Glucose-6-phosphate dehydrogenase activity, structure, molecular characteristics and role in neonatal hyperbilirubinemia in cord blood in Çukurova region

Ferda Özlü[1], Mehmet Satar[1], Şule Menziletoğlu-Yıldız[2], İsa Ünlükurt[2], Kıymet Aksoy[2]

Departments of [1]Neonatology and [2]Biochemistry, Çukurova University Faculty of Medicine, Adana, Turkey

SUMMARY: Özlü F, Satar M, Menziletoğlu-Yıldız Ş, Ünlükurt İ, Aksoy K. Glucose-6-phosphate dehydrogenase activity, structure, molecular characteristics and role in neonatal hyperbilirubinemia in cord blood in Çukurova region. Turk J Pediatr 2011; 53: 130-136.

The most common causes of neonatal indirect hyperbilirubinemia are blood incompatibility and erythrocyte enzyme defects. Glucose-6-phosphate dehydrogenase (G6PD) is a guarantee of erythrocyte stability and capability of existence of red cells. We present here the results of a study on the effect of enzyme kinetics and different mutations on neonatal hyperbilirubinemia in the Çukurova region.

Two hundred healthy term male neonates born in Çukurova University Balcalı Hospital, Adana Maternity Hospital and Çukurova Maternal and Children's Hospital between 1 November 2004 and 30 November 2007 were consecutively studied. Nanogen® DNA microarray was used to determine Gd Union, Gd San, Gd Mediterranean, and Gd San Antonio mutations. Quantitative G6PD enzyme assays were performed.

Glucose-6-phosphate dehydrogenase deficiency was detected in six out of 200 male neonates (3%). The other 194 neonates had normal G6PD activity, with a mean of 8.3±2.1 IU/g hemoglobin (Hb) (5.2-12.7 IU/g Hb). Clinical follow-up, enzyme kinetics and genetic studies were performed in the G6PD-deficient neonates.

Differences were observed in clinical outcomes, rates of bilirubin decline and maximum total bilirubin levels in the neonates having the same mutation. These differences might be caused by the effects of kinetic variant on the hyperbilirubinemia without the direct effect of the mutation. In future studies, mutation analyses of further G6PD-deficient cases may address the genotype differences and their clinical effects in G6PD-deficient patients.

Key words: glucose-6-phosphate dehydrogenase, deficiency, hyperbilirubinemia, neonatal.

The most common causes of neonatal indirect hyperbilirubinemia are blood incompatibilities and erythrocyte enzyme defects. Glucose-6-phosphate dehydrogenase (G6PD) deficiency was first discovered in the mid-1950s. G6PD is the rate-limiting enzyme and catalyzes the first reaction in the pentose phosphate pathway. G6PD is a guarantee of erythrocyte stability and capability of existence of red cells. The incidence of G6PD deficiency in the Çukurova region was reported as 10-11.5% in previous studies[1].

Four hundred forty-four different G6PD variants are documented on the basis of the World Health Organization (WHO) criteria (biochemical, kinetic and clinical parameters)[2]. The most common variant in the world that is used to describe the other variants is Gd B+. Gd Mediterranean is another mutation frequent in Caucasians in the Mediterranean region and has a normal movement in electrophoresis and 0-10% activity[3].

A study from Malaysia demonstrated that although G6PD Viancghan, G6PD Mahidol and

This study was supported by Çukurova University Scientific Research Project Unit.

G6PD Mediterranean are common mutations in the cord blood of neonates in Malaysia, there is no significant difference in the incidence of jaundice among the neonates with different mutations[4].

We present here the results of a study of the effects of enzyme kinetic and mutation differences on the severity of neonatal hyperbilirubinemia and the incidence of G6PD variants among neonates in the Çukurova region.

