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## Ethanol Induced Oxidative Stress and Membrane Injury in Rat Erythrocytes

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**Summary:** The aim of this study was to observe membrane injury and to investigate the mechanism of antioxidant defence systems against acute ethanol toxicity. Erythrocyte superoxide dismutase and  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase activities were significantly decreased and catalase levels were significantly increased one hour after ethanol intoxication of male swiss albino rats. These data demonstrated that superoxide dismutase and catalase are susceptible to lipid peroxidation and that these enzymes protect tissues from free radicals. The possible mechanism involved in  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase and  $\text{Ca}^{2+}$ -ATPase inhibition are discussed in relation to the development of ethanol toxicity and the role of lipid peroxidative processes.

### Introduction

One of the most frequently encountered types of chemically induced tissue injury in man is alcoholic liver disease (1). Recently, it was proposed that ethanol toxicity involves free radicals, constituting evidence for increased lipid peroxidation during ethanol poisoning (2–5). Stimulation of lipid peroxidation in liposome membranes and in subcellular organelles has been shown to increase membrane rigidity. In addition to changes in fluidity, lipid peroxidation causes an increase in ionic permeability and affects the surface potentials of the membranes (4). It is known that the viability of erythrocytes depends in large part on the functional state of its active sodium and potassium transport system, which in turn depends on the structural integrity of the membrane (6). A growing body of evidence indicates that the fluidity of the lipid environment influences the activity of the physiologically important membrane enzyme,  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase<sup>1)</sup> (7).

In this communication, we report experiments to identify the effects of acute ethanol intoxication on red cell membrane lipid composition,  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase, and the activities of superoxide dismutase<sup>1)</sup> and catalase<sup>1)</sup> in haemolysates.

### Materials and Methods

#### Animals

Two months old male Swiss albino rats weighing 180–220 g were used in these experiments. They were fed with standard pelleted diet and tap water ad libitum.

The ethanol group ( $n = 6$ ) received a solution of ethanol in water, volume fraction 0.5, by gavage in a dose of 3 g/kg body weight in 3 minutes. The control group ( $n = 6$ ) received water alone.

#### Sampling

Fifty minutes after ethanol administration, blood samples were taken by left ventricle puncture into EDTA-containing centrifuge tubes. After separation of plasma, the packed erythrocytes were washed two times 9 g/l NaCl solution and haemolysed with ice-cold water.

#### Enzyme assays

Superoxide dismutase and catalase activities were determined immediately in haemolysates. The haemoglobin values of these haemolysates were determined with *Drabkin's* method. Superoxide dismutase activities were measured according to *Misra & Fridov-*

<sup>1)</sup> Enzymes:

ATP phosphohydrolase, EC 3.6.1.4

$\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Mg}^{2+}$  dependent ATPase

$\text{Mg}^{2+}$  dependent ATPase

$\text{Ca}^{2+}$  ATPase

Superoxide dismutase, EC 1.15.1.1

Catalase, EC 1.11.1.6

ich based on the inhibition of autoxidation of epinephrine by superoxide dismutase at 480 nm in a LKB Ultraspec 2 spectrophotometer. The level of enzyme that causes 50% inhibition of epinephrine autoxidation is defined as 1 unit (8, 9). Catalase levels were determined as described by *Aebi*. The catalase-mediated decomposition of  $H_2O_2$  was followed directly at 240 nm. One unit of catalase activity is defined as the level of enzyme required to decompose 1  $\mu\text{mol}$   $H_2O_2$  in 1 minute (10–12). Aspartate amino transferase and alanine aminotransferase activities of plasma samples were measured in a Hitachi 705 autoanalyser.

#### Membrane preparation

Red cell membranes were prepared according to *Beutler & Wood* (13). The packed ghosts were freeze-thawed 3 times and stored at  $-70^\circ\text{C}$  until assay. Protein determinations were performed according to *Lowry et al.* (14).

#### $Na^+$ , $K^+$ -ATPase assay

The standard medium for total ATPase was used and the assay was performed according to *Muriel & Mourelle* (7).  $Mg^{2+}$ -activated ATPase was measured in an identical medium that included 1 mmol/l ouabain. The difference between the two reactions represented  $Na^+$ ,  $K^+$ -ATPase. Inorganic phosphate was determined by the vanadate-molybdate method.  $Ca^{2+}$ -ATPase activity was determined according to *Muriel & Mourelle* (7).

