IN VITRO EFFECTS OF ETHANOL AND ASPIRIN COMBINED ADMINISTRATION ON ERYTHROCYTE MEMBRANE PROPERTIES

ERİTROSİT MEMBRANINDA ETANOL VE ASPİRİNİN BİRLİKTE VERİLMESİNİN İN VİTRO ETKİLERİ

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Abstract
This report describes in vitro effect of ethanol and combined administration of ethanol with aspirin on erythrocyte membrane properties. Furthermore, we evaluated the possible protective role of s-Adenosylmethionine (SAMe) and for this aim, we determined plasma malondialdehyde (MDA), sialic acid (SA) levels and the activity of membrane-bound Na^+-K^+ATPase.
Concentration doses were adjusted as control (0% ethanol), ethanol (0,5 %), aspirin (4 mg/ml) and SAMe (2.5 nM). Blood samples were divided into five groups as control, ethanol, [ethanol+aspirin], [ethanol+SAMe] and [ethanol+SAMe] group and incubated on 25°C for 16 h. After this, we measured Na^+-K^+ ATPaz, MDA, Sialik Asit levels.
Na^+-K^+ATPase activities were decreased in the ethanol and 0,5% [ethanol+SAMe] groups as compared to the control group ($p<0.05$). Plasma MDA levels in the [ethanol+aspirin] and [ethanol+aspirin+SAMe] groups were higher than in the control group ($p<0.001$), also the [ethanol+aspirin] group was higher than in the ethanol group ($p<0.001$). SA levels in the [ethanol+SAMe] groups were increased significantly as compared to the control group ($p<0.01$).
We assume that aspirin protected ethanol-induced inactivation of Na^+-K^+ ATPase activities and also decreased sialic acid levels. But, combined administration of aspirin with ethanol increased peroxidative injury of erythrocyte membranes. We think that protective role of SAMe may be dose-dependent. (Pam Med J 2009;2(1):1-7).
Key words: Acetylsalicylic acid ,s- adenosylmethionine, ethanol, Na^+K^+ATPase, Sialic Acid, MD

Özet
Bu çalışmanın amacı; etanolün ve etanol ile aspirinin birlikte verilmesinin eritrosit membranındaki in vitro etkilerini açıklamaktır. Bununla birlikte s-adenozilmetiyonin'in (SAMe) muhtemel koruyucu etkisini değerlendirdik ve bu amaç için membrana bağlı Na+-K+ ATPaz'ın aktivitesini ve plazma malondialdehit (MDA), sialik asit (SA), düzeylerini tespit ettik.
Konsantrasyon oranları, etanol (%0.5), aspirin (4 mg/ml) ve SAMe (2.5 nM) olarak ayarlanan kan örnekleri [etanol+aspirin+SAMe], [etanol+SAMe], [etanol+aspirin], etanol ve kontrol grubu olmak üzere 5 gruba ayrıldı ve bu kan örnekleri 16 saat boyunca 25°C'de inkübasyona bırakıldı. Daha sonra bu numunelerde Na^+-K^+ ATPaz, MDA, Sialik Asit düzeyleri çalışıldı.
Etanol ve [etanol+SAMe] grubunun Na^+-K^+ ATPaz aktiviteleri kontrol grubu ile mukayese edildiğinde azaldığı görüldü ($p<0.05$). [Etanol+aspirin] ve [etanol+aspirin+SAMe] gruplarında plazma MDA düzeyleri kontrol grubundan ($p<0.001$), [Etanol+aspirin] grubu da etanol grubundan yüksekti ($p<0.001$). [Etanol+SAMe] gruplarında SA düzeyleri kontrol grubu ile mukayese edildiğinde anlamlı bir şekilde artmıştı ($p<0.01$).
Aspirinin etanolle oluşturulmuş Na^+-K^+ ATPaz aktivitelerinin inaktivasyonunu koruduğunu aynı zaman da sialik asit düzeylerini azalttığını, ancak etanolle aspirinin birlikte verilmesi eritrosit membranındaki peroksidatif hasarı arttırdığını tespit ettik. Biz SAMe'nin koruyucu rolünün doza bağlı olabileceğini düşünmekteyiz. (Pam Tıp Derg 2009;2(1):1-7).
Anahtar kelimeler: Asetil salisilik asit, s-adenozilmetiyonin, etanol, Na^+K^+ ATPaz, Sialik Asit, MDA

