Original Article

Plasma Ascorbic Acid Levels and G-6-PD Activities in Symptomatic, Asymptomatic Malaria and Malaria Negative Subjects

C.E.J. Udiong[1], E. C. Azodoh[2], P. C. Inyang-Etoh[3] and A. E. Usanga[3]

[1]*Department of Chemical Pathology, College of Medical Sciences, University of Calabar, Calabar, Cross River State.*
[2]*Department of Chemical Pathology, School of Medical Sciences, University of Calabar Teaching Hospital, Calabar, Cross River State.*
[3]*Department of Medical Laboratory Science, Faculty of Allied Medical Sciences, College of Medical Sciences,University of Calabar, Calabar, Nigeria.*

Malaria parasite endemicity and development have been associated with ascorbic acid availability and the activity of G-6-PD. There is no record of studies that relate the two parameters in malaria subjects. Plasma ascorbic acid levels and glucose-6-phosphate dehydrogenase (G-6-PD) activity in symptomatic malaria, asymptomatic malaria and malaria negative subjects in Calabar Municipality were measured to evaluate the relationship. The level of ascorbic acid in malaria subjects (2.22±1.82m/dl) was significantly lower (P<0.05) than the level in control subjects (3.47±0.97mg/dl). However, the level of ascorbic acid in symptomatic malaria (2.22 ± 1.82 mg/dl) was significantly lower than the level in control subjects (P<0.05). The G-6-PD activities in malaria positive subjects, (asymptomatic, 12.75 ± 6.2U/gHb and symptomatic malaria, 18.5 ± 6.1U/gHb) were significantly higher than the activity in control subjects (8.34 ± 6.6U/gHb, P<0.05). The G-6-PD/ascorbic acid ratios in non-malaria (2.40), asymptomatic (3.73), and symptomatic (8.33) malaria subjects differed significantly (P<0.05). A positive correlations was observed between ascorbic acid level and G-6-PD activity in asymptomatic malaria subjects (r =0.444, p<0.05), while a negative correlation was observed in non-malaria subjects (r = 0.239, P<0.05). These results show an inverse relationship between ascorbic acid level and G-6-PD activity in malaria subjects. The G-6-PD/Ascorbic acid ratios differentiate between non-malaria, asymptomatic and symptomatic malaria. Both the levels of ascorbic and the activity of G-6-PD vary significantly with malaria status and with level of malaria parasitaemia. From these finding we advice that G-6-PD activity results should be interpreted in relation to malaria status in malaria endemic zones.

Key words: Malaria parasite, Ascorbic acid, G-6-PD, Symptomatic, Asymptomatic.

Introduction

Malaria remains endemic in 102 countries, placing over half of the world population at risk with about 100 million malaria infections and perhaps a million death yearly (WHO 1989). Low levels of ascorbic acid in malaria has been documented (Carter et al 2011, Akpotuzor et al 2012, Uzuegbu, 2011, Onyeseli, 1990, Azeuike, 1991, Oselisi, 1992, O'Brien *et al*., 1994 and Shahabuddin *et al*., 1994). Ascorbic acid (Vitamin C) is a carbohydrate whose structure is reminiscent of glucose. Ascorbic acid is a reducing agent capable of reducing compounds such as molecular oxygen, nitrate and cytochrome a and c and maintains metallic cofactors such as $Cu^{2+,}$ and Fe^{2+} in reduced state required for enzyme activity (Mayes, 1994, Blanchard, 1991). Glucose-6-phosphate

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Correspondence: Christopher Udiong, Department of Chemical Pathology, College of Medical Sciences, University of Calabar, Calabar, Cross River Sta
Email: chrisudiong@yahoo.com

dehydrogenase (G-6-PD) is an enzyme responsible for the initial dehydrogenation of glucose into the pentose phosphate pathway (PPP) to form 6-phosphogluconate, a reaction that provides $NADPH_2$ in erythrocytes for the conversion of oxidized gluthathione to the reduced form, a form essential for the maintenance of haemoglobin in the reduced state (Baron, 1994).

