

Full Length Research Paper

Comparative osmotic fragility of three erythrocyte genotypes (HbAA, HbAS and HbSS) of male participants administered with five antimalarial drugs

P. C. Chikezie^{1*}, A. A. Uwakwe² and C. C. Monago²

¹Department of Biochemistry, Imo State University, Owerri, Imo State, Nigeria.

²Department of Biochemistry, University of Port Harcourt, Port Harcourt, Rivers State, Nigeria.

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In vivo study was carried out to ascertain the mean corpuscular fragility (MCF) index and corresponding stability of three erythrocyte genotypes (HbAA, HbAS and HbSS) before (control; t = 0 h) and after (tests; that is, at t = 3, 6 and 18 h) five (5) antimalarial drugs (FansidarTM, HalfanTM, Quinine, CoartemTM, and Chloroquine phosphate) were administered to male participants. Clinically confirmed healthy non-malarious and malarious male participants enrolled for this study. Erythrocytes obtained from these individuals were suspended in two separate sets of Phosphate buffer saline (PBS) solution of decreasing concentrations in the following order: 0.9, 0.7, 0.6, 0.4, 0.3 and 0.2 g / 100 ml. Spectrophotometric method was used to determine the level of erythrocyte osmotic fragility. The mean (\pm S.D) MCF values of the three genotypes were in the order: HbAA<HbAS<HbSS irrespective of the malarial status of participants. However, there was no significant difference ($p > 0.05$) between the MCF values of HbAA and HbAS erythrocytes. Comparatively, parasitized erythrocytes exhibited significantly ($p < 0.05$) increased MCF values. The five antimalarial drugs were agents of erythrocyte destabilization in both categories of participants. However, the overall capacities of the drugs to disturb erythrocyte stability diminished as the experimental time progressed.

Key words: Antimalarials, erythrocytes, mean corpuscular fragility, genotypes, osmotic fragility.

INTRODUCTION

When red blood cells are placed in hypotonic solution in which osmolarity is diminished the gain in red blood cell water is both, instant and quantitative. This phenomenon is put into practical use in the red blood cell osmotic fragility test, which determines the release of haemoglobin from red blood cells in hypotonic sodium chloride (NaCl) solution. Therefore, osmotic fragility index is a measure of the resistance of red blood cells to lysis by osmotic stress (Oyewale and Ajibade, 1990). The test is generally useful to ascertain the level of stability and functionality of plasma membrane (Krogmeier et al., 1993), erythrocyte Mean Cell Volume (MCV) and Surface Area-to-Volume Ratio (SAVR) and diagnosis of hereditary spherocytosis

(Alderich et al., 2006; Kumar, 2002).

Certain xenobiotics such as primaquine and *Fava* beans extracts are agents that can interfere with the redox status of red blood cells, specifically in those individuals with impaired glucose-6-phosphate dehydrogenase activity (Mayes, 1983; Champe et al., 2005; Ojo et al., 2006). Other compounds may act in such a way that the activity of certain erythrocyte redox enzymes, such as glutathione reductase (Berker et al., 1995; Forchetti et al., 2006), glutathione peroxidase (Mayes, 2003) and glucose-6-phosphate dehydrogenase (Mayes, 2003; Champe et al., 2005; Ojo et al., 2006), that are required for membrane integrity are compromised. Therefore, this present study intend to ascertain the capacity of five (5) commonly prescribed antimalarial drugs (FansidarTM, HalfanTM, Quinine, CoartemTM and Chloroquine phosphate) to alter/distort

*Corresponding author. E-mail: p_chikezie@yahoo.com. Tel: +23408038935327.

erythrocyte membrane integrity and by implication, functionality.

MATERIALS AND METHODS

Anti-malarial drugs

Five (5) antimalarial drugs were used in this study: Fansidar™ {Swiss (Swipha) Pharmaceuticals Nigeria Ltd}, Coartem™, (Beijing Norvatis Pharmaceutical Company, Beijing, China) Chloroquine phosphate (May and Baker, Pharmaceutical Company, Nigeria Plc), Halfan™ (Smithkline Beecham Laboratories Pharmaceutical Company, France) and Quinine (BDH, UK).