Introduction

Aspirin (acetylsalicilic acid), which is one of the widely used non-steroidal anti-inflammatory drugs, is probably the most highly consumed worldwide pharmaceutical product which has gained greater importance as a cardioprotective drug. However, because of adverse effects on

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Yazının dergiye gönderilme tarihi: 22.07.2008 Yazının basıma kabul tarihi:24.12.2008

multiple tissues, the use of aspirin is associated with significant morbidity and mortality. Long-term consumption of aspirin is related to gastrointestinal ulcerations, nephrotoxicity, hepatotoxicity and even renal cell cancer [1,2].

Chronic ethanol consumption represents one of the most serious socio-economic and health problems in the world and causing a significant mortality [3]. Ethanol metabolism in the different animal tissues and cells is via oxidation to acetaldehyde by alcohol dehydrogenase, ethanol-inducible cytochrome P-450 (CYP2E1), and catalase [4]. Furthermore, because of the highly reactive properties of acetaldehyde, which can modify proteins and other molecules the oxidation of ethanol to acetaldehyde is deleterious. In addition to the effects of ethanol metabolites, unmetabolized ethanol may also have direct effects on cells, and it has been shown that it can permeate membranes and disrupt normal cell structure and metabolism. Investigators reported that no detectable metabolism of ethanol was found in erythrocytes, although ethanol itself caused an elevated rate of spontaneous haemolysis in erythrocyte preparations. The results from the haemolysis studies on erythrocyte stability indicate that ethanol has a destabilizing effect on erythrocytes [5-7].

Visual examination of the ethanol-exposed erythrocytes revealed no obvious morphological changes in the preparations, although it is known that after longer periods of exposure morphological changes can occur [8].

Ethanol metabolism causes oxidative stress and lipid peroxidation not only in liver but also in extra-hepatic tissues. Malondialdehyde (MDA) is one the markers of peroxidative injury [9,10]. This compound is a reactive aldehyde and is one of the many reactive electrophile species that cause toxic stress in cells and form advanced glycation endproducts [11]. The production of this aldehyde is used as a biomarker to measure the level of oxidative stress in an organism [12,13].

The flux of ions across membranes is largely dependent on transmembrane ion specific proteins such as the Na+-K+ATPase. Due to the close interaction of Na^+-K^+ATPase with the membrane lipids, several lipophilic molecules are known to have toxic effects on this pump [14]. Current evidence indicates that ethanol and aspirin affects membrane ATPases and membrane lipid composition [9,14]. SA is one of the components in biological fluids in cell membranes as non-reducing terminal residues of glycoproteins and glycolipids [15]. Ponnino et al [16] reported that SA is a valuable marker for detecting and monitoring alcohol abuse and concentrations in female and male alcoholics were significantly higher in serum. SAMe serves as a methyl donor for most biolgical transmethylation reactions [17] and prevents ethanol-related liver damage and it contributes to normalization of erythrocyte membrane fluidity by affecting membrane lipids and membrane ATPases [18].

In our study, to evaluate erythrocyte membrane alterations, the in vitro effects of ethanol and ethanol plus aspirin administration were investigated. Furthermore, the possible protective role of SAMe as methyl donor on human erythrocyte membrane was evaluated.

Material and Methods

Experimental design and preparation of erythrocytes:

For in vitro experiments on isolated erythrocytes in the department of Biochemistry in Eskişehir Osmangazi University, cells were donated by Healty adult males. Four different erythrocyte preparations were used in all experiments. Bloods samples in this study was obtained from haematology department at Osmangazi University Medical Faculty and informed consent of all donors for blood sample collection. Blood samples were collected in the anticoagulant tubes including NaEDTA and 40 samples were divided into five equal groups and incubated at 25°C, during 16 h. for: (1) the 0% ethanol group as a control group, (2) 0,5% ethanol group, (3) 0.5% ethanol plus aspirin group (4) 0.5% ethanol plus SAMe group (5) 0.5% ethanol, aspirin plus SAMe group. In the control group equal volume of 0.9% NaCl was administrated into tubes. 0.5 % ethanol concentration was given according to Tyulina et al [7]. Aspirin (4mg/ml) was administrated in addition to the ethanol as described by Bilto [19]. SAMe (2.5mM) was incubated in same conditions according to Seyoum [17].

