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BIOLOGICAL MARKERS OF OXIDATIVE STRESS INDUCED BY ETHANOL AND IRON OVERLOAD IN RAT

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Abstract. Studies on rats treated for 15 months with ethanol (10%, w/v, solution in drinking water) revealed that the stimulation of hepatic cytochrome P-450 monooxygenases activity was accompanied by enhanced microsomal malondialdehyde formation, a lipid peroxidation index and a decreased level of the antioxidant, α-tocopherol. The other components of the prooxidant/antioxidant system, diene conjugates and catalase, glutathione peroxidase and superoxide dismutase activities were unaffected. Oxidative stress in blood was shown by a significant decrease in the α-tocopherol level whereas lipid peroxidation and antioxidant enzyme activity remained unchaged. The prooxidative effect of ethanol was catalytically promoted by an iron overload (Fe-saccharate, 100 mg Fe^{3+}/kg body wt. intraperitoneally, 2, 5 and 7 day before test) to simulate the effect of alcoholic hemochromatosis. Thus, the level of malondialdehyde and α-tocopherol in the serum may be recommended as biological markers of ethanol-provoked oxidative stress, which is especially useful in the evaluation of the combined effect of ethanol and other chemicals that affect the redistribution of active iron complexes.

INTRODUCTION

The role of lipid peroxidation of biomembranes in several pathological effects and in the course of biotransformation of xenobiotics has been commonly acknowledged. Although various cellular defensive mechanisms act as effective antioxidants, a disturbance in the prooxidant/antioxidant systems in favour of the former, determined as "oxidative stress" may contribute to oxidative injury as a common factor in xenobiotics intoxication (21).

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The concept of an increased lipid peroxidation rate in hepatocytes provoked by ethanol was put forward by Di Luzio (7) as a possible mechanism in the development of a fatty liver and this has been confirmed in many reports (9, 11, 14). It has been reported that heavy consumption of ethanol led to a lowering of the level of α-tocopherol (5) and an increase level of malondialdehyde (22) in the serum, as well as to a decrease of glutathione and an increase of diene conjugates content in the liver biopsies (19) in a human alcoholic. Some controversial findings favoured the adaptive mechanism of scavenging free radicals by antioxidative enzmyes (15).

It is commonly accepted that prolonged ethanol ingestion induces microsomal cytochrome P-450 monooxygenase (referred to as the microsomal ethanol oxidizing system, MEOS) and thereby, ethanol may be metabolized in the system at a higher rate (11). Ethanol oxidation by MEOS leads to increased production of hydroxyl radicals and other reactive oxygen species, promoting peroxidation of the membranous lipids (14). Iron ions play a major role in initiation and propagation of the reaction of enzymatic lipid peroxidation (3). In the course of the initiating reactions, they contribute to the conversion of primary reactive oxygen species into hydroxyl or hydroxyl-like radicals, while in the course of propagation reactions, they catalyse a decomposition of hydroperoxides into reinitiating radical species. Recently it has been found that ionized iron is involved in xenobiotic-induced lipid peroxidation (28).

It is known that excessive iron is progressively deposited in the parenchymal cells of the liver in chronic disease hemochromatosis and the resulting liver damage has been partly attributed to membrane lipid peroxidation. In addition to its primary genetic form, secondary hemochromatosis may result from an excess of iron deposits which accumulate as a complication of alcoholism. Iron overload in alcoholics may enhance the susceptibility of the liver to other toxic agents (29).

The enhancement of the proxoidative effect of ethanol was previously observed in rats in cases of combined exposure to ethanol and to the industrial solvents, carbon disulphide (26) and xylene (24) and also in human volunteers exposed jointly to ethanol and xylene or toluene at the concentration nearby the industrial threshold limit value (TLV) (25). Ethanol/xylene-provoked stimulation of malondialdehyde formation in the hepatic microsomes in rats and in the serum in huamn volunteers was related to the increased activity of hepatic monooxygenases and the metabolic clearance of antipyrine, respectively (24, 25).

The study undertaken was an attempt to assess the extent of the prooxidative effect of chronic ethanol ingestion and iron overload against a background of antioxidative potential in the liver and blood in animal experiments in order to find biological markers of oxidative stress.

The study showed that oxidative stress provoked by ethanol is manifested by an increased level of lipid peroxidation final product, malondialdehyde and a decreased level of the antioxidant, α-tocopherol, both in the liver and the blood.

