

## Protective effect of green tea on electric properties of rat erythrocyte membrane during ethanol intoxication

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**Abstract:** Food ingredients, such as, ethanol that is metabolized with reactive aldehyde and free radicals formation can modify cell membrane components and its physicochemical properties. The destructive action of free radicals can be neutralized by administration of antioxidants, especially natural ones like green tea. For this reason, the purpose of this work was to determine the effect of green tea on concentration of ionizable groups, amount of phospholipids and level of lipid peroxidation products of erythrocyte membrane of rats, chronically intoxicated with ethanol. The surface charge density as a pH function used for the above calculations was measured by electrophoresis. Qualitative and quantitative composition of phospholipids in the membrane was determined by HPLC, while the extent of lipid peroxidation was determined by spectrophotometric measurement of the level of lipid peroxidation products as thiobarbituric reactive substances (TBARS). It was shown that ethanol intoxication caused an increase in the above parameters. Green tea significantly prevented changes caused by ethanol. The authors proposed the theoretical description for the experimental curves.

**Key words:** Surface charge density, Phospholipids, Lipid peroxidation, Erythrocyte, Ethanol, Green tea.

### Introduction

Every cell of a living organism is enclosed by cell membrane constituting its integral part. Like at any interface, there is an electric double layer between the cell membrane and the cell environment. The structure and properties of the layer are determined by the components of the outer membrane layer and at equilibrium between its components and the substances present in its environment. Any perturbation in the action of the cell is manifested by variations in the action of the cell membrane, i.e. in its electric double layer. An essential property of the electric double layer is its electric charge which can be altered by various xenobiotics or by metabolic transformations. For this reason, studies of the electric charge can furnish much information on equilibrium existing within the membrane and between the membrane and its environment, both in physiological and in non-physiological conditions (Gennis, 1989; Dołowy, 1984).

Structure of a natural membrane is very complex implying numerous equilibrium between the cell components and between the cell components and the cell environment. It is impossible to determine the parameters of so numerous equilibria numerically. Therefore, a small number of parameters should be adopted to characterize the equilibrium between membrane components and the environment that would cover the values averaged for all equilibria. Both the concentration sum of the functional acidic and as well as of sum basic groups on the membrane surface and their averaged association constants with hydrogen or hydroxyl ions can be used as such parameters.

It is the purpose of the present work to propose the above magnitudes as parameters characterizing the cell

membrane, to check their utility for following changes in the membrane surface caused by toxic agents, and to elaborate the method to determine the parameters from electrophoretic mobility of cells. This work is also aimed at evaluation of the effect of green tea on the proposed parameters, the content of phospholipids and the level of lipid peroxidation products of rat erythrocyte membrane in chronic ethanol intoxication.

**Theory:** Dependence of surface charge density of cell membrane on pH of electrolyte solution can be described in terms of acid-base equilibrium. The membrane components, mainly phospholipids and proteins, have ionizable groups which can adsorb the H<sup>+</sup> or OH<sup>-</sup> ions on the membrane surface. The existing equilibrium can be written in the form:



Association constants of the H<sup>+</sup> and OH<sup>-</sup> ions with ionizable groups are expressed by equations (Harris, 1996):

$$K_A = \frac{a_{AH}}{a_{A^-} \cdot a_{H^+}} \quad (3)$$

$$K_B = \frac{a_{BOH}}{a_{B^+} \cdot a_{OH^-}} \quad (4)$$

Here:

$K_A$ ,  $K_B$  - mean association constants of acidic and basic groups (A<sup>-</sup> and B<sup>+</sup>), respectively,

$a_{A^-}$ ,  $a_{AH}$ ,  $a_{B^+}$ , and  $a_{BOH}$  - surface concentrations of corresponding groups on the membrane surface,

$a_{H^+}$  and  $a_{OH^-}$  - corresponding concentrations by volume of the H<sup>+</sup> and OH<sup>-</sup> ions in solution. Surface charge density is

expressed as follows:

$$\delta = (a_{B^+} - a_{A^-}) \cdot F \quad (5)$$

Here:

$$\delta \left[ \frac{C}{m^2} \right] - \text{surface charge density,}$$

$$F = 96487 \left[ \frac{C}{mol} \right] - \text{Faraday constant.}$$

Ionizable group concentration balances are expressed as follows:

$$C_{TA} = a_{A^-} + a_{AH} \quad (6)$$

$$C_{TB} = a_{B^+} + a_{BOH} \quad (7)$$

Here:

$$C_{TA}, C_{TB} \left[ \frac{mol}{m^2} \right] - \text{total surface concentrations of}$$

ionizable groups.