Following incubation, blood samples were centrifuged at 1500 x g for 10 minutes at room temperature. Supernatant were separated as plasma samples and erythrocyte pellets were washed three times with 0.9 % NaCl for Na^+-K^+ ATPase activity of the erythrocyte membrane. MDA and SA levels were determined on the plasma samples.

Erythrocyte ghost preparation:

Erythrocytes were hemolyzed with 10 mmol/L Tris, 1 mmol/EDTA (pH 7.4) at 4^o C. Ghosts were

sedimented at 27.000 x g (20 min, 4°C). Pellets were washed with 10 mmol/L tris, (pH 7.4) three times. Membranes were resuspended in the buffer and stored -70°C. Membrane proteins were measured using to Biuret method [20].

Assay of membrane-bound ATPase activities:
The assay links the hydrolysis of ATP by ATPase with NADH oxidation in the presence of excess pyruvate kinase, lactate dehydrogenase (LDH) and phosphoenolpyruvate (PEP), thus allowing continuous spectrometric recording at 340 nm. The activity of Na^+-K^+ ATPase was measured in the following reaction mixture: 30 mmol/L imidazole (pH 7.3), 100 mmol/L NaCl, 10 mmol/L KCl, 2.5 mol/L $MgCl_2$, 0.5 mol/L ethylene glycol-bis (β-aminoethylether)-N, N, N', N_2-tetraacetic acid (EGTA), 1 mol/L Na_2-ATP, 1 mol/L PEP, 0.15 mol/L NADH, and 50 μg/ml LDH. The enzyme reaction was started by the addition of 25 μl of the membrane suspension in reaction mixture. NADH oxidation was monitored by measuring the absorbance at 340 nm every 30 s for 10-15 min. The results were expressed as units per miligram protein described in Matteucci et al [21].

Plasma malondialdehyde levels:
Lipid peroxidation was assayed by the measurement of MDA reacted with thiobarbituric acid (TBA), according to Ohkawa et al [22]. Absorbances were measured at 532 nm. Plasma MDA levels were expressed as nmol/dl.

Plasma sialic acid levels:
Sialic acid levels were determined described in Denny et al [23]. Ten µl of plasma samples were hydrolyzed in 0.05 mol/l H_2SO_4 in a final volume of 0.1ml for 1 h at 80°C to release sialic acid. Standards contain SA and those without SA serving as blanks, were also arranged to a volume of 0.1 ml to achieve a final concentration of 0.05 mol/l H_2SO_4. 0.25 ml periodate solution (0.025 mol/L periodic acid in 0.25 mol/L HCl) was added into standart and samples tubes and incubated at 37°C for 30 min. The excess periodate was reduced by adding 0.25ml of the sodium thiosulfate solution (5g/dl $Na_2S_2O_3.5H_2O$) directly into the sample solution and mixing without delay. The reaction was completed by the addition of 1.25 ml of TBA solution (0,1 mol/l TBA adjusted to pH:5.0 -7.0 with NaOH) and was heated at 100°C for 15 min to achieve colour production. The samples were cooled to room temperature. Following the addition of 2.2 ml acidic butanol (5 ml/dl 12 mol/l HCl in butan-1-ol), the tubes were capped and shaken. After centrifugation at 400 x g for 5 min, the butanol phase was removed and absorbances were measured at 549 nm. Sialic acid levels were expressed as μmol/ml.
Chemicals:
All chemicals were obtained from Sigma Chemical Co. (St Louis, Mo).

Statistical analysis:
In this study, Shapiro-Wilk tests of normality were made for all of the variables, Na^+-K^+ ATPase, Sialic acid, MDA had a normal distribution and one-way ANOVA were used. To make a parametric multiple comparison, the Tukey HSD test was used. For this analysis, spss 12.0 package program in the Statistic Department of Osmangazi University was used.

