

## THE EFFECTS OF ETHANOL ON GLUCOSE 6-PHOSPHATE DEHYDROGENASE ENZYME ACTIVITY FROM HUMAN ERYTHROCYTES *IN VITRO* AND RAT ERYTHROCYTES *IN VIVO*

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**Abstract** — **Aims:** The effects of ethanol on erythrocyte glucose 6-phosphate dehydrogenase (G6PD) activity were investigated under *in vitro* and *in vivo* conditions. **Methods:** For *in vitro* studies, glucose 6-phosphate dehydrogenase was purified from human erythrocyte and rats were used for *in vivo* studies. Enzyme activity was determined spectrophotometrically by the Beutler method. **Results:** The *in vitro* study showed that the  $I_{50}$  value was 17 mM for ethanol. In the case of the *in vivo* study, a 2 ml/kg dose of ethanol significantly inhibited the G6PD activity. The inhibition rate after ethanol administration was 59%, 40% and 6% at 1, 3 and 6 h after, respectively. **Conclusions:** The results of this study suggest that ethanol has a significant inhibitory effect on the G6PD activity both *in vivo* and *in vitro*.

### INTRODUCTION

The most common red blood cell enzyme defect throughout the world is glucose 6-phosphate dehydrogenase (G6PD) deficiency (Weksler *et al.*, 1990). G6PD deficiency is an X chromosome-linked trait, fully expressed in males and homozygous females and is variably expressed in heterozygous females (Aksoy *et al.*, 1987). G6PD deficiency is frequently seen in African, Mediterranean, Middle Eastern and Far Eastern populations and their lineages with a frequency ranging from 5 to 40% (Berkow, 1987; Weksler *et al.*, 1990; Laurence *et al.*, 1997). Sometimes, G6PD deficiency disorder is also referred to as primaquine sensitivity or favism (Weksler *et al.*, 1990; Beutler, 1994; Kayaalp, 1998). In Turkey, cases of this disorder are present in Çukurova Region and Başkale district of Van and the highest incidence is seen in Jewish Kurds (62% of males) (Kayaalp, 1998). Some drugs (primaquine, aspirin, sulphonamids etc.) or other chemicals (methylene blue, naphthalene) lead to production and accumulation of toxic peroxides, causing oxidation of haemoglobin and red blood cell membranes and resulting in excessive haemolysis in patients with G6PD deficiency.

G6PD (D-glucose 6-phosphate: NADP<sup>+</sup> oxidoreductase, EC 1.1.1.49) is the key enzyme which catalyses the first step of the pentose phosphate metabolic pathway (Shreve and Levy, 1977). A major role of NADPH in erythrocytes is regeneration of reduced glutathione, which prevents haemoglobin denaturation, preserves the integrity of red blood cell membrane sulphhydryl groups, and detoxifies hydrogen peroxide and oxygen radicals in and on the red blood cells (Deutsch, 1983; Weksler *et al.*, 1990). A decrease of G6PD results in NADPH and reduced glutathione deficiency in erythrocytes; scarcity of reduced glutathione causes early haemolysis in spleen (Andrews and Mooney, 1994).

Ethanol is a widely consumed sedative–hypnotic drug throughout the world (Lee and Becker, 1989). It has been shown that ethanol intake may lead to oxidative damage in several tissues

such as brain, stomach, liver or erythrocyte (Bondy and Guo, 1994; Sozmen *et al.*, 1994; Lindi *et al.*, 1998; Hernandez-Munoz *et al.*, 2000). Ethanol increases the generation of reactive oxygen species in these tissues and its acute intake decreases reduced glutathione levels in plasma and erythrocytes (Loguercio *et al.*, 1997).

Since ethanol has oxidant effects in erythrocytes, it was considered important to reveal the effect of ethanol consumption on erythrocyte G6PD activity, which has not been studied before. Therefore the objective of this study was to investigate the effect of ethanol on G6PD enzyme activity *in vitro* in human, and *in vivo* in rat, erythrocytes.

### MATERIALS AND METHODS

#### Materials

2',5'-ADP–Sephacrose 4B was purchased from Pharmacia, Sweden. NADP<sup>+</sup>, glucose 6-phosphate, protein assay reagent, and chemicals for electrophoresis were purchased from Sigma, St Louis, MO, USA. All other chemicals used were analytical grade and were purchased from either Sigma or Merck, Germany. Ethanol was obtained from Atatürk University, Medical Faculty, drugs and chemical store.

#### *In vitro* studies

**Preparation of the haemolysates.** Fresh human blood collected in tubes with EDTA (5 mM) was centrifuged at 2500 g for 15 min and the plasma and leucocyte coat were removed by aspiration. The packed red cells were washed with 0.16 M KCl solution thrice each time, the samples were centrifuged at 2500 g and the supernatants were removed. One vol of erythrocytes was haemolysed with 5 vol of ice-cold water and centrifuged at 4°C and 10 000 g for 30 min to remove the ghosts and intact cells (Shreve and Levy, 1977; Ninfali *et al.*, 1990; Weksler *et al.*, 1990).