The Eqns. from (3) to (7) were treated as an equation system; the  $a_{A^-}$ ,  $a_{AH}$ ,  $a_{B^+}$ , and  $a_{BOH}$  values were eliminated yielding the equation:

$$\frac{\delta}{F} = \frac{C_{TB} \cdot a_{H^+}}{a_{H^+} + K_B \cdot K_w} - \frac{C_{TA}}{K_A \cdot a_{H^+} + 1} \quad (8)$$

It is difficult to carry out the regression of Eqn. (8) to determine the  $C_{TA}$ ,  $C_{TB}$ ,  $K_A$ , and  $K_B$  constants.

Simplifying to one fraction and making transformations described in this work (Brzozowska and Figaszewski, 2003), we can receive the equation of a straight line for high and low ion concentration  $H^+$ , from which  $C_{TA}$ ,  $C_{TB}$ ,  $K_A$ ,

$K_B$  values can be established.

The coefficients could be determined by linear regression and they could be used to determine the  $C_{TA}$ ,  $C_{TB}$ ,  $K_A$ , and  $K_B$  values. In each case, it should be determined which points should be considered in the regression, both in a high and low  $H^+$  concentration range.

### Materials and Methods

**Animal:** Twelve month-old (520-550g b.w.) male Wistar rats were used for all experiments. Rats were fed the liquid diet for 5 weeks before death. Dietary intake was comparable in all groups, with all rats demonstrating consistent weight gain throughout the 5 weeks feeding period. Ethanol fed rats had slightly decreased rates of weight gain (about 10% less weight gain compared with control-fed rats), consistent with the well-studied effects of isocaloric ethanol feeding on intermediate metabolism. All experiments were approved by the Local Ethic Committee in Białystok (Poland) referring to the Polish Protection of Animals Act of 1997. Rats were housed in individual cages and pair-fed with either nutritionally control - adequate liquid Lieber DeCarli diet containing 47% of total

energy as carbohydrate, protein 18%, lipid 35% or the identical diet with ethanol substituted isocalorically for carbohydrate (36% of total energy) (Lieber and DeCarli, 1970). Liquid diet (control and ethanol) containing 7g green tea extract/l diet was also prepared. Green tea - *Camellia sinensis* (Linnaeus) O. Kuntze (standard research blends - lyophilized extract) was provided by TJ Lipton (Englewood Cliffs, NJ). Green tea extract contained epigallocatechin gallate (97mg/g dried extract), epigallocatechin (82mg/g dried extract), epicatechin (90mg/l), epicatechin gallate (15mg/g dried extract) and caffeic acid (10mg/g dried extract), determined by HPLC (Maiani et al., 1997).

The animals were divided into the following groups:

Control group was fed for 5 weeks on a control Lieber DeCarli liquid diet (n=6).

Green tea group was fed for 5 weeks on a control liquid Lieber DeCarli diet containing green tea (7g/l) (n=6).

The ethanol group was fed for one week on a control liquid Lieber DeCarli diet and for the next 4 weeks on ethanol Lieber DeCarli liquid diet (n=6).

Ethanol and green tea group were fed for one week on a control Lieber DeCarli liquid diet containing green tea (7g/l) and next for 4 weeks with ethanol Lieber DeCarli liquid diet containing also green tea (7g/l) (n=6).

After 5 weeks of the experiment all rats were sacrificed under ether anaesthesia (six animals in each group) and the blood was taken into containers with heparin.

**Electrochemical methods:** In order to determine surface charge density of cell membrane, erythrocytes were put into the measuring vessel, and then electrophoretic mobility was measured by using DTS5300 Zetasizer 3000 apparatus (Malvern instruments).

