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Keywords: glutathione S-transferase activity, erythrocytes, pyrimethamine/sulphadoxine, quinine, 1-chloro-2, 4- dinitrobenzene (CDNB).

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Glutathione *S*-Transferase Activity of Three Erythrocyte Genotypes of Human Participants Treated with Pyrimethamine/Sulphadoxine and Quinine

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Abstract- Studies to ascertain levels of erythrocyte glutathione *S*-transferase (Ery-GST) activity of non-malarious and malarious male participants of HbAA, HbAS and HbSS erythrocyte genotypes treated with pyrimethamine/sulphadoxine mixture and quinine were carried out. Incubation of erythrocytes with 1-chloro-2, 4-dinitrobenzene (CDNB) caused quantitative conjugation of reduced glutathione (GSH) to produce S-(2, 4-dinitrophenyl) glutathione, which formed the bases for the measurement of Ery-GST activity using a spectrophotometer. Blood samples were drawn from treated non-malarious and malarious participants at time intervals of 0, 3, 6 and 18 h and measured for Ery-GST activity. The control values of Ery-GST activity of non-malarious and malarious participants were within the ranges of 3.27 ± 0.13 – 12.50 ± 1.58 IU/gHb and 2.75 ± 0.16 – 12.21 ± 1.20 IU/gHb respectively. Ery-GST activity of malarious participants was significantly ($p < 0.05$) lower than that of the malarious participants, except that of parasitized HbSS erythrocytes. Generally, Ery-GST activities in the presence of the two antimalarials exhibited a biphasic profile. The first phase showed decreasing levels of Ery-GST activity at $t < 6$ h following the administration of pyrimethamine/sulphadoxine mixture and quinine to the non-malarious and malarious participants. In the second phase, Ery-GST activity increased when experimental $t > 6$ h. The overall pattern of Ery-GST activity within the experimental time ($0 < t < 18$ h) showed evidence of antimalarial induced disturbance of erythrocyte homeostasis, which could be of relevance from toxicological standpoints and for monitoring therapeutic events in malarial disease.

Keywords: glutathione S-transferase activity, erythrocytes, pyrimethamine/sulphadoxine, quinine, 1-chloro-2, 4-dinitrobenzene (CDNB).

I. Introduction

Pyrimethamine (250 mg)/sulphadoxine (50 mg) mixture is commonly used for prophylaxis and treatment of certain strains of *Plasmodium falciparum* that are resistant to chloroquine (Bray *et al.,* 1998) and usually sold under the trade mark name of Fansidar™. The drug combination effectively blocks two enzymes involved in the biosynthesis of folinic acid within the parasite (Milhous *et al.,* 1985). Quinoline blood schizontocides behave as weak bases when concentrated in food vacuoles of susceptible *Plasmodia*, where it causes increase in vacuolar pH, inhibits peroxidase activity of haem and thereby, disrupts non-enzymatic polymerization of ferroprotoporhyrin IX (FPIX) - haemin to haemozoin. The failure to inactivate FPIX kills the parasite via oxidative damage to membranes, digestive proteases and possibly other critical biomolecules of the parasite (Ducharme and Farinotti, 1996).

Enzyme studies have revealed a collection of protein molecules with common characteristic high affinity for reduced glutathione (GSH). Several of these protein molecules have been isolated from rat and human liver (Ketley *et al.,* 1975; Awasthi *et al.,* 1981; Hayes and Pulford, 1995), pigeon, locust gut, housefly and other sources (Ketley *et al.,* 1975) and characterized. These protein molecules are classified based on their enzymatic activities as glutathione *S*-transferase (GST: EC: 2.5.1.18) (Jacoby, 1976). The functions of GSTs are classified into two general categories (Harvey and Beutler, 1982).As intracellular binding proteins (Mannervik and Danielson, 1988; Hiller *et al.,* 2006), GSTs on a broad scale function as solubilizing and transport proteins, analogous to the extracellular functions of albumin (Boyer and Oslen, 1991; Oakley *et al.,* 1999). Also, GSTs catalyze the conjugation of electrophilic groups of hydrophobic drugs and xenobiotics to form glutathione-thioethers (Board *et al.,* 1990). Thio-ethers are eventually converted to mercapturic acid by the sequential actions of gamma-glutamyl transpeptidase, depeptidase and N-acetylase (Habig *et al.,* 1974; Mannervik and Danielson, 1988).