**Ammonium sulphate fractionation and dialysis.** Ammonium sulphate (35–65%) precipitation was performed on haemolysates. Ammonium sulphate was slowly added to complete dissolution. The mixture was centrifuged at 5000 g for 15 min and the precipitate was dissolved in 50 mM

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phosphate buffer (pH 7.0), then dialysed at 4°C in 50 mM K-acetate/50 mM K-phosphate buffer (pH 7.0) for 2 h with two changes of buffer (Ninfali *et al.*, 1990).

**Preparation of affinity gels.** Two grams of dried 2',5'-ADP-Sepharose 4B gel were used for a 10 ml column volume. The gel was washed with distilled water to remove foreign bodies and air in the swollen gel was eliminated. The gel was suspended in 0.1 M K-acetate/0.1 M K-phosphate buffer (pH 6.0), then packed in a small column (1 × 10 cm) and equilibrated with the same buffer. The gel was washed with equilibration buffer. The flow rates for washing and equilibration were adjusted by a peristaltic pump to 50 ml/h (Ninfali *et al.*, 1990).

**Purification of G6PD by affinity chromatography.** The dialysed sample was loaded on the 2',5'-ADP-Sepharose 4B affinity column and the gel was washed with 25 ml of 0.1 M K-acetate/0.1 M K-phosphate (pH 6.0), with 25 ml of 0.1 M K-acetate/0.1 M K-phosphate (pH 7.85), and finally with 0.1 M KCl/0.1 M K-phosphate (pH 7.85) buffer. Washing continued up to an absorbance of 0.05 at 280 nm. Elution was carried out with 80 mM K-phosphate + 80 mM KCl + 0.5 mM NADP<sup>+</sup> + 10 mM EDTA (pH 7.85) solution at 20 ml/h flow rate. Eluates were collected in 2 ml tubes and the activity of each was separately calculated. Active fractions were collected. All procedures were performed at 4°C (Morelli *et al.*, 1978; Delgado *et al.*, 1990; Ninfali *et al.*, 1990).

**Protein determination.** Quantitative protein determination was performed spectrophotometrically at 595 nm by the method of Bradford (1976), with bovine serum albumin as standard.

**Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis.** This was performed after the purification of enzyme by the method of Laemmli (1970). It was carried out in 3% and 10% acrylamide concentrations for gel stacking and running respectively, containing 0.1% SDS.

#### *In vitro* inhibitor studies

Ethanol was used as the inhibitor. Activities were measured at 0.242, 0.484, 0.968, 1.936 and 2.904 mM cuvette concentrations of ethanol. Drug concentrations which produce 50% inhibition ( $I_{50}$ ) were calculated from graphs drawn from data with five different ethanol concentrations.

#### Measurements of G6PD activity

G6PD activity was measured at 37°C by the method of Beutler (1971), which depends on the reduction of 2 mM NADP<sup>+</sup> by G6PD, in the presence of glucose 6-phosphate. The activity measurement was made by monitoring the increase in absorption at 340 nm at 37°C. One enzyme unit was defined as the reduction of 1 μmol of NADP<sup>+</sup>/min at 37°C, pH 8.0.

#### *In vivo* inhibitor studies

Ten adult male Sprague-Dawley rats with a weight of 200–250 g were used for the experiment. The animals were housed individually and were fed with standard laboratory chow and water before the experiment. The animal laboratory was windowless with controlled temperature (22 ± 1°C) and lighting controls (14 h light/10 h dark cycles). Twenty-four hours before the experiments, the rats were starved, but were allowed access to water *ad libitum*. For control measurements,

a 0.5 ml blood sample was taken from a tail-vein before drug administration. Then, 2 ml/kg of ethanol (96%) was administered by gavage. At 1, 3 and 6 h after ethanol administration, 0.5 ml blood samples were taken again. All blood samples were added to EDTA tubes. Haemolysates were prepared as described for the *in vitro* studies. G6PD activity was measured at 37°C according to Beutler's method (Beutler, 1971).

#### Statistical analysis

Results are given as means ± SD. Data were analysed by Student's *t*-test and analysis of variance (ANOVA).  $P < 0.05$  was considered significant.

## RESULTS

G6PD was purified 9300-fold with a yield of 51.6% by using ammonium sulphate precipitation and 2',5'-ADP-Sepharose 4B affinity gel. SDS-polyacrylamide gel electrophoresis was performed after purification of the enzyme and the electrophoretic pattern obtained is shown in Fig. 1.

