Lipid peroxidation and antioxidant capacity of G6PD-deficient patients with A-(202G>A) mutation

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ABSTRACT. Glucose-6-phosphate dehydrogenase (G6PD) deficiency is an enzymopathy in which reduced NADPH concentrations are not maintained, resulting in oxidative damage. We evaluated G6PD activity, oxidative stress levels and Trolox equivalent antioxidant capacity in individuals with the A-(202G>A) mutation for G6PD deficiency. Five hundred and forty-four peripheral blood samples were screened for G6PD deficiency; we also analyzed lipid peroxidation products measured as thiobarbituric acid reactive species and Trolox equivalent antioxidant capacity. Men with the A-(202G>A) mutation
had lower G6PD activity than women with the same mutation. Individuals with the A-(202G>A) mutation also differed in mean Trolox equivalent antioxidant capacity values but not for thiobarbituric acid reactive species values. We concluded that A-(202G>A) mutation is associated with reduced G6PD activity and increased Trolox equivalent antioxidant capacity.

**Key words:** G6PD deficiency; TBARS; TEAC; Oxidative stress

**INTRODUCTION**

Glucose-6-phosphate dehydrogenase (G6PD), a vital enzyme in all cells, catalyzes the first reaction of the pentose phosphate pathway allowing the conversion of glucose-6-phosphate to 6-phosphogluconolactone. In this reaction, nicotinamide adenine dinucleotide phosphate (NADP) is reduced to NADPH, which is also used in the protective process against physiologically high levels of oxidative damage (Bilmen et al., 2001). In red blood cells, this is the only source of NADPH, making them more vulnerable than other cells to destruction by oxidative stress (Mason et al., 2007). NADPH is a key hydrogen donor for the reduction of oxidized glutathione (GSSG) to a tripeptide known as reduced glutathione (GSH). This tripeptide is used as a reducing agent by glutathione peroxidase, which is involved in the detoxification of hydrogen peroxide. In this process, GSH is converted to GSSG leading to a decrease in GSH. The regeneration of GSH occurs by the action of glutathione reductase, which catalyzes the reduction of GSSG to GSH in the presence of NADPH (Njålsson and Norgren, 2005). Since there are no other sources of NADPH in red blood cells, G6PD is essential in protecting hemoglobin sulfhydryl groups and preventing red blood cell membrane oxidation (Pandolfi et al., 1995).

G6PD deficiency is the most common red blood cell enzyme disorder in humans, affecting more than 400 million people worldwide (Beutler, 1994; Mason et al., 2007). It is characterized by a hemolytic crisis after ingestion of oxidizing agents such as antimalarial drugs, sulfanilamide, acetanilide and some sulfones (Beutler, 2008). This change is caused by mutations in the gene coding for G6PD, located in the telomeric region of the X-chromosome long arm, Xq28 (Martini et al., 1986). Most of the mutations show a marked reduction in enzyme stability due to changes in protein folding. The regions affected are usually sites of contact between the protein subunits or regions that interact with the NADP molecule (Mason et al., 2007). Phenotypic expression of G6PD deficiency affects men more than women according to the pattern of sex-linked recessive inheritance. The illness is completely expressed in men, while in heterozygous women G6PD enzyme activity may be normal, moderately low or very deficient, depending on the cell population distribution (Cappellini and Fiorelli, 2008).

In G6PD deficiency, normal NADPH concentrations cannot be maintained and GSH levels decrease, resulting in red blood cell oxidative damage due to endogenous and exogenous agents and leading to a hemolytic crisis (Frank, 2005; Mason et al., 2007). Thus, in view of the possibility of oxidative stress and hemolysis in individuals with G6PD deficiency, this study aimed to determine the levels of lipid peroxidation and Trolox equivalent antioxidant capacity in red blood cells from individuals without hemolytic crisis and a specific G6PD mutation.
MATERIAL AND METHODS

Population

A total of 544 peripheral blood samples were drawn and analyzed, after consent, from individuals of the northwestern region of São Paulo State. Three hundred and forty-six (63.60%) were males and 198 (36.40%) were females, with age ranging from 16 to 85 years. From this sample 382 (70.22%) were Caucasians, 74 (13.60%) African descendants and 88 (16.18%) from different ethnic origins. The total group was composed of 426 healthy blood donors from the Blood Bank of São José do Rio Preto, São Paulo State, Brazil (HEMOCENTRO), and 118 individuals from an educational institute.

G6PD deficiency tests

Positive samples for G6PD deficiency were screened using the Brewer qualitative method, based on methemoglobin (Fe$^{3+}$) formation by the action of nitrite on hemoglobin (Fe$^{2+}$). Methylene blue stimulates the phosphate pentose pathway resulting in the production of NADPH, which reconverts the oxidized hemoglobin to the reduced form. The rate of reduction is proportional to erythrocyte G6PD activity (Brewer et al., 1960).

