Gender differences in alcohol-induced oxidative stress and altered membrane properties in erythrocytes of rats

Kindinti Rameshwar Reddy, Vaddi Damodara Reddy, Pannuru Padmavathi, Godugu Kavitha, Bulle Saradamma and N C Varadacharyulu*

*Department of Biochemistry, Sri Krishnadevaraya University, Anantapur 515055, Andhra Pradesh, India

bDepartment of Medicine, Center for Systems Biology & Oxygen Sensing, University of Chicago, Chicago-60637, IL, USA

cDepartment of Medicine, Section of Hematology/Oncology, University of Illinois at Chicago, Chicago - 60612, IL, USA

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Alcohol-induced oxidative stress leads to imbalance between reactive oxygen species (ROS) and the antioxidant defense system, resulting in oxidative damage to membrane components such as lipids and proteins, ultimately altering membrane properties. In this study, we assessed oxidative stress status and alterations in erythrocyte membrane properties in alcohol-administered rats with respect to gender difference. Alcohol (20% v/v) administered rats of both genders showed significant changes in plasma lipid profile with elevated nitrite/nitrate levels. Furthermore, alcohol-administration significantly decreased erythrocyte antioxidant enzymes and enhanced erythrocyte membrane lipid peroxidation, cholesterol/phospholipid (C/P) ratio and Na$^+/K$-ATPase activity in both males and females. Besides, anisotropic studies revealed that alcohol-administration significantly decreased erythrocyte membrane fluidity. In conclusion, alcohol-administration significantly increased oxidative stress by decreasing antioxidant status, and subsequent generation of ROS altered membrane properties by altering fluidity and Na$^+/K$-ATPase activity. Female rats were more vulnerable to alcohol-induced biochemical and biophysical changes in plasma and erythrocyte including oxidative stress than male rats.

Keywords: Alcohol, Erythrocyte membrane, Fluidity, Gender, Nitric oxide

Chronic alcohol consumption causes metabolic disorders, leading to a broad spectrum of deleterious health effects, resulting in alcohol liver disease (ALD), coronary heart disease (CHD) and other diseases in both males and females$^{1,2}$. There has been a marked increase of female alcoholics worldwide, including India in recent years$^3$. Females absorb and metabolize alcohol differently than males$^4$. In general, females have less body water than males of similar body weight, thus achieve higher concentrations of alcohol in the blood after drinking equivalent amounts of alcohol$^5$. Previous studies also suggested that females are more susceptible than males to alcohol-related organ damage$^6$. Blood and its components are exposed to alcohol for longer time after consumption and red cells are more susceptible to alcohol-induced oxidative damage, due to high content of iron and polyunsaturated fatty acids. Hence blood cell is ideal model to study alcohol-induced biochemical and biophysical changes. So far, no systematic study has been carried out on alcohol-induced oxidative stress in plasma and erythrocytes with respect to gender differences.

Nitric oxide (NO) is a signaling molecule involved in diverse cellular functions, and mediates various physiological and pathological processes$^7$. Previous studies indicated that alcohol-induced NO plays a pivotal role in biochemical changes of erythrocyte membranes in male rats$^8$. Removing superoxide ion and hydroxyl radical is probably one of the most effective defense mechanisms against a variety of diseases. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are among most important antioxidant enzymes in free radical scavenger system in normal conditions$^9$.

Previous studies have suggested that alcohol impairs the antioxidant defense mechanisms of the cell, leading to membrane deformity, specifically by changing the lipid fluidity in the middle zone of the bilayer$^{8,10}$. Phospholipids are exquisitely capable of regulating membrane protein activity$^{11}$. Alcohol affects the physical properties and functions of membranes, which leads to alterations in ion...
channels, receptors and disruption of membrane lipids\textsuperscript{12}. The activity of most membrane-bound enzymes is regulated by the physiochemical state of their membrane lipid environment.

Alcohol-induced generation of free radicals affects membrane-linked enzyme activity through modification of membrane fluidity\textsuperscript{8}. Limited studies available on females are confined to behavioral and psychological aspects ignoring other health related criteria, such as biochemical and biophysical changes\textsuperscript{13}. Under pathological conditions, alterations in chemical composition, properties and functions of bio-membranes and antioxidant status serve as indices to assess the etiology and progression of pathology. Thus, studies on females are warranted to understand the biochemical perturbations associated with chronic alcohol intake and to develop suitable gender-specific therapeutic strategies. The present investigation has evaluated alcohol-induced oxidative stress in plasma and erythrocytes with respect to gender differences.

