

# G6PD ENZYME ACTIVITY IN NORMAL TERM MALAYSIAN NEONATES AND ADULTS USING A OSMMR2000-D KIT WITH H<sub>B</sub> NORMALIZATION

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**Abstract.** Glucose-6-phosphate dehydrogenase (G6PD) deficiency is one of the commonest causes of neonatal jaundice in Malaysia. Screening of cord blood for G6PD deficiency by the semiquantitative fluorescent spot test (FST) is performed in Malaysia but this test can miss cases of partial G6PD deficiency. The OSMMR-D kit assay measures G6PD activity and hemoglobin (Hb) concentration, allowing direct expression of results in U/gHb. We evaluated this method and established the normal range for G6PD activity in normal term neonates and adults. EDTA blood from 94 neonates and 295 adults (age 15-59 years old) with normal Hb and FST were selected. The normal means for G6PD activity for neonates and adults were  $12.43 \pm 2.28$  U/gHb and  $9.21 \pm 2.6$  U/gHb, respectively; the reference ranges for normal G6PD activity in neonates and adults were 10.15-14.71 U/gHb and 6.61-11.81 U/gHb respectively. There were no significant differences in mean normal G6PD activity between the Malays and Chinese racial groups or between genders. The upper and lower limit cut-off points for partial deficiency in neonates were 7.4 U/gHb (60% of the normal mean) and 2.5 U/gHb (20% of the normal mean), respectively. For adults, the upper and lower limit cut-off points for partial deficiency in adults were 5.52 U/gHb (60% of the normal mean) and 1.84 U/gHb (20% of the normal mean), respectively. The quantitation of G6PD enzymes using this OSMMR-D kit with Hb normalization was simple since the Hb was analyzed simultaneously and the results were reproducible with a CV of less than 5%.

**Key words:** glucose-6-phosphate dehydrogenase, enzymes assay, OSMMR-D kit with hemoglobin (Hb) normalization

## INTRODUCTION

Glucose-6-phosphate dehydrogenase (G6PD) is an important enzyme in the hex-

ose monophosphate oxidative pathway where it maintains the production of the cofactor NADPH. NADPH protects red cells from oxidative damage. G6PD deficiency increases the vulnerability of erythrocytes to oxidative stress. In Malaysia, the overall incidence of G6PD deficiency among males is 3.1% and is a very important cause of severe neonatal jaundice (Hon *et al*, 1989). It is more common in Chinese and Malays than in Indians. Screening of cord blood for G6PD defi-

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ciency in carried out in all hospitals in this country using the semiquantitative fluorescent spot test (FST). However this method, being a qualitative test, detects only cases with severe G6PD deficiency, but misses cases of partial G6PD deficiency when the enzyme levels are more than 20% of the mean normal level (Ainoon *et al*, 2003). Individuals with partial G6PD deficiency are usually asymptomatic, however when exposed to oxidative stress, such as infections, exposure to certain drugs or following fava bean ingestion, they may develop acute hemolytic anemia. Enzyme quantitation using a colorimetric method is relatively simple, and is able to quantitate the level of the G6PD enzyme activity. A G6PD kit from Randox Laboratories Ltd (Randox) has been used in our hospital to detect partial G6PD deficiency in female heterozygotes missed by the regular screening method (Ainoon *et al*, 2003). However, this method is laborious, time consuming, has a long turnaround time with the hemoglobin measurement being done separately on an automated blood cell counter. The OSMMR-D G6PD kit assay (Reclos *et al*, 1999) is another method for G6PD activity assay that employs the hemoglobin normalization procedure. The principle is the same as the previous method. Red cell G6PD activity is tested by the production of NADPH in the reaction mix. The NADPH reacts with a color reagent and produces a distinct color that can be measured at 550nm using a spectrophotometer, and is directly proportional to the concentration of G6PD in the blood sample. This is followed by measurement of Hb concentration again at 450 nm, hence allowing the simultaneous measurement of G6PD activity and Hb concentration. We evaluated a method for G6PD enzyme quantitation using the OSMMR-D kit with Hb normalization and

established the normal range for G6PD activity and cut-off points for G6PD deficiency for normal term neonates and adults.

