Original Article

Association between alcohol-induced erythrocyte membrane alterations and hemolysis in chronic alcoholics

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The present study aimed to understand the association between erythrocyte membrane alterations and hemolysis in chronic alcoholics. Study was conducted on human male volunteers aged between 35–45 years with a drinking history of 8–10 years. Results showed that plasma marker enzymes AST, ALT, ALP and γGT were increased in alcoholic subjects. Plasma and erythrocyte membrane lipid peroxidation, erythrocyte lysate nitric oxide (NOx) levels were also increased significantly in alcoholics. Furthermore, erythrocyte membrane protein carbonyls, total cholesterol, phospholipid and cholesterol/phospholipid (C/P) ratio were increased in alcoholics. SDS-PAGE analysis of erythrocyte membrane proteins revealed that increased density of band 3, protein 4.2, 4.9, actin and glycophorins, whereas glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and glycophorin A showed slight increase, however, decreased ankyrin with no change in spectrins (α and β) and protein 4.1 densities were observed in alcoholics. Moreover, alcoholics red blood cells showed altered morphology with decreased resistance to osmotic hemolysis. Increased hemolysis showed strong positive association with lipid peroxidation ($r = 0.703$, $p<0.05$), protein carbonyls ($r = 0.754$, $p<0.05$), lysate NOx ($r = 0.654$, $p<0.05$) and weak association with C/P ratio ($r = 0.240$, $p<0.05$). Bottom line, increased lipid and protein oxidation, altered membrane C/P ratio and membrane cytoskeletal protein profile might be responsible for the increased hemolysis in alcoholics.

***Key Words*: alcohol, erythrocyte membrane, hemolysis, oxidative/nitrosative stress, SDS-PAGE**

Chronic alcohol consumption affects all tissues of the body including blood. The pathological and toxicological effects of alcohol on blood were studied by earlier researchers and they found that increased oxidative/nitrosative stress and altered biochemical profile are responsible for these effects.[1] Reports also revealed that intake of moderate alcohol has protective effects by decreasing coronary heart disease mortality,[2,3] while excessive alcohol consumption has detrimental effects on cardiovascular system which leads to coronary heart disease or hypertension and hemorrhagic stroke.[4,5] Besides, alcohol also exerts a number of deleterious effects which include the hyper fluidization of membranes and denaturation of proteins,[6] by increased production of reactive oxygen species (ROS), which can oxidize the lipids, proteins and DNA.[7] Red blood cells are first line of cells affected by ethanol after stomach mucosa cells. Due to high content of iron and polyunsaturated fatty acids present in erythrocytes make more susceptible to oxidative damage.[8]

Chronic and excessive alcohol consumption leads to various diseases, the progression and severity of those diseases reflects in blood biochemical profile. Blood patho-physiological conditions contribute to erythrocyte morpho-functional alterations that enhance platelet reactivity, RBC procoagulant activity, adhesiveness to endothelium and reduce erythrocyte deformability.[9] Alterations in the red blood cell structure resulting in the impaired blood flow, chronic anaemia, endothelial dysfunction, ischemia, hypertension, risk for cardiovascular diseases and lysis of red blood cells at physiological conditions.[10–12] Studies revealed that increased red blood cell breakdown impairs functions of erythrocytes and it lead to anaemia, jaundice, sickle cell disease, reticulocytosis and deficiency of glucose-6-phosphate dehydrogenase.[13,14] Previous reports revealed that chronic alcoholics showed increased hemolysis,[10,15] however, the exact underlying mechanism is not known. Furthermore, red blood cells are major storage site for nitric oxide (NOx).[16] NOx is an important mediator of many physiological functions, and its role in the pathogenesis of many diseases is gaining recognition.[17] Higher levels of NOx potentially toxic, interacts with superoxide thereby forming peroxynitrite, a strong oxidant which reacts with most biological molecules and make them functionally inactive.[18] Previous reports revealed that alcohol enhances the NOx levels. However, role of NOx on erythrocyte lysis was not explained in deatailed.

Maintenance of erythrocyte morphology depends on the membrane lipid and protein composition. Red blood cell lipid bilayer is a complex and dynamic protein-lipid structures and microdomains that serve as functional platform for interacting signalling lipids and proteins.[19,20] Ethanol directly can interact with membrane proteins and bring about conformational changes, thereby influencing their structure and functions.[21,22] It has been reported that un-metabolized ethanol can induce hemolysis by altering erythrocyte membrane fluidity and forming membrane pores.[23] The two dimensional cytoskeleton composed of an approximately hexagonal network of cross linked spectrin protein molecules. Erythrocyte membrane integrity is critical for maintaining the erythrocyte characteristic shape and is based on vertical and horizontal interactions among the cytoskeletal proteins, integral membrane proteins and phospholipid bilayer.[24] Vertical interactions are based on spectrin, ankyrin, band 3, protein 4.1 and glycophorin, while horizontal interactions are mainly based on spectrin, protein 4.1 and actin.[25] Hence, we hypothesized that

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increased hemolysis in alcoholics is due to by alterations in erythrocyte membrane lipid and protein profile.

Materials and Methods

Subjects. Adult healthy human male volunteers ($n = 30$ in each group) aged between 35–45 years were selected based on a questionnaire. A written consent was obtained from all the volunteers. The male subjects who have regular practice of consuming alcohol everyday for at least 70–80 g for the last 8–10 years were selected. Fasting blood samples were collected into heparinized tubes. These samples were served as alcoholic groups, simultaneously males with the same age and nutritional status without any clinical abnormality and who do not consume alcohol were chosen as controls. The present study was carried with the prior approval of institutional ethics committee.

