Chapter 4

Glucose-6-Phosphate Dehydrogenase Deficiency and Malaria: A Method to Detect Primaquine-Induced Hemolysis *in vitro*

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1. Introduction

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common enzymopathological disease in humans. This disease is described as a widespread, heritable, X-chromosome linked abnormality (Reclos, *et al*., 2000). It is estimated that it affects approximately 400 million people worldwide (Noori-Daloii, *et al*., 2004). This disease is seen most frequently in approximately all of Africa, Asia, and the countries near the Mediterranean Sea (Frank, 2005). G6PD enzyme was demonstrated to play an active role in survival of erythrocytes. It is known that in the pentose phosphate pathway of erythrocytes, glucose-6 phosphate dehydrogenase (G6PD) enzyme provides the production of NADPH and GSH. GSH, produced by pentose phosphate pathway can react with H_2O_2 and reduce it to H_2O. This prevents the generation of oxidative stress within red blood cells; oxidative stress can be induced in erythrocytes whose G6PD enzymes are deficient. In this situation, GSH is not produced and H_2O_2 is not reduced to H_2O, leading to oxidative stress and hemolysis. This is the only mechanism available for the erythrocyte in order to generate reducing equivalence, therefore making it essential for the survival of erythrocytes. In individuals whose G6PD enzyme is deficient, different kinds of hemolysis from mild to severe are seen bound to differences in variants of the disease (Beutler, 1983, Luzzatto, 1989).

In epidemiological studies, it was shown that the prevalence of G6PD deficiency significantly related to malaria. Malaria is known as a parasitic disease that affects 300-500 million people all over the world. It is widespread in tropical and subtropical regions of Asia, Africa and American continents. Five different types of *Plasmodium* species—*P.*

falciparum, P. vivax, P. ovalae, P. malariae and P.knowlesi—lead to this disease by infecting erythrocytes. Malaria can become a life-threatening condition when it is not treated. Each year, malaria leads to deaths of millions of people all around the world and a large percentage of deaths are seen in Sub-Saharan regions of Africa. As it can be easily seen, malaria and G6PD deficiency share the same geographic distribution. It was shown that G6PD enzyme has various genetic variants and polymorphic frequencies. Highly polymorphic frequencies, which are indicators of G6PD deficiency, are seen in endemic regions for malaria such as Asia, Africa, Central and South America, while in non-endemic regions, these rates decrease, suggesting the relationship between G6PD deficiency and malaria (Haworth, *et al.*, 1988, Organization, 2009, Sutherland, *et al.*, 2010). This relationship reveals two important results. One of them is that G6PD deficiency provides great protection from malaria infection, especially for falciparum infections (Motulsky, 1961, Siniscalco & Bernini, 1961, Ganczakowski, *et al.*, 1995). On the other hand, G6PD deficiency has been recently demonstrated to cause serious problems in fighting against malaria. Primaquine, which is the only drug currently, used in the treatment of Plasmodium infections leads to severe hemolysis in G6PD-deficient patients. This drug may even cause death in G6PD-deficient patients. When primaquine is administered to individuals with G6PD deficiency, its metabolites lead to more severe hemolysis by inducing oxyhemoglobin generation, GSH depletion and stimulation of the hexose monophosphate pathway (Beutler, *et al.*, 1955, Bolchoz, *et al.*, 2002, Beutler & Duparc, 2007).

Therefore, investigations on detection of G6PD deficiency have a vital importance for malaria patients before their treatment with primaquine. On the other hand, the methods that are used for diagnosing G6PD deficiency are unreliable. Even worse is that it is very difficult to distinguish heterozygously-deficient patients from healthy individuals (Peters & Noorden, 2009). All these data indicate that there is an urgent need to develop new methods for reliable detection of G6PD deficiency in order to prevent hemolysis in patients treated with primaquine. Current methods cannot determine primaquine sensitivity in patients with G6PD deficiency every time. However, in our previously researches, we developed a new method for the determination primaquine induced hemolysis *in vitro*. This method provides the determination of G6PD deficiency patients that are susceptible to primaquine independently from the variants of G6PD deficiency. In our studies, it was determined that this method demonstrated high sensitivity for detection of primaquine-induced hemolysis before treatment of malaria patients with primaquine. This chapter aims to represent the relationship between G6PD deficiency and malaria, and to demonstrate the method that has high sensitivity for detection of primaquine-induced hemolysis in patients with malaria whose G6PD enzyme is deficient before their treatment with primaquine.

This chapter aims to represent the problems in treatment of malaria patients with G6PD deficiency by using primaquine, different methods for determination of G6PD deficiency and a new method to determine primaquine induced hemolysis before treatment of patients with G6PD deficiency.

2. Genetic basis of G6PD

G6PD deficiency was identified in 1956 by Carson *et al.* (Alving, *et al.*, 1956), and its X-chromosomal inheritance was discerned in the 1950s by Childs *et al.* (Childs, *et al.*, 1958). G6PD was cloned and sequenced by Persico *et al.*(Persico, *et al.*, 1986, Persico, *et al.*, 1986) in 1986 and independently by Takizawa and Yoshida (Takizawa, *et al.*, 1986) G6PD (Misumi, *et al.*, 1982) is in the hexose monophosphate pathway, the only NADPH-generation process in mature erythrocytes, which lack the citric acid cycle. Deficiency of this enzyme in erythrocytes causes various forms of illnesses such as favism, anemia, chronic nonspherocytic hemolytic anemia, drug-sensitive hemolytic anemia, primaquine sensitivity and jaundice in newborns (Beutler, *et al.*, 1968).

By virtue of fact that G6PD is found in all cells, functional and structural studies have revealed properties of this housekeeping gene (Luzzatto, 2006). G6PD expression level is regulated by hormonal and nutritional factors in only a few tissues. G6PD expression is regulated in liver and adipose tissue, and its activity depends on the rate of fatty acid biosynthesis (Greene, 1993). The G6PD gene region is one of the first regions of the human genome to be completely sequenced (Chen, *et al.*, 1996). The gene encoding G6PD is located near the telomeric region of the distal arm of the X chromosome (Pai, *et al.*, 1980, Szabo, *et al.*, 1984, Patterson, *et al.*, 1987) (band Xq28) and a valuable X-linked genetic marker for determination of X chromosome inactivations (Migeon, 1983). G6PD has various polymorphism sites at the G6PD locus like the colorblindness, Xg blood group and the hemophilia A locus and has close linkage at the X chromosome (Boyer & Graham, 1965, Adam, *et al.*, 1967). G6PD is one of a group of genes including fragile X, (Oberle, *et al.*, 1987) color vision (Motulsky, 1988, Filosa, *et al.*, 1993) hemophilia A (Boyer & Graham, 1965) clasped-thumb mental retardation syndrome (MASA), (Macias, *et al.*, 1992) and dyskeratosis congenita (Arngrimsson, *et al.*, 1993) existing on the distal long arm of the X chromosome.

