GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND PYRUVATE KINASE IN SICKLE CELL ANEMIA

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SUMMARY : The glucose-6-phosphate dehydrogenase (G-6-PD) and pyruvate kinase (PK) were determined in sickle cell anemia patients using kits from Boehringer Mannheim. The level was found to be 155±62 mU/10^9 erythrocytes and 414.3±188 mU/10^9 erythrocytes, for G-6-PD and PK respectively. These levels were significantly higher than the levels obtained in normal individuals. The enzyme levels were subjected to regression and correlation analysis with the hematological parameters in these patients. A negative correlation was obtained between the enzyme level (G-6-PD, PK) and total hemoglobin, red blood cell count and packed cell volume, while a positive correlation was demonstrated with MCV, MCH and white blood cells. No correlation was demonstrated between these enzymes and MCHC, reticulocytes, Hb A_2 and Hb F. These results show that G-6-PD and PK levels are elevated in patients with sickle cell anemia. This may be a consequence of associated elevation in neocytes, or due to a real increase in G-6-PD level or due to a modified phenotypic expression of G-6-PD. Thus an increase in the anemic state may result in an increase in the G-6-PD and PK activity, thereby masking any associated enzyme deficiency, particularly in patients who have a partial enzyme deficiency and when commercially available kits are used.

Key Words: Glucose-6-phosphate dehydrogenase, pyruvate kinase, sickle cell anemia.

INTRODUCTION

Sickle cell disease occurs at a high prevalence in regions of Saudi Arabia that have a past or present history of malaria endemicity (1-4). In previous studies a high frequency of glucose-6-phosphate dehydrogenase deficiency (G-6-PD) was demonstrated in patients suffering from sickle cell disease and sickle cell heterozygotes (Hb AS) in all regions of Saudi Arabia (5-7). Similar results have been reported from Ghana (8), and in Afro-American sickle cell anemia patients (9,10). However, other reports contradicted these findings and showed no interactions between sickle cell anemia and G-6-PD deficiency (11,12). These contradictory reports have been explained by the suggestion that in sickle cell disease, due to the preferential survival of younger red cell population and due to an increase in the reticulocyte count, which have a higher level of G-6-PD, the G-6-PD deficiency may be easily masked (12,13). Furthermore, Efrandson *et al.* (14) and Piomelli *et al.* (9) have suggested the possibility that sickle cell anemia modifies the phenotype expression of G-6-PD deficiency, thus normal of slightly increased G-6-PD activity may be found depending on the severity of hemolysis. This had led to the suggestion that a G-6-PD level higher than the normal may be found in sickle cell disease patients, even those with G-6-PD deficiency.

In order to clarify these reports, we initiated the present study on sickle cell anemia patients to correlate the level of red cell G-6-PD and pyruvate kinase (PK) to hematological parameters and subjected the result to regression and correlation analysis. This paper present the level of G-6-PD and PK in sickle cell disease patients and shows the correlation between these red cell enzymes and hematological parameters. It also discusses the disadvantage of using commercially available kits for estimation of red cell G-6-PD activity.

MATERIALS AND METHODS

Blood samples were collected from 74 known cases of sickle cell anemia who were attending outpatient clinics at King Khalid University Hospital and Ministry of Health hospitals in Riyadh. Two to five millimeters of venous blood was collected in hepari-

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nized tubes and hematological parameters were estimated in fresh blood using a Coulter Counter ZF6 with a hemoglobinometer attachment. Red cell indices: mean cell volume (MCV), mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) were obtained either from the Coulter of were calculated using standardized procedures (15).

The blood was centrifuged and the red cells were carefully separated from the plasma and buffy coat. The red cells were hemolysed by addition of five volumes of 0.02% digitonin at 4°C and the clear supernatant was immediately analyzed to determine the activities of red cell G-6-PD and PK, using kits from Boehringer Mannheim GmbH. For the measurement of G-6-PD activity the increase in absorbance (ΔA/min) was measured at 365 nm using a Zeiss Spectrophotometer and the units of G-6-PD were calculated as follows:

$$mU/10^9 \text{ erythrocytes} = \frac{5417 \times \Delta A/\min \times 10^9}{\text{No. of erythrocytes}/ml}$$

where 15477 is the factor correcting for the extinction coefficient and the dilution (Boehringer Mannheim Instructions).

