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COMBINED EXPOSURE TO M-XYLENE AND ETHANOL: OXIDATIVE STRESS IN THE RAT LIVER

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Abstract
Objectives: Ethanol may be a significant combined factor in human solvent toxicity. Lipid peroxidation has been suggested to be an important contributing mechanism involved in experimental alcohol-induced liver injury. The aim of the study was to investigate whether a short-term ethanol ingestion in rats chronically exposed to m-xylene vapor may influence the lipid peroxidation rate in the intracellular hepatic membranes, the level of glutathione and the activity of glutathione-related enzymes in the liver. **Materials and Methods:** Experiments were performed on male Wistar rats (outbred IMP:WIST) exposed to m-xylene (5 months, 5 h/day), at a low concentration (400 mg/m^3), and/or acute ethanol administration (6 oral doses of 0.25 g/100 g b.w. at 12 h intervals, for the last 3 days of xylene exposure). To estimate the oxidative stress in the liver, lipid peroxidation rate in microsomal and lysosomal membranes, glutathione sulfhydryls levels, and glutathione-S-transferase activity were determined. **Results:** The studies indicated that combined exposure to ethanol and m-xylene, as distinct from the chronic exposure to m-xylene alone, led to the increased lipid peroxidation rate in microsomal and lysosomal membranes with a simultaneous decrease in the levels of glutathione sulfhydryls and glutathione-S-transferase activity. **Conclusions:** We conclude that the enhanced lipid peroxidation rate in the intracellular hepatic membranes may be an important agent in combined ethanol/xylene-induced hepatotoxicity.

Key words:
m-Xylene, Ethanol, Lipid peroxidation, Glutathione, Glutathione-S-transferase

INTRODUCTION

Xylene, as an organic solvent, is widely used for industrial (e.g., paint, rubber) and domestic purposes. The solvent absorbed by employees at workplaces is slowly excreted from the human body due to its high solubility in the lipids [1]. Because of consumption of alcoholic beverages rather frequent in the working population, the simultaneous presence of xylene and ethanol in tissues is quite common. Thus, ethanol can be a significant combined factor in human solvent toxicity.

It is well known that ethanol, owing to its inductive effect on cytochrome P-450 monooxygenases, may evoke disorders in metabolism of drugs and xenobiotics [2]. Metabolized xenobiotics in this system may be converted into active/toxic derivatives. Thus, the ethanol-induced microsomal enzymes can enhance xenobiotic toxicity. The combined exposure of rats to toxic chemicals and ethanol, resulting in the additive stimulatory effect on the activity of hepatic monooxygenases and cytochrome P-450 concentration, has already been described [3–5].

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Due to recent data, it has been suggested that lipid peroxidation could be an important contributing mechanism involved in experimental alcohol-induced liver injury [6–8]. Evidence that lipid peroxidation is increased after ethanol ingestion was found not only in experimental animals, but it was also supported by data gathered in the studies of human tissues. The depletion of glutathione level and the increased formation of diene conjugates were found in the liver biopsies of ethanol-abused people [9]. It is now established that ethanol metabolized by cytochrome P 450 2E1, owing to its inductive effect on the microsomal monooxygenase system, stimulates reactive oxygen species (ROS) formation. It is generally accepted that this mechanism could be involved in the initiation of lipid peroxidation [6].

In our previous studies it was indicated that the liver deterioration provoked by combined exposure to ethanol and high concentration of m-xylene (4000 mg/m^3) may be related to the disturbance of liver oxidant status [5].

The aim of the study was to assess whether ethanol ingestion in rats chronically exposed to m-xylene at low concentration (400 mg/m^3) aggravates the solvent-induced derangement of the liver oxidant status, i.e., depletion of glutathione level and glutathione-related enzymes activity, diminishing the liver antioxidative potential and thus enhancing lipid peroxidation.

MATERIALS AND METHODS

Treatment

Experiments were carried out on male Wistar rats (outbred IMP:WIST) of 200–250 g b.w., fed with a laboratory Murigram diet (Agropol, Motycz, Poland) with free access to water.