The X-linkage of the G6PD gene has important implications. This linkage is very stable and linkage with other group locuses is similar in all mammals (Luzzatto & Battistuzzi, 1985, Group, 1989, Luzzatto, 1989, Beutler, 1990). In mice, X-linkage of G6PD was shown by Epstein (Epstein, 1969). Epstein concluded that the G6PD gene is X-linked in the mouse; its synthesis occurs in the oocyte and is dosage-dependent. G6PD is a sex-linked and very polymorphic gene in populations in which males have only one allele (hemizygous) and females have two G6PD alleles. Thus, females can be either normal or deficient (homozygous), or intermediate (heterozygous) phenotypes, whereas males can be either normal or G6PD-deficient phenotype (Luzzatto, 2006). The frequency of the deficient phenotype is higher in males than females owing to males being hemizygous, in which one allele of the gene expresses the deficient phenotype; to arise in females, G6PD-deficiency needs two deficient alleles. However, hemizygous deficient males and homozygous express the same degree of enzyme deficiency level. Since deactivation of one X-chromosome in embryological development in heterozygous females have two populations of red cells (G6PD-normal and G6PD-deficient), with a wide range of total G6PD enzyme activity depending on the relative proportions. If one of the alleles contains deficiency, as a result of

random deactivation of X-chromosomes, about half of the cells will be normal and the other half will be deficient, although there is a wide range of variation around that average (Nance, 1964, Rinaldi, *et al.*, 1976). For this reason, total G6PD activity in heterozygous females can show variability between near-normal to near-deficient (Luzzatto & Battistuzzi, 1985, Segel, 2000). Deactivation of X-chromosome actualizes at random. Correspondingly, binomial distribution would be expected in deficiency level; the extent of this distribution depends on X-inactivation time in embryonic tissue and the number of cells in the embryo. Furthermore, random deactivation of one X-chromosome engenders genetic mosaics in heterozygous females (Luzzatto, 2006). As a result, G6PD mutations show the typical mendelian X-linked inheritance (Adam, 1961), severe G6PD deficiency is much more common in males than in females, and X-chromosome inactivation in heterozygous females for two different G6PD alleles indicate somatic cell mosaicism (Beutler, *et al.*, 1962, Gall, *et al.*, 1965).

The total length of the gene is about 18.5 kb on the X chromosome (xq28) and contains 13 exons. Exon 13 is about 800 nucleotides long and contains the translation stop codon (Nagel & Roth, 1989, Greene, 1993). The protein-coding region is divided into 12 segments, ranging in size from 12 to 236 bp (Martini, *et al.*, 1986). Exon and intron numbers and the exon sizes and sequences are conserved in higher eukaryotes (Nagel & Roth, 1989, Greene, 1993). The first exon contains no coding sequence and intron 2 between exons 2 and 3 is extraordinarily long, extending for 9,857 bp. The function of this long intron is unknown; it may be important for transcription or processing because compressed versions of the G6PD gene still have this largest intron in some species (Chen, *et al.*, 1991, Mason, *et al.*, 1995).

The sequence of the whole G6PD gene is known (Chen, *et al.*, 1991). G6PD sequence analogy between humans and mice or rats is 87%. The analogy between the mouse and rat cDNA sequences is greater than humans with 93% similarity. Most of the sequence dissimilarity is in the 3′- UTR region, which has 600 nucleotides on average and contains a single polyA site (Nagel & Roth, 1989, Greene, 1993). G6PD gene promoter is embedded in a CpG island that starts about 680 nucleotides upstream of the transcription initiation site, extending about 1,050 nucleotides downstream of the initiation site, and ends at the start of the first intron (Chen, *et al.*, 1991). CpG island is conserved between some species (Martini, *et al.*, 1986, Toniolo, *et al.*, 1991), and has highly enriched guanine and cytosine residues, like characteristically in other housekeeping genes and this island appears to be preserved between humans and mice (Toniolo, *et al.*, 1991).

The promoter of the G6PD gene contains a TATA-like, TTAAAT sequence, and a great number of stimulatory protein 1 (Sp1) elements (Philippe, *et al.*, 1994, Rank, *et al.*, 1994, Franze, *et al.*, 1998, Hodge, *et al.*, 1998). These Sp1-binding sites are essential for promoter activity (Philippe, *et al.*, 1994). Deletion analysis has uncovered that the "essential" segment of the promoter is only about 150 bp (Ursini, *et al.*, 1990).

The transcribed region from the initiation site to the poly(A) addition site covers 15,860 bp. (Chen, *et al.*, 1991). The major 5′-end of mature G6PD mRNA in several cell lines is located

177 bp upstream of the translation-initiating codon (Martini, *et al.*, 1986). The G6PD activity and mRNA quantity differ between tissues (Nagel & Roth, 1989, Greene, 1993). S1 nuclease and primer extension analyses of mouse G6PD mRNA indicate that when the transcriptional start site regulated with lipogenesis in liver and adipose tissue, in kidney G6PD is expressed constitutively (Ho, *et al.*, 1988); this quantity potentially depends on oxidative stress, tissue specific differences and reductive biosynthesis reactions (Nagel & Roth, 1989, Greene, 1993). Some different mRNA forms of G6PD mRNA have been found, but their functions are completely unknown. The alternatively spliced form has been documented (Hirono & Beutler, 1988, Hirono & Beutler, 1989, Cappellini, *et al.*, 1993), but this mRNA frame contains 138 nucleotides (Mason, *et al.*, 1988, Persico, *et al.*, 1989, Bautista, *et al.*, 1992, Tang, *et al.*, 1994). Some researchers (Kanno, *et al.*, 1989) suggested that in reality, G6PD translation product made from two separate mRNAs as a result of study to be based on an artifact (Henikoff & Smith, 1989, Beutler, *et al.*, 1990, Mason, *et al.*, 1990, Yoshida & Kan, 1990).

Up to 450 G6PD variants have been identified depending on the enzyme kinetics, physicochemical characteristics, and other parameters (Luzzatto & Battistuzzi, 1985, Chen, *et al.*, 1991). Nearly 300 variants of these have been confirmed by the World Health Organization (1967). Point mutations and small deletions trigger defects in the enzyme structure. These structural defects cause altered activity, instability of the enzyme or decreased affinity of G6PD for its substrates (Luzzatto, 2006).