#### Lipid assays

Lipids were extracted according to the modified method of *Folch* (15). An antioxidant, 2,6 di-*tert*-butyl-4-methyl phenol, was added at a concentration of 5 mg/l to all solvents as described by *Pohl et al.* (16). After the extraction with chloroform-methanol, the lower phase was filtered. Following evaporation, the lipid residue was used for phospholipid phosphorus and cholesterol determinations. Phospholipid phosphorus was determined by colorimetric molybdate and vanadate reaction, and cholesterol was determined by the *Liebermann-Buchardt* reaction.

#### Reagents

All reagents were analytical grade and purchased from Sigma Chem. Co (St. Louis) and Merck Darmstadt (Germany).

#### Liver examination

The livers were excised, the liver slices were fixed in buffered formalin and stained with haematoxylin eosin for histopathological examination. Liver homogenates in phosphate-buffered solution were used to determine the aspartate aminotransferase and alanine aminotransferase activities.

#### Results

Table 1 shows the results for osmotic fragility of erythrocytes in the control and acute ethanol-treated groups. Erythrocyte fragility was increased in the acute ethanol group. The cholesterol/phospholipid molar ratio was increased after acute ethanol administration, due to an increased cholesterol content. As seen in table 2, there was a significant decrease in  $Na^+$ ,  $K^+$ -ATPase activity ( $p < 0.05$ ) and superoxide dismutase activity ( $p < 0.01$ ). Catalase levels increased two fold when

compared with the control group ( $p < 0.01$ ). We also found a non-significant decrease in the activity of  $Ca^{2+}$ -ATPase. Histo-pathological examination of the livers showed no difference between the ethanol-treated and control groups under light microscopy. Liver homogenate and plasma aspartate aminotransferase and alanine aminotransferase activities were the same in both groups (tab. 3).

#### Discussion

There are conflicting reports on the ability of acute ethanol administration to induce hepatic lipid peroxidation (2, 4, 17). Ingestion of ethanol causes considerable metabolic dearrangement as well as changes in the structure and function of hepatocellular organelles (18–20). Typical of such changes are those affecting the ultrastructure of the hepatic plasma membrane and its associated enzyme activities such as  $Na^+$ ,  $K^+$ -ATPase (21). Another proposed mechanism of ethanol toxicity is based on the involvement of free radicals. Free radical mechanisms are now well recognized as being involved in several types of toxic liver damage. Several authors have presented evidence for increased lipid peroxidation during ethanol poisoning (2, 3, 17).

We found a two fold increased activity of catalase in livers from ethanol-treated animals. It has been reported that ethanol is oxidized in the liver by three different enzyme systems: alcohol dehydrogenase, the microsomal ethanol oxidizing system and catalase (22). *Koechling et al.* found a relationship between blood catalase activity and alcohol consumption in a human population and they suggested that catalase activity measurement would be a strong positive determinant of alcohol intake (23). On the other hand, *Bradford et al.* demonstrated that catalase is the predominant enzyme of alcohol metabolism in mice treated with the alcohol dehydrogenase inhibitor, 4-methylpyrazole. Their results showed that while the contribution of catalase was 50% in alcohol dehydrogenase (+) mutant mice at low doses of ethanol, it became 100% as the ethanol concentration was elevated (24).

We found a significant decrease in superoxide dismutase activity in the acute ethanol-treated group. Similar results were also observed by *Ledig et al.*, who reported a decrease of superoxide dismutase activity following the intraperitoneal injection of ethanol (17). They suggested that the inhibition of superoxide dismutase activity by ethanol may allow an accumulation of cytotoxic  $O_2$  radicals, which could partially explain the toxicity of ethanol for cellular metabolism (17). This decrease in super-

**Tab. 1** The results of osmotic fragility tests in the control and ethanol-treated groups.

Sodium chloride		Fraction of erythrocytes haemolysed (%)		
Volume fraction of 9 g/l NaCl (%)	Concentration (g/l)	Control group	Ethanol group	Significance
30	2.70	94.25 ± 0.85	96.75 ± 1.25	p > 0.05
40	3.60	79.00 ± 1.73	75.75 ± 5.89	p > 0.05
45	4.05	49.50 ± 2.50	61.50 ± 1.70	p < 0.01
50	4.50	18.50 ± 0.96	26.75 ± 2.01	p < 0.01
55	4.95	1.00 ± 0.41	10.50 ± 4.11	p < 0.05
60	5.40	0	3.00 ± 0.41	p < 0.01
70	6.30	0	0.50 ± 0.29	p < 0.05

Data were given as the mean ± SEM.

