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Evaluation of effect of some corticosteroids on glucose-6-phosphate dehydrogenase and comparative study of antioxidant enzyme activities

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Abstract

Corticosteroids are anti-inflammatory drugs that are similar to the natural corticosteroid hormones produced by the cortex of the adrenal glands. The objective of this study was to scrutinize effects of some corticosteroids on glucose-6-phosphate dehydrogenase (G6PD) and some antioxidant enzymes. Initially, G6PD was purified from human erythrocytes by using ammonium sulphate precipitation and affinity chromatography. The two drugs, dexamethasone phosphate and prednisolone, investigated on the purified enzyme inhibited the enzyme activity.

Comparative *in vivo* studies were performed to determine the effects of dexamethasone phosphate on the antioxidant enzyme activities using Spraque-Dawley rats. G6PD and catalase (CAT) activities were found significantly lower than in the control, whereas glutathione peroxidase (GP) activity was significantly increased in the erythrocytes of rats the receiving drug; glutathione reductase (GR) activity was unaffected. The results imply that dexamethasone phosphate may affect oxidative stress by changing antioxidant enzyme activities.

Keywords: *Glucose-6-phosphate dehydrogenase, antioxidant enzymes, purification, corticosteroids, inhibition*

Introduction

The pentose phosphate metabolic pathway (PPP) is the only source of NADPH in erythrocytes. NADPH is crucial in the protection of cells from oxidative stress, caused by free radicals, of a number of molecules in cells, including membrane lipids, proteins, and nucleic acids [1,2]. Glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) catalizes the genesis reaction, reducing NADP to NADPH. G6PD deficiency belongs to the most common human disorders of metabolism [3]. In affected patients generation of free radicals causes life-threatening hemolytic crises, for example, after consumption of certain drugs and foods or after infections [4]. These free radicals are neutralized by an elaborate antioxidant defense system consisting of enzymes such as glutathione reductase (GR, EC 1.6.4.2), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GP, EC 1.11.1.9) and superoxide dismutase (SOD, EC 1.15.1.1). GP removes peroxide from the erythrocyte [5]. Reduced glutathione (GSH) serves as a substrate for this enzyme and, because NADPH is required for the reduction of oxidized glutathione and protein sulfhydryl groups, it is an essential factor in the chain of reactions that defends the erythrocyte against peroxide [6]. Regeneration of GSH and reduction of the oxidized glutathione (GSSG) is accomplished by glutathione reductase (GR, EC 1.6.4.2) [7]. GSH is one of the important antioxidants and apart from scavenging free radicals it also plays a role in the reduction of various disulfide linkages and maintenance of proteins in the proper oxidized/reduced state [8]. Erythrocytes are a particularly rich source of catalase (CAT, EC 1.11.1.6), but this enzyme is relatively inefficient at removing low levels of peroxide. Moreover, catalase has the ability to bind NADPH tightly. The activity of the PPP serves to

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remove peroxide not only through the action of GP, but also by activating CAT [9]. Clearly, both enzymes may have a role to serve as backup mechanisms for each other. Under normal conditions, reductive and oxidative capacities of the cell (redox state) favor oxidation [10]. If the generation of free radicals in cells impairs antioxidant defences or exceeds the ability of the antioxidant defence system to eliminate them, then this causes oxidative stress [11]. Many drugs and chemicals can increase the rate of free radicals formation in specific organs of the body [12].

G6PD deficiency has a polymorphic frequency that is second only to the haemoglobinopathies, 400 variants having been described [3]. Deficiency of the enzyme in erythrocytes causes haemolytic anaemia. Drug-induced haemolysis has attracted the most attention. Numerous reports attest to the importance of drugs in causing hemolytic anemia and changing of enzyme activities [13]. The present study was undertaken to evaluate the effects of some corticosteroids on G6PD and antioxidant enzyme activities.

Materials and methods

Materials

2′,5′ ADP-Sepharose 4B was obtained from Pharmacia. All other chemicals were obtained from either Sigma Chemical Co. or Merck and were analytical grade. Medical drugs were from the Hospital of Ataturk University.

Preparation of the haemolysate

Fresh blood collected from one person under the study was centrifuged (15 min, 2500 × *g*). Preparation of the haemolysate was done as described elsewhere [14,15].

Measurements of enzymes activities and protein assay

G6PD, GR and GP activities were spectrophotometrically assayed by measuring the alteration in absorption at 340 nm as described by Beutler [16]. *Catalase activity* was measured by following the reduction of hydrogen peroxide at 240 nm [17]. Quantitative protein concentrations were determined by the method of Bradford [18].

