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Erythrocyte Glutathione Depletion Impairs Resistance to Haemolysis in Women Consuming Alcohol

Ekambaram Padmini*, and Balasubramaniam Thiripura Sundari

P.G and Research Department Of Biochemistry, Bharathi Women's College, Affiliated to the University of Madras, Chennai-600 108, Tamilnadu, India

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Summary **Alcohol abuse is known to cause an array of ethanol induced abnormalities in men but very few reports are available on the effect of alcohol in women. None of them discuss the effect of ethanol consumption on erythrocyte membrane. In the present study, erythrocytes in women who consume alcohol showed significant decrease in their ability to resist haemolysis with HPLC studies. Erythrocyte membrane indicates decreased phospholipid ($p<0.05$) levels, which increased the cholesterol/phospholipid ratio significantly ($p<0.01$) in women who consume alcohol. This can decrease the fluidity of membrane, which appears to be related to the effect of ethanol on erythrocyte membrane. Also the protection against exogenous and endogenous peroxides in the erythrocytes of alcoholic women is considerably affected due to decreased ($p<0.05$) activity of catalase, glucose-6-phosphate dehydrogenase, protein–SH group and glutathione (GSH). Enhanced free radical generation induced oxidation of oxyHb to metHb in alcoholics. Increased methemoglobin leads to significant reduction in membrane GSH, which may cause protein thiol oxidation. Thus peroxidative damage to membrane lipids and oxidation of membrane protein thiols potentially harmful to membrane fluidity and flexibility is responsible for decreased resistance to haemolysis as demonstrated in women who consume alcohol.**

Key Words: **erythrocyte membrane, haemolysis, glutathione, alcoholics, phospholipid, protein carbonyl**

Introduction

Much of our knowledge of alcoholism has been gathered from studies conducted with predominance of male subjects. Recent study involving more female subjects, reveal that effect of drinking differs between men and women. These studies also indicate that among drinkers women equal or surpass men in the number of problems that result from their drinking [*1*]. The present trend in the Indian Society shows increase in women who consume alcohol both in higher and lower strata of life. But very few reports are available on the toxic side effect of alcohol consumption in these women, most of them focusing only on liver damage.

After alcohol is consumed, it diffuses uniformly into all body water, both inside and outside the cells. For a given amount of alcohol consumed, blood levels are higher in women than in men for two reasons, the smaller body size of women and the distribution of alcohol in smaller water space because of their higher proportion of fat [*2*]. Furthermore among people under the age of 50 years, women have less gastric alcohol dehydrogenase activity than men: with less gastric alcohol broken down in the stomach, a proportionally larger amount enters the blood stream [*3*]. This leads to heightened vulnerability of women to physiological condi-

*To whom correspondence should be addressed.
Tel: +091-044-26213748 Fax: +091-044-25280473
E-mail: epi622001@yahoo.com

tions that cause various pathological changes. The changes seen in individual drinking alcohol are influenced not only by amount drunk but by the individuals nutritional state, the diet taken with alcohol drinking, individual's genetic predisposition, sex and hormonal status. Hence reliable research is required to assess the risks of developing adverse health consequences in women due to alcohol intake.

In the present investigation the effect of ethanol on erythrocytes in women who consume alcohol is studied in detail because the blood ethanol is known to diffuse in to the erythrocytes. Although alcohol abuse is known to cause an array of ethanol induced red blood cell (RBC) abnormalities in men the underlying mechanism remain poorly understood. The deleterious effects of ethanol consumption on erythrocytes *in vivo* may be at least in part, the result of direct effects of unmetabolised ethanol on erythrocyte components. Since blood alcohol content in women who consume alcohol is more than men, the effect of the unmetabolised ethanol on erythrocytes in women may be more pronounced. The aim of the present study was to evaluate the acid haemolysis of erythrocytes in women who consume alcohol, to evaluate the composition and fluidity of erythrocyte membrane in women who consume alcohol and to hypothesize possible mechanism that may be responsible for the erythrocyte membrane alterations or damage in women who consume alcohol with minimal or no liver damage.

Materials and Methods

Erythrocyte source and preparation

Healthy adult females age between 30–40 years were selected. During initial screening, height, weight, alcohol consumption and physical activity questionnaires were completed.