For PK the decrease in absorbance at 340 nm was measured and the mean absorbance change per minute (ΔA/min) x 10 was used for the calculation of PK units:

$$mU/10^9 \text{ erythrocytes} = \frac{5417 \times \Delta A/\min \times 10^9}{\text{No. of erythrocytes}/ml}$$

where 5417 is a factor correcting for the extinction coefficient and the dilution (Boehringer Mannheim Instructions). The hemoglobin phenotype was confirmed by electrophoresis at alkaline (16) and acid pH (17). Hemoglobins A_2 and F were estimated using an elution technique following cellulose acetate electrophoresis (16) and alkali denaturation (18), respectively.

The results of hematological parameters and the G-6-PD and PK units were fed on the computers at the King Saud University Computer Center. The mean and standard deviation was obtained for each parameter using the Statistical Analysis System (SAS). Regression and correlation analysis were conducted using the General Linear Model (GLM) program and regression lines were obtained between each enzyme and the hematological parameter.

RESULTS

The value of hematological parameters and G-6-PD and PK levels in the sickle cell anemia patients are presented in Table 1. The results show significantly elevated G-6-PD and PK activities when compared with normal values established for the normal population i.e. 97.5±32.5 mU/10^9 erythrocytes for G-6-PD and 166±32.5 mU/10^9 erythrocytes for PK (19, 20). The G-6-PD level was subjected to regression analysis with each of the hematological parameters. The correlation coefficient (r), intercept and slope value are presented in Table 2. The G-6-PD level was plotted against the hematological parameters and Figures 1 and 2 show the correlation between G-6-PD level and hemoglobin (Hb) and red cell count (RBC), respectively. With each of the hematological parameters a statistically significant negative correlation was obtained, except for white blood cell count with there was a positive correlation which was statistically significant. With MCV and MCH a positive correlation was obtained, while with reticulocytes, MCHC, Hb A_2 and F, no statistically significant correlation was demonstrated.

Between PK and each hematological parameters a statistically significant negative correlation was obtained except with WBC for which no correlation could be demonstrated. A positive correlation was obtained between PK and MCV and MCH. No significant correlation was obtained between PK and MCHC, reticulocytes, Hb A_2 and Hb F. The correlation coefficient (R), intercept and slope values between PK and haematological parameters are presented in Table 3. Figure 3 presents the correlation between red cell PK and RBC.

Table 1: Hematological parameters, G-6-PD and PK levels in patients with sickle cell disease.

Parameters	Sickle cell disease patients (Mean±SD)
RBC (x10^{12}/l)	3.70±0.95
Hb (g/dl)	8.36±1.23
PVC (l /l)	0.22±0.045
MCV (fl)	79.70±10.40
MCH (pg)	28.38±5.87
MCHC (g/dl)	37.02±4.58
Reticulocytes (%)	22.36±11.87
Hb A_2 (%)	3.43±1.24
Hb F	11.16±6.80
WBC (x10^9/dl)	15.40±8.96
G-6-PD mU/10^9 ery.	155.0±62.0
PK mU/10^9 ery.	414.0±188.0

Table 2: Regression analysis and correlation coefficient between G-6-PD level and hematological parameters.

Correlation between G-6-PD level and:	r	Intercept	Slope	p
RBC	0.335	221.93	-26.7	< 0.05
Hb	0.230	252.66	-13.3	0.05
PCV	0.276	237.8	-4.3	< 0.05
MCV	0.345	-70.20	2.64	< 0.05
MCH	0.3147	29.887	3.85	< 0.05
MCHC	0.211	26.85	3.069	0.09
Reticulocytes	0.0129	141.25	0.0635	0.9422
Hb A_2	0.01	137.1	-0.744	0.938
Hb F	0.045	114.97	-0.46	0.728
WBC	0.148	114.97	1.84	< 0.05

