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A Comparison of Methanol and Ethanol Effects on the Activity and Distribution of Lysosomal Proteases

E. Skrzydlewska[1], A. Roszkowska[1], J. Moniuszko-Jakoniuk[2]

[1] Department of Analytical Chemistry and [2] Department of Toxicology,
Medical University of Białystok, 15-230 Białystok, ul. Mickiewicza 2, Poland

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Abstract

The activity of lysosomal proteolytic enzymes (cathepsin A, B, C, D and E) in cytosol, in the liver homogenate, and in the blood plasma of rats intoxicated with methanol and ethanol was measured 6, 12 and 24 h; and 2, 5, and 7 days after intoxication. The activity of all proteases was increased in the cytosol from 12h to 5 days of alcohol intoxication, whereas the activity of these enzymes was decreased in the liver homogenate during the same time. Ethanol caused a higher increase in cytosol proteases activity than methanol. The magnitude of the decrease in proteolytic activity in the liver homogenate depends on the amino acid active center of the enzyme and on the kind of alcohol. The greatest decrease was observed for sulfhydryl and hydroxyl proteases and a smaller one for carboxyl proteases. Moreover, methanol caused a greater decrease than ethanol. It was shown that the lysosomal protease activity in the plasma was increased from 12 h to 5 days after alcohol intoxication and ethanol caused fewer changes than methanol. The increase in the liver lipid peroxidation products examined as tiobarbituric acid reactive substances was also observed at the same time. These results indicate that during methanol and ethanol intoxication the cellular and lysosomal membranes are impaired and proteases are translocated into the blood. However, changes in proteases activities and proteases distribution within the hepatocytes may lead to disturbances in the catabolism of cell proteins and to the destruction of liver cells.

Keywords: methanol intoxication, ethanol intoxication, proteases, liver, lipid peroxidation

Introduction

The action of alcohols on human and animal organisms is characterized by a severe derangement of subcellular metabolism and structural alteration of liver cells. In this tissue, methanol and ethanol are oxidized to aldehydes and acids [1, 2]. These processes cause an increase in NADH concentration and owing to it stimulate the conversion of xanthine dehydrogenase into its oxidase form, which is a superoxide anion-generating enzyme [3]. Free radicals generated during the metabolism of alcohols can react with proteins and lipids changing their structure and functions [4, 5]. In addition, released leukotrienes from infiltrated PMN leukocytes of the liver in the presence of excess of aldehydes are formed during lipid peroxidation of membrane. It is known that products of this process are toxic to the liver [6]. Formaldehyde (and to a lesser degree acetaldehyde) react with many endogenous small molecular compounds such as urea, amino acids, and peptides, and macromolecular compounds such as proteins and nucleic acids [7, 8, 9, 10]. A result of free radicals and aldehyde action may be the modification of biologically active proteins, and damage to biological membranes. The translocation of enzymes from organelles of hepatocytes into the intercellular compartments and into the blood stream could confirm this suggestion. Until now, the increase in concentration of AST and ALT hepatic cytosol enzymes has been shown in methanol intoxication [11, 12].

Formic acid formed in methanol intoxication inhibits the activity of cytochrome oxidase which is the reason of

optic nerve degeneration and blindness [13]. It may also cause metabolic acidosis and in a consequence death [2, 13]. In connection with action of its metabolites methanol is recognized as a toxic compound [2, 13]. Because ethanol possesses more affinity to alcohol dehydrogenase and is oxidized first so methanol is removed in nonmetabolized form. Therefore ethanol is used as antidote in methanol intoxication [14]. However ethanol oxidation is accompanied by a generation of metabolites which can effect liver cells, similar to methanol.

The aim of this study has been the estimation of lysosomal proteolytic enzymes, their activity and distribution, and determination of lipid peroxidation products in the liver of rats intoxicated with methanol and ethanol.