The women who have regular practice of consuming alcohol everyday for atleast last two years were selected. These women were not hospitalized for past one year. The selected women did not have any complications like diabetes, hypertension or liver disorders. They consume about 200 ml to 300 ml of alcoholic drink everyday after work. Fasting blood samples were collected in the morning from these women in vacutinized tubes. These samples served as alcoholic group. Simultaneously women with same age and nutritional status without any clinical abnormality were chosen. These group women did not consume alcohol, so they served as control group.

Preparation of erythrocytes

Blood drawn was anticoagulated with heparin. Erythrocytes were prepared by three centrifugation washes in 10 volumes of phosphate buffered saline (PBS) in deionized water pH 7.4. The buffy coat of leukocytes was carefully removed from the surface of erythrocyte pellet with each wash.

Acid haemolysis of erythrocytes

The stability of erythrocytes was determined by the time course of its haemolysis in the presence of 2 mM HCl as described by Stusj [*4*].

Measurement of methemoglobin

Methemoglobin in hemolysate was determined according to Winter bourn 1990 method [*5*]. The results were expressed as the percent methemoglobin relative to total hemoglobin (Hb).

Assay of intracellular glutathione (GSH), catalase, glucose-6-phosphate dehydrogenase (G6PDH) and sialic Acid

Reduced GSH levels, G6PDH activity, catalase and sialic acid were estimated by established methods [*6*].

Isolation of red cell membrane

Red cell membranes were isolated by haemolysing the cells with 10 mmol/l Tris-HCl buffer and 0.1 mmol/l EGTA, pH 7.7 centrifugation at 22000 × g of 10 mins at 4°C and washing the ghosts in the same icecold buffer three times by resuspension and centrifugation as mentioned above. Ghosts were analyzed for cholesterol [*7*], phospholipids [*8*], protein content [*9*], sialic acid [*10*], GSH [*11*], and lipid peroxide [*12*].

Estimation of protein carbonyls

Protein carbonyl's in the erythrocytes membrane was determined by the method of Levine *et al.* [*13*]. The results were expressed as μg/mg protein.

Estimation of available protein sulphydryl

Erythrocytes membrane available protein sulphydryl content was estimated by the method of Habeeb [*14*]. The results were expressed as μg/mg protein.

Estimation of total protein sulphydryl

Total protein sulphydryl content in the erythrocyte membrane was determined by the method of Habeeb [*15*]. The results were expressed as μg/mg protein.

HPLC studies of phospholipids

Phospholipids of ghosts were extracted by addition of solution of chloroform/methanol 2:1 v/v in a ratio 20:1 and used for HPLC studies.

Phospholipids were analyzed by the method of Becart *et al.* [*16*]. 5 μm diol bonded silica column, (250 × 4.6 mm) was used. The solvents used are chloroform, methanol, and 26% ammonium hydroxide. The sample injection volume was 20–40 μl and the flow rate was adjusted to be 1 ml/minute. The total run time of the experiment was 30 minutes Evaporative light scattering detector was used. The results were expressed as μg/mg protein.

Erythrocyte morphology determination

The shapes of erythrocytes were characterized by light microscopy after staining.

Statistical analysis

Hemolysis and results are presented as the mean ± SD. Haemolysis curves were judged to be significantly different from each other. Significant differences ($p<0.05$) existed between each data set on the curves from 3 min to 7 min of haemolysis time. $p<0.05$ was used as evidence of a significant difference between data sets. All results are expressed as mean ± S.D: statistical differences were determined by student's *t* test.

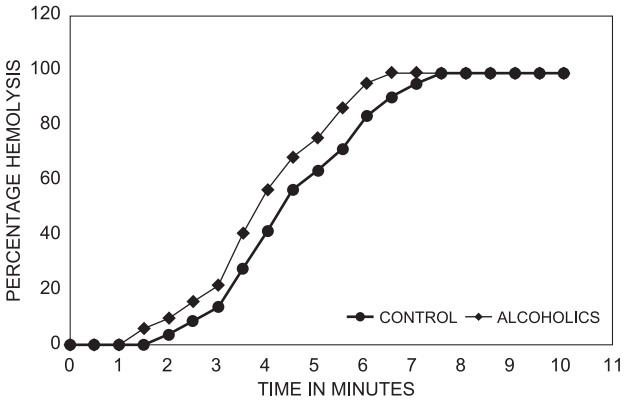

Fig. 2. Acid hemolysis of erythrocyte in the control and the alcoholic groups.

