

УДК 576.32/.36:(547.262 : 616.36-002-099)

BIOCHEMICAL MARKERS OF PREDISPOSITION TO ETHANOL-INDUCED LIVER DAMAGE*Mikhail I. Bushma, Sergey M. Zimatkin*, Yuri G. Ambruskevich, Tatiana V. Bushma*

Grodno State Medical University, Belarus;

Institute of Biochemistry, National Academy of Sciences of Belarus

A new approach to identification of the factors, responsible for predisposition to ethanol-induced liver damage, was developed. It is based on the examination of the liver in the same animals both before (biochemistry) and after (histology, blood markers of liver damage) chronic alcohol administration and comparison the biochemical and histological sets of data, using the methods of multiple stepwise regression, correlation, canonical analyses and ANOVA. The experiments were carried out in 118 male Wistar rats (250-300 g). In 94 animals the central and left lateral hepatic lobes (~65-70% of the liver weight) were removed under anaesthesia with a ligation of the lobar bases. Two months later, after liver recovery (confirmed by biochemistry and histology), they were administered ethanol (30% water solution, 5 g/kg, intragastrically, once a day, for 57 days). Control animals after the same hepatectomy operation received the same volumes of water instead of ethanol. It was found that animals with initially higher activity of alcohol dehydrogenase and lipid peroxidation system and lower contents of reduced glutathione, retinols and cytochrome b₅, lower activity of UDP-glucuronyl-, glutathione-S-transferases and rate of NADH oxidation in the liver had higher ethanol-induced liver damage indicated by histology and increased alanine and aspartate aminotransferase activities in blood. Those rats were also more sensitive to the hypnotic effect of ethanol. The proposed animal model and approach can be used in the finding of other biomarkers of the sensitivity to the hepatotoxic effect of ethanol (predisposition to the alcohol-induced liver damage) as well as biomarkers of the sensitivity to other hepatotoxins.

Keywords: animal models, ethanol, hepatotoxicity, liver damage, predisposition, biomarkers.

Introduction

Ethanol can be considered as a hepatotoxin and alcohol liver disease (ALD) is a common consequence of chronic alcohol consumption (Ishak *et al.*, 1991; Lieber and DeCarli, 1991; Lieber, 1994). ALD includes hepatic steatosis, hepatitis, perivenular fibrosis and cirrhosis. The high variations in individual sensitivity to alcohol-induced liver damage and ALD development are well known. Only about 20% of alcoholics develop serious liver disease, but others heavy drinking for decades do not (Mezey, 1982). After the same dose of ethanol the degree of individual morphological changes in the liver is significantly different (Zimatkin *et al.*, 1997; Sherlock and Doolie, 1999). Despite of many investigations, the biochemical mechanisms responsible for the predisposition of human or animal liver to ethanol-induced injuries remains largely unclear. The elucidation of those mechanisms may be critically important for understanding of ALD early pathogenesis and development of therapeutic measures (Lindros, 1995). One group of the biochemical mechanisms which can be responsible for the ethanol-induced liver injury are free radical mechanisms. The generation of the excess amount of free radicals takes place through ethanol and acetaldehyde metabolism (Albano *et al.*, 1996). The increased generation of oxygen- and ethanol-derived free radicals occurs through the microsomal CYP450

IIЕ1, cytosol xanthine and/or aldehyde oxidases and the mitochondrial respiratory chain. The resulting oxidative stress can lead to enhanced lipid peroxidation and affects the important cellular components, such as proteins or DNA. Lipoperoxidation is the crucial mechanism in the pathogenesis of ethanol toxicity in the liver (Kawase *et al.*, 1989; Nordmann *et al.*, 1992). It may be suggested that the risk of development of alcoholic liver injury is to a considerable extent genetically predisposed and depends on the biochemical individuality of animals. The inborn features of the liver metabolism may be a decisive factor of its vulnerability to ethanol action.

The aim of the present study was the elucidation of the biochemical factors responsible for the individual sensitivity of liver to the toxic effect of ethanol (predisposition to the ethanol-induced liver damage). The previous studies did not provide a direct and clear answer to the question, since they investigated mostly the consequences of alcohol intoxication. In the present paper we propose a new rat model for the examination of the biochemical markers of the predisposition to ethanol-induced damage of liver. It is based on the examination of the liver in the same animals both before (biochemistry) and after (histology, blood markers of liver damage) chronic alcohol administration and comparison the biochemical and histological sets of data.

