

# Effects of high dose of long-term ethanol exposure on oxidative damage of spleen in experimental rat model

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## ABSTRACT

**Background:** Alcoholic beverages have been used in human societies at least since the beginning of recorded history. Research has demonstrated sufficiently to our understanding of the relation of drinking to specific disorders, and has shown that the relation between alcohol consumption and health outcomes is complex and multidimensional. **Materials and Methods:** In this study, we had demonstrated the role of oxidative stress in long-term ethanol-induced spleen damage in 16 to 18-week-old male albino rats of Wistar strain weighing 200–220 g. **Results:** Ethanol exposures (1.6 g ethanol/ kg body wt/ day for 12 weeks) caused a significant decrease in reduced glutathione (GSH) concentration and activities of superoxide dismutase and glutathione reductase; while a significant increase in thiobarbituric acid reactive substances (TBARS) content and glutathione s-transferase activity in spleen homogenate. **Conclusion:** Our study revealed that high dose of ethanol (1.6 g/ kg body weight/ day) for a long period caused significant oxidative stress in the spleen. Intermediates of oxygen reduction may be associated with the development of ethanol-induced organ damage.

**Keywords:** Ethanol, Glutathione, Oxidative stress, Spleen, Thiobarbituric acid reactive substances.

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## INTRODUCTION

The physical, psychological, social and legal harms of excessive alcohol use represent a major public health problem.<sup>1</sup> Excessive consumption of alcohol may lead to a variety of gastrointestinal, neurologic, cardiovascular, and malignant diseases.<sup>2,3</sup> Alcohol intoxication can lead to different types of disorders like gastritis, gastric ulcer, fatty liver, liver cirrhosis, alcoholic hepatitis, etc. Pancreatitis is very common in chronic alcoholics. Alcohol in heavy doses causes muscular weakness and acute alcoholic myopathy. It can lead to elevation of blood pressure. It can change the membrane fluidity. In binge drinkers, the chance of atherosclerosis is very high.<sup>4</sup> Alcohol administration is associated with hypoparathyroidism and low testosterone and other sex steroids levels, resulting in low serum calcium level and low reproducibility in both males and female.<sup>5,6</sup> Heavy drinking increases the risks of carcinoma of tongue, mouth, larynx, and esophagus and liver. Even in small doses alcohol produces ataxia, alcohol neuropathy, and Wernicke's encephalopathy.<sup>5</sup> Alcohol consumption during pregnancy leads to fetal alcohol syndrome that affects in infants- low birth weight, greater susceptibility to infection due to immune deficiency, congenital malformation, tortuosity of the retinal artery and vein and high serum uric acid level.<sup>5,7</sup> Alcohol stimulates the pancreatic  $\beta$  cells to produce insulin. Thus, a high level of insulin secretion reduces blood sugar levels and produces hypoglycemia. Low blood sugar level inhibits the formation of liver glycogen. Alcohol also inhibits glucose formation from lactate and from other glucogenic amino acids in the liver.<sup>5,6</sup> Chronic alcoholism is associated with derangement in sulfur amino acid metabolism producing ethanol-induced hyperhomocysteinemia. Homocysteine induces neuronal cell death. Alcohol in high doses induces DNA damage and impairs with DNA repair system.<sup>8</sup> These mechanisms induce

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apoptosis and early aging.<sup>9</sup> It is associated with a three to seven-fold increase in premature mortality,<sup>2,3</sup> as well as up to 80% of cases of chronic liver disease and approximately 30 to 50% of traumatic injuries and drowning; as well as imposes a heavy economic burden.<sup>10</sup>

Chronic ethanol consumption is associated with an increased incidence of a variety of illnesses. The precise mechanisms leading to alcohol related diseases are still imprecisely known. The metabolic effects of alcohol are due both to its direct action and to that of its first metabolite acetaldehyde, and can also be connected with the changes in redox state. Ethanol increases the rate of generation of free radicals, decreases the antioxidant levels, and potentiates oxidative stress. Cells are protected against oxidation by the action of certain enzymes, vitamins, and other substances, known collectively as antioxidants.<sup>11</sup>

