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G6PD Enzyme Activity in Newborns and Children: Reference Values by the Quantitative Colorimetric Method and a Comparison with the Fluorescent Spot Test

Suphara Manowong, Narumon Tachawong, Sugunya Waiyo and Pimlak Charoenkwan

Department of Pediatrics, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand

Correspondence: Pimlak Charoenkwan, MD, Department of Pediatrics, Faculty of Medicine, Chiang Mai University, 110 Intawarorot road, Sriphum, Muang, Chiang Mai 50200, Thailand
E-mail: pimlak.c@cmu.ac.th

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ABSTRACT

OBJECTIVE This study aimed to establish G6PD enzyme activity reference levels in newborns and children by the quantitative colorimetric method and to compare the results with the fluorescent spot test.

METHODS Leftover blood samples from newborns and children that were sent for G6PD measurement and tested by the fluorescent spot test at the Pediatric Laboratory, Faculty of Medicine, Chiang Mai University were further tested by the quantitative colorimetric method. The values were analyzed according to age group and the two methods were compared.

RESULTS There were 111 newborns (76 males, mean age 3.9±3.5 days) and 182 children (81 males, mean age 7.4±4.2 years). The mean G6PD enzyme activity levels in normal male and female newborns using the colorimetric method were 12.7±2.9 and 13.2±2.0 IU/g Hb, and in normal male and female children were 10.7±4.0 and 11.3±3.2 IU/g Hb, respectively. By the fluorescent spot test, 33 (11.3%), 7 (2.4%) and 253 (86.3%) samples were classified as G6PD deficient, intermediate-deficient and normal G6PD status, respectively. The sensitivity and specificity by the fluorescent spot test of males were 93.3, 98.4% and of female were 45.0, 99.1%, respectively.

CONCLUSIONS G6PD enzyme activity reference levels in newborns and children by a colorimetric method were established. The fluorescent spot test shows a good performance in males, but a lower performance in intermediate-deficient females. Therefore, females should be tested by the colorimetric method.

KEYWORDS colorimetric method, G6PD, Glucose-6-phosphate dehydrogenase, fluorescent spot test, G6PD enzyme activity, newborns, children

INTRODUCTION

Glucose-6-phosphate dehydrogenase (G6PD) is an essential enzyme in the pentose phosphate pathway (PPP)(1). G6PD deficiency is one of the most common inherited enzymopathies in malaria-endemic areas including Southeast Asia (2). G6PD deficiency is common in the Thai population, with a prevalence of 3-18% in males (3). The condition is an X-linked disorder with more than 400 identified variant enzymes (4). Non-synonymous mutations in the gene can decrease enzyme activity or reduce the stability of the enzyme, resulting in different degrees of G6PD deficiency (1). G6PD enzyme is involved in the protection of erythrocytes from oxidative injury and a deficiency can result in mild to severe hemolysis (5). Symptomatic patients are mostly male and, less commonly, homozygous females. G6PD deficiency is the most common enzyme disorder that can cause neonatal hyperbilirubinemia, which can lead to kernicterus and spastic cerebral palsy or death. In older children and adults, G6PD deficiency can cause acute episodic hemolysis triggered

by exposure to oxidizing agents, e.g., those in specific drugs such as primaquine and other antimalarial drugs, and also in fava beans (5-7).

G6PD deficiency is diagnosed by a low G6PD enzyme level in enzyme assays. Qualitative tests such as the fluorescent spot test are simple to use and have diagnostic performance comparable to quantitative tests (8). However, the fluorescent spot test has a threshold ability to distinguish enzyme activity of around 30% of normal, which is sufficient to identify G6PD-deficient homozygous females and hemizygous males, but inadequate for heterozygous females with intermediate enzyme activities above 30% of normal but below a pre-defined considered safe threshold such as 70-80% of normal (9,10). Thus diagnosis of this higher threshold requires a quantitative test for G6PD activity.

Presently, there are many G6PD quantitative diagnostic tests available. An enzymatic colorimetric method is among the most frequently used quantitative measurement of G6PD enzyme activity in a whole blood specimen. The enzymatic colorimetric method has a high sensitivity for measuring NADPH which is a product in the G6PD-mediated pathway (11). This study aimed to determine the reference values for G6PD activity levels in newborns and children of both sexes using a quantitative colorimetric method, and to compare the fluorescent spot test and the colorimetric method.