Materials and methods

Animals

118 male Wistar rats (250-300 g) were used. They were maintained in the animal facilities with 12 hr light/12 hr dark cycle and 20-24°C room temperature, in individual plastic cages; with a constant access to standard laboratory food and tap water. The consumption of food and water was under the control. The appropriate approval from the University Committee for experimental animals had been obtained.

Experimental design

In 94 animals the central and left lateral hepatic lobes (~65-70% of the liver weight) were removed under the ether anaesthesia with a ligation of the lobar bases (Higgins and Anderson, 1931). Then the removed liver samples were examined biochemically. Two months later, after the liver recovery (as confirmed by the liver biochemistry and histology), those rats were administered ethanol (30% water solution, 5 g/kg, intragastrically, once a day, for 57 days). After that the animals were decapitated and their livers were studied histologically. In addition, the trunk blood samples were taken for the biochemical examination of the blood plasma markers of the liver damage. Animals of the control group (16 rats) were treated similarly, but received the same volumes of water instead of ethanol. In the additional 8 control rats we confirmed the structural and metabolic restoring of the liver after the partial hepatectomy in our experiments. We examined and compared (by the same histological and biochemical methods) the initial (in the removed part of liver) and final (in the regenerated liver 2 months following the operation) state of the liver in the same animals.

Liver sampling

For the subcellular fractions biochemistry the liver samples were perfused by the cold KCl to remove the blood. The subcellular fractions were obtained from homogenates by differential centrifugation (Karuzina and Archakov, 1977) and freshly examined. Other parts of the liver were frozen and stored in liquid nitrogen before examination.

Liver biochemistry

In the hepatic lobes removed two months before the ethanol administration, the activity of enzymes involved in ethanol and acetaldehyde metabolism, the state of lipid peroxidation (LPO) and antioxidant defence systems, the parameters of the xenobiotics biotransformation were examined. In general, 33 biochemical parameters were measured. In liver homogenates the catalase activity (Koroliuk *et al.*, 1988), malondialdehyde (MDA) content and activities of its generation in ascorbate- and NADPH-dependent reactions (Buege and Aust, 1978), the level of the reduced glutathione (GSH) (Sedlak and Lindsay, 1968), carotins (Gatautus *et al.*, 1987), conjugated

diens and ketotrienes ketons were examined spectrophotometrically (Kostiuk *et al.*, 1984); the levels of tocopherols, retinols and coenzyme Q were examined spectrofluorometrically (Taylor, 1976). In mitochondrial hepatic fraction the activities of alcohol dehydrogenase (ADH) (Tottmar *et al.*, 1973), aldehyde dehydrogenase (Litterst *et al.*, 1975) glutathion reductase (Gerasimov *et al.*, 1976) and superoxide dismutase were examined spectrophotometrically (Kostiuk *et al.*, 1990); the glutathione peroxidase activity was examined colorimetrically (Moin, 1986). In liver microsomes the activity of cytochrome P450 2E1 (Kato and Gillette, 1965), NADPH- cytochrome P450 and NADH-cytochrome b₅ reductases (Dallner, 1963), the rate of NADH and NADPH oxidation (Gillette *et al.*, 1957), cytochrome b₅, P450 and P420 contents (Omura *et al.*, 1965) and activities of UDP-glucuronyl (Isselbacher, 1956) and glutathione S-transferases were examined spectrophotometrically (Habig *et al.*, 1974). In postmicrosomal fraction the activity of UDP-glucose dehydrogenase (Strominger *et al.*, 1957), CDNB and BSL-glutathione-S-transferase were examined spectrophotometrically (Habig *et al.*, 1974). The intensity of "rapid bevest" of chemiluminescence was examine by chemiluminescence method (Abakumov *et al.*, 1988). Finally, the individual "biochemical passport of the liver" including all the above parameters values was developed for every experimental animal.

