Long-term ethanol consumption leads to lung tissue oxidative stress and injury

Subir Kumar Das1,* and Sukhes Mukherjee2

1Department of Biochemistry; ESI-PGMSR, Joka, Kolkata; 2Amrita Institute of Medical Sciences; Cochin, Kerala India

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Abbreviations: ARDS, acute respiratory distress syndrome; ECM, extracellular matrix; GSH, reduced glutathione; GSSG, oxidized glutathione; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione S-transferase; LPO, lipid peroxidation; MMP, matrix metalloproteinase; ROS, reactive oxygen species; SOD, superoxide dismutase; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TCA, trichloroacetic acid

Background: Alcohol abuse is a systemic disorder. The deleterious health effects of alcohol consumption may result in irreversible organ damage. By contrast, the ravages of alcohol abuse have been viewed as relatively sparing the lung. More than two centuries ago, Benjamin Rush, the first Surgeon General of the United States, noted that pneumonia and tuberculosis were part of the ethanol is metabolized primarily in the liver by alcohol dehydrogenase, a cytosolic enzyme with multiple isoforms. Alcohol can also be metabolized in microsomes via the cytochrome P-450 component CYP2E1. This enzyme complex has a lower affinity for alcohol than the hepatic alcohol dehydrogenase and therefore may not contribute significantly to overall alcohol metabolism following occasional use. Alcohol is metabolized in the lung through the cytochrome P-450 system.
In addition, during alcohol ingestion, alcohol freely diffuses from the bronchial circulation directly through the ciliated epithelium where it vaporizes as it moves into the conducting airways. Moreover, vaporized alcohol can deposit back into the airway lining fluid to be released again into the airways during exhalation. This “recycling” of alcohol vapor results in repeated exposure of the airway epithelium to high local concentrations of alcohol.12

Therefore, we investigated long-term effects of ethanol in the lung in this study.

### Results

Body weight of ethanol exposed rats increased significantly after 12 weeks compared to the control group (0 week) and continued to increase (Table 1). However, there was no significant change in relative weight (g/100 g body weight) of lung with duration of ethanol exposure (Table 1). Once exposed to ethanol, plasma alcohol levels remain unchanged in rats (Table 1).

Ethanol exposure significantly increased nitrite and protein carbonyl content after 4 weeks; thiobarbituric acid reactive substances (TBARS) level, oxidized glutathione (GSSG) content and redox ratio (GSSG/GSH) after 12 weeks; while significantly decreased reduced glutathione (GSH) level after 12 weeks in comparison to the control group in the lung homogenate (Table 2).

Compared to control group, ethanol exposure significantly reduced GPx and Na\(^+\)K\(^+\)ATPase activities after 4 weeks, while glutathione reductase (GR), catalase and superoxide dismutase (SOD) activities after 12 weeks in the lung homogenate (Table 3). However, GST activity increased significantly after 12 weeks of ethanol exposure compared to the control (0 weeks) or 4 weeks of ethanol exposed groups (Table 3). Moreover, there were significant differences in glutathione peroxidase (GPx), glutathione s-transferase (GST) and catalase activities between 12 weeks and 24 weeks of ethanol exposure (Table 3). Interestingly, no significant change in these oxidative stress related parameters was observed between 24 weeks and 36 weeks of ethanol exposed lung homogenates of rats (Table 2).

Total matrix metalloproteinase (MMP) activity increased significantly in ethanol exposed lung tissues. The activity showed a progressive increase, attaining maximum on 24\(^{th}\) week after ethanol exposure (Fig. 1A and B). Histopathological analysis showed that the broncholar and normal alveolar structure was preserved in the control specimen (Fig. 2A), whereas degenerative alveolar structures and leukocytic infiltration were observed in the lung tissues of the ethanol exposed groups (Fig. 2B–E). Severity of inflammation increased with duration of ethanol exposure (Fig. 2B–E). In the controls, median scores of leukocyte infiltration were mainly under 0 and 1, whereas this score and severity of infiltration increased with duration of ethanol exposure (Table 4).

