Gaziantep Tıp Dergisi 1999; 1,2: 58-63

Evaluation of erythrocyte G6PD activity unit; it is better defined as a function of RBC count

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SUMMARY

Glucose 6-phosphate dehydrogenase (G6PD) deficiency, one of the most common red cell abnormalities, is characterized by wide clinical, biochemical and molecular heterogeneity. In clinical laboratories erythrocyte G6PD activity is defined either as a function of hemoglobin (Hb) concentration or red blood cell (RBC) count and seldom hematocrit (Hct). These parameters are variable for every individual and misinterpretation of the results cannot be avoided with reference interval establishment studies unless the best approach that define erythrocyte G6PD activity is selected. Hb concentration is frequently prone to the effects of genetic, physiological and environmental factors where G6PD activity should correlate with RBC count even when it is altered.

In this study relations of the erythrocyte G6PD activity with RBC count, Hb concentration of the erythrocyte suspensions and RBC indices of 242 individuals were studied. Additionally, relations with platelet and white blood cell (WBC) counts were evaluated to determine possible interference. Erythrocyte G6PD activity was determined with kinetic spectrophotometric method according to the ICSH at 30°C. RBC, WBC, platelet counts, hemoglobin concentration of the erythrocyte suspensions and erythrocyte indices were determined with an automatic complete blood counter.

Enzyme activity was correlated with RBC count (r: 0.53, $p<0.001$), there was no correlation with Hb ($p>0.05$). The negative correlation of the enzyme activity with MCV and MCH (r: -0.44 and r: -0.29, $p<0.001$), disappeared as a ratio of RBC count ($p>0.05$) and strengthened as a ratio of Hb concentration (r: -0.61 and r: -0.44, $p<0.001$). In this study a correlation of erythrocyte suspentions' platelet, leucocyte count and the enzyme activity as a ratio of Hb (r: 0.33 and r: 0.25, $p<0.001$) was observed. On the other hand enzyme activity as a ratio of RBC was only poorly correlated to WBC count (r: 0.15, $p<0.01$).

Results of this study indicate that erythrocyte G6PD activity is related to RBC count and has several advantages over Hb in assessing G6PD status. It gives more reliable results in conditions that could affect erythrocyte morphology and Hb concentration.

Key Words: Erythrocyte G6PD, RBC, hemoglobin, MCV

INTRODUCTION

Glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate; NADP oxidoreductase; EC 1.1.1.49; G6PD) is a constitutive enzyme that catalyzes the first and the rate-limiting step of the pentose phosphate pathway. It's key role in red blood cell (RBC) metabolism is to provide educing power in the form of reduced nicotine adenine dinucleotide phosphate (NADPH) (1) . NADPH serves as a cofactor for RBC primary anti-oxidant defense system enzymes like glutation reductase, catalase and NADPH dependent methemoglobin reductase (2-4). NADPH itself and via reduced glutatione, detoxifies hydrogen peroxide and organic peroxides, impedes methemoglobin formation and protects membrane and enzyme proteins of RBC. Pentose phosphate pathway is the only source of NADPH in these cells (5).

Deficiency of G6PD activity is the most common disease producing enzyme deficiency of human beings and usually results from decreased catalytic activity, accelerated breakdown or both. Acute hemolytic anemia associated with oxidative stress is the most characteristic clinical manifestation of G6PD deficiency. G6PD is a subject of public health, because avoiding hemolytic crisis is possible with a preventive approach (6,7).

