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ORIGINAL PAPER

Influence of the Inherited Glucose-6-phosphate Dehydrogenase Deficiency on the Appearance of Neonatal Hyperbilirubinemia in Southern Croatia

Anet Papazovska Cherepnalkovski[1], Eugenija Marusic[2], Katica Piperkova[1], Bernarda Lozic[2], Ana Skelin[2], Todor Gruev[3], Vjekoslav Krzelj[2]

[1]University Pediatric Clinic, Medical Faculty, University "St. Cyril and Methodius", Skopje, Republic of Macedonia
[2]University Hospital Split and School of Medicine, University of Split, Croatia
[3]University Clinic of Clinical Biochemistry, Medical Faculty, University "St. Cyril and Methodius", Skopje, Republic of Macedonia

Corresponding author: Anet Papazovska Cherepnalkovski, M.D. Ms.sci, Department of Neonatology, University Pediatric Clinic, Vodnjanska 17, 1000 Skopje, Republic of Macedonia, Tel. number: +389 2 3147 717, E-mail: anet.cherepnalkovski@gmail.com

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ABSTRACT

Background: Neonatal hyperbilirubinemia is a common clinical manifestation of the inherited glucose-6-phosphate dehydrogenase (G6PD) deficiency. **Aim of the study:** The aim of this study was to investigate the influence of the inherited G6PD deficiency on the appearance of neonatal hyperbilirubinemia in southern Croatia. **Methods:** The fluorescent spot test (FST) was used in a retrospective study to screen blood samples of 513 male children who had neonatal hyperbilirubinemia, of unknown cause, higher than 240 μmol/L. Fluorescence readings were performed at the beginning and at the fifth and tenth minute of incubation and were classified into three groups: bright fluorescence (BF), weak fluorescence (WF) and no fluorescence (NF). Normal samples show bright fluorescence. All NF and WF samples at the fifth minute were quantitatively measured using the spectrophotometric method. **Results:** Bright fluorescence was present in 461 patients (89.9%) at the fifth minute. The remaining 52 (10.1%) were quantitatively estimated using the spectrophotometric method. G6PD deficiency was observed in 38 patients (7.4%). **Conclusions:** Prevalence rate of G6PD deficiency among male newborns with hyperbilirubinemia in southern Croatia is significantly higher ($p < 0.01$) compared with the previously reported prevalence rate among male in general population of southern Croatia (0.75%). We recommend FST to be performed in hyperbilirubinemic newborns in southern Croatia.

Key words: G6PD deficiency, neonatal hyperbilirubinemia, fluorescent spot test, southern Croatia

1. INTRODUCTION

Glucose-6-phospahte dehydrogenase (G6PD), the key regulatory enzyme of the hexose monophospahte shunt, catalyses the oxidation of glucose-6-phospate to 6-phosphogluconolactone and the production of reducing equivalents in the form of nicotinamide adenine dinucleotide phosphate (NADPH) to meet the cellular needs for reductive biosynthesis (1). This pathway is the only source of NADPH in the red cells that protects the cell and its haemoglobin from the oxidative stress mediated through reduced glutathione (GSH) (2).

G6PD deficiency is the most common enzymopathy affecting more than 400 million people worldwide (1, 3). To date, more than 400 variants have been described based on their biochemical properties and grouped into five classes according to the level of residual enzyme activity and clinical manifestations. Several variants considered different based on their biochemical properties, were shown to be identical at the molecular level (2). Molecular analysis of the X-linked gene for G6PD identified 191 mutations or combination of mutations (4).

Acute acquired hemolytic anemia, congenital nonspherocytic hemolytic anemia, favism and neonatal hyperbilirubinaemia are the most frequent clinical manifestations of G6PD deficiency. Severe G6PD- associated neonatal hyperbilirubinemia that can be complicated with kernicterus was identified in the 1960s and was subsequently well documented (5-9). World Health Organization (WHO) data suggest that the global infant mortality due to G6PD-associated neonatal jaundice is in the range of 0.7–1.6 per 1000 of all births. Infants, who survive the acute hyperbilirubinemia, may develop severe athetoid cerebral palsy, auditory dysfunction, intellectual and other handicaps associated with chronic bilirubin encephalopathy (7, 9). The frequency of neonatal jaundice in G6PD-deficient

infants varies between studies, with approximately one third being affected (5, 10, 11).

G6PD- associated neonatal jaundice was believed to be a consequence of haemolysis; however, modern techniques have shown only modest shortening of red cell lifespan. The main cause of neonatal icterus in G6PD-deficient infants is the inability of the liver to adequately conjugate bilirubin (6, 12). In addition, this problem is compounded when the infant inherits the uridine 5'-diphospho-glucuronosyltransferase (UDP glucuronosyltransferase) promoter polymorphism associated with Gilbert's disease (6, 13).