Measurement of plasma marker enzymes. Plasma and red cells were separated by centrifugation at 800 × *g* for 10 min and were used for analysis. The activities of AST, ALT and ALP in plasma were determined using commercially available kits (Span diagnostics, Surat, India) and plasma γGT was estimated as described previously.[26]

Determination of erythrocyte total nitrate and nitrite levels. Erythrocyte nitrite and nitrate levels were determined as described previously.[27] Briefly, red cell lysate samples were treated with 30% zinc sulphate to deproteinize samples followed by centrifugation at 4,000 × *g* for 5 min. Nitrite was determined from 1.0 ml aliquots of erythrocyte lysate using Griess reagent (1% sulphanilamide, 2.5% phosphoric acid, and 0.1% 1-naphthylethylene diamine). One millilitre aliquots of the supernatant were swirled for 90 min separately with activated cadmium granules for the conversion of nitrite to nitrate and then Griess reagent was added. Nitrite concentrations were estimated using a standard curve developed with sodium nitrite.

Erythrocyte membrane preparation. Erythrocyte membranes were prepared as described previously.[1] Erythrocyte suspension was washed with phosphate buffered saline (pH 7.4) and the cells were lysed with 10 mM phosphate buffer (pH 8.0) and RIPA lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.4% deoxy cholate, 1% NP-40 containing protease inhibitors including 1 mM phenylmethylsulfonylfluoride (PMSF), and phosphatase inhibitors including 10 mM β-glycerophosphate, 10 mM NaF, 0.3 mM Na3Vo4 and 0.3 mM aprotinin and spun at 15,000 × *g* for 30 min. Hemoglobin free ghosts obtained by another wash with 5 mM phosphate buffer were used for analysis.

Determination of erythrocyte membrane TBARS and protein carbonyls. TBARS were measured by the formation of malondialdehyde. The samples were treated with 2 ml of thiobarbituric acid reagent as described previously.[28] Protein carbonyls were determined as described previously using 2,4-dinitrophenylhydrazine (DNPH) assay method.[28]

Determination of erythrocyte membrane total cholesterol, phospholipids and C/P ratio. Lipids extraction and total cholesterol and phospholipids were determined as described previously.[29] To the lysed membrane preparations, methanol and chloroform was added in 1:2 ratio. After 30 min the filtrate was collected from the mixture, and the residue was used again for another extraction. The pooled filtrates were used for estimation of cholesterol and phospholipids.

Analysis of red blood cell morphology. Red blood cell morphology was assessed in freshly prepared wet-mount preparations. Blood samples were diluted (1:200 dilution) in PBS were transferred onto glass slide having silicone grease well of ~1 mm depth and covered with a cover slip and cells were observed under microscope and images were captured at 10× magnification.

Determination of erythrocyte osmotic stability. Release of hemoglobin from erythrocytes was determined as described previously.[30] Diluted red cell suspension was added to tubes containing NaCl (0.9%) and allowed to stand at room temperature for 30 min and centrifuged at 2,000 × *g* for 5 min and absorbance of the supernatant was measured at 540 nm against blank.

SDS-polyacrylamide gel electrophoresis. The SDS-PAGE was done according to the method of Laemelli *et al.*[31] The separating gel (12%) was made of 30% acrylamide, 0.8% *N*,*N*-methylene bisacrylamide, 10% sodium dodecyl sulphate (SDS) and 1.5 M Tris-HCl, pH 8.8. The Various components were mixed and polymerization was initiated by adding 10% ammonium persulfate and 0.02% TEMED. The solution was mixed and quickly poured into glass plate and water layered on top. After polymerization stacking gel (4%) containing 30% acrylamide, 0.8% *N*,*N*-methylene bisacrylamie, 10% SDS and 1 M Tris, pH 6.8 was poured on the separating gel layer and allowed to polymerize after addition of 10% ammonium sulphate and TEMED. The tank buffer contained 0.025 M Tris, 0.192 M glyceine, 0.2% SDS, pH 8.3. The sample buffer for SDS-PAGE was 1 M Tris-HCl, pH 6.8, 10% glycerol, 20% SDS, 5% β-mercaptoethanol, 0.1% bromophenol blue. The samples were denatured with sample buffer in water bath for 5 min. After cooling samples were loaded into the gel. Protein separation was achieved by running the sample through the stacking gel at a low voltage (50 mV) and then increasing the voltage (150 mV) once the dye front has reached the resolving gel. The electrophoresis was continued until the bormophenol blue reached the bottom of the gel.

Silver staining. This was carried out by the method of Okley *et al.*[32] with slight modification. After removing the gel, it was placed in 5 gel volumes of 30% ethanol and incubated for 30 min at room temperature with gentle shaking and this step was repeated two times. Later ethanol was discarded and 10 gel volume of distilled water was added and incubated for 10 min at room temperature and this step was repeated for 3–4 times. After discarding distilled water 5 gel volumes of 0.1% solution of $AgNO_3$ (freshly prepared) was added and incubated for 30 min at room temperature with gentle shaking. Then $AgNO_3$ solution was discarded and both sides of the gels were washed with distilled water. Finally 5 gel volumes of freshly made aqueous solution (2.5% sodium carbonate, 0.02% formaldehyde and 0.001% sodium thiosulfate) was added and incubated with gentle agitation. As soon as bands started appearing, glacial acetic acid was poured to the run until bubbles were stopped. Then gels were washed 3–4 times with distilled water and stored in a solution containing 6 ml of acetic acid and 2.5 ml of methanol in 100 ml of distilled water.