Selection of volunteers/experimental design

One hundred and thirty one (131) male (61-73kg) participants of confirmed HbAA, HbAS and HbSS erythrocyte genotypes doses were administered in the following specifications (Table 1). The participants administered with single dose of each of five antimalarial drugs, were grouped according to their individual genotype and malarial status. The doses were administered in the following specifications:

Blood samples were withdrawn from these participants at time intervals of 3, 6 and 18 h after dosage and analyses were carried out to ascertain for erythrocyte osmotic fragility. The determinations of the red blood cell parameter prior to the administration of the five antimalarial drugs to participants constituted the control sample analysis.

Ethics

The institutional review board of the Department of Biochemistry, University of Port Harcourt, Port Harcourt, Nigeria, granted approval for this study and all participants involved signed an informed consent form. This conducted study was in accordance with the ethical principles that have their origins in the Declaration of Helsinki. Individuals drawn were from Imo State University, Owerri, Nigeria and environs. The research protocols were in collaboration with registered and specialized clinics and medical laboratories.

Collection of blood samples/preparation of erythrocyte haemolysate

Five milliliters (5.0 ml) of human venous blood of HbAA, HbAS, and HbSS genotypes obtained from participants by venipuncture was stored in EDTA anticoagulant tubes. Blood of HbSS genotype and malarious blood samples were from patients attending clinics at the Federal Medical Center (FMC), Imo State University Teaching Hospital (IMSUTH), Orlu, St. John Clinic / Medical Diagnostic Laboratories, Avigram Medical Diagnostic Laboratories and Qualitech Medical Diagnostic Laboratories. These centers are located in Owerri, Imo State, Nigeria.

The erythrocytes were washed by methods as described by Tsakiris et al. (2005). Within 2 h of collection of blood samples, portions of 1.0 ml of the samples were introduced into centrifuge test tubes containing 3.0 ml of buffer solution pH = 7.4:250 mM Tris-HCl / 140 mM NaCl / 1.0 mM MgCl₂ / 10 mM glucose). The erythrocytes were separated from plasma by centrifugation at 1200 g for 10 min, washed three times by three similar centrifugations with this buffer solution. The erythrocytes were re-suspended in 1.0 ml of this buffer and the test carried out with these washed and intact erythrocytes.

Determination of erythrocyte osmotic fragility

Osmotic fragility of three red blood cell genotypes, HbAA, HbAS, and HbSS was determined by a measure of haemoglobin released from red blood cells when placed in an environment containing serial dilutions of Phosphate Buffer Saline (PBS) solution as described by Oyewale (1993), with minor modifications (Mafuvadze et al., 2008).

Twenty microliters (20 µl) portion of red blood cells suspended in 1.0 ml buffer solution: pH = 7.4 (TrisHCl/140 MmNaCl/1.0 mM MgCl₂/10.0 mM glucose), was added to test tube containing 5.0 ml of PBS solution, pH = 7.4- {NaCl (9.0 g)/Na₂HPO₄.2H₂O(1.71 g)/ NaH₂PO₄.2H₂O(2.43 g) per 1 L of distilled water}, of serial concentrations in the order of 0.9, 0.7, 0.6, 0.4, 0.3 and 0.2 g/100 ml. The seventh test tube contained distilled water. The test tubes were allowed to stand for 30 min at room temperature (24°C). Subsequently, the contents of test tubes were centrifuged at 1200 g for 10 min. The supernatant was decanted and haemoglobin content determined spectrophotometrically at λ_{max} = 540 nm using PBS (0.9 g/100 ml) solution as blank. Haemolysis in each test tube was expressed as a percentage, taking as 100% the maximum value of absorbance of the test tube that contained erythrocytes suspended in distilled water (0.0 g/100 ml).

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.

Evaluation of percentage erythrocyte haemolysis and stabilization

The quotient of absorbance of the content of each test tube (1st - 6th) and the seventh test tube was multiplied by a factor of 100. The range of values represented the percentage of erythrocyte lysis at each corresponding PBS concentration (0.9 - 0.2 g/100 ml). The corresponding concentration of PBS solution that caused 50% lysis of red blood cells defined the mean corpuscular fragility (MCF) index (Dewey et al., 1982, Krogmeier et al., 1993). The cumulative erythrocyte osmotic fragility curve, the plot of percentage of erythrocyte lysis versus concentrations of PBS solution was used to obtain the MCF values.