METHODS

This study was approved by the Research Ethics Committee, Faculty of Medicine, Chiang Mai University. Leftover blood samples obtained from newborns and children that had been sent for G6PD measurement and tested by a fluorescent spot test at the Pediatric Laboratory Faculty of Medicine, Chiang Mai University, were further tested using a quantitative colorimetric method. The whole blood samples were collected in EDTA tubes and were either immediately tested or were kept in a refrigerator at 4°C until tested. All samples were tested within 48 hours. G6PD enzyme activity levels were measured by two methods: the R&D Diagnostics® G6PD fluorescent spot test (R&D Diagnostics, Athens, Greece) and the Standard™ G6PD quantitative colorimetric test (SD Biosensor, Gyeonggi-do, Republic of Korea). G6PD values were analyzed by gender and age group and the two methods were compared using a quantitative method as the gold standard.

The fluorescent spot test was performed according to the manufacturer's recommendations using either a dried blood spot or whole blood. In brief, 5 µL of whole blood was mixed in a microtube with 100 µL of reagents containing glucose-6-phosphate, NADP, oxidized glutathione, saponin, and tris(hydroxymethyl)-aminomethane in dilution buffer. The normal control and sample tests were left at room temperature for ten minutes, after which 10 µL of the mixture was transferred onto filter paper. Deficiency controls were transferred to a filter paper immediately. The dried blood spots were examined under ultraviolet light. The normal controls showed strong fluorescence and the deficient controls showed very weak or no fluorescence. The fluorescence intensity of the tested samples were graded independently by two trained laboratory staff. In cases of discrepancy in grading, the test results were judged by a third trained staff member. Samples with weak to moderate fluorescence were graded as an intermediate deficiency.

The colorimetric test was performed according to the manufacturer's recommendations using a G6PD code chip. In brief, the test device was inserted into the slot in the G6PD analyzer. Ten µL of whole blood was mixed with an extraction buffer containing glucose-6-phosphate, NADP, 5-bromo-4-chloro-3-indoyl-phosphate (BCIP), and nitroblue tetrazolium (NBT). Then 10 µL of the mixture was applied to the specimen application hole of the test device. The G6PD level was read from the analyzer's screen after two minutes. G6PD normal and deficient controls were tested along with each lot of reagents. The measuring ranges of the tests were: total Hb 4-25 g/dL and G6PD 0-20 U/g Hb. Interpretation of the test results was done following WHO guidelines. The adjusted male median of G6PD activity was used as a reference of normal activity. The adjusted male median of G6PD activity in newborns and children was calculated by excluding results from males with severe G6PD deficiency (activity

less than 10% of normal) (9,10,12). In males, red cell G6PD activity less than 30% of the adjusted male median was regarded as G6PD deficient. Any males who had red cell G6PD activity of 30% or more of the adjusted male median were regarded as G6PD normal. In females with red cell G6PD activity, less than 30% of the adjusted male median was regarded as G6PD deficient. Any females who had red cell G6PD activity of 80% or more of the adjusted male median were regarded as G6PD normal. Red cell G6PD activity between 30% and 80% of the adjusted male median was regarded as intermediate deficiency.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics for Windows, version 22.0 IBM Corp, 2013 (Armonk, NY, USA). The G6PD levels were analyzed by gender and age group The continuous variables were analyzed using descriptive statistics: mean and standard deviation for normally-distributed variables and median and interquartile range (IQR) for non-normally-distributed variables. Sensitivity, specificity, accuracy, positive predictive value, and negative predictive value of the fluorescent spot test were also calculated.

RESULTS

Blood samples from 111 newborns (76 males, mean age 3.9±3.5 days) and 182 children (81 males, mean age 7.4±4.2 years) were analyzed. G6PD enzyme activity levels in newborns and children by sex and proposed cut-off values for deficient, intermediate, and normal G6PD activity are shown in Table 1. Table 2 shows G6PD enzyme activity levels measured by the colorimetric test classified using WHO guideline recommended cutoff values. The mean normal levels in male and female newborns were 12.7±2.9 and 13.2±2.0 IU/g Hb, and in male and female children were 10.7±4.0 and 11.3±3.2 IU/g Hb, respectively. The distribution of samples from newborns and children by G6PD enzyme activity level is shown in Figure 1. By the colorimetric method, 31 (10.6%) and 40 (13.6%) samples were classified as G6PD deficient and intermediate-deficient. The fluorescent spot test showed 33 (11.3%) and 7 (2.4%) as G6PD deficient and intermediate-deficient, respectively. The classification of G6PD status by the colorimetric method and the fluorescent spot test is shown in Table 3. The sensitivity, specificity, accuracy, positive and negative predictive values by the fluorescent spot test in each age group are shown in Table 3.