MATERIALS AND METHODS

The experiments were carried out on male Wistar rats (outbred stock Imp: DAK) with an initial body weight of 230–250 q fed on diet Murigran laboratory pellets (Fodder Factory, Motycz, Poland) and given either tap water or 10% (w/v) ethanol (Rectified Spirit, POLMOS, Poland) ad lib. as the only drink for 3–15

months. A model of iron overload (28) resembling secondary hemochromatosis in alcoholics (29) was employed in order to study its prooxidative action: control and ethanol-exposed rats received three injections of Fe-saccharate as a stabilized colloid (Ferrum Lek. LEK, Ljubjana, Yugoslavia) at a dose 100 mg Fe^{3+} per kg of body weight each, 2, 5 and 7 days before test.

Rats, fasted overnight, were sacrificed by decapitation and the blood and livers were immediately processed. Liver microsomes and cytoplasm were obtained from the postmitochondrial supernatant by ultracentrifugation at 105000 g at 4°C for 75 min and the protein concentration was determined (12).

Heptic microsomal monooxygenases activity (EC, 1, 14, 14, 1) was assessed based on the determination of 7-ethoxycoumarin o-deethylase (2) and 7-ethoxyresorufin o-deethylase (18) activity and the level of cytochome P-450 (17). The prooxidant effects were evaluated on the basis of the determination of lipid peroxidation products, diene conjugates (8) and malondialdehyde in microsomes (13) and serum (27).

The antioxidant potential was assayed in the hepatic microsomes, cytoplasm or homogenate and in the blood on a basis of the determination of α-tocopherol level (23) and the activity of supperoxide dismutase (SOD, EC 1.15.1.1) (4), glutathione peroxidase (EC 1.11.1.9) (10) and catalase (EC 1.11.1.6) (1), all measured at 25°C with kinetic registration.

Homoglobin was determined with a standard diagnostic reagent kit. All biochemical assays were done within 48 h of necropsy.

The statistical analysis of the results was performed using Student's t-test.

RESULTS

Metabolic effect of ethanol

The model of long term ethanol ingestion at 10% solution in drinking water assured a steady intake of ethanol, on average 0,7 q $\approx$ 5.0 kcal/100 g of body weight daily as the caloric value of ethanol amounts to 7.1 kcal/g, yielding its mean blood level of 8.2 mml/l (former denotation 38 mg/100 ml) without any symptoms of nutritional anomaly (weight gain 0.8 g/day, as in the control). On this regimen the daily consumption of ethanol by rats is equivalent to 1.2 l of 40% vodka per day for a human. Since the rate of ethanol oxidation in the rat (0.3 g per kg per h) is three times faster than in humans (0.1 g per kg per h) the level of exposure of the rat to ethanol may be tantamount to alcohol abuse in man.

The stimulatory effect of ethanol on the metabolic capacity of the liver as evaluated on the basis of the activity of the microsomal monooxygenase system was confirmed only in rats exposed for 15 months: the level of cytochrome P-450 increased by 36% and the activity of 7-ethoxycoumarin deethylase by 70%, whereas the minute activity of 7-ethoxyresorufin deethylase remained unchanged. Iron-overload did not essentially affect the activity of cytochrome P-450 monooxygenases both in the control and ethanol-treated rats (Table 1).

Table 1. Effect of prolonged ethanol ingestion and/or short-term iron-overload on the activity of the microsomal monooxygenase system in the liver of rats

Groups		Microsomal	Microsomal monooxygenases		
Exposure	Treatment	protein mg/g liver	Cytochrome P-450 nmol/mg protein	7-ethoxycoumarin o-deethylase	7-ethoxyresorufin o-deethylase
				pmol/min/mg protein	
0 (Control)	0	22.30 ± 0.80	0.75 ± 0.03	not determined	not determined
Ethanol, 3 months	0	24.80 ± 0.71	0.72 ± 0.05	not determined	not determined
0	0	21.20 ± 0.61	0.69 ± 0.06	840 ± 101	71 ± 9
Ethanol, 15 months	0	22.48 ± 0.49	0.94 ± 0.03[b]	1420 ± 113[b]	77 ± 14
0	Fe^{3+}	21.62 ± 0.65	0.67 ± 0.04	668 ± 95	60 ± 14
Ethanol, 15 months	Fe^{3+}	23.04 ± 0.90	0.80 ± 0.03[a]	998 ± 100[a]	30 ± 12

Regimen of exposure: Ethanol, 10% water solution, as the only drink for 3 to 15 months.
Fe^{3+} as ferric-saccharate complex, three intraperitoneal injections at dose 100 mg Fe per kg, 2, 5 and 7 days before the test.
[a,b] significantly different from the concurrent control group at p 0.05 and 0.01, respectively.
Results are the mean ± SE for 7 rats.

Prooxidative effect of ethanol in the liver

Lipid peroxidation in the microsomes was measured at two stages of the process: diene conjugates represented the intermediate and malondialdehyde final products.

In the rats treated with ethanol for 15 months the level of diene conjugates in the microsomes was unaffected but the formation of malondialdehyde increased by 27%. Short-term iron overload at the end of the 15-month ethanol drinking period led to a further, 60% increase of malondialdehyde formation (Table 2).