Results

Fig. 1 shows that in subjects who consume alcohol there was a significant increase ($p<0.01$) in serum Sialic acid as compared to control group. Increase in serum Sialic acid was seen in all alcoholic samples analyzed. Since Sialic acid is marker for alcohol abuse all the samples analyzed for the study are from women who consume alcohol regularly and did not abstain from alcohol abuse.

Fig. 2 shows the hemolytic curve for erythrocytes from normal individuals and alcoholic women. It was found that erythrocytes from alcoholic subject have a significantly decreased resistance to hemolysis in comparison to the cells from normal subjects as evidenced by the left shift of the curve.

Table 1 shows that the erythrocytes isolated from women who consume alcohol exhibited significant decrease ($p<0.05$) in catalase and glucose-6-phosphatedehydrogenase activity. Reduced glutathione was found to be significantly low ($p<0.05$), whereas methemoglobin in erythrocytes of alcoholic women was significantly increased ($p<0.001$) when compared to control samples.

Table 2 presents the lipid profile and oxidative status of erythrocyte membrane in control and alcoholic women. Cholesterol levels are significantly increased ($p<0.05$) in alcoholic group while phospholipid levels are decreased. Enhanced lipid peroxidation with significantly decreased ($p<0.001$) glutathione is also observed in women who consume alcohol.

The composition of the erythrocyte membrane in control

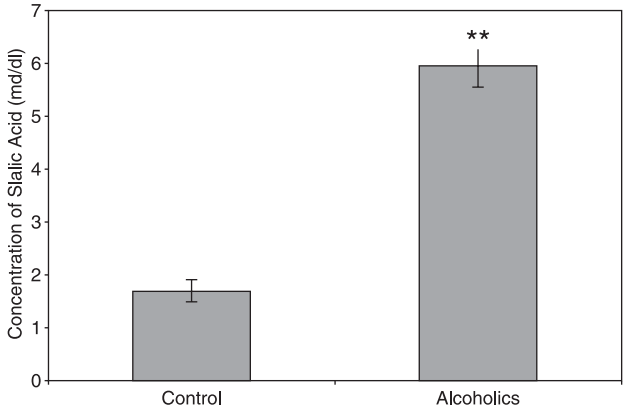

Fig. 1. Level of sialic acid in the serum of control and the alcoholic groups.
**$p<0.001$ highly significant when compared to controls

Table 1. Levels of catalase, glucose-6-phosphate dehydrogenase, reduced glutathione and methemoglobin in the control and the alcoholic group.

Parameter	Control	Alcoholics
Catalase[a]	3449 ± 458	3046 ± 652*
Glucose-6-phosphate dehydrogenase[b]	11.8 ± 0.5	8.2 ± 0.4*
Reduced glutathione[c]	417.8 ± 23.4	308.5 ± 22.7*
Methemoglobin %	2.1 ± 0.08	10.4 ± 0.56**

Values are expressed as mean ± SD.
[a] k/gHb, [b] IU/gHb, [c] mol/gHb, * $p<0.05$, ** $p<0.001$

Table 2. Levels of Cholesterol, Phospholipid, lipid peroxide and reduced glutathione in the erythrocyte membrane of the control and the alcoholic group.

Parameter	Control	Alcoholics
Cholesterol[a]	502.7 ± 18.5	592 ± 23.4*
Phospholipid[a]	618.6 ± 21.2	554.7 ± 16.4*
Lipid Peroxide[a]	30.04 ± 11.65	103.67 ± 14*
Reduced glutathione[b]	5.59 ± 0.82	2.71 ± 0.83**

Values are expressed as mean ± SD.
[a] nanomoles/mg protein, [b] micromoles/mg prtoein, * $p<0.05$, ** $p<0.001$

