UNIT 3 GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENCY (G6PD)

Contents

Learning Objectives

After reading this unit, you will be able to:

- Understand the importance of G6PD in health and disease; and
- To know various methods used for the diagnosis of G6PD deficiency.

3.0 INTRODUCTION

Glucose- 6- phosphate dehydrogenase (EC 1. 1. 1. 49) (G6PD) is an enzyme belonging to the class of oxidoreductases. G6PD gene is located on the long arm (q) of X chromosome. The length of G6PD gene is 18.5 Kilobase pairs and contains 13 exons. This enzyme is expressed in the cytoplasm of all cells of human body. G6PD, in pentose phosphate (phosphogluconate/hexose monophosphate shunt) pathway (PPP), catalyzes the oxidation of glucose-6-phosphate into 6 phosphogluconate and reduction of nicotinamide adenine

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dinucleotide phosphate (NADP) to NADPH (Figure 3.1). PPP is the only mechanism available in RBC for the production of NADPH. Using NADPH as a reducing co-factor, Glutathione reductase enzyme converts the oxidized glutathione (GSSG) to its reduced form (GSH). GSH by scavenging free radicals (unpaired electrons) such as superoxide (O2-) and Hydroxyl radical (OH) and acting as electron donor, protects the RBC from oxidative stress. Impairment of this function of GSH may cause vulnerability of RBC to oxidative stress resulting in hemolysis and subsequent anaemia, finally landing the affected individual into severe to fatal condition.

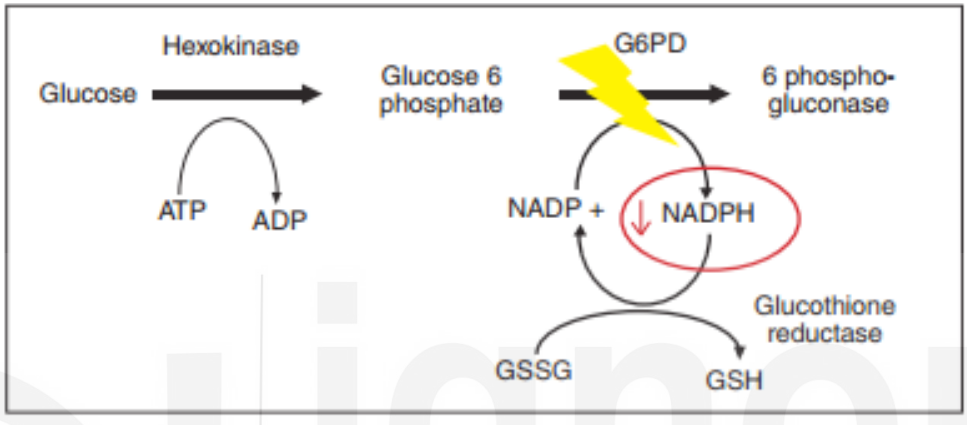

Fig. 3.1: Pentose Phosphate Pathway (Source: WHO RDT Guide, 2018)

G6PD deficiency is due to the mutations in the G6PD gene mostly resulting in single amino acid substitutions. Mutations in G6PD gene reduce the stability and catalytic efficiency of the enzyme. G6PD deficiency came to the attention of scientific community in 1950s when the administration of antimalarial drug such as primaquine caused haemolytic anaemia. In India, G6PD deficiency was reported among Parsi community of Mumbai in 1963 by Baxi and co-workers. Till now 217 mutations have been reported in G6PD gene in protein coding regions, introns and 5' and 3' untranslated regions and are affecting 400 million people around the globe. Higher frequency of G6PD deficiency was observed in the populations of Africa, Mediterranean, Asia and Middle East. The most common mutations in G6PD worldwide are of G6PD Mediterranean and G6PD African types. The prevalence of G6PD deficiency ranged from 0-27% in caste, tribal and ethnic groups of India. In India the most common variants associated with G6PD deficiency are G6PD Mediterranean, G6PD Kerala-Kalyan and G6PD Odisha. The other less frequent variants found are G6PD Nilgiri, G6PD Namoru, G6PD Chattam, G6PDInsuli, G6PD Coimbra, G6PD Rohini, G6PD Jammu and G6PD Jamnagar.