A previous study showed that prevalence of G6PD deficiency in the southern Croatia, which is situated in the Mediterranean basin, in both sexes is 0.44% and 0.75% in males (14). Molecular characterisation of G6PD-deficient patients in the area revealed at least six mutations, among which one is a new variant described as G6PD Split (15). To date, no studies were performed to comparison of the frequency of G6PD-deficiency in a selective group of newborns with hyperbilirubinemia with the incidence of G6PD-deficiency in southern Croatia.

2. MATERIALS AND METHODS

2.1. Participants

The study included 513 male subjects from southern Croatia who were treated in the neonatal period at the University Hospital Split for hyperbilirubinemia greater than 240 µmol/L. Medical records were retrospectively analysed and the patients were subsequently invited to enrol in the study.

Medical records of healthy term newborns without any major congenital anomalies were included through selection process only. Newborns with prematurity defined as <37 gestational weeks and low birth weight of <2500 g were excluded as were newborns with other identifiable risk factors for jaundice. These included sepsis, blood group incompatibility with positive Coombs test, ecchymoses, bruising and cephalhaemathoma due to birth trauma, as well as Down syndrome, hypothyroidism, impaired gastrointestinal motility and maternal diabetes.

2.2. Fluorescent spot test (FST)

The subjects were screened using the FST as the most acceptable method for screening recommended by the International Committee for Standardization in Haematology (16).

This method detects the fluorescence of NADPH under long-wave (365 nm) ultraviolet light in complete darkness. Reduction of nicotinamide adenine dinucleotide (NADP) to NADPH occurs in the presence of G6PD; the rate of NADPH formation is proportional to G6PD activity. Blood used for the test was anti-coagulated in KT3-EDTA (Becton Dickinson, Plymouth, UK) and stored at 4°C up to 7 days. Commercially available kits (Cat. No. 203, Sigma Diagnostics, Taufkirchen, Germany) were used. Ten microliters of whole blood was incubated with 200 µL of the reagent mixture. Spots were made on the filter paper at the beginning (zero time), fifth and tenth minute after blood incubation with reagent mixture. Normal samples show bright fluorescence, whereas deficient samples show little or no fluorescence. The results were classified into three groups: bright fluorescence (BF), weak fluorescence (WF) and no fluorescence (NF).

All NF and WF samples at the fifth minute were quantitatively measured using the spectrophotometric method.

Quantitative spectrophotometric method

Enzyme activity was determined by measuring the rate of absorbance change at 340 nm, due to the reduction of NADP to NADPH when a sample was incubated with G6P. Glucose-6- phosphate dehydrogenase activity was calculated in relation to erythrocyte count. Commercially available kits (Cat. No. PD 410, Randox Laboratories Ltd, Crumlin, Great Britain) were used as previously published (14). Values of $131^{+}/_{-}13$ mU/10^{9} erythrocytes were considered normal.

The results were interpreted as percentage of the normal G6PD activity. Deficient individuals have <60% of the normal G6PD activity. Enzyme activity <10% of the normal activity was classified as severe deficiency, and the activity between 10% and 60% of the normal activity was classified as moderate deficiency (3).

2.3. Statistical analysis

All data analyses were performed using the statistical package Statistical Package for the Social Sciences (SPSS) 17.0 for Windows (SPSS Inc., Chicago, IL, USA). The result was considered significant if probability value was ≤0.01. All parents of children who were included in the study signed an informed consent.

The study has been approved by an institutional Ethics Committee in accordance with the Declaration of Helsinki.

3. RESULTS

Blood samples of all 513 patients were analysed with the FST. In 461 (89.9%) patients, bright fluorescence was noted at the fifth and tenth minute of incubation that indicated normal activity of G6PD. The remaining 52 (10.1%) patients did not present with bright fluorescence at the fifth minute of the test.

All 52 WF and NF samples at the fifth minute were quantitatively measured using the spectrophotometric method.

According to the spectrophotometric G6PD activity measurement 38 samples (7.4%) were G6PD deficient and 12 (2.3%) were false positive.

Three of 38 deficiency patients (7.9%) showed severe deficit of <10% of normal activity. The remaining 35 (92.1%) showed moderate enzyme deficit in the range of 10%–60% of the normal activity. All three samples with severe G6PD deficiency were NF at the fifth and tenth minute of the FST. At the fifth minute eight samples with moderate enzyme deficiency were NF and 27 were WF. Bright fluorescence (BF) appeared in 17 (44.7%) of moderate deficient samples at the tenth minute (Table 1).