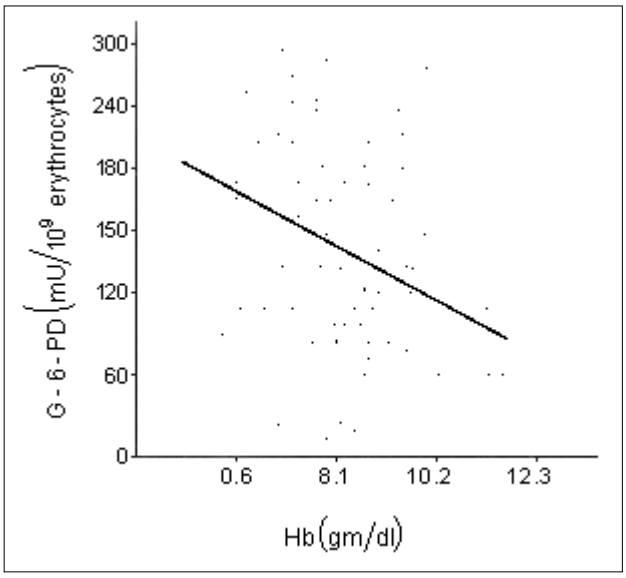

Figure 1: Correlation between erythrocyte G-6-PD level and Hb level in sickle cell anaemia patients.

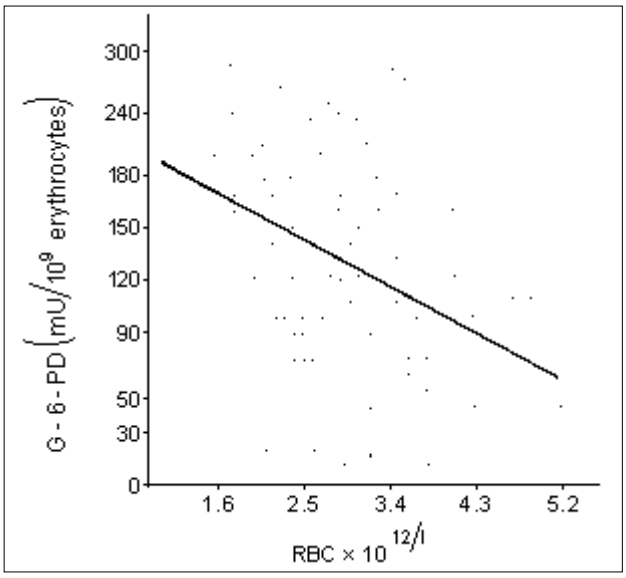

Figure 2: Correlation between erythrocyte G-6-PD level and RBC count in sickle cell anaemia patients.

DISCUSSION

The G-6-PD and PK ranges obtained in the sickle cell disease patients are significantly higher ($p<0.05$) when compared to the normal references values, established for these cell enzymes in healthy Saudi individuals (19, 20). The sickle cell disease patients were all anemic and as the values of Hb, RBC and PCV decreased both G-6-PD and PK levels increased. An effect which may be due to (a) increase in the reticulocyte count, (b) a real increase in the number of young erythrocytes or (c) a real increase in the enzyme activity. Correlation between G-6-PD and

Table 3: Correlation between red cell pyruvate kinase and hematological parameters.

Correlation between G-6-PD level and:	r	Intercept	Slope	p
RBC	0.448	682.33	-90.86	< 0.05
Hb	0.245	22.3	-2.2	< 0.05
PCV	0.387	769.3	-16.37	< 0.05
MCV	0.457	-262.9	8.4	< 0.05
MCH	0.379	60.429	12.0	< 0.05
MCHC	0.2712	-4.782	10.9	< 0.05
Reticulocytes	0.249	295.58	3.69	0.116
Hb A_2	0.205	499.1	-30.1	0.087
Hb F	0.109	429.8	-2.9	0.346
WBC	0.063	328.8	1.3	0.583

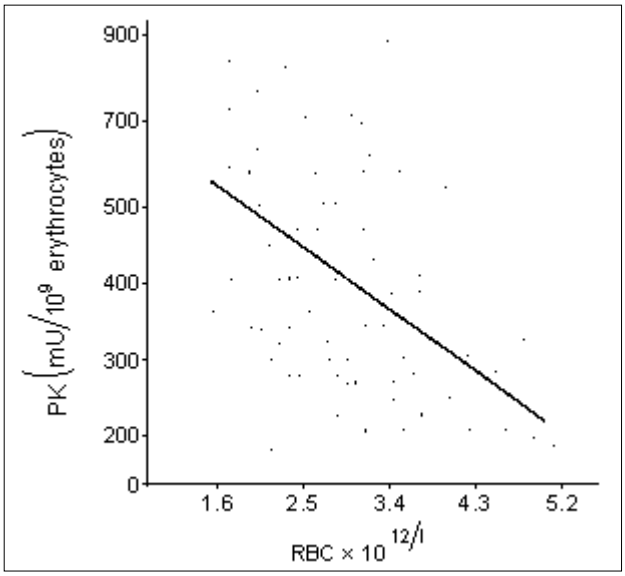

Figure 3: Correlation between erythrocyte, PK level and RBC count in sickle cell anaemia patients.