GST activity has been implicated in the acquisition of drug resistance (Black and Wolf, 1991). However, the role of GST activity in malaria resistance has not been studied, except by Dubois *et al.,* (1995) who reported that drug-resistant *Plasmodium berghei* resulted from altered GST activity (Srivastava *et*

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al., 1999). The present study ascertained the comparative levels of erythrocyte glutathione *S*-transferase (Ery-GST) activity of non-malarious and malarious male participants of HbAA, HbAS and HbSS erythrocyte genotypes treated with pyrimethamine/ sulphadoxine mixture and quinine.

II. Materials and Methods

a) Anti-malarials

Fansidar™ (Swiss (Swipha) Pharmaceuticals Nigeria Ltd) and quinine (BDH, UK), were purchased from Cimpok Pharmaceuticals, Amakhohia, Owerri, Nigeria.

b) Selection of participants/experimental design

Fifteen (n = 15) malarious males (59 - 79 kg) infected with antimalarial susceptible strain of *Plasmodium falciparum* and twenty (n = 20) non-malarious male participants (61 - 73 kg), both of confirmed HbAA, HbAS and HbSS genotypes enrolled for this study. The malarious participants were individuals attending clinics at the Federal Medical Center (FMC), St. John Clinic/Medical Diagnostic Laboratories, Avigram Medical Diagnostic and Research Laboratories, and Qualitech Medical Diagnostic Laboratories. All laboratory investigations were carried out at Avigram Medical Diagnostic and Research Laboratories, Owerri, Imo State, Nigeria. The malarious participants were within the age brackets of 21 - 34 years old, whereas the non-malarious participants were within the age brackets of 20 - 28 years old. All participants were administered with single dose of pyrimethamine/sulphadoxine mixture and quinine, each according to the following specifications, [pyrimethamine] = 14.9 mg/kg: [sulphadoxine] = 2.9 mg/kg and [quinine] = 5.9 mg/kg. Specifically, nine (n = 9) and six (n = 6) of the malarious participants received pyrimethamine/sulphadoxine mixture and quinine respectively. For comparative study, the 20 non-malarious participants were administered with the same doses of the two antimalarials, of which eleven (n = 11) and nine (n = 9) of the participants received pyrimethamine/sulphadoxine mixture and quinine respectively.

The participants were randomly selected between June and August 2012. Exclusion criteria include; gastrointestinal tract infection, protein energy malnutrition, renal diseases, cirrhosis, hepatitis, obstructive jaundice, cancer, diabetes mellitus, hypertension, obesity, smoking, alcoholism, persons living with HIV, patients taking anti-malaria drugs and vitamin supplements, patients who have been treated for malaria in the past 2 months (Onyesom and Onyemakonor, 2011; Idonije*et al.,* 2011).

c) Ethics

The Ethical Committee of University of Port Harcourt, Port Harcourt, Nigeria, approved the study in compliance with the Declaration on the Right of the Patient (WMA, 2000). Before enrolment for the study, the patients/participants involved signed an informed Consent Form.