## MATERIALS AND METHODS

One hundred six neonates (57 males; 49 females) born at the UKM Medical Centre and 295 adults (152 males, 143 females, age range 15-59 years old) were studied. The adult patients were either in-patients or those from the follow-up clinics at UKM Medical Centre whose EDTA treated cord blood or venous blood samples were sent to the hematology laboratory for a routine full blood count. Patients with normal hemoglobin levels and a normal fluorescent spot test were included in the study. A G6PD activity assay was performed on all cord blood and peripheral blood samples.

For the fluorescent spot test, samples were spotted and dried on Whatman's filter paper. The test was carried out according to the method described by Beutler and Mitchell (1968). This technique is based on the visualization of fluorescence of reduced pyridine nucleotide (NADPH) when activated by UV light. Samples that did not show fluorescence were classified as G6PD-deficient and those showing fluorescence were classified as normal.

For the quantitation of G6PD activity, a OSMMR-D G6PD assay kit with hemoglobin normalization from R & D Diagnostics (Holargos, Greece) was used. The measurement of enzyme activity was done using a spectrophotometer (Ultramicroplate Reader EL808, Bio Tek, Instruments). Five microliters of cord or peripheral blood was mixed with 75  $\mu$ l of elution buffer in the well of a microplate; 75  $\mu$ l of the reagent was put in a different well of the microplate. The microplate were placed on an orbital shaker in an incubator and was slowly

Table 1  
G6PD activity levels for normal Malay and Chinese neonates.

Ethnic group	No. of cases	Mean G6PD activity (U/gHb)	Upper limit of total deficiency (20% of mean value) U/gHb	Upper limit of partial deficiency (60% of mean value) U/gHb
Malay	48	12.44	2.49	7.46
Chinese	46	12.42	2.48	7.45
Total	94	12.43	2.49	7.46

There were no significant differences in mean G6PD activity between both racial groups ( $p > 0.05$ ).

warm to 37°C for 20 minutes. Fifteen microliters of the sample was added to the reagent, then the sample was read at 405 nm in a single measurement mode using a spectrophotometer for Hb evaluation. Eighty microliters of the color reagent mixture (from OSMMR-D Kit) was then added to the sample. This reagent contains substrate, coenzyme and buffer. After mixing, the microplate was again read at 550 nm in kinetic mode. Two readings were taken, at 0 and 15 minutes. The total change in optical density ( $\Delta OD$ ) for each sample and control was calculated using the following formula, with the enzyme activity assay results expressed in U/gHb.

$$\frac{(\delta OD_{\text{sample}550\text{nm}}/\text{min}) / (\delta OD_{\text{control}550\text{nm}}/\text{min})}{\frac{OD_{\text{sample}405\text{nm}}}{OD_{\text{control}405\text{nm}}}} \times \text{Control} = \text{sample (U/gHb)}$$

The means, SD, ranges of G6PD activity were determined. A 60% cut-off point for the diagnosis of G6PD deficiency was established for each group (WHO Working Group, 1989). The G6PD activity assay results were also compared with the fluorescent spot test.

## RESULTS

### Neonatal population

The results for partial and total G6PD deficiency in 94 cases of neonates (48 Malays and 46 Chinese) are shown in Table 1. There were no significant difference in the mean G6PD activity between the two racial groups. There were also no significant differences in mean normal G6PD activity between male and female neonates in each racial group (Table 2).

The overall mean value for G6PD activity was  $12.43 \pm 2.28$  U/gHb. The reference range for normal G6PD activity in neonates was 10.15-14.71 U/gHb. The upper and lower limit cut-off points for partial deficiency were 7.4 U/gHb (60% of the normal mean) and 2.5 U/gHb (20% of the normal mean), respectively. The upper limit for total or severe deficiency was 2.5 U/gHb (20% of the normal mean G6PD activity for neonates). The intrabatch CV for samples with normal G6PD activity was 4.6%.

### Adult population

The results for mean normal G6PD activity, partial and total G6PD deficiency for all 295 adults (151 Malays and 144 Chinese) are shown in Table 3. There were no significant differences in mean normal

Table 2  
G6PD activity levels for normal male and female neonates.

