CLINICAL STUDY

Erythrocyte antioxidant enzymes in patients with alcohol dependence syndrome

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Abstract

Background: **The role of free radicals and hydrogen peroxides in the metabolism and toxicity of alcohol is supported by many studies, therefore, many autors have tried to use the enzymes, metabolizing highly reactive chemical compounds as biological markers of alcoholism.**
Methods: **Erythrocyte antioxidant enzymes were measured in 37 male patients with alcohol dependence syndrome, without severe liver disease, aged between 18 and 59 years, with different duration (years) of alcohol abuse.**
Results: **Superoxide dismutase (SOD) activity was statistically significantly increased in alcoholics. Glutathione peroxidase (GPX) activity has shown no significant difference in alcoholics compared to the control group. Catalase (CAT) activity was significantly decreased in alcoholics. Specific activity of CAT was positively correlated with the duration of alcohol abuse (years).**
Conclusions: **Catalase activity has shown statistically significant decrease in patients with alcohol dependence syndrom.** ***(Tab. 3, Fig. 4, Ref. 31.)***
Key words: erythrocyte catalase, superoxide dismutase, glutathione peroxidase, alcoholics.

Ethanol oxidation involves three main metabolic pathways localized in three different subcellular compartments of the liver cell: alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) in the cytosol; the microsomal ethanol oxidizing system (MEOS) in the endoplasmatic reticulum and the catalase in both peroxisome and mitochondria.

Several studies showed that the first stable product of ethanol metabolism *via* aldehyde oxidase (AO), acetaldehyde, may play a role in ethanol-induced free radical injury (1, 2). The enzyme can use several electron acceptors, but molecular oxygen is the physiological oxidant that is divalently reduced to produce H_2O_2. However, a part of the oxygen is univalently reduced, generating superoxide anion radical ($O^{2\cdot-}$) (3). Acute ethanol (via production of NADH) and chronic ethanol (induction of P-450II E1 uncoupling) administration may increase microsomal generation of oxygen radicals (ethoxyl, hydroxyethyl). Both acute and chronic ethanol intoxications can increase local production of reactive oxygen species. Iron mobilization from ferritin by ethanol-stimulated $O^{2\cdot-}$ can supply reactive $^{\cdot}OH$ (hydroxyl) radicals. A direct increase of membrane permeability induced by ethanol may cause an increased susceptibility to lipid peroxidation. The development of oxidative stress in the liver may contribute to hepatotoxic action of alcohol (4).

The role of free radicals and hydrogen peroxide in the metabolism and toxicity of alcohol is supported by many studies, some of them suggesting that cellular injury may develop when reactive chemical specieses are produced in amounts sufficient to overcome the antioxidant defense mechanisms (5, 6). Therefore, some authors have tried to use the enzymes, metabolizing highly reactive chemical compounds, as biological markers of alcoholism (7, 8, 9). Among these enzymes, superoxide dismutase (SOD; EC.1.15.1.1) catalyzes dismutation of the superoxide anion ($O^{2\cdot-}$) into hydrogen peroxide (H_2O_2), both catalase (CAT; EC.1.11.1.6) and its catalatic activity detoxifies H_2O_2, and glu-

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Acknowledgements: We greatly acknowledge the help of the entire medical staff of the Skopje Hospital in Skopje especially of V. Avramovska MD and V. Ortakov MD for their participation in this study. We would like to thank to B. Kozarov for technical assistance.

tathione peroxidase (GPX; EC.1.11.1.9) detoxifies H_2O_2 and converts lipid hydroperoxides to nontoxic alcohols.

In some clinical studies, catalase alone, or together with other antioxidant enzymes, was measured in blood as a possible biological indicator for alcoholism (10, 11, 12). However, the results seem somewhat conflicting. Therefore, the aim of our study was to:

1. Measure erythrocyte antioxidant enzymes superoxide dismutase, glutathione peroxidase and catalase in patients with alcohol dependence syndrome (alcoholics);

2. Evaluate the effect of duration of alcohol abuse on the activity of the examined antioxidant enzymes activity;

3. Examine the possibility that erythrocyte superoxide dismutase, glutathione peroxidase and catalase may be possible biological indicators for alcoholism;

4. Examine possible inter-relationships between AOE activities in alcoholics as well as between AOE activities and biochemical laboratory tests routinely performed in alcoholics.