Blood biochemistry

In the whole blood obtained from the retroorbital sinus 1 hour following the first ethanol administration ethanol (Pronko *et al.*, 1987) and acetaldehyde (Pronko *et al.*, 1993) were determined by gas chromatography. The activities of blood plasma enzymes, the markers of the liver damage (Kolb and Kamyshnikov, 1982) were determined spectrophotometrically. Alkaline phosphatase, alanine amino transferase (ALAT) and aspartate amino transferase (AsAT) were determined using the kits from Lachema Bio-test (Brno, Czech Republic) and Reakhim (Russia).

Histology and histochemistry

For histological examination the liver samples were fixed in a 10% neutral formalin and embedded in paraffin. Five-micrometers thick sections were ordinary stained by hematoxylin and eosin. The histological preparations were studied microscopically and degree of some morphological parameters typical for the alcohol liver damage (Wight, 1993) was expressed in points (from 0 – no, up to 4 – very high expression): the extension of the blood capillaries and veins, the expression of the inflammatory infiltration; the degree of hepatocytes vacuolisation as well as the degree of destruction and depth of hepatocytes.

For lipid histochemistry and fat inclusions estimation the liver samples were frozen and stored in liquid nitrogen. Twenty micrometers thick sections

were prepared in a cryostat at 20°C, mounted on a microscope slides, dried at room temperature, fixed for 1 h in cold 4% formaldehyde with 1% CaCl₂, stained with sudan black (1% in 70% ethanol) and, following rinsing in 70% ethanol and distilled water, mounted in glycerol. Slices from all samples were treated under the identical conditions. The degree of parenchymal fatty infiltration was expressed in points (from 0 – no, up to 4 – very high expression).

Sensitivity to hypnotic effect of ethanol

The sensitivity of rats to hypnotic effect of ethanol was estimated by the duration of alcohol-induced sleep (the lost of the righting response) following intragastric alcohol administration during the experiment.

Chemicals

All chemicals were of analytical grade. Sodium pyrophosphate was purchased from Fisher; MgCl₂, NAD, HADH, caproaldehyde (hexanal) and nitro blue tetrazolium from Sigma, pyrazole from Aldrich, agarose powder from Bio-Rad Labs, acetaldehyde from Baker Inc and benzaldehyde from Eastman Kodak Co. Acetaldehyde was distilled over nitrogen before use. All reagents were dissolved in deionized distilled water; hexanal and nitro blue tetrazolium were predissolved in methanol and dimethyl formamide, accordingly.

Statistical analyses and mathematical modelling

The relationships between the individual biochemical indices in rat livers before the ethanol administration and pattern and degree of the subsequent alcohol-induced liver damage (by blood biochemistry and liver histology) were established using the methods of multiple stepwise regression, correlation, canonical analyses and ANOVA (Afifi and Eisen, 1982; Urbakh, 1975).

Results

No significant biochemical or histological differences were found in the regenerated liver of rats two months following the partial hepatectomy, as compared to the initial state of liver in the same animals. It confirms the full structural and metabolic restoring of the liver after the partial hepatectomy and adequacy of the present animal model to the papers of the study.

The significant individual variations (interindividual variability) of the parameters studied were found in rats before (liver biochemistry) and after (blood plasma biochemistry and liver histology) chronic alcohol administration. In particular, the blood acetaldehyde level (0.3-7.8 μM/L), coenzyme Q (0.03-14.0 mg/g), “rapid bevest” of chemiluminescence (10-87 imp/sec), UDP-glucose dehydrogenase (0.54-13.2 nanomoles/min/mg pr.) demonstrated the highest variations.

Chronic ethanol administration induced a significant liver damage, including the microscopic features of hepatic steatosis and hepatitis. The

individual histological and histochemical pictures of liver after chronic alcohol administration varied significantly. All individual histological parameters of alcohol-induced liver damage varied from 0 to 4 points.

A significant correlation between some individual metabolic parameters before chronic ethanol administration and severity of the liver damage after it was found (Table). In particular, the increased MDA level, low content of cytochrome b₅ and UDP-glucuronyl transferase activity correlated positively with the high inflammatory infiltration of the hepatic parenchyma. The low initial hepatic contents of retinols and cytochrome b₅, as well as slow NADH oxidation, correlated with high degree of hepatocytes vacuolisation. The later animals were also more sensitive to hypnotic effect of ethanol (Table).