The relative capacity of the five antimalarial drugs to stabilize or destabilize red blood cell membrane was evaluated as percentage of the quotient of the difference between MCF values of test and control samples to the control sample (Parpart et al., 1947; Chikezie, 2007). Thus,

$$\text{Relative Stability (\%)} = \frac{\text{MCF}_{\text{control}} - \text{MCF}_{\text{test}}}{\text{MCF}_{\text{control}}} \times \frac{100}{1}$$

RESULTS

The mean corpuscular fragility (MCF) index represented and interpreted level of erythrocyte membrane stability. The mean (±S.D) MCF values of the three erythrocyte genotypes (HbAA, HbAS and HbSS) of blood samples obtained from non-malarious and malarious participants before being administered with the corresponding five

Table 1. The participants administered with a single dose of each of five antimalarial drugs.

Male participants	Drugs	Doses administered (mg/kg)
Non-malarious (n = 70)	Fansidar	
	Pyrimethamine	14.9
	Sulphadoxine	2.9
	Halfan	
	Halofantrine base	13.9
	Quinine	5.9
	Coartem	
	Artemether	1.2
	Lumefantrine	7.2
	Chloroquine phosphate	14.9
Malarious (n = 61)	Fansidar	
	Pyrimethamine	14.5
	Sulphadoxine	2.9
	Halfan	
	Halofantrine base	13.5
	Quinine	5.8
	Coartem	
	Artemether	1.2
	Lumefantrine	7.0
	Chloroquine phosphate	14.5

N = Number of male participants.

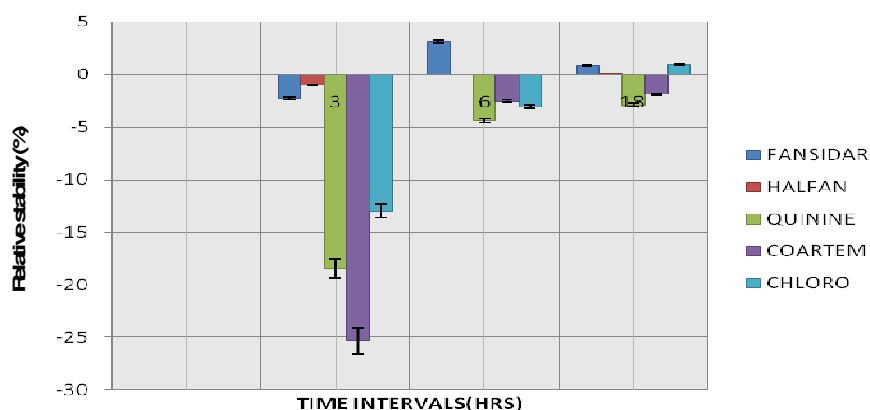


Figure 1. A time (18 h) dependent effect of five antimalarial drugs on relative stability of HBAA erythrocyte of non-malarious male participants.

antimalarial drugs (control/reference values) is presented in Table 2 and illustrated in Figures 1, 2, 3, 4 and 5.

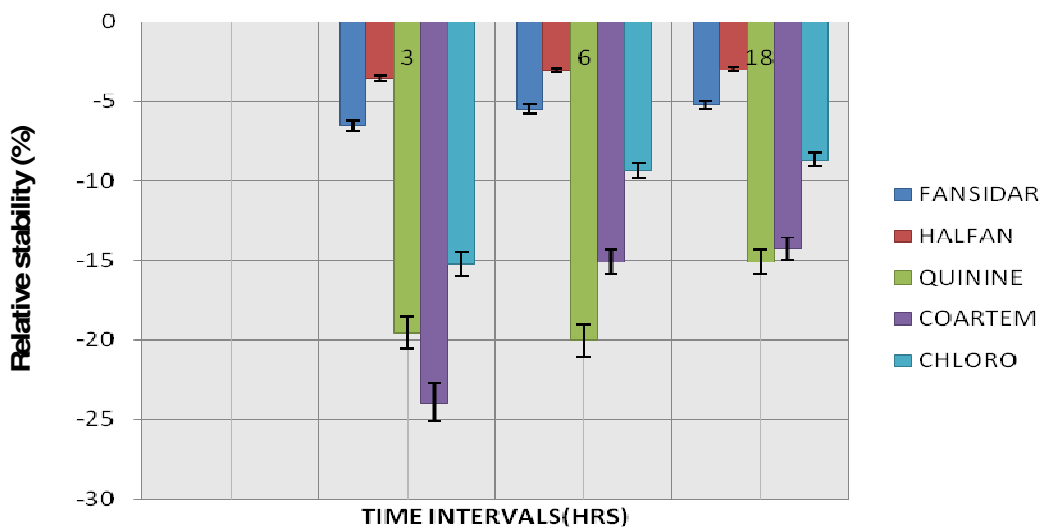
The mean (\pm S.D) MCF values of the three genotypes