Materials and Methods

Chemicals

The synthetic substrates (CBZ-Glu-Tyr, Bz-DL-Arg-pNA, Gly-Phe-pNA) and human plasma albumin were purchased from Sigma Chemical Co. (St. Louis, USA) and bovine hemoglobin was obtained from Difco (Detroit, USA).

Animals

Male Wistar rats (approximately 230 g b.w.) fed on a standard diet (0.55% cysteine and methionine) were used in all experiments. All procedures were in accordance with a guide for care and use of laboratory animals and the protocol was approved by the local Animal Care Committee. The intoxicated rats were divided into 2 groups of 36 animals each. Rats were dosed with a 50% solution of ethanol or methanol in isotonic saline. The alcohols were administered orally through a plastic tube with a syringe. The first group received 6.0 g methanol/kg body weight, the second - 8.6 g ethanol/kg body weight (the quantity of given methanol in moles corresponds with given ethanol in moles). An equivalent volume of saline was given orally to ten control rats. 6, 12 and 24 h and 2, 5 and 7 days after alcohol administration, the blood was taken into the 0.1 M sodium citrate and the animals were killed under ether anaesthesia (ten animals in each group).

Tissue and Plasma Preparation

Livers were removed quickly and placed in iced 0.15 M NaCl solution, perfused with the same solution to remove blood cells, blotted on filter paper, weighed and homogenized (1:9, w/v) in a glass-teflon Potter homogenizer in 0.25 M sucrose without and with 0.2% Triton X-100. The homogenates were centrifuged at 100,000 x g (4°C) for 60 min to settle the organelles and their membranes. The supernatant received from homogenate prepared in sucrose was called cytosol. The supernatant received from homogenate prepared in sucrose but with Triton X-100 was called the liver homogenate.

The blood was centrifuged at 3,000 x g (4°C) and plasma was used to determine protease activity.

Biochemical Analyses

The substrates and pH of activity determination were as follows: cathepsin A - CBZ-Glu-Tyr, pH 5.0 [15], cathepsin B - Bz-DL-Arg-pNA, pH 6.0 [16], cathepsin C - Gly-Phe-pNA, pH 6.0 [17], cathepsin D - urea-denatured hemoglobin, pH 4,0 [18] and cathepsin E - albumin from human blood, pH 2.5 [19].

The extent of lipid peroxidation was assayed with thiobarbituric acid (TBA). The chromogenous condensation product of TBA with malondialdehyde (thiobarbituric acid-reactive substances - TBA-rs) was extracted from the aqueous phase into butanol and then an absorption at 532 nm was monitored [20].

The results were expressed as mean ± SD. Statistical analysis was performed using Student's t test for unpaired data, and values from $p < 0.001$ to $p < 0.05$ were considered significant.

Results

Ethanol as well as methanol intoxication causes a transient increase in lysosomal protease activities: cathepsin A, B, C, D and E in the liver cytosol. This effect is observed from 12h to 5 days of intoxication. Methanol causes the highest increase in activity of cathepsin C and E (about 28% and 34%), and less for cathepsin A, B and D (about 17%, 13% and 22%). Ethanol causes the highest increase in activity of cathepsin C and B (about 45% and 42%) and less for cathepsin E (about 35%). The smallest changes refer to cathepsin A and D (about 12% and 25%). The activity of lysosomal proteases in the liver homogenats was decreased from 12 h to 7 days after intoxication. After methanol intoxication the highest decrease was observed for: cathepsin A - 14%, cathepsin B - 36%, cathepsin C -17% and cathepsin D and cathepsin E - 13%, while after ethanol intoxication the higher increase was observed for: cathepsin A - 14%, cathepsin B - 22%, cathepsin C -12% and cathepsin D - 10% and cathepsin E - 12%. Alcohol intoxication also causes transient increase in lysosomal proteases activity in the plasma. This activity was elevated from 12h to 5 days after intoxication and was the greatest after 2 days. After methanol intoxication the highest increase for cathepsin A and B was 32%, for cathepsin C- 47% , for cathepsin D - 42% and for cathepsin E - 36%. However, after ethanol intoxication the highest increase for cathepsin A was 25% and for cathepsin B - 16%, for cathepsin C - 35%, for cathepsin D - 32% and for cathepsin E - 22%.