d) Collection of blood specimen and preparation of erythrocyte haemolysate

Blood samples were drawn, using 5.0 mL capacity disposable syringes, from treated non-malarious and malarious participants at time intervals of 0, 3, 6 and 18 h. Erythrocytes were separated from the blood samples and washed by centrifugation methods of Tsakiris *et al.,* (2005) with modifications according to Chikezie, (2011). Within 15 min of collection of blood samples, portions of 3.0 mL of the samples were introduced into centrifuge test tubes containing 3.0 mL of buffer solution pH = 7.4: 250 mM tris (hydroxyl methyl) amino ethane-HCl (Tris-HCl)/140 mM NaCl/1.0 mM $MgCl_2$/10 mM glucose). The erythrocytes were separated from plasma by centrifugation at 1200 x *g* for 10 min and washed 3 times by the same centrifugation method with the buffer solution. The pelleted erythrocytes were re-suspended in 3.0 mL of phosphate buffer saline (PBS) solution and passed twice through newly packed columns (3.5 cm in a 30 mL syringe) of cellulose-microcrystalline cellulose (ratio 1:1; *w/w*) to obtain erythrocyte suspension sufficiently devoid of leucocytes and platelets. The pelleted erythrocytes were finally re-suspended in 6.0 mL of PBS to obtain approximately 10% haematocrit according to Chikezie *et al.,* (2012). A 2.0 mL portion of the separate pelleted erythrocyte genotypes were lysed by freezing/thawing as described by Galbraith and Watts, (1980) and Kamber *et al.,* (1984). The erythrocyte haemolysate was used for the measurement Ery-GST activity.

e) Malaria parasite density test

Portion of 2.0 mL of the blood samples were collected into EDTA bottles for malaria parasite tests. Measurement of parasite density of peripheral blood smear was by Giemsa stained techniques. The films were examined microscopically using ×100 objective under oil immersion (Cheesbrough, 1998). Participants with parasitaemia levels within the range of 1000 to 9999/µL were used for the present study.

f) Erythrocytes haemolysate haemoglobin concentration

A modified method (Baure, 1980), based on cyanomethaemoglobin reaction was used for the determination of haemolysate haemoglobin concentration. The expressed values were in grams per deciliter (g/dL). A 0.05 mL portion of erythrocyte haemolysate

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NaCN and 300 mg $K_4Fe(CN)_6$ per liter). The mixture was left to stand for 10 min at room temperature ('25' '±'and '5°C') and absorbance read at λmax = 540 nm against a blank. The absorbance was used to evaluate haemolysate haemoglobin concentration by comparing the values with the standards.

g) Erythrocyte glutathione S-transferase

Ery-GST activity was measured by the method of Habig *et al.,* (1974) as described by Pasupathi *et al.,* (2009) with minor modifications according to Chikezie *et al.,* (2009). The reaction mixture contained 1.0 mL of 0.3 mM phosphate buffer (pH = 6.5), 0.1 mL of 30 mM 1-chloro-2, 4-dinitrobenzene (CDNB) and 1.7 mL of distilled water. After pre-incubating the reaction mixture at 37°C for 5 min, the reaction was started by the addition of 0.1 mL of erythrocyte haemolysate and 0.1 mL of GSH substrate. The absorbance was measured at time intervals of 30 s for 5 min at λmax = 340 nm. Ery-GST activity was expressed in international unit per gram haemoglobin (IU/gHb) using an extinction coefficient (∑) of 9.6 mM^{-1} cm^{-1} in a reaction mixture in which 1 mole of GSH was oxidized (Equation 1).

Calculation of Ery-GST activity

$$E_A = \frac{100}{(Hb)} \times \frac{OD/min}{\sum} \times \frac{V_C}{V_H} \quad \textit{Equation 1}$$

Where,

E_A = Enzyme activity in IU/gHb

[Hb] = Haemolysate haemoglobin concentration (g/dL)

OD/min = Change per min in absorbance at 340 nm.

V_C = Cuvette volume (total assay volume) = 3.0 mL.

V_H = Volume of haemolysate in the reaction system (0.05 mL).

h) Statistical analyses

The experiments were designed in a completely randomized method and data collected were analyzed by the analysis of variance procedure while treatment means were separated by the least significance-difference (LSD) incorporated in the statistical analysis system (SAS) package of 9.1 version (2006). The correlation coefficients between the results were determined with Microsoft Office Excel, 2010 version.