Diagnosis of G6PD deficiency is based on either qualitative or quantitative analysis of the enzyme activity, demonstration of the enzyme protein with immunochemical approaches or methods depending on reducing power of NADPH. Today qualitative tests are used only for screening

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purposes as most of them have limited sensitivity and/or specificity. Immunochemical methods are not in use, owing to inefficiency for variants with decreased catalytic activity. Isolation and biochemical characterization of deficient variants and gene sequence analyses are subject of research laboratories (6,8,9). In clinical laboratories similar quantitative kinetic methods are performed that are based on the same principle. The distinction between these methods are the reaction temperature, the reaction period, selection of RBC lysing reagent, contents of the blank tube, concentration of the reaction components etc. and definition of the enzyme activity as a function of different complete blood count (CBC) parameters (haemoglobin (Hb) concentration, RBC count or seldom hematocrit (hct)) (8,10,11). A possible confusion about interpretation of the results that may originate from such differences can be eliminated with reference interval establishment study of each laboratory, as the variables are the same for every patient except for the CBC parameter. For this reason, best approach for defining erythrocyte G6PD activity takes an additional attention. The best parameter in this sense should not only determine the G6PD activity but should not be affected from distinct genetic, pathologic, physiological and environmental conditions as well.

Today automated complete blood counters have wide acceptance that offer fast, easy and accurate determination of RBC count and RBC indices. Although since 1967 WHO recommended G6PD and Hb should be assayed on the same hemolysate, there are methods that define G6PD activity as a function of RBC count (10,12). In this study, the relation of G6PD activity to RBC count and RBC indices are evaluated to ascertain possible advantages of this approach over Hb that is greatly influenced by nutritional and metabolic states.

MATERIAL AND METHODS

The Reference Sample Group

In this study, 242 reference individuals (123 F, 119 M) referred to national health units for routine biochemical investigation were studied after their informed consent according to the Helsinki declaration as revised in 1996. Mean age of the subjects were 39.5 (range: 1-80). None of them were erythrocyte G6PD deficient according to the reference interval of our laboratory (6.4 - 13.2 U/ g Hb or 178 - 354 mU/ 10^9 RBC, 30^{O}C) or had reticulocyte count over 0.5%. Infants were not included due to the shorter lifespan, characteristic morphology and different metabolic pattern of neonatal and infantile erythrocytes (13,14).

Specimen Collection and Preparation

Three ml of blood from antecubital vein was collected into heparinized tubes and stored at $+4^{O}$C until the analysis which was performed within three days. Erythrocyte pellets were prepared after centrifugation at 5000×g, $+4^{O}$C for 10 minutes and discarding the plasma followed by two washing steps with isotonic NaCl solution in the same manner. Erythrocyte pellet was suspended in isotonic NaCl solution with a standard ratio. Hemolyzates were prepared with addition of EDTA-β-mercaptoethanol solution to erythrocyte suspension and the complete lysis was confirmed with microscopic examination (8).

RBC Count and Hemoglobin Concentration

RBC, WBC, platelet count, hemoglobin concentration of the erythrocyte suspensions and erythrocyte indices were determined with an automatic complete blood counter: Medonic CA 160 (Sweden). Blood cell classification in Medonic CA 160 depends on direct current impedance measurement for the identification of the blood cell classes and Hb analysis on Drabkin's method.

Erythrocyte G6PD Activity Determination

Erythrocyte G6PD activity was determined with kinetic spectrophotometric method according to the ICSH at 30^{O}C, except for water blank that gives exactly the same results either with the reagent or the sample blanks (data not shown) (8). One unit of G6PD activity is defined as the quantity of the enzyme that involves in transforming one micromole of oxidized coenzyme ($NADP^+$) into reduced form ($NADPH+H^+$) per one minute, at the conditions mentioned above and proportioned to RBC count or hemoglobin concentration of the erythrocyte suspension (10). All chemicals were obtained from Sigma (St Louis, USA) and Merck (Darmstadt, Germany). A semiautomathic spectrophotometer (Unifast 3 analyser,

Sclavo Dignostics) was used for G6PD determination. For control of accuracy, Precinorm® E and Precipath® E (Boehringer Mannheim) were included in each analytical run. One human whole blood pool was used to assess the within-day precision of the assay. The coefficient of variation (CV) was %0.9.

Statistical Evaluation

Evaluation of the relations were performed with correlation, partial correlation and regression analyses. Two tailed p values lower than 0.05 were taken into consideration (15,16). Statistical analyses and scatter diagrams were performed with Microsoft Excel and Statgraphics® programs.