World Health Organization in 1967 categorized variants related to G6PD deficiency into five classes based on their activity in RBC and their related clinical characteristics. They are: class 1: severe G6PD deficiency along with chronic non-spherocytic (non sphere shaped RBC) haemolytic anaemia; Class 2: <10% of normal G6PD activity; Class 3: 10 - 60% of normal G6PD activity; Class 4: 60-100% of normal G6PD activity; and Class 5: twice the level of normal G6PD activity. Due to the higher frequency of G6PD deficiency in regions endemic to malaria, hypothesis of malaria protection in individuals

with G6PD deficiency was flagged but recent meta-analysis by Mabanefo and co-workers (2017) showed that protection among G6PD enzyme deficient individuals against uncomplicated malaria is restricted to the people of African countries and among the heterozygous females only.

G6PD deficiency is an X chromosome linked inherited disorder. As males inherit one X chromosome, they may be G6PD normal or G6PD deficient. In the latter group of males, <10% of normal G6PD activity is seen. Females have two X chromosomes, they may be heterozygotes, homozygous deficient or homozygous normal. Less than 10% of normal G6PD activity in homozygous deficient females and 30%-80% of normal G6PD activity in heterozygous females is observed. In heterozygous females, half of cells express a deficient variant while other half of cells exhibits wild type enzyme due to random inactivation of X chromosome. As a result of which the diagnosis of G6PD is challenging in heterozygous females and G6PD deficient cells are susceptible to oxidizing agents (drugs, bacteria and virus) (Figure 3.2). Most of the G6PD deficiency carriers have no symptoms till they are exposed to haemolysis inducing agents (typhoid fever, pneumonia, cytomegalovirus, hepatitis virus A and B, consumption of fava beans and intake of antimalarial, antibacterial and analgesic drugs) which cause haemolysis. Drug related induction of haemolysis is seen in the carriers of African type mutation in G6PD. Among the carriers of class 1 genetic variants of G6PD gene require long-term blood transfusion due to the chronic non-spherocytic (non sphere shaped RBC) haemolytic anaemia. Neonates carrying the dual mutations in the G6PD and uridine diphosphate glucoronosyl transferase I genes, develop jaundice.

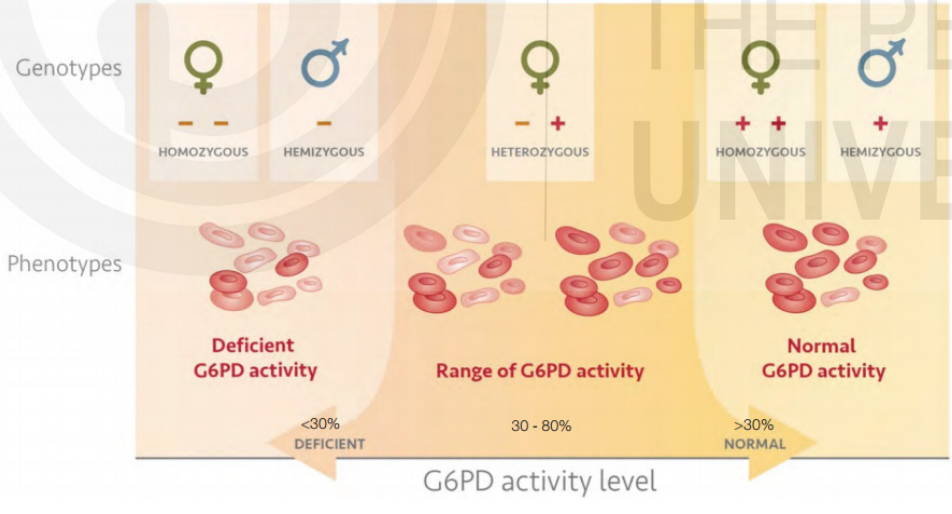

Fig. 3.2: G6PD Genotypes and Phenotypes
(Sources: WHO RDT Guide, 2018)

3.1 METHODS FOR DIAGNOSIS OF G6PD DEFICIENCY

Due to the risk of haemolysis in G6PD deficient individuals, the screening for G6PD deficiency is recommended to prevent morbidity and avoidable mortality. Various methods are used for the diagnosis of G6PD deficiency

such as fluorescent spot test, dye decolourisation, cytochemical assay, rapid diagnosis test (RDT), quantitative assay, G6PD quantitative one substrate assay and molecular tests (PCR-restriction fragment length polymorphism, sanger sequencing and next generation sequencing). Each method has its own merits and demerits. In this unit, Rapid Diagnosis Test (RDT) recommended by WHO (WHO, 2018), Fluorescent Spot Test (FST), dye decolourisation and G6PD quantitative one substrate assay (Roper et al.2020) have been described for the diagnosis of G6PD deficiency.