Material and Methods

Two hundred healthy term male neonates born in Çukurova University Balcalı Hospital, Adana Maternity Hospital and Çukurova Maternal and Children's Hospital between 1 November 2004 and 30 November 2007 were consecutively studied. Neonates who were born preterm or small-for-gestational age or with congenital malformations, meconium aspiration, or prenatal asphyxia due to blood gases in the first hour of life were excluded. Five ml of cord blood samples collected in EDTA from each neonate in the delivery room for routine screening of G6PD deficiency were used for determination of G6PD kinetics, and peripheral blood leukocytes were used for DNA analysis.

Hemogram, direct Coombs test, serum total bilirubin, direct bilirubin levels, reticulocyte count, and blood group of the infants diagnosed with G6PD deficiency and blood groups of their mothers were studied. Serum total bilirubin, direct bilirubin and hematocrit levels were studied at the 3rd, 5th, 7th, 10th and 15th days of life.

Therapeutic indications for phototherapy and exchange transfusion for jaundiced neonates were based on the recommendations of the American Academy of Pediatrics[5].

Nanogen® DNA microarray was used to determine Gd Union, Gd San, Gd Mediterranean, and Gd San Antonio mutations.

Quantitative G6PD enzyme assays were performed according to the method described by Beutler[6].

Glucose-6-phosphate dehydrogenase activity assays were performed in accordance with the WHO recommendations[2]. Five ml of citrated blood was centrifuged at 3000 rpm for 5 minutes (min), and the supernatant was washed with normal saline twice with removal of buffy coat. The hemolysate supernatant was used for G6PD enzyme activity study by adding 50 μl of hemolysate to a 950 μl assay containing buffer A (1 M Tris buffer pH 8.0, 0.1 M $MgCl_2$), glucose-6-phosphate (6 mM, Sigma) and NADP (2 mM, Sigma). The rate of NADPH generation was measured at 340 nm at 30°C for 10 min. The average change of optical density per minute was calculated to determine the activity of the G6PD enzyme. The G6PD activity was calculated and reported as IU per gram hemoglobin (Hb).

Purification of the Enzyme with DE-52 Cellulose

The columns (2x10 cm) were filled with DE-52 cellulose gel and washed with buffer A until the pH of the column was 7.0. After the addition of the hemolysate supernatant, proteins not attached to columns were washed with buffer A, while column-attached proteins were washed with 0.25 M KCl-added buffer A and collected as 3 ml fractions. The partially purified enzyme was studied for kinetic parameters.

Kinetic Study

Km G6P and NADP were determined at the Lineweaver-Burk curve with utilization of 20-200 μL G6P and 40 μL NADP. 6 mM 2dG6P, 6 mM Gal6P, 2 mM NAD, and 2 mM dNADP were used to determine usage of the % and optimum pH.

In our laboratory, the normal value of cord blood G6PD activity was 8± 2.2 IU/g Hb in normal neonates (mean ± standard deviation [SD]). G6PD deficiency was identified when the activity was <5 IU/g Hb.

Results

Glucose-6-phosphate dehydrogenase deficiency was detected in six out of 200 male neonates (3%). The other 194 neonates had normal G6PD activity, with a mean of 8.3±2.1 IU/g Hb (5.2-12.7 IU/g Hb). Clinical follow-up, enzyme kinetics and genetic studies were performed in the G6PD-deficient neonates. Clinical characteristics are given in Table I. Three enzyme-deficient neonates had Gd

Mediterranean mutation (Fig. 1). Two of the neonates with Gd Mediterranean mutation had pathological hyperbilirubinemia and had phototherapy while one neonate had physiological hyperbilirubinemia without any need for treatment. Hemolysis proven by a hematocrit decrease or reticulocyte count increase was not detected in any of the mutant neonates. One of the Gd Mediterranean mutation-negative G6PD-deficient neonates had pathological hyperbilirubinemia necessitating exchange transfusion, while two other non-mutant neonates did not need any treatment for jaundice.