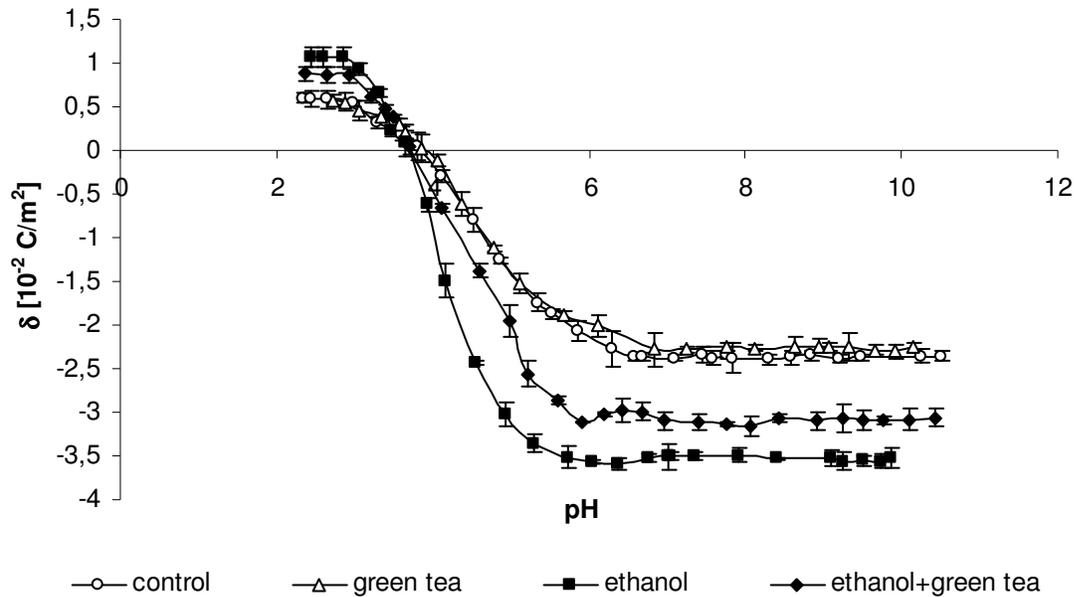
The surface charge density has been determined using the equation:  $\sigma = \eta u / d$ ; here  $u$  – electrophoretic mobility,  $\eta$  - viscosity of solution,  $d$ - diffuse layer thickness (Krysinski and Tien, 1986). The diffuse layer thickness was determined from

$$\text{the formula (Sobczyk et al., 1982) } d = \sqrt{\frac{\epsilon \cdot \epsilon_0 \cdot R \cdot T}{2 \cdot F^2 \cdot I}}$$

$R$  is the gas constant,  $T$  is the temperature,  $F$  is the Faraday number and  $I$  is the ionic strength of 0,9 % NaCl,  $\epsilon \epsilon_0$  – permeability electric medium.

**Isolation and analysis of phospholipids by HPLC method:**

Ten milliliters of blood were centrifuged at 1500 g for 30 min. The plasma and leukocytes forming a layer at the surface of erythrocytes were removed with a micropipette. The cells were suspended in Tris buffer (osmolality 310 mmol/kg, pH 7,6). The sample was centrifuged at 1500 g for 30 min. The supernatant was removed and washing with buffer solution was repeated twice. The washed cells were resuspended in Tris buffer to 10 ml. The erythrocytes were lysed by adding 30ml Tris buffer (osmolality 20 mmol/kg, pH 7-6) to 5 ml of the cell suspension. The mixture was allowed to stand for 5 min, and then it was centrifuged at 4000 x g for 40 min. The supernatant was



**Fig. 1:** Effect of green tea on changes in electric properties of erythrocyte membrane of rats receiving ethanol and/or green tea as function pH.

removed. The membranes were washed a further three times with 20 mmol/kg Tris buffer (Alling *et al.*, 1984). Then a method of Folch was applied to extraction of phospholipids. Separations and analysis of phospholipids was achieved by HPLC method (Ostrowska *et al.*, 2000).