DISCUSSION

There are several measurement methods available for G6PD assay. Some methods are more suited for population studies and others are more suited for individual case management decision-making (10). The reference method for the quantification of G6PD enzyme activity is spectrophotometry, which is based on the colorimetric detection of NADPH (1,12,13).

Table 1. G6PD enzyme activity levels in newborns and children by sex and proposed cutoff values for deficient, intermediate, and normal G6PD activity

G6PD enzyme activity level (IU/g Hb)	Male newborns (N=76)	Adjusted male newborns (N=73)	Female newborns (N=35)	Male children (N=81)	Adjusted male children (N=80)	Female children (N=101)
Median (IQR)	11.8 (5.0-13.8)	12.0 (7.1-13.9)	12.1 (9.7-14.5)	9.2 (6.9-13.2)	9.4 (7.1-13.2)	8.6 (6.6-11.6)
Deficient (< 30% of adjusted male median)	-	≤ 3.6	≤ 3.6	-	≤ 2.8	≤ 2.8
Intermediate (30-80% of adjusted male median)	-	-	3.7-9.6	-	-	2.9-7.5
Normal (> 30% of adjusted male median in males) (> 80% of adjusted male median in females)	-	≥ 3.7	≥ 9.7	-	≥ 2.9	≥ 7.6

Table 2. Glucose-6-phosphate dehydrogenase enzyme activity levels as measured by colorimetric test and classified by the proposed cutoff values

	Glucose-6-phosphate dehydrogenase enzyme activity level (IU/g of Hb)							
	Male				Female			
	N	Mean±SD	Min	Max	N	Mean±SD	Min	Max
Newborn								
Normal	58	12.7±2.9	4.4	> 20.0	27	13.2± 2.0	9.7	16.9
Intermediate	-	-	-	-	7	6.9±1.1	5.5	8.7
Deficient	18	2.0±1.0	0	3.6	1	1.8	-	-
Children								
Normal	72	10.7±4.0	3.0	19.8	65	11.3±3.2	7.6	19.9
Intermediate	-	-	-	-	33	5.8 ± 1.3	3.0	7.5
Deficient	9	1.4±0.3	0.9	2.0	3	2.5	2.5	2.6