Rats were divided into four groups:

- **Control group** – rats were kept in dynamic inhalation chambers with air flow for 5 months (5 h/day, 5 days/week);
- **Ethanol group** – rats were kept in dynamic inhalation chambers with air flow for 5 months (5 h/day, 5 days/week) and treated with ethanol for the last 3 days of xylene exposure. Ethanol (reactified Spirit, Polmos, Poland) was administered by gavage (6 oral doses of 0.25 g/100 g b.w. at 12 h intervals);
- **m-Xylene group** – rats were exposed for 5 months (5 h/day, 5 days/week) to m-xylene (POCH, Poland) at a concentration of 400 mg/m^3 in dynamic inhalation chambers;
- **m-Xylene + Ethanol group** – rats were exposed for 5 months (5 h/day, 5 days/week) to m-xylene (POCH, Poland) at a concentration of 400 mg/m^3 in dynamic inhalation chamber and jointly treated with ethanol (6 oral doses of 0.25 g/100 g b.w. at 12 h intervals) for the last 3 days of exposure.

Each group consisted of 6 animals.

Concentration of xylene in the chamber was controlled by means of gas chromatograph (Varian 1440), three times during the exposure period.

In all experiments, the Polish law on the protection of animals was followed [10].

Preparation procedures

Rats were killed by decapitation and livers were homogenized with three volumes of KCl (150 mmol) buffered with Tris-HCl (10 mmol), pH 7.4 in a glass Potter-Elvehjem homogenizer with a teflon pestle. Microsomes were prepared by the $CaCl_2$ aggregation method, according to Kamath et al. [11]. Lysosomal fraction was isolated according to Sawant et al. [12]. In brief, 10% (w/v) homogenate of fresh liver in 0.25 M sucrose-0.01 M Tris-HCl, pH 7.4, was successively centrifuged at 4°C at 900 g for 10 min, 7000 g for 15 min and 20 400 g for 35 min to sediment nuclei, mitochondria and lysosomes, respectively. After washing with 150 mmol KCl and re-centrifugation at 20 400 for 35 min the lysosomal fraction was suspended by re-homogenization in 150 mmol KCl.

Biochemical assays

Glutathione sulfhydryls (GSH) was determined by the method of Ellman et al. [13] and glutathione-S-transferase (E.C. 2.5.1.18) by the method of Habig et al. [14]. Protein was determined according to Lowry et al. [15]. Activity of β-glucuronidase [E.C. 3.2.1.31] was assayed using the method of Nimmo-Smith et al. [16], with p-nitrophenol glucuronide as a substrate; p-nitrophenol was determined

spectrophotometrically at 400 nm. Lipid peroxidation in the fresh microsomal and lysosomal fractions *in vivo* and after *in vitro* stimulation with NADPH-Fe^{2+} or ascorbate-Fe^{2+} (denoted as *ex vivo*) was evaluated on the basis of the detection of thiobarbituric acid reactive substances (TBARs) such as malondialdehyde (MDA) according to Mihara et al. [17]. An extinction coefficient of 156 000 $mol^{-1}cm^{-1}$ according to Wills was used for MDA formation [18]. The chemical stimulation permits to measure multiplied biological effects of chemicals acting *in vivo*.

Statistical analysis

For comparisons of the exposed groups to the control or ethanol group, Student's t-test was used. The differences were considered statistically significant at $p < 0.05$.

RESULTS

General effect of exposure. Exposure to m-xylene did not influence the food consumption or weight gain, which was similar in all groups (0.8 g per day). There were no statistically significant differences in the rat body weight, liver wet weight, protein contents of the liver microsomes and lysosomes, or β-glucuronidase activity in lysosomes (marker of the lysosomes activity) between the control and experimental groups (Tables 1–3).

Effects of ethanol. In rats exposed to ethanol no statistically significant changes in the level of lipid peroxidation in microsomal and lysosomal fractions were observed, as compared to the control group (Tables 1–3).

Effects of m-xylene. m-Xylene vapors did not evoke any statistically significant changes in the hepatic gluthatione-S-transferase activity and GSH content (Table 1), or in lipid peroxidation in microsomal and lysosomal fractions (Tables 2 and 3) in comparison with controls.

Combined effect of ethanol and m-xylene. The lipid peroxidation in lysosomal and microsomal fractions of the liver (Tables 2 and 3) without and after enzymatic stimulation of microsomal fraction with NADPH-Fe^{2+} and Ascorbate-Fe^{2+} (Table 2, Fig.1) were increased in rats exposed to m-xylene vapors and ethanol ($p < 0.05$). This increase was concomitant with decrease in the GSH levels and

Table 1. Changes in the activity of glutathione-S-transferase and glutathione sulfhydryls (GSH) content in the liver of rats chronically exposed to m-xylene and/or acute administration of ethanol

Group	Glutathione sulfhydryls (μmol/g tissue)	Glutathione--S-transferase (μmol/g tissue/min)
Control	6.11 ± 0.14	65.40 ± 1.58
Ethanol	5.80 ± 0.21	61.11 ± 2.75
m-Xylene	6.40 ± 0.15	69.09 ± 2.71
m-Xylene + Ethanol	5.05 ± 0.26[b]	57.01 ± 2.75[a]

Results are the mean ± SE.
[a,b] Significantly different from the control at $p < 0.05$ and 0.01, respectively.