Confirmation of G6PD deficiency was carried out by assaying for G6PD activity using the method recommended by Beutler (1984). The diagnosis is made by adding a measured amount of hemolysate to an assay mixture containing substrate (glucose-6-phosphate) and cofactor (NADP). The test measures the reduction of NADP to NADPH, which is proportional to the increase in absorbance at 340 nm. Enzyme activity is expressed in IU·gHb$^{-1}$·min$^{-1}$ at 37°C and pH 8.0, where one enzyme unit (IU) is defined as the amount of enzyme that reduces 1 μmol NADP$^+$/min at 37°C (Beutler, 1984).

DNA from G6PD-deficient blood samples was isolated by the phenol/chloroform method (Pena et al., 1991). The A-192G>A G6PD gene mutation was confirmed by polymerase chain reaction and restriction fragment length polymorphism (PCR/RFLP) analysis (Saiki et al., 1988; Hamel et al., 2002). The forward primer used was 5’ CAG CCA CTT CTA ACC AC 3’ and the reverse primer was 5’ AAC CAG GCT GGG GGA G 3’. PCR amplification was performed using a preheating step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 50 s, with final extension at 72°C for 5 min. The PCR fragment produced was 314 bp in length. Samples were digested with the NlaIII enzyme (New England BioLabs) in order to detect the normal allele fragment (314 bp) and the mutant allele fragments (218 and 96 bp).

Oxidative stress and antioxidant status determination

Oxidative stress was evaluated by determining the level of plasma thiobarbituric acid reactive species (TBARS), which are end products of lipid peroxidation. In red blood cells, lipid peroxidation causes disorganization of the lipid moiety of cell membranes resulting in lethal damage to the cell. TBARS quantification is based on the reaction of malondialdehyde and other aldehydes with thiobarbituric acid at low pH and high temperature to form a complex where absorbance can be read at 535 nm. Normal values were considered to be up to 440 ng/mL (Mihara and Uchiyama, 1978; Percario et al., 1994).
The antioxidant capacity of the samples was determined according to their equivalence to the antioxidant activity of Trolox (6-hydroxy-2,5,7,8-tetramethylychroman-2-carboxylic acid), a vitamin E synthetic analogue used as an antioxidant standard. Plasma Trolox equivalent antioxidant capacity (TEAC) was determined using a technique based on the colorimetric reaction between the ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt and potassium persulfate (K$_2$S$_2$O$_8$), which produces the green/blue colored ABTS$^•+$ radical. In the reaction, the addition of the sample containing antioxidant molecules reduces the ABTS$^•+$ radical thus causing a discoloration that can be determined at 734 nm (Miller et al., 1993; Re et al., 1999).

**Statistical analysis**

Data were tested for normal distribution by graphic inspection of the residues and for homogeneity of variances using the Levene test. Thus, two-way ANOVA followed by multiple comparison using the Tukey test was employed to assess the effects of gender (male and female) and group (control and G6PD-deficient) on enzyme activity, TBARS and TEAC values (Zar, 1999). Statistical analysis was carried out using the Statistica 7 software (Statsoft Inc.), and the level of significance was set at P < 0.05.

**RESULTS**

The screening test for G6PD deficiency suggested a possible alteration in enzyme activity in 57 samples (10.48%). Among these, 27 (4.96%) displayed G6PD deficiency confirmed by assaying enzyme activity, and none of them showed hemolytic crisis. With the PCR/RFLP test for identifying the A-(202G>A) mutation, 22 samples were positive, which represented 81.14% of the G6PD deficiency group, and 4.04% of the total group studied, comprising nine (40.91%) African descendants, six (27.27%) Caucasians and seven (31.82%) of different ethnic origins. The test group used in assessing enzyme activity was composed of samples with positive results in all three G6PD deficiency tests. The control group was formed by 45 samples, randomly allocated, with normal G6PD activity.

The results of G6PD enzyme activity separated by gender are shown in Table 1. For the control group, G6PD activity ranged from 8.03 to 14.80 IU·gHb$^{-1}$·min$^{-1}$ at 37°C and was set as a reference for the deficiency group. The group with G6PD deficiency showed lower values, with a significant difference in relation to the control group ($F_{(1,63)} = 319.15, P < 0.01$). Moreover, an effect of gender was found on the G6PD activity values ($F_{(1,63)} = 11.26, P < 0.01$) for the G6PD-deficient group. Men with G6PD deficiency showed lower enzyme activity than did women ($P < 0.01$, Tukey test), while there was no difference between men and women in the control group ($P = 0.86$, Tukey test).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>G6PD-deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Males (N = 29)</td>
<td>Females (N = 16)</td>
</tr>
<tr>
<td>G6PD</td>
<td>9.87 ± 1.35</td>
<td>10.24 ± 1.79</td>
</tr>
<tr>
<td>(IU·gHb$^{-1}$·min$^{-1}$ at 37°C)</td>
<td>(8.03-13.34)</td>
<td>(8.06-14.80)</td>
</tr>
</tbody>
</table>

Test significance | NS | $P < 0.01$

Table 1. Erythrocyte glucose-6-phosphate dehydrogenase (G6PD) activity of control and G6PD-deficient groups, separated by gender.