Material and methods

Subjects. Our group included 37 male individuals with alcohol dependence syndrome from the Hospital Skopje, hospitalized for the rehabilitation program. They were aged between 25 and 58 years and showed no clinical or biological evidence of liver cirrhosis. The study also included 111 healthy male blood donors. Liver biopsies were not performed for ethical reasons. Alcoholic patients (patients with alcohol dependence syndrome) were defined as subjects consuming alcohol of more than 100 g/day for the last two years, who continued drinking despite harm, with an inner stimulus to consume alcohol. Subjects were asked to abstain from alcohol, or any other drug usage except for nicotine, coffeine or prescribed medication, for 24 hours prior testing. Two questionnaires were used; Concordia Alcohol Screening Questionnaire (CASQ) (13, 11) and Michigen Alcoholism Screening Test (MAST) (14). The (CASQ) provided medical history, alcohol drinking history, prescribed and nonprescribed drug usage and familiar history of alcoholism. The latter was used to quantifiy alcohol consumption in a 30-day period. An index of daily alcohol use, Q value, was calculated (total alcohol consumed/30) for the recent 30-day period (recent Q-value). All information was based on self-reports in the presence of medical doctor.

All patients consumed excessive quantities of alcohol until the day of hospitalization (mean±SD=179.4±126.3) g/day. The duration of alcohol abuse was from 5–40 years with (mean±SD=6.0±3.4). Only one of them declared family drinking history. 92 % of them were smokers.

The liver enlargement was examined by manual palpation (0=no, 1=yes). Blood was collected within the first two days of hospitalization and the last drink was reported two days before the collection of the blood samples.

The control group consisted of 111 healthy male blood donors selected without known bias, aged between 18 and 59 years, consuming less than 13 g pure alcohol a day (15).

All individuals were informed about the study and the collection of blood samples by scribed information list, and the agreement was obtained in the presence of a medical doctor. The Ethnic Committee of Doctor's Chamber of the Republic of Macedonia approved all procedures that have been undertaken during the project.

Blood samples. Venous blood samples for measuring the enzyme activities were collected into heparinized tubes between 08.00 and 9.00 h. Hemoglobin concentration was determined in K3EDTA blood samples with a Coulter Microdif 18.

The blood was centrifuged at 2600 rpm for 15 minutes and plasma was separated. The erythrocytes were carefully sampled from the bottom of the tubes, to minimize contamination with leukocytes, washed three times with isotonic saline solution, and leased by addition (1/4, by vol.) of doubly distilled water containing 5 ml/L Triton x-100, followed by vigorous vortex-mixing and storage on ice for 10 minutes. Membrane-free hemolysate was obtained by centrifugation at 10,000x g for 5 minutes.

Antioxidant enzymes (AOE) were measured within the same day of collecting.

Enzyme Assays. Assay of superoxide dismutase (SOD; EC.1.15.1.1) activity: SOD activity was measured with RANSOD kits (cat. No. SD 125; Randox Labs., Crumlin, North Ireland). This method employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye. The superoxide dismutase activity is then measured by the degree of inhibition of this reaction. All diluted sample rates were converted into percentages of the sample diluent rate, and substracted from 100 % to give a percentage inhibition. The activity was measured at 37 °C on a PU 8630 UV-VIS spectrophotometer, and absorbency was monitored at 505 nm for 3 minutes. The unit of activity is defined as the amount of enzyme that inhibits the rate of the formazan dye formation by 50 %.

SOD units were obtained from standard curve using percentage inhibition of the samples (SOD units/ml of whole blood-absolute activity) and were converted to SOD units/g hemoglobin (specific activity). Standards were prepared by diluting a commercial SOD preparation (cat. No SD 125) in order to obtain standard curve.

Assay of glutathione peroxidase (GPX; EC.1.11.1.9) activity: GPX activity was determined with Ransel kits (cat. No RS 505; Randox Labs., Crumlin, North Ireland) at 37 °C on a PU 8630 UV-VIS spectrophotometer at 340 nm for 3 minutes. This assay, based on the method of Paglia and Valentine, requires cumene hydroperoxide as a substrate (16). Before the analysis were the samples diluted 40 fold to a hemolysate by adding Drabkin's reagent (double strength) to inhibit the peroxidase activity of hemoglobin (16). The final concentrations of reagents in the assay were those recommended by the manufacturer. The activity mmof GPX was expressed in mol/L/min for absolute activity and mol/gHb/min for specific activity.