\section*{Materials and Methods}

\subsection*{Chemicals}
The chemicals used in the study were of analytical reagent grade procured from Sigma Chemicals Co., St. Louis MO, USA and SISCO Research Laboratories Ltd., Mumbai, India.

\subsection*{Animals and experimental design}
Two-month old male and female albino Wistar rats weighing about 120-130 g were procured from Sri Venkateswara Enterprises, Bangalore, India. Animals were divided into 4 groups of eight animals in each group according to gender and alcohol treatment. Alcohol (20\% v/v) was administered orally with the help of a stomach tube at a dose of 4 g/kg b.wt/day for a period of 60 days. The dose of alcohol used in the present study was chosen based on earlier studies\textsuperscript{14-16}. Control rats received iso-caloric amounts of glucose equivalent to that of alcohol. Animals were maintained on standard pellet diet (Hindustan Lever Ltd., Bombay, India) and water \textit{ad libitum} for 60 days. At the end of experimental period, the rats in each group were fasted overnight. The blood was collected by cardiac puncture into heparinized tubes, separated into plasma and cells and analyzed immediately. Tissues were collected and stored at -80\degreeC for further analysis. The present work on animal models was undertaken with prior approval by our departmental as well as Institutional Ethics Committee.

\subsection*{Plasma analysis}
Plasma triglycerides\textsuperscript{17}, total cholesterol\textsuperscript{18} and HDL-cholesterol\textsuperscript{19} were determined by the enzymatic colorimetric methods using appropriate kits (Span diagnostics, Surat, India) according to the manufacturer’s instructions. The concentration of LDL-cholesterol was calculated according to the formula: LDL-C = (TC-HDL-C)-(TG/5)\textsuperscript{20}. Atherogenic index was calculated as described previously\textsuperscript{21}. Plasma thiobarbituric acid reactive substances (TBARS)\textsuperscript{22}. Total nitrite and nitrate levels were also determined\textsuperscript{23}.

\subsection*{Erythrocyte membrane lipid analysis}
Erythrocyte membranes were prepared according to the method described previously\textsuperscript{24}. Lipids were extracted from erythrocyte membrane\textsuperscript{25}. To the erythrocyte membrane preparations, 5 ml of methanol was added, followed by chloroform. After 30 min, the same was filtered to collect filtrate and the residue was again subjected to same step and filtered again. The filtrates were pooled and used for cholesterol\textsuperscript{26} and phospholipids\textsuperscript{26} analysis.

\subsection*{Erythrocyte antioxidant enzyme activities}
Erythrocytes were washed thrice with 0.9\% NaCl and suspended in 1 vol. of 0.9\% NaCl. The packed cell volume was adjusted to 5\% with PBS, pH 7.5 (10 mM phosphate buffer saline). Hemoglobin content in erythrocytes was determined\textsuperscript{27}. Reduced glutathione (GSH) content was estimated and expressed as \textmu moles/g Hb\textsuperscript{28}. The superoxide dismutase (SOD) activity was measured based on the ability of the enzyme to inhibit the autoxidation of adrenaline and activity was expressed as Units/mg Hb/min\textsuperscript{29}. The catalase (CAT) activity in hemolysate was also estimated and the activity of the enzyme was calculated using the extinction coefficient of H\textsubscript{2}O\textsubscript{2} as 0.071 cm\textsuperscript{-1} mol\textsuperscript{-1} and expressed as IU x 10\textsuperscript{4}/g Hb at 37\degreeC\textsuperscript{30}. The glutathione peroxidase (GPx) activity was measured and the activity was expressed as \textmu moles of glutathione oxidized/min/mg Hb\textsuperscript{31}.

\subsection*{Erythrocyte membrane fluidity studies using DPH}
The quantitative measurement of erythrocyte membrane fluidity was performed by the fluorescence polarization technique with 1,6 diphenyl 1,3,5 hexatriene (DPH) as fluorescence probe as described earlier\textsuperscript{32}. Briefly, erythrocyte preparations (50 \textmu g protein) were suspended in 50 mM DPH solubilized in tetrahydrofuran and incubated at 37\degreeC for 30 min. Fluorescence polarization was determined using a
Hitachi fluorescence spectrophotometer (Hitachi, Japan) equipped with rotating polarizing filters with samples held at 25°C. Samples were excited at 360 nm and the emission intensity was read at 435 nm. Polarization (P) and fluorescence anisotropy (γ) were calculated using the equation:

\[ P = \frac{I_{VV} - I_{VH}}{I_{VV} + I_{VH}} \]

\[ G \]

in which \( I_{VV} \) and \( I_{VH} \) represent the intensities measured parallel and perpendicular to the vertical axis of excitation beam and \( G \) is the correction factor \( \frac{I_{VH}}{I_{HH}} \gamma \) calculated using the formula \( \gamma = \frac{2P}{3-P} \).