Data are reported as means ± SD and minimum-maximum values in parentheses. NS = not significant.
The normal values for the TEAC method were established under local analysis conditions in 81 samples with normal TBARS values, randomly selected. The established mean level for TEAC was $2.12 \pm 0.10 \text{mM}$, ranging from 1.85 to 2.31 mM. The values corresponded to the average obtained from the group more or less three times the standard deviation. This interval corresponds theoretically to 99.73% of all data with Gaussian distribution.

As the G6PD activity value was lower in men compared to women in the G6PD deficiency group, we also evaluated the effects of gender on TBARS and TEAC assays. However, no gender differences were noted in the TBARS ($F_{(1,94)} = 0.14, P = 0.70$) and TEAC ($F_{(1,94)} = 0.01, P = 0.93$) results.

Comparison between the control and the G6PD deficiency groups, regardless of gender, indicated that there was no significant difference for TBARS ($F_{(1,94)} = 0.01, P = 0.96$). In the G6PD deficiency group, only one sample showed TBARS value above the normal range. However, all values of TEAC were within the normal range, as previously mentioned, and a higher average value in the G6PD deficiency group was found in relation to the control group, showing a significant difference ($F_{(1,94)} = 14.02, P < 0.01$). The means, standard deviation, and maximum and minimum values for TBARS and TEAC, in both control and G6PD deficiency groups, are presented in Table 2.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (N = 81)</th>
<th>G6PD-deficient (N = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBARS (ng/mL)</td>
<td>260.86 ± 92.40 (74-428)</td>
<td>259.94 ± 117.07 (97-480)</td>
</tr>
<tr>
<td>Test significance</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>TEAC (mM)</td>
<td>2.12 ± 0.10 (1.85-2.31)</td>
<td>2.22 ± 0.06 (2.12-2.31)</td>
</tr>
<tr>
<td>Test significance</td>
<td>$P &lt; 0.01$</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Thiobarbituric acid reactive species (TBARS) and Trolox equivalent antioxidant capacity (TEAC) values in serum of control and glucose-6-phosphate dehydrogenase (G6PD)-deficient groups.

Data are reported as means ± SD and minimum-maximum values in parentheses. NS = not significant.

**DISCUSSION**

In the present study, we found a moderate frequency of G6PD deficiency (4.96%). Previous reports have shown a prevalence between 1 and 10% in Brazil. The frequency of the A-(202G>A) mutation confirms previous studies showing that it is the most common variant in the country, mainly in the Southeast region and reinforces the important contribution of people from Africa, Southern Italy, Spain, Portugal, and Arabian Peninsula, where this mutation is frequent (Saad et al., 1997; Compri et al., 2000; Hamel et al., 2002; Castro et al., 2006).

In this study, there was no difference in G6PD activity between genders in the control group. However, for the G6PD deficiency group, hemizygous men had a lower G6PD average than women. Since the heterozygous women are genetic mosaics resulting from random X-chromosome inactivation, the phenotype may be asymptomatic or moderate or show severe acute hemolytic anemia. Thus, hemizygous men have deficient G6PD activity and women have an enzyme activity that may vary from normal to severely deficient levels (Beutler, 2008; Brandt et al., 2008; Cappellini and Fiorelli, 2008).
As G6PD activity was lower in men than in women with G6PD deficiency, a greater oxidative damage and/or a higher antioxidant capacity was expected. However, males and females did not differ in lipid peroxidation pattern, a marker of cellular oxidative damage, as observed in the comparison between genders in the control group. Only TEAC was higher in the G6PD deficiency group, which may be a response to oxidative stress.

Previous in vitro studies demonstrated the importance of NADPH in maintaining antioxidant enzymes, such as catalase (Scott et al., 1993). Gaetani et al. (1996) reported a decrease in catalase levels and reduced glutathione in red blood cells with G6PD deficiency during hemolytic crises. Therefore, it was expected that erythrocyte with G6PD deficiency would show a greater susceptibility to damage caused by reactive oxygen species, since there would be less maintenance of NADPH-dependent enzymes, such as catalase and glutathione reductase in G6PD-deficient individuals without hemolytic crisis. However, as observed in other studies, differences were not found in TBARS values between men and women with G6PD deficiency, as well as in the control group (Gerli et al., 1982; Bilmen et al., 2001). Despite this finding, it is possible that other oxidative stress markers may provide additional information that was not detected in this study, since there is controversy about the choice of convenient marker (Del Rio et al., 2005).

According to the literature, there are not many studies that have examined oxidative stress in G6PD deficiency due to a specific mutation. Our results show that people with G6PD deficiency due to the A-(202G>A) mutation, may differ in G6PD activity and TEAC values but not in relation to lipid peroxidation, when compared to individuals without G6PD deficiency.

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REFERENCES

G6PD deficiency and antioxidant system