Races	Gender	No. of cases	Mean G6PD activity (U/gHb)	Upper limit of total deficiency (20% of mean value) U/gHb	Upper limit of partial deficiency (60% of mean value) U/gHb
Malay	Male	25	12.72	2.54	7.60
	Female	23	12.13	2.42	7.28
Chinese	Male	24	12.95	2.59	7.77
	Female	22	11.85	2.37	7.11
Total	Male	49	12.83	2.57	7.70
	Female	45	11.99	2.40	7.19

There were no significant differences in mean G6PD activity between males and females in each racial group ( $p > 0.05$ ).

Table 3  
G6PD activity levels for Malay and Chinese adults.

Ethnic group	No. of cases	Mean G6PD activity (U/gHb)	Upper limit of total deficiency (20% of mean value) U/gHb	Upper limit of partial deficiency (60% of mean value) U/gHb
Malay	151	9.43	1.87	5.66
Chinese	144	8.94	1.79	5.36
Total	295	9.20	1.84	5.52

There were no significant differences in mean G6PD activity between both racial groups ( $p > 0.05$ ).

Table 4  
G6PD activity levels for male and female adults.

Races	Gender	No. of cases	Mean G6PD activity (U/gHb)	Upper limit of total deficiency (20% of mean value) U/gHb	Upper limit of partial deficiency (60% of mean value) U/gHb
Malay	Male	84	9.26	1.85	5.60
	Female	67	9.65	1.93	5.79
Chinese	Male	68	9.07	1.81	5.44
	Female	76	8.82	1.76	5.29
Total	Male	152	9.17	1.85	5.50
	Female	143	9.21	1.83	5.52

There were no significant differences in mean G6PD activity between males and females in each racial group ( $p > 0.05$ ).

Table 5  
G6PD activity in different age group in adults.

Age group	No. of cases	Mean G6PD activity (U/gHb)	Upper limit of total deficiency (20% of mean value) U/gHb	Upper limit of partial deficiency (60% of mean value) U/gHb
15 - 29	74	9.60	1.92	5.76
30 - 39	78	8.84	1.77	5.30
40 - 49	68	9.02	1.80	5.41
50 - 59	75	9.34	1.87	5.60
Total	295	9.20	1.84	5.52

There were no significant differences in mean G6PD activity among different age groups in adults ( $p > 0.05$ ).

G6PD activity between the two racial groups. There was also no significant difference in mean normal G6PD activity between male and female adults in each racial group as shown in Table 4. The mean G6PD activities in both age groups are shown in Table 5; on comparison between these 2 groups there were no significance differences.

The mean value for G6PD activity was  $9.21 \pm 2.6$  U/gHb. The reference range for normal G6PD activity in adults was 6.61-11.81 U/gHb. The lower and upper limit cut-off points for partial deficiency were 1.84 U/g Hb (20% of the normal mean) and 5.52 U/g Hb (60% of the normal mean), respectively. The cut-off point for total or severe deficiency was 1.84 U/gHb (20% of the normal mean G6PD activity for adults).

## DISCUSSION

The gene which encodes for G6PD is located on the X-chromosome. In female heterozygotes, red cell mosaicism arising from random X chromosome inactivation results in G6PD-deficient and G6PD-normal cell types, the proportion of these two cells can vary enormously ranging from

completely normal activity to complete deficiency. Many female heterozygotes may have overall G6PD activity that can range from 20 to 60%, classified as partial deficiency (WHO). For many years, G6PD deficient heterozygotes were not regarded as being at risk. However, many studies have shown that female heterozygotes are at risk of developing severe neonatal hyperbilirubinemia (Davidson *et al*, 1963; Meloni *et al*, 1983; Kaplan *et al*, 1999; Reclos *et al*, 2000). Therefore, heterozygotes should be warned as early in life as possible and treated as if she is totally G6PD deficient.

