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Effects of Some Drugs on Enzymatic Activity of Glucose 6-Phosphate Dehydrogenase from Chicken Erythrocytes *in vitro*

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in vitro inhibitory effects of some important drugs on glucose 6-phosphate dehydrogenase, the $NADP^+$ depending enzyme, from the chicken erythrocytes were investigated in the peresent study. Chicken erythrocytes glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: $NADP^+$ oxidoreductase, EC 1.1.1.49) (G6PD) was purified by using 2',5'-ADP Sepharose 4B affinity chromatography. During the overall purification steps, the enzyme having a specific activity of 17.90 EU/mg proteins was obtained 5343-fold with a yield of 52 %. A constant temperature (4 °C) was maintained during the purification process. Enzyme activity was determined with the Beutler's method by using a spectrophotometer at 340 nm. This method was utilized for all kinetic studies. Then, metamizol, ceftriaxone, prilocaine, meloksikam, lidocaine were investigated for *in vitro* inhibition of this $NADP^+$ depending enzyme. The enzyme was strongly inhibited by these drugs. In addition, I_{50} values of the drugs were determined by plotting activity *% vs.* drug concentrations. I_{50} values were 0.57 mM for metamizol, 0.33 mM for ceftriaxone, 28.17 mM for prilocaine, 0.0012 mM for meloksikam and 1.54 mM for lidocaine.

Key Words: G6PD enzyme, Chicken, inhibition, Drug.

INTRODUCTION

Glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate: $NADP^+$ oxidoreductase, EC 1.1.1.49; G6PD) is the first enzyme in the hexose monophosphate shunt (HMPS). The enzyme catalyzes the oxidation of D-glucose 6-phosphate to D-glucose-O-lactone 6-phosphate[1,2] in the presence of $NADP^+$. It is the first step of the HMPS for the metabolism of glucose in the red blood cell. The major role of the pathway is to produce NADPH, which is widely used in biosynthetic processes and also plays an important role in maintaining glutathione in the reduced state (GSH), in drug detoxification and in the removal of peroxides[3-6]. GSH contains tripeptide that has a free thiol group. With this form, GSH acts as a scavenger of H_2O_2, a

central reactive oxygen species, by means of the glutathione-ascorbate cycle, which is very important in stress protection[7]. In the case of NADPH lack, the concentration of glutathione in a living system declines, resulting in cell death. For this reason, G6PD can be defined as an indirect antioxidant enzyme[8,9]. As a result, this enzyme is very important for the plants to grow, to develop and to be prevented against oxidative stress. NADPH is also a coenzyme participating in the synthesis of a number of biomolecules such as fatty acids, steroids and some amino acids[10-12].

For medical treatment of the animal disease are being used a lot of drugs such as antienflamatuar antibiotics and anesthetic. However, we have few reports about effects on activities of different enzyme of the drug. For instance, inhibition effects of metamizole, amikacin sülfate, sodium ampicillin and netilmicin sulfate as drugs were investigated in rat erythrocyte *in vitro* and *in vivo* 6PGD enzyme activity[13]. However, no reports could be encountered in the literature on the *in vitro* effects of metamizol, ceftriaxone, prilocaine, meloksikam, lidocaine on chicken erythrocyte G6PD. This paper was focused at purifying chicken erythrocyte G6PD and to determination effects of these drugs on chicken red blood cell G6PD activity.

EXPERIMENTAL

2',5'-ADP Sepharose 4B was purchased from Pharmacia. $NADP^+$, glucose 6-phosphate, protein assay reagent were purchased Sigma Chem. Co. All other chemicals used were analytical grade and purchased from either Sigma or Merck.

Preparation of the hemolyzate: Blood samples collected in EDTA were centrifuged (15 min, 2,500xg) and plasma was removed. The pack of red cells was washed three times with KCl (0.16 M) and hemolyzed with 5 volume of ice-cold water and then centrifuged (4 ºC, 10,000xg, for 0.5 h) to remove the ghosts and intact cells[14].