Assay of catalase (CAT; EC.1.11.1.6) activity: Catalase activity was measured in hemolysates at 250C with PU 8630 UV VIS spectrophotometer by the method of Aebi (17). The decom-

Tab. 1. Descriptive statistics of selected variables of the control subjects and alcoholics, mean±SD.

Variables	Controls (n=111)	Alcoholics (n=37)
Cigarette consumption (cig/day)	8.1±10.3	25.5±19.8*
Alcohol intake (g/day)	5.4±6.3	179.4±126.3***
AST	25.6±8.7	30.7±19.2
ALT	27.8±11.9	31.9±14.5
Bilirubin (mol/L)	10.6±4.4	18.09±1.6***
Direct bilirubin (mol/L)	4.3±0.8	8.56±4.8***
Hemoglobin (g/L)	143.89±20.88	135.03±15.7*

n — number of cases; AST — aspartate-aminotransferase; ALT — alanine-aminotransferase; * p<0.05; *** p<0.001.

Tab. 2. Antioxidant enzymes activity in male alcohol abusers versus control group, mean±SD.

	Alcohol abusers group (n=37)	Control group (n=111)
SOD U/ml	265.7±141.5**	217.5±47.4
SOD U/gHb	1984±1045***	1501±308.7
GPX U/L	6290±2796	7369±3246
GPX U/gHb	47.0±21.0	51.5±23.4
CAT k	25.2±6.5***	29.5±5.83
CAT k/gHb	188.9±48.3*	205.4±43.3

n — number of cases; SOD U/ml — superoxide dismutase expressed in absolute activity; SOD U/gHb — superoxide dismutase expressed in specific activity; GPX U/L — glutathione peroxidase expressed in absolute activity; GPX U/gHb — glutathione peroxidase expressed in specific activity; CAT k — catalase expressed in absolute activity; CAT k/Hb — catalase expressed in specific activity; k — a rate constant of the first order reaction as defined by Aebi; * p<0.05; ** p<0.01; *** p<0.001.

position of the substrate H_2O_2 was monitored spectrophotometrically at 240 nm for 3 minutes. Activity was expressed as k (rate constant of the first order reaction as defined by Aebi) (absolute activity) and k/g hemoglobin (specific activity).

Enzyme assays of each sample were performed in duplicate on 8630 PU UV-VIS spectrophotometer.

Total and direct bilirubin were measured with commercial Randox kits (BR 411). The activity of aspartate-aminotransferase (AST) and alanine-aminotransferase (ALT) was measured at 30 °C with the commercial Randox kits (AS 2359 and AL 2360).

Data analyses. Statistical analyses were performed by using the commercial statistical package, Statistica for Windows, Version 5.0. Standard analyses to study inter-relationships between enzyme activities and their potential determinants were used. Unpaired Student t-test was used between independent variables. Pirson's correlation coefficient was used to investigate associations between parametric variables. Spearman's rank correlation coefficient was used to investigate associations between enzyme activities and non-parametric variables. Partial correlation of the various enzyme activities was studied after adjustment for the effects of the confounding factors. The level of significance was p<0.05.

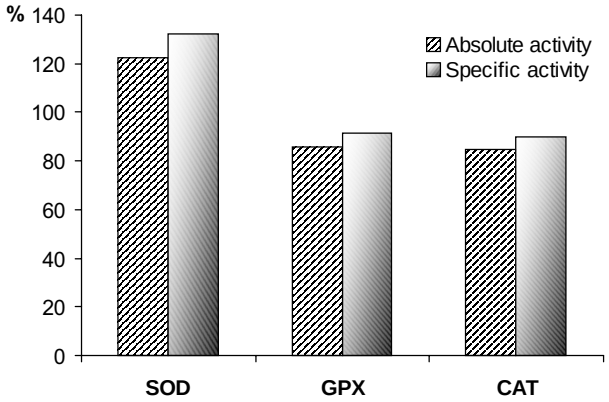


Fig. 1. Percentual ratio of the activity of erythrocyte antioxidant enzymes (absolute and specific activity) in patients with alcohol dependence syndrome, compare to the control group (100 %).