### Discussion

The intragastric ethanol infusion technique allowed maximal ethanol consumption and absolute control over ethanol-induced...
organ injury. In the present study, a dose of ethanol 1.6 g/kg body
weight/day was used based on our previous observation,\textsuperscript{15} where
we found that this amount of ethanol exposure was tolerable
for long period, causing organ damage and is partially reversible
during abstinence.\textsuperscript{14} However, continuous ethanol exposure
maintained plasma ethanol level in a steady state in our study (Table 1)
is in agreement with other report.\textsuperscript{15} It is also assumed that alcohol metabolism through the cytochrome p-450 system
in the lung is significant\textsuperscript{11} and may be sufficient to exert significant oxidative stress in the lung,\textsuperscript{16,17} due to their unique structure
and function.\textsuperscript{18}

Reactive oxygen species (ROS) are constantly produced in the cells, but under normal physiological conditions the enzymatic and non-enzymatic antioxidant mechanisms of the cell overcome the destructive potential of ROS. There is a delicate balance between the production of ROS and endogenous protection mechanisms. Overproduction of ROS or a decrease in antioxidants results in oxidative stress, and may cause cellular damage by peroxidation of membrane lipids, sulphydryl enzyme inactivation, protein cross-linking and DNA breakdown. This damage may be involved in the etiology of diverse human diseases.\textsuperscript{16,18-22} Consequently, organ damage may further increase ROS production.

One of the proposed mechanisms of chronic ethanol induced-toxicity is the membrane damage due to the direct effect of lipid peroxidation products,\textsuperscript{13} i.e., TBARS, which was found to be increased in the ethanol exposed rats in the present study (Table 2). Chronic alcohol ingestion enhanced superoxide generation in the lung tissue.\textsuperscript{23} Protein nitration has been suggested to be a final product of highly reactive nitrogen oxide intermediates (e.g., peroxynitrite) formed in reactions between NO and oxygen-derived species such as superoxide. Nitrite is a stable metabolite of NO in vivo. Increased nitrite level was observed in lung homogenate of ethanol exposed rats in the present study (Table 2) through the

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Table 3. Effect of ethanol on glutathione reductase (GR), glutathione peroxidase (GPx), glutathione s-transferase (GST), catalase, superoxide dismutase (SOD) and Na\textsuperscript{+}K\textsuperscript{−}-ATPase activities in lung homogenate

|                  | GR\textsuperscript{*} | GPx\textsuperscript{*} | GST\textsuperscript{*} | Catalase\textsuperscript{‘} | SOD\textsuperscript{‘} | Na\textsuperscript{+}K\textsuperscript{−}-ATPase\textsuperscript{‘} |
|------------------|------------------------|------------------------|------------------------|---------------------------|------------------------|----------------------------------|
| Control (0 week) | 14.95 ± 1.26           | 24.82 ± 0.71           | 4.43 ± 0.08            | 3.26 ± 0.08               | 1.36 ± 0.08            | 223 ± 9.55                        |
| 4 week           | 13.38 ± 1.07           | 19.57 ± 1.24\textsuperscript{‘} | 4.5 ± 0.09            | 3.1 ± 0.09                | 1.28 ± 0.09            | 171 ± 10.4\textsuperscript{‘}      |
| 12 week          | 11.35 ± 0.89\textsuperscript{‘} | 15.67 ± 1.5\textsuperscript{‘} | 6.58 ± 0.26\textsuperscript{‘} | 2.91 ± 0.11\textsuperscript{‘} | 1.08 ± 0.11\textsuperscript{‘} | 134.3 ± 7.2\textsuperscript{‘}     |
| 24 week          | 10.12 ± 0.77\textsuperscript{‘} | 13.15 ± 0.79\textsuperscript{‘} | 7.13 ± 0.28\textsuperscript{‘} | 2.65 ± 0.1\textsuperscript{‘} | 0.97 ± 0.1\textsuperscript{‘} | 124 ± 8\textsuperscript{‘}        |
| 36 week          | 9.07 ± 1.04\textsuperscript{‘} | 11.67 ± 1.42\textsuperscript{‘} | 7.5 ± 0.37\textsuperscript{‘} | 2.58 ± 0.11\textsuperscript{‘} | 0.93 ± 0.08\textsuperscript{‘} | 118.5 ± 12.4\textsuperscript{‘}    |
| F variance       | 41.465                 | 113.506                | 212.244                | 47.752                    | 23.292                 | 120.875                          |
| Significance     | <0.001                 | <0.001                 | <0.001                 | <0.001                    | <0.001                 | <0.001                           |

Values are mean ± SD of 6 rats in each group. p values: *<0.001, **<0.01, *<0.05 compared to control group (Group 1); *<0.001, **<0.01, *<0.05 compared to 4 week ethanol treated group (Group 2); *<0.001, **<0.01, *<0.05 compared to 12 week ethanol treated group (Group 3). No significant change was observed between 24 weeks and 36 weeks of ethanol exposed rats. \textsuperscript{*}, nmole NADPH breakdown/min/mg protein; \textsuperscript{‘}, μmole CDNB conjugate formed/min/mg protein; \textsuperscript{‘}, One unit of the enzyme was the amount of SOD capable of inhibiting by 50% the rate of NADH oxidation observed in the control. The specific activity was expressed as units/mg protein; \textsuperscript{‘}, nmole Pi/mg/protein/h.