In man, it is involved in collagen synthesis, degradation of tyrosine, synthesis of adrenaline from tyrosine, bile formation, steroidogenesis, iron absorption. It serves as water-soluble antioxidant (Mayes, 1994, McCromic and Green; 1999). It plays an important role as free ionized radical scavenger. The effects of ascorbic acid deficiency include increased fragility of vascular wall, poor wound healing, depleted bone matrix; suppressed fracture healing, scurvy and anaemia due to depressed erthropoiesis (Mayne, 1998). Deficiency of G-6-PD is common in African patients with malaria (Carter et al 2011, Gelpi 1967, Peters and Noorden, 2000). Malaria parasite density has also been directly associated with G-6-PD activity (Shahabuddin, 1994). This study determined the levels of Vitamin-C and G-6-PD in symptomatic, asymptomatic malaria subjects and non-malaria subjects and evaluated the relationship between vitamin-C levels and G-6-PD activities in those subjects.

Materials and Methods

Subjects and Consent

Symptomatic malaria blood samples were collected from 100 patients that presented with malaria symptoms and tested positive for malaria parasites in the University of Calabar Teaching Hospital. Two hundred apparently healthy, age matched volunteers were included in the study after obtaining a verbal consent from them. These comprised of 100 non-malaria subjects that were positive for malaria parasite on testing (asymptomatic), and 100 non-malaria subjects (control) that tested negative for malaria parasites. The subjects were not receiving vitamin-C supplement, and were not on malaria treatment prior to blood sample collection. All samples were screened for G-6-PD deficiency using the brilliant cresyl blue test (Motulsky et al, 1959). Hemizygous and heterozygous subjects were excluded from the study (Ruwende et al, 1995).

Malaria Parasite Detection

Thick and thin blood films were made from each blood sample on clean glass slides. Both films were place on a flat surface to air dry. There after the thin films were fixed in absolute methanol for 5 seconds and allowed to air dry. Both the thick and the thin films were then stained in freshly prepared 2% Giemsa's stain for 30 minutes (Payne, 1988). At the end of the staining, the films were removed from the stain, rinsed in buffered water (pH 7.2) and stood vertically to dry. Both the stained thick and thin films were examined microscopically using x 100 objective lens with oil immersion. The thick blood films were used for the detection of malaria parasite while the thin films were used for speciation of plasmodium.

Haemoglobin Estimation

Haemoglobin was estimated using cyanmethaemoglobin technique. 20 µl each of well-mixed blood sample, or haemoglobin standard was transferred into 4ml Drabkin solution, mixed, and allowed to stand at room temperature for 4 minutes. The absorbance was read against Drabkin solution (blank) at 540nm comparing it with the absorbance of haemoglobin standard.

Ascorbic Acid Assay

Plasma was separated from the cells and 20mg of oxalic acid was added per ml of plasma as preservative. The samples were then stored frozen at about 0^0 C. Ascorbic acid was assayed using the method of Roe (1961). The assays were performed within 5 days of sample collection using the Roe's (1961) methodology. The absorbances thereof were read at 520nm in a Spectrophotometer (Spectronic – 21 UVD).

G-6-PD Assay

The methaemoglobin reduction method (Motulsky et al, 1959) was employed to screen all blood specimens for G-6-PD deficiency, while G-6-PD assay was performed on red blood cell haemolysates by measuring the rate of decrease in absorbance at 340nm. Commercial reagent kits manufactured by Biolabo Inc. of France were used for the determination of G-6-PD. The contents of Vial R1 and Vial R2 were reconstituted with 30ml and 3ml of distilled water respectively. These were allowed to stand for 10 minutes before their contents were mixed.

Haemolysates for G-6-PD determination were prepared by washing 0.1ml of whole blood with 1ml of 0.9% sodium chloride solution 3 times. The washed cells were suspended in 0.9ml of reagent in Vial R3 and stood at 4^0C before it was centrifuged. Twenty-five micro-litre (25µl) supernatant from there was added to 1.5ml of the reagent from Vial R1, mixed and incubated at room temperature for 5 minutes. Fifty micro-litre (50µl) of reagent from Vial R2 was added and mixed and the absorbance read after 30 seconds, then after 1, 2, and 3 minutes.

G-6-PD activity in U/gHb was calculated as shown below.

$$\text{G-6-PD activity in U/gHb} = \frac{\Delta\text{Absorbance/minute x 500 at room temperature}}{\text{Hb in g/dl}}$$

Milton Roe Spectronic 21 UVD spectrophotometer (USA) was used for the reading of absorbance at the specified wavelengths. Hetich bench centrifuge (Germany) was used for centrifugation.