3. Structure of G6PD and enzymatic properties

G6PD is a typical cytoplasmic, housekeeping enzyme and has been found in all cells from liver to kidney and organisms, from prokaryotes to yeasts, to protozoa, to plants and animals (Luzzatto & Battistuzzi, 1985, Antonenkov, 1989, Glader, 1999, Notaro, *et al.*, 2000). Inactive form of G6PD is a monomer with 515 amino acids and has a molecular weight of over 59 kDa (Rattazzi, 1968). The primary structure of the G6PD enzyme in humans has been determined from the sequence of full-length cDNA clones (Persico, *et al.*, 1986). Furthermore, the tertiary structure of the enzyme has been determined (Au, *et al.*, 1999). Dimer structure of the two subunits in the enzyme are symmetrically located across a complex interface of β-sheets (Au, *et al.*, 1999).

Activation of the enzyme requires $NADP^+$ tightly binding to dimer or tetramer formation of enzyme. G6PD catalyses the first step of the oxidative pentose phosphate pathway and controls reaction velocity (Wrigley, *et al.*, 1972). In this first step, while G6PD catalyses the conversion of glucose 6-phosphate (G6P) to 6-phosphogluconolactone, at the same time it reduces NADP to NADPH (Au, *et al.*, 1999, Turner, 2000). Human G6PD has no activity with nicotinamide adenine dinucleotide (NAD) as coenzyme. Also, G6P is very specific for its substrate compared to other hexose phosphates (e.g., galactose 6-phosphate or mannose 6-phosphate) (Luzzatto & Battistuzzi, 1985, Glader, 1999). The G6P binding site is nearby lysine 205 in tertiary structure of the enzyme, and this amino acid has a critical role in electron transfer (Bautista, *et al.*, 1995). The NADP binding site is located nearby 38 to 43 amino acids; this region constitutes the N-terminus in tertiary structure encoded in exon 3. This site is important for stability of G6PD (Au,

et al., 1999). As an inhibitory effect, one of the products of G6PD reaction NADPH is an effective inhibitor (Luzzatto, 1967). Increase in NADP and decrease in NADPH as a result of whichever oxidative event in cells effect prepotently to increase G6PD activity (Luzzatto & Testa, 1978). Consequently, G6PD is the most important enzyme in biosynthesis reactions owing to enzyme property as NADPH reducer in its critical role in the cytoplasm (Koehler & Van Noorden, 2003).

4. The effect of G6PD on erythrocyte metabolism

4.1. Erythrocytes

Erythrocytes, which contain hemoglobin, are blood cells that perform the transfer of oxygen and carbon dioxide between tissues. G6PD is an important enzyme that performs vital functions within all cells of the body (Greene, 1993). The quantity of active G6PD decreases during the life of an erythrocyte and also the older erythrocytes become vulnerable to oxidative stress. G6PD, an enzyme in the oxidative pentose phosphate pathway, converts the nicotinamide adenine dinucleotide phosphate ($NADP^+$) into its reduced form NADPH. It is necessary for the protection against oxidative stress in erythrocytes. The cells cannot eliminate this stress, which causes hemolysis of erythrocytes. Because H_2O_2 and other reactive oxygen species cannot be reduced, oxidation of hemoglobin to methemoglobin and membrane damage occur (Ruwende & Hill, 1998, Peters & Van Noorden, 2009).

4.2. The importance of pentose phosphate pathway for erythrocytes

G6PD is the key enzyme in the oxidative pentose phosphate pathway. The first step of the pentose phosphate pathway is catalyzed by G6PD. In this step, $NADP^+$ is reduced to NADPH, and ribulose-5-phosphate, a precursor of DNA, RNA, and ATP, emerge from G6P (Turner, 2000). The most important reducing agent in the cytoplasm of cells is NADPH (Koehler & Van Noorden, 2003). The second enzymatic step in this pathway is NADPH production as a consequence of reactions that reduce oxidized glutathione (GSSG) to reduced glutathione (GSH). The only defense against oxidant stress in the red blood cell (RBC) is GSH production (Friedman, 1979, Group, 1989, Peters & Van Noorden, 2009). In unstressed, normal erythrocytes, the G6PD activity is only about 2% of total capacity (Group, 1989, Peters & Van Noorden, 2009). The pentose phosphate pathway's main function is the generation of reducing capacity through the production of NADPH and ultimately, GSH. This is essential for cell survival and is available in the erythrocyte for generating reducing capacity (Greene, 1993).

4.3. Classification of Glucose-6-Phosphate Dehydrogenase variants

More than 400 variants of G6PD have been distinguished based on their biochemical characteristics, enzyme kinetics, physicochemical characteristics, and other parameters (Luzzatto & Battistuzzi, 1985, Chen, *et al.*, 1991, Greene, 1993). G6PD B^+ is the most commonly found enzyme type and it is used as a standard for normal enzyme activity and electrophoretic mobility. For identification of other variants, G6PD B^+ is used. The rate at

which $NADP^+$ is reduced by glucose-6-phosphate with G6PD B^+ as the catalyst is the standard for activity. Based on this, enzyme activity relative to G6PD B^+ variants are classified as fast, normal, and slow in terms of electrophoretic mobility and as Classes I—V (Luzzatto, 1989, Beutler, 1990, Greene, 1993, Segel, 2000, Betke K, Brewer GJ, Kirkman HN, Luzzatto L,Motulsky AC, Ramot B, and Siniscalco M 1967). There are 5 classes for these variants. Class I includes chronic nonspherocytic hemolytic anemia with a severe enzyme deficiency (e.g., G6PD Minnesota, G6PD Tokyo, G6PD Campinas). Class II variants have severe enzyme deficiency without chronic nonspherocytic hemolytic anemia (e.g., G6PD mediterrian, G6PD Canton, G6PD Union, G6PD Kaiping). Class III variants includes medium or mild enzyme deficiency, with the activity at 10-60% of G6PD B^+ (e.g., G6PD A^-). Class IV variants have a weak or no enzyme deficiency. The activity is 60-100 % of G6PD B^+ (e.g., G6PD A^+). Class V variants have increased enzyme activity (e.g., G6PD Hektoen) (Beutler, 1994, Segel, 2000).

4.3.1. Some Important G6PD Variants

4.3.1.1 G6PD A^+ is the most widely seen variant worldwide and also the first variant in which the nucleotide mutation and amino acid substitution were determined (Beutler, 1990). This Class IV variant has 90% of the enzyme activity of G6PD B^+ (Luzzatto, 1989). This variant also called for the African variant cause widely seen in Africa; 20-40% of African men and 20% of African American men have this variant. It is faster than G6PD B^+ electrophoretically and it does not cause hemolysis (Beutler, 1989). G6PD A^+ derives from a single amino acid substitution of aspartic acid for asparagine at amino acid number 126, and this was the result of an adenine to guanine mutation at nucleotide number 376.

