean age 52.3±10.2 y (range 42 to 62 y), who drank only occasionally. Their histories were negative for diabetes, hypertension, cancer, heart disease, alcohol, cigarette (16 were current smokers) or drug abuse, and their serum cholesterol and triglyceride levels were within the normal levels. Control subjects had not received antioxidant therapy or vitamin supplements in the previous 12 mo. All subjects had similar socioeconomic status and dietary habits. Each study participant was informed and the study was approved by the local ethics committee.

Blood sampling. Blood samples were obtained within 24 h of hospital admission. Venous blood was taken following an overnight fasting period into dry and ethylenediaminetetraacetic acid (EDTA)-containing tubes. EDTA-plasma and serum were obtained by centrifugation at 1,500 ×g for 10 min. They were stored at −80°C until they were analysed.

Biochemical analyses in serum/plasma. Serum alanine transaminase (ALT), aspartate transaminase (AST) and γ-glutamyl transferase activities (GT), total bilirubin, total cholesterol (TC) and triglyceride levels were determined by using an autoanalyzer. Determination of plasma HDL-C concentration with dextran sulphate-magnesium precipitation was followed by enzymatic determination of cholesterol and LDL-C was calculated by Friedewald’s formula (18). The degree of endogenous lipid peroxidation in serum was assessed by two different methods: a) Serum diene conjugate (DC) formation was determined spectrophotometrically at 365 nm (19). For this reason, serum lipids were extracted with a chloroform/methanol (2:1) mixture. The extracted lipids were redissolved in cyclohexane and the approximate amounts of hydroperoxides were calculated using a molar extinction coefficient of 2.52×10−4 M−1. b) Malondialdehyde (MDA) levels were determined according to the method of Buege and Aust (19). Plasma HC levels were determined with the AxSYM System (Abbott, Diagnostics Division). This is a Fluorescence Polarization Immunoassay (FPIA) for the quantitative measurement of total HS in human serum or plasma on the AxSYM Analyzer. Serum folate and vitamin B12 levels were determined by using Simul-TRAC-SNB radioassay kit B12 (57Co)/Folat (125I) obtained from ICN. Serum paraoxonase activity was measured using paraoxon as substrate by the method of Furlong et al. (20). The absorbance was continuously monitored at 405 nm. A molar extinction coefficient of 18.05×103 was used for calculations of activity. A unit of paraoxonase activity is defined as 1 nmol of 4-nitro phenol formed per minute under the assay conditions.

Lipoprotein analyses. Apo B 100-containing lipoproteins (LDL+VLDL) were precipitated from EDTA-plasma by dextran sulfate and MgCl2 solution, pH 7.0. The pellet was suspended in 0.9% NaCl and reprecipitated by adding precipitation reagent, vortexing and centrifuging in order to remove EDTA from the non-HDL fraction. The pellet was redissolved with phosphate buffered saline (0.68 M NaCl, 10 mM Na2HPO4, pH 7.0) (21) and the baseline DC and lipid hydroperoxide (LOOH) levels were estimated. To determine baseline DC, lipids were extracted from LDL+VLDL samples with chloroform-methanol (2:1), dried under nitrogen, then redissolved in cyclohexane, and analysed spectrophotometrically at 234 nm (22). LOOHs were measured by iodometric assay (23). This assay is based on the capacity of LOOHs to convert free I2 to I3−, which can be measured photometrically at 365 nm.

For the in vitro LDL+VLDL oxidation experiment, the oxidation of the LDL+VLDL fraction (200 µg cholesterol/mL) was initiated by the addition of CuSO4 at a final concentration of 46 nmol/mL and DC formation was followed in 5 min intervals for 4 h at 25°C by monitoring the change in absorbance at 234 nm. The lag phase was defined as the intercept of the baseline and the slope of the absorbance curve in the propagation phase and was expressed in minutes (21, 24). Maximum DC formation was calculated using the difference between the maximum and minimum absorbance at 234 nm (ΔA234). The rate of DC production was also calculated using the slope of the absorbance curve during the propagation phase.

Statistical analyses. Values were expressed as means±SD. A comparison between groups was performed by Student’s t-test and the Mann Whitney U-test. Correlation coefficients were determined by Pearson’s correlation test. In addition, a multiple linear regression analysis was carried out to find an independent determinant which influenced the dependent variable.

RESULTS

The results obtained from patients with chronic alcoholism were compared to those of control subjects (Tables 1, 2).