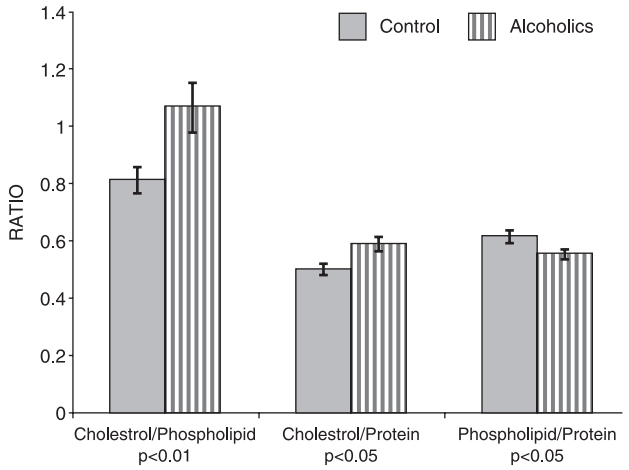

Fig. 3. Levels of cholesterol/phospholipid, cholesterol/protein and phospholipid/protein ratios in erythrocyte membrane of control and alcoholic groups.

and alcoholic group is presented in Fig. 3. The latter group shows a significant increase in cholesterol/phospholipid ($p<0.01$) and cholesterol/protein ratio ($p<0.05$) while there is a decrease in phospholipid/protein ratio ($p<0.05$).

Fig. 4 shows the levels of individual phospholipids estimated by HPLC in erythrocyte membrane of control and alcoholic group. The levels of individual phospholipids namely PE, PI, PS, PC and SM are significantly decreased in alcoholic group (4c and 4d) when compared to control group erythrocyte membrane (4b). 4a shows the standard curve for the above mentioned phospholipid.

Table 3. Levels of Phospholipids in the erythrocyte membrane of the control and the alcoholic group.

Parameter	Control	Alcoholics
Phosphatidyl ethanolamine	292.96 ± 2.6	209.56 ± 15.78*
Phosphatidyl inositol	139.63 ± 8.9	115.98 ± 9.38*
Phosphatidyl serine	137.04 ± 6.28	115.05 ± 4.29*
Phosphatidyl choline	144.08 ± 4.71	121.10 ± 10.7*
Sphingomyelin	284.44 ± 4.18	270.70 ± 20.91[a]

Values are expressed as microgram/milligram protein.
Values are expressed as mean ± SD.
* $p<0.05$, [a] Nonsignificant.

Table 3 shows the significant increased ($p<0.05$) levels of individual fractions of phospholipids in the erythrocyte membrane of control and alcoholic group.

