Formazan ring method: a simple test for screening of glucoce-6-phosphate dehydrogenase (G-6-PD) deficiency in the neonates

Purnomo Suryantoro
Department of Child Health, Faculty of Medicine, Gadjah Mada University,
Yogyakarta, Indonesia.

ABSTRAK
Purnomo Suryantoro – Metode ciri-ciri formazan : suatu uji karya sederhana defisiensi glukosa-6-fosfat dehidrogenase pada neonat

Keturangan glukosa-6-phosphate dehydrogenase merupakan suatu penyakit keturunan yang bersifat x-linked yang berupa anemia hemolitik yang muncul secara paroksit. Incidensinya bisa dihubungkan dengan terjadinya epizooti malaik pada suatu darah, misalnya di Mediterania, Afrika dan Asia. Terakhir AIDS Tenggara!

Metode formazan ring yang dikembangkan oleh Nakahara merupakan metode yang mudah, murah, tidak menyakitkan dan bisa dipakai untuk skrining. Dari 145 bayi baru lahir yang diteksi ternyata sebanyak 9 orang (6,2 %) mempunyai defisiensi aktivitas enzim yang rendah, bahkan 2 orang (1,4 %) kekurangan glukosa-6-phosphate dehidrogenase.

ABSTRACT

Glucose-6-phosphate dehydrogenase deficiency, is an x-linked inherited disease which is responsible for episodic hemolytic anemia. The incidence correlates with the malari epidemic in many area like Mediterranean, Africa and Asia including South East Asia.

This paper reports a simple, cheap, transmotic, and reliable method screening test called formazan ring developed by Nakahara. Among 145 newborn infants in this report the low enzyme activity was as high as 93.82% including 7.14% G-6-PD deficiency.

Key words: glucose-6-phosphate dehydrogenase deficiency – x-linked inherited disease – formazan ring test – neonatal screening

Glucose-6-phosphate dehydrogenase (G-6-PD) deficiency is an X-linked inherited anemoly which is responsible for episodic hemolytic anemia induced by infection or certain drug and spontaneous nonspherocytic hemolytic anemia\(^1\). The role of this enzyme is to catalyse the conversion of glucose-6-phosphate to 6-phospho glucoseate, maintaining a high intracellular level of NADPH which is important to defend against oxidative stress by detoxification of peroxides\(^2\). Without any nucleus contained DNA so far, G-6-PD synthesis in the erythrocytes is not possible and shortened life time will result if deficiency exists. In the neonate it may be one of many causes of hyperbilirubinemia.WHO report showed these disease subjects were not less than 100 millions people\(^3\) in the world especially around Mediterranean sea and Asia\(^4\) correlated with some mechanisms of protection against malarial infection\(^5\). It is estimated that in Indonesia the frequency G-6-PD deficiency 3 - 6%, but this figure has not been clarified yet.

The number of screening procedures have been introduced and found effectively to detect...
G-6-PD deficient persons. These include the spot test of Fairbanks and Beutler, the dye decolouration test of Montell and Campbell-Kraut and methylene blue reduction test of Oki and Grewney. All micro screening tests are performed with a few drops of capillary blood. The methemoglobin reduction test of Brewer et al. The acetic acid-cyanate test of Yacob and Jandt and the THNH fluorescence test of Beutler require venous blood which is more difficult. These tests are psychologically and physically more traumatic for the individuals during the screening programs.

Recently, Knudsen and Brewer \(^\text{1}\) introduced a micro-modification of the methemoglobin reduction test, based on the inability of G-6-PD deficient erythrocytes to generate NADPH (TPNH). Through peptone phosphate shunt simulation by glucose and methylene blue, the NADPH generation reduces sodium nitrite-induced methemoglobinemia in normal red cells whereas methemoglobinemia persists in G-6-PD deficient erythrocytes.

The purpose of this study was to determine the potential usefulness of the formazan ring method for the screening test of G-6-PD deficiency in the neonates. This technique will be briefly explained in the material and method mentioned below.

**MATERIALS AND METHODS**

After informed consent was obtained from their parents, 145 blood spots were collected from the newborn babies not less than 3 days of age delivered in Parit Raja Hospital Yogyakarta. These blood spots on filter papers were dried up in the room air and then kept in the 4°C refrigerator. This sample was then round cutted 4 mm in diameter and applied on the formazan gel before incubated for 5 hours on 37°C. The blue ring would appear around the paper.