Results
Membrane-bound Na^+-K^+ ATPase activities, plasma MDA and SA levels are shown in Figure 1, 2 and 3 respectively. Na^+-K^+ ATPase activities were decreased in the 0.5% ethanol and 0.5% (ethanol + SAMe) groups as compared to the control group (0.018±0.002 U/mg protein; 0.017±0.002 U/mg protein; 0.027± 0.003 U/mg protein, respectively) ($p<0.05$).

Plasma MDA levels in the 0.5% (ethanol+ aspirin) and 0.5% (ethanol+aspirin+SAMe) groups) were higher than in the control group and 0.5% ethanol group (5.0±0.74 nmol/dl; 4.81±0.63 nmol/dl; 1.09±0.09 nmol/dl;1.69±0.46 nmol/dl, respectively) ($p<0.001$; $p<0.001$, $p<0.01$). The 0.5% (ethanol+aspirin) group was higher than in the 0.5% (ethanol + SAMe) group (5.0±0.74 nmol/dl; 2.57±0.24 nmol/dl, respectively) ($p<0.05$).

Plasma SA levels in the 0.5% ethanol and 0.5% (ethanol + SAMe) groups were higher than in the control group (2.66±0.13μmol/ml; 2.94±0.24 μmol/ml; 1.97±0.20μmol/ml, respectively) ($p<0.05$; $p<0.01$). The 0.5% of (ethanol+SAMe) group were increased as compared to the 0.5% (ethanol + aspirin) group (2.94±0.24μmol/ml; 2.15±0.11μmol/ml) ($p<0.05$). 0.5% (ethanol+ aspirin+SAMe) group were lower than in the 0.5% (ethanol+SAMe) group (2.02±0.12μmol/ml; 2.93±0.24μmol/ml, respectively) ($p<0.05$).

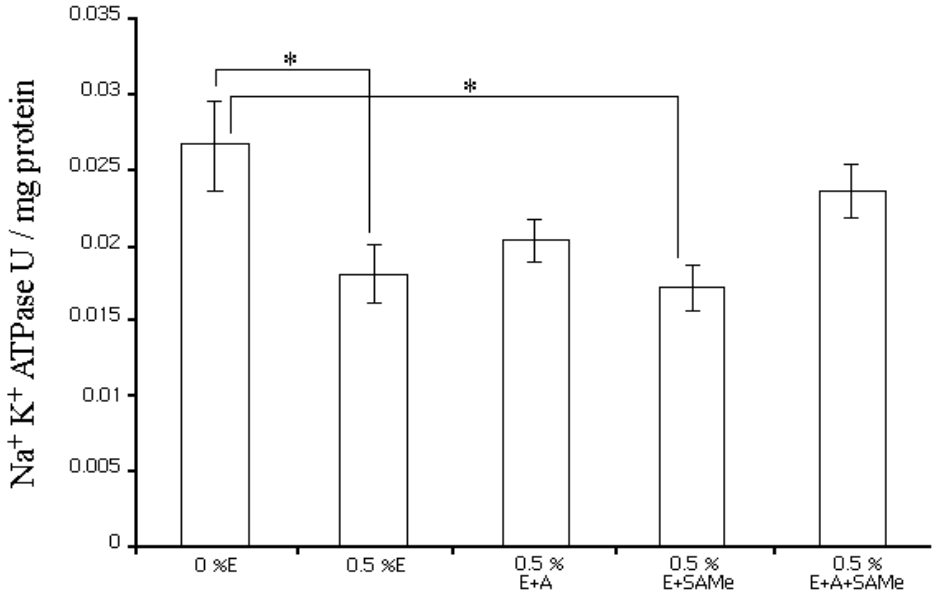

Figure 1. Membrane-bound Na^+-K^+ ATPase activities of human erythrocyte membrane.
The bar charts show the data mean ± SD (bars) for the effects of 0.5 % Ethanol, 0.5% Ethanol+aspirin, 0.5% Ethanol+SAMe and Ethanol+aspirin+SAMe on erythrocyte Na^+-K^+ATPase activities at 25°C for 16 h incubation period. The asterisk indicates the differences of groups (* $p<0.05$).