were in the order: HbAA<HbAS<HbSS irrespective of the malarial status of participants. Whereas there was no significant difference ($p > 0.05$) between the MCF values

Table 2. Osmotic fragility: Mean corpuscular fragility (MCF) index of erythrocyte of non-malarious and malarious male participants.

Genotype	MCF values (g/100ml)	
	Non-malarious	Malarious
HbAA (n = 25 ^{NM} , 24 ^M)	0.352 ± 0.06 ^a	0.445 ± 1.01 ^a
HbAS (n = 25 ^{NM} , 25 ^M)	0.356 ± 0.92 ^a	0.472 ± 1.09 ^b
HbSS (n = 20 ^{NM} , 12 ^M)	0.422 ± 1.49 ^b	0.497 ± 1.11 ^c

*M and NM= number of malarious and non-malarious blood samples respectively. *Means in the column with the same letter are not significantly different at $p < 0.05$ according to LSD.

**Figure 2.** A time (18 h) dependent effect of five antimalarial drugs on relative stability of hbaa erythrocyte of malarious male participants.

of non-malarious blood samples of HbAA and HbAS erythrocytes, the values between HbAA and HbSS erythrocytes exhibited significant difference ($p < 0.05$).

In addition, parasitized erythrocytes exhibited significantly ($p < 0.05$) increased MCF values. This was an obvious reflection of higher fragility index of these *Plasmodium falciparum* infected erythrocytes. Specifically, whereas MCF values of erythrocytes obtained from non-malarious participants ranged between 0.352 ± 0.06 and 0.422 ± 1.49 g/100 ml, malarious individuals presented values between the range of 0.445 ± 1.01 and 0.497 ± 1.11 g/100 ml.

The five antimalarial drugs showed similar pattern but varied capacities to destabilize HbAA erythrocyte membrane at the end of the first three (3) h. At the 6th h, Coartem, Quinine and Chloroquine Phosphate exhibited diminished capacities to destabilize erythrocyte membrane integrity. In a different characteristic manner, Halfan and Fansidar showed neutral and stabilizing effect respectively (Figure 1). In addition, Figure 1 showed the administration of Fansidar, Halfan and Chloroquine Phosphate caused the stabilization of the red blood cells by 0.85, 0.10 and 0.95% after 18 h respectively.

However, the stabilizing effect of Fansidar and Chloroquine Phosphate on the red blood cell membrane was not significantly different ($p > 0.05$) when compared with the control/reference values. At an earlier hour of 6 h after dosing these individuals, Fansidar stabilized the erythrocytes by 3.13%. This level of stabilization was significantly different ($p < 0.05$) compared to the control/reference values.

The administration of the five antimalarial drugs caused destabilization of erythrocytes to individuals in this group (malarious male). The level of destabilization with respect to the drug administered was in the order: Halfan < Fansidar < Chloroquine Phosphate < Coartem < Quinine. Figure 2 showed the capacities of these drugs to destabilize the red blood cells attenuated as the experimental time progressed. Quinine and Chloroquine Phosphate did not significantly ($p > 0.05$) alter the level of destabilization of the erythrocytes between (3 - 6 h) and (6 - 18 h) after dosage respectively (Figure 2).