Ammonium sulphate fractionation and purification of G6PD

Haemolysate was brought to 35–65% $(NH_4)_2SO_4$ saturation with solid $(NH_4)_2SO_4$. All assays and dialyses were done according to Ninfali [14].

Dry 2′,5′ ADP-Sepharose 4B was resuspended in 0.1 M K-acetate/0.1 M K-phosphate buffer (pH 6.0), then used to pack a small column (1 × 10 cm) which was equilibrated in the same buffer. The dialysed enzyme solution was loaded on the column. All other procedures were performed as described in previous studies [14,19]. In eluates, the activity of G6PD was determined in all fractions. Protein determination at 280 nm in eluates was not performed since $NADP^+$ absorbance masked the actual protein absorbance [14,19].

SDS polyacrylamide gel electrophoresis

SDS-PAGE was carried out in 10% and 4% acrylamide concentrations containing 0.1% SDS for the running and the stacking gel, respectively, according to Laemmli [20]. The electrophoretic pattern was photographed.

In vitro *inhibitor studies*

Dexamethasone phosphate and prednisolone were used as the corticosteroids. In order to determinate I_{50} values (the inhibitor concentrations causing up to 50% inhibition), inhibition percent values were obtained from five different inhibitor concentrations with 0.6 mM constant substrate concentration. Enzyme activities were measured at 1–5 mM cuvette concentration for dexamethasone phosphate, and 2–5.52 mM cuvette concentration for prednisolone. Control cuvette activity in the absence of drug was taken as 100%. Regression analysis graphs were drawn using inhibition percent values by a statistical packing program on a computer. I_{50} values were determined from the graphs.

To determine K_i values, G6P (glucose-6-phosphate) was used as substrate at five different concentrations. In the media with inhibitor or without inhibitor, the substrate concentrations were 0.06–0.6 mM. For Lineweaver-Burk graphs, 1/V vs1/[S], regression analysis was carried out and the equations obtained were used to draw graphs for each fixed inhibitor concentration. K_i values were calculated from these Lineweaver-Burk graphs.

In vivo *inhibitor studies*

Six Spraque-Dawley rats were selected from adult (200–250 g) rats which were raised for *in vivo* studies. The rats were kept separately in specious cages and were fed with standard commercial laboratory feed and water before the experiment with temperature at 25°C and lighting controls (14 h light/10 h dark). Blood samples of 0.5 ml were taken from the tail of each rat in the light and put in tubes containing EDTA. Then, 3 mg/kg of dexamethasone phosphate (which has a lower I_{50} value than the I_{50} value of prednisolone) was intraperitoneally injected to each rat [21]. The daily dose for humans is the same as for animals (3 mg/kg). Blood samples were taken from

each rat at 2, 4, and 6 h after injection. All blood samples were centrifuged in 2500 × *g* and the haemolysate was prepared. Studies were carried out at 4°C. Determination of enzyme activity was made at 25°C according to the above mentioned methods.

Statistical analysis

All statistical analysis was performed using Statistica software release 6.0. Analysis of variance was performed and means were separated using Fisher's protected least significant differences (LSD) test at $P \leq 0.001$, $P \leq 0.01$ and $P \leq 0.05$.

Results and discussion

Antioxidant enzymes are important in oxidative defence because they metabolize either free radicals or reactive oxygen intermediates to nonradical products (Figure 5). Antioxidant enzymes, such as SOD, GP, CAT and GR are indispensable in both maintaining cellular stability and scavenging free radicals. GP metabolizes H_2O_2 and lipid peroxides to non-toxic products and, in so doing, it leads to the oxidation of GSH to GSSG. GSSG is reduced to GSH by GR. This reaction enzymatically needs NADPH produced by G6PD and 6-phosphogluconate dehydrogenase in the PPP [22].

In this study, the effects are examined of some corticosteroids on antioxidant enzymes and G6PD in *in vivo* and *in vitro.* G6PD was purified from human erythrocytes by ammonium sulphate precipitation and 2′,5′-ADP Sepharose 4B affinity chromatography, respectively. The purification factor was 9,811 fold in a yield of 42.4%; all purification steps are shown in Table I. The enzyme pattern was photographed and as shown in Figure 1, the enzyme had a single band after SDS-PAGE gel for the determination of purity of the enzyme from the one person under study.

The inhibition effects of dexamethasone phosphate and prednisolone as corticosteroids was investigated. Dosage requirements of corticosteroids vary among individuals and the diseases being treated but in general, the lowest possible effective dose is used.