PK enzyme level and the reticulocytes count was not found to be statistically significant. However, since the sickle cell disease patients suffer from a state of chronic hemolysis and the older red cells are removed from the circulation more than the younger red cells, which are rich in the G-6-PD and PK content, the overall level of these enzymes may be increased in the patients suffering from anemia. This would suggest that in anemic cases associated G-6-PD deficiency could be significantly masked, since the proportion of young red cells is larger than that of the mature red cells.

Furthermore, a positive correlation was found to exist between the red cell G-6-PD level and WBC count. The sickle cell disease patients often suffer from infections and the WBC level is considerably higher than in normal individuals. An increase in the level of G-6-PD could also

be due to the increased WBC count, and would influence the results unless the buffy coat is carefully removed, as was done in this study. Within this group, PK showed no correlation with WBC level since majority of the patients had high WBC count. In an earlier study on anemic and non-anemic individuals a positive correlation was obtained between PK and WBC (21). Furthermore, since PK level was significantly higher in these patients this may be due to the presence of a higher proportion of neocytes and WBC count which are rich in the enzymes.

No significant correlation was demonstrated between the G-6-PD and PK level and MCHC. A positive correlation with MCV and MCH could be due to associated anemic state in these patients. In addition, with the Hb F and Hb A_2 level no correlation was seen. These results also suggest that in thalassaemic cases G-6-PD and PK level may be significantly altered if there was associated anemia.

It is clear from the results of this study that G-6-PD and PK levels are significantly elevated in sickle cell anemic patients. This appears to be a consequence of reticulocytosis and increase WBC in sickle cell disease patients. Since in an earlier study on anemic and non-anemic individuals a positive correlation was reported between the PK and G-6-PD level and the reticulocyte count and WBC (21, 22). Furthermore, it can be stated on the basis of these results that sickle cell anemia is possibly modifying the phenotypic expression of G-6-PD and PK and significantly elevated levels of these enzymes are obtained, which are directly related to the anemic state. This is in line with the suggestion raised by Efrandson *et al.* (14), and Piomelli *et al.* (9) and would explain why the correlations between G-6-PD deficiency and sickle gene in different studies have been contradictory. In some patients with severe anemia the phenotypic expression of G-6-PD deficiency may have been altered thus masking the G-6-PD deficiency. On the other hand, patient with a mild disease like those in our previously reported studies from the eastern province of Saudi Arabia (5, 7), the phenotypic expression of G-6-PD deficiency is not altered significantly due to a mild anemic state and thus an increased frequency of G-6-PD deficiency is seen in patients with sickle cell anemia. This is also confirmed if one studies the increased frequency of G-6-PD deficiency in sickle cell heterozygotes, who are generally asymptomatic (5-7).

Commercially available kits for qualitative and quantitative estimation of G-6-PD are widely in use in both hospital and research laboratories. These kits do not include in the procedure any steps to remove the leucocytes and may give elevated G-6-PD and PK level particularly in patients with leucocytosis and reticulocytosis. Hence it is necessary that during routine G-6-PD and PK estimations, prior to using the kits the buffy coat be carefully removed and RBC are taken from the bottom of the centrifuge tube to avoid contamination by young cells. For research purpose it may be necessary to use Ficol gradient to separate the young cells.

In conclusion, this study has shown that G-6-PD and PK levels are significantly higher in sickle cell disease patients. It has also confirmed the suggestion made by Nance (13), Naylor *et al.* (12), Efrandson *et al.* (14) and Piomelli *et al.* (9) that G-6-PD deficiency could be masked in sickle cell anemia patients due to reticulocytosis and/or modified phenotypic of G-6-PD deficiency due to sickle cell anemia. It has also shown that leucocytosis is a factor resulting in elevation of G-6-PD and PK level and the leucocytes must be removed carefully prior to the estimation of red cell PK and G-6-PD. It is thus necessary that determination of G-6-PD and PK level in sickle cell anemia patients be made with care. Furthermore, it must be taken into account that G-6-PD deficiency cannot be entirely ruled out, if G-6-PD level is found to be normal or slightly reduced in patients showing a certain degree of anemia. Particularly in those patients who have G-6-PD variants that results only in a partial reduction of G-6-PD activity.

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