Ammonium sulphate precipitation and dialysis: Ammonium sulphate precipitation was done according to previous study[1]. The enzyme was observed to precipitate at 30-50 % precipitation. The enzyme solution was dialyzed in 50 mM K-acetate plus 50 mM K-phosphate buffer (pH: 7,0) for 2 h with two changes of buffer[1].

Preparation of 2',5'-ADP Sepharose 4B affinity column and enzyme elution: The dialyzed enzyme solution that obtained[15] was loaded on the 2',5'-ADP Sepharose 4B affinity chromatography column and the flow rate was adjusted as 20 mL/h. The washing and elution solutions was prepared as reported by Yilmaz *et al.*[15]. The enzyme activity was measured in final fractions and the activity-having tubes were collected together. In resultant solution, the protein was determined. During all procedures[14,16,17], the temperature was kept at 4 ºC.

Activity determination: The enzymatic activity was measured by Beutler's method[18]. One enzyme unit was defined as the enzyme amount reducing 1 μmol $NADP^+$ per 1 min.

Protein determination: Quantitative protein determination was spectrophotometrically measured at 595 nm according to Bradford's method, with bovine serum albumin being used as a standard[19].

SDS polyacrylamide gel electrophoresis (SDS-PAGE): The control of enzyme purity, using Laemmli's procedure, was carried out in 3 and 8 % acrylamide concentrations for running and stacking gel, respectively. The staining was made for about 2 h in the solution of 0.1 % Coommassie Brillant Blue R-250 + 50 % methanol + 10 % acetic acid. Finally, the washing was carried out in the solution of 50 % methanol + 10 % acetic acid + 40 % distilled water until protein bands were cleared[20].

***in vitro* drugs effects:** In order to determine the effects of metamizol, ceftriaxone, prilocaine, meloksikam, lidocaine in several cuvette concentrations on G6PD were added to separate tubes containing purified enzyme.

To determine I_{50} values, activities were calculated with a 0.60 mM constant substrate (G6P) and different inhibitor concentrations. The enzyme activity was measured in these tubes taking the tubes containing no drug as control (100 % activity). The experiments were repeated three times. For each drug activity %-[drug] graph was drawn at different inhibitor concentrations. Regression analysis graphs were drawn using inhibition % values by a statistical package (SPSS-for windows; version 10.0) on a computer. Drug concentrations that produce 50 % inhibition (I_{50}) were calculated from graphs.

RESULTS AND DISCUSSION

The purification process of chicken erythrocyte G6PD enzyme was summarized in Table-1. G6PD was purified 5343-fold in a yield of 52 % by using ammonium sulfate precipitation and 2',5'-ADP Sepharose 4B affinity chromatography. Purity of the enzyme was confirmed by SDS-PAGE (Fig. 1). [Drug] *vs.* activity % graphs were drawn for the drugs (Figs. 2-6). I_{50} values were calculated as 0.57, 0.33, 28.17, 0.0012 and 1.54 mM from the graphs for metamizol, ceftriaxone, prilocaine, meloksikam, lidocaine, respectively.

A number of chemicals and drugs have adverse effects on the organism when administered at relatively low doses affect metabolism by altering normal enzyme activity, particularly through inhibition of a specific enzyme. The effects can be dramatic and systemic[21]. For example, dantrolene sodium has inhibitory effect on human erythrocyte G6PD enzyme activity *in vitro* and rat erythrocyte *in vivo*[22]. Beydemir *et al.*[22]

TABLE-1
PURIFICATION SCHEME OF GLUCOSE 6-PHOSPHATE DEHYDROGENASE FROM CHICKEN ERYTHROCYTES

Purification step	Activity (EU/mL)	Total volume (mL)	Protein (mg/mL)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification factor
Haemolyzate	0.051	150	15.20	2280	7.65	0.00335	100	1
Ammonium sulphate precipitation (30-50) %	0.381	14	2.11	29.54	5.33	0.180	69	53.73
2′,5′-ADP sepharose 4B affinity chromatography	0.505	8	0.0282	0.2256	4.04	17.90	52	5343