Activity G6PD	Time of fluorescence appearance (minute)								
	0			5			10		
	-	+/-	+	-	+/-	+	-	+/-	+
Severe deficiency N° samples (3)	3			3			3		
Moderate deficiency N° samples (35)	35			8	27		8	10	17

Table 1. G-6-PD activity and the time of fluorescence appearance in deficient samples. - No fluorescence (NF), +/-Weak fluorescence (WF), + Bright fluorescence (BF). Normal G6PD activity: 131+/–13 mU/109 erythrocytes.Severe deficiency: <10% of the normal activity. Moderate deficiency: 10%–60% of the normal activity

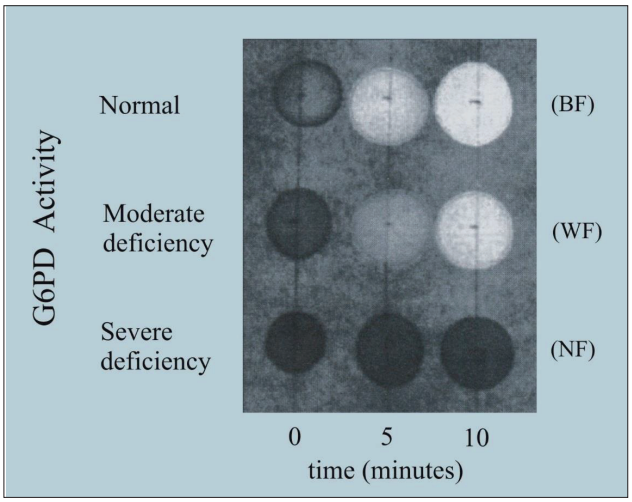

Figure 1. G6PD activity and the time of fluorescence appearance in G6PD normal and deficient samples. Line 1, a sample with normal G6PD activity showing bright fluorescence (BF) at the 5 and 10th minute. Line 2, a sample with moderate G6PD deficiency showing weak fluorescence (WF) at the 5th and BF at the 10th minute. Line 3, a sample with severe deficiency showing no fluorescence (NF) at both measurements.

The prevalence rate of patients with G6PD deficit corresponds to 7.4% of the study population of 513 male patients with neonatal hyperbilirubinemia, which is statistically significantly higher ($p < 0.01$) compared with the previously reported prevalence rate among males in southern Croatia (0.75%).

4. DISCUSSION

G6PD deficiency is the most common hereditary enzyme deficiency affecting hundreds of millions of people worldwide (1, 3). The human G6PD gene that comprised 13 exons has been mapped to the Xq28 region (17, 18). Almost all known G6PD mutations affect the coding region of the gene, the majority being single base missense mutations (3, 4).

The most common clinical manifestations of G6PD deficiency include: acute drug or infection mediated hemolytic anemia, favism, neonatal hyperbilirubinemia and chronic non-spherocytic hemolytic anemia (2, 3).

Neonatal hyperbilirubinemia defined as total serum bilirubin level of >86 µmol/L is a frequent problem presenting in up to 60% of term and 85% of preterm newborn infants [19].

Severe neonatal hyperbilirubinemia associated with G6PD deficiency has the potential of causing bilirubin encephalopathy and kernicterus (5-9). In the last update of the AAP (American Academy of Paediatrics) recommendations, G6PD deficiency was included as a risk factor for neurotoxicity [20]. Higher exchange transfusion rates in G6PD-deficient infants have been reported (21).

The G6PD-associated neonatal hyperbilirubinemia was traditionally considered hemolytic in origin and in some instances, known triggers of haemolysis, such as menthol or Chinese remedies applied to the umbilicus for antisepsis, have been implicated. In addition, fava is transmitted through mother's breast milk, henna is applied to the newborn's skin in some Middle Eastern cultures, bacterial or viral infections and chemical cleansers have been considered as triggers of haemolysis. Contrary to these findings, haemoglobin and hematocrit values as well as reticulocyte count were infrequently diminished in G6PD-deficient hyperbilirubinemic infants (5, 10). Using modern techniques only a modest and inconsistent shortening of red cell lifespan has been shown, which could contribute to a limited extent to jaundice (5, 12). It was shown that the crucial component of neonatal icterus in G6PD-deficient infants was the inability of the liver to adequately conjugate bilirubin (6, 12). Serum total conjugated bilirubin as well as monoconjugated and diconjugated bilirubin fractions were significantly lower in G6PD-deficient newborns that developed hyperbilirubinemia in comparison to the non-hyperbilirubinemic G6PD-deficient neonates signifying the decreased bilirubin conjugation as a key factor for development of their hyperbilirubinemia (12). In addition, this problem is compounded when the infant inherits the UDP glucuronosyltransferase promoter polymorphism associated with Gilbert's disease (6, 13). The presence of these two genes mutations in combination was shown to significantly increase the incidence of hyperbilirubinemia (13).