Ethanol as well as methanol causes an increase in the amount of lipid peroxidation products reacting with thiobarbituric acid. The highest increase was observed 24h after methanol intoxication (about 42%) and 12h after ethanol intoxication (about 35%).

Discussion

The degradation rate of protein is mainly dependent on proteases activity and on the integrity of cellular organelle membranes. Cellular protein degradation takes place mainly in lysosomes and is mainly caused by cathepsins B and D. The other proteases (such as cathepsin A, C and E) participate in protein degradation, too [21]. The above mentioned proteases have different active centres as follows: sulfhydryl (cathepsin B and C), hydroxyl (cathepsin A) and carboxyl (cathepsin D and E).

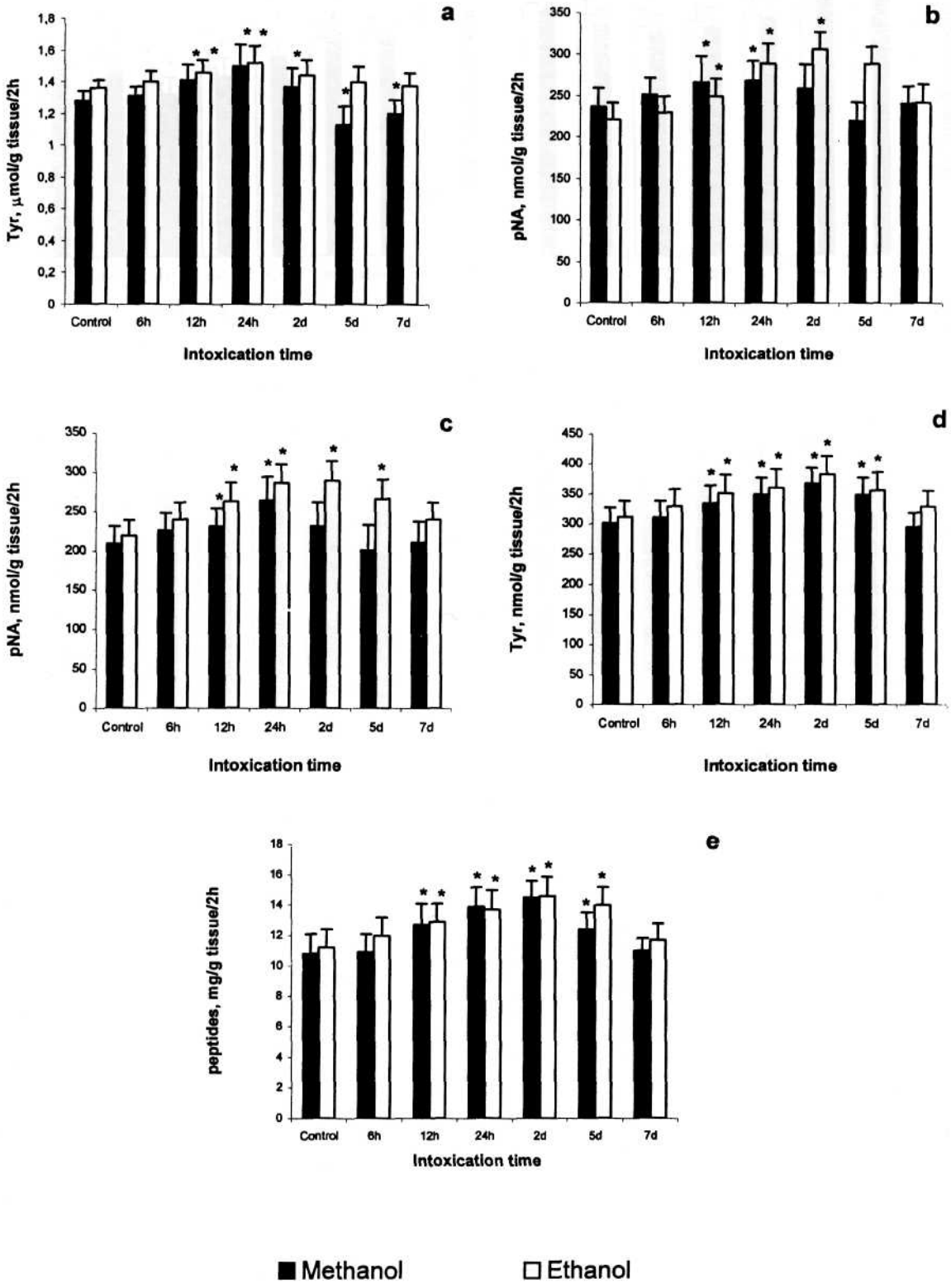

Fig. 1. Activity of cathepsin A (a), cathepsin B (b), cathepsin C (c), cathepsin D (d), cathepsin E (e) in the liver cytosol of the rats intoxicated with methanol and ethanol.
* - significantly different from control value; $p < 0.05$.