Serum ALT, AST and γ-GT activities were significantly higher in patients with chronic alcoholism, but no change in serum total bilirubin levels was observed. Higher triglyceride and HDL-C levels, lower LDL-C levels and unchanged TC levels were found in the serum of heavy alcohol drinkers. Serum paraoxonase activity was also observed to be higher and this activity was correlated with HDL-C levels (r=0.468, p<0.01) in chronic alcoholics. Serum MDA, DC, HC and vitamin B12 levels were also higher, whereas folic acid levels were lower in heavy drinkers as compared to controls.

Endogenous DC and LOOH levels were higher in LDL+VLDL fractions in heavy raki drinkers. When in vitro LDL+VLDL oxidation kinetics was investigated, the propagation rate and maximum DC formation of
LDL+VLDL oxidation were not found to be changed in heavy drinkers, but the lag phase was shortened. There was a negative correlation between endogenous (LDL+VLDL)-DC levels and lag time values \( (r=-0.465, p<0.01) \) in patients. Although a positive significant correlation was found to exist between lag time values and serum paraoxonase activity \( (r=0.393; p<0.05) \), there was no correlation between endogenous (LDL+VLDL)-DC levels and serum paraoxonase activity \( (r=-0.146; p>0.05) \) in heavy raki drinkers. Similarly, we could not observe any significant correlation between plasma HC levels and LDL+VLDL oxidizability parameters. However, the multiple linear regression analysis between dependent factors [endogenous (LDL+VLDL)-DC levels or lag time values] and independent factors (plasma TC, LDL-C, HDL-C, triglyceride and HC levels and POX activity) were not statistically significant in patients.

**DISCUSSION**

Epidemiological studies suggest that moderate alcohol consumption, irrespective of beverage type, can reduce the risk of atherosclerotic cardiovascular disease \( (1, 2) \). Wine, especially red wine with its abundant content of phenolic acids, polyphenols, and flavonoids has received special attention in this regard. It has been reported that red wine decreases the susceptibility of LDL to lipid peroxidation \( (25-27) \) and it has an antiatherosclerotic effect in cholesterol-fed animals \( (27,28) \) and apolipoprotein E-deficient mice \( (29) \).

Contrarily, heavy alcohol consumption has been considered to increase atherosclerosis risk \( (1, 4, 11, 12) \). Since alcohol can induce oxidative stress and reduce levels of endogenous antioxidants in the liver and circulation \( (5, 6, 30, 31) \), LDL is likely to be more sensitive to oxidative damage following heavy alcohol ingestion. Indeed, some investigators have shown that LDL oxidation increased in humans \( (11, 12) \) and baboons \( (32) \) following heavy alcohol intake. In addition, heavy alcohol consumption has been reported to decrease plasma HDL-cholesterol levels \( (12) \). However, the effect of raki consumption on LDL oxidation and HDL-cholesterol levels has not been investigated previously. In this study, endogenous DC levels and LOOH levels as well as the
oxidation kinetic of LDL+VLDL, a parameter showing their susceptibility to copper-induced lipid peroxidation, were determined in heavy raki drinkers. According to our results, endogenous DC and LOOH levels increased in LDL+VLDL, whereas the lag phase was shortened in heavy raki drinkers.

As known, the protective effect of HDL against the development of coronary heart diseases is complex (2, 3) and HDL-associated enzyme paraoxonase may play an important role in the inhibition of LDL-oxidation (2, 33). In our study, we found that both plasma HDL-C and HDL-associated enzyme paraoxonase activity increased, having a positive correlation between them, in heavy raki drinkers. Although we observed that there was a significant positive correlation between serum paraoxonase activity and lag time values, negative but insignificant correlation between serum paraoxonase and (LDL+VLDL)-DC levels were detected in chronic alcoholics. Therefore, our findings indicate that the elevation in serum paraoxonase enzyme activity may be related to high HDL levels and that HDL-associated protective mechanism is inadequate to prevent LDL+VLDL oxidation in heavy raki drinkers.

Hyperhomocysteinemia may be another factor increasing the incidence of vascular disease in chronic alcoholics. HC is a thiol-containing amino acid derived from the metabolism of methionine. HC can be metabolized by two pathways, either remethylated to methionine or catabolized by the transsulfuration pathway to cysteine (16). Remethylation is catalyzed by methionine synthase, which requires vitamin B12 as a cofactor and 5-methyltetrahydrofolate as a methyl donor. It has been reported that acetaldehyde, the main metabolite of ethanol, inhibits methionine synthase in the liver (34) and that this inhibition may result in the impairment of remethylation of HC and cause an increase in plasma HC levels following alcohol intake (14). The degradation of HC to cysteine occurs by two vitamin B6-dependent enzymes, cystathionine ß-synthase and cystathionine γ-lyase. Since folate, vitamin B12 and vitamin B6 are coenzymes of methionine-HC metabolism, their deficiencies may influence serum HC levels. Indeed, plasma folate and vitamin B6 levels have been usually reported to decrease, but B12 levels increased in chronic alcoholics (15). However, liver concentrations of vitamin B12 have been reported to be low in spite of high circulating vitamin B12 levels (35). This situation has been explained by the impaired retention of vitamin B12 by peripheral tissues due to alcohol intake.