The formazan gel composition for 20 ml was as follows:

- G-6-PD, Na2 (Nacalai Co) 25mg
- 8 NaDP (Oriental yeast Co) 5mg
- Agar (Doshindo Co) 150mg
- MTT (Nacalai Co) 5mg
- MS (Wako Chem) 5mg
- 0.1 Tris-HCl-0.001M MgCl2 (pH 6.5)

As shown in FIGURE 1, two blood spot as control were used. The first was part of an episodic hemolytic anemia case induced by infection (Parotitic Epidemic), the diameter of blue were 7±7 mm and had been proved to have 77 µl G-6-PD/10^\(^{10}\) erythrocytes. The second was normal in diameter (6±8 mm) containing 115 µl G-6-PD/10^\(^{10}\) erythrocytes, the measurement of the G-6-PD level was done using laboratory (Proda). According to this laboratory, the normal range is 110-136 µl/10^\(^{10}\) erythrocytes.

**RESULTS**

One hundred forty five blood spots from newborn infants were collected consisting of 71 males and 74 females. The blue color diameter of the males were (8.2 ± 0.7) mm and of the females were (8.6 ± 0.7) mm.

As seen in Table I, there were 9 (12.6%) of the males that had low G-6-PD levels possibly of G-6-PD levels below 77 µl/10^\(^{10}\) erythrocytes, this include 2 cases of G-6-PD deficient. Among the females neither low nor deficient G-6-PD was detected in this screening test.

![FIGURE 1. - The result of formazan ring test of dried blood spot after 5 hours incubation at 37°C. Small diameter (7mm) contains G-6-PD activity: 77 µl/10^\(^{10}\) erythrocytes and normal diameter (6mm) similar 115 µl/10^\(^{10}\) erythrocytes. Normal range activity were 110-136 µl/10^\(^{10}\) erythrocytes.](image-url)
small blue ring will result. It is also possible that the ingredients do not dissolve homogeneously in the gel, therefore the diameter of the blue ring is not perfectly accurate.

Eventhough, this technique is suitable for screening test in the childhood.

The incidence of G-6-PD deficiency in Indonesia as reported by Injo Luang Eng (1964) was 1.1% with the highest in West Iriau (8%) and Kalimantan (6-30%), while Notopuwo and Donosepuro found out 2 cases of lowered G-6-PD activity among 105 cases studied.

This report showed that the incidence of lowered G-6-PD levels was as high as 6%. This finding is similar to the number estimated previously. Deficiency occurred in 2 (1.4%) cases, close to those reported by Injo Luang Eng.

Since the G-6-PD deficiency is X-linked, low or deficient case may exist depending on a double or single X-chromosome affected in females. This report fails to show this phenomenon. In males only single X chromosome is available, therefore males should be homozygote. Manifestation of deficiency will exist especially if the deficient indiviual is exposed to infection or drugs. This is the case we use as small-size sample control. Among neonates the risk of male (12%) is twice compare to those of all over (6%), eventhough we cannot find any correlation with jaunice occurrence during the first week of life.

G-6-PD molecule consists of 514 amino acids accounted for 400 variants which are differ in severity, clinical expression and biochemical properties. Variation of the enzyme activity is still unexploated yet, for instance among class I type such as A-type which is common in Africa. The activity of the enzyme is ranging from at 10 to 60%. At the molecular levels 58 different mutations have been identified accounting for 97 variants. The mutations are almost exclusively missense mutation causing single amino acid substitution. They are spread throughout the coding region of the gene, appeared to be a cluster of mutation that causes a more severe clinical phenotype towards the 3'end of the gene.

**SUMMARY**

The formazan Ring method is simple, less traumatic and reliable method for the screening of
G-6-PD deficiency in the neonates. The frequency of lower G-6-PD activity is 6.2% and deficiency is 1.4%. The risk of male neonate is twice (12.4%) than all over the case (6.2%). This figures close to those estimated previously (3.6%) and or reported by Ino Luang Eng in Surabaya (1.1%). Further study are needed to detect the enzyme abnormality of activity at the molecular levels.

REFERENCES