**Biochemical analysis:** The erythrocytes were separated by centrifugation, washed twice with 5 ml PBS solution and lysed by the addition of 2.0 ml of ice-cold distilled water. Cell membranes and hemoglobin were removed from the obtained mixtures by extraction with 1.0 ml of cold ethanol and 0.6 ml of cold chloroform. Next centrifugation was done at 31,000 x g for 60 min at 4 °C. The supernatant was used for the analysis.

The extent of lipid peroxidation in erythrocytes was assayed with thiobarbituric acid (TBA). Chromogenous condensation product of TBA with malondialdehyde thiobarbituric acid-reactive substances -(TBARS) was extracted from the aqueous phase into butanol and then an absorption at 532 nm was monitored (Buege and Aust, 1978). The concentration of malondialdehyde was expressed in nmoles TBARS/ml erythrocytes lysates.

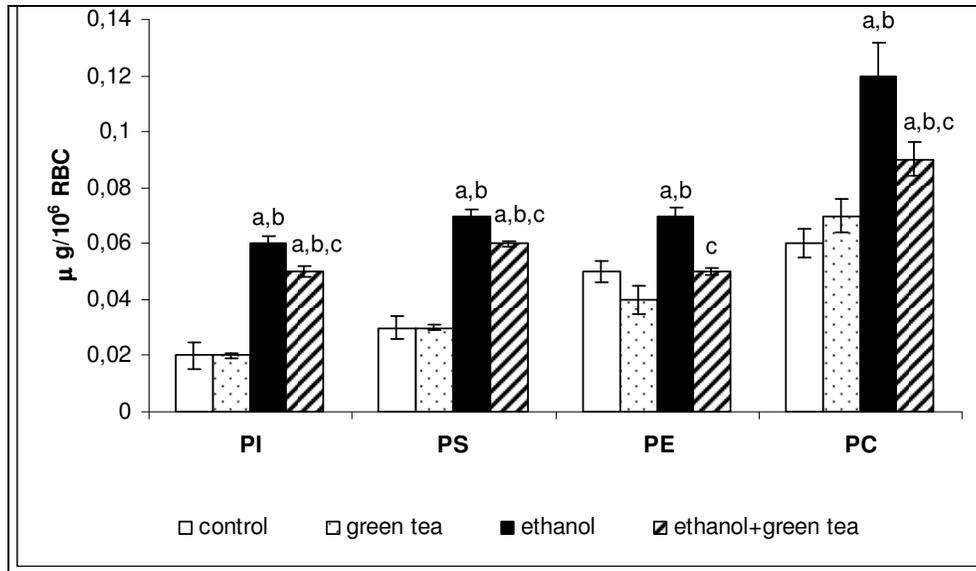
**Statistical analysis:** The data obtained in this study are expressed as mean  $\pm$  SD. The data were analyzed by the use of standard statistical analyses: one way ANOVA with Scheffe's F test for multiple comparisons to determine significance between different groups. The values for  $p < 0.05$  were considered significant.

### Results and Discussion

The experimental results indicate that a chronic ethanol intoxication of rats provokes an increase in negative and positive charge numbers at the erythrocytes surface. It

corresponds to an increased surface concentration of acidic groups ( $C_{TA}$ ) and basic groups ( $C_{TB}$ ) (Table 1). Changes in functional groups' composition on the membrane surface are due to the appearance of new functional groups and/or to disappearance of the existing ones as the result of reaction of the alcohol or of its metabolism products with membrane components. Variations in the number and kind of functional groups bring about variations in  $C_{TA}$  and  $C_{TB}$  and, in turn, in their association constant values. Ethanol intoxication makes the association constant of negatively charged groups ( $K_A$ ) lower and the association constant of positively charged ones ( $K_B$ ) higher (Table 1).