Table 4 shows the significant increased ($p<0.001$) levels

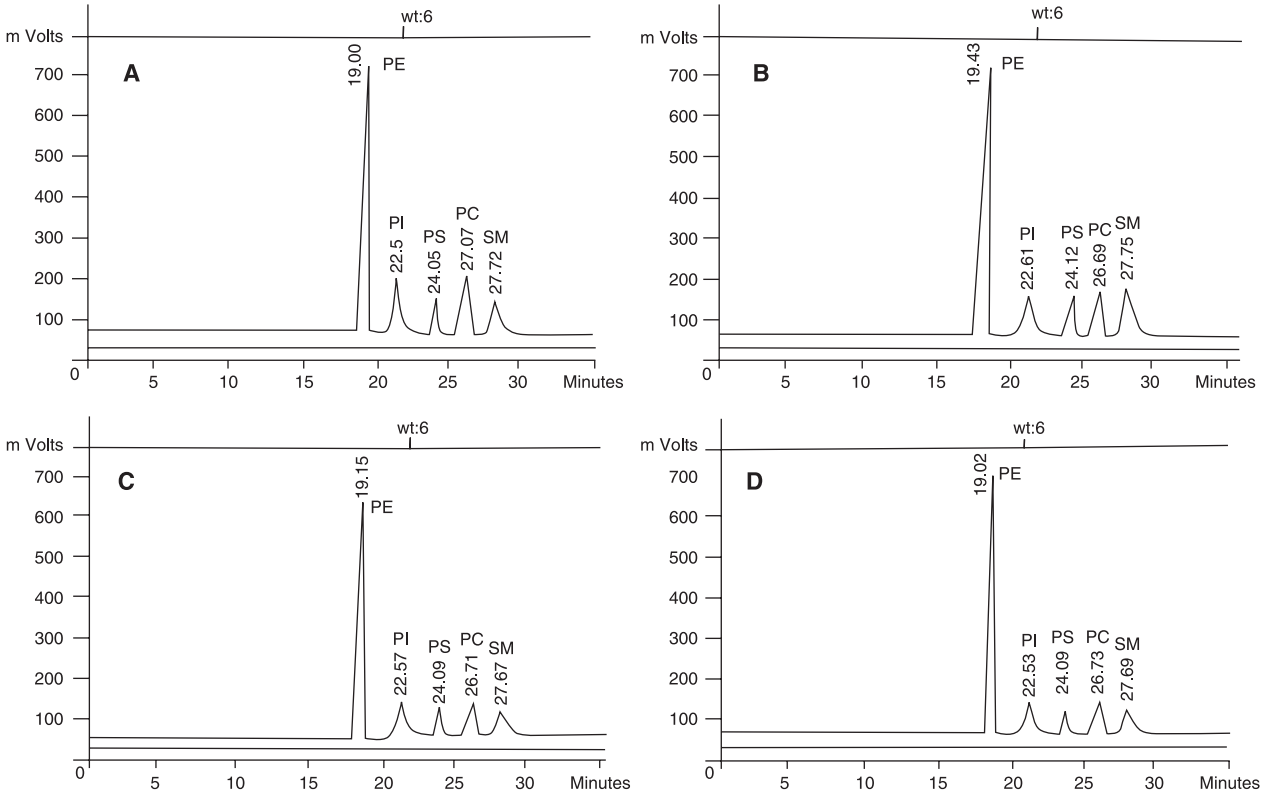

Fig. 4. The levels of individual phospholipids in the erythrocyte membrane of the control and the alcoholic group. A: Standard phospholipids. B: Control. C & D: Alcoholics.

of protein carbonyl, protein available sulfhydryl group and protein total sulfhydryl group in the erythrocyte membrane of control and the alcoholic group.

Erythrocyte morphology changes between the control and alcoholic group are presented in Fig. 5 and Fig. 6.

Discussion

A number of laboratory markers are suggested for the detection and monitoring of alcohol abuse. One of them is sialic acid the acyl derivative of neuraminic acid occurs as non-reducing terminal residue of glycoproteins or glycolipid in biological fluids and cell membrane. The sensitivity of sialic acid as marker is 95.5% in women who consume alcohol [*17*]. Maritta Ponnio [*18*] suggested that serum sialic acid levels are significantly elevated during drinking and would serve as potential marker for alcohol abuse. In our studies the Sialic acid levels in serum increased significantly in alcoholic group compared to control. The elevated Sialic acid level clearly demonstrates the alcohol abuse in test samples hence the erythrocytes from these samples are used for further membrane studies along with control samples.

In the present investigation acid hemolysis difference between alcoholic and control samples are clearly demonstrated in erythrocytes. The increased alcoholic hemolysis is related to the effects of ethanol on erythrocyte Ion transport system and to changes in the fluidity of the erythrocyte membrane caused by ethanol consumption [*19*, *20*]. Such changes to the properties of the membrane may be caused by direct effect of ethanol on the membrane [*21*] or by formation of reactive metabolites of ethanol, which can damage membrane components oxidatively [*22*]. To investigate these changes further studies were carried out in erythrocytes and its membrane.