Table 3. Lipid peroxidation and β-glucuronidase activity in the liver lysosomal fractions in the control and exposed groups

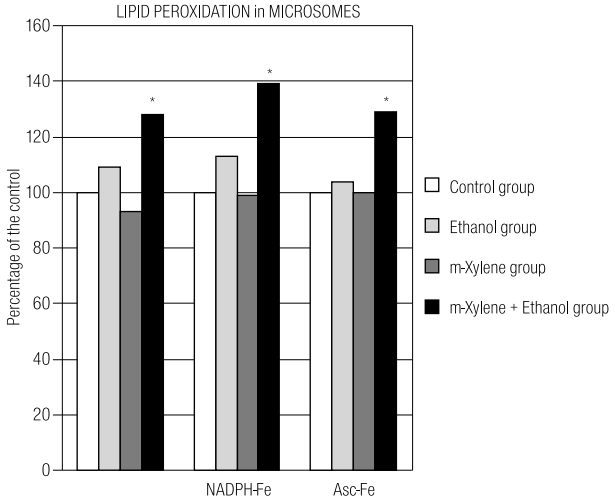
Group	Lipid peroxidation (nmol MDA/g tissue)	β-glucuronidase (μmol/15 min/g tissue)
Control	21.6 ± 1.78	5.69 ± 0.20
Ethanol	26.2 ± 1.41	5.92 ± 0.33
m-Xylene	19.7 ± 1.05	5.90 ± 0.20
m-Xylene + Ethanol	30.8 ± 1.09[b]	6.20 ± 0.20

Results are the mean ± SE.
[b] Significantly different from the control at $p < 0.01$.
MDA – malondialdehyde.

Table 2. The rate of lipid peroxide formation in the liver microsomal fraction in the control and exposed groups

Group	Lipid peroxidation		
	Without stimulation (nmol MDA/g tissue)	After stimulation *in vitro* with	
		NADPH-Fe^{2+} (nmol MDA/20 min/g tissue)	Ascorbate-Fe^{2+} (nmol MDA/20 min/g tissue)
Control	51.97 ± 3.26	110.9 ± 9.33	418.5 ± 23.7
Ethanol	56.57 ± 2.21	130.3 ± 7.94	434.4 ± 31.3
m-Xylene	47.70 ± 1.13	109.5 ± 2.03	418.0 ± 25.9
m-Xylene + Ethanol	66.75 ± 4.30[a]	154.4 ± 7.30[b]	540.6 ± 29.6[a]

Results are the mean ± SE.
[a,b] Significantly different from the control at $p < 0.05$ and 0.01, respectively.
MDA – malondialdehyde.



* Statistical significance in comparison with control group ($p < 0.05$).

Fig. 1. Lipid peroxidation levels in the liver microsomal fraction of rats exposed to ethanol and m-xylene after stimulation (in vitro) with NADPH-Fe^{2+} and Ascorbate-Fe^{2+} (as percentage of control).

glutathione-S-transferase activity, statistically significant compared to the control group (Table 1).

DISCUSSION

The present report clearly demonstrates that in rats chronically exposed to low concentration of m-xylene (400 mg/m^3), lipid peroxidation, GSH content and glutathione-S-transferase activity were not affected. However, the combined exposure of rats to m-xylene at low concentration and acute ethanol ingestion brought about a biological effect manifested by the enhanced lipid peroxidation rate and depletion of the GSH levels in the liver.

In our previous studies of the combined exposure to ethanol + m-xylene (6- and 12-week exposure to m-xylene at a concentration of 4000 mg/m^3, and for the last 3 days of exposure jointly treated with ethanol – 6 oral doses of 0.25 g/100 g b.w. at 12 h intervals), additive stimulatory effects on the activity of hepatic cytochrome P-450 monooxygenases (aniline p-hydroxylase, microsomal ethanol oxidizing system, NADPH-cyt. c reductase, cytochrome P-450) and lipid peroxidation in the hepatic microsomal fraction in rats were documented [5].