RESULTS

Investigation of the Factor That Determines G6PD Activity

Correlation of G6PD activity with Hb concentration and RBC count of the erythrocyte suspensions were investigated. Enzyme activity was positively correlated with RBC count (r:0.53, $p<0.001$). There was no correlation with Hb concentration ($p>0.05$) (Table 1).

Investigation of Interferences

WBC, platelet contents and RBC morphology of the erythrocyte suspensions were analyzed. Statistically significant correlations were observed between the enzyme activity with WBC and platelet counts (r: 0.17 and r: 0.29, $p<0.001$). MCV and MCH were negatively correlated with the enzyme activity (r: -0.44 and r: -0.29, $p<0.001$) (Table 1).

A significant correlation between MCV and MCH values (r: 0.61, $p<0.05$) was observed. To eliminate the affect of MHC on correlation between MCV and the enzyme activity and vice versa partial correlation analysis was performed. The partial correlation coefficient of the enzyme activity with MCV was r: -0.35, $p<0.01$ where there was no correlation with MCH ($p>0.05$) (Table 2).

Another significant correlation was observed between RBC and platelet counts (r: 0.48, $p<0.05$). After elimination of indirect effect of this relation with partial correlation analysis, platelet count and the enzyme activity were comprehended to be unrelated ($p>0.05$).

How far these undesired factors affect G6PD activity when proportioned to RBC count and Hb concentration, were investigated (Table 3). G6PD activity as a function of Hb concentration was negatively correlated with MCV and MCH (r: -0.61 and r: -0.44, $p<0.001$). On the other hand G6PD activity as a function of RBC count was no more correlated with erythrocyte indices ($p>0.05$). Distribution of G6PD activity as a function of Hb concentration against MCV values is shown in Figure 1.

Affect of WBC and platelet count on G6PD activity are different according to the parameter proportioned. G6PD activity as a function of Hb concentration is positively correlated with WBC and platelet counts (r:0.25 and r: 0.33, $p<0.001$). Correlation of the enzyme activity as a function of RBC count with platelet disappears ($p>0.05$) and with WBC decreases (r:0.15, $p<0.01$) (Table 3).

DISCUSSION

G6PD is a housekeeping enzyme that is widely distributed in human tissues and blood cells. About 400 million individuals are thought to be affected from genetically polymorphic G6PD deficiency (6,11). A G6PD mutation introduced in mouse cells makes them exquisitely sensitive to oxidative stress, indicating that this ubiquitous metabolic enzyme has a major role in the

Table 1. Correlation of the G6PD activity with RBC, WBC, Platelet counts, Hb concentration of the erythrocyte suspensions and erythrocyte indices.

	Correlation Coefficients	Significance Level
RBC (10^9/µl)	0.53	p<0.001
Hb (g/dl)	-0.01	p>0.05
WBC(10^3/µl)	0.17	p<0.001
Platelet(10^3/µl)	0.29	p<0.001
MCV (fl)	-0.44	p<0.001
MCH (pg)	-0.29	p<0.001

Table 2. Partial correlation coefficients of erythrocyte G6PD activity with MCV, MCH and platelet counts.

	Partial Correlation Coefficients	Significance Level
MCV (fl)	-0.35	p<0.01
MCH (pg)	-0.02	p>0.05
Platelet (10^3/µl)	0.01	p>0.05