Fig. 1. SDS-PAGE bands of G6PD Lane 1: Rainbow trout erythrocytes G6PD; Lane 2: Chicken erythrocytes G6PD; (Lane 3: Standards: Rabbit myosin (205,000), *E. coli* bgalactosidase (116,000), rabbit phosphorylase B (97,400), bovine albumin (66,000), chicken ovalbumin (45,000) and bovine carbonic anhydrase (29,000))

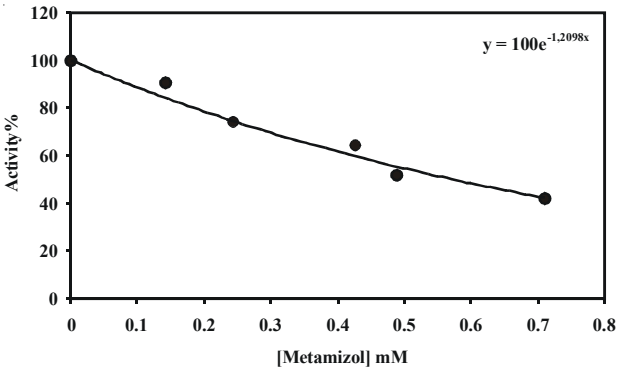

Fig. 2. Activity *% vs.* [Metamizol] regression analysis graphs for G6PD in the presence of 5 different metamizol concentrations

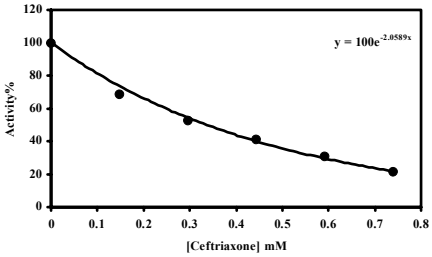

Fig. 3. Activity *% vs.* [Ceftriaxone] regression analysis graphs for G6PD in the presence of 5 different ceftriaxone concentrations

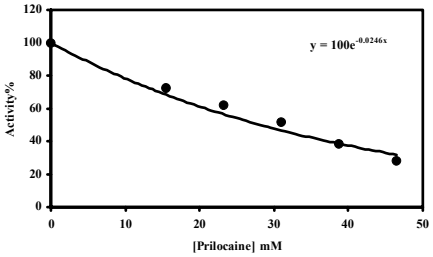

Fig. 4. Activity *% vs.* [Prilocaine] regression analysis graphs for G6PD in the presence of 5 different prilocaine concentrations

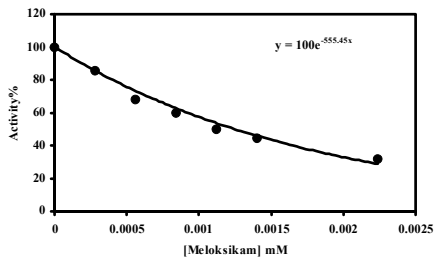

Fig. 5. Activity *% vs.* [Meloksikam] regression analysis graphs for G6PD in the presence of 6 different meloksikam concentrations

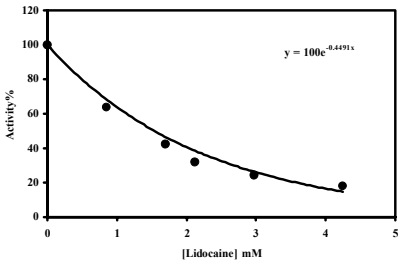

Fig. 6. Activity *% vs.* [Lidocaine] regression analysis graphs for G6PD in the presence of 5 different lidocaine concentrations

reported vancomycin hydrochloride and gentamicin sulphate inhibited sheep lens G6PD avtivity and ceftriaxone and sodium cefazolin were observed to activate this enzyme. In addition, it was stated that the inhibitory effects of melatonin hormone on carbonic anhydraze from rainbow trout erythrocyte *in vitro* and *in vivo* enzyme activity[23]. In many tissues, especially erythrocytes have been expressed in previous studies that oxidative stress occurs in administration of some drugs in medical treatment[24,25].