3.1.1 Rapid Diagnosis Test (RDT)

This is a lateral flow chromatographic test for qualitative detection of G6PD deficiency on capillary blood drawn by pricking the finger. The method described below is based on the guide on rapid diagnosing test of World Health Organization published in 2018.

3.1.1.1 Principle

In this method, blood followed by buffer is added to the nitroblue tetrazolium impregnated nitrocellulose strip housed in plastic cassette and incubated for 10 minutes. G6PD present in the blood convert the nitroblue tetrazolium into formazon which gives purple colour. Absence of colour in the plastic casette is determined as G6PD deficient sample.

3.1.1.2 Materials Required for Drawing of Capillary Blood by Pricking of Finger

A pair of sterile gloves, Alcohol (70%-100%), Sterile cotton wool, Lancet/ sterile needle, A sharp disposable container, A non-sharp disposable bin, Pipette/ cup/10µl micropipette.

3.1.1.3 Procedure Involved in Drawing of Capillary Blood

1) Wash your hands and wear a new pair of gloves for each volunteer/patient.

2) Choose fourth finger from the thumb. If a person is right handed then select left hand or vice versa to avoid inconvenience.

3) Wipe the pulp (ball) and sides of the fingertip with alcohol swab and allow it to air dry. Open the lancet or sterile needle.

4) Stab the centre of the fingertip with lancet or sterile needle and by squeezing along the finger towards the prick, gently push downwards the tip of the finger.

5) Dispose the used lancet/sterile needle into a sharp disposal container.

6) Wipe the first drop of the blood with a sterile cotton wool and discard the used cotton wool in non-sharp discarding bin.

7) Collect the second blood drop until the 2 µl mark on the pipette or rim of the cup when a good size drop of blood is on the finger.

3.1.1.4 Materials Required for Rapid Diagnostic Test

A new pair of sterile gloves, Test cassette, G6PD assay buffer (provided by the manufacturer), Watch, Pencil, A sharp disposable container and a non-sharp disposal bin.

3.1.1.5 Procedure

1) Open the test package and discard the desiccant sachet.
2) Write the name of the volunteer/patient on test cassette with pencil.
3) Dispense the blood in to the square hole (s) of the test casette holding vertically the pipette or cup.
4) Discard the pipette or cup into the sharp container.
5) Add 2 drops of buffer into the A well of test cassette.
6) Incubate the contents in a test cassette for 10 minutes.
7) Discard the used gloves into the non-sharp disposable bin.
8) Read the results in the test cassette. Presence of purple colour in the test cassette shows normal G6PD enzyme activity and no colour change or faint colour demonstrates G6PD deficiency and no migration of blood confirms invalid result.