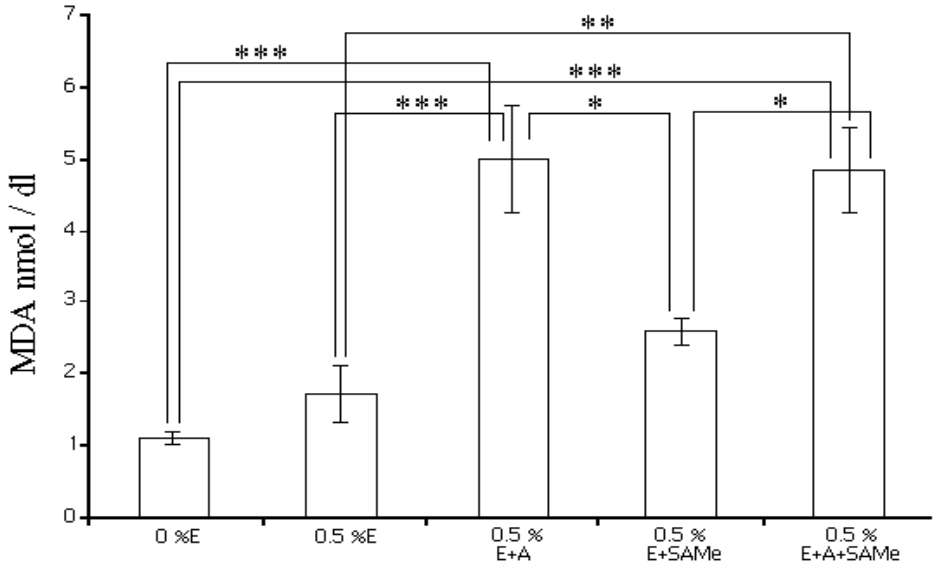

Figure 2. Plasma MDA levels
The bar charts show the data mean ± SD (bars) for the effects of 0.5% Ethanol, 0.5% Ethanol+aspirin, 0.5% Ethanol+SAMe and Ethanol+aspirin+SAMe on plasma MDA levels at 25°C for 16 h incubation period. The asterisk indicates the significant differences of groups (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

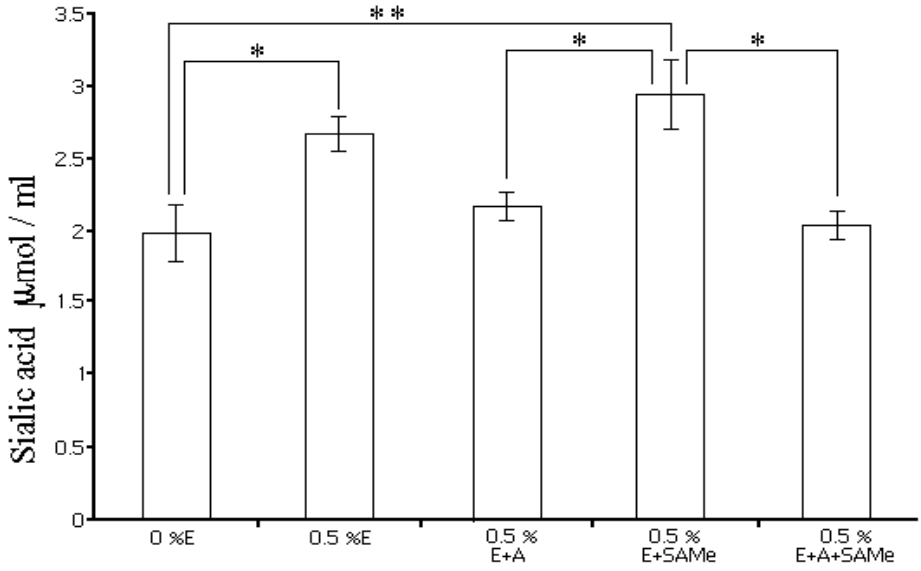

Figure 3. Plasma Sialic Acid Levels
The bar charts show the data mean ± SD (bars) for the effects of 0.5% Ethanol, 0.5% Ethanol+aspirin, 0.5% Ethanol+SAMe and Ethanol+aspirin+SAMe on plasma sialic acid levels at 25°C for 16 h incubation period. The asterisk indicates the significant differences of groups (* $p<0.05$, ** $p<0.01$).