Statistical analysis. Data were subjected to statistical analyses; values are mean ± SD of 30 subjects in each group. The data was normally distributed and Student *t* test was performed for finding significant difference between the groups. A $p<0.05$ was considered statistically significant. Correlation coefficients (r) were also made between certain parameters.

Results

Alcohol consumption is known to cause an array of alcohol-induced biochemical changes in plasma. In the present study, we investigated the effect of alcohol on plasma marker enzymes and the data was presented in Fig. 1a–d. Activities of commonly used pathological and biochemical marker enzymes such as AST, ALT, ALP and γGT were showed significant increase in alcoholic subjects compared to control subjects.

Erythrocytes are more susceptible to alcohol-induced oxidative stress, TBARS and protein carbonyls are markers for oxidative stress status. In the present study, erythrocyte membrane of alcoholic subjects showed significantly increased TBARS (Fig. 2a) and protein carbonyls (Fig. 2b) compared to controls. NOx production was assessed by measuring the total nitrite and nitrate

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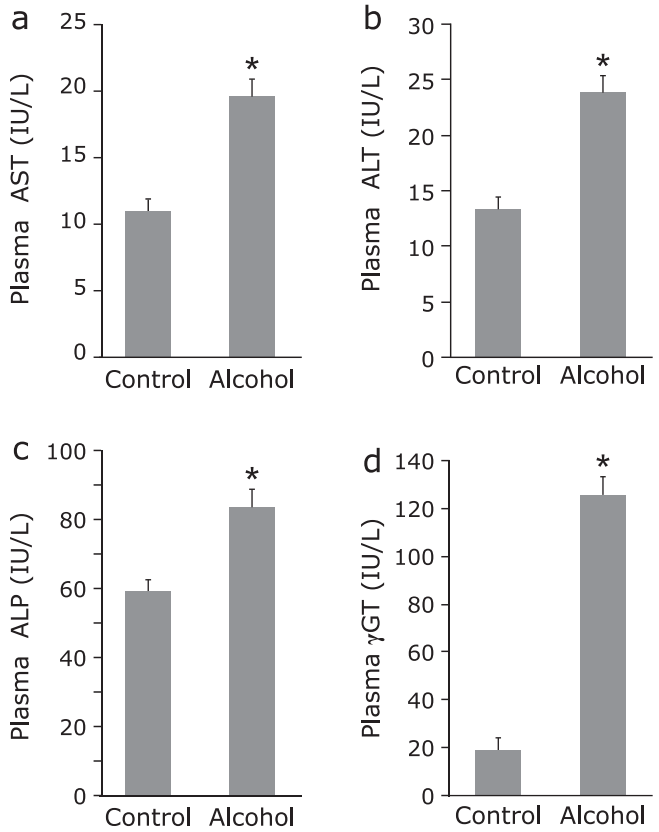

Fig. 1. Effect of alcohol on liver marker enzymes a) AST, b) ALT, c) ALP, d) γGT. All enzyme activities were expressed as IU/L. Values are expressed as mean ± SD. A $p<0.05$ statistically significant in between groups and *indicates statistically significant from control group.

levels in erythrocyte lysate from alcoholic and control subjects. Results of the present study showed significantly increased nitrite and nitrate levels in erythrocyte lysate (Fig. 2c) of alcoholics when compared to controls.

Erythrocyte membrane total lipid composition was determined by measuring membrane total cholesterol and phospholipids. The data was presented in Table 1, reveals significant increase in total cholesterol, phospholipids and the consequent C/P ratio in alcoholic group compare to controls.

Table 1. Effect of erythrocyte membrane total cholesterol, phospholipids and C/P ratio

Parameter	Control	Alcohol
Total cholesterol	99 ± 2.5	148 ± 6.2*
Total phospholipids	113 ± 4.2	130 ± 3.2*
C/P ratio	0.88 ± 0.04	1.14 ± 0.07*

All the values are expressed as μg/mg protein. Values are expressed as mean ± SD of each group. A $p<0.05$ statistically significant between groups and *indicates statistically significant from control group.

Red cell morphology was studied in freshly prepared wet-mount preparations using PBS. Red blood cells of alcoholics and controls were observed using microscope and images were depicted in Fig. 3. Results showed that red cell morphology was changed into stomatocyte and spike like protrusion in alcoholics, however, controls red blood cells showed normal phenomena.

Erythrocyte membrane protein profile was analyzed by using SDS-PAGE and the data was presented in Fig. 4. Since, erythrocyte membrane contains few number of proteins we have performed SDS-PAGE analysis rather than western blotting, and also availability of antibodies are also limited. Erythrocyte membrane proteins were classified according to their degree of migration along the gels from cathode to anode end. SDS-PAGE analysis of erythrocyte membrane proteins revealed that the density of band 3, protein 4.2, p58, demantin, actin and glycophorins were significantly increased in alcoholics than the controls. Moreover, glycophorin A and GAPDH were also slightly increased compared to controls. However, we noticed decreased ankyrin with no change in spectrins and protein 4.1 densities in alcoholics.

Hemolysis in blood samples were determined by exposing red blood cells to 0.9% NaCl and the data was presented in Fig. 5. Blood cells of alcoholics were showed less resistance which reflects in increase in osmotic hemolysis compared to controls.

The association between variables in alcoholics were determined using Pearson correlation analysis and the data presented in Fig. 6a–d. Hemolysis showed strong positive association with lipid peroxidation ($r = 0.703$, $p<0.05$), protein carbonyls ($r = 0.754$, $p<0.05$) and RBC lysate NOx ($r = 0.754$, $p<0.05$), moreover, weak association with C/P ratio ($r = 0.240$, $p<0.05$) were noticed in alcoholics.