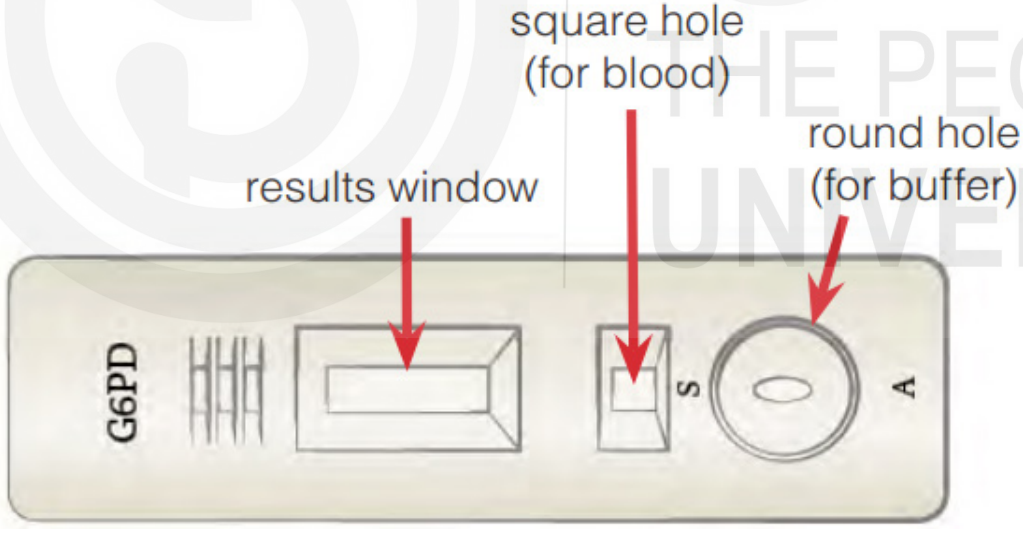

Fig. 3.3: Test Cassette (Source: WHO RDT Guide, 2018)

Fig. 3.4: G6PD normal

3.5A

3.5B

Fig. 3.5A and 3.5B: G6PD Deficient

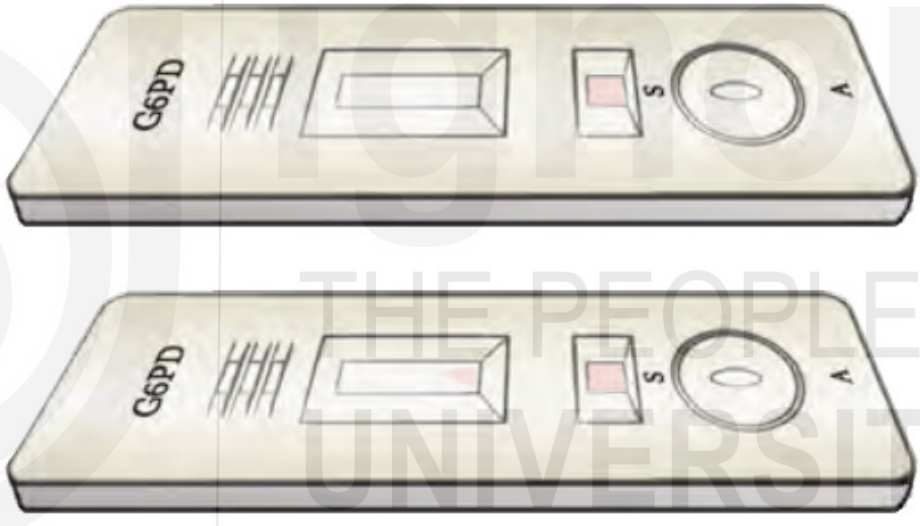

Fig. 3.6: Invalid Results

3.1.2 Fluorescent Spot Test (FST)

It is a widely used qualitative test for detection of G6PD deficiency.

3.1.2.1 Principle

Patient's/volunteer's blood is incubated with reaction mixture of glucose-6-phosphate, non-fluorescent NADP and detergent to lyse the RBC, for standard time, then spotted on the filter paper, dried and examined under ultraviolet light of 350-420 nm. The rate of appearance of bright fluorescence due to NADPH is directly proportional to the activity of G6PD.

3.1.2.2 Material Required

1) UV light source (350-420nm).

2) Anticoagulated (Ethylene diamine tetra acetic acid (EDTA), sodium or potassium salt, heparin or Citrate Dextrose stored at 25ºC for 5 days or 21

days at 4ºC or dried blood spots of filter paper. Dilute the blood samples (if they contain high concentration of haemoglobin if cord blood used) with 0.9% saline to bring the sample in the range of normal haemoglobin.

3) 20 mL of reaction mixture (β-NADP (7.5mmol/L)-2mL; Glucose-6-phosphate (10mmol/L)- 4mL; Saponin (10g/L)- 4mL; Tris-HCl buffer (0.75mol/L) pH7.8-6mL; Oxidized Glutathione (GSSG) (8mmol/L)- 2mL; Double distilled water or ultra-pure water-2mL). The reaction mixture of 20mL stored in volume of 0.2mL (200µl) into 100 microcentrifuge tubes of 1mL at -20 ºC for one year. Before starting the experiment, keep the stored microcentrifuge tubes till they reach room temperature.

4) Micropipette (100 µl).