Ethanol acts as an immunomodulator. The ingestion of ethanol was shown to be associated with immunodeficiency.<sup>12</sup> Researchers have shown that alcohol administration is directly related with immunosuppression. Alcohol intake increases the susceptibility of the individual to different types of infections. Both humoral and cell-mediated immunity have been shown to be suppressed in chronic

alcoholics.<sup>13</sup> Ethanol reduces the number of lymphocytes and phagocytosis by macrophages.<sup>14,15</sup> It can suppress the host defense mechanisms to bacterial infections<sup>12</sup> and inhibit neutrophil function.<sup>16</sup> Ethanol modifies the specificity of antibody functions against a defined epitope, probably due to changes of the conformation of the antibodies and this effect is concentration-dependent.<sup>17</sup> Ethanol administration impaired cell-mediated immune response, probably by inhibiting early events in T-lymphocyte activation.<sup>18,19</sup> Ethanol is involved in the impairment of IgM synthesis and secretion by plasma cells, especially in the mesenteric lymph node.<sup>20</sup> It also suppresses the synthesis of IL-1, 2, and 4 in the spleen, probably by inhibiting the translation of mRNA for the cytokines.<sup>21</sup>

The spleen is an organ located in the abdomen, where it functions in the destruction of old red blood cells and holding a reservoir of blood. It is regarded as one of the centers of activity of the reticuloendothelial system (part of the immune system). It is increasingly recognized that its absence leads to a predisposition to certain infections.

Ethanol produces progressive inhibition of antibody-dependent cell-mediated cytotoxicity (ADCC). The binding of spleen cells to antibody-sensitized target cells was not inhibited by comparable concentrations of ethanol. Kinetic analysis revealed decreased rates of lysis with increasing concentrations of ethanol. Changes of effector to target cell ratios revealed inhibition of maximum lysis and decreased lytic efficiency in the presence of 88 mM ethanol. Preincubation experiments showed the inhibitory effect of ethanol to be reversible. Macrophage-depleted spleen cells appeared to be as susceptible to inhibition by ethanol as unfractionated spleen cells. Ethanol also inhibited natural killer and alloimmune cytotoxic T-cell activity.<sup>22</sup>

Experimental evidence on the immunomodulating effects of ethanol is contradictory and, in animals, the immunological effects of long-term alcohol intake may depend on the age of the animal, amount of alcohol consumed, and nutritional composition of the administered diet. Food intake, rate of gain in body weight, and percentages of B cells, T-cells, and T-cell subtypes were not affected by ethanol intake. Ethanol consumption had no significant influence on spleen weights.<sup>23</sup>

Chronic ethanol (ETOH) ingestion adversely affects the immunocompetence of alcohol abusers. Ethanol directly impairs host defense mechanisms and indirectly modulates immunocompetence by interfering with the nutritional status of the alcoholic. Spleen cell number and secretions of immunoreactive interleukin-2 and tumor necrosis factor were totally independent of the diet, being affected only by ethanol. Body, spleen, and thymus weights, interferon-gamma secretion, and natural killer cell and phagocytic activities were modulated by ethanol as well as by diet. Natural killer cell and phagocytic activities were also directly affected by the nutritional quality of the diet.<sup>24</sup>

Spleen cell number, and interleukin-2, and tumor necrosis factor (TNF) secretion were independent of the diet

consumed but were affected by the consumption of ethanol. Body and spleen weights and interferon-gamma secretion were modulated by ethanol as well as by diet. The results indicate that the nutritional composition of the diet consumed during concurrent administration of ethanol modulates the immunotoxic effects of chronic ethanol ingestion.<sup>25</sup>

Therefore, in this study, we had examined the role of oxidative stress in long-term ethanol-induced spleen damage.

## MATERIALS AND METHODS

### Materials

#### Chemicals

Ethanol was purchased from Bengal Chemicals, Kolkata. Fine chemicals were purchased from Sisco Research Laboratory (SRL), India; and Sigma Chemical Co., St. Louis, USA; and analytical grade chemicals from E. Merck or SRL.

#### Animal maintenance and experimental procedure

Male albino rats of the wistar strain (16–18 week-old weighing 200–220 g) were used for the current study. The animals were housed in plastic cages inside a well-ventilated room. The room was maintained under standard husbandry conditions (25 ± 2°C temperature, 60–70% relative humidity, and 12-hour light/dark cycle). All rats had free access of standard diet containing 31% Bengal gram, 30% gingelly oil cake, 28% wheat, 10% polished rice, 0.5% salt mixture, 0.3% vitamin-mineral mixture, 0.2% yeast with fish or liver oil. Food and water were given *ad libitum*.<sup>26</sup> The animals were weighed daily and its general condition was recorded including their daily intake of liquid. The Animal Ethics Committee of Amrita Institute of Medical Sciences, Cochin approved the procedures in accordance with the CPCSEA guideline. The rats were divided into two groups of 6 each – Control group (rats without any exposure to ethanol) and the treated group (rats with 1.6 g ethanol exposure/kg body weight/day for 12 weeks). Ethanol was diluted with distilled water to get the desired concentration and fed orally. At the end of the experimental period, the animals were sacrificed after an overnight fast, by applying intra-peritoneal thiopentone. The spleen was dissected out and cleaned with ice-cold saline, blotted dry, and immediately transferred to the ice chamber. Spleen samples were homogenized in an ice-cold suitable buffer and centrifuged to remove the tissue debris. The supernatants were used for the biochemical estimation.