This is the first study examining HC and folic acid and B12 levels in the plasma of heavy raki drinkers. We found that plasma HC levels increase together with decreased folate and increased vitamin B12 levels in the plasma of heavy raki consumers as reported in earlier studies in chronic wine and spirit drinkers (9, 15).

In conclusion, our results indicate that increases in LDL+VLDL oxidizability and plasma HC levels may enhance the susceptibility to vascular diseases in heavy raki drinkers.

Acknowledgments
This work was supported by the Research Fund of the University of Istanbul (No:BYP-114/12122002).

REFERENCES
1) Puddey IB, Croft K. 1997. Alcoholic beverages and lipid peroxidation: relevance to cardiovascular disease. Add Biol 2: 269–276.
2) Van Tol A, Hendriks HJ. 2001. Moderate alcohol consumption: effects on lipids and cardiovascular disease risk. Curr Opin Lipidol 12: 19–23.
3) Hannuksela ML, Lissansantti MK, Savolainen MJ. 2002. Effect of alcohol on lipids and lipoproteins in relation to atherosclerosis. Crit Rev Clin Lab Sci 39: 225–283.
4) Belleville J. 2002. The French paradox: Possible involvement of ethanol in the protective effect against cardiovascular diseases. Nutrition 18: 173–177.
5) Mutlu-Türköğlu Ü, Doğru-Abbasoğlu S, Aykaç-Toker G, Mırsal H, Beyazıyık M, Uysal M. 2000. Increased lipid and protein oxidation and DNA damage in patients with chronic alcoholism. J Lab Clin Med 136: 287–291.
6) Sun AY, Ingelman-Sundberg M, Neve E, Matsumoto H, Nishitani Y, Minowa Y, Fukui Y, Bailey SM, Patel VB, Cunningham CC, Zima T, Fialova L, Mikulikova L, Popov P, Malbooban L, Janebova M, Nesper K, Sun KY. 2001. Ethanol and oxidative stress. Alcohol Clin Exp Res 25: 237S–243S.
7) Suzukawa M, Ishikawa T, Yoshiida H, Hosoi K, Nishio E, Yamashita T, Nakamura H, Hashizume N, Suzuki K. 1994. Effects of alcohol consumption on antioxidant content and susceptibility of low-density lipoprotein to oxidative modification. J Am Coll Nutr 13: 237–242.
8) De Rijke YB, Demacker PNM, Assen NA, Sloots LM, Katan MB, Stalenhoef AFF. 1996. Red wine consumption does not affect oxidizability of low-density lipoproteins in volunteers. Am J Clin Nutr 63: 329–334.
9) De la Vega MJ, Santolaria F, Gonzalez-Reimers E, Aleman MR, Milena A, Martinez-Riera A, Gonzalez-Garcia C. 2001. High prevalence of hyperhomocysteinemia in chronic alcoholism: the importance of the thermolabile form of the enzyme methylenetetrahydrofolate reductase (MTHFR). Alcohol 25: 59–67.
10) Van der Gaag MS, Van Tol A, Sceek LM, James RW, Urgert R, Schaafsma G, Hendriks HJF. 1999. Daily moderate alcohol consumption increases serum paraoxonase activity; a diet-controlled, randomised intervention study in middle-aged men. Atherosclerosis 147: 405–410.
11) Wehr H, Milewski B, Pozniak M, Rodó M. 1997. Anti-low-density lipoprotein antibodies in alcoholics without and with liver disease and in social drinkers. Alcohol Alcohol 32: 43–49.
12) Zima T, Fialova L, Mestek O, Janebova M, Crkovska J, Malbooban I, Stipek S, Mikulikova L. 2001. Oxidative stress, metabolism of ethanol and alcohol-related diseases. J Biomed Sci 8: 59–70.
13) Stickel F, Choi SW, Kim YI, Bagley PJ, Seitz HK, Russell RM, Selhub J, Mason JB. 2000. Effect of chronic alcohol consumption on total plasma homocysteine level in rats. Alcohol Clin Exp Res 24: 259–264.
14) Barak AJ, Beckenauer HC, Khurbanda KK, Tuma DJ. 2001. Chronic ethanol consumption increases homocysteine accumulation in hepatocytes. Alcohol 25: 77–81.
15) Cravo ML, Gloria LM, Selhub J, Nadeau MR, Camilo ME,
Resende MP, Cardoso JN, Leitao CN, Mira FC. 1996. Hyperhomocysteinemia in chronic alcoholism: correlation with folate, vitamin B-12, and vitamin B-6 status. *Am J Clin Nutr* **63**: 220–224.