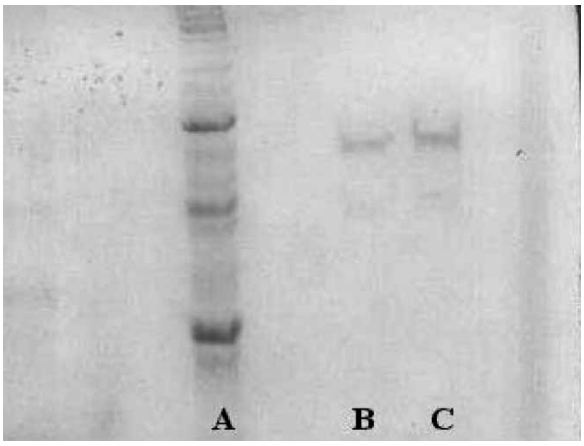

Figure 1. SDS-polyacrylamide gel electrophoresis of G6PD from human erythrocytes. Line A: mixture of standard proteins and their approximate molecular weight (β Galactosidase, *Erischeria coli*, 116.000; Phosphorylase B, rabbit, 97.400; Albumin, bovine, 66.000; Ovalbumin, chicken, 45.000; carbonic anhydrase, bovine, 29.000), line B and C; band for enzyme purified from human erythrocytes.

Corticosteroids given in multiple doses throughout the day are more effective, but also more toxic, than if the same total dose is given once daily, or every other day. In order to characterise the inhibition effects, I_{50} and K_i parameters of these corticosteroids for G6PD were determined. The inhibitor concentrations causing up to 50% inhibition for dexamethasone phosphate and prednisolone were determined as 3.53 and 5.02 mM, respectively, from the regression analysis graphs (Figure 2). K_i values of the drugs were determined as 3.99 and 4.89 mM, respectively, by Lineweaver-Burk graphs (Figure 3). I_{50} and K_i value for G6PD is shown in Table II. The Inhibition type for dexamethasone phosphate was non-competitive in respect to G6P; prednisolone inhibited the enzyme in a competitive manner.

In *in vivo* studies, together with G6PD activity, we also examined the variations of activity in the enzymatic systems involved in cell antioxidant defence, e.g. GR, GP, CAT. After blood samples of 0.5 ml were taken from the tail of each rat,

Table I. Purification scheme for human glucose 6-phosphate dehydrogenase from person with normal activity.

Purification step	Activity (EU/mL)	Total volume (mL)	Protein (mg/mL)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification factor
Haemolysate	0.45	300	85	25500	135	0.0053	100	1
Ammonium sulfate precipitation (35–65%)	0.6	120	57	6840	72	0.01	53	1.89
2′,5′-ADP Sepharose-4B affinity chromatography	5.2	11	0.1	1.1	57.2	52	42.4	9,811

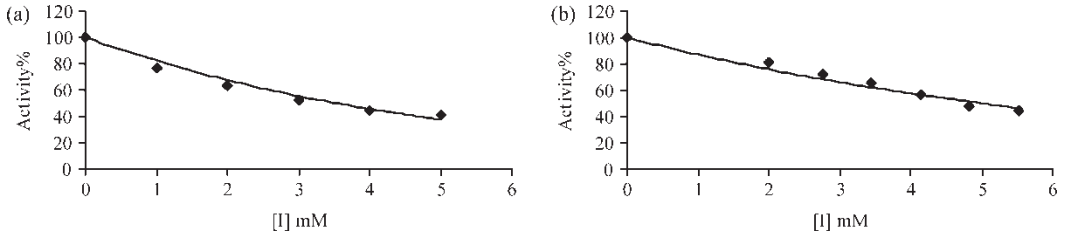

Figure 2. Percentage activity-[I] regression analysis graphs in 1 M Tris-HCl for dexamethasone phosphate (a) and prednisolone (b).

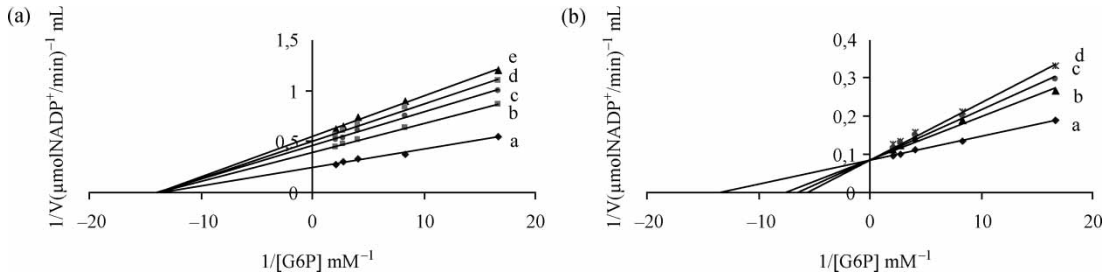

Figure 3. Lineweaver-Burk graph for 5 different G6P concentrations and (a) 4 different dexamethasone phosphate concentrations (a; control, b; 3.4 mM, c; 3.5 mM, d; 3.7 mM, e; 3.9 mM) and (b) 3 different prednisolone concentrations (a; control, b; 4.8 mM, c; 5.3 mM, d; 5.7 mM).