4.3.1.2 G6PD A^- is a Class II variant that has 10 to 20% of the activity of G6PD B^+ and the same electrophoretically mobility as G6PD A^+ (Luzzatto, 1989); 11% of African American men have this variant. Its half-life is 13 days. Three types of mutations have arisen with molecular studies. The most common mutation being at nucleotide number 202 is a result of a guanine to adenine mutation at amino acid number 68 substitution of valine to methionine (Beutler, 1989, Luzzatto, 1989, Beutler, 1990). The second one occurs at nucleotide number 680 as a result of a guanine to thymine mutation at amino acid number 227 substitution of arginine to leucine. And the third mutation occurs at the nucleotide number 968, as a result of a thymine to cytosine mutation at amino acid number 323 substitution of leucine to proline (Beutler, 1989). G6PD A^+ and G6PD A^- variants are defined as unique to Africa, but they can also be seen in Caucasian populations from Italy, Spain, Southeast Asia, Middle East and South America (Beutler, 1990).

4.3.1.3 G6PD Mediterranean is a widely seen variant in the Mediterranean region and Middle East. In addition, it is seen in the Indian subcontinent and other regions of the Americas (Beutler, 1991). This Class II variant has less than 10% of the enzyme activity of G6PD B^+ and the electrophoretical mobility is similar with G6PD B^+ (Luzzatto, 1989). Its half-life is only 8 days and DNA analysis identified two different point mutations. The first mutation is a result of a cytosine to thymine mutation at nucleotide number 563, at amino

acid number 188 substitution of serine to phenylalanine (Vulliamy, *et al.*, 1988). Second is a silent mutation result of a cytosine to thymine mutation at nucleotide number 1311 (Beutler, 1990). There are many similar Class II variants in the Mediterranean region (Cagliari, Sassari, El Fayoum), South Asia (Hong Kong, Canton, Mahidol), and elsewhere. Most of these emerge as a consequence of point mutations resulting in single amino acid mutations that have variable effects on activity and electrophoretic mobility (Luzzatto & Battistuzzi, 1985, Luzzatto, 1989, Beutler, 1990, Beutler, 1991, Beutler, 1992).

5. Clinical tables on G6PD deficiency

Depending on G6PD enzyme deficiency are: Hemolytic Anemia (Drug-induced hemolysis), Diabetes mellitus-induced hemolysis and Infection-induced hemolysis; chronic nonspherocytic anemia, Favism and Neonatal jaundice.

5.1. Hemolytic anemia

5.1.1 Mechanism of hemolysis. In some people, for example, the Mediterranean-type, G6PD deficiency from drug intake occurs, although not a permanent hemolytic condition. In erythrocytes, NADPH cannot form with G6PD deficiency and unformed NADPH creates a deficiency in conversion of the oxidized form of glutathione (GSSG), to its reduced form (GSH) (Lachant, *et al.*, 1984, Beutler, 1994). There is normally plenty of GSH in erythrocytes and it protects the cell from oxidizing agents. If G6PD is deficient, hemoglobin is oxidized by oxidative substances to be eliminated and it returns methemoglobin that cannot function normally. Also, hemoglobin precipitates with denaturation in the cytoplasm forms Heinz bodies. These structures attach to the membrane with disulfide bonds and disrupt its normal structure. The erythrocytes that contain Heinz bodies in their cytoplasm are sequestered by macrophages in the spleen and removed from the circulation. G6PD deficiency hemolysis occurs like that in the extravascular compartment and also occurs again as a result of membrane defects (Alving, *et al.*, 1956). Thus, drug-induced hemolysis is the first and best-known morbid effect of G6PD deficiency. After a 1- or 2-day delay in such drug administration, a fall in the hemoglobin (Hb) concentration occurs.

The red blood cell (RBC) membrane was adhered to by Heinz bodies, which are particles of denatured protein. These appear in the early stages of drug administration and disappear as hemolysis progresses. Hemolysis usually occurs in blood vessels and hemoglobinuria follows. The increase of reticulocytes emerges in response to this situation and the hemoglobin level begins to increase again within 8-10 days (Beutler, 1994). In severe hemolysis, the patient may complain of back and stomach pain and the urine turns dark. The hemolytic anemia is self-limited when G6PD deficiency is relatively mild because only the older RBCs are destroyed and the young RBCs have normal or nearly-normal enzyme activity (Beutler, 1994).

Table 1 lists the drugs and chemicals that cause clinically significant hemolytic anemia.

5.2. Diabetes mellitus-induced hemolysis. Hemolysis in G6PD deficiency individuals might initiate diabetic ketoacidosis. This situation is not exactly accepted. However, hemolysis formation has been reported when blood glucose levels are normal in diabetic individuals (Beutler, 1994). It has also has been reported that hypoglycemia might precipitate hemolysis in patients with G6PD deficiency (Beutler, 1994).

5.3. Infection-induced hemolysis. Infections are probably the most common cause of hemolysis in people with G6PD deficiency. There are numerous reports about the importance of infection in causing hemolytic anemia. A large number of bacterial, viral and rickettsial infections have been reported as predisposing factors. Infectious hepatitis (hepatitis A), pneumonia and typhoid fever are known to trigger hemolysis. Involving the upper respiratory tract and gastrointestinal system, viral infections have been reported to cause a more severe hemolysis (Luzzatto, 2001). The mechanism of infection-induced hemolysis is not clear, but it is thought to be that during the infection, superoxide anion and H_2O_2 production by macrophages causes the hemolysis (Glader, 1999, Luzzatto, 2001).

5.4. Chronic nonspherocytic anemia

Class I G6PD variants, such as the absence of precipitating factors in the occurrence of excessive hemolytic anemia, lower still further the remaining enzyme activity. This is observed in people with chronic hemolytic anemia and oxidative stress, even if unstable conditions occur as a result of insufficient enzyme activity in erythrocytes. Granulocyte dysfunction is seen in some cases. In these cases, more severe hemolysis is due to increased susceptibility to infection (Beutler, 1994, Luzzatto, 2001).