5) Microcentrifuge tubes (1mL).

3.1.2.3 Procedure

1) Add 20 µl of anticoagulated blood to the microcentrifuge tube (1mL) containing 0.2mL (200 µl) of reaction mixture and mix the contents with micropipette.

2) Take a drop of mixed content and spot on the whatman filter paper (No.1) and keep it (incubate) at room temperature for 10 minutes.

3) Examine the dried filter paper under UV light source.

4) Sample containing normal G6PD fluoresce due to NADPH. Delayed or absence of fluoresce in the sample is considered as G6PD deficient.

3.1.3 Dye decolourisation Screen Test

This is also a qualitative test used for the detection of G6PD deficiency.

3.1.3.1 Principle

Patient's/volunteer's hemolysate is incubated with reaction mixture containing glucose-6-phosphate, NADP, Tris-HCl, and brilliant cresyl blue (dye). The rate of decolourisation of dye in reaction mixture due to the NADPH is proportional to the G6PD content of the RBC.

3.1.3.2 Materials Required

1) Anticoagulated blood (EDTA) collected within 24hr.

2) Hemolysates (20 µl of blood + 1mL of distilled or ultrapure water) prepared within 6 hours.

3) Control sample with normal or reduced G6PD activity.

4) Liquid Paraffin (store at room temperature).

5) β-NADP (0.7mmol/L)-1mL (store at -20ºC).

6) Glucose-6-phosphate (30mmol/L)-1mL.

7) Buffer-dye mixture (Tris-HCl (0.7mol/L) pH 8.5, 320mg/L)-4.5 mL (store at – 20ºC).

8) Working mixture (mix NADP, G6P and buffer-dye mixture).
9) Waterbath.
10) Micropipette (1mL).
11) Microcentrifuge tubes (1mL).

Thaw the reagents (NADP, G6P and buffer-dye mixture) and bring them to the room temperature before starting the experiment

3.1.3.3 Procedure

1) To the hemolysates of test and control (350 µl), add 0.65mL (650 µl) of working mixture in microcentrifuge tubes (1mL). Cover the caps of microcentrifuge tubes with a layer of liquid paraffin.
2) Keep the microcentrifuge tubes in water bath at 37ºC and note the decolourisation of dye.
3) Sample containing normal G6PD activity, discolouration of dye occurs within 35-60 minutes and decolourisation of G6PD deficient sample may range from one and half hour to 24 hours.

3.1.4 Quantitative G6PD One Substrate Assay

3.1.4.1 Principle The rate of formation of NADPH is proportional to the G6PDH activity and is measured in spectrophotometer as an increase in absorbance at 320nm (Trinity Biotech, 2013).

3.1.4.2 Materials Required

1) Anticoagulated (EDTA, heparin and citrate dextrose) blood samples even stored at 25ºC for 5 days or 21 days at 4 ºC.
2) Hemolysing agent (EDTA (27mmol/L) pH 7.0 and 0.7mmol/L 2-Mercaptoethanol) prepared by mixing 100mg of disodium EDTA salt and 5µl of 2-mercaptoethanol in 100ml of water and adjusting pH to 7 with either HCl or NaOH).
3) Hemolysates.
4) Tris-HCl (0.1mol/L), EDTA (0.5 mmol/L) buffer pH 8.0.
5) MgCl2 (100mmol/L).
6) NADP (2mmol/L).
7) Glucose-6-phosphate (6mmol/L).
8) Labtop centrifuge.
9) Sodium chloride (0.15mmol/L).
10) Micropipette (1mL).
11) Microcentrifuge tubes (2mL).
12) Microcentrifuge stand.
13) Falcon tubes (5mL).

14) Water bath.

15) UV spectrophotometer.

3.1.4.3 Preparation of Hemolysates

They are prepared by two methods. In the first method, hemolysates are prepared by removing white cells and platelets using mixed cellulose column. In the second method, plasma and buffy coat of the blood present in 5mL falcon tubes are removed by centrifugation at 1000rpm for 5 minutes, the cold sodium chloride (0.15mmol/L) is added to RBC, centrifuged at1000rpm and supernatant is removed. This step is repeated twice. The RBC are resuspended in an equal volume of cold sodium chloride (0.15mmol/L). From this suspension, 02mL of cell suspension is pipetted with micropipette (1mL) and added to the microcentrifuge (2mL) containing 1.8mL hemolysing agent. This cell suspension is kept on ice for 10 minutes and mixed before use. If no lysis is observed in the microcentrifuge tube, place it on ice and alcohol mixture, freeze and thaw by keeping the microcentrifuge tube on a microcentrifuge stand on water bath at room temperature. Measure the concentration of haemoglobin. Place the hemolysate at 4ºC and use within 2 hours.