#### Determination of lipid peroxidation

Sample supernatant (0.3 mL) was mixed thoroughly with 2 mL of TCA-TBA-HCl [trichloroacetic acid (TCA) 15% w/v, thiobarbituric acid (TBA) 0.375% w/v, and hydrochloric acid (HCl) 0.25 N]. The solutions were heated for 15 minutes in a boiling water bath, cooled, the flocculent precipitates were removed by centrifugation at 1000 g for 10 minutes, and the absorbances were recorded at 535 nm. The extent of

lipid peroxidation was calculated using molar extinction coefficient ( $\epsilon$ )  $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$  for malondialdehyde.<sup>27</sup>

#### Estimation of glutathione content

For glutathione content, the sample supernatants were immediately mixed with sulfosalicylic acid, shaken well, centrifuged at 3000 rpm for 10 minutes, and 0.5 mL aliquot of the supernatant was mixed with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB 0.01 mM, in 0.01 M phosphate buffer, pH 8.0) and after 2 minutes absorbance were recorded at 412 nm.<sup>28</sup> Glutathione content was determined from a standard curve obtained from pure GSH.

#### Determination of Catalase (EC 1.11.1.6) activity:

The rate of decomposition of  $\text{H}_2\text{O}_2$  (2  $\mu\text{L}$ , 30%) in 0.05 M phosphate buffer (1-mL, pH 7.0) at 240 nm after the addition of sample supernatant was noted. The activity of the enzyme was calculated assuming molar extinction coefficient  $40 \text{ M}^{-1} \text{ cm}^{-1}$  for  $\text{H}_2\text{O}_2$  at 240 nm.<sup>26</sup>

#### Assay of glutathione reductase (GR; EC 1.6.4.2) activity

To 2.6 mL of phosphate buffer, 0.1 mL 15 mM EDTA, 0.1 mL distilled water and 0.1 mL of sample supernatant were added, mixed, waited for 5 minutes. 0.05 mL of 9.6 mM NADPH was added to the mixture. The reaction was initiated by adding oxidized glutathione (GSSG, 65.3 mM). Change in absorbance was monitored at 340 nm, and the specific activity of the enzyme was determined using a molar extinction coefficient for NADPH of  $6.22 \text{ cm}^{-1} / \mu\text{mole}$ .<sup>29</sup>

#### Assay of Glutathione S-transferase (GST; EC 2.5.1.18) activity

To 200  $\mu\text{L}$  of phosphate buffer, 20  $\mu\text{L}$  of 1-chloro-2,4-dinitrobenzene (CDNB, 25 mM in 95% ethanol) and 680  $\mu\text{L}$  of distilled water was added and incubated at  $37^\circ\text{C}$  for 10 minutes. 50  $\mu\text{L}$  of 20 mM GSH was added, mixed well; and then 50  $\mu\text{L}$  of sample supernatant was added, and the change in absorbance was monitored at 340 nm per min, and calculated from extinction coefficient  $9.6 \text{ mM}^{-1} \text{ cm}^{-1}$ .<sup>30</sup>

#### Determination of Superoxide dismutase (EC 1.15.1.1) activity

The superoxide dismutase activity was measured by the inhibition of autooxidation of 0.2 mM pyrogallol (air equilibrated) in 50 mM Tris-HCl buffer (pH 8.2) containing 1 mM diethylenetriamine pentaacetic acid. The rate of autooxidation was monitored at 420 nm. The percentage inhibition of the rate of autooxidation of pyrogallol was initiated by the addition of sample supernatant.<sup>31</sup>

#### Statistical Analysis

All data were analyzed with t-test using the statistical package SPSS (version 11.0, SPSS Inc., Chicago, IL).