Discussion

Chronic alcohol consumption causes liver damage because higher amounts of alcohol metabolism takes place in liver finally

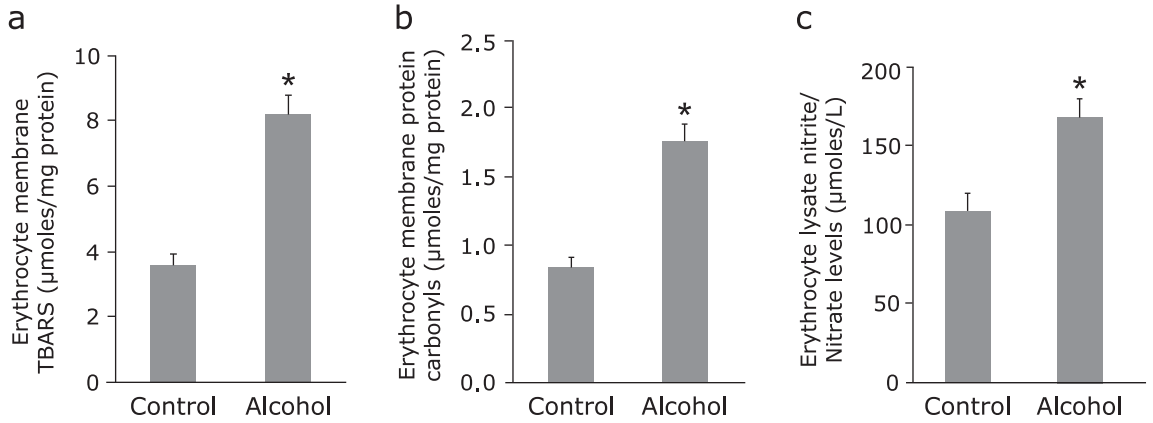

Fig. 2. Effect of alcohol on erythrocyte membrane a) TBARS, b) protein carbonyl levels, c) erythrocyte lysate nitrite and nitrate levels. Values are expressed as mean ± SD in each group. A $p<0.05$ statistically significant in between groups and *indicates statistically significant from control group.

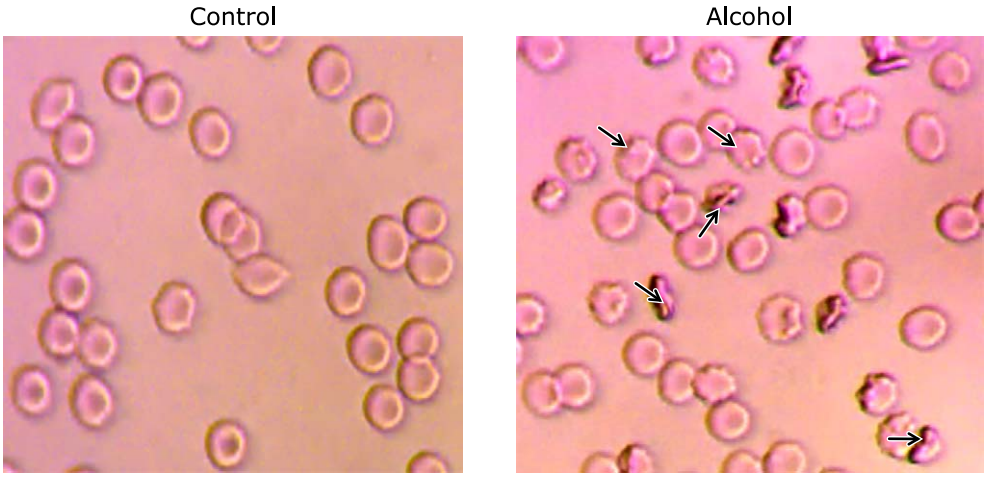

Fig. 3. Effect of alcohol on red cell morphology, arrow mark indicating the altered red cell structure and shape.