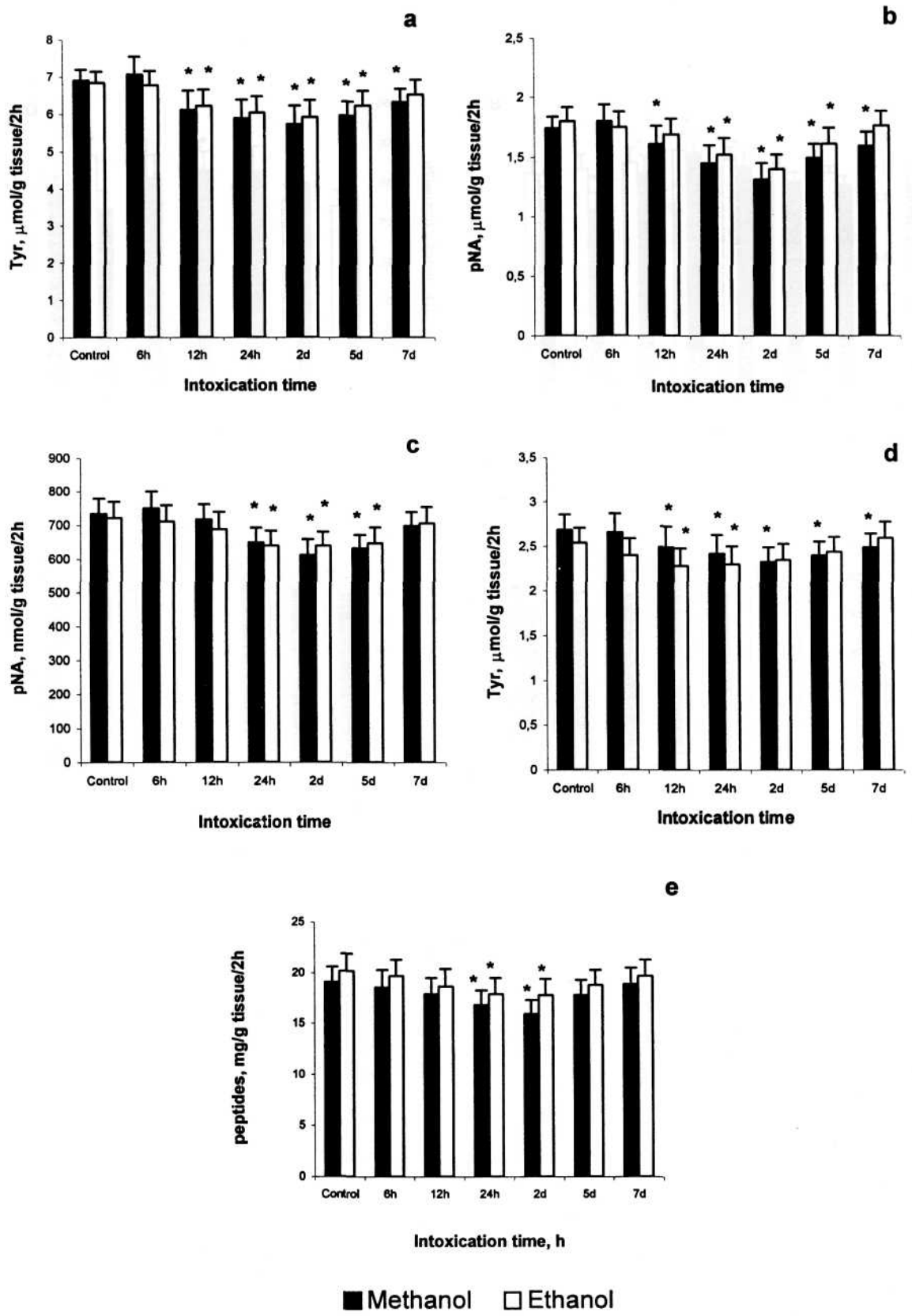

Fig. 2. Activity of cathepsin A (a), cathepsin B (b), cathepsin C (c), cathepsin D (d), cathepsin E (e) in the liver homogenate of the rats intoxicated with methanol and ethanol.
* - significantly different from control value; $p < 0.05$.