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Figure 1. Changes in the total activity of matrix metalloproteinases in ethanol exposed rat lung with time. Rats were fed with ethanol (1.6 g/kg body weight/day). (A) Extracts of lung tissue samples (100 μl) from control and ethanol exposed rats of different time interval (up to 36 weeks) were copolymerised with acrylamide-bisacrylamide containing gelatin in Tris buffer (pH 8.8). After polymerization, the gels were then incubated in substrate buffer, stained with Coomasie brilliant blue and destained with methanol-acetic acid-water. A blank was prepared without enzyme. (B) Activity measured by densitometric analysis. Values given are average of 3 experiments ± SD.
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 oxidized to reduced glutathione may then be indication of this stress. Decreased GSH level, increased GSSG level and redox ratio with duration of ethanol exposure indicated time dependent elevation of oxidative stress in the lung (Table 2).

Chronic ethanol ingestion resulted in significant decrease in GPx activity in the lung may be due to either free radical dependent inactivation of enzyme or depletion of its substrates i.e., GSH and NADPH. Glutathione s-transferase (GST) plays an essential role by eliminating toxic compounds by conjugating them with glutathione. Increased GST activity, and decreased GPx and GR activities (Table 3), followed by thiol depletion (Table 2) are important factors in sustaining a pathogenic role for oxidative stress.

The presence of superoxide dismutase (SOD) in various compartments of human body enables SOD to dismute superoxide radical immediately. The cytochrome P450 2E1 was demonstrated to generate higher amounts of H₂O₂, and is linked to increased generation of hydroxyl radicals. Decreased SOD and catalase activities with duration of ethanol exposure in the lung in this study (Table 3) may be due to loss of NADPH, or generation of superoxide, or increased lipid peroxidation or combination of all.

Leukocyte infiltration was evaluated to determine the severity of oxidative damage in the lung. Each section was divided into subsections, and leukocyte infiltration was assessed using the scale for comparison at a magnification of X400: 0 = no extravascular leukocytes; 1 ≤ 10 leukocytes; 2 = 10 - 45 leukocytes; and 3 ≥ 45 leukocytes.

| Group     | Leukocyte infiltration score |
|-----------|-----------------------------|
| 0         | 1 2 3 total                |
| Control   | 5 1 2 - 6                 |
| 4 week    | - 2 3 1 6                 |
| 12 week   | - 1 3 2 6                 |
| 24 week   | - 2 3 2 7                 |
| 36 week   | - 1 4 3 8                 |

Leukocyte infiltration score of the control and ethanol exposed groups

Figure 2. Lung tissues fixed in formalin, processed for hematoxylin and eosin stain to assess morphological changes under microscope. (A) Normal texture of lung in control animals; (B) 4 weeks ethanol treated lung with mild inflammation; (C) 12 weeks ethanol treated rat-Lung alveoli with marked interstitial inflammation with inflammatory exudates in some of the alveoli; (D) 24 weeks ethanol treated rats-Lung alveoli filled with inflammatory exudates; (E) 36 weeks ethanol treated rat-severe inflammation.

Table 4. Leukocyte infiltration score of the control and ethanol exposed groups
Na’-K’ ATPase participates in lung fluid clearance by creating the active transport of sodium.³⁻⁹ Oxidative stress plays a role in mediating the ethanol-induced downregulation of lung Na’-K’ ATPase in this study. GSH depletion seems to be a major determinant of this effect.³² Increased lipid peroxidation combined with decreased tissue Na’-K’ ATPase activity in the lung in this study (Table 3) may be associated with impairment of membrane phospholipids.³⁹ The decreased enzyme activity gives rise to the disintegration of the cells and consequently to the thickening of the air-blood barrier, alveolar degeneration and leukocyte infiltration,³¹ as observed histopathologically in the lungs of the ethanol-exposed rats (Fig. 2 and Table 4). Ethanol-induced reactive species causes phenotypic alterations in the lung and alters the lung’s response to inflammatory stimuli.³⁶ These inflammatory mediators lead to leukocyte activation, expression of endothelial adhesion molecules and vascular endothelial damage.³⁴

The alveolar extracellular matrix (ECM) is considered to be a static structural component of the lung tissue. It serves as a modulator of cell growth and development, inflammation, angiogenesis, cell migration, tissue differentiation and repair. There is growing evidence that aberrant remodeling of the ECM contributes both to the early inflammatory phase as well as to the later fibroproliferative phase of the syndrome.³⁵,³⁶ Evidence suggests that degradation of the ECM by (MMPs) may contribute to the development of lung injury,³⁷ as observed in this study (Fig. 1).