**Erythrocyte membrane TBARS and Na\(^+\), K\(^+\)-ATPase activity**

Erythrocyte membrane TBARS were determined as described earlier\(^{22}\). Na\(^+\)/K\(^+\)-ATPase activity was determined indirectly by estimating the phosphorus liberated after the incubation of erythrocyte membrane in a reaction mixture containing the substrate ATP with the co-substrate elements at 37°C for 15 min\(^{33}\). The reaction arrested by adding 1.0 ml of 10% TCA. The phosphorus content from the TCA supernatants was then determined\(^{34}\). Membrane protein concentration was estimated as described by Lowry \textit{et al}.\(^{35}\).

**Statistical analysis**

Data were subjected to Duncan multiple range test (DMRT), followed by student ‘t’ test and presented as mean ± SD of each group. A **p<0.01 and *p<0.05 was statistically significant between groups.

**Results**

**Effect of alcohol on plasma lipid profile with respect to gender**

In the present study, we investigated the effect of alcohol on plasma lipid profile with respect to gender and the data are presented in Table 1. Results revealed that alcohol-administered male and female rats showed significantly (\( p<0.05 \)) increased plasma total cholesterol, triglycerides and low HDL-cholesterol compared to respective controls. However, alcohol-administered female rats showed slightly higher triglyceride levels, though not significantly different compared to male rats. Further, alcohol-administered male and female rats showed significantly (\( p<0.05 \)) higher LDL and VLDL-cholesterol levels as well as AI with respect to their controls.

**Effect of alcohol on TBARS, total nitrite and nitrate levels and antioxidant status with respect to gender**

Measurement of TBARS reveals the oxidative stress status. In the present study, we measured TBARS levels in plasma (Fig. 1) and erythrocyte membrane (Fig. 2) of controls and alcohol-administered rats in both genders. Both plasma and erythrocyte membrane of alcohol-administered rats showed significantly (\( p<0.05 \)) increased TBARS

### Table 1—Gender specific changes in plasma lipid profile of alcohol-administered rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Male Control</th>
<th>Alcohol</th>
<th>Female Control</th>
<th>Alcohol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>77 ± 4.13</td>
<td>88 ± 3.12*</td>
<td>74 ± 2.14</td>
<td>99 ± 3.36*#</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>75 ± 5.38</td>
<td>92 ± 6.59*</td>
<td>72 ± 3.43</td>
<td>97 ± 7.12*</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>36 ± 2.05</td>
<td>23 ± 3.61*</td>
<td>37 ± 1.91</td>
<td>25 ± 2.17*</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td>27 ± 1.83</td>
<td>47 ± 4.22*</td>
<td>23 ± 1.97</td>
<td>57 ± 1.24*#</td>
</tr>
<tr>
<td>VLDL-cholesterol</td>
<td>15 ± 1.31</td>
<td>19 ± 1.84*</td>
<td>14 ± 1.81</td>
<td>18 ± 1.65*</td>
</tr>
<tr>
<td>Atherogenic index</td>
<td>1.11 ± 0.14</td>
<td>2.64 ± 0.16*</td>
<td>1.04 ± 0.13</td>
<td>3.02 ± 0.08*#</td>
</tr>
</tbody>
</table>

All values are expressed as mg/dL. A \( p<0.05 \) is statistically significant between groups. *Indicates statistically significant from controls. #Indicates statistically significant from alcohol-administered male rats.
compared to their respective controls. However, there was a significant \((p<0.05)\) increase in erythrocyte membrane TBARS of alcohol-administered male rats as compared to female rats.

Total nitrite and nitrate levels reveal the nitric oxide production and the data are presented in Fig. 3. Our results showed significantly \((p<0.05)\) increased nitrite and nitrate levels in alcohol-administered rats of both genders compared to their control rats, while alcohol-administered female rats showed more nitrite and nitrate levels compared to male rats.