5.5. Favism is an illness that occurs in G6PD deficiency individuals with acute hemolysis by eating raw beans (Vicia fabu). Wet, dry or frozen fava bean ingestion of grains, even if the mother eats fava beans can cause hemolysis in newborn infants through breast milk may occur (Luzzatto, 2001). Individuals with G6PD deficiency hemolytic effect caused by the beans contained many glycosides that are toxic due to the visin and konvisin (Beutler, 1994, Akhter, *et al.*, 2011). In addition, β-glucosides in bean seeds, maturity stage of fava beans attain very high amounts causing a severe course of hemolytic crisis (Katz & Schall, 1979, Greene, 1993, Beutler, 1994). Often, in the G6PD Mediterranean variant, acute and a very severe hemolytic crisis are seen due to fava bean ingestion, even capable of causing death (Fairbanks, 1999, Luzzatto, 2001). In favism, damage in erythrocytes is similar to oxidative damage of drugs. Fava beans include visin, konvisin, ascorbic acid and L-Dopa, which have oxidative properties. The most commonly cited konvisin and visin glycosides during digestion fava beans by β-glycosidase or acid hydrolysis demolished to the active agents, which are converted to "divisine" and "izouramil." Divisine and izouramilin reduce the level of the GSH and NADPH in vitro conditions and damage the cell membrane by the formation of cross-connection with Heinz bodies; it also has been shown to inhibit Ca^{2+}-ATPase and catalase (Arese & De Flora, 1990, Beutler, 1994, Gaetani, *et al.*, 1996, Luzzatto, 2001). 24-48 hours after ingesting foods like fava beans, characteristic symptoms occur in the form of pallor, jaundice and hemoglobinuria (Ninfali, *et al.*, 2000). In addition, jaundice,

headache, backache, nausea, fever, and chills are all signs of acute hemolysis (Tyulina, *et al*., 2000). Favism is most common seen in children between the ages of 2-5, and is also 2-3 times more common in boys than in girls (Luzzatto, 2001). Clinical signs of favism begin earlier and are more severe than drug-induced hemolytic crises. Rarely, as a result of pollen of fava inhalation, hemolysis may occur within hours (Beutler, 1994). While each favism patient must have G6PD deficiency, hemolytic reactions may not occur after ingestion of fava beans in each person with G6PD deficiency. Each individual with G6PD deficiency of the same family could not be affected in the same way when they eat fava bean. On the other hand, changes are observed in the same person at different times. Genetic variations between individuals, differences of fava bean active metabolites may be responsible for these variable characteristics (Meloni, *et al*., 1983, Group, 1989, Luzzatto, 2001).

5.6. Neonatal jaundice

One of the most threatening consequences of G6PD deficiency is neonatal jaundice (Beutler, 1994). Jaundice in babies with G6PD enzyme deficiency could be mild or severe enough to cause kernicterus, a spastic type of cerebral palsy, and may even cause death (Luzzatto, 1993). In addition, infants with G6PD deficiency, hyperbilirubinemia is more remarkable than anemia. It facilitates this because of the inadequate physiological conjugation in liver in the neonatal period (Moskaug, *et al*., 2004). G6PD A^{-}, G6PD mediterrian, G6PD Canton variants are known as types that cause kernicterus and hyperbilirubinemia (Luzzatto, 2001). Clinically, the jaundice, the level of G6PD in the normal physiological jaundice in newborns occur on the same days, or a little earlier, but it takes as long as 2-3 weeks (Tan, 1981, Luzzatto, 2001). There are two major differences between jaundice due to incompatibility of blood groups and jaundice due to G6PD deficiency. First, the presence of jaundice in G6PD deficiency is very rare during childbirth and usually it begins in the second or third day. Second, according to anemia, jaundice is more pronounced and it is encountered with severe anemia very rarely in the absence of the enzyme (Luzzatto, 1993, Luzzatto, 2001).

6. Malaria and glucose-6 phosphate dehydrogenase deficiency

As we mentioned above, there is a strong relationship between malaria and G6PD deficiency diseases. In several epidemiological studies, it was shown that distribution of malaria was nearly the same with distribution of G6PD deficiency (Motulsky, 1961, Siniscalco & Bernini, 1961, Ganczakowski, *et al.*, 1995). This situation reveals two important facts. One of them is that G6PD deficiency provides great protection from malaria, especially for falciparum infections. On the other hand, using antimalarial drugs can cause life-threatening hemolytic anemia in patients with G6PD deficiency. Hence, malaria patients should be screened for their tendency to G6PD deficiency before their treatment with antimalarial drugs. In this part, we will first summarize the importance of malaria for the world. Then, we will explore the relationship between these two diseases in detail.

As it is known, malaria is a parasitic disease that threatens 300-500 million people all over the world. Malaria can be defined as the most deadly vector-borne disease in the world

(Myrvang & Godal, 2000). It is widespread in tropical and subtropical regions of Asia, Africa and the American continents. Each year, malaria leads to deaths of millions of people all around the world and a large percentage of deaths are seen in Sub-Saharan regions of Africa. The causative agents of malaria are the Plasmodium parasites, which are transmitted to humans by the bites of infected mosquitoes. If patients are not treated with antimalarial drugs, malaria can easily lead to death. Five different types of Plasmodium species—*P. falciparum, P. vivax*, *P. ovalae*, *P. malariae* and *P.knowlesi*—lead to this disease (Wernsdorfer & McGregor, 1988, Sutherland, *et al.*, 2010).

Plasmodium falciparum (P. falciparum) is the most serious and life-threatening form of the disease. 80% of death cases are reported from patients that have been infected with *P. falciparum*. It was also demonstrated that resistance has been developed in this type of parasites against current antimalarial drugs. It is generally seen in Africa, specifically in sub-Saharan regions. Interestingly, falciparum-derived malaria cases have been recently reported in various parts of the world where this parasite species was believed to be completely eradicated.

Plasmodium vivax (P. vivax) constitutes a milder form of the disease. Vivax infections generally do not cause death. However, individuals that suffer from vivax infection also need to be treated. Among all *Plasmodium* species, *P. vivax* is the one that shows the broadest geographic distribution worldwide. Causative agents for 60% of malaria infections are reported as *P. vivax* infections in India. This parasite has a liver stage and can remain in the body for years without causing sickness. If the patient is not treated, the liver stage may re-activate and cause relapses—malaria attacks—after months, or even years without symptoms.

Plasmodium ovale (P. ovale) is known as one of the other milder form of the disease. Like *P. vivax*, it generally does not commonly lead to death. Nevertheless, infected individuals require medical therapy. This parasite, similar to *P.vivax*, can live in the liver for long periods without causing symptoms. Therefore, if it is not treated, reactivation of parasites can be observed in the liver and this leads to relapse of the disease

Plasmodium malariae (P. malariae) is also another milder form of the disease. It does not commonly lead to death. However, it still requires treatment. This type of *Plasmodium* parasites are reported to stay in the blood of some individuals for several decades.

Plasmodium knowlesi (P. knowlesi) causes malaria in macaques, but can also infect humans (Mendis, *et al.*, 2001, Singh, *et al.*, 2004, Mueller, *et al.*, 2007).