It seems that the above-said effects caused by alcohol intake may affect xylene metabolism and elimination of its derivates. It is known that metabolism of xylene to xylenols proceeds via arene oxidation in the cytochrome P-450-dependent monooxygenase system. Other pathways of xylene metabolism, proceeding via cytoplasmic dehydrogenases, lead to the formation of toluic acids, which conjugated with glycine form methylhippuric acids [1,3,4,19]. Recently, phenylmercapturic acid (DPMA) isomers have been identified as metabolites of xylenes in human urine [20]. These results demonstrate that aromatic epoxides may be produced as intermediates of xylene metabolism. Earlier, aromatic epoxides were identified in toluene metabolism [21]. Epoxides may influence not only the overall damage to biological systems but also to the site of injury.

The mechanism responsible for the potentiating effect of ethanol on xylene-induced hepatotoxicity is not yet elucidated. However, the following mechanisms could be considered: 1) enhancement of the free radicals generation and prooxidative activity related to cytochrome P-450 induction; 2) formation of reactive intermediate metabolites (arene oxides, aromatic epoxides); 3) decrease in the glutathione level and/or activity of the glutathione-dependent enzymes; and 4) decrease in the detoxification of xylene metabolites (e.g., xylenols) associated with GSH catalyzed by glutathione-S-transferase.

Over the last years, it was suggested that lipid peroxidation can play an important role in the liver damage induced by ethanol. An increased level of lipid peroxides has been demonstrated in the liver of animals acutely and chronically treated with ethanol [6,8,22]. Recently, a quantitative immunohistochemical analysis has revealed a significant, fivefold, increase in the level of lipid peroxidation end-products (malondialdehyde and 4-hydroxynonenal) in the liver sections prepared from rats treated with ethanol [23]. The results of the present study also showed that short-term ethanol treatment of rats chronically exposed to m-xylene at a concentration of 400 mg/m^3 provoked the potentiation of lipid peroxidation in hepatic microsomal and lysosomal membranes (Tables 2–3). It has been claimed that ethanol as well as xylene may act as pro-oxidants via ROS formation.

It has also been suggested that ethanol-induced lipid peroxidation may be a consequence of the impaired

hepatocellular defence system, i.e., glutathione and glutathione-related enzymes. Glutathione, as a nucleophilic agent, plays an important protective role against oxidant and free-radical mediated cell injury [6]. In addition, glutathione depletion may favor the lipid peroxidation process [24]. The depletion of hepatic glutathione level in rats exposed to xylene or to ethanol has been documented [6,8,9,22,24]. Our study confirmed these findings. The activity of glutathione-S-transferase and GSH content in the liver of rats exposed jointly to ethanol and xylene at a concentration of 400 mg/m^3 were lower. Conjugation of electrophilic xenobiotic metabolites with glutathione, catalyzed by glutathione-S-transferase, is known as detoxification pathways [25]. We evidenced that the changes in the hepatic defence system, namely the decreased GSH content and glutathione-S-transferase activity, were associated with lipid peroxidation. However, the role of lipid peroxidation in ethanol/xylene potentiation of hepatotoxicity still remains open.

Ethanol has been reported to cause damage to subcellular organelles (membrane lipids) through lipid peroxidation [6]. Since peroxidation in membranes leads to the decrease in its integrity (fluidity), it may also affect the membrane-bound enzymes (e.g., lysosomal). Wills and Wilkinson [26] suggest that lipid peroxide formation leads to rupture of the lysosomal membrane and allows the release of the latent enzymes. Exposure to m-xylene at a concentration of 400 mg/m^3 and short time ethanol ingestion did not produce evident changes in the activity of lysosomal β-glucuronidase (a marker of the lysosomes function) as related to the increased lipid peroxidation in the lysosomal membranes, whereas a significantly enhanced activity of lysosomal enzymes and lysosomal protein content in rats exposed to ethanol and m-xylene at a concentration of 4000 mg/m^3 were observed. The changes were accompanied by proliferation of the smooth endoplasmic reticulum and the development of large vacuoles (comprised giant secondary lysosomes) in the hepatocyte cytoplasm (unpublished data). Such a large vacuole in the cytoplasm of sublethally injured liver cells is often an indication of heightened autophagic and heterophagic activity [27].

Propagation of lipid peroxidation by ethanol is considered as one of the mechanisms involved in toxic effects of several organic solvents (e.g., toluene, benzene, CCl_4, CS_2) [5,28–30]. Recently, it has been observed that the mixture of xylene with methanol is able to potentiate lipid peroxidation in the liver of exposed rats. Moreover, coenzyme Q10 and vitamin E are efficient antioxidants reducing this effect [31]. Taking into consideration our data as well as those of other authors, one can conclude that the stimulation of lipid peroxidation in microsomal and lysosomal membranes may be an important factor in combined ethanol/xylene-produced hepatotoxicity.

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