Table 2 shows erythrocyte antioxidant enzyme activities and the content of GSH. Results showed significant \((p<0.05)\) decrease in erythrocyte CAT, SOD, GPx and GSH content in alcohol-administered rats of both genders with respect to their controls. Interestingly, the decrease in the observed antioxidant status was more prominent in alcohol-administered female rats compared to male rats.

Effect of alcohol on erythrocyte membrane fluidity with respect to gender

Alcohol affects membrane fluidity, thus assaying membrane total cholesterol and total phospholipids provides an idea about membrane fluidity. We analyzed erythrocyte membrane total cholesterol and total phospholipids in control and alcohol-administered rats (Table 3). We found significant \((p<0.05)\) increase in total cholesterol, phospholipids and the consequent C/P ratio in alcohol-administered rats of both genders compared to their respective controls. The increased total cholesterol, phospholipids and the consequent C:P ratio was more prominent in alcohol-administered female rats than male rats.

We further analyzed erythrocyte membrane fatty acyl chain fluidity using hydrophobic fluorescent probe DPH, which reveals the movement of fatty acyl chains. Results showed significant \((p<0.01)\) increase in anisotropic value \((\gamma)\) in both genders of alcohol-administered rats compared to control rats (Fig. 4).
However, alcohol-administered female rats showed significantly ($p<0.05$) higher $\gamma$ value than male rats.

**Effect of alcohol on erythrocyte membrane Na$^+$/K$^+$-ATPase activity with respect to gender**

Alcohol-administration affects membrane transport. Analysis of Na$^+$/K$^+$-ATPase activity reveals the effect of alcohol on membrane-bound enzymes. The activity of Na$^+$/K$^+$-ATPase was measured in controls and alcohol-administered rats and the data were presented in Fig. 5. Alcohol-administered rats of both genders showed significantly ($p<0.05$) increased erythrocyte membrane Na$^+$/K$^+$-ATPase enzyme activity compared to their respective controls. However, we noticed alcohol-administered female rats showing significantly ($p<0.05$) higher activity than alcohol-administered male rats.

**Discussion**

Studies on alcoholism have clearly indicated that gender differences play a major role in alcohol intoxication and other adverse effects of alcohol use$^{36}$. In the present study, we studied alcohol-induced oxidative stress status on plasma, erythrocytes and erythrocyte membrane with respect to gender. Our results revealed that alcohol-administered rats showed increased plasma total cholesterol, triglycerides, LDL-cholesterol, VLDL-cholesterol and atherogenic index when compared to their controls, but the increase was more pronounced in female alcoholic rats than males.

Previous study in humans has revealed that moderate alcohol consumption increases HDL-cholesterol and is beneficial; however, chronic or heavy alcohol consumption decreases HDL-cholesterol levels$^{37}$. In the present study, chronically alcohol-administered rats showed decreased HDL-cholesterol with increased atherogenic index, which was more pronounced in alcohol-administered female rats, suggesting female alcoholic rats are likely more prone to atherosclerosis than males. Reports have revealed that alcohol metabolizing enzyme content, as well as the gastric alcohol dehydrogenase activity have been found to be
lower in females than males, leading to high blood alcohol levels in females\textsuperscript{38}. Gender-specific differences in the body composition, estrogen levels and ethanol metabolism might also contribute to differences in ethanol elimination\textsuperscript{39}. Alcohol elimination rates and vulnerability to ethanol toxicity are still not clear in female rats.

Sex differences in susceptibility to alcohol-induced oxidative injury have been observed in experimental animal models\textsuperscript{3,40,41}. ROS are continuously produced in red blood cells (RBC) due to the high oxygen tension in arterial blood and their abundant iron content. Free radicals can directly damage RBC membranes by peroxidation of membrane polyunsaturated fatty acids and cause considerable changes in the structural organization and functions of cell membranes and making the membrane leaky\textsuperscript{42}. Furthermore, a number of pathological phenomena, such as increased membrane rigidity in case of erythrocyte membrane, decreased cellular deformability, reduced erythrocyte survival and lipid fluidity have been reported in alcoholic rats\textsuperscript{43}. In the present study, increased TBARS levels and decreased erythrocyte antioxidant status observed in alcohol-administered rats reflected the enhanced oxidative stress in both genders, however, the effect was more pronounced in females, indicating females were more susceptible to alcohol-induced oxidative damage than males.