Studies of this group (HbAS genotype) showed that there was no defined pattern of magnitude of the five antimalarial drugs to destabilize red blood cells. For instance, at the 3rd h, administered Fansidar and Coartem

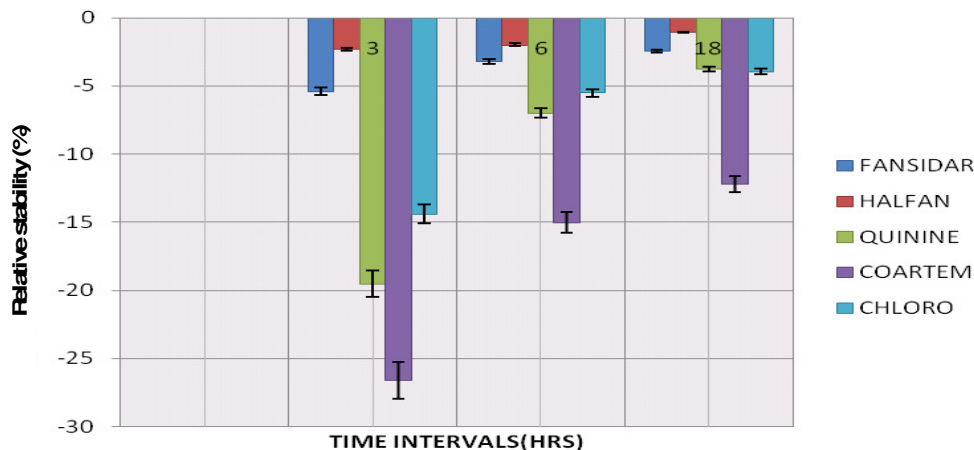


Figure 3. A time (18 h) dependent effect of five antimalarial drugs on relative stability of HBAS erythrocyte of non-malarious male participants.

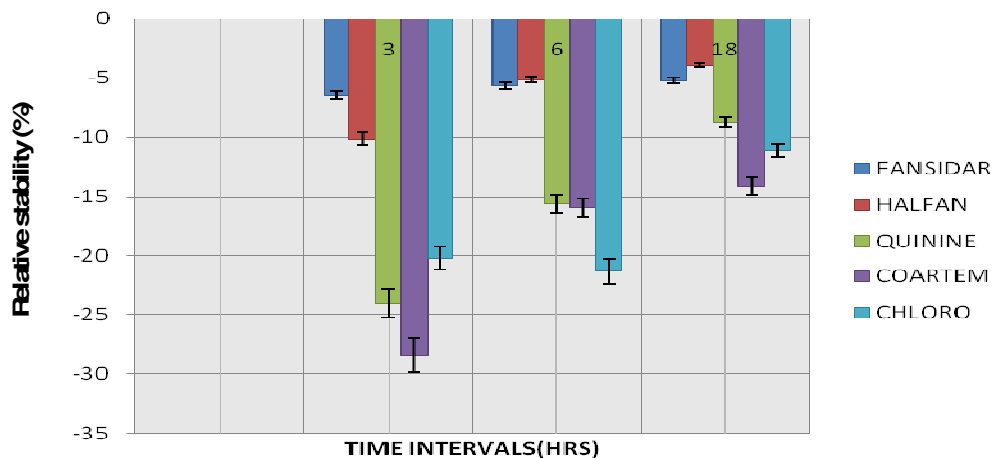


Figure 4. A time (18 h) dependent effect of five antimalarial drugs on relative stability of hbas erythrocyte of malarious male participants.

elicited the lowest and highest destabilizing effect respectively. The second 3rd h showed the order to be Halfan < Fansidar < Quinine < Coartem < Chloroquine Phosphate. Finally, at the 18th hour, Coartem and Halfan exhibited the highest and lowest capacities to destabilize the red blood cells respectively.

An overview of the results showed that administered Quinine did not significantly ($p > 0.05$) cause destabilization of the erythrocytes when compared with control/reference values of individuals in this category. Administration of the five antimalarials to corresponding groups of male participants of HbSS genotype showed that Quinine exhibited the highest destabilization property at the 3rd h. There was no sustained capacity of the same drug to destabilize the red blood cells throughout the duration of this present investigation. The level of destabilization decreased from -29.11 to -14.04% at the

3rd and 18th h, respectively. Whereas Fansidar showed the lowest capacity to destabilize the erythrocyte (stabilization = -8.54% at 3 h), chloroquine phosphate exhibited the lowest destabilization effect at the end of the experiment (stabilization = -3.98%). A look at Figure 5 illustrated that the destabilization effect of Halfan was more profound with this class of human erythrocyte genotype compared to other classes described above.