the dexamethasone phosphate intraperitoneal injection was performed to the test groups. The enzyme activities of the groups after the drug injection were measured at 2, 4 and 6 h. It is seen that G6PD and CAT were significantly inhibited by dexamethasone phosphate (Table III, Figure 4). GP enzyme was activated by this drug. It was observed that the activities in the absence of drug, 2, 4 and 6 h was respectively determined as 9.63 ± 1.476, 5.1 ± 0.98, 7.2 ± 0.95 and 7.5 ± 3.3 EU g^{-1} Hb for G6PD; 6.3 ± 1, 6.9 ± 0.73, 6.4 ± 1.6 and 6.8 ± 0.43 EU g^{-1} Hb for GR; 33.1 ± 6.8, 57.3 ± 16.8, 46.6 ± 16.9 and 36 ± 8 EU g^{-1} Hb for GP; 14578 ± 1542, 10855 ± 1589, 13834 ± 376 and 14977 ± 1561 g^{-1} Hb for CAT.

The enzyme activities changed 2 h after injection (Table III, Figure 4). These results indicate that dexamethasone phosphate entered into the erythrocytes. Thus, the *in vivo* studies related to this drug supported *in vitro* studies with G6PD.

Table II. I_{50} and K_i values obtained from regression analysis graphs for G6PD in the presence of different corticosteroids.

Drug	I_{50} values (mM)	Mean K_i values (mM)	Inhibition type (with respect to G6P)
Dexamethasone phosphate	3.53	3.99	Noncompetitive
Prednisolone	5.02	4.89	Competitive

I_{50}; the inhibitor concentrations causing up to 50% inhibition, Cuvette concentration for dexamethasone phosphate; 1 mM, 2 mM, 3 mM, 4 mM and 5 mM, prednisolone 2 mM, 2.76 mM, 3.45 mM, 4.14 mM, 4.83 and 5.52 mM.

G6PD showed subnormal activity in the erythrocytes of rats receiving the drug ($P \leq 0.001$ and $P \leq 0.01$), which corresponded to 53% and 75% of control. Dexamethasone phosphate should not be taken by G6PD-deficient patients. In the body of patients there is insufficient enzyme and existing enzyme is also inhibited to a large extent. For this reason, these drugs can cause undesirable results since G6PD deficiency is the cause of neonatal haemolysis and jaundice. If a recipient is challenged against the active hemolytic metabolite of a drug, hemolysis will not be apparent [3]. Moreover, even when a drug does shorten erythrocyte life span, the degree of hemolysis may be so modest as to be not of clinical significance (Figure 5) [23].

CAT activity in the erythrocytes significantly decreased compared to the control. This decrease corresponded to 74.5% and 95% of the control.The primary role of CAT is to abolish H_2O_2 that has been generated by free radicals or by SOD in removal of superoxide anions and to convert it to water [24]. Decreased CAT activity may be due to enzyme protein oxidation as a result of accumulation of H_2O_2 and other radicals. The observed decrease in CAT activity after administration of dexamethasone phosphate may be related to oxidative inactivation of enzyme protein [12]. In the follow-up study, CAT activity returned to its normal level. This may indicate a relief from oxidative stres.

GP activity significantly increased in the erythrocytes of the rats ($P \leq 0.01$) at 2 h after dexamethasone phosphate treatment, which corresponded to 173% of

Table III. Statistical values obtained from *in vivo* studies for dexamethasone phosphate.

	Time (h)			
	0	2	4	6
G6PD (EU/gHb)	9.63 ± 1.476	5.1 ± 0.98***	7.2 ± 0.95**	7.5 ± 3.3
GR (EU/gHb)	6.3 ± 1	6.9 ± 0.73	6.4 ± 1.6	6.8 ± 0.43
GP (EU/gHb)	33.1 ± 6.8	57.3 ± 16.8**	46.6 ± 16.9*	36 ± 8
CAT (EU/gHb)	14578 ± 1542	10855 ± 1589*	13834 ± 376	14977 ± 1561

*Statistically significant at $P \leq 0.05$; **statistically significant at $P \leq 0.01$; ***statistically significant at $P \leq 0.001$.