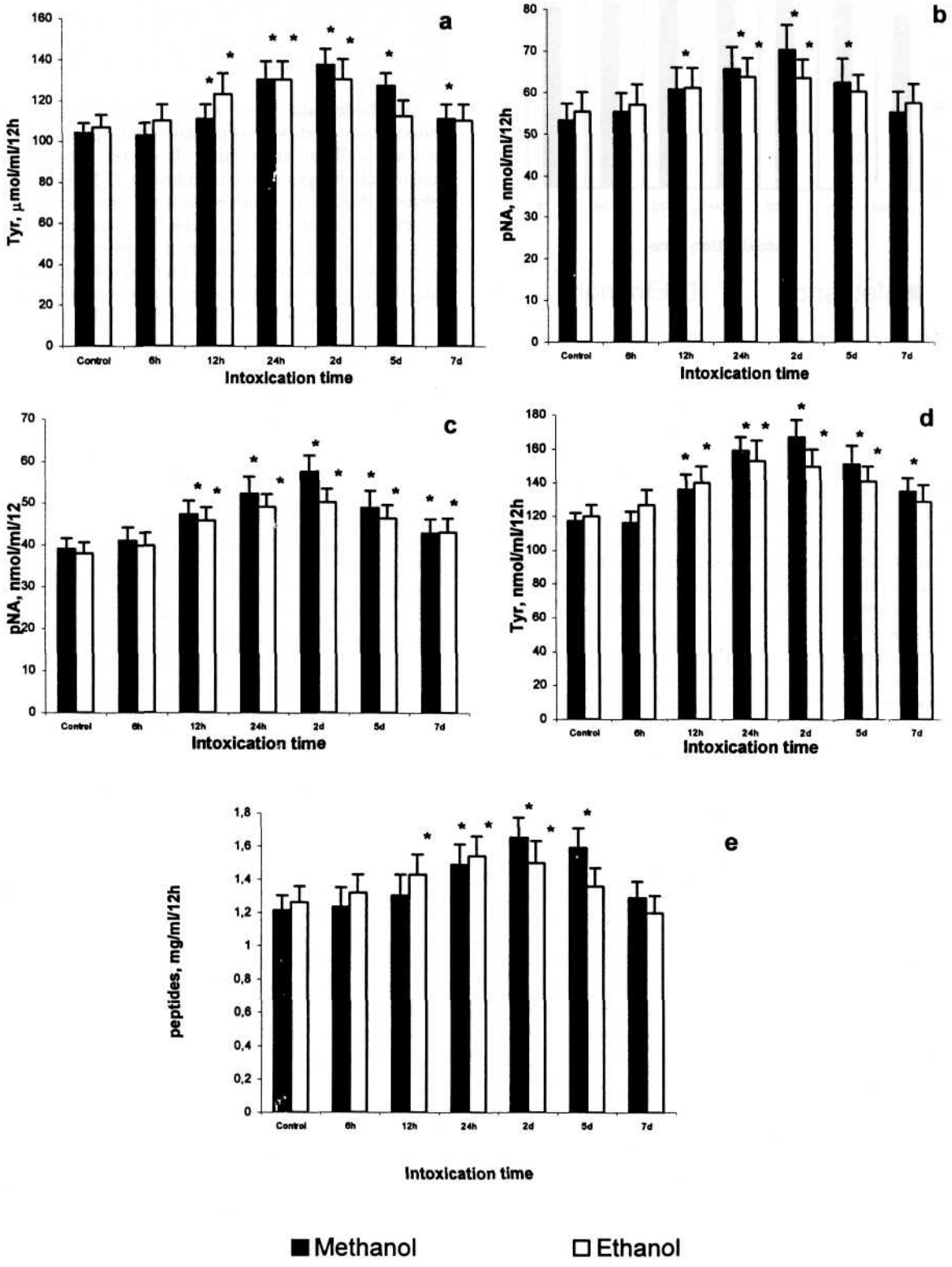

Fig. 3. Activity ofcathepsin A (a), cathepsin B (b), cathepsin C (c), cathepsin D (d), cathepsin E (e) in the plasma of the rats intoxicated with methanol and ethanol.
* - significantly different from control value; $p < 0.05$.