The erythrocytes have multiple defenses against oxidation, which may protect different components particularly the membrane components of the cell. Scott *et al.* [*23*] suggested that Nicotinamide adenine dinucleotide phosphate (reduced) (NADPH) is significant antioxidant for the protection of intracellular Hb. Since the activity of G6PDH is decreased in alcoholic samples the NADPH protection for Hb may be considerably reduced. Normally erythrocytes appear to have high catalase activity because of great exposure of erythrocytes to molecular oxygen. Catalase has a dual functional role, a true catalytic role in the decomposition of H_2O_2 and a peroxidic role in which the peroxide is utilized to oxidize a range of hydrogen donors. Since the catalase activity is significantly reduced in alcoholic samples peroxidic protection and H_2O_2 decomposition within the cell may be considerably reduced. In this situation the increased peroxy radicals would significantly reduce the intracellular GSH apart from GSH insufficiency arising from reduced NADPH levels. Indeed in our studies we found a significant reduction in the GSH levels of erythrocytes in women who consume alcohol. Sandhu *et al.* [*24*] reported that reduced intracellular GSH could play a major role in the erythrocyte hemolytic event. Baton [*25*] proposed that catalase and NADPH protect erythrocyte against acute high exogenous levels of peroxide, where as GSH is needed to protect the cell against the low levels that are continuously generated endogenously. It is evident from our study that the protection against exogenous and endogenous peroxides in the erythrocytes of alcoholic women are considerably affected due to decreased activity of catalase, glucose-6-phosphate dehydrogenase and GSH. As the protection reduced the free radical generation would cause noticeable oxidation of oxyHb to metHb. Our studies also demonstrate increased methemoglobin levels in erythrocytes of alcoholic women indicating the depletion of GSH may be mainly responsible for Hb oxidation. Lusini *et al.* [*26*], who concluded the thiol oxidation of RBC was directly related to methemoglobin formation, support this observation.

Erythrocyte membranes are the first working point on which absorbed alcohol affects erythrocytes. Alcohol directly acts on cholesterol and phospholipid of the membrane and affect the fluidity of the membrane and its haemolysis. Phospholipid, the backbone of all cellular membrane is the primary target of peroxidation and can be strikingly altered by ethanol [*27*]. Our HPLC studies clearly show decreased level of erythrocyte membrane phospholipid in alcoholic women compared to control. Benedetti *et al.* [*19*] reported that altered composition of fatty acids in erythrocyte membrane in alcoholics would directly affect its membrane fluidity. Significant increase in the ratios cholesterol/phospholipid and cholesterol/protein in membrane would decrease the membrane fluidity in women who consume alcohol, where membrane fluidity was studied using diphenyl hexatriene as probe. Chin [*28*] also reported that fluidity could be influenced by changes in cholesterol/phospholipid ratio. Decreased membrane fluidity of erythrocytes from women who consume alcohol could be the main cause for increased haemolysis is evident from our studies.

Increased lipid peroxide in the erythrocyte membrane indicates the peroxidative damage to this membrane. GSH levels are significantly reduced in the membrane of women alcoholic. Kosower *et al.* [*29*] reported that when cellular GSH decreases membrane protein thiols are oxidized with the formation of intrachain and interchain disulfides. They established a direct link between the thiol status of the cell membrane and cellular GSH. GSH serves as a reductant of membrane protein disulfides in addition to averting membrane thiol oxidation and depletion of GSH leads to the destabilization of membrane skeleton through oxidation of membrane protein thiols [*30*]. Also Zarodnik *et al.* [*31*] have reported clearly that membrane changes preceded GSH depletion and were observed simultaneously with methemo-

globin in human red blood cells. Increased methemoglobin leads to hemi chrome formation, which can release heme after binding to the membrane. This leads to significant reduction in membrane GSH, which may cause protein thiol oxidation. Thus peroxidative damage to membrane lipids and oxidation of membrane protein thiols potentially harmful to membrane fluidity and flexibility is demonstrated in women who consume alcohol. Decreased levels of GSH are believed to be the main reason for the above mentioned changes. Hence the present results reveal for the first time a mechanism that could account for increased haemolysis.

Oxidative modification of protein in erythrocyte membrane is of great importance because of their biological role in transport, enzyme activity and membrane fluidity. Introduction of carbonyl groups into amino acid residues of protein is a hallmark of oxidative modification. In the present study, the comparison of protein carbonyls protein total and available sulphydryl content of control and women alcoholic reveal a significant reduction in available and total sulphydryl and increased protein carbonyls content. The modification of protein content may be due to the increased protein oxidation caused by depletion of glutathione and which in turn leads to destabilization of membrane skeleton [*32*]. Loguercio *et al.* [*33*] reported the decreased total sulphydryl group in chronic alcoholics. Our histopathological study confirms that the shape of the erythrocytes is significantly altered in the erythrocytes in women who consume alcohol (Burr cell formation).

The minimal alteration of liver function test (data not shown) in the group of alcoholics considered in the present study suggests that changes in the properties of erythrocyte membrane appear early and reflect the diffuse effect of ethanol on biological membrane organization.

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