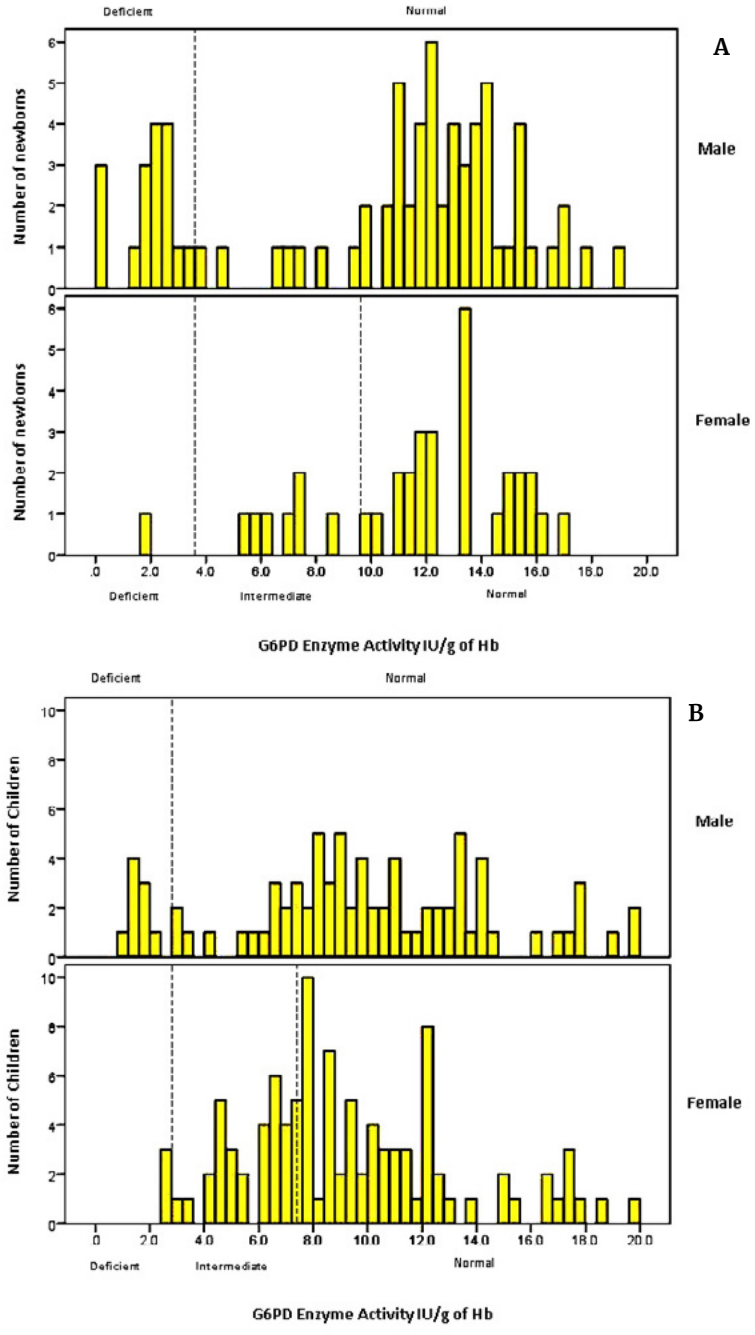

Figure 1. Distribution of samples from newborns (A) and children (B) by G6PD enzyme activity level

Table 3. Comparison of Glucose-6-phosphate dehydrogenase enzyme activity levels as measured by fluorescent spot test and the colorimetric test

	Colorimetric test			
Fluorescent spot test	**Deficient**	**Intermediate**	**Normal**	**Total**
Newborn				
Deficient	17	0	2	19
Intermediate	0	1	0	1
Normal	2	6	83	91
Total	19	7	85	111
Children				
Deficient	11	0	3	14
Intermediate	0	6	0	6
Normal	1	27	134	162
Total	12	33	137	182
Male				
Deficient	28	-	2	30
Intermediate	-	-	-	-
Normal	2	-	125	127
Total	30	-	127	157
Female				
Deficient	3	0	0	3
Intermediate	1	5	1	7
Normal	2	9	115	126
Total	6	14	116	136

Newborn: sensitivity 69.2%, specificity 97.6%, accuracy 91.0%, PPV 90.0%, NPV 91.2%
Children: sensitivity 37.8%, specificity 97.8%, accuracy 83.0%, PPV 85.0%, NPV 82.7%
Male: sensitivity 93.3%, specificity 98.4%, accuracy 97.4%, PPV 93.3%, NPV 98.4%
Female: sensitivity 45.0%, specificity 99.1%, accuracy 91.2%, PPV 90.0%, NPV 91.3%

It is essential to provide reference and cutoff values for each equipment platform and reagent. Also, the G6PD enzyme activity level for different age groups needs to be established for each method as G6PD levels in newborns and children are reported to be higher than in adults (14,15). Newborns with G6PD deficiency are at risk of hyperbilirubinemia, which can progress to kernicterus. G6PD enzyme activity reference levels in newborns and children were established in this study. The normal values of all groups were higher than the reported oriental adult reference values determined using a colorimetric method: 8.92±2.65, 8.99±2.29 U/g Hb in males and females, respectively (16).

In males, G6PD status is classified as either normal or deficient. The distribution pattern of G6PD enzyme levels is typically in two groups; however, in our study, the pattern of distribution was not clear. There were some male newborns and children with G6PD enzyme levels in the intermediate deficiency range of females. This may be caused by the falsely elevated G6PD levels associated with reticulocytosis during acute hemolysis or by previous blood transfusions (14,15,17-24). G6PD levels in females are classified as normal, intermediate deficiency and deficiency. In this study, the G6PD levels in females were distributed normally (25,26). The results show that fluorescent spot test can be used to diagnose G6PD deficiency in males, but the efficiency was low in females with intermediate deficiency. Limitations of this study include that the clinical information and reticulocyte count were not available. Additionally, the WHO methemoglobin reduction assay and molecular characterization of G6PD gene was not done.

Both the sensitivity and specificity of the fluorescent spot test to diagnose G6PD deficiency were high in males. However, the test showed low performance in discriminating intermediate deficiency in females. The children group had a higher percentage of females than the newborn group; as a result, the overall sensitivity was lower. Use of a quantitative method is suggested for diagnostic testing for G6PD intermediate deficiency in females. The risks

of hemolysis in females with intermediate deficiency when exposed to medications are largely uncertain. Knowledge of enzyme levels can help guiding decisions regarding whether medications can be safely given (27).

CONCLUSION

G6PD enzyme activity reference levels in newborns and children as measured by the colorimetric method were established. G6PD enzyme activity is age-dependent, with higher levels in newborns than in children as measured by the colorimetric method. As males usually have either normal or deficient activity, qualitative tests perform well in males. However, qualitative tests have lower performance in heterozygous females with intermediate deficiency (27). For that reason, this study suggests that females should be tested by the colorimetric quantitative method.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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