III. Results

The reference values of Ery-GST activity of male participant of HbAA, HbAS and HbSS genotypes is presented in Table 1. Ery-GST activity of the human erythrocyte genotypes was in the order: HbSS > HbAS > HbSS. The control values of Ery-GST activities of non-malarious and malarious male participants were within the ranges of 3.27 ± 0.13 – 12.50 ± 1.58 IU/gHb and 2.75 ± 0.16 – 12.21 ± 1.20 IU/gHb respectively.

Table 1 : Erythrocyte glutathione *S*-transferase activity of male participants administered with pyrimethamine/sulphadoxine mixture and quinine at $t = 0$ h

	Ery-GST Activity (IU/gHb)			
	NMAL		MAL	
Genotype	PS ($n = 11$)	Q ($n = 9$)	PS ($n = 9$)	Q ($n = 6$)
HbAA	$3.40 \pm 0.05^{b,c}$	$3.27 \pm 0.13^{b,c}$	$2.81 \pm 0.76^{b,c}$	$2.52 \pm 0.23^{b,c}$
HbAS	4.25 ± 0.10^{b}	4.30 ± 0.07^{b}	2.75 ± 0.16^{b}	2.79 ± 0.11^{b}
HbSS	12.50 ± 1.58^{a}	11.65 ± 1.20^{a}	12.19 ± 1.76^{a}	12.21 ± 1.13^{a}

Means with the different letters are significantly different at $p > 0.05$. NMAL: non-malarious participants; MAL: malarious participants; PS: pyrimethamine/sulphadoxine mixture; Q: quinine; n: number of male participants.

A comparative overview of Ery-GST activities of three erythrocyte genotypes of participants treated with pyrimethamine/sulphadoxine mixture and quinine, within the experimental time intervals of 0 h < t < 18 h, are summarized in Figures 1, 2 and 3. The Ery-GST activity was presented as relative enzyme activity (%) at the given experimental time intervals to that of the enzyme activity at $t = 0$ h.

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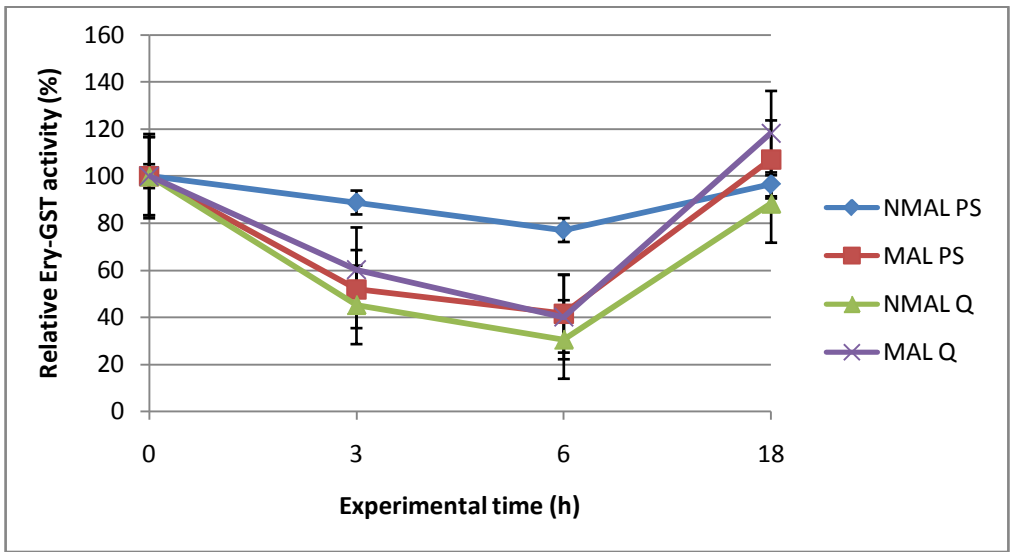