Though biochemical alterations and oxidative stress related parameters respond early in alcoholism than the histopathological changes;³⁸ leukocyte infiltration and matrix metalloproteinases activation were observed in ethanol-treated lungs and its severity increased with duration of ethanol exposure in the present study. In conclusion, our results suggest that long-term ethanol administration aggravates systemic and local oxidative stress, which is one of the leading causes of lung injury.

**Methods**

**Materials.** Ethanol was purchased from Bengal Chemicals, Kolkata. Chemicals from Sisco Research Laboratory (SRL), India, Sigma Chemical Co., St. Louis, MO; and E. Merck were used. Protein-carbonyl content.

**Animals and treatment.** The male albino rats (16–18 weeks old) of Wistar strain weighing 200–220 g were housed in plastic cages inside a well-ventilated room, with the room temperature maintained at 25 ± 2°C, with a 12 h light/dark cycle. Animals had free access of standard diet containing (% bengal gram, 31; gingelly oil cake, 30; wheat, 28; polished rice, 10; salt mixture, 0.5; vitamin-mineral mixture, 0.3; and yeast with fish or liver oil, 0.2. Food and water were given ad libitum. Animals were weighed daily and their general condition and behavior were recorded, including their daily intake of food.

The rats were divided into the following five groups of 6 animals each. Group 1: control—were fed normal diet and water; Group 2: ethanol (1.6 g ethanol/kg body wt/day) treated for 4 wks; Group 3: ethanol (1.6 g ethanol/kg body wt/day) treated for 12 wks; Group 4: ethanol (1.6 g ethanol/kg body wt/day) treated for 24 wks; Group 5: ethanol (1.6 g ethanol/kg body wt/day) treated for 36 wks.

At the end of the experimental period, blood samples were collected from retro-orbital plexus of rats. The animals were then sacrificed after overnight fast, by intraperitoneal injection of sodium pentobarbital (50 mg/kg body wt). The lung was dissected out, cleaned with ice-cold saline, blotted dry and immediately transferred either to the ice chamber for biochemical studies or fixed in 10% buffered formal saline for histopathological examinations. Various oxidative stress related non-enzymes such as TBARS, nitrite, protein carbonyl, GSH, GSSG; and enzymes activities such as GR, GPX, GST, catalase, SOD and Na’K’ATPase were estimated. The Animal Ethics Committee of the Institution approved the procedures in accordance with the CPCSEA guideline.

**Biochemical methods.** Blood collected from retro-orbital plexus was used for blood ethanol concentration estimation using an ethanol assay kit (Sigma).

Lung samples were homogenized in 0.25 M sucrose solution, and were used to estimate tissue protein.³⁹

**Determination of thiobarbituric acid reactive substances (TBARS).** Lung samples were homogenized in ice-cold 0.25 M tris buffer (pH 7.4). 0.3 ml of this homogenate 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 min in a boiling water bath, cooled; the flocculent precipitates were removed, and the absorbance was recorded at 535 nm. The extent of lipid peroxidation was calculated using molar extinction coefficient 1.56 x 10⁵ M⁻¹cm⁻¹.⁴⁰

**Nitrite estimation.** Sulfanilamide (1%, 50 μl) in 2.5% orthophosphoric acid (Griess reagent 1), followed by N-(1-naphthyl) ethylenediamine (0.1%, 50 μl) in distilled water (Griess reagent 2) were added to the tissue homogenate, incubated in dark at room temperature for 10 min. The absorbance was measured at 540 nm. The concentration of nitrite was measured by using NaNO₂ as a standard.⁴¹

**Protein-carbonyl content.** Proteins were precipitated with 20% trichloroacetic acid and centrifuged. The precipitates were resuspended in 2,4-dinitrophenylhydrazine (10 mM) and vortexed at 10 min intervals for 1 h at room temperature. The pellets were washed thrice with ethanol/ethyl acetate, resuspended in 0.6 ml of 6 M guanidine hydrochloride, incubated at 37°C for 15 min and centrifuged at 5,000 g for 3 min. The absorbance of supernatant was measured at 366 nm for carbonyl content, and calculations were performed with a value of 22,000 M⁻¹cm⁻¹.⁴²