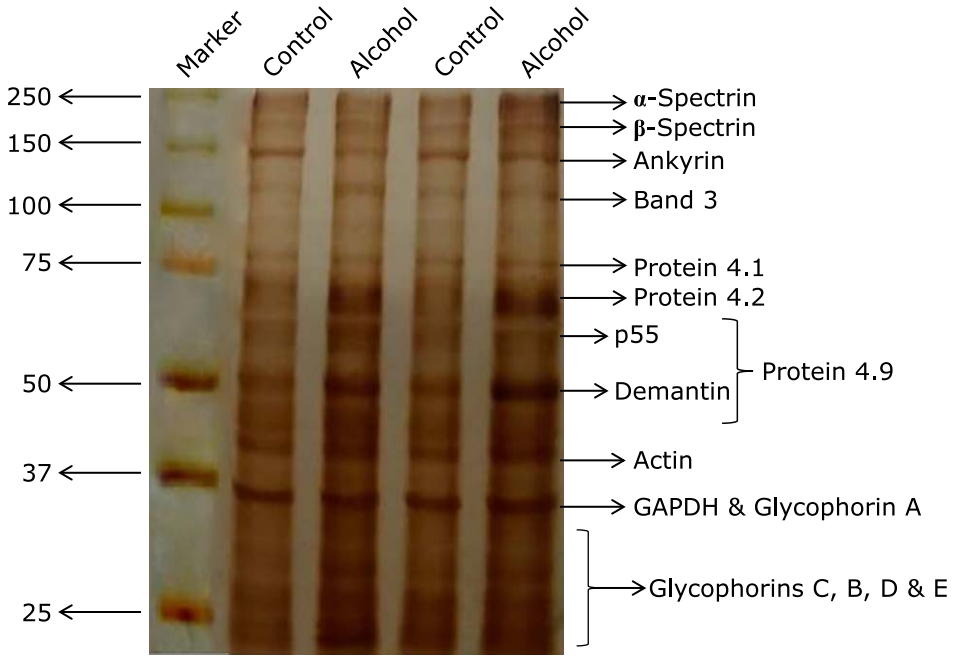

Fig. 4. SDS-PAGE analysis of alcohol-induced alterations in erythrocyte membrane proteins prepared from control and alcoholic subjects. Erythrocyte membrane proteins were resolved in 12% polyacrylamide gel and protein bands were visualised by using silver staining as described in materials and methods.

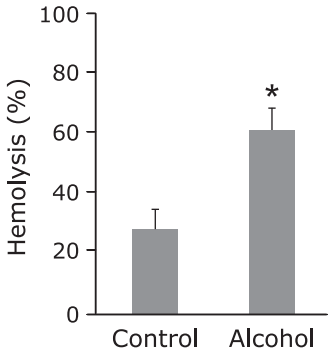

Fig. 5. Effect of alcohol on red cell resistance at 0.9% NaCl. A $p<0.05$ statistically significant between groups *indicates statistically significant from controls.

contributing to liver damage which leads to spill of cellular contents into plasma.[33] Measurement of plasma marker enzyme levels was employed in the analysis of hepatic damage and diseases. In this study plasma marker enzymes AST, ALT, ALP and γGT were increased significantly in alcoholics compared with controls. Erythrocyte membranes are more susceptible to alcohol induced ROS and oxidative damage and they are considered as the best models to verify the effects of oxidative conditions in living cells. ROS and RNS are continuously produced in erythrocytes due to the high oxygen tension in arterial blood and their abundant iron content. These free radicals can directly damage red blood cell membrane by lipid peroxidation of membrane poly unsaturated fatty acids.[34] Our results clearly demonstrated that chronic alcohol consumption resulted in sustained damage to erythrocytes as evidenced by increased TBARS in erythrocyte membrane of alcoholic subjects than controls. Oxidative alteration of erythro-

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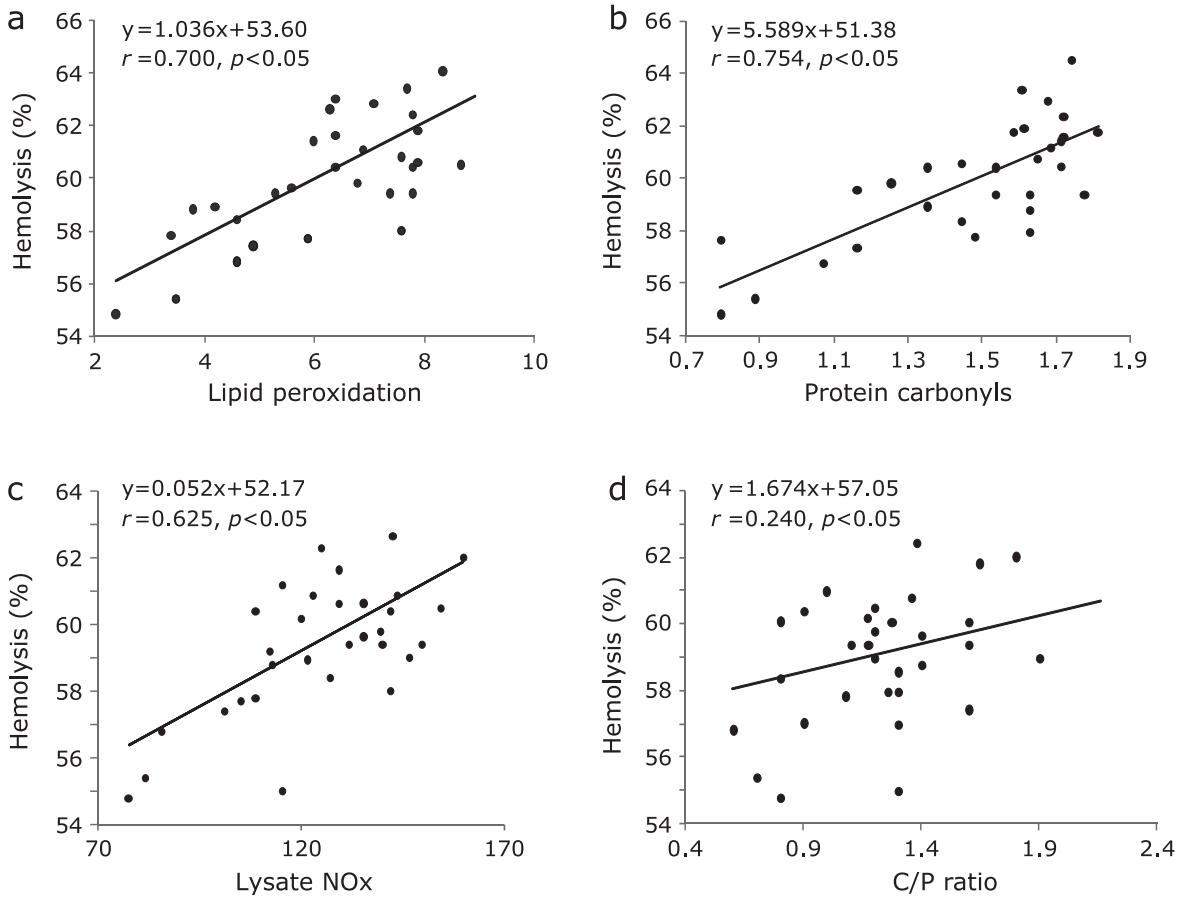