Our study clearly revealed that increased oxidative stress, decreased antioxidant status, and imbalance in NO homeostasis were responsible for the adverse changes due to alcohol intake and it corroborated with previous studies\textsuperscript{8,44}. It is well-known that NO regulates virtually every cellular function and also mediates cellular damage in a wide range of conditions. NO often interacts with superoxide, thereby forming peroxynitrite. Although peroxynitrite is a strong oxidant, it reacts at a relatively slow rate with most biological molecules. Peroxynitrite is able to cross cell membranes through anion channels, where it reacts slowly and selectively throughout the cell to produce downstream biological and pathological effects\textsuperscript{45}. Nitric oxide synthase (NOS), mainly the iNOS isoform is responsible for NO production in alcohol-treated rats\textsuperscript{46}. Further, NO production is known to itself regulate rheological behavior, preserves cellular mechanics and modulates membrane fluidity\textsuperscript{47,49}. Alcohol-induced over-production of NO could bind to hemoglobin and reduce oxygen carrying capacity. Consequently, the affected cells undergo hypoxia, which is known to augment NO production itself\textsuperscript{50}. In the present study, increased NO could be due to either alcohol alone or alcohol-induced hypoxia. Furthermore, due to long-term presence of alcohol in plasma, the involvement of NO leading to decreased erythrocyte membrane fluidity among alcohol-administered rats could not be ruled out. Experimental data suggested NO might mediate alcohol-induced biochemical and biophysical changes in a gender-specific manner. However, further studies are required to confirm the action of NO and metabolized and/or unmetabolized ethanol on alcohol-induced changes.

The presence of acute alcohol in vitro demonstrates fluidizing effect of erythrocyte membranes by altering the structural arrangement of lipid bilayer. In chronic alcoholism, there is a resistance of the membranes to the ethanol fluidizing effect\textsuperscript{51}. Cholesterol and phospholipid contents serve as membrane fluidity indices. In the present study, alcohol administration affected the membrane cholesterol and phospholipids by increasing C/P (cholesterol/phospholipids) ratio significantly and consequently reducing membrane fluidity with irrespective of gender. Our earlier study has revealed a decrease in membrane fluidity in alcoholic animal erythrocyte membranes of male gender\textsuperscript{9}. Alcohol-induced decrease in erythrocyte membrane fluidity in male and female rats was further confirmed by fluorescent anisotropic experimentation. Increase in anisotropic value observed in alcohol-treated female and male rats suggested decreased fluidity. However, the decreased fluidity among alcohol-administered female rats appeared more prominent when compared with males.

Membrane tolerance development is also accompanied by biochemical changes, such as alterations in Na\textsuperscript{+}/K\textsuperscript{+}-ATPase like membrane-bound enzymes and lipid profile. In the present study, increase in the activity of Na\textsuperscript{+}/K\textsuperscript{+}-ATPase in both genders was observed in alcohol-administered rats. Na\textsuperscript{+}/K\textsuperscript{+}-ATPases are sensitive to changes in membrane fluidity\textsuperscript{52}. Erythrocyte Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activity is closely correlated to C/P ratios of the erythrocyte membrane\textsuperscript{3}. Erythrocyte Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activity depends largely on the membrane and serum lipid profiles\textsuperscript{13}. In the present study, the increased C/P ratio of erythrocyte membrane produced a marked elevation of Na\textsuperscript{+}/K\textsuperscript{+}-pump activity. Further, the lipid composition of erythrocyte membrane is known to reflect strongly the plasma lipid profiles\textsuperscript{53}. Studies
suggested that cholesterol and phospholipid content of the cell membrane participate in the regulation of Na\(^{+}\), K\(^{+}\) transport and Na\(^{+}\)/K\(^{+}\)-ATPase activity\(^{54}\). Alcohol consumption induces changes in the membrane and plasma lipid profiles, affecting the Na\(^{+}\)/K\(^{+}\)-ATPase activity\(^{8,21}\). These transport alterations are often related to abnormal lipid metabolism and and/or disturbances in cell membrane lipid composition.

In conclusion, administration of alcohol to rats increased oxidative stress by decreasing antioxidant status and subsequent generation of ROS altered membrane properties by altering membrane fluidity and Na\(^{+}\)/K\(^{+}\)-ATPase activity. Overall, these effects were more pronounced in alcohol-administered female rats, suggesting that female rats were more vulnerable to alcohol-induced biochemical and altered membrane properties. Further studies are required to find out the molecular mechanisms underlying enhanced female susceptibility to alcohol-induced oxidative damage and altered membrane properties.

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