Our study revealed that ethanol inhibited the G6PD activity of rat and human red blood cells in *in vivo* and *in vitro* conditions, respectively. In the *in vitro* study with human red cell haemolysates (Table 1), there was a concentration-dependent decrease in G6PD activity, which was statistically significant ( $P < 0.005$ ). The  $I_{50}$  value obtained from *in vitro* studies was 0.968 mM ethanol.

The data from the *in vivo* studies (Table 2) using rat erythrocytes show that ethanol significantly inhibited the activity of G6PD. The percentage inhibition by ethanol was 59%, 40% and 6% at 1, 3 and 6 h, respectively after administration.

## DISCUSSION

Ethanol is a widely consumed sedative-hypnotic drug throughout the world (Lee and Becker, 1989). The ethanol contents of various beverages varies between 2.5% (beer) and

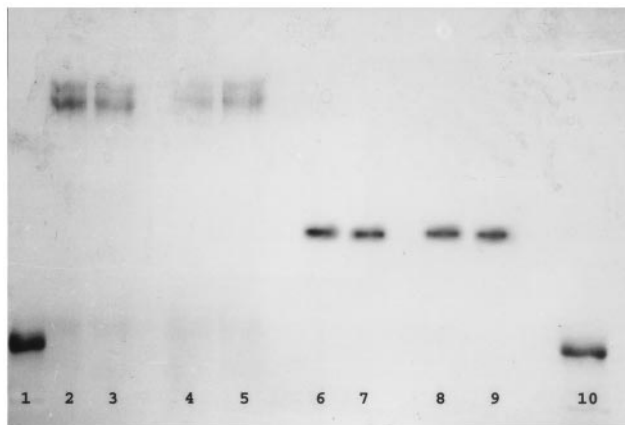


Fig. 1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of glucose 6-phosphate dehydrogenase (G6PD) purified by affinity gel chromatography.

Lanes 1 and 10: human carbonic anhydrase-1; lanes 2–5: buffalo milk lactoperoxidase; lanes 6–9: human G6PD.

Table 1. Effects of various concentrations of ethanol *in vitro* on purified glucose 6-phosphate dehydrogenase (G6PD) from human erythrocyte

Ethanol concentration (mM)	G6PD activity
0 (drugless) <sup>a</sup>	100 ± 0
0.242	90 ± 0*
0.484	68 ± 1*
0.968	50 ± 0*
1.936	39 ± 0*
2.904	15 ± 2*

Values are means ± SD for three determinations per group activity (EU/ml).

<sup>a</sup>Drugless cuvette was accepted as the control.

\*P < 0.005 vs control, by analysis of variance.

Table 2. Effect of ethanol administration on rat erythrocyte glucose 6-phosphate dehydrogenase activity

Time (h)	Activity (EU/g Hb) (mean ± SD)	Significance* (P)
0	7.233 ± 0.540	—
1	2.972 ± 0.309	<0.0001
3	4.275 ± 0.373	<0.0001
6	6.806 ± 0.533	<0.0001

\*Versus control, by Student's *t*-test.

55% (strong spirit). Together with some beneficial effects, ethanol exerts serious side-effects in the CNS, liver and other tissues (Rang *et al.*, 1999). It is known that ethanol increases the generation of reactive oxygen species and leads to oxidative damage in erythrocytes (Sozmen *et al.*, 1994; Soszynski and Schuessler, 1998). In fact, ethanol causes several haemolytic disorders due to both direct and indirect effects (Lee and Becker, 1989). Additionally, the ethanol metabolite acetaldehyde inside of the erythrocyte has the ability to generate free radical species and to cause deleterious effects on erythrocytes (Tyulina *et al.*, 2000).

After ethanol intake, its blood level rises to a peak within 30–90 min (Laurence *et al.*, 1997). The half-life of ethanol in plasma is highly variable, but a single dose of ethanol will not all be disposed of for 6–8 h or even more (Laurence *et al.*, 1997). In our study, maximal inhibition of G6PD activity was within 1 h after ethanol administration, and inhibition continued significantly after 3 h (Table 2). These results suggest a correlation between plasma peak level of ethanol and maximal inhibition of G6PD enzyme activity, and between the plasma half-life of ethanol and continuity of inhibition. Moreover, the results of our study show that the ethanol itself is a highly potent inhibitor of erythrocyte G6PD enzyme activity *in vitro*. Based on our results, we believe that the inhibitory effect of ethanol on G6PD enzyme activity at least may be one of the causes of ethanol-induced haemolysis.

In conclusion, the present study suggests that patients with G6PD deficiency should avoid ethanol intake. If they consume any ethanol, they should not be administered oxidant drugs such as analgesics and antipyretic, which frequently inhibit G6PD activity. If it is required to give these drugs to the patient with G6PD deficiency, their dosage should be strictly determined to minimize the haemolytic side-effects.

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