Kinetic study results are given in Table II. Km G6P was between 6.8-555.7 μM and Km NADP was 10.6-167.8 μM. Analog use for dNADP, NAD, Gal6P and 2dG6P was also studied, and the results were 46.5-94, 0-182, 18-142 and 6.8-100, respectively.

Gd Union, Gd San and Gd San Antonio mutations were not found. Enzyme kinetic results of the patients were compared with the mean levels of GdB+ and Gd Mediterranean of WHO criteria and are also given in Table II.

Discussion

Glucose-6-phosphate dehydrogenase deficiency is one of the most common inherited disorders of mankind, with more than 400 million people being affected worldwide, predominating in the tropics and subtropics of Asia, part of the Mediterranean, and Papua New Guinea. The world distribution of malaria is remarkably similar to the world distribution of mutant G6PD alleles, extending around the tropical belt. Epidemiological and in vitro studies indicate that this results from the selection advantage period provided by G6PD deficiency during Plasmodium falciparum infection[7-9].

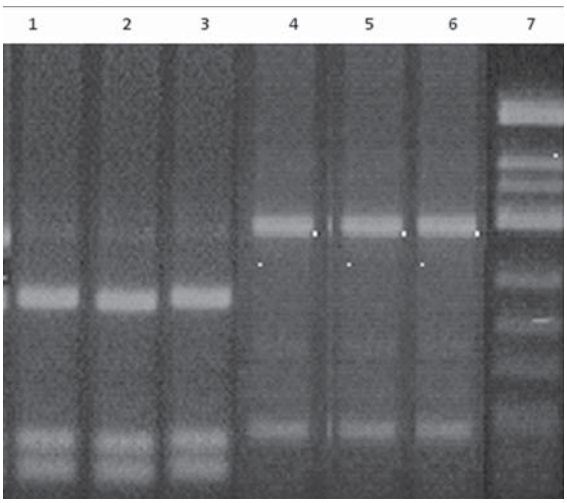

Fig. 1. Electrophoresis appearance of Gd Med mutation
1. Male, hemizygote case with 3 bands, NEONATE O.K.
2. Male, hemizygote case with 3 bands, NEONATE İ
3. Male, hemizygote case with 3 bands, NEONATE K
4. Male, normal case with 2 bands, NEONATE G
5. Male, normal case with 2 bands, NEONATE D
6. Male, normal case with 2 bands, NEONATE F
7. Marker

Although most affected individuals are asymptomatic, there is a risk of neonatal jaundice due to acute hemolytic anemia, triggered by infection and ingestion of certain drugs and notably through eating broad beans (favism).

The mechanism of the hemolysis is not yet exactly defined. Hemolysis of G6PD-deficient red cells most probably results from an increased susceptibility to oxidative damage. During this hemolysis, erythrocyte damage causes hemoglobinuria. A rare but more severe form of G6PD deficiency is found throughout the world and is associated with chronic nonspherocytic hemolytic anemia[10-13].

One of the most important complications of G6PD deficiency and neonatal hyperbilirubinemia is the high risk of developing kernicterus. Therefore, it is important to describe the relation of enzyme deficiency and increased bilirubin in G6PD-deficient jaundiced neonates.

Glucose-6-phosphate dehydrogenase deficiency is the most common X-linked enzymopathy in the world. The prevalence rate varied from 2% to 27% in different population groups[14,15]. Prevalence in neonates with hyperbilirubinemia is 10-11.5%[16]. In our study, the incidence in male neonates was found as 3%.

Neonatal jaundice occurs in G6PD deficiency, but it is likely that it is largely due to impairment of liver function rather than to hemolysis[17,18]. In our patients, although increased bilirubin levels indicating treatment were demonstrated, hematocrit decrease due to hemolysis was not noted.