Fig. 6. Correlation analysis (*r* values) between the following parameters in alcoholics a) hemolysis vs lipid peroxidation, b) hemolysis vs protein carbonyls, c) hemolysis vs RBC lysate NOx, and d) hemolysis vs C/P ratio. Pearson correlation analysis method was used to determine the correlation coefficient.

cyte membrane polyunsaturated fatty acids generates a number of degradation products in alcoholics. Alcohol-induced lipid peroxidation causes depolarization of lipid bilayer and yields changes in the structural organization of membrane lipids which can disturb the membrane fluidity.[35] A strong positive association were noticed between lipid peroxidation and hemolysis. Moreover, the influence of lipid peroxidation on hemolysis cannot be ruled out in alcoholics. Oxidation of proteins can lead to a whole variety of amino acid modifications. The attack of ROS against proteins modifies lysine, arginine, proline and histidine residues generating carbonyl moieties.[36] Elevation of protein carbonyl content is a major indicator of protein oxidation caused by oxidative stress.[35] In the present study, alcohol-induced oxidative radicals could have oxidised amino acid side chains, cleavage of peptide bonds and formation of covalent protein-protein cross link derivatives in erythrocyte membrane which leads to destabilisation of the membrane in alcoholics.

Alcohol metabolism generates a variety of NOx radicals. Nitrosative stress occurs when generation of these radicals exceeds the system ability to neutralize and eliminate. NOx produced by vascular endothelial cells may reach the erythrocytes, diffuse across erythrocyte membrane, and react with molecular oxygen to form nitrite, or with superoxide anion to form peroxynitrite ($ONOO^-$).[37,38] Peroxynitrite interacts with erythrocyte membrane lipids and proteins and make them functionally inactive in alcoholics. In the present study, red cell lysate nitrite and nitrate levels were increased in alcoholics than controls. Previous reports also suggested that NOx participates in the regulation of erythrocyte membrane properties and rheological behaviour.[39] Our lab reports revealed that NOx significantly alters erythrocyte membrane fluidity, membrane bound proteins, enzymes and transport mechanisms.[40]

Lipids play multiple roles in biologic membranes by providing the proper polarity and spacial arrangement necessary for optimal activity of membrane-bound enzymes and proteins.[41] In this study, erythrocyte membrane cholesterol, total phospholipids were significantly increased in alcoholics than the controls. As reported by earlier researchers alcohol enhances lipid synthesis and its insertion in erythrocyte membrane.[34] Alcohol-induced oxidative damage also causes alterations in phospholipids and resulting changes in structural characteristics and dynamics of the lipid bilayer with functional impairment in membrane fluidity, ion permeability, membranous enzyme activity, and cell signalling.[39] Furthermore, alcohol consumption alter the organization of membranes with altered fluidity and reacting with free amino, sulfhydryl and other functional groups leading to oxidative modification of proteins and consequently changes in the properties of membranes.[42,43]

Cytoskeletal proteins interact with membrane integral proteins and lipids to maintain structural integrity of erythrocyte membrane.[44] Erythrocyte cytoskeleton network mainly composed of spectrin, ankyrin, actin and protein 4.1. Ankyrin binds to spectrin tetramers to form a link to the membrane protein band 3.[45] In this study, alcoholics showed decrease in ankyrin, this could due to increased proteolytic degradation, and ROS/RNS mediated protein oxidation by alcohol or its metabolite acetaldehyde.[46] In the

present study, increased density of band 3, protein 4.2 and 4.9, actin, glycophorins and GAPDH might be due to the enhanced synthesis and insertion or altered signalling mechanisms might have played a role to adopt alcohol-induced alterations in the red cell membrane. Protein 4.1 promotes and regulates actin-spectrin association, and also links to another transmembrane protein glycophorin. Band 3 anchors the spectrin/actin cytoskeleton to the lipid bilayer, organizes and regulates a complex of glycolytic enzymes and also participates in control of several important membrane-spanning proteins.[47] Furthermore, band 3, serves as a docking site for multiple peripheral membrane proteins, including protein 4.1 and protein 4.2.[48] Tyualina *et al.*[43] was reported that plasma and erythrocyte membrane protein profile was increased in chronic alcoholics and also *in situ* acetaldehyde treated red blood cell membranes. However in-depth confirmative studies were required to understand the molecular events underlying in chronic alcohol-induced alterations in erythrocyte membrane proteins and their interactions.

Effect of alcohol on red cell morphology were studied and results showed that red cell morphology was changed in to stomatocyte and echinocytes, this might be due to increased lipid peroxides, protein carbonyls, altered membrane lipid and protein composition. Further, we extended our studies to test membrane resistance by measuring hemolysis. Results revealed that alcoholics showed less resistance with increased hemolysis than the controls. Increased hemolysis might have resulted from ROS/RNS mediated membrane oxidation of lipids and proteins in the membrane and cytoskeleton proteins. Strong positive association was noticed between hemolysis and NOx levels and protein carbonyls in alcoholics. Researchers also reported that oxidative modification of erythrocyte membrane proteins would lead to increased hemolysis in chronic alcoholics.[10,15] Alimi *et al.*[23] reported that alcohol increased the number of red cell stomatocytes and echinocytes which enhances the red cell fragility due to incorporation of excess cholesterol into erythrocyte membrane of rats. Further, decreased ankyrin levels in alcoholics might have played a role in the destabilization and anchorage of cytoskeleton to the erythrocyte membrane which might have lead to altered shape, membrane destabilisation and lysis of red blood cells.