Results

Table 1 presents some relevant data concerning the characteristics of control subjects and alcoholics. Patients smoked three times more than controls and consumed alcohol about 30 times more than controls did, which was statistically significant (t=1.98 146 df, p<0.05 and t=8.37 146 df, p<0.00). The classical biochemical analyses performed in alcoholics such as aspartate-aminotransferase (AST) and alanine-aminotransferase (ALT) showed no significant increase in activity compared to the control group. In alcoholics total and direct bilirubin showed a statistically significant increase in the concentrations, compared to the control group (t=15 and t=5.3 df 146, p<0.001). As statistically significant decrease of the hemoglobin concentration was noticed in alcoholics, compared to the control group (t=3.39 df 146, p<0.05).

The results, concerning the erythrocyte antioxidant enzyme activities in alcoholics versus control group, are presented in Table 2. We have found a statistically significant changes of the activity of SOD (t=4.0 df 146, p<0.01 for absolute activity and t=4.33 df 146, p<0.001 for specific activity) and CAT in alcoholics (t=-3.61 df 146, p<0.001 for absolute activity and t=-2.14 df 146, p<0.05 for specific activity) compared to the control group. Glutathione peroxidase showed no significant changes of the activity in alcoholics, compared to the control group. In alcoholics the superoxide dismutase activity showed a significant increase (20 % for absolute activity and 30 % for specific activity) (Fig. 1). Conversely, catalase showed a significant decrease in the activity in alcohol abusers group, compared to the control group (about 20 % decrease) (Fig. 1). Although the activity of GPX was decreased in alcoholics (15 % for absolute activity and 8.8 % decrease for specific activity), this decrease was not statistically significant, compared to the control group (Fig. 1).

Our patients had different duration of alcohol abuse, ranging from 5 to 40 years (mean=6.0±3.4). The activity of antioxidant erythrocyte enzymes in alcohol abuser group with the duration of alcohol abuse less than 20 years and from 20 to 40 years,

Tab. 3. Antioxidant enzyme activity in control subjects and alcoholics with diffrerent duration of alcohol abuse (less than 20 years and more than 20 years) and between two groups of alcoholics with different duration of alcohol abuse, mean±SD.

Antioxidant erythrocyte enzymes	Control group (n=111)	Alcohol abusers group (duration of alcohol abuse <20 years) (n=13)[a]	Alcohol abusers group (duration of alcohol abuse >20 years) (n=24)[b]	a vs b
SOD U/ml	217.5±47.4	289.2±141.4	244.4±136.9	n.s.
SOD U/gHb	1501±308.7	2095.4±1057.8	1882.3±1059.5	n.s.
GPX U/L	7369±3246	6200±3046	6440±2720	n.s.
GPX U/gHb	51.5±23.4	44.8±22.6	49.2±21.1	n.s.
CAT k	29.5±5.83	25.6±5.1*	25.3±7.5*	n.s.
CAT k/gHb	205.4±43.3	185.1±31.6*	192.8±57.6	n.s.

n — number of cases; SOD U/ml — superoxide dismutase expressed in absolute activity; SOD U/gHb — superoxide dismutase expressed in specific activity; GPX U/L — glutathione peroxidase expressed in absolute activity; GPX U/gHb — glutathione peroxidase expressed in specific activity; CAT k — catalase expressed in absolute activity; CAT k/Hb — catalase expressed in specific activity; k — a rate constant of the first order reaction as defined by Aebi; Statistical significances are given for comparisons between controls and alcoholics with different duration of alcohol abuse and between alcoholcs with different alcohol abuse (a vs b); * $p<0.05$.

versus control group, is presented in Table 3. Catalase has shown significant changes in the activity in both groups, compared to the control group. In the group of alcoholics with the duration of alcohol abuse less than 20 years (mean±SD=12.31±3.22) was catalase statistically significantly decreased, compared to the control group ($t=2.55$ df 122, $p<0.05$ for absolute activity and $t=2.09$ df 122, $p<0.05$ for specific activity). In the group of patients with the duration of alcohol abuse for more than 20 years (mean±SD=26.8±4.95) only total activity of catalase showed a significant decrease compared to the control group ($t=2.58$ df 133, $p<0.05$).