Glucose-6-phosphate dehydrogenase deficiency has genetic heterogeneity. The same mutation in different patients may cause different clinical symptoms; therefore, the same mutation

Table I. Clinical Findings of the Cases

Name of case		Neonate O.K.	Neonate F	Neonate İ	Neonate K	Neonate D	Neonate G
Blood group		A Rh(+)	O Rh(+)	A Rh(-)	A Rh(+)	O Rh(+)	A Rh(+)
Blood group of mother		A Rh(+)	O Rh(+)	A Rh(-)	A Rh(+)	O Rh(+)	A Rh(+)
Direct Coombs		(-)	(-)	(-)	(-)	(-)	(-)
Total bilirubin (mg/dl)	Cord blood	3	5	4	4.3	4	3.6
	2nd day	20	18	20			
	3rd day	11.2	35	22	11	10.8	11
	5th day	17.2	20	22	8.4	7	6.5
	7th day	14.2	18	25.7	6	6	5.5
	10th day	10.2	15	11	4.2	3	3
	15th day	13.6	10	5	2.7	2	3
Hematocrit (%)	Cord blood	50	47	45	50	56	60
	2nd day	48	48	50			
	3rd day	49	58	53	52	54	57
	5th day	46	53	54	55	54	56
	7th day	48	54	57	53	55	54
	10th day	49	55	54	54	55	56
3rd day reticulocyte %		3	3	2.8	2.5	3	3
Treatment	Phototherapy	Phototherapy	Exchange transfusion	Phototherapy	None	None	None
Gd Med mutation		(+)	(-)	(+)	(+)	(-)	(-)

Table II. Comparison of the Kinetic Findings of the Cases with GdB^+ and Gd Mediterranean Mutation

No	Case	G6PD activity (U/g Hb)	Km (μM)		Analog Use				Opt pH	Change in first 20 min (%)
			G6P	NADP	dNADP	NAD	Gal6P	2dG6P		
1	NEONATE O. K.	0	6.8	42.4	27	0	142	87	8	68
2	NEONATE F	0	229.3	23.8	46.5	0	18	16	8	82
3	NEONATE İ	0	172	15.1	81	119	44	100	8	250
4	NEONATE K	0	72.5	10.6	66	10	32	6.8	8	44
5	NEONATE D	0	555.7	77.1	94	112	71	77	8	81
6	NEONATE G	0	78.2	167.8	82	182	59	29	6-8	100
	Gd B+	8.3±3.3	50-70	2-4	55-60	<1	7-15	<4		
	Gd Mediterranean	0-7	19-26	1.2-1.6	350		20	23-37		

may cause chronic hemolytic anemia or non-hemolysis[19,20]. One of the three neonates in this study with Gd Mediterranean mutation did not have jaundice, with only two neonates progressing to severe hyperbilirubinemia requiring phototherapy. Two of three neonates with mutations other than Gd Mediterranean did not have jaundice, with only one neonate progressing to severe hyperbilirubinemia requiring exchange transfusion.

As the prevalence of G6PD deficiency became appreciated, so did the degree of heterogeneity of this enzyme, culminating in the publication of biochemical characteristics of more than 400 reported variants and 140 molecular variants, spreading through a wide geographic area. While Gd Mediterranean is the most common mutation in Mediterranean countries, Middle Asia and Indian regions, Gd A- mutation is the most common mutation in the African region[21]. As predicted early in this process, it appears that the number of genuine variants is less than the number defined through biochemical analysis. This discrepancy has arisen because some common variants have been given different names when encountered in different parts of the world, e.g. Gd Mediterranean and Gd Dallas are shown to have the same molecular mutations. On the other hand, the opposite is also seen: Gd A-, which was thought to be a single mutation, is shown to have genetic heterogeneic structure. In addition, without any enzyme deficiency, some structural variants have been shown[22].

Gd Mediterranean is the most studied mutation thus far among the 140 mutations. Keskin and colleagues[23] reported 79% 563 T Mediterranean mutations in G6PD-deficient subjects in the Denizli region of Turkey. Luzzatto and colleagues[22] described the variant as a change of C →T in the 563rd nucleotide on the 6th exon of the enzyme[3,24].