In summary, chronic alcohol consumption increases oxidative/nitrosative stress which in turn alters erythrocyte membrane lipid composition and protein profile. These alterations might be responsible for the increased hemolysis.

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Conflict of Interest

No potential conflicts of interest were disclosed.

References

1 Maturu P, Reddy VD, Padmavathi P, Varadacharyulu N. Ethanol induced adaptive changes in blood for the pathological and toxicological effects of chronic ethanol consumption in humans. *Exp Taxicol Pathol* 2012; **64**: 697–703.
2 Gaziano JM, Gaziano TA, Gynn RJ, *et al*. Light to moderate alcohol consumption and mortality in the Physicians' Health Study enrolment cohort. *J Am Coll Cardiol* 2000; **35**: 96–105.
3 Englund Ogge L, Brohall G, Behre CJ, Schmidt C, Fagerberg B. Alcohol consumption in relation to metabolic regulation, inflammation and adiponectin in 64-year-old Caucasian women: a population-based study with a focus on impaired glucose regulation. *Diabetes Care* 2006; **29**: 908–913.
4 Klatsky AL, Friedman GD, Armstrong MA, Kipp H. Wine, liquor, beer and mortality. *Am J Epidemol* 2003; **158**: 585–595.
5 Durazzo TC, Gazdzinski S, Banys P, Meyerhoff DJ. Cigarette smoking exacerbates chronic alcohol-induced brain damage: a preliminary metabolic imaging study. *Alcohol Clin Exp Res* 2004; **28**: 1849–1860.
6 Baker RC, Kramer RE. Cytotoxicity of short-chain alcohols. *Annu Rev Pharmacol Toxicol* 1999; **39**: 127–150.
7 Song BJ, Abdelmegeed MA, Henderson LE, *et al*. Increased nitroxidative stress promotes mitochondrial dysfunction in alcoholic and non-alcoholic fatty liver disease. *Oxid Med Cell Longev* 2013; **2013**: 781050.
8 Reddy KR, Reddy VD, Padmavathi P, Kavitha G, Saradamma B, Varadacharyulu NC. Gender differences in alcohol-induced oxidative stress and altered membrane properties in erythrocytes of rats. *Indian J Biochem Biophys* 2013; **50**: 32–39.
9 Straat M, van Bruggen R, de Korte D, Juffermans NP. Red blood cell clearance in inflammation. *Trandus Med Hemother* 2012; **39**: 353–361.
10 Tyulina OV, Huentelman MJ, Prokopieva VD, Boldyrev AA, Johnson P. Does ethanol metabolism affect erythrocyte hemolysis? *Biochem Biophys Act* 2000; **1535**: 69–77.
11 Maio R, Siacqua A, Bruni R, *et al*. Association between hemoglobin level and endothelial function in uncomplicated untreated hypertensive patients. *Clin J Am Sco Nephrol* 2011; **6**: 648–655.
12 Mozos I. Mechanisms linking red blood cell disorders and cardiovascular diseases. *BioMed Res Int* 2015; **2015**: 682054.
13 Ballard HS. The haematological complications of alcoholism. *Alcohol Health Res World* 1997; **21**: 42–52.
14 Büyükokuroğlu ME, Altikat S, Ciftçi M. The effect of ethanol on glucose-6-phosphate dehydrogenase enzyme activity from human erythrocytes *in vitro* and rat erythrocytes *in vivo*. *Alcohol Alcoholism* 2002; **37**: 327–329.
15 Minneci PC, Deans KJ, Zhi H, *et al*. Hemolysis-associated endothelial dysfunction mediated by acceleration NO inactivation by decompartmentalized oxyhemoglobin. *J Clin Invest* 2005; **115**: 3409–3417.
16 Kleinbongard P, Schulz R, Rassaf T, *et al*. Red blood cells express a functional endothelial nitric oxide synthase. *Blood* 2006; **107**: 2943–2951.
17 Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. *Physiol Rev* 2007; **87**: 315–424.
18 Owusu BY, Stapley R, Honavar J, Patel RP. Effects of erythrocyte aging on nitric oxide and nitrite metabolism. *Antioxid Redox Signal* 2013; **19**: 1198–1208.
19 Gov N, Safran SA. Red blood cell shape and fluctuations: cytoskeleton confinement and ATP activity. *J Biolgic Phys* 2005; **31**: 453–464.
20 Escribá PV, González-Ros JM, Goñi FM, *et al*. Membranes: a meeting point for lipids, proteins and therapies. *J Cell Mol Med* 2008; **12**: 829–875.
21 Fadda F, Rossetti ZL. Chronic ethanol consumption: from neuroadaptation to neurodegeneration. *Prog Neurobiol* 1998; **56**: 385–431.
22 Tóth ME, Vigh L, Sántha M. Alcohol stress, membranes and chaperones. *Cell Stress Chaperones* 2014; **19**: 299–309.
23 Alimi H, Hfaeidh N, Bouoni Z, Sakly M, Ben Rhouma K. Protective effect of Opuntia ficus indica f. Inermis prickly pear juice upon ethanol-induced damages in rat erythrocytes. *Alcohol* 2012; **46**: 235–243.
24 Gallagher PG, Jarolim P. Red blood cell membrane disorders. In: Hoffman R, Benz EJ Jr., Shattil SJ, *et al.*, eds. *Hematology: Basic Principles and Practice*. Philadelphia, PA: Churchill Livingstone, 2009; 623–643.
25 Ciccoli L, De Felice C, Paccagnini E, *et al*. Erythrocyte shape abnormalities, membrane oxidative damage and β-actin alterations: an unrecognised triad in classical autism. *Mediators Inflamm* 2013; **2013**: 432616.
26 Padmavathi P, Damodara Reddy V, Narendra M, Varadacharyulu N. Bidis--hand-rolled, Indian cigarettes: induced biochemical changes in plasma and red membranes of human male volunteers. *Clin Biochem* 2009; **42**: 1041–1045.
27 Sastry KV, Moudgal RP, Mohan J, Tyagi JS, Rao GS. Spectrophotometric determination of serum nitrite and nitrate by copper-cadmium alloy. *Anal Biochem* 2002; **306**: 79–82.
28 Reddy VD, Padmavathi P, Hymavathi R, Maturu P, Varadacharyulu NCh. Alcohol-induced oxidative stress in rat liver microsomes: protective effect of *Emblica officinalis*. *Pathophysiology* 2014; **21**: 153–159.
29 Kavitha G, Damodara Reddy V, Paramahamsa M, Akhtar PM, Varadacharyulu