Quality Control

We could not acquire a commercial control serum with ascorbic. Never the less, we performed recovery experiment 10 times on a pooled serum sample with an initial ascorbic acid level of 3.0mg. To an aliquot of this was then added 3.0mg/dl ascorbic acid for the recovery. Our average recovery for the ascorbic acid was 96.4%. Our within and between batch coefficient of variations were 3 ± 1.2% and 5 ± 2.1% respectively.

Data Analysis

Comparison of paired data from the three groups of subjects was done using T-test, while correlations between groups were analyzed using Pearson correlation formula. SPSS and Microsoft excel programmes were used for T-test and correlation coefficient calculations respectively. A two-tailed p-valve of <0.05 was considered indicative of a statistically significance difference.

Results

The entire malaria positive subjects included in this analysis had Plasmodium falciparum parasiteemia. Table 1 shows that among non-malaria subjects 60% were carriers of malaria parasites. More male (64%) were asymptomatic malaria carriers compared to females (36%). Table 2 shows that ascorbic acid level in symptomatic malaria (2.22 ± 1.82mg/dl) was significantly lower (P<0.05) than the level in malaria-negative subjects (3.47 ± 1.97mg/dl). The G-6-PD activities in symptomatic and asymptomatic malaria subjects (18.5 ± 6.1U/gHb, 12.75 ± 6.2U/gHb respectively) were significantly higher (P<0.05) than the activity in malaria negative (8.34 ± 6.64U/gHb) subjects.

Fig. 1 shows that there was a significant positive correlation between ascorbic acid level and G-6-PD activity in asymptomatic malaria subjects (r=.444, p<.001), while a similar analysis (Fig. 2) between ascorbic acid level and G-6-PD activity in non-malaria subjects showed a significant negative correlation (r=0.269, P=<0.05). Table 3 shows significant differences (P<0.05) between the ratios of the mean G-6-PD (UgHb/ascorbic acid ascorbic acid (mg/dl) in symptomatic versus asymptomatic malaria ($p=3.35 \times 10^{-18}$), asymptomatic malaria versus malaria negative subjects ($p=2.57 \times 10^{-8}$) and symptomatic malaria versus malaria negative subjects ($P=6.58 \times 10^{-5}$).

Table 1 Distribution of malaria infection among Malaria and Non-malaria subjects

Subject	All	Symptomatic	Asymptomatic Malaria	Malaria negative
Total	300(100%)	100(100%)	100(100%)	100(100%)
Male	160(66%)	50(50%)	64(64%)	46(56%)
Female	140(44%)	50(50%)	36(36%)	54(64%)

Table 2 Acorbic acid level and G-6-PDactivity in symptomatic, asymptomatic malaria and malaria negative subjects

Malaria status of subjects	Assorbic acid mg/dl (M+SD)	P	Inference	G-6-PD U/gHb (M+SD)	P	Inference
Sympt.(n=100) v Asym. (n=100)	2.22±1.82 v 3.42±1.89 (12.23±10.03 v 18.79±10.41)	<0.z	S	18.5±6.1 vs 12.75±6.2	P<0.05	S
Sympt.(n=100) v non-mal. (n=100)	2.22±1.82 v 3.47±1.97 (12.23±10.03 v 19.12±8.85)	<0.05	S	18.5±6.1 vs 8.34±6.64	P<0.05	S
Asysmpt.(n=100) v non-mal. (n=100)	3.42±1.89 v 3.47±1.97 (18.79±10.41 v 19.12±8.85)	>0.05	NS	12.75±6.2 vs 8.34±6.64	P<0.05	S

Table 3 G-6-PD / Ascorbic acid ratios in symptomatic, asymptomatic malaria and malaria negative subjects

G-6-PD U/gHb/ Ascorbic acid mg/100ml			
	M + SD	p-value	Inference
Symptomatic vs asymptomatic	8.33±3.35 vs 3.73±3.28	3.35×10^{-18}	S
Asymptomatyic vs Malaria negative	7.43±4.88 vs 2.40±3.37	2.57×10^{-8}	S
Symptomatic vs Malaria negative	8.33±3.35 vs 2.4±3.37	6.58×10^{-5}	S