The most common mutation in our region is the Gd Mediterranean variant. Although the Gd Mediterranean variant causes severe enzyme deficiency, it may sometimes not cause hemolysis. In this study, the relation between genotype and phenotype in G6PD-deficient and especially Gd Mediterranean-mutant neonates was investigated. The most severe phenotype is reported to be due to Gd Mediterranean and GD A- mutations. Three of our neonates

had Gd Mediterranean, two of whom had higher bilirubin levels. On the other hand, the exchange transfused neonate did not have Gd Mediterranean mutation, but might have had some other mutations we could not demonstrate.

Many parameters like G6PD activity, kinetic studies in partial purified enzyme, use of substrate analogs, thermo stability, and optimal pH are used in variant analysis of the G6PD enzyme. In this report, results of kinetic analysis performed in neonates with decreased enzyme activity are shown in Table II.

One of our Gd Mediterranean mutation-negative neonates had biphasic optimal pH reaction, which is a characteristic of Gd Mediterranean mutation[25].

Analog use like Km G6P and Km NADP in neonates with Gd mutation was different than the standards recommended by WHO. This may be due to different kinetic variants of the same mutation. The differences in clinical outcomes, rate of bilirubin decrease and maximum total bilirubin levels in the same-mutant neonates might be caused by the effects of kinetic variant on the hyperbilirubinemia without the effect of the mutation directly. Furthermore, some other G6PD mutations that we could not study may have had a role in the different clinical presentations.

The limitation of this study is the number of the patients. More patients could be included in another study investigating G6PD mutations in the severity of neonatal hyperbilirubinemia.

A neonate with undetected enzyme activity is not able to live, in fact, but we did not have any fatal complications in our neonates. There may be some other proteins inhibiting the enzyme activity and causing different kinetic properties in the erythrocytes.

In future studies, more cases and more mutation analysis may address the cause of genotype differences in G6PD-deficient patients.

REFERENCES

1. Satar M, Atici A, Oktay R. The influence of clinical status on total bilirubin binding capacity in newborn infants. J Trop Pediatr 1996; 42: 43-45.
2. World Health Organization. Scientific group on the standardization of procedures for the study of glucose-6-phosphate dehydrogenase. Geneva: WHO Techn Rep Ser No 366; 1967.
3. Vives-Corrons JL, Kuhl W, Pujades MA, Beutler E. Molecular genetics of the glucose-6-phosphate dehydrogenase (G6PD) Mediterranean variant and description of a new G6PD mutant, G6PD Andalus1361A. Am J Hum Genet 1990; 47: 575-579.
4. Ainoon O, Yu YH, Amir Muhriz AL, Boo NY, Cheong SK, Hamidah NH. Glucose-6-phosphate dehydrogenase (G6PD) variants in Malaysian Malays. Hum Mutat 2003; 21: 101.
5. American Academy of Pediatrics. Management of hyperbilirubinemia in the newborn infant 35 or more weeks of gestation. Subcommittee on Hyperbilirubinemia. Pediatrics 2004; 114: 297-316.
6. Beutler E. Red Cell Metabolism: A Manual of Biochemical Methods (3rd ed). Orlando: Grune & Stratton Inc; 1984: 68-71.
7. Ganczakowski M, Town M, Bowden DK, et al. Multiple glucose 6-phosphate dehydrogenase-deficient variants correlate with malaria endemicity in the Vanuatu archipelago (southwestern Pacific). Am J Hum Genet 1995; 56: 294–301.
8. Beutler E, Westwood B, Prchal JT, Vaca G, Bartsocas CS, Baronciani L. New glucose-6-phosphate dehydrogenase mutations from various ethnic groups. Blood 1992; 80: 255-256.
9. Vulliamy TJ, Othman A, Town M, et al. Polymorphic sites in the African population detected by sequence analysis of the glucose-6-phosphate dehydrogenase gene outline the evolution of the variants A and A-. Proc Nat Acad Sci 1991; 88: 8568-8571.
10. Glader B, Lukens JN. Glucose-6-phosphate dehydrogenase deficiency and related disorders of hexose monophosphate shunt and glutathione metabolism. In: Lee GR, Foerster J, Lukens J, et al. (eds). Wintrobe's Clinical Hematology (10th ed). Egypt: Mass Publishing; 1999: 1176-1190.
11. Gaetani GF, Galiano S, Melani C, et al. A new glucose-6-phosphate dehydrogenase variant with congenital nonspherocytic hemolytic anemia (G6PD Genova). Biochemical characterization and mosaicism expression in the heterozygote. Hum Genet 1990; 84: 337-340.
12. Beutler E, Luzzatto L. Hemolytic anemia. Semin Hematol 1999; 36: 38-47.
13. Kılınç Y. The incidence of glucose 6 phosphate dehydrogenase deficiency in cord blood in midsouth part of Turkey. Ç Ü Tıp Fak Dergisi 1982; 3: 229-232.
14. Niazi GA, Adeyokunnu A, Westwood B, Beutler E. Neonatal jaundice in Saudi newborns with G6PD Aures. Ann Trop Paediatr 1996; 16: 33-37.
15. Kwok CJ, Martin AC, Au SW, Lam VM. G6PDdb, an integrated database of glucose-6-phosphate dehydrogenase (G6PD) mutations. Hum Mutat 2002; 19: 217-224.
16. Nuchprayoon I, Sanpavat S, Nuchprayoon S. Glucose-6-phosphate dehydrogenase (G6PD) mutations in Thailand: G6PD Viangchan (871G>A) is the most common deficiency variant in the Thai population. Hum Mutat 2002; 19: 185.