## RESULTS

Effects of high dose of ethanol exposure on various oxidative stress-related parameters are presented in Table 1 and Figures 1-3. A significant decrease in spleen-reduced glutathione (GSH) content was found (Table 1 and Figure 1)

on ethanol exposure. The total GSH content of the spleen was reduced by 50% in the treated group compared to the control group. Similarly, there was a nearly 39% decrease in GSH per unit protein. While, the level of lipid peroxidation, presented as thiobarbituric acid reactive substances (TBARS), was found to be increased significantly in both total (Table 1, approximately 26% increase) as well as per unit protein (Figure 1, approximately 55% increase). Upon long-term exposure to a high dose of ethanol, the catalase activity of the spleen remained unaltered (Table 1 and Figure 2). Even though there was more than 19% decrease in total catalase activity of the spleen, the difference between the means was not statistically significant. Similarly, the observed 28% increase in total splenic SOD activity was not found to be statistically significant (Table 1). On the contrary, SOD activity of both groups was found to be significantly different when it is expressed as per unit protein value (Figure 2). Likewise, insignificantly higher total GST activity was observed in the spleen of ethanol-exposed animals (Table 1). When the splenic GST activities of both the animal groups were presented in terms of per unit protein, a significant difference was noted between the groups with 60% higher values in the exposed group in comparison to the control group (Figure 3). In addition, the glutathione reductase activity was found to be significantly decreased when expressed as either total activity (Table 1; lowered by 37% in the treated group) or per unit protein (Figure 3; lowered by 23% in the treated group).

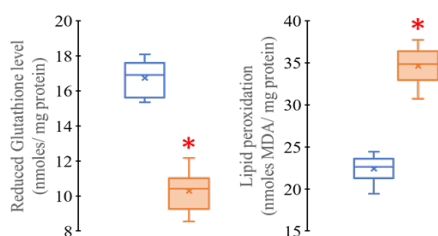
## DISCUSSION

The orogastric ethanol exposure technique allowed maximal ethanol consumption and absolute control over ethanol-induced spleen injury. As ethanol can supplement a large part of energy requirement therefore nutritional disorders may occur in these groups of rats.

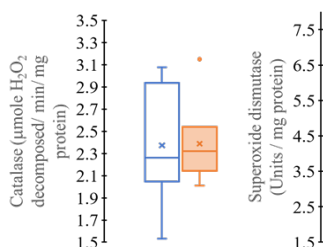
**Table 1:** Comparison of total oxidative stress parameters of spleen of both experimental groups of rats.

Oxidative study parameters	Control	Treated
Total GSH content of spleen (nmoles)	73.60 $\pm$ 7.16	36.69 $\pm$ 7.46*
Total TBARS content of spleen (nmoles)	98.16 $\pm$ 6.09	123.56 $\pm$ 20.82*
Total Catalase activity of spleen ( $\mu\text{mole H}_2\text{O}_2$ decomposed/min)	10.42 $\pm$ 2.48	8.40 $\pm$ 0.92
Total superoxide dismutase activity of spleen (U)	17.48 $\pm$ 3.15	22.35 $\pm$ 5.63
Total glutathione reductase activity of spleen (nmole NADPH breakdown/min)	79.10 $\pm$ 8.92	49.81 $\pm$ 14.59*
Total glutathione S-transferase activity of spleen ( $\mu\text{mole CDNB conjugate formed/min}$ )	16.99 $\pm$ 3.99	22.17 $\pm$ 5.01

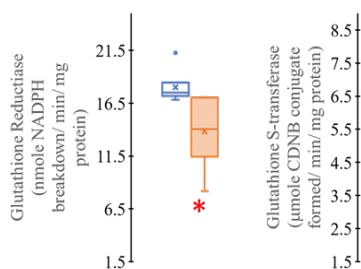
Values are presented as mean  $\pm$  SD of 6 rats in each group. \* indicates a significant difference ( $p < 0.05$ ) between the experimental study groups.



**Figure 1:** Box and whisker plots showing effects of ethanol exposure on levels of reduced glutathione and lipid peroxidation in spleen of experimental groups of rats. □ - Control group, ■ - Treated group, \* indicates  $p < 0.05$  compared to the control group.



**Figure 2:** Box and whisker plots showing effects of ethanol exposure on activities of catalase and superoxide dismutase in spleen of experimental groups of rats. □ - Control group, ■ - Treated group, \* indicates  $p < 0.05$  compared to the control group.