Figure 1 : Relative erythrocyte glutathione *S*-transferase activity of HbAA genotype of male participants administered with pyrimethamine/sulphadoxine mixture and quinine. NMAL PS: non-malarious male participants administered with pyrimethamine/sulphadoxine mixture; MAL PS: malarious male participants administered with pyrimethamine/sulphadoxine mixture; NMAL Q: non-malarious male participants administered with quinine; MAL Q: malarious male participants administered with quinine

Prior to administration of the two antimalarials to the participants, Ery-GST activities of the three genotypes were in the increasing order: HbSS > HbAS > HbSS. The profiles of Ery-GST activities of the three erythrocyte genotypes were irrespective of malarial status of the participants. However, there was no significant difference ($p > 0.05$) in Ery-GST activity between HbAA and HbAS erythrocytes (Figures 1 and 2). Furthermore, Ery-GST activities of parasitized erythrocytes were significantly ($p < 0.05$) lower than that of non-malarious participants, except Ery-GST activity of HbSS erythrocyte genotype.

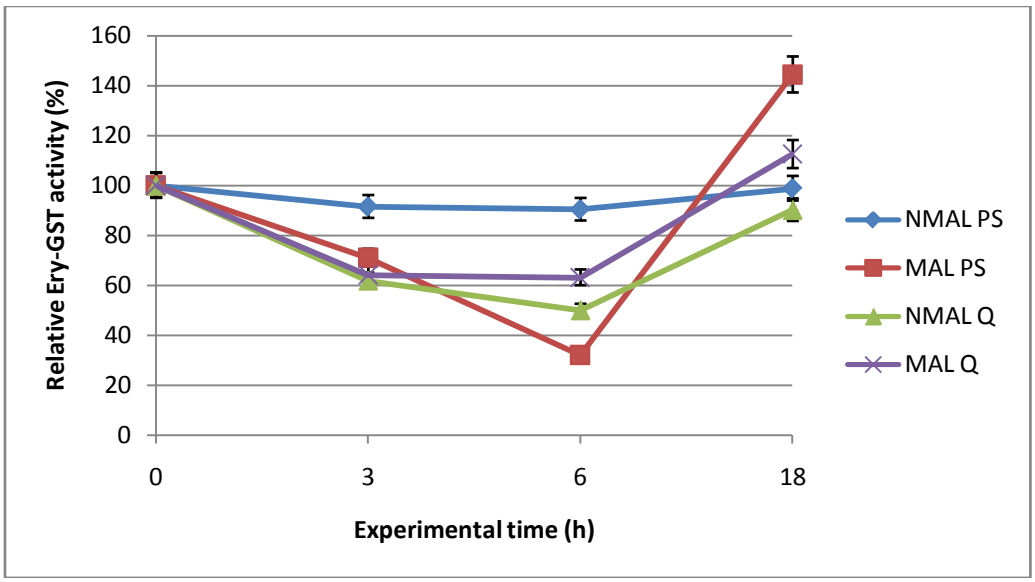

Figure 2 : Relative erythrocyte glutathione S-transferase activity of HbAS genotype of male participants administered with pyrimethamine/sulphadoxine mixture and quinine. NMAL PS: non-malarious male participants administered with pyrimethamine/sulphadoxine mixture; MAL PS: malarious male participants administered with pyrimethamine/sulphadoxine mixture; NMAL Q: non-malarious male participants administered with quinine; MAL Q: malarious male participants administered with quinine

Ery-GST activity of malarious participants of HbAS genotype gave the lowest level enzyme activity at $t = 6$ h following the administration of quinine (Figure 2), whereas malarious participants of HbSS genotype gave peak Ery-GST activity = 12.58 ±1.50 IU/gHb at $t = 18$ h (Figure 3).