The initially high ADH activity, as well as high MDA content and activated systems of MDA generation in ascorbate- and NADH-dependent reactions, promoted hepatocyte destruction and death after alcohol administration. Initial hepatic GSH content, rate of NADH oxidation and microsomal glutathione S-transferase activity correlated negatively with fatty infiltration of the hepatic parenchyma and were more sensitive to hypnotic effect of ethanol (Table).

The low initial rate of NADH oxidation and activity of cytosolic glutathione S-transferase correlates with higher blood ALAT activity after ethanol treatment. Low initial hepatic GSH level, low rate of NADH oxidation and glutathione S-transferase activity correlates with the increased blood AsAT activity after alcohol administration. These later animals were also more sensitive to hypnotic effect of ethanol (Table).

A multiple stepwise regression analysis indicate that the relationship between the MDA (x) and cytochrome b₅ content (y) and degree of inflammatory infiltration (z), may be described by the linear equation of multiple regression: $z = 3.666*x - 0.959*y$. The

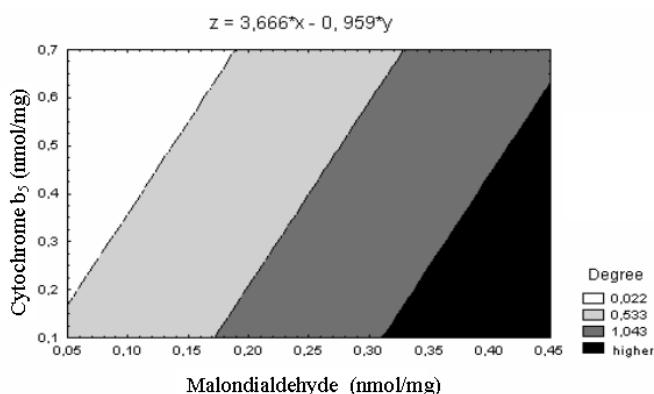


Figure 1. Relationship between the degree of hepatic inflammatory infiltration (z; after chronic alcohol administration) and the initial cytochrome b₅ (y) and malondialdehyde contents in the rat liver (x; before alcohol administration).

Note: The degree of ethanol-induced liver damage is shown in Figures 1-4 by color intensity and values rightwards of the figure.

Table. Correlations between the parameters of the initial liver biochemistry and final ethanol-induced liver damage.