**Figure 3:** Box and whisker plots showing effects of ethanol exposure on activities of glutathione reductase and glutathione S-transferase in spleen of experimental groups of rats. □ - Control group, ■ - Treated group, \* indicates  $p < 0.05$  compared to the control group.

The thiobarbituric acid reactive substances (TBARS) level in spleen was increased due to ethanol exposure. One of the proposed mechanisms of chronic ethanol induced toxicity is membrane damage due to the direct effect of lipid peroxidation products,<sup>11</sup> i.e., TBARS, which was found to be increased in the ethanol-treated rats in the present study. The reduced glutathione (GSH) content of spleen decreased on ethanol exposure. Glutathione is an important antioxidant; it protects cells against damage from free radicals and toxic endogenous and exogenous compounds either by reacting directly with these toxins or by facilitating the reduction of protein disulfide bridges (Griffith, 1985).<sup>32</sup> Glutathione in mitochondria is the only defense available to metabolize  $H_2O_2$ . One study demonstrated that the mitochondrial glutathione system is depressed by chronic ethanol consumption in animals.<sup>33</sup> Mitochondria are not only one of the main cellular sources of reactive oxygen species (ROS), but they also are a key target of ROS. Depletion of GSH in

this organelle renders the cell more susceptible to oxidative stress originating in mitochondria. One of the antioxidant properties of glutathione is mediated by the enzyme glutathione peroxidase. During the detoxification of lipid and other peroxides produced by free radical attack, glutathione peroxidase converts glutathione from a reduced state (GSH) to an oxidized one (GSSG).<sup>34</sup> The NADPH-dependent enzyme glutathione reductase converts GSSG back to GSH, and so almost all intracellular glutathione is reduced. During an oxidative stress, there will be a flux of glutathione to the oxidized form, and the ratio of reduced to oxidized glutathione may then be an indication of this stress.<sup>32</sup> GSH may also exist in another oxidized form, as mixed disulfides with protein thiols. Thiol-sulfide exchange reactions between GSSG and protein thiols are potentially important in biological samples, causing artifacts in the measurement of glutathione. Concentrations of total glutathione therefore give an indication of the intracellular oxidative state.

Catalase activity was unchanged in the present study. The amount of SOD is organ-specific. Three types of SOD have been purified; CuZn-SOD, Mn-SOD<sup>35</sup> and extracellular SOD (ECSOD).<sup>36</sup> CuZn-SOD consists of two protein subunits each unit has an active site containing one Cu ion and one Zn ion.<sup>35</sup> Cu ion serves as an active redox site and Zn ions maintain the protein structure.<sup>37</sup> CuZn-SOD is found abundantly in the cytosol. Mn-SOD is located in the mitochondrial matrix. It has four subunits with Mn in each subunit. EC-SOD is a tetrameric glycoprotein, which contains Cu and Zn. The presence of SOD in various compartments of our body enables SOD to dismutate superoxide radicals immediately. In the present work, superoxide dismutase activities increased on ethanol exposure. Increased expression of SOD results in increased dismutation of superoxide to  $H_2O_2$ . Evidence is accumulating that intermediates of oxygen reduction may in fact be associated with the development of ethanol-associated organ damage.

Ethanol exposure caused a significant decrease in glutathione reductase (GR) activity and an increase in glutathione S-transferase (GST) activity. A significant decrease in hepatic GR activity and glutathione level after ethanol treatment is indicative of impaired reduction of oxidized glutathione to reduced form.<sup>38</sup> Glutathione s-transferase (GST) plays an essential role in eliminating toxic compounds by conjugating them with glutathione. Increased glutathione s-transferase activity and decreased glutathione reductase activity, followed by thiol depletion, are important factors sustaining a pathogenic role for oxidative stress.<sup>11, 39</sup>

## CONCLUSION

The current study confirmed that higher doses of ethanol exposure (1.6 g/ kg body weight/ day) for a long period caused significant oxidative stress in the spleen as a result of the generation of superoxide radicals by ethanol metabolism. Intermediates of oxygen reduction may in fact be associated with the development of ethanol-induced organ damage.



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## **PEER-REVIEWED CERTIFICATION**

During the review of this manuscript, a double-blind peer-review policy has been followed. The author(s) of this manuscript received review comments from a minimum of two peer-reviewers. Author(s) submitted revised manuscript as per the comments of the assigned reviewers. On the basis of revision(s) done by the author(s) and compliance to the Reviewers' comments on the manuscript, Editor(s) has approved the revised manuscript for final publication.