Neonatal screening for G6PD deficiency utilizing the semiquantitative fluorescent spot has long been carried out in many countries, including Malaysia. In a previous study we showed the fluorescent spot test failed to detect a substantial proportion (46.3%) of female heterozygotes with G6PD deficiency (20-60% of normal G6PD activity) and in our own experience many of these neonates had severe neonatal jaundice. The FST was not able to detect individuals with red cell G6PD activity of  $>2.92$  U/gHb (20% of mean normal G6PD activity). We established that it

is important to identify partially G6PD-deficient neonates as part of our strategy to manage severe neonatal hyperbilirubinemia. These individuals have been shown to be exclusively females, were missed by the routine fluorescent spot test. Male G6PD-deficient patients were easily diagnosed by the FST because male hemizygotes are fully expressed phenotypically, similar to female homozygotes and therefore are almost always severely deficient (G6PD activity < 20% mean normal).

We have since introduced in our laboratory, the G6PD assay kit from Randox Laboratories to screen neonates with hyperbilirubinemia. The kit utilizes the chemical reaction described by Beutler *et al* (1968). The NADPH produced is measured using a kinetic mode on the Hitachi 717 Autoanalyser (Boehringer Menheim, Germany). However, we find this method laborious as it involves multiple steps, beginning with the elution stage for red cell lysis, followed by incubation of supernatant with reagents containing substrate and cofactor NADP and subsequently the photometric measurement of the kinetic reaction at 340 nm. In this method, the sample hemoglobin is measured separately on an automated cell counter and G6PD activity is derived by manual calculation and expressed as U/gHb. This affects the accuracy of the test and is time consuming. In the present study we evaluated the OSMMR-D kit for G6PD, a rapid G6PD assay kit where the measurement of enzyme activity is performed on a ultramicroplate spectrophotometer (Ultramicroplate Reader EL808, Bio Tek, Instruments) using a kinetic mode. The wide wavelength of the spectrophotometer allows determination of G6PD activity and Hb concentration simultaneously. Hb normalization does away with manual

calculation of enzyme activity, a potential source of error and allows direct expression of the results in U/gHb. We find the method less laborious and it gives faster results. The method shows good reproducibility with intrabatch CV of 4% for normal samples.

In this study, we established the normal range, the mean and the standard deviations for G6PD activity for both neonates and adults. The mean values for G6PD activity for the two major ethnic groups, Malays and Chinese, were not significantly different from each other ( $p>0.05$ ). There were no significant differences in mean G6PD activity between females and males for both ethnic groups ( $p>0.05$ ). This lack of difference in red cell G6PD activity level among ethnic and gender groups has been reported previously in Malays and Chinese in Malaysia using the Sigma kit (Ainoon *et al*, 2003). Comparison of mean values among different age groups in adults also showed no significant differences ( $p>0.05$ ). The overall means for normal neonates and normal adults were 12.43 U/gHb and 9.20 U/gHb, respectively. It was expected to find mean red cell G6PD activity to be higher in neonates than adults because of the higher number of circulating young red cells in neonates. We found the mean G6PD activity for neonates using this method was lower than the mean G6PD activity previously established in our laboratory using the Randox G6PD kit (14.55 U/gHb), but the mean for adults was higher compared to the Randox G6PD assay (7.47 U/gHb) (Ainoon, 1999). It is crucial to determine the 60% cut-off point for mean normal residual G6PD activity to diagnose G6PD deficiency (WHO Working Group, 1989). With the establishment of mean normal G6PD activity we were able to determine the 60% cut-off point to diagnose G6PD

deficiency and determine the 20% to 60% range to establish partial deficiency. The partial deficiency ranges (20-60% of normal) for G6PD activity in neonates and adults were 2.5-7.4 U/gHb and 1.84-5.52 U/gHb, respectively; the partially deficient ranges with the Randox G6PD assay were 2.92-8.7 U/gHb for neonates and 1.54-4.63 U/gHb for adults (Ainoon *et al*, 2003).

In conclusion, the OSMMR-D G6PD kit assay is rapid, easy to perform, less laborious and has good reproducibility. We established the normal ranges and the 60% and 20% cut-off points to be used for the diagnosis of G6PD deficiency term neonates and adults age 15-59 years old. We hope the establishment of a rapid G6PD assay that is cost effective, can replace the semiquantitative fluorescent spot test as a more accurate and sensitive method for screening neonates for G6PD deficiency.

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