**Tab. 2** Effect of ethanol on erythrocyte antioxidant and membrane enzymes.

	Control group (n = 6)	Ethanol group (n = 6)	Significance
Superoxide dismutase (U/gHb)	7164.8 ± 378	2068 ± 320	p < 0.01
Catalase (U/gHb)	5724.0 ± 317	10826 ± 669	p < 0.01
Na <sup>+</sup> , K <sup>+</sup> -ATPase (P <sub>i</sub> , μmol/h · mg protein)	0.63 ± 0.04	0.43 ± 0.08	p < 0.05
Ca <sup>2+</sup> -ATPase (P <sub>i</sub> , μmol/h · mg protein)	1.52 ± 0.12	1.43 ± 0.10	p > 0.05
Cholesterol/phospholipid molar ratio	0.52 ± 0.22	0.72 ± 0.12	p > 0.05
Cholesterol (μmol/mg protein)	0.28 ± 0.03	0.36 ± 0.06	p > 0.05
Phospholipid (μmol/mg protein)	0.45 ± 0.04	0.49 ± 0.05	p > 0.05

Data were given as the mean ± SEM.

**Tab. 3** The activities of liver marker enzymes in plasma and liver homogenates of control and ethanol-treated animals.

	Control group (n = 6)	Ethanol group (n = 6)
Plasma aspartate aminotransferase (U/l)	123.30 ± 8.21	104.50 ± 3.32
Plasma alanine aminotransferase (U/l)	44.10 ± 3.30	41.80 ± 4.23
Liver aspartate aminotransferase (U/mg protein)	1.23 ± 0.11	1.47 ± 0.21
Liver alanine aminotransferase (U/mg protein)	0.76 ± 0.11	0.72 ± 0.07

oxide dismutase activity might be due to inactivation of the enzyme by the increased concentration of hydroxy radicals arising from ethanol metabolism.

Free radicals can cause cellular injury when produced in amounts sufficient to overcome the normally efficient protective mechanism. Lipid peroxidation is a free radical mediated chain reaction which is enhanced as a consequence of oxidative stress, and it results in an oxidative deterioration of membrane polyunsaturated fatty acids (25). It is known that O<sub>2</sub> radicals inactivate Na<sup>+</sup>, K<sup>+</sup>-ATPase, as we also observed. On the other hand, fatty acyl composition and cholesterol content of the plasma membrane can also affect Na<sup>+</sup>, K<sup>+</sup>-ATPase activity. Since free fatty acids can alter cellular functions by their effects on transport mechanisms, it has been proposed that they alter Na<sup>+</sup>, K<sup>+</sup>-ATPase activity, Ca<sup>2+</sup> movement and guanylate cyclase activity (25). In order to determine the alterations of fluidity of erythrocyte

membranes due to oxidative stress, we investigated the osmotic fragility of erythrocytes. The results (tab. 1) show that haemolysis of erythrocytes from ethanol-treated rats started when the medium consisted of 6.3 g/l NaCl, while haemolysis of controls started at about 4.95 g/l NaCl. *Comporti et al.* confirmed that lipid peroxidation in liver homogenates was increased by acute ethanol intoxication (26). The increased lipoperoxides can be considered as responsible, at least in part, for the red cell injury, since we observed increased osmotic fragility of erythrocytes in the acute ethanol-treated group. Peroxidative attacks on membrane proteins have been reported as responsible for both cation leakage and haemolysis. Lipid peroxidation alters membrane constituents and affects their interactions in such a way that membrane microviscosity and fluidity are also altered. In ethanol-treated rats we found an increase in the cholesterol/phospholipid molar ratio due to an increased

cholesterol content, although the change was not statistically significant. Some investigators have reported an increase in the cholesterol content of erythrocyte membranes in animals treated with ethanol (27, 28). Although chronic ethanol administration causes an increase in acyl CoA-cholesterol acyltransferase, the enzyme responsible for the intracellular production of cholesteryl esters (29), the mechanism of increase in

membrane cholesterol in acute ethanol ingestion is still obscure.

We therefore suggest that several pathological processes are related to the membrane alterations that occur after acute ethanol administration, even when there is no histopathological evidence of cell injury. We consider it highly probable that lipid peroxidation is an initiating factor in the pathogenesis of membrane injury.

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