17. Beutler E. G6PD: population genetics and clinical manifestations. Blood Rev 1996; 10: 45-52.

18. Kaplan M, Herschel M, Hammerman C, Hoyer JD, Heller GZ, Stevenson DK. Neonatal hyperbilirubinemia in African American males: the importance of glucose-6-phosphate dehydrogenase deficiency. J Pediatr 2006; 149: 83-88.

19. Bruggen R, Bautista JM, Petropoulou T, et al. Deletion of leucine 61 in glucose-6-phosphate dehydrogenase leads to chronic nonspherocytic anemia granulocyte dysfunction and increased susceptibility to infections. Blood 2002; 100: 1026-1030.

20. Maffi D, Pasquino MT, Caforio MP, Sorrentino F, Cappabianca MP, Salvatti AM. Identification of G6PD Mediterranean mutation by amplification refractory mutation system. Clinica Chimica Acta 2002; 321: 43-47.

21. Sukumar S, Mukherjee MB, Colah RB, Mohanty D. Molecular basis of G6PD deficiency in India. Blood Cells Mol Dis 2004; 33: 141-145.

22. Luzzatto L, Mehta A, Vulliamy TJ. Glucose 6-phosphate dehydrogenase deficiency In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds). The Metabolic and Molecular Bases of Inherited Disease (8th ed). New York: McGraw-Hill; 2001: 4517–4553.

23. Keskin N, Ozdes I, Keskin A, Acikbas I, Bagci H. Incidence and molecular analysis of glucose-6-phosphate dehydrogenase deficiency in the province of Denizli, Turkey. Med Sci Monit 2002; 8: 453-456.

24. Aksoy K, Yuregir GT, Dikmen N, Unlukurt I. Three new G6PD variants, G6PD Adana, G6PD Samandag, and G6PD Balcalı in Cukurova, Turkey. Hum Genet 1987; 76: 199-201.

25. Corcoran CM, Calabro V, Tamagnini G, et al. Molecular heterogeneity underlying the G6PD Mediterranean phenotype. Hum Genet 1992; 88: 688-690.