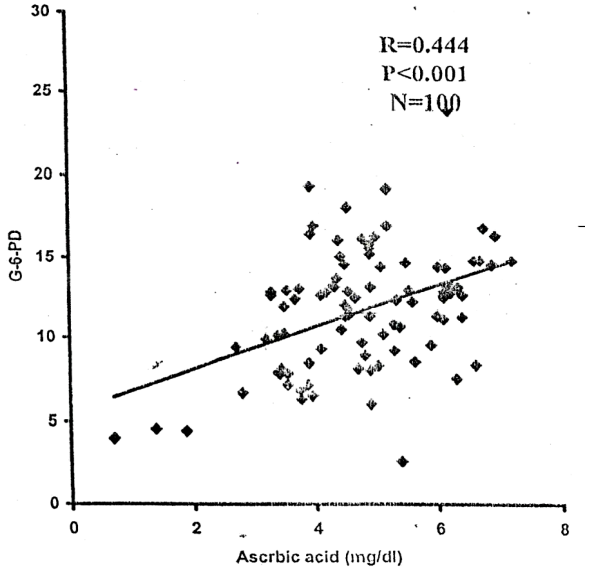

Fig.1 Correlation plot between G-6-PD and Ascorbic Acid in asymptomatic malaria snbjects

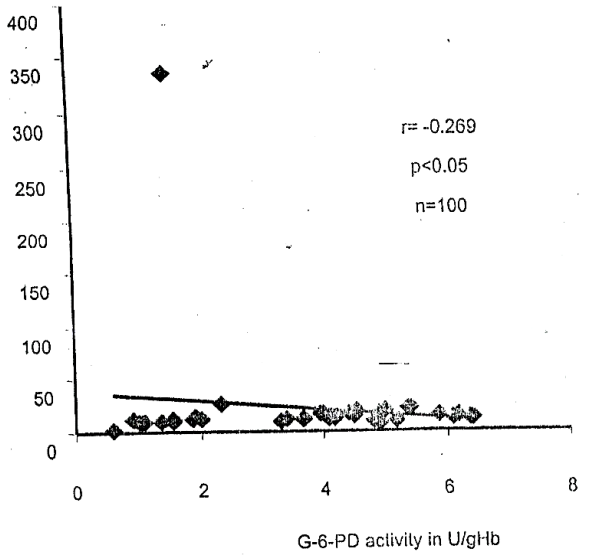

Fig.2 Correlation plot between ascorbic acid and G-6-PD in non-malaria subjects

Discussion

Low levels of Ascorbic Acid and G-6-PD deficiency have been reported in malaria endemic zones. This study examined the relationship between ascorbic acid levels and G-6-PD activity in non-malaria, asymptomatic and symptomatic malaria subjects.

The prevalence of malaria parasitaemia in symptomatic male and female subjects was similar while the prevalence in asymptomatic malaria subjects was higher in males than in females. The sex selective carriage of malaria may be associated with the differences in haemoglobin levels in male and female subjects (Dacie and Lewis, 1975; Painter et al, 1999). This finding suggests that subjects with high levels of haemoglobin are more prone to malaria parasitaemia, probably due to increase chance of parasite survival in such hosts.

Ascorbic acid levels seem to vary widely in different countries, even when the same technique of analysis is employed. In this study, we recorded levels of 3.47 ± 1.97mg/dl (19.12 ± 8.85m mol/l) for healthy non-malaria subjects; a rather low value in an area where vitamin C rich foods abound. In USA, Lee (1988) reported varying levels of ascorbic acid with age and sex which covered the range 58.5 – 134.4 μmol/l (10.6-24.4mg/dl) for subjects on ascorbic acid supplement and 20-80μmol/l for subjects not receiving supplement, the minimum risk for developing clinical signs of ascorbic acid deficiency is 2.9mg/dl. In Canada levels of 2.0 – 3.9mg/dl is regarded as low values. In Nigeria, Osilesi (1992) reported 0.40 – 40mg/dl as marginal deficiency for supplement subjects. Such low values may be due to ascorbic acid depression by P. falciparum infection variously recorded among malaria subjects (Mayne 1998).

Environmental factors such as malaria endemicity are probably an important factor responsible for the variation in reference values recorded in different studies and different countries.