**Glutathione content.** The lung (~100 mg) samples were homogenized in ice-cold 0.1 M phosphate buffer (pH 7.4). For reduced glutathione (GSH) content, the homogenates were immediately mixed with sulfosalicylic acid, shook well and centrifuged. Each supernatant fraction was mixed separately with 5,5’-dithiobis(2-nitrobenzoic acid) (in 0.01 M phosphate buffer, pH 8) and absorbance was recorded at 412 nm.⁴³ For oxidized glutathione, 200 μl supernatant was added to 3.78 ml of water to which 40 μl of 2-vinylpyridine was mixed to mask the GSH and left at room temperature for 3 h before estimation as described above.⁴⁴
Glutathione reductase (GR, EC 1.6.4.2) activity. The tissues were homogenized in phosphate buffer (0.12 M, pH 7.2), and were mixed to 15 mM EDTA in phosphate buffer and 9.6 mM NADPH. The reaction was initiated by adding oxidized glutathione (GSSG, 65.3 mM). Change in absorbance was monitored at 340 nm; and the specific activity was determined using extinction coefficient for NADPH of 6.22 cm⁻¹/μM.¹⁵

Glutathione peroxidase (GP, EC 1.11.1.9) activity. Glutathione peroxidase activity was measured based on the principle that oxidized glutathione produced by GP is reduced at a constant rate by glutathione reductase with NADPH as a cofactor. NADPH allows maintaining predictable levels of reduced glutathione. The oxidative rate of NADPH was monitored at 340 nm.¹⁶

Glutathione-s-transferase (GST; EC 2.5.1.18) activity. The tissues were homogenized using phosphate buffer (0.05 M, pH 6.5). 1-chloro-2,4-dinitrobenezene (CDNB) in phosphate buffer was mixed with reduced glutathione, and then tissue extract was added. The change in absorbance was monitored at 340 nm, and calculated from extinction coefficient 9.6 mM⁻¹ cm⁻¹.¹⁷

Catalase (EC 1.11.1.6) activity. The tissues were homogenized in 0.05 M phosphate buffered saline (pH 7.0). The rate of decomposition of H₂O₂ (2 μl, 30%) in 0.05 M phosphate buffer (1 ml, pH 7.0) at 240 nm after addition of homogenized tissue was noted. The specific activity was calculated assuming molar extinction coefficient of 22,000 M⁻¹ cm⁻¹ at 240 nm.¹⁸

Superoxide dismutase (SOD, EC 1.15.1.1) activity. Superoxide dismutase activity was measured by the inhibition of auto-oxidation of 0.2 mM pyrogallol (air equilibrated) in 50 mM at Tris-HCl buffer (pH 8.2) containing 1 mM diethylene-triamine pentaacetic acid. The rate of autooxidation was inhibited at 420 nm. The inhibition of pyrogallol autooxidation was initiated by addition of tissue homogenate.¹⁹

Na⁺-K⁺ ATPase activity. Specific activity of Na⁺-K⁺ ATPase estimation is based on the principle that the inorganic phosphate is released from protein in presence of 3 mM disodium adenosine 5'-triphosphate in incubation medium.²⁰

Multiwell zymogram (Total MMP activity). 100 μl tissue samples were placed in 24-well containing plate and incubated at 37°C for 30 mins for enzyme activation. Zymo gel (15 mg gelatin dissolved in 3.75 ml of Tris buffer pH 8.8, 3.75 ml acrylamide-bisacrylamide (30:0.3), 7.125 ml double distilled water, 150 μl 10% ammonium persulphate (freshly prepared) and 15 μl TEMED) was added and allowed to settle for 1 h. The gels were then placed in 6-well containing plates with zymo buffer (calcium chloride buffer, pH 7.5: 3.03 g Tris-HCl and 0.36 mg CaCl₂ dissolved in 500 ml double distilled water) and incubated overnight. After removing the zymo buffer, the gels were stained with Coomasie brilliant blue for 3 to 4 h. The gels were finally destained with methanol-acetic acid-water.²¹

Histopathological examination. Lung tissues fixed in 10% formalin, routinely processed and embedded in paraffin. Sections were cut (4 μm thick) and stained with hematoxylin and eosin to assess morphological changes under microscope. Leukocyte infiltration was evaluated to determine the severity of oxidative damage that resulted from ethanol intoxication. Each section was divided into 10 subsections, and leukocyte infiltration was assessed using the following scale for comparison at a magnification of x400: 0 = no extravascular leukocytes; 1 ≤ 10 leukocytes; 2 = 10–45 leukocytes; and 3 ≥ 45 leukocytes.

Statistical analysis. Results were expressed as mean ± SD (standard deviation). All statistical analyses were performed by one-way analysis of variance (ANOVA) with multiple comparison tests and student’s t-test using the Statistical Package for Social Sciences, version 11 (SPSS, Chicago, IL). The values of significance were evaluated with p<0.05. The differences were considered significant at p<0.05.

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