Initial biochemical parameters	Parameters of the final ethanol-induced liver injury					
	Liver				Plasma	
	Inflammatory infiltration	Hepatocyte vacuolation	Hepatocyte destruction and death	Parenchymal fatty infiltration	ALAT activity	AsAT activity
Ethanol metabolic system						
1. Alcohol dehydrogenase (ADH)	+0,24	-0,07	+0,43	-0,11	-0,12	-0,22
2. Cytochrome P450 2E1	-0,08	-0,16	-0,03	+0,32	-0,20	-0,12
3. Catalase	-0,05	-0,24	-0,03	-0,13	+0,02	-0,16
4. Aldehyde dehydrogenase	+0,24	+0,31	-0,04	+0,48	+0,12	+0,32
5. Ethanol (blood)	-0,27	-0,06	-0,19	-0,09	-0,34	-0,24
6. Acetaldehyde (blood)	-0,15	-0,21	-0,26	-0,35	-0,17	-0,10
Hepatic antioxidant system						
7. Glutathione peroxydase	+0,18	+0,32	+0,02	+0,30	+0,32	+0,31
8. Glutathion reductase	+0,33	+0,31	+0,10	+0,32	+0,38	+0,29
9. Superoxide dismutase	-0,09	+0,18	-0,32	+0,38	-0,14	+0,30
10. Reduced glutathione	-0,27	-0,27	+0,02	-0,71	+0,02	-0,46
11. Retinols	-0,21	-0,70	-0,27	-0,09	+0,22	+0,31
12. Tocopherols	+0,16	+0,08	-0,02	+0,03	-0,22	-0,16
13. Ubiquinone	-0,11	+0,001	-0,39	+0,09	+0,12	+0,30
14. Carotins	+0,16	-0,12	+0,32	+0,23	-0,36	-0,35
15. Antioxidative activity	+0,12	+0,001	-0,001	+0,10	+0,15	+0,10
Lipid peroxidation system						
15. Conjugated diens	-0,30	-0,34	+0,14	-0,28	+0,09	-0,33
16. Ketotrienes ketons	+0,29	+0,21	+0,25	+0,35	-0,23	-0,01
17. Malondialdehyde (MDA)	+0,42	+0,39	+0,52	+0,34	-0,03	-0,08
18. Ascorbate-dependent LPO	+0,001	-0,26	+0,44	-0,11	-0,32	-0,35
19. NADPH-dependent LPO	+0,20	-0,39	+0,41	-0,22	-0,35	-0,38
20. "Rapid bevest" of chemiluminescence	+0,22	-0,11	+0,15	-0,06	-0,32	-0,39
System for xenobiotic metabolism						
22. NADPH oxidation	+0,23	-0,05	+0,21	+0,33	-0,31	-0,17
23. NADH oxidation	+0,05	-0,69	+0,36	-0,51	-0,47	-0,49
24. NADPH-cytochrome P450 reductase	-0,03	+0,08	+0,16	+0,23	+0,28	-0,08
25. NADH- cytochrome b ₅ reductase	-0,39	-0,35	-0,03	-0,25	+0,33	-0,13
26. Cytochrome b ₅	-0,69	-0,44	-0,31	-0,24	+0,36	-0,02
27. Cytochrome P450	-0,08	+0,20	+0,09	-0,07	+0,29	-0,14
28. Cytochrome P420	+0,35	+0,24	-0,06	+0,22	-0,14	+0,35
29. UDP- glucose dehydrogenase	-0,23	+0,27	+0,06	+0,29	-0,25	-0,28
30. UDP- glucuronyl transferase	-0,40	-0,04	-0,26	-0,35	+0,33	-0,01
31. Microsomal CDNB- glutathione-S- transferase	-0,35	-0,39	-0,25	-0,41	-0,26	-0,42
32. Cytosolic CDNB- glutathione-S- transferase	+0,06	-0,33	+0,33	-0,15	-0,48	-0,69
33 Cytosolic BSL- glutathione-S- transferase	-0,29	+0,33	-0,23	+0,24	-0,20	-0,25

NOTES: The most significant correlations are indicated by bold characters.

Abbreviations: LPO - lipid peroxidation; GSH - reduced glutathione; UDP - uridine diphosphate; ALAT - alanine amino transferase; AsAT - aspartate amino transferase; BSL - bromsulfolefin; CDNB - 1-chlor-2,4-dinitrobenzene.

hepatic ethanol-induced inflammatory infiltration was most prominent in rats with high initial hepatic MDA content and low initial cytochrome b₅ level (Fig. 1).

Hepatocyte vacuolation (z) was more marked in

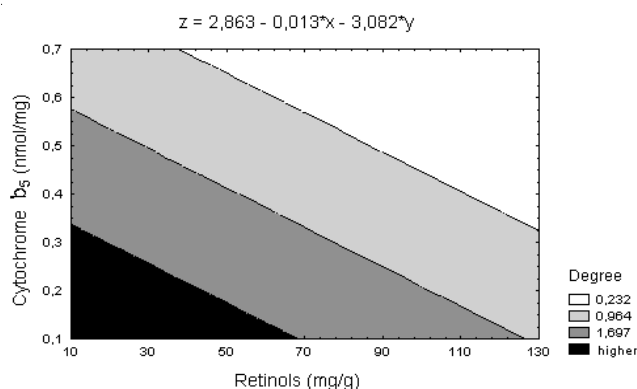


Figure 2. Relationship between the degree of hepatocytes vacuolisation (z; after chronic alcohol administration), cytochrome b₅ (y) and retinols contents in the rat liver (x; before the alcohol administration).

animals with low initial hepatic levels of retinols (x) and cytochrome b₅ (y). This relationship may be described by the linear equation of multiple regression: $z = 2.863 - 0.013*x - 3.082*y$ (Fig. 2).

Hepatocyte destruction and death (z) under the chronic ethanol influence was more expressed in rats with initially activated system of MDA generation through the ascorbate-dependent way (x) and high initial hepatic MDA level (y). A relationship between these parameters may be described by the non-linear equation of multiple regression: $z = 3.071 - 1.519*x - 4.465*y - 1.672*x*x + 19.711*x*y - 29.718*y*y$ (not shown).