When life cycles of Plasmodium parasites are investigated, it is seen that the parasites multiply in the liver of the human body, and then infect erythrocytes. As we mentioned before, *Plasmodium* parasites enter the human body when bitten by an infective female mosquito, which is called Anopheles. These mosquitoes become infected with malaria when they take *Plasmodium*-containing blood from an infected person. Approximately one week later, these parasites mix with the mosquito's saliva when the mosquito takes its next blood meal from another person and this individual is injected with *Plasmodium* parasites when they are being bitten (Bozdech, *et al.*, 2003).

Multiplication of the parasites within erythrocytes enhances the severity of the disease and cause symptoms such as anemia, fever, chills, nausea, flu-like illness, and, in severe cases, coma, and death. Treatment of this disease can be achieved by using antimalarial drugs. Primaquine, which is the most common antimalarial drug, can be used as a primary prophylactic because it prevents primary parasitemia of *Plasmodium* species by destroying these parasites in the liver before they reach the bloodstream and cause disease (Yazdani, *et al.*, 2006).

As we pointed out before, according to epidemiological studies, the prevalence of malaria deeply relates to glucose-6 phosphate dehydrogenase (G6PD) enzyme deficiency. In these studies, it was demonstrated that 66 of 77 genetic variants that have reached polymorphic frequencies were seen in populations living in tropical and subtropical areas where malaria was endemic. On the other hand, this genetic diversity does not occur in populations living in non-endemic regions of the world for malaria, indicating that high polymorphism is the indicator of G6PD deficiency.

When investigated in terms of cellular biology, we can see that Plasmodium parasite that causes malaria use erythrocytes as host cells. Erythrocytes are also the most affected cells from G6PD deficiency. This situation also suggests the relationship between the two diseases. In several studies, it was demonstrated that G6PD deficiency provides a protection against malaria infections. In one of the early studies, it was indicated that *P. falciparum* and *P. vivax* parasites preferred to invade younger erythrocytes, which possessed high levels of G6PD enzyme. Since enzyme levels are diminished in older erythrocytes, parasites do not prefer to invade these erythrocytes. These studies suggested the protective effect of G6PD deficiency from parasitemia (Allison & Clyde, 1961, Kruatrachue, *et al.*, 1962). In the recent past, Ruwando et al. also carried out a case-control study on more than 2,000 African children and exhibited that risk of contracting malaria in patients that have the African form of G6PD deficiency decreased at a rate of 46 to 58%. In this study, it was suggested that the selective advantage of resistance to malaria was counterbalanced with selective disadvantageous results of G6PD deficiency, and this stopped the rise of malaria frequencies in endemic regions (Ruwende, *et al.*, 1995). In another study, Ninokata et al. (2006) investigated 345 healthy adults for G6PD deficiency on Phuket Island, which had been determined to be a malaria-endemic region and found out that 10% of these individuals had G6PD deficiency. Interestingly, it was observed that none of the individuals had molecular evidence of malaria infection. According to this study, researchers postulated that G6PD deficiency provided an advantageous genetic trait against malaria (Ninokata, *et al.*, 2006).

The exact mechanism of this protection is still unknown. However there are two postulated explanations. According to the first suggestion, it was found that parasites that cause malaria can only survive in conditions with low oxygen levels (Clark, *et al.*, 1989). This demonstrates that these parasites are very susceptible to oxidative stress. It is known that in the pentose phosphate pathway of erythrocytes, glucose-6 phosphate dehydrogenase (G6PD) enzyme has an important role in production of NADPH and GSH. This is the only

mechanism for erythrocytes to survive. GSH that is produced by NADP+ reduction reacts with H_2O_2 and reduce it to H_2O. This prevents the generation of oxidative stress within red blood cells. Since oxidative stress is the most important factor for the disruption of red blood cells, these cells are protected from this effect. However, in G6PD deficient erythrocytes, G6PD activity is significantly reduced. In G6PD A (-) variant, enzyme activity level reduces to 10 or 20% of normal levels, while enzyme activity completely disappears in G6PD variant. Therefore, oxidative stress can be induced in erythrocytes whose G6PD enzymes are deficient. In this situation, GSH is not produced and H_2O_2 is not reduced to H_2O and leads to oxidative stress. Hence, it is thought that since malaria parasites are susceptible to oxidative stress, they do not live within the erythrocytes where their maturation occurs (Toncheva & Tzoneva, 1985, Greene, 1993). Additionally, during oxidative stress, the loss of potassium from the cell and from the parasite can cause the death of the parasite (Friedman & Trager, 1981).

According to the second suggestion, *Plasmodium* parasites oxidize NADPH and reduce the level of reduced glutathione (GSH) in erythrocytes. In the situation of G6PD deficiency, this effect becomes more severe and induces oxidative-induced damage within erythrocytes. Moreover, *Plasmodium* parasites break down hemoglobin and release toxic components like iron and these substances lead to hemolysis. Hence, the development rates of *Plasmodium* parasites are diminished. Additionally, red blood cells that are affected by oxidative stress and are damaged are eliminated by the immune system via phagocytosis. This elimination decreases the growth of parasites much more since it occurs during an early ring-stage of parasites' maturation. Therefore, all of these data indicate that G6PD deficiency can provide protection against malaria infections. Considering the relationship between G6PD deficiency and plasmodium infections, research has aimed to develop antimalarial drugs that decrease the level of GSH within erythrocytes and then produce hydrogen peroxide and the other free radical species in order to enhance the inhibition of Plasmodium species (Mehta, *et al.*, 2000, Fortin, *et al.*, 2002, Kwiatkowski, 2005, Prchal & Gregg, 2005).

Primaquine is the only effective antimalarial drug that provides inhibition of persistent liver stages of *P. falciparum, P. vivax,* and *P. ovalae* parasites that lead to relapses of malaria (Phompradit, *et al.*, 2011).

However, as we initially mentioned, using primaquine in order to prevent the relapse of malaria can be very dangerous for G6PD deficiency patients since its usage results in very severe hemolysis. In all G6PD variants, activity levels of the enzyme have been diminished and this partially prevents the defense of erythrocytes against oxidative attack. However, when primaquine is administered, its metabolites lead to more severe hemolysis than oxidative damage by inducing oxyhemoglobin generation, GSH depletion and stimulation of the hexose monophosphate pathway. Moreover, primaquine can also induce the generation of Heinz bodies, which are insoluble aggregates that attach to the surfaces of erythrocytes. The most probable mechanism of primaquine-induced hemolysis is the generation of oxyhemoglobin, which forms hydrogen peroxide. Since G6PD enzyme level is low in G6PD-deficient

erythrocytes, these peroxides accumulate and lead to denaturation of hemoglobin. Peroxides also generate Heinz bodies that attach to cell membranes of red blood cells. Hemolysis occurs when damaged erythrocytes pass through the spleen. In each pass, red blood cells lose a portion of the cell membrane. After additional passes, membranes of cells completely lose their competency (Beutler, *et al.*, 1955, Bolchoz, *et al.*, 2002, Beutler & Duparc, 2007).