It has been demonstrated hitherto that ethanol poisoning provokes modifications in lipid double layer membrane (Klemm and Yuritas, 1992). Penetrating into the lipid bilayer, alcohol breaks hydrogen bonds, assumes the role of water, and at the same time reduces membrane surface hydration (Klemm and Yuritas, 1992). The alcohol molecule can form only one hydrogen bond in which its hydrogen atom is involved, either with a protein or with a lipid. A functional group is then liberated and may provoke a change in the cell membrane electric charge. Consequently, surface concentration of basic and/or acidic groups on the membrane can increase. Change in the group number can bring about change in association constants of positively and negatively charged groups. Interaction of alcohol molecule with the outer membrane surface consisting in its penetration between the phospholipid molecules heads results in increased membrane fluidity (Chin *et al.*, 1979; Zerouga and Beauge, 1992; Waring *et al.*, 1981). These changes are accompanied by a decrease in the sialic acid amount, both free and bonded in glycolipids and in glycoproteins, in the liver cell membrane (Beauge *et al.*, 1994;



**Fig. 2:** Content of four phospholipid classes of erythrocyte membrane of rats receiving ethanol and/or green tea.

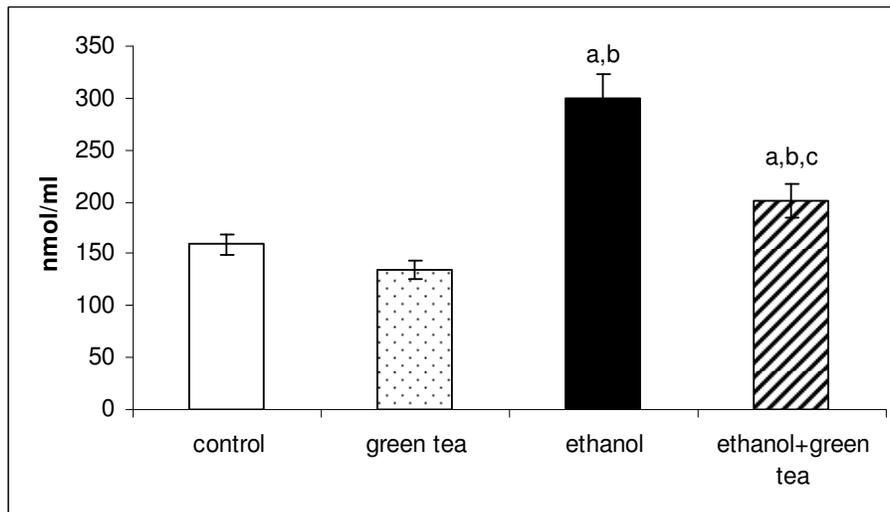
Statistically significant differences for  $p < 0.05$

<sup>a</sup> In comparison with control

<sup>b</sup> In comparison with green tea group

<sup>c</sup> In comparison with ethanol group

PI-phosphatidylinositol, PS- phosphatidylserine, PE- phosphatidylethanolamine, PC- phosphatidylcholine;



**Fig. 3:** Effect of green tea on the lipid peroxidation products measured as thiobarbituric reactive substances (TBARS) in erythrocytes of rats receiving ethanol and/or green tea.

Statistically significant differences for  $p < 0.05$

<sup>a</sup> In comparison with control

<sup>b</sup> In comparison with green tea group

<sup>c</sup> In comparison with ethanol group

Cherian and Klemm, 1991). A lower amount of this acid is supposed to expose positive and negative groups of lipids or proteins. It can change association constants. The results obtained in this work suggest that the negatively charged groups appearing on the surface are more acidic and the

positively charged ones are more basic than groups localized on the membrane surface of cells from erythrocyte of control rats.

In alcohol intoxication, the cell membrane components are also subjected to the action of alcohol metabolites. The first ethanol metabolite – acetaldehyde being very reactive can

**Table – 1:** Effect of green tea on the value  $C_{TA}$ ,  $C_{TB}$ ,  $K_A$ ,  $K_B$  of erythrocyte membrane intoxicated with ethanol.