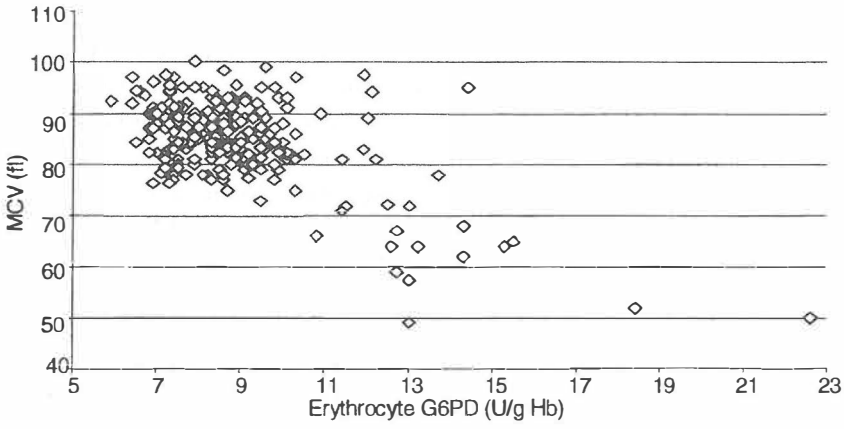

Figure 1. Scatter diagram of G6PD activity as a function of Hb concentration against MCV values.

defense against oxidative stress, even in eukaryotic nucleated cells, which have several alternative routes for providing the same protection (17). Although an old enzyme, G6PD still arouse a widespread interest. New evidence has been presented to suggest that G6PD activity plays a critical role in cell growth (18). Recent studies indicate that leukocyte G6PD is decreased in majority of patients with acute non-lympocytic leukemia (ANLL) and acute lympocytic leukemia (ALL), dependent on the percentage of myelocytes and increased in chronic myeloid leukemia (CML) patients (19,20). The hypothesis of lower mortality from cancer and cardiovascular diseases among men expressing G6PD deficiency is still a controversy (21).

As G6PD has found new clinical fields, it is required that G6PD activity will not inform roughly about only enzyme deficiency but accurate informing G6PD status. Either Hb or RBC would be the denominator of the units of measurement of the enzyme activity; it is necessary to find which always correlate with G6PD. As long as the gene expression of the G6PD is not altered it is expected that G6PD activity should correlate with RBC count. It is well known that G6PD deficiency is a result of decreased catalytic activity, accelerated breakdown or both. Diminished rate of synthesis of G6PD due to gene deletion is not reported since (22-25). Stimulation of G6PD gene expression is an essential component of the metabolic adaptation to oxidative stress. Nutritional regulation of the G6PD gene expression mediated by a nuclear posttranscriptional mechanism is also noted. But the contrast of this situation, an alteration of RBC count discordant with G6PD expression is not known (26,27).

Table 3. Correlation of erythrocyte G6PD activity proportioned to RBC count and Hb concentration with WBC, Platelet counts, erythrocyte indices of the erythrocyte suspensions

	G6PD (mU/10^9RBC)	G6PD (U/g Hb)
MCV (fl)	-0.03	-0.61*
MCH(pg)	-0.11	-0.44*
WBC (10^3/µl)	0.15**	0.25*
Platelet (10^3/µl)	0.04	0.33*

*p<0.001, **p<0.01

In our study, direct enzyme activity was positively correlated with RBC count where no relation was observed with Hb concentration, suggesting that the determining factor for erythrocyte G6PD activity is RBC count (Table 1). On the other hand Hb concentration is regulated by different mechanisms. It is expected that situations with discordance of RBC count and Hb concentration can prompt misinterpretation of G6PD status. Genetically decreased production of Hb with increased RBC count is seen in thalassemia major and thalassemia minor combined with hemoglobinopathy syndromes. In course of iron deficiency anemia, Hb is relatively lower than the RBC count like some other hypochromic anemias: pyridoxine deficiency, lead poisoning, anemia of chronic inflammation and cancer. This discordance is also a characteristic of childhood. After birth, the RBC does not fall to the degree that the

Hb does, MCV and MCH decreases abruptly then gradually and the lowest value is reached at about one year. Between ages of one year and 15 years they gradually rise and reach adult values. Hyperchromic anemias are a second class of discordance in which RBC number can fall below 500 000/µl where the decrease of Hb is less prominent: Pernicious anemia, folate and/or cobalamine deficiency, alcohol intoxication, anemia in liver disease and medications with antimetabolite drugs, phenytoine and estrogens etc. (10,11,17).