In this study, we found G-6-PD activity of 8.34 ±

6.4U/gHb in non-malaria subjects, an activity which is lower than the activity (12.1 ± 2.09U/gHb) reported by Faribenk and Glee after (1999) in the USA. Pretreatment of glucose-6-phosphate dehydrogenase with ascorbic acid in normal cells caused a slight enhancement in malaria parasite development. In G-6-PD deficient cells, a suppressive effect on parasites development was found (Marva, 1989). In this study, significantly lower levels of ascorbic acid were found in subject with malaria symptoms when compared with the levels in malaria negative subjects. The level of ascorbic acid in asymptomatic malaria subject though lower than the level in non-malaria did not differ significantly, suggesting that there is greater ascorbic acid depression during malaria attack. Such depressed values may be due to increase utilization of ascorbic acid by malaria parasites or due to high demand by stressed host body system (Azuike 1991). The correlation coefficients between ascorbic acid levels and G-6-PD activity in symptomatic ($r=0.041$ $P>0.05$), a symptomatic ($r=0.444$, $P<0.05$) and none=malaria ($r= - 0.269$, $P<0.05$) subjects differed from one another showing that malaria parasitaemia alters the association between ascorbic acid levels and G-6-PD activity. Heavy parasitaemia results in decreased ascorbic acid levels and raised G-6-PD activity.

There was a significant increase ($P<0.05$) in G-6-PD activity in symptomatic malaria subjects compared with the levels in asymptomatic malaria and non-malaria subjects suggesting that the increase in G-6-PD activity may be associated with the malaria status of the subjects probably because malaria parasites synthesize their own G-6-PD (Marva 1989). Such parasite G-6-PD activity is measured together with host G-6-PD activity in the assay of host enzyme. This agrees with earlier work which reported that P.falciparum synthesizes it own G-6-PD which has a molecular weight double the size of the enzyme derived from human tissues in an invitro study (Shahabuddin et al, 1994).

The ratios of the mean G-6-PD (UgHb)/ascorbic acid (mg/dl) in symptomatic and asymptomatic malaria ($p = 3.35 \times 10^{-18}$), asymptomatic malaria and malaria negative subjects ($P = 2.57 \times 10^{-8}$) and symptomatic malaria versus malaria negative subjects ($p = 6.58 \times 10^{-5}$) discriminate between symptomatic, asymptomatic and non-malaria subjects, as shown by a widening gap between G-6-PD activity/ascorbic acid level ratios in malaria and non malaria subjects. In malaria subjects the parasite utilizes ascorbic acid for the development of itself, a process that also enhances the development of its own G-6-PD content, which was measured together with the subjects' G-6-PD.

The marked difference between the levels of ascorbic acid in asymptomatic and symptomatic malaria subjects may be responsible for some symptoms of malaria experienced during malaria attacks. Such symptoms include body and joint pains, increased vascular fragility and haemolysis (anaemia) since such symptoms are associated with ascorbic acid deficiency (Mayes 1994). In malaria endemic zones, where vitamin C rich food such as citrus fruits, and green vegetables abound, the prevalence of malaria also abound indicating a mutual relationship between the two. These rich sources of ascorbic acid could be the enhancing or sustenance factors for the survival of parasites in endemic areas. The malaria belt of the world (tropical regions) with rich source of vitamin C constitutes malaria endemic zones. Apart from dietary sources, ascorbic acid supplement may act as booster to malaria parasites in carriers. Moreover, the marked increase in the activity of G-6-PD in malaria subject may falsify G-6-PD screening tests and assay results in such subjects. The result of this study suggests that ascorbic acid ingestion in asymptomatic P. falciparum carries may actually enhance malaria parasite multiplication.

We conclude that ascorbic acid levels are reduced while the activity of G-6-PD is raised in symptomatic malaria subjects compared to the levels in non-malaria and asymptomatic malaria subjects. The level of ascorbic is inversely related to the level of malaria parasitaemia while G-6-PD is directly related to the level of P. falciparum parasitaemia. The association between ascorbic acid and G-6-PD differ in symptomatic, asymptomatic and in malaria negative subjects. The G-6-PD/ascorbic acid ratio discriminates between symptomatic, asymptomatic and non-malaria subjects. We suggest caution in the interpretation of G-6-PD results in malaria subjects as P.falciparum parasitaemia in such subjects alters both the screening and the quantitative test results for G-6-PD in individuals carrying the parasites. Specific assay of human G-6-PD which excludes P.falciparum G-6-PD is recommended.

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