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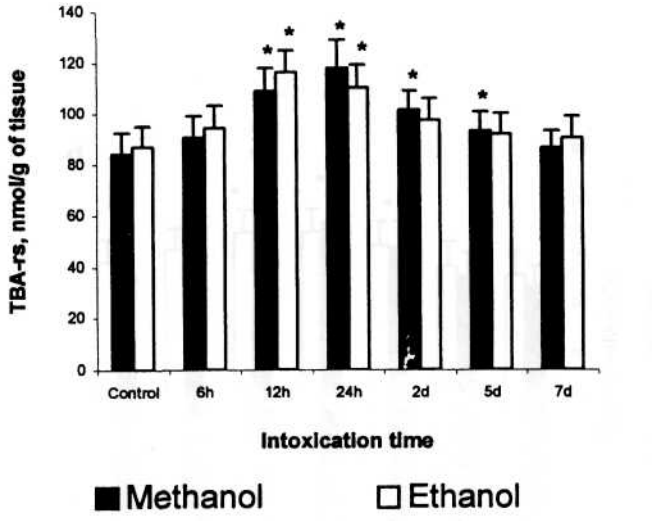

Fig. 4. TBA-rs content in the plasma of the rats intoxicated with methanol and ethanol.
* - significantly different from control value; $p < 0.05$.