There were no significant differences in erythrocyte antioxidative enzyme activities between the two groups of alcoholics with different duration of alcohol abuse (Tab. 3).

In order to find out whether antioxidant enzyme activity could serve as a biological marker for drinking history in alcoholics we evaluated the correlation of the duration of alcohol abuse and daily alcohol intake with the activity of antioxidant enzymes. We found out that only specific activity of catalase had statistically significant positive correlation with the duration of alcohol abuse (mean±SD=6.0±3.4]) ($r=+0.37$ and $p<0.05$) (Fig. 2). The positive correlation between the duration of alcohol intake (years) and catalase activity (absolute and specific activity), was also noticed in the group of alcoholics with the duration of alcohol abuse from 5 to 20 years ($r=+51$, $t=2.68$, $p<0.05$) (Fig. 3), and in the group with the duration of alcohol abuse from 20 to 40 years ($r=+0.58$, $t=3.22$ and $p<0.01$) (Fig. 4).

We have found no significant correlation between daily alcohol intake, Q value (recent Q value), and AOE activities. No correlation was found between the activities of ALT and AST with AOE activities as well as between bilirubin concentrations (total and direct) and AOE activities in alcoholics. The presence of palpable liver was also not significantly correlated with AOE activities. Cigarette consumption statistically positively correlated only with the GPX activity ($r=+0.58$, and $p<0.001$ for absolute activity and $r=+0.23$ and $p<0.01$ for specific activity).

There were no significant differences in the antioxidant enzyme activities between the groups of alcoholics with the presence of palpable and without. We have found no significant correlation between antioxidant enzymes in alcoholics, although there was a significant negative correlation between CAT and SOD in the control group (data not presented).

Discussion

Both in animals and humans, chronic alcohol intake causes many cellular alterations, particularly in the liver. Biochemical studies revealed an increase of reactive oxygen intermediates generation during the alcohol metabolism. The role of free radicals and hydrogen peroxide in the metabolism and toxicity of alcohol is supported by some studies (5, 6). Therefore, many authors have tried to use the enzymes metabolizing these highly reactive chemical compounds as biological markers of alcoholism (11, 12, 13).

Regarding the fact that literature data on variations of blood antioxidant enzymes in alcoholics are contradictory, we evaluated erythrocyte activity of superoxide dismutase, glutathione peroxidase and catalase in male alcoholics without clinical evidence of severe liver disease.

We have found a statistically significant increase of superoxide dismutase (absolute and specific activity) in alcoholics, compared to the control groups. We did not find significant differences in erythrocyte SOD activity between the groups of alcoholics with and without palpable liver. Del Villano et al have found an increased E-SOD activity in black alcoholics, statistically significant compared to the activity of E-SOD in control black subjects, that seemed not to be related to the liver damage, what is consistent with our findings (7). An increase of the E-SOD activity was reported also by Emerit et al in restricted population of 12 alcoholics (7 male and 5 female) aged between 28 and 75 years with severe liver injury and in alcoholics with liver disease presumably linked with the liver injury reported by Ledig

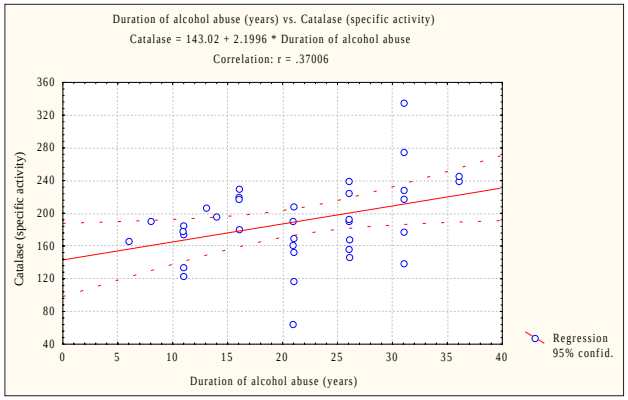
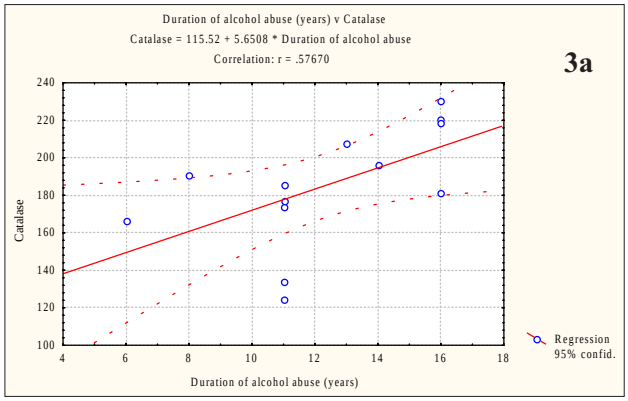