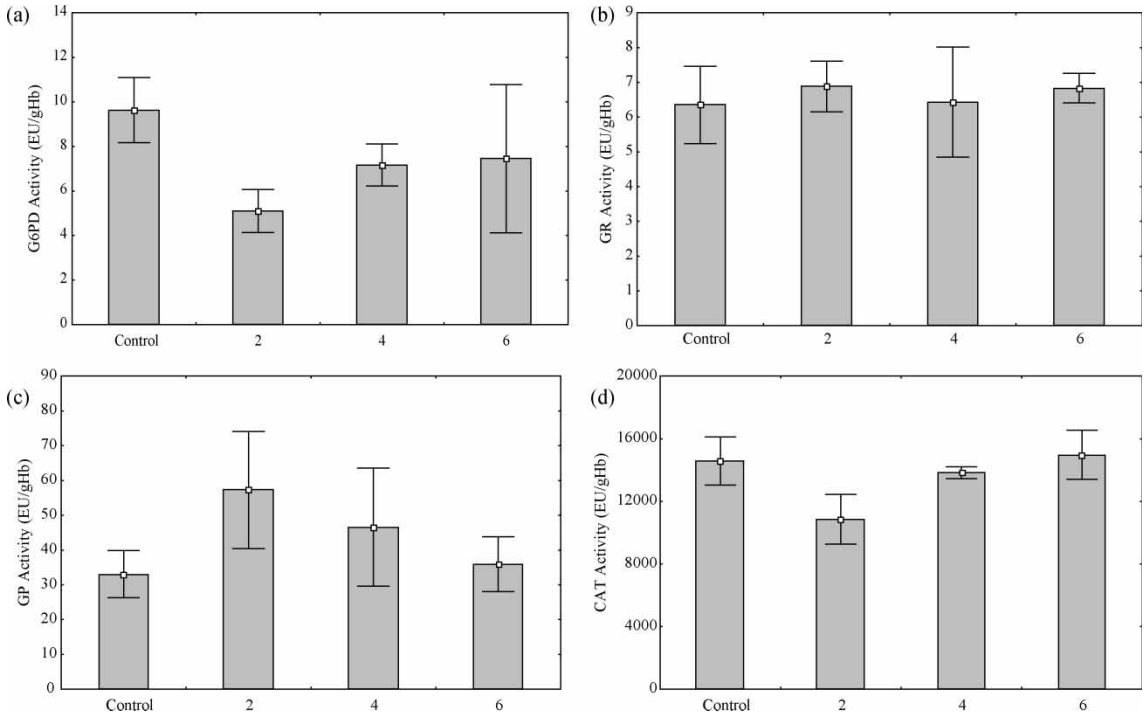

Figure 4. Effects of dexamethasone phosphate on G6PD (a), GR (b), GP (c) and CAT (d) in the rat erythrocytes. One unit of G6PD represents 1 μmol of $NADP^+$ utilized per minute. One unit of GR represents 1 μmol of NADPH utilized per minute. One unit of GP represents 1 μg GSH utilized per minute. One unit of CAT represents the amount of enzyme that decomposes 1 μmol of H_2O_2 per minute. □ = Mean; ⊤ = mean ± SD (n = 6).

the control. This is indicative of scavenging of excess peroxides due to drug-induced oxidative stress. GP acts to nonspecifically scavenge and decompose excess hydroperoxides, including H_2O_2, which may be prevalent under oxidative stress [25,26]. The increase of GP activity may reflect persistent oxidative stress. The increased GP activity may be a compensatory mechanism to remove excess peroxides due to low CAT activity. The depression of GP activity may reflect the oxidative tissue injury due to treatment [27].

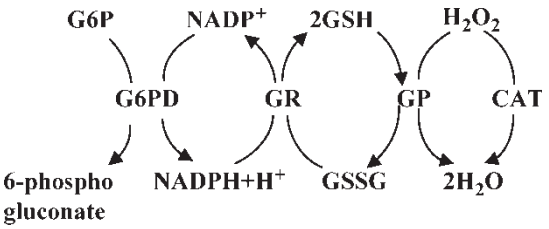

Figure 5. The catalyzing reactions by G6PD, GR, GP and CAT. H_2O_2 can be detoxified by CAT and GP, the latter of which is dependent upon GSH maintained by NADPH-dependent reduction of glutathione disulfide (GSSG) by GR. NADPH levels in turn are maintained by G6PD.

We conclude that dexamethasone phosphate can be linked to oxidative stress by having effects on the antioxidant defence system. This situation may be associated with an increased influx of free radicals after metabolism of dexamethasone phosphate.

Acknowledgements

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