Table 2. Effect of prolonged ethanol ingestion and/or short-term iron-overload on the microsomal lipid peroxidation in the liver of rats

Groups		Lipid peroxidation products	
Exposure	Treatment	Diene conjugates A_{240}	Malondialdehyde nmol/mg protein
0 (Control)	0	0.141 ± 0.036	7.00 ± 0.28
Ethanol, 3 months	0	0.160 ± 0.040	7.98 ± 0.46
0	0	0.278 ± 0.070	6.38 ± 0.49
Ethanol, 15 months	0	0.218 ± 0.031	0.13 ± 8.61[a]
0	Fe^{3+}	0.261 ± 0.022	7.41 ± 0.70
Ethanol, 15 months	Fe^{3+}	0.236 ± 0.033	11.90 ± 1.07[b]

Malondialdehyde was estimated in fresh microsomes on a basis of the reaction with thiobarbituric acid. Diene conjugates were estimated on a basis of the ultraviolet absorption of chloroform-methanol (2:1) extract of lipids of the microsomal fraction equivalent to 0.1 g of liver per ml of extract.
Other details as in Table 1.

Table 3. Effect of prolonged ethanol ingestion and short-term iron-overload on the antioxidant potential of the liver of rats

Groups		Enzymatic antioxidants			Nonenzymatic antioxida	
Exposure	Treatment	Catalase k*/mg protein	GSH**-Peroxidase μmol NADPH/min/mg protein substrate: hydrogen peroxide	substrate: cumene hydroperoxide	SOD*** units/mg protein	α-tocopherol μg/mg protein
			Mocrosomes			
O (Control)	0	0.105 ± 0.009	0.031 ± 0.001	0.044 ± 0.002	3.93 ± 0.35	not determined
Ethanol, 3 months	0	0.125 ± 0.010	0.028 ± 0.002	0.038 ± 0.03	3.71 ± 0.24	not determined
0	0	0.148 ± 0.013	0.036 ± 0.003	0.052 ± 0.006	3.48 ± 0.45	0.205 ± 0.037
Ethanol, 15 months	0	0.139 ± 0.013	0.037 ± 0.002	0.044 ± 0.008	4.02 ± 0.16	0.119 ± 0.010[a]
0	Fe^{3+}	0.120 ± 0.021	0.029 ± 0.003	0.051 ± 0.004	3.19 ± 0.21	0.146 ± 0.010
Ethanol, 15 months	Fe^{3+}	0.115 ± 0.018	0.028 ± 0.001	0.039 ± 0.004	3.62 ± 0.30	0.113 ± 0.009[a]
			Cytoplasm			Homogenate
0 (Control)	0	0.249 ± 0.015	0.201 ± 0.013	0.322 ± 0.009	636 ± 47	not determined
Ethanol, 3 months	0	0.270 ± 0.012	0.226 ± 0.009	0.355 ± 0.015	708 ± 42	not determined
0	0	0.298 ± 0.019	0.309 ± 0.045	0.491 ± 0.032	742 ± 52	0.148 ± 0.010
Ethanol, 15 months	0	0.343 ± 0.020	0.301 ± 0.038	0.442 ± 0.018	759 ± 65	0.114 ± 0.010[a]
0	Fe^{3+}	0.305 ± 0.019	0.230 ± 0.020	0.336 ± 0.025	638 ± 54	0.141 ± 0.009
Ethanol, 15 months	Fe^{3+}	0.323 ± 0.031	0.249 ± 0.025	0.404 ± 0.028	509 ± 59	0.109 ± 0.010[a]

* rate constant of a first order reaction.
** glutathione.
*** superoxide dismutase.
Other details as in Table 1.

Enhanced lipid peroxidation led to an increased consumption of endogenous antioxidant, α-tocopherol, resulting in its significant decrease in the microsomes and in the whole homogenate, on average by 30%, although antioxidative enzymes, catalase, glutathione peroxidase and superoxide dismutase, remained unaffected. In such cases iron overload did not continue to affect the antioxidant potential of the liver, modified by ethanol drinking i.e. the α-tocopherol level, which remained at the level depressed by prior ethanol treatment (Table 3).

Prooxidative effect of ethanol in blood

The results of the analysis of blood, accepted as noninvasive assay with perspectives of clinical application, revealed a decrease in the level of α-tocopherol by 20% in rats treated with ethanol for 15 months. This was the only sign of the prooxidative effect of ethanol in the blood as the other components of the antioxidant system – catalase, glutathione peroxidase and superoxide dismutase – were unchanged. The level of malondialdehyde in the blood was also unchanged. Iron-overload promoted the prooxidative effect of ethanol in blood with both a significant increase of malondialdehyde and a further decrease in α-tocopherol levels (Table 4).