Fig. 2. Linear regression and correlation of catalase (CAT) specific activity with duration of alcohol abuse (years).



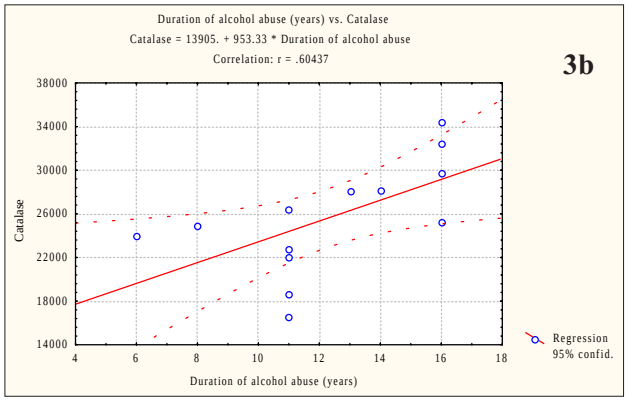
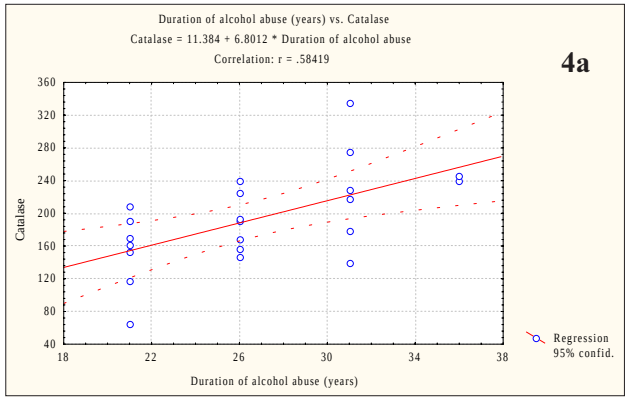


Fig. 3. Linear regression and correlation of catalase (CAT) activity with the duration of alcohol abuse less than 20 years (a — absolute activity, b — specific activity)



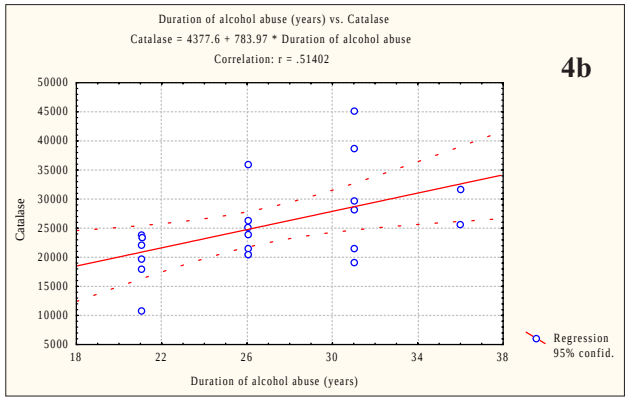


Fig. 4. Linear regression and correlation of catalase (CAT) activity with the duration of alcohol abuse more than 20 years (a — absolute activity, b — specific activity).

et al (18, 19). Conversely to our, Del Villano et al (7) and Emerit et al (8) findings, Guemouri et al have found a statistically significant 6.8 % decrease of E-SOD activity in alcoholics before withdrawal, compared to control group (12). A decrease of plasma SOD activity was reported by Kubota et al in the case of alcoholic liver disease (20). On the other hand, Bjorneboe et al and Akkus et al have found there was no significant difference of erythrocyte SOD between alcohol consumers and healthy non-drinkers, suggesting still sufficient availability of trace elements (zinc and copper) for the enzyme synthesis (8, 21).