The negative correlation between G6PD activity and erythrocyte indices could be evaluated as a result of hematological diseases that affect erythrocyte morphology (Table 1). But in our study almost a fixed Hct was obtained with a standard ratio of dilution (Mean ± SD: 44-50%). With a fixed Hct, decreased MCV analytically means increased RBC count and supports the determining role of RBC count. The correlation of MCH that disappears with elimination of the indirect effect of MCV is possibly a result of a presence of low MCH along with low MCV in a number of situations. The third observation of our study supporting this hypothesis is the increasing correlation coefficient of erythrocyte indices with G6PD activity when proportioned to Hb, again due to the presence of low MCV and MCH along with low Hb concentration in certain pathological and physiological conditions like iron deficiency anemia, β-thalassemia major, minor and physiological anemia of childhood. On the other hand, G6PD as a function of RBC count is no longer correlated with erythrocyte indices.

Like other tissue and cells, WBCs and platelets also have G6PD activity. Although erythrocyte and WBC enzyme is thought to be under same genetic control, in G6PD deficiency relatively shorter life span of WBCs and inability of erythrocytes to synthesize new protein leads to lower G6PD activity of erythrocytes than other cells (28,29). In our study platelet count was positively correlated with the enzyme activity and enzyme activity as a function of Hb concentration suggesting company of platelets with RBCs rather than the interference of enzyme content of the platelets. But the correlation of the enzyme activity with WBC was independent from RBC or Hb content of the blood.

It can be concluded that erythrocyte G6PD activity is related to RBC count and has several advantages over Hb in assessing G6PD status. It gives more reliable results in conditions that could affect erythrocyte morphology and Hb concentration. Presence of WBC in analytical medium could interfere with the results.

REFERENCES

1. Naylor CE, Rowland P, Basak AK, Gover S, Mason PJ, Bautista JM, et al. Glucose 6-phosphate dehydrogenase mutations causing enzyme deficiency in a model of the tertiary structure of the human enzyme. Blood 1996; 87:2974-2982.

2. Gaetani GF, Ferraris AM, Rolfo M, Mangerini R, Arena S, Kirkman HR. Predominant role of catalase in the disposal of hydrogen peroxide within human erythrocytes. Blood 1996; 87:1595-1599.

3. Martinez Cayuela M. Oxygen free radicals and human disease. Biochimie 1995; 77:147-161.

4. Nelson DA, Davey FR. Erythrocytic disorders. In: Henry JB (ed). Clinical Diagnosis and Management by Laboratory Methods (18th ed). Philadelphia, WB Saunders, 1991: 660-662.

5. Lehninger AL, Nelson DL, Cox MM. Principles of Biochemistry (2nd ed). New York, Worth Publishers, 1993: 436-437.

6. Luzzatto L, Mehta A. Glucose 6-phosphate dehydrogenase deficiency. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds). The metabolic and molecular bases of inherited disease (ed). USA, 1995: 3367-3398.

7. Yüreğir GT, Aksoy K, Arpacı A, Ünlükurt İ, Tuli A. Studies on red cell glucose 6-phosphate dehydrogenase: evaluation of reference values. Ann Clin Biochem 1994; 31:50-55.

8. Luzzatto L. Investigation of hereditary haemolytic anaemias: membrane and enzyme abnormalities. In: Dacia JV, Lewis SM (eds). Practical Haematology (7th ed). Singapore, Longman Publishers, 1993: 204-214.

9. Scopes DA, Bautista JM, Naylor CE, Adams MJ, Mason PJ. Amino acid substitutions at the dimer interface of human glucose 6-phosphate dehydrogenase that increase thermostability and reduce the stabilising effect of NADPH. Eur J Biochem 1998; 251:382-388.

10. Bauer JD. Clinical Laboratory Methods (9th ed). USA, C V Mosby Company, 1982: 96-100, 220-221.

11. Fairbanks VF, Klee GG. Biochemical aspects of hematology. In: Burtis CA, Ashwood ER (eds). Tietz Textbook of Clinical Chemistry (2nd ed). Philadelphia, WB Saunders Company, 1994: 1977-1986.