Parameters Group	$C_{TA}$ ( $10^{-7}$ mol/m <sup>2</sup> )	$C_{TB}$ ( $10^{-7}$ mol/m <sup>2</sup> )	$K_A$ (m <sup>3</sup> /mol)	$K_B$ ( $10^6$ m <sup>3</sup> /mol)
Control	2.46 ± 0.12	0.65 ± 0.07	21.51 ± 0.9	1.32 ± 0.08
Green tea	2.33 ± 0.11	0.64 ± 0.04	23.31 ± 1.1 <sup>a</sup>	1.09 ± 0.06 <sup>a</sup>
Ethanol	3.70 ± 0.11 <sup>a,b</sup>	1.19 ± 0.07 <sup>a,b</sup>	15.68 ± 0.9 <sup>a,b</sup>	2.07 ± 0.07 <sup>a,b</sup>
Ethanol +green tea	3.20 ± 0.07 <sup>a,b,c</sup>	0.96 ± 0.07 <sup>a,b,c</sup>	19.49 ± 0.8 <sup>a,b,c</sup>	1.9 ± 0.07 <sup>a,b,c</sup>

Statistically significant differences for  $p < 0.05$

<sup>a</sup>In comparison with control, <sup>b</sup>In comparison with green tea group, <sup>c</sup> In comparison with ethanol group

modify amino acid residues of membrane proteins by reacting with free amino and sulfhydryl groups (Skrzydłowska and Roszkowska-Jakimiec, 1992; Sprince *et al.*, 1975). Consequently, it can reduce the positive charge of proteins and charge of lipids of the erythrocyte membrane.

Membrane lipids and proteins can also be modified by reactive oxygen species (ROS) appearing as the result of alcohol metabolism (Sevanian and Ursini, 2000). The ROS action results in lipid peroxidation and to lipid hydroperoxide formation. The enhanced level of lipid peroxidation products was observed in this study after ethanol intoxication (Fig. 3). As the consequence, phosphatidylserine molecules, physiologically present on the inner side of the membrane become exposed on its outer side (Jain, 1985; Tyurina *et al.*, 2000). Exposing the phosphatidylserine molecule to the outer side of the membrane makes an additional negatively charged group appear there. It provokes an increase in acid group surface concentration as it has been confirmed in this work (Table 1). The presence of additional ionizable groups of phosphatidylserine can result in a decreased association constant of negatively charged groups of the membrane and in increased association constant of positively charged ones because their  $K_A$  value is lower and the  $K_B$  value is higher than those of original cell membrane groups (Petelska and Figaszewski, 2002).

Proteins also undergo oxidative modifications in the presence of ROS, particularly residues of aromatic amino acid containing sulfhydryl groups are particularly sensitive (Davies *et al.*, 1987b; Wolff *et al.*, 1986). Changes in the primary structure of protein bring about modifications of secondary and tertiary structure which can induce either aggregation or fragmentation of protein macromolecules (Davies *et al.*, 1987a; Dean, 1995). Protein fragmentation yields new ionizable groups, both positively and negatively charged. The process can also result in exposing phospholipid ionizable groups which are present in the membrane; it can result in an increased surface concentration of acid and basic groups on the cell surface. Such a fact has been confirmed in this work (Table 1). All these phenomena can modify the association constant of both negatively and positively charged groups.

On the other hand, the results presented in this work (Fig. 2) demonstrate that ethanol intoxication brings about an increase in the level of all phospholipids in erythrocyte

membrane. It has been supported by the literature data (Branchey and Buydens-Branchey, 1990; Slater *et al.*, 1993). Therefore, the number of acid and basic functional groups is reduced during ageing of an organism because of decrease of phospholipid amount resulting in lower  $C_{TA}$  and  $C_{TB}$  values as well as in changes in the  $K_A$  and  $K_B$  values.

It is considered that most changes in the cell membrane occurring during alcohol intoxication are due to an increasing formation rate of reactive oxygen species and to reduced antioxidant abilities of the organism. For this reason, it was attempted to prevent these changes by administering green tea extract to the ethanol-intoxicated animals. Green tea is an example of such a natural antioxidant preparation. It contains a large amount of polyphenols, mainly catechin and its derivatives of strong antioxidant properties (Lee *et al.*, 2000; Skrzydłowska *et al.*, 2002). For this reason, the ingested catechins can exert biological effects. Consumption of green tea by ethanol-intoxicated rats significantly prevented changes caused by ethanol.

The following parameters  $C_{TA}$ ,  $C_{TB}$ ,  $K_A$  and  $K_B$ , can be used to show changes occurring at the surface of membrane in different pathological situation e.g. alcohol intoxication. Changes in this parameters enable of discussion concerning causes above modifications up to molecular level.

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