These conditions reach life-threatening scenarios for all G6PD deficiency patients with different genetic variants. Hence, individuals that are required to use antimalarial drugs should be screened very carefully for their tendency to have G6PD deficiency. For effective control and treatment, either a reliable test for detecting G6PD deficiency or an anti-malarial drug that can be safely given to G6PD deficiency patients is required.

7. Detection methods of G6PD deficiency

Currently, primaquine, which causes hemolysis in G6PD-deficient patients, is the only radical cure of *Plasmodium vivax* infections (Burgoine, *et al.*, 2010). Therefore, screening to detect G6PD deficiency is very important. Various tests can be used for the detection of G6PD deficiency, which are based on the assessment of the NADPH production capacity of G6PD. The most frequently used tests that measure NADPH production are the fluorescent spot test, cytochemical assay and spectrophotometric assay. However, fluorescent spot test and the spectrophotometric assay are not reliable for the detection of heterozygous females. In addition, DNA analysis can be done to detect G6PD deficiency for the homozygous, hemizygous, and heterozygous-deficient patients. However, we have to design primers for all mutations (Peters & Van Noorden, 2009).

7.1. Fluorescent spot test

Fluorescence is a form of luminescence that uses the physical change of emission of light upon excitation of molecules. There are various different types of luminescence, classified depending on the style of excitation: chemo-luminescence (ending in a chemical reaction) photo-luminescence (fluorescence, phosphorescence and delayed fluorescence), bio-luminescence (via a living organism) and others (Bernard, 2002).

Nicotinamide Adenine Dinucleotide Phosphate (NADPH) is the reduced form of NADP, with absorption maximum at 340 nm and a maximum emission at 460 nm. NADPH concentrations have been studied in great detail using optical methods. A parameter for direct measurements of the G6PD activity is the fluorescence of NADPH. When G6PD shows enough functional activity in erythrocytes, two molecules of $NADP^+$ are reduced to NADPH. After the addition of glucose 6-phosphate and $NADP^+$, blood spot fluoresces at *340 nm* if NADPH is produced (Beutler & Baluda, 1966).

7.2. Spectrophotometric assay

Spectrophotometric methods are greatly used in biological sciences for quantitative and qualitative measurements due to the fact that these methods do not break down the

molecules analyzed and enable us to assay small quantities of matter fundamentally (Lehninger, 2000). Spectrophotometric techniques allow detection of the concentration of a solution by evaluating its absorbance of a specific wavelength by way of a spectrophotometer, which produces light at a chosen wavelength and passes it directly through the sample. Because every molecule have a specific absorption spectrum, we can recognize and characterize its properties or detect its current concentration in the presence of other compounds (Lehninger, 2000).

In the case of enzyme activity measurements, the assay solution contains some other compounds that are required for the reaction to occur. Other compounds in the reaction mix may absorb light at the same wavelength with the enzyme being analyzed. To eliminate the interference of other compounds, the absorbance of a sample solution is compared with blank solution, which is taken as the reference. The blank contains everything found in the sample solution except the substance to be assayed.

In the matter of protein (enzymatic activity or protein concentration) measurements, colorimetric methods are used. Colorimetric measurements are performed by way of quantitative assessment of a colored complex, which is mostly formed by the reaction of a colorless compound and a dye reagent. However, the compound that will be analyzed can be naturally colored and can be read directly spectrophotometrically.

Glucose-6-phosphate dehydrogenase catalyzes the first step in the pentose phosphate shunt, oxidizing glucose-6-phosphate (G-6-P) to 6-phosphogluconate (6-PG). The enzyme activity can be determined quantitatively by spectrophotometer assay method, which is based on the rate of NADPH production from NADP+ in G6PD-deficient patients (Kornberg, *et al.*, 1955, Lohr & Waller, 1974).

These reactions are illustrated below:

$$\text{Glucose-6-phosphate} + \text{NADP}^+ \xrightarrow{\text{G6PD}} \text{6- phosphogluconate} + \text{NADPH} + \text{H}^+$$

Nictotinamide adenine dinucleotide phosphate (NADP) is reduced by G6PD in the presence of G-6-P. The rate of formation of NADPH is proportional to the G6PD activity and is measured spectrophotometrically as in increase in absorbance at 340 nm. Production of a second molar equivalent of NADPH by erythrocyte 6-phosphogluconate dehydrogenase (6-PGDH) occurs according to the reaction:

$$\text{6- phosphogluconate} + \text{NADP}^+ \xrightarrow{\text{6PGD}} \text{Ribulose-5-Phosphate} + \text{NADPH} + \text{H}^+ + \text{CO}_2$$

This is prevented by use of maleimide, an inhibitor of 6-PGDH.

The Enzyme Commission of the International Union of Biochemistry recommends expressing this in international units (IU) and defines 1 IU as the amount of an enzyme that catalyzes the transformation of 1 micromole of substrate per minute under standard

conditions of temperature, optimal pH, and optimal substrate concentration. Specific activity relates activity to total mass of protein to avoid bias through individual differences in weight (Bairoch, 1993). Therefore, G6PD activity was expressed as units (micromoles of NADP reduced per minute) per miligram of soluble protein at 37°C.

7.3. Cytochemical staining assay

The Cytochemical staining assay is based on the intracellular reduction of the tetranitro blue tetrazolium (TNBT) by the G6PD via exogenous electron carrier 1-methoxyphenazine methosulfate and TNBT is reduced to dark-colored water-insoluble formazan, which can be determined by light microscopy (Peters & Van Noorden, 2009).

7.4. *In vitro* primaquine-induced hemolysis methods

3 cc of venous blood anti-coagulated by 2% heparin solution (126 mM NaCl, 14 mM Na_2HPO_4, 1 mM KH_2PO_4, 13,2 mM glucose, pH 7.4) was collected from healthy and G6PD-deficient persons. The blood was washed three times with sterile heparin solution at 3000 rpm for 10 min. Erythrocytes were resuspended in PBS after that hematocrit was adjusted to 2%. This is the one of the most important steps for detection of *in vitro* primaquine-ınduced hemolysis. Primaquine solution was prepared in 0.1 M Tris buffer (pH 7.4). Primaquine concentration was used between 1 and 4 mM in experiments. Different concentrations of primaquine were added into tubes containing 2% erythrocytes that were prepared before. Tubes were then placed and rotated in a rotator tube for 2 hours at 37°C. The rotation speed was less than 2 rpm. This is another important step for detection of *in vitro* primaquine-induced hemolysis. After 2 hours, the supernatant was collected for the heme concentration, which was then determined spectrophotometrically. Hemoglobin released from erythrocyte induction of primaquine-induced hemolysis and compared with complete lysis (100% hemolysis, control group) obtained by adding 5 mM Tris-HCl (Fig. 1) (Allakhverdiev & Grinberg, 1981).