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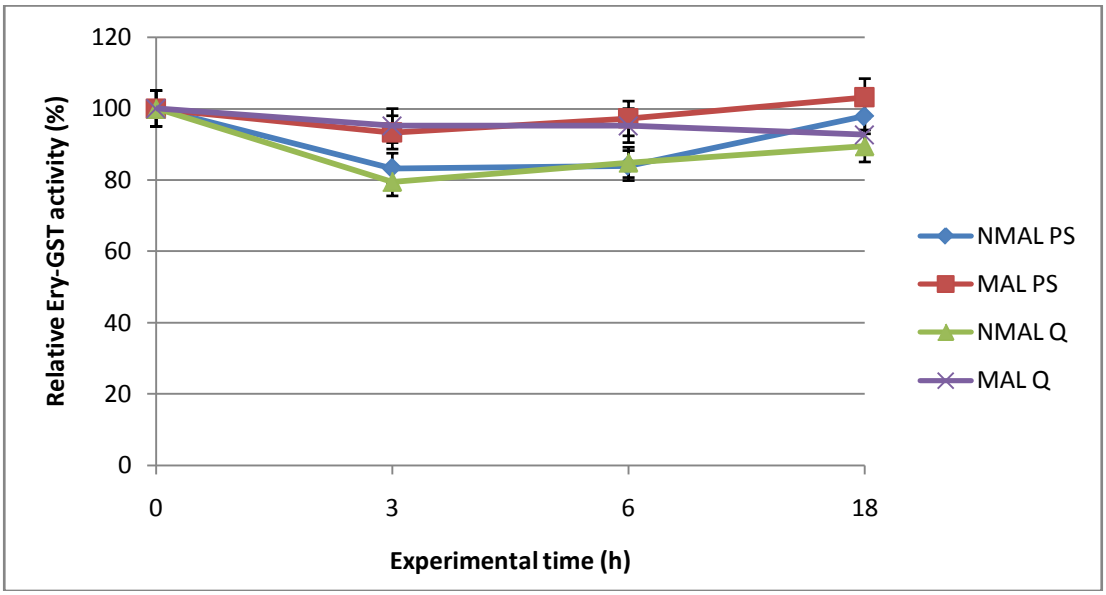

Figure 3 : Relative erythrocyte glutathione S-transferase activity of HbSS genotype of male participants administered with pyrimethamine/sulphadoxine mixture and quinine. NMAL PS: non-malarious male participants administered with pyrimethamine/sulphadoxine mixture; MAL PS: malarious male participants administered with pyrimethamine/sulphadoxine mixture; NMAL Q: non-malarious male participants administered with quinine; MAL Q: malarious male participants administered with quinine

Generally, the patterns of Ery-GST activities in the presence of the two antimalarials exhibited a biphasic profile. The first phase showed decreasing levels of Ery-GST activity within $t < 6$ h following the administration of pyrimethamine/sulphadoxine mixture and quinine to the non-malarious and malarious male participants. In the second phase, Ery-GST activity increased when the experimental $t > 6$ h.

IV. Discussion

Human GST activity, though not routinely assayed in clinical laboratories, could serve as a useful marker enzyme in diagnostic pathology. For instance, over-expression of GST in erythrocytes of patients with chronic renal failure (Galli *et al.,* 1999) and uremia (Galli *et al.,* 1999; Carmagnol *et al.,* 1981) have received immense attentions and documentations. Patients with hepatocellular damage present elevated plasma GST activity (Mulder *et al.,* 1999; Beckett and Hayes, 1993). In addition, low GST activity consequent upon impaired placental detoxification pathways may represent a risk factor for recurrent early pregnancy loss (Zusterzeel *et al.,* 2000) and as an indicator of oxidative stress at birth (Neefjes *et al.,* 1999). The level of expression of GST could provide useful diagnostic parameter in carcinoma of the breast (Forrester *et al.,* 1990) and bladder (Engel *et al*., 2002).

The present report showed that Ery-GST activity of the human erythrocyte genotypes was in the order: HbSS > HbAS > HbSS (Table 1), which was in concordance with previous findings (Anosike *et al.,* 1991). According to Shalev *et al.,* (1995), comparative raised levels of Ery-GST activity of HbSS genotype was the outcome of corresponding raised levels of oxidants in this erythrocyte genotype. The intermediate level of Ery-GST activity of HbAS erythrocytes was a reflection of the hybrid nature of heterogeneous erythrocyte (Anosike *et al.,* 1991).