3.1.4.4 Procedure

1) This assay should be conducted either at 37ºC or 30ºC.
2) One mL reaction mixture is prepared in the cuvette of test and blank as shown in the table 3.1.
3) In the test cuvette, except substrate (G6P) all mixture contents are added and incubated for 10 minutes.
4) After adding the substrate to the test cuvette record the absorbance for 10-15 minutes in UV spectrophotometer at 340nm.
5) Subtract the value of blank from the test reaction.
6) The absorbance initially increases for first few minutes, then become linear and finally decreases as the substrate is consumed in test cuvette.
7) Absorbance data of 10 minutes is used for calculation. Draw the straight line in the graph using recorded points then read the increase in absorbance and divide by 10 to get the increase in one minute.

Table 3.1: Contents of Test and Blank Reaction Mixture

Reagents	Test(µl)	Blank(µl)
Tris-HCl (0.1mol/L), EDTA (0.5 mmol/L) buffer pH 8.0	100	100
MgCl2 (100mmol/L)	100	100
NADP (2mmol/L)	100	100
1:20 hemolysate	20	20
Double distilled water or ultrapure water	580	680
Glucose-6-phosphate	100	-

The activity of G6PD in the hemolysate is calculated from the initial rate of change of NADPH accumulation Enzyme activity in IU/g haemoglobin=

$$\frac{\text{Change in absorbance at 340nm /minute } \mathbf{X} \text{ dilution factor X } 100}{6.22 \qquad \text{Hb}}$$

Hb=haemoglobin

6.22= mmol extinction coefficient of NADPH at 340nm

Values are expressed per 1012 cells or per mL RBC or per g Hb or per g Hb

Abbreviations

mmol=millimol; L=Litre; g=grams, mg=milligram

3.2 REFERENCES

Frank, J.E. (2005). Diagnosis and management of G6PD deficiency. *Am Fam Physician. 2005 Oct 1;72(7):*1277-82.

Glucose -6-phosphate dehydrogenase. Clinical chemistry catalogue. Trinity Biotech, (2013). Available at https://www.trinitybiotech.com/area/g-6-pdh/.

World Health Organization; (2018). How to use a G6PD rapid diagnostic test (for detecting glucose-6-phosphate dehydrogenase deficiency): A guide for training at health facility level. Geneva.

Mbanefo, E.C. Ahmed, A.M. Titouna, A. Elmaraezy, A. Trang NT, Phuoc Long, N. Hoang Anh, N. Diem Nghi, T. The Hung, B. Van Hieu, M. Ky Anh, N. Huy, NT. Hirayama, K. (2017). Association of Glucose-6-Phosphate Dehydrogenase Deficiency and Malaria: A Systematic Review and Meta-Analysis. *Sci Rep. 2017 Apr 6;7:45963.* doi: 10.1038/srep45963.

Mukherjee, M.B. Colah, R.B. Martin, S. Ghosh, K. (2015). Glucose-6-phosphate dehydrogenase (G6PD) deficiency among tribal populations of India - Country scenario. *Indian J Med Res. 2015 May;141(5):*516-20. doi: 10.4103/0971-5916.159499.

Roper, D. Layton, M. Rees, D. Lambert, C. Vulliamy, T. De la Salle, B. D'Souza, C. (2020). British Society for Haematology. Laboratory diagnosis of G6PD deficiency. A British Society for Haematology Guideline. Br J Haematol. 189 (1):24-38.

Standardization of procedures for the study of glucose-6-phosphate dehydrogenase. Report of a WHO Scientific Group. World Health Organ Tech Rep Ser 1967; 366:1-53.

Tripathy, V. Reddy, B.M. (2007). Present status of understanding on the G6PD deficiency and natural selection. *J Postgrad Med. 53* (3):193-202.