Our study indicates that methanol and ethanol intoxication cause a decrease in proteolytic enzyme activity in the liver. The magnitude of this decrease depends on amino acid residues found in the active centre of enzymes. The activities of sulfhydryl and hydroxyl proteases are more decreased than those of carboxyl proteases. It has been shown that methanol intoxication also causes decrease in proteins sulfhydryl groups in the liver [11, 12]. Because a decrease in protease activity and changes in protein concentration are only transient, this may suggest that only posttranslation modification of proteins takes place during alcohol intoxication, whereas their synthesis is probably unchanged. The de novo synthesised enzymes have unchanged activity. The decrease of protease activity results from the high reactivity of aldehydes and oxygen radicals which are produced during alcohol intoxication. Formaldehyde is the most chemically reactive aldehyde, so it cannot be found as a free compound in methanol intoxication [22]. It reacts most easily with cysteinyl, methionyl, lysyl, arginyl and tyrosyl residues, and to a smaller degree with other amino acid residues and peptide bonds of proteins [9, 23]. Generated hydroxymethyl derivatives may further react with nucleophilic groups and form methylene bridges in whose formation participate mainly lysine residues and (rarely) arginine and other residues [24]. Moreover, hydroxymethyl derivatives of lysine residues can react with phenolic rings of tyrosine [25]. As a result, intra- and intermolecular bonds are formed in the proteins [26]. The greater reactivity of formaldehyde than of acetaldehyde is a reason for bigger changes in protein structure and proteolytic activity after methanol than ethanol intoxication.

Superoxide anions and hydroxyl radicals formed during aldehyde oxidation can oxidatively modify amino acid residues of proteins, in particular residues of aromatic and sulfhydryl amino acids [27, 28]. Oxygen radicals can also cause formation of the protein peroxides [29]. Changes of the primary structure of proteins cause modification in their secondary and tertiary structures. Free radicals result also in denaturation, aggregation and fragmentation of protein molecules [27].

An increase in lipid peroxidation after alcohol intoxication is shown. Products of lipid peroxidation such as alkoxyl and peroxyl radicals can also modify the protein structure. They react easily with histydyl and prolyl residues of proteins [30]. They react with lisyl residues of apolipoprotein B of LDL [6]. It is known that a product of lipid peroxidation - 4-hydroxynonenal reacts as well with the active center of cathepsin B and forms the eter-thiol adducts [31].

Aldehydes and free radicals generated during alcohol intoxication modify also proteins and lipids in the lysosomal and cellular membranes. It causes the structural and functional changes of the membranes [32]. First of all, membrane fluidity is changed and an increase in their permeability is observed [33]. Like the plasmalemma, lysosomes are subject to the potential membrane-disrupting effects of free radicals, causing increased lysosomal fragility and the release of lysosomal hydrolases into the cytosol [34]. The decrease in ATP synthesis also participates in destabilization of membranes during alcohol intoxication [35]. Furthermore, the increase in lipids peroxidation products and disruption of lysosomal membranes were confirmed by ultrastructure examinations [36]. The increase in quantity of autophagosomes containing fragments of disturbed organelles and the increase in secondary lysosomes with different density content have been also examined.

In rats, ethanol metabolism occurs mainly due to alcohol dehydrogenase, while methanol oxidation is caused mainly by catalase. In such a situation ethanol oxidation is accompanied by higher NADH generation and, as a consequence, higher free radical generation than methanol oxidation; so in ethanol intoxication lysosomal membrane in the liver may be damaged more intensively, and the proteases in a higher degree may be translocated into the cytosol.

Proteolytic enzymes passing from the organelles into the cytosol may cause the non-controlled proteolysis in the liver cells. The increase in the blood AST and ALT activity, the markers of liver destruction, indicates such processes [11, 12]. Such a situation is also confirmed by the increase in the quantity of autophagosomes in the liver [36]. However, the reduction of proteolytic enzyme activity in the lysosomes may cause accumulation of modificated proteins.

In the physiological conditions only very small amounts of cellular proteolytic enzymes are translocated into the blood plasma [21]. This is done through exocytosis, or by disruption of old cells during aging. In pathological conditions translocation of cellular proteases into the blood takes place due to damage or disruption of cellular and lysosomal membranes. Such a situation was observed in alcohol intoxication.

These results clearly suggest that intoxication of rats with methanol and ethanol causes the changes in proteases activity and in distribution within the hepatocytes, which may lead to disturbances in the cell proteins catabolism, and to the destruction of liver cells. In conclusion, ingestion of ethanol during methanol intoxication may intensify damage to liver cells caused by methanol.

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