12. Löhr GW, Waller HD. Glucose 6 Phosphate Dehydrogenase. In: Bergmeyer HU (ed). Methods of Enzymatic Analysis. New York and London, 1974: 636.

13. Travis SF, Kumar SP, Paez PC, Delivoria-Papadopoulos M. Red cell metabolic alterations in postnatal life in term infants: glycolytic enzymes and glucose 6-phosphate dehydrogenase. Pediatr Res 1980; 14:1349-1352.

14. Vetrella M, Barthelman W. Enzyme activities in the erythrocytes of human fetuses. Z Kinderheilk 1971; 110: 99-103.

15. Kahn SE, Jandreski MA. Laboratory Statistics. In: Kaplan LA, Pesce AJ, (eds). Clinical Chemistry Theory, Analysis, Correlation (3rd ed). USA, Mosby Company 1996: 342-364.

16. Kingle O. Statistical procedures. In: Burtis CA, Ashwood ER (eds). Tietz Textbook of Clinical Chemistry (2nd ed). Philadelphia, 1994:384-484.

17. Martini G, Ursini MW. A new lease of life for an old enzyme. Bioassays 1996; 18:631-637.

18. Tian WN, Braunstein LD, Phang J, Stuhlmeier KM, Xi OC, Tian X, et al. Importance of glucose-6-phosphate dehydrogenase activity for cell growth. J Biol Chem 1998; 273:10609-10617.

19. Devi GS, Prasad MH, Reddy PP, Rao DN. Leukocyte glucose 6-phosphate dehydrogenase as a prognostic indicator in children with acute lymphocytic leukemia. Indian J Exp Biol 1997; 33:829-832.

20. Devi GS, Prasad MH, Reddy PP, Rao DN. Leukocyte glucose 6-phosphate dehydrogenase as a prognostic indicator in ANLL and CML. Indian J Exp Biol 1997; 35:155-158.

21. Cocco P, Todde P, Fornera S, Manca MB, Manca P, Sias AR. Mortality in a cohort of men expressing the glucose-6-phosphate dehydrogenase deficiency. Blood 1998; 91:706-709.

22. Adediran SA. Kinetic and thermodynamic properties of two electrophoretically similar genetic variants of human erythrocyte glucose-6-phosphate dehydrogenase. Biochimie 1996; 78:165-170.

23. Beutler E, Kuhl W, Gelbart T, Forman L. DNA sequence abnormalities of human glucose - 6- phosphate dehydrogenase variants. The Journal of Biological Chemistry 1991; 226:4145-4150.

24. Luzzatto L. Genetic heterogeneity and pathophysiology of G6PD deficiency. British Journal of Haematology 1974; 28:151-154.

25. Morelli A, Benatti U, Geatani GF, De Flora A. Biochemical mechanisms of glucose 6-phosphate dehydrogenase deficiency. Proc Natl Acad Sci 1978; 75:1979-1983.

26. Hodge DL, Salati LM. Nutritional regulation of the Glucose 6-phosphate dehydrogenase (G6PD) gene mediated by a nuclear posttranscriptional mechanism. Arch Biochem Biophys 1997; 348:303-312.

27. Spolarics Z. Endotoxemia, pentose cycle, and the oxidant/antioxidant balance in the hepatic sinusoid. J Leukoc Biol 1998; 63:534-541.

28. De Flora A, Morelli A, Benatti U. Entrapment of normal and mutant glucose 6-phosphate dehydrogenase (G6PD) within G6PD deficient erythrocytes. Biblthca Haemat 1985; 51:50-56.

29. Mamlock MR, Mills GC, Goldblum RM. Glucose 6-phosphate dehydrogenase Beaumont: A new variant with severe enzyme deficiency and CNSHA. Enzyme 1985; 34:15-21.