Study conducted on a sample of 2726 high-school examinees of both sexes in southern Croatia, showed the prevalence of 0.44% of G6PD-deficient individuals (0.75% in males) (14). An isolate was described in the small town of Komiza on the island Vis with a significantly higher prevalence of G6PD deficiency (5.96% in males) compared with the general population of southern Croatia (22). In southern Croatia, cases of favism have been describedand an association was found between Factor V Leiden and G6PD deficiency (14, 22, 23). This study aimed to determine the prevalence of G6PD deficiency in male newborns with hyperbilirubinaemia in southern Croatia. For this purpose, medical records of 513 male subjects from central Dalmatia with neonatal hyperbilirubinemia of >240 µmol/L were retrospectively analysed. All subjects were screened using the FST according to the recommendation by the International Committee for Standardization in Haematology as being the most acceptable method for screening (16).

All 52 samples that showed no fluorescence (NF) and weak fluorescence (WF) at the fifth minute of FST were quantitatively measured using the spectrophotometric method. According to the spectrophotometric G6PD activity measurement 38 (73%) of the FST positive samples at the fifth minute were G6PD deficient and 12 (27%) were false positive. The degree of deficiency influences the reliability of FST. Moderate enzyme deficiency can be identified more accurately with an early reading of fluorescence appearance and also by taking weak fluorescence into consideration (14). The International Committee for Standardization recommends only one examination, which should be carried out in the course of the period from the fifth to the tenth minute of the FST (16). In this study bright fluorescence (BF) appeared even in 17 (44.7% of deficient samples) at tenth minute. These individuals were moderate G6PD deficient. If the test had been taken only at the tenth minute then 44.7% of our positive cases would not have been registered (Table 1). It is obvious that in heterozygotes and hemizygotes with moderate deficiency fluorescent spot test is more reliable in the case of early fluorescence reading, but then there are more false positive results.

Because neonatal hyperbilirubinemia is one of the most common and well documented clinical manifestations of G6PD deficiency, we hypothesised a higher frequency of

occurrence of G6PD deficiency among hyperbilirubinemic patients compared with the general population. We managed to prove a significantly higher prevalence rate among male newborns with hyperbilirubinemia (7.4%) compared with the previously reported prevalence rate among males in southern Croatia (0.75%) (14). Depending on the prevalence of G6PD deficiency in a particular population and according to the WHO recommendations, neonatal screening should be universally performed when G6PD is common (3%–5% of males) (7). In South Croatia population only a small town of Komiza on the island Vis has a significantly higher prevalence of G6PD deficiency (5.96% in males) compared with the general population of southern Croatia.

Certain studies have recommended screening for infants of both sexes (24). G6PD-deficient heterozygotes were shown to be at increased risk for neonatal hyperbilirubinemia (25). Although percentage of jaundice among G6PD-deficient newborns ranges between 20%–40% in different studies (5, 10, 11), an early increase of bilirubin in the immediate perinatal period with a possible intrauterine start was reported (26). Neonates with G6PD deficiency may develop severe hiperbilirubinemia that may lead to kernicterus and death. Therefore, an early therapy and careful monitoring is necessary in newborns with G6PD-associated jaundice compared with jaundice from other causes (22).

In the group of deficient patients we discovered three (7.9%) with severe deficit of <10% of the normal activity. The remaining 35 (92.1%) showed moderate enzyme deficit in the range of 10%–60% of the normal activity. Moderate G6PD deficiency newborns are at increased risk for neonatal hyperbilirubinemia. Within the African American neonates, a subgroup of G6PD-deficient infants mainly of G6PD A- molecular background was observed to have a higher incidence of hyperbilirubinemia and a greater requirement for phototherapy (11).

5. CONCLUSION

In this study, G6PD deficiency in neonatal pathologic hyperbilirubinemia in southern Croatia males was evaluated. G6PD deficiency is recognized as one of the risk factors with high frequency in neonatal hyperbilirubinemia. Prevalence rate of 7.4% G6PD deficiency among male newborns with hyperbilirubinemia in southern Croatia is significantly higher ($p < 0.01$) compared with rate among male in general population of southern Croatia (0.75%). Therefore, we recommend selective screening with FST to be performed in all hyperbilirubinemic neonates of unknown cause in southern Croatia. FST is more reliable in the case of early fluorescence reading.

CONFLICT OF INTEREST: NONE DECLARED

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