16) Mangoni AA, Jackson SHD. 2002. Homocysteine and cardiovascular disease: current evidence and future prospects. *Am J Med* **112**: 556–565.

17) American Psychiatric Association. 1994. Diagnostic and Statistical Manual of Mental Disorders, 4th ed. American Psychiatric Association Press, Washington, DC.

18) Bauer JD. 1982. Clinical Laboratory Methods. C. V. Mosby Company, St Louis.

19) Buege JA, Aust JD. 1978. Microsomal lipid peroxidation. *Methods Enzymol* **52**: 302–310.

20) Furlong CE, Richter RJ, Siedel SL, Costa LG, Motulsky AG. 1989. Spectrophotometric assays for the enzymatic hydrolysis of the active metabolites of chlorpyrifos and parathion by plasma paraoxonase/arylesterase. *Anal Biochem* **180**: 242–247.

21) Zhang A, Vertommen J, Van Gaal L, De Leeuw J. 1994. A rapid and simple method for measuring the susceptibility of low-density-lipoprotein and very-low-density-lipoprotein to copper-catalysed oxidation. *Clin Chim Acta* **227**: 159–173.

22) Ahotupa M, Ruutu M, Mantyla E. 1996 Simple methods of quantifying oxidation products and antioxidant potential of low density lipoproteins. *Clin Biochem* **29**: 139–144.

23) El-Saadani M, Esterbauer H, El-Sayed M, Nasar AY, Jürgens GA. 1989. Spectrophotometric assay for lipid peroxides in serum lipoproteins using a commercially available reagent. *J Lipid Res* **30**: 627–630.

24) Sattler W, Malle E, Kostner GM. 1998. Methodological approaches for assessing lipid and protein oxidation and modification in plasma and isolated lipoproteins. *Method Mol Biol* **110**: 167–191.

25) Rišiç VA, Stephen EM, Schneider SH, Khachadurian AK. 1999. Red wine inhibits the cell-mediated oxidation of LDL and HDL. *J Am Coll Nutr* **18**: 137–143.

26) Wakabayashi Y. 1999. Effect of red wine consumption on low-density lipoprotein oxidation and atherosclerosis in aorta and coronary artery in Watanabe heritable hyperlipidemic rabbits. *J Agric Food Chem* **47**: 4724–4730.

27) Vinson JA, Teufel K, Wu N. 2001. Red wine, deacetylated red wine, and especially grape juice, inhibit atherosclerosis in a hamster model. *Atherosclerosis* **156**: 67–72.

28) Kluerfeldt DM, Kritchevsky D. 1981. Differential effects of alcoholic beverages on experimental atherosclerosis in rabbits. *Exp Mol Pathol* **34**: 62–71.

29) Hayek T, Fuhrman B, Vaya J, Rosenblat M, Belinsky P, Coleman R, Ellis A, Avram M. 1997. Reduced progression of atherosclerosis in apolipoprotein E-deficient mice following consumption of red wine, or its polyphenols quercetin or catechin, is associated with reduced susceptibility of LDL to oxidation and aggregation. *Arterioscler Thromb Vasc Biol* **17**: 2744–2752.

30) Balkan J, Kambargh O, Aykaç-Toker G, Uysal M. 2002. Taurine treatment reduces hepatic lipids and oxidative stress in chronically ethanol-treated rats. *Biol Pharm Bull* **25**: 1231–1233.

31) Arteel G. 2003. Oxidants and antioxidants in alcohol-induced liver disease. *Gastroenterology* **124**: 778–790.

32) Navder KP, Baraona E, Leo MA, Lieber CS. 1999. Oxidation of LDL in baboons is increased by alcohol and attenuated by polyenylphosphatidylcholine. *J Lipid Res* **40**: 983–987.

33) Laplaud PM, Dantoine T, Chapman MJ. 1998. Paraoxonase as a risk marker for cardiovascular disease: Fact and hypotheses. *Clin Chem Lab Med* **36**: 431–441.

34) Kenyon SH, Nicolau A, Gibbons WA. 1998. The effect of ethanol and its metabolites upon methionine synthase activity in vitro. *Alcohol* **15**: 305–309.

35) Kanazawa S, Herbert V. 1985. Total corrinoid, cobalamin (vitamin B12), and cobalamin analogue levels may be normal in serum despite cobalamin depletion in liver in patients with alcoholism. *Lab Invest* **53**: 108–110.