doi: 10.3164/jcbn.16-16

NC. Role of nitric oxide in alcohol-induced changes in lipid profile of moderate and heavy alcoholics. *Alcohol* 2008; **42**: 47–53.
30 Pannuru P, Vaddi DR, Kindinti RR, Varadacharyulu N. Increased erythrocyte antioxidant status protects against smoking induced hemolysis in moderate smokers. *Hum Exp Toxicol* 2011; **30**: 1475–1481.
31 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**: 680–685.
32 Oakley BR, Kirsch DR, Morris NR. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal Biochem* 1980; **105**: 361–363.
33 Dey A, Cederbaum AI. Alcohol and oxidative liver injury. *Hepatology* 2006; **43 (2 Suppl 1)**: S63–S74.
34 Reddy VD, Padmavathi P, Paramahamsa M, Varadacharyulu N. Modulatory role of *Emblica officinalis* against alcohol induced biochemical and biophysical changes in rat erythrocyte membranes. *Food Chem Toxicol* 2009; **47**: 1958–1963.
35 Maturu P, Vaddi DR, Pannuru P, Nallanchakravarthula V. Alterations in erythrocyte membrane fluidity and Na^+/K^+-ATPase activity in chronic alcoholics. *Mol Cell Biochem* 2010; **339**: 35–42.
36 Catalá A. Lipid peroxidation of membrane phospholipids generates hydroxy-alkenals and oxidized phospholipids active in physiological and/or pathological conditions. *Chem Phys Lipids* 2009; **157**: 1–11.
37 Pandey KB, Rizvi SI. Markers of oxidative stress in erythrocytes and plasma during aging in humans. *Oxid Med Cell Longev* 2012; **3**: 2–12.
38 Tsuda K, Kinoshita-Shimamoto Y, Kimura K, Nishio I. Nitric oxide is a determinant of membrane fluidity of erythrocytes in postmenopausal woman: an electron paramagnetic resonance investigation. *Am J Hypertens* 2003; **16**: 244–248.
39 Maturu P, Vaddi DR, Pannuru P, Nallanchakravarthula V. Modifications of erythrocyte membrane proteins, enzymes and transport mechanisms in chronic alcoholics: an *in vivo* and *in vitro* study. *Alcohol Alcohol* 2013; **48**: 679–686.
40 Deng XS, Deitrich RA. Ethanol metabolism and effects: nitric oxide and its interaction. *Curr Clin Pharmacol* 2007; **2**: 145–153.
41 Muriel P, Castañeda G, Ortega M, Noël F. Insights into the mechanism of erythrocyte Na^+/K^+-ATPase inhibition by nitric oxide and peroxynitrite anion. *J Appl Toxicol* 2003; **23**: 275–278.
42 Stibler H, Beaugé F, Leguicher A, Borg S. Biophysical and biochemical alterations in erythrocyte membranes from chronic alcoholics. *Scand J Clin Lab Invest* 1991; **51**: 309–319.
43 Tyulina OV, Prokopieva VD, Boldyrev AA, Johnson P. Erythrocyte and plasma protein modification in alcoholism: a possible role of acetaldehyde. *Biochim Biophys Acta* 2006; **1762**: 558–563.
44 Zennadi R, Moeller BJ, Whalen EJ, *et al*. Epinephrine-induced activation of LW-mediated sickle cell adhesion and vaso-occlusion *in vivo*. *Blood* 2007; **110**: 2708–2717.
45 Ferru E, Giger K, Pantaleo A, *et al*. Regulation of membrane-cytoskeletal interactions by tyrosine phosphorylation of erythrocyte band 3. *Blood* 2011; **117**: 5998–6006.
46 Celedón G, González G, Pino J, Lissi EA. Peroxynitrite oxidizes erythrocyte membrane band 3 protein and diminishes its anion transport capacity. *Free Radic Res* 2007; **41**: 316–323.
47 Pantaleo A, Giribaldi G, Mannu F, Arese P, Turrini F. Naturally occurring anti-band 3 antibodies and red blood cell removal under physiological and pathological conditions. *Autoimmun Rev* 2008; **7**: 457–462.
48 Chu H, Breite A, Ciraolo P, Franco RS, Low PS. Characterization of the deoxyhemoglobin binding site on human erythrocyte band 3: implications for O_2 regulation of erythrocyte properties. *Blood* 2008; **111**: 932–938.