DISCUSSION

The data obtained shows that prolonged ethanol ingestion by rats results in a stimulation of the activity of the microsomal monooxygenase system in the liver (Table 1), accompanied by enhanced lipid peroxidation and α-tocopherol consumption both in the liver and blood (Tables 2–4). The inductive potential of monooxygenases under the influence of ethanol is related to the types of cytochrome P-450 involved (16): 7-ethoxycoumarin o-deethylase depends on the mixed types of cytochrome P-450 inducible by 3-methylcholanthrene, phenobarbital and ethanol (subfamilies IA, IIB and IIE1, respectively), whereas 7-ethoxyresorufin o-deethylase depends on cytochrome P-448 inducible typically by 3-methylcholanthrene (subfamily IA).

The induction of cytochrome P-450 IIE1 and the activity of 7-ethoxycoumarin o-deethylase by ethanol may contribute to the prooxidative effects i.e, an increase in the generation of the superoxide an hydroxyl anions in NADPH/NADH-dependent microsomal electron transfer systems related to the xenobiotics biotransformation processes (6, 9). Free radicals are known to promote lipid peroxidation and iron ions catalytically potentiate the process (20, 28). Chronic alcohol abuse, as in human alcoholism corroborates the assumption, since ethanol: 1) stimulates the hepatic monooxygenase system, a source of free radical formation, and 2) predisposes to excessive iron accumulation resulting in a secondary type of hemachromatosis (11, 20, 29).

The comparative assessment of the markers of prooxidative and antioxidative inbalances due to oxidative stress under the influence of long-term alcoholization of rats has not yet been reported.

The evidence presented here on the increased level of malondialdehyde, a final product of lipid peroxidation in the hepatic microsomes, and the depression of the level of α-tocopherol, a nonenzymatic antioxidant in the liver and blood, despite

Table 4. Evaluation of lipid peroxidation and antioxidant potential in the blood as a marker of oxidative stress provoked by prolonged ethanol ingestion and/or short-term iron-overload in rats

Groups		Lipid peroxidation in serum	Antioxidants in blood				Hemoglobin
Exposure	Treatment	Malondialdehyde nmol/ml	Catalase k/ml	GSH-peroxidase μmol NADPH/min/ml substrate: hydrogenperoxide	SOD J/ml	α-tocopherol μg/ml	g/l
0	0	3.28 ± 0.26	7.93 ± 0.33	17.60 ± 1.25	12375 ± 538	not determined	136 ± 5.4
Ethanol, 3 months	0	3.17 ± 0.21	6.93 ± 0.51	20.20 ± 1.65	12885 ± 411	not determined	132 ± 6.1
0	0	3.32 ± 0.20	5.48 ± 0.79	18.60 ± 1.17	10237 ± 306	6.18 ± 0.22	137 ± 8.5
Ethanol, 15 months	0	3.39 ± 0.27	6.46 ± 0.79	19.29 ± 0.44	9996 ± 420	5.20 ± 0.28[a]	136 ± 7.3
0	Fe^{3+}	2.83 ± 0.20	6.14 ± 0.50	19.35 ± 1.11	12185 ± 580	5.85 ± 0.35	154 ± 8.8
Ethanol, 15 months	Fe^{3+}	3.50 ± 0.17[a]	7.02 ± 0.33	20.52 ± 1.15	10625 ± 300	3.60 ± 0.21[c]	163 ± 6.9

The levels of diene conjugates were at the limit of detection: $A_{240}^{\%} \sim 0.050$ for lipid extract from 0.5 ml of the blood.
Details as in Table 1–8

normal catalase, glutathione peroxidase and superoxide dismutase activities, supported the concept that ethanol does provoke oxidative stress under conditions that resemble alcohol abuse in humans. Moreover, the short-term iron overload of rats to simulate the effect of alcoholic hemachromatosis potentiates the prooxidative effect of ethanol, which leads to a further malondialdehyde increase and a decrease of α-tocopherol levels in the liver and blood (Tables 2–4). In the case of exposure to some hepatotoxic industrial organic solvents, accompanied by ethanol ingestion, an enhanced rate of lipid peroxidation has been noted (24, 25, 26). It seems most likely that such agents, either by the induction of cytochrome P-450 and the increased generation of the free radicals related to biotransformation or activation of iron deposits may initiate supplementary lipid peroxidation.

The experiments performed indicate the need for monitoring the biological effect of ethanol abuse, especially if it is an extra-factor to occupational exposure to solvents. Monitoring should be based on the determination of the two markers of oxidative stress in blood, i.e. the lipid peroxidation final product, malondialdehyde and the antioxidant, α-tocopherol.

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