Fatty infiltration of hepatic parenchyma (z) was most strongly expressed in rats with low initial rate of NADH oxidation (x) and low initial level of GSH (y). The relationship between these indices may be described by the linear equation of multiple regression: $z = 1.656 - 0.39*x + 0.01*y$ (Fig. 3).

Rats with high plasma ALAT activity after chronic ethanol intoxication (z) had low hepatic rate of NADH oxidation (x) and low baseline activity of cytosolic chlorodinitro-benzene (CDNB) glutathione S-transferase (y). The

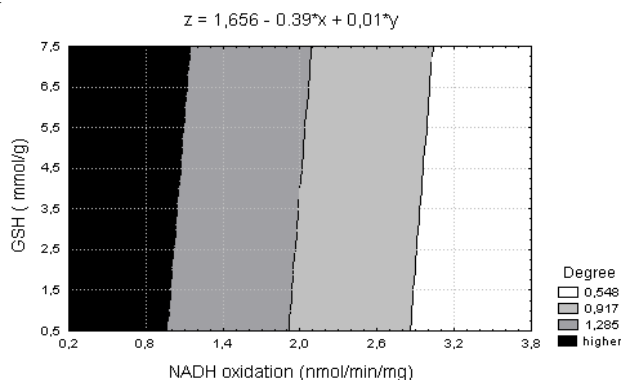


Figure 3. Relationship between the degree of hepatic parenchymal fatty infiltration (z; after the chronic alcohol administration), reduced glutathione content (y) and rate of NADH oxidation in the rat liver (x; before the alcohol administration).

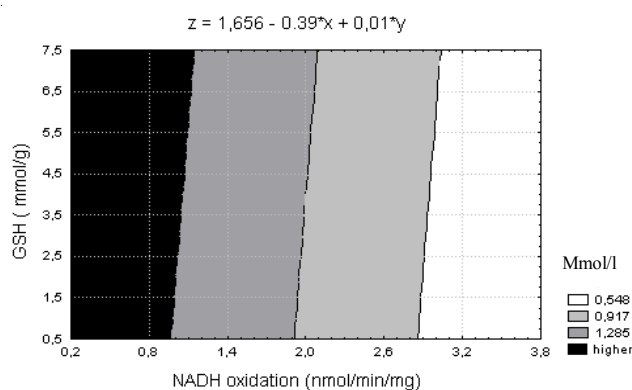


Figure 4. Relationship between the degree of plasma ALAT activity (z; after the chronic alcohol administration), activity of cytosolic glutathione-S-transferase (y) and rate of NADH oxidation in the rat liver (x; before the alcohol administration).

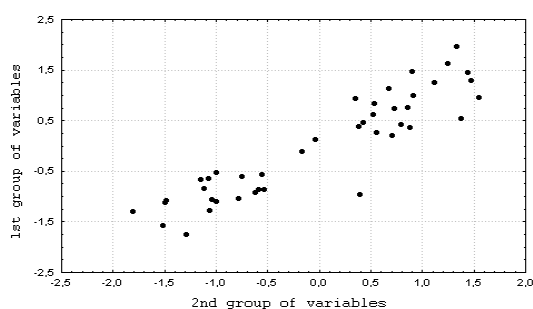


Figure 5. Set of animals studied in coordinates of the 1st and the 2nd groups of variables.

The 1st group of variables is the parameters studied before the chronic alcohol administration (liver biochemistry); the 2nd group is the parameters characterizing the liver damage (liver histology, plasma enzyme biochemistry).

The parameters are presented in the table.

The individual rats are designated by points.

relationship between these parameters may be described by the linear equation of multiple regression: $z = 2.094 - 0.142*x - 0.645*y$ (Fig. 4).

The results of the analysis of variants (ANOVA) indicate the high informativity of the constructed models. The model data had a statistical significance ($P < 0.05$).

The results of the canonical analysis confirmed a strong significant correlation between the parameters studied before the intoxication and parameters of alcohol-induced liver damage (Fig.5). It means that alcohol-induced liver damage was significantly related with the initial biochemical indices shown in the Table. The calculated $r=0.92$ and $p=0.0002$ indicate that the predisposition to alcohol-induced liver damage determined by the morphological (liver histology) and biochemical (blood plasma enzymes) studies was due to the state of the investigated initial biochemical hepatic parameters in 85% of cases (see table). In 15% of cases the damage was induced by the unclear and unstudied in the present experiments casual factors.