Previous investigations by Sarin *et al.,* (1993) revealed that parasitaemia caused decreased levels of enzyme activities associated with the glutathione system such as glutathione peroxidase (GPx), glutathione reductase (GRx) and GST activities of erythrocyte lysates. Accordingly, the present study showed that Ery-GST activities of parasitized erythrocytes of HbAA and HbAS genotypes exhibited significant ($p < 0.05$) decreased levels of enzyme activity compared to corresponding Ery-GST activity of non-malarious participants (Figures 1 and 2), which corroborated the findings of Sohail *et al.,* (2007). Therefore, low level of Ery-GST activity probably served as host defense strategy against the malarial parasites through up-regulation of oxidative protection mechanisms. In addition, Ery-GST activity served as a biomarker for diagnostic and therapeutic events in malaria. For similar purposes and reasons, reports have equally shown that patients infected with the malarial parasites (Becker *et al.,* 2004; Kavishe *et al.,* 2006) and causative organism of visceral Leishmaniasis (Neupane *et al.,* 2008) exhibited lower plasma levels of reactive oxygen and nitrogen species (RONS) antagonist such as glutathione (GSH), catalase and α-tocopherol than in the control groups. Therefore, inoculation of malarial parasites into biologic systems, most probably,elicitsthe production of reactive

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oxygen species (ROS) as a part of host defense strategy against the invading parasites (Becker *et al.,* 2004). The non-significant difference ($p > 0.05$) in Ery-GST activity between the non-malarious and malarious participants of HbSS genotype (Figure 3), implied that the host HbSS erythrocytes did not turn on the oxidative up-regulatory pathways that are involved in the control measures and elimination of the parasite. Expectedly, the perpetual high oxidative state of HbSS erythrocytes (Anosike *et al.,* 1991) provided and sustained the requisite anti-fecundity capabilities of this erythrocyte genotype against the malarial parasites.

Furthermore, low levels of Ery-GST activity of malarious participants was in connection with malarial pathophysiology described elsewhere (Dubios *et al.,* 1995, Liebau *et al.,* 2002). The ingestion and degradation of large quantities of haemoglobin by malarial parasite elicits the generation of potentially parasitotoxic FPIX. Accordingly, FPIX efficiently binds to *P. falciparum* GST (pfGST) (Harwaldt *et al.,* 2002) as well as to Ery-GST, preferably to the GST-GSH complex (Hiller *et al.,* 2006), and thereby, engenders uncompetitive inhibition of the GSTs.

In the first phase enzyme activity profile, Ery-GST showed decreasing level of activity with progression of experimental time, which was in concordance with previous reports (Mannervik and Danielson, 1988; Ayalogu *et al.,* 2001; Hiller *et al.,* 2006). The second phase showed evidence of recovery and activation of Ery-GST activity, exemplified by increasing level of the enzyme activity with increasing experimental time. The positive activation of Ery-GST activity in the second phase of Ery-GST activity profile was the outcome of generation and accumulation of ROS associated with the molecular events of the first phase enzyme activity profile. Therefore, ROS induced positive activation of Ery-GST activity served as a measure to detoxify and neutralize the cytotoxic ROS, in efforts to restore erythrocyte homeostasis. In agreement with the present findings, Hayes and Pulford, (1995) had proposed that cellular GST activity was under the regulatory mechanism of ROS and activation of GST activity can be considered as an adaptive response for the detoxification of cytotoxic carbonyl-, peroxide and epoxide-containing metabolites released in the cell by oxidative stress.

The overall pattern of Ery-GST activity within the experimental time ($0 < t < 18$ h) showed evidence of antimalarial induced disturbance of erythrocyte homeostasis, which could be of relevance from toxicological standpoints and for monitoring therapeutic events in malarial disease.

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