Discussion

One of the toxic effects of alcoholism is an elevated erythrocyte haemolysis and chronic anaemia. The ethanol-mediated haemolysis is related to the effects of ethanol on erythrocyte membrane ion transport systems and to changes in the fluidity of the erythrocyte membrane caused [24]. Such impairment of the membrane structure may be caused by direct effects of ethanol on the membrane [25].

The direct inhibitory action of aspirin on the ATPase activity of erythrocytes and both, in vivo and in vitro on the cell size and/or cell volume has been reported [26,27]. Aspirin could play a rheologically active role on erythrocytes and these effects could be explained by acetylation of intracellular proteins [19].

SAMe plays an important role in phospholipid metabolism and in maintenance of membrane structure and function. SAMe-induced removal of excess cholesterol from the erythrocyte membranes of cirrhotic patients is accompanied by a return of membrane fluidity towards the normal range. It is possible that SAMe might directly contribute to normalization of erythrocyte membrane fluidity by stimuling the methylation of phosphatidylethanolamine to more fluid lecithin molecule [18].

The Na^+-K^+ ATPase is ubiquitously expressed in the plasma membrane to maintain the sodium and potassium gradients across the cell membrane. Our results showed that enzyme activities were elevated by aspirin administration in to the ethanol treated erythrocytes, such that they were the same as for the control group levels.

Reactive oxygen species (ROS) and free radicals are generated during ethanol metabolism, causing oxidative stress and lipid peroxidation in liver, brain, heart, and skeletal [28]. Our findings showed that plasma MDA levels slightly elevate in 0.5% ethanol group. But, this increase was not statistically significant ($p>005$).

Interestingly, aspirin led to peroxidative injury whilst aspirin significantly increased MDA together with 0.5% ethanol. Kirkova et al [29]. reported that aspirin showed a biphasic dependence on concentration in vitro low concentrations (1.0mM) stimulated the spontaneously formed malondialdehyde in the liver homogenate and the high concentration (5.0mM) inhibited it. Our results showed that the protective effect of SAMe was not seen aspirin plus ethanol-induced peroxidative injury on erythrocyte membrane.

SA, which is non-reducing terminal residues of glycoproteins and glycolipids, in serum were elevated depending on ethanol consumption. Sillanaukee et al [15] reported that sialic acid levels were elevated by high alcohol

consumption and reduced during abstinence. Ponnio et al. [16] also indicated that serum sialic acid is a valuable marker for detecting and monitoring alcohol abuse. Studies on erythrocyte membranes of alcholics showed also a decrease in sialic acid content and a disorganization of the outher membrane leaflet and reduction in erythrocyte sialic acid is a mechanism of erythrocyte destruction and may be important in erythrocyte senescence [30,31]. Investigators reported that administering ethanol (7.9 g/kg of body of weight) every day to wistar rats for 60 days resulted in significantly elevated levels of liver and brain sialic acid levels [32]. Since, sialic acid levels in the 0.5% ethanol group were higher than in the control group ($p<0.05$). Our findigs supported these reports. Aspirin appears to be prevents ethanol-induced elevated sialic acid levels. But, ethanol-induced sialic acid levels were not decreased by SAMe administration.

In conclusion, we found decreased Na^+-K^+ ATPase activities and increased plasma sialic acid levels at the concentration of 0.5% ethanol. But, this dose of ethanol did not lead to peroxidative injury. The effect of aspirin on eryhrocyte membrane properties together with ethanol was different. Aspirin protected ethanol-induced inactivation of Na^+-K^+ ATPase activities and also was decreased sialic acid levels. On the other hand, aspirin plus ethanol was increased peroxidative injury of erythrocyte membranes. In this paper, the protective role of SAMe may be dose-dependent but this still needs further investigations.

Acknowledgements: We would like to express our gratitude to Dr. Zafer Gülbaş,the Head of the Haematology Department, as to help us provide the blood samples and to Dr. Fezan Sahin for helping with the statistical analysis.

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