Discussion

Our control experiment indicated that the initial and final (following chronic administration) parameters studied in the liver of the same animals did not differ

significantly. It confirms the full structural and metabolic restoring (recovery) of the liver (on the parameters studied) during two months after the partial hepatectomy. It indicates the adequacy of the proposed animal model to the papers of the study.

The results obtained confirm both the high interindividual variability of the initial liver metabolism (biochemical parameters) in naive Wistar rats and the subsequent various expression of ethanol-induced liver damage (as liver histological and plasma biochemical examinations demonstrated). The correlations between the initial biochemical parameters in the liver and degree of the signs of the consequent ethanol-induced liver damage in the same animals were demonstrated.

The close relationship between the high initial hepatic ADH activity and hepatocyte destruction and death in our experiments can be explained by faster oxidation of ethanol to very toxic and reactive metabolite, acetaldehyde. The important role of acetaldehyde in the pathogenesis of ALD and mechanisms of hepatotoxicity is widely accepted (Lauterberg and Bilzer, 1988). Acetaldehyde can bind with proteins, phospholipids, amino acids and thiols. The formation of protein adducts results in autoantibody production, enzyme inactivation and decreased DNA repair. It causes the plasma membrane damages mediated by protein depolymerization, and finally, hepatocyte destruction and death (Lieber, 1994; Sherlock and Doolie, 1999; reviewed by Lieber, 1999).

It is known that chronic alcohol administration significantly induces MEOS in liver smooth endoplasmic reticulum. During the oxidation of ethanol by cytochrome P450 2E1 a great amount of reactive oxygen intermediates (superoxide, hydrogen peroxide) is generated (reviewed by Lieber, 1999) Hence, animals with initially activated lipid peroxidation processes (as indicated by the increased MDA amount and its accelerated generation in ascorbate- and NADPH-dependent reactions) or low antioxidant capacity (lower GSH and retinol contents, low activity of glutathione-S-transferase) probably had a higher initial sensitivity to alcohol-induced oxidative stress and subsequent liver injury.

Oxidation of ethanol and acetaldehyde by the ADH and ALDH in the liver significantly increases the intracellular redox-potential and hepatocytes NADH/NAD⁺ ratio, shown to be responsible for many metabolic effects of ethanol (Lieber, 1998). It may stimulate lipogenesis and decrease in fatty acid oxidation, accumulation of lipids in the liver and plays an important role in the pathogenesis of fatty dystrophy of hepatocytes, enhanced hepatic collagen generation and another structural and functional injuries of the organ (Day and Yeaman, 1994; Simpson, 1996; Sherlock and Doolie, 1999). It explains a more significant ethanol-induced liver fatty infiltration and damage in animals with low rate of NADH oxidation

and shift of NADH/NAD⁺ ratio to NADH. The lower content of cytochrome b₅ - electron acceptor in NADH-dependent microsomal electron-transferring chain (Archakov, 1975) may contribute into the slower rate of NADH oxidation in those animals.

The higher hypnotic effect of ethanol can also indicate the higher behavioural sensitivity of these animals to alcohol. It is interesting that it is associated with higher sensitivity of hepatocytes to ethanol-induced injury.

Thus, the results of our study indicate that the initial individual specificity of the biochemical processes in rat liver play an important role in the sensitivity to hepatotoxic effect of ethanol. A more active alcohol dehydrogenase and lipid peroxidation system, low antioxidant potential, low UDP-glucuronyl transferase and glutathione S-transferase activities, low cytochrome b₅ content and low rate of NADH oxidation predispose to the alcohol-induced liver damage.

The proposed animal model and approach can be used in the finding of new biomarkers of the sensitivity to the hepatotoxic effect of ethanol (predisposition to the alcohol-induced liver damage) as well as biomarkers of the initial sensitivity to other hepatotoxins.

Acknowledgements - Supported by the Foundation for Basic Studies of Belarus (grant 96-335). The authors are grateful to Dr. Vechevskiy Buko and Dr. Kai Lindros for the revision of the manuscripts and valuable comments.

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