The increased levels of Cu, Zn-SOD may result either from the presence of a target compound which may be sensitive to a metal ions (Cu^{2+}, Zn^{2+}) contained in SOD or probably from alcohol induced modifications of the cell membrane. Our results suggest that the high E-SOD activity may protect the cell membranes by scavenging the $O^{2\cdot-}$ radicals, increased after chronic alcohol consumption and causing liver injuries.

We have found no correlation between SOD activity and the duration of alcohol abuse (years) or daily drinking quantity (Q value). Contradictive to our finding is a statistically significant negative correlation of SOD and daily drinking quantity reported by Zhou and Chen (22).

We found a mild, but not statistically significant decrease of the GPX activity in alcoholics, compared to the control group. Our results are consistent with those reported by Akkus et al and earlier by Tanner et al and Bjorneboe et al (21, 23, 8). On the other hand, a statistically significant 13 % decrease of E-GPX activity was reported by Guemouri, Zima, and Zhou and Chen (12, 24, 22). Girre et al (9) observed a decrease of plasma GPX activity in alcoholics that is in contrast with the results of Guemouri et al (10) who have found an increased plasma GPX activity in alcoholics, compared to the control group. A statisti-

cally significant decrease of blood GPX activity in rats fed with ethanol versus control rats was reported by Ozaras et al (25).

Pirson's correlation coefficient showed that only cigarette consumption had a positive correlation with E-GPX activity, that coincides with the results reported by Luty-Frackiewicz A et al, who have found low blood selenium concentration in alcoholics, positively associated with the activity of GPX in male smokers (26). GPX activity in our study of alcoholics suggests that the availability of selenium, that is required for synthesis of the enzyme, is still sufficient. There were no statistically significant correlations between the duration of alcohol abuse (years) and GPX activity, as well as between daily drinking quantity and GPX activity, in contrast to the negative, statistically significant correlation between daily drinking quantity and GPX activity reported by Zhou and Chen (22).

We have found a statistically significant decrease of catalase activity in alcoholics compared to the control group. There was a significant positive correlation between catalase activity and alcohol abuse duration (years). Our results corresponde to the results reported by Zhou et al who have found that catalase activity was decreasing with the increasing time and quantity of alcohol abuse (27). Later, the same authors (Zhou and Chen, 2001) reported catalase activities were significantly decreased in alcoholics (22). Presented linear regression correlation in their study confirmed their previous finding-negative correlation between catalase activity and daily drinking quantity, what is different from our results. Our results are consistent with the results of Tarasova who has found a decreased catalase activity in alcoholics which was in a reverse correlation with lipid peroxidation activation (28). Previously, no variation of catalase activity in alcoholics was reported previously by Tanner et al, and Bjorneboe et al (23, 8). Guemouri et al (1991, 1993) found no statistically significant effect of alcohol consumption on catalase activity (10, 12). In contrast to our results and to results of Taner et al (23), and Bjorneboe et al (8) is a significant increase of catalase activity reported by Negru et al and Temel et al (29, 30). The study of Temel at al indicated that an increased erythrocyte catalase activity was a possible protective mechanism against alcohol-inducts oxidative stress (30). A strong relationship between the alcohol drinking and the catalase activity was also presented in the study of Temel et al what was in accordance with our results.

In contrast to our finding and to the results of Temel et al, is nor significant association between alcohol consumption and any of the measured blood enzymes reported by Mulholland et al (31).

We have found no significant association between Q values (daily alcohol consumption) and catalase activity, what was not consistent with the positive correlation between alcohol intake and catalase activity observed in both FH- (family history of alcoholism negative) and FH+ (family history of alcoholism positive) individuals (13, 11). On the contrary, Zhou and Chen have found a statistically significant negative correlation between catalase activity and daily drinking quantity (g) (22).

In conclusion, the activity of antioxidant enzymes observed in patients with alcohol dependence syndrome has a limited amplitude and does not allow the use either of them as markers of alcohol abuse. The only antioxidant enzyme that has shown significant changes in alcohol dependence syndrome was catalase and it might serve as biological indicator of duration of alcohol abuse (years). To support this hypothesis further studies on catalase activity are necessary including subjects with or without family history of alcoholism.

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Received December 10, 2004.
Accepted February 20, 2005.