HMPS has an important role to protect oxidative stress in living metabolism. The pathway acts in production of NADPH as an important molecule in the preservation of the structure of the cell membrane in the living cells[15,26]. NADPH generation is provided by activities of the two enzymes that is G6PD and 6-phosphogluconate dehydrogenaze (6PGD) in the oxidative phase of the hexose monophosphate shunt. The dinucleotide has also a vital role in maintaining glutathione in the reduced state (GSH), in drug detoxification and in the removal of peroxides[4,5]. GSH is very important for scavenging of nonfree oxygen spices as H_2O_2[27]. In the case of NADPH lack, the concentration of glutathione in a living system declines, resulting in cell death. For this reason, G6PD can be defined as an indirect antioxidant enzyme[8,9]. For this important property in hexose monophosphate shunt, G6PD has been purified from many human, animal and plant tissues. For example, the enzyme has been first isolated from human erythrocytes by Yoshida[28]. In following years, the enzyme was purified by using different chromatography techniques, column gels and characterized[14,29]. However, it has been done few experiments on the enzyme activity of medical drugs and different chemical. Whereas, inhibition of the G6PD enzyme activity is vital especially in patients that have G6PD deficiency. Particularly, in humans cataract risk increases by G6PD deficiency in lens. A number of studies have revealed the relationship between cataractogenesis and G6PD deficiency[30]. Therefore, G6PD deficiency may found in all tissues, including the red blood cells[31] and has a vital importance in living systems. Because, the life span of cases with G6PD deficiency is short due to complications caused by chronic hemolysis. Older erythrocytes that are exposured to some drugs such as some antibiotics and chemical substances. For example, sulfonamides can have risk of hemolysis. Anemia may lead to hemoglobinuria and acute renal failure[32]. Due to the reasons, to determine *in vitro* effects on G6PD activity of some drugs used oftenly veterinary medicine and human medical treatments, the enzyme was purified 5343-fold by using ammonium sulphate fractionation (30-50 %) and 2',5'-ADP Sepharose 4B affinity chromatography (Table-1). SDS-PAGE confirmed the purity of the enzyme. As seen in the gel photograph, a high-purity enzyme was obtained with this method (Fig. 1).

In this study, metamizol, ceftriaxone, prilocaine, meloksikam, lidocaine were chosen as inhibitor. Metamizol have been used as an analgesic and an antipretic. Its side effects include born marrow depression and water and salt retention. Ceftriaxone is an antibiotic. They kill bacteria by interfering with the ability of bacteria to form cell walls. It belongs to the family of antibiotics known as cephalosporins. Prilocaine is a local anesthetics and anti arrhythmic drug. It has some side effects such as anxiety, restlessness, difficulty breathing and shortness of breath. Meloxikam is a nonsteroid,

antiinflammatory, analgesics and antipyretic drug. Lidocaine is a drug used as local anestesic. It can performe local tromphlebits with long-time intravenous use. When it was used for local anesthesia, it has been stated that allergic contact dermatitits, urtiker and anaflactia was seen[32-34]. For this drugs, activity % values of G6PD for five different concentration of each drug were determined. The enzyme activity without a medical drug was accepted as 100 % activity. The inhibitor concentrations causing up to 50 % inhibition (I_{50}) were determined from the regression analysis graphs (Fig. 2-6). By using the obtained I_{50} values, undesirable side effects on G6PD activity and body metabolism and fatty acid synthesis can be reduced.

In conclusion, since these drugs may damage the health of living metabolism and they have hemolytic side effects. If it is necessary to give these drugs, their dosage should be carefully determined.

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Antibacterial Activity of Some 4-Pyridinone Derivatives Synthesized from 4-Pyrones

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