The principle of this method is based on conversion of Hemoglobin (Hb) to cyanmethemoglobin by the addition of KCN and ferricyanide, whose absorbance is measured spectrophotometrically as cyanmethemoglobin at 540 nm versus a standard solution. Supernatant of hemolyzed red blood was diluted four-fold (v/v) with distilled water. On the other hand, the control group was diluted twenty-fold (v/v) with distilled water. After that, 50 μL KCN (10% w/v) and 50 μL potassium ferricyanide (2% w/v) were directly mixed and the color was measured at 540 nm. The standard curve was constructed using the standard cyanmethemoglobin solutions in different concentrations (Bhaskaram, *et al.*, 2003).

This method demonstrated that the *in vitro* model of primaquine-induced hemolysis can be only maintained by using 2% hematocrit in physiological conditions. Primaquine leads to hemolysis at concentrations between 1 and 4 mM. Other factors that induce primaquine-derived hemolysis are exposure time, incubation temperature, drug

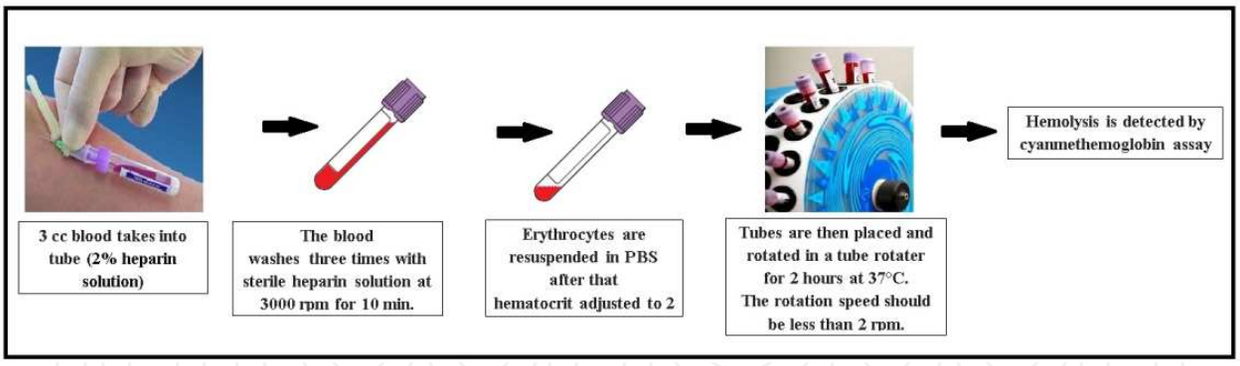

Figure 1. *In vitro* Primaquine-Induced hemolysis

concentration and amount of oxygen. Despite the fact that there are several methods in order to diagnose G6PD deficiency, these methods do not determine primaquine sensitivity in patients with G6PD deficiency every time. Therefore, lack of primaquine-based treatment by considering only G6PD deficiency can be very dangerous in terms of health of patients with malaria and the epidemiology of the disease. On the other hand, treatment of primaquine-sensitivive individuals with primaquine can cause death. Hence, in the Centers for Disease Control and Prevention (CDC) report (Hill, *et al.*, 2006), it was highlighted that there was an urgent need to develop new *in vitro* methods for determining hemolysis that indicate primaquine sensitivity before treatment of patients with this drug. By considering primaquine-induced hemolysis in patients with G6PD deficiency, it can be determined whether these patients may be treated with primaquine or not. The advantage of this method is that it can determine primaquine-induced hemolysis before treatment with primaquine and its capacity to determine G6PD deficiency.

8. Conclusion

This chapter has aimed to represent the relationship between G6PD deficiency and malaria and to suggest a sensitive method for detection of primaquine-induced hemolysis in patients with G6PD deficiency. As mentioned above, G6PD deficiency is the most common enzymopathologic disorder in humans and it affects 400 million people worldwide. In patients with G6PD deficiency, oxidative stress cannot be prevented since G6PD enzyme is the initial catalyst of the pentose phosphate pathway in erythrocytes that reduces the peroxides to H_2O. This situation leads to mild to severe hemolysis, changing depending on genetic variants of the disease. As we mentioned before, according to epidemiological studies, the prevalence of G6PD deficiency deeply relates to malaria. In these studies, it was demonstrated that 66 of 77 genetic variants, which have reached to polymorphic frequencies, were seen in populations living in tropical and subtropical places where malaria was endemic. On the other hand, this genetic diversity does not occur in populations living in non-endemic regions of the world for malaria, indicating that high polymorphism is the indicator of G6PD deficiency and distribution of malaria is nearly the same with distribution of G6PD deficiency. This situation reveals two important results.

One of them is that G6PD deficiency provides partial protection from malaria infections, especially for falciparum infections. In several studies, it was demonstrated that risk of contracting malaria in patients that have G6PD deficiency decreased at a rate of 46 to 58%. On the other hand, using antimalarial drugs can cause life-threatening hemolytic anemia in patients with G6PD deficiency. Since G6PD deficiency does not provide exact protection, these patients still have a risk of contracting malaria. However, using primaquine, which is the only radical cure of Plasmodium infections, can induce more severe hemolysis by generating oxyhemoglobin, GSH depletion and Heinz bodies and enhancing oxidative attack. This threatens the lives of patients with G6PD deficiency. Hence, patients with malaria should be screened for their tendency to G6PD deficiency before their treatment with antimalarial drugs. Common methods that are used for diagnosing G6PD deficiency are unreliable. Even worse is that it is very difficult to distinguish heterozygously-deficient patients from healthy individuals. Additionally, current methods cannot accurately indicate hemolysis, even though they give information about activity of the enzyme. Also, these methods do not determine primaquine sensitivity in patients with G6PD deficiency every time. However, the method that we developed provides the determination of primaquine sensitivity in patients with G6PD deficiency *in vitro* independently from the variants of G6PD deficiency. The principle of the method is based on the quantitative detection of hemolysis by incubation of erythrocytes obtained from G6PD-deficient patients with primaquine in low hematocrit while rotating the culture in a hybridization oven for 2 hours at 37°C. By considering primaquine-induced hemolysis in patients with G6PD deficiency, it can be determined whether these patients may be treated with primaquine or not. The advantages of this method are that it can determine the primaquine-sensitivity in patients with G6PD deficiency before treatment with primaquine. Using this method not only on G6PD deficiency patients but also on patients that suffer from other diseases that may cause primaquine-induced hemolysis constitutes another advantage of the method.

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