The five antimalarial drugs caused red blood cell destabilization. The decreasing tendency of erythrocyte destabilization in the presence of the antimalarials was time dependent. The increasing capacity of these drugs to cause destabilization at the 3rd hour was in the order: Halfan < Fansidar < Chloroquine Phosphate < Coartem < Quinine. The destabilizing effect of Halfan on the red blood cells was not statistically significant ($p < 0.05$) between the 3rd and 6th h (Figure 6).

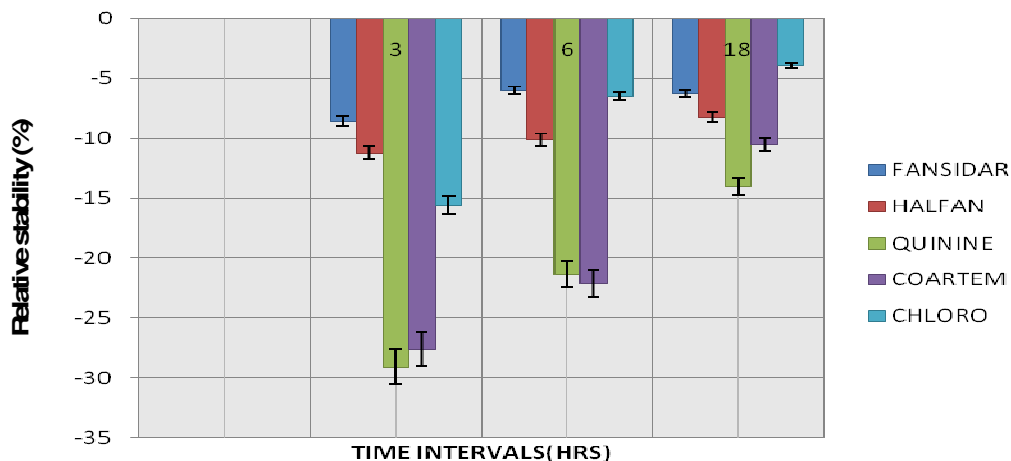


Figure 5. A time (18 h) dependent effect of five antimalarial drugs on relative stability of HBSS erythrocyte of non-malarious male participants.

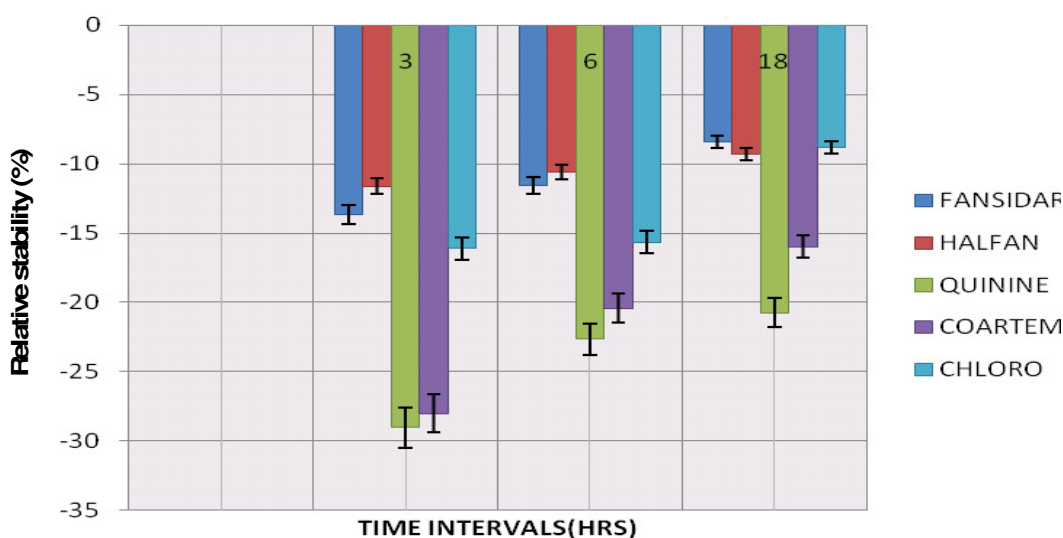


Figure 6. A time (18 h) dependent effect of five antimalarial drugs on relative stability of HBSS erythrocyte of malarious male participants. *Chloro means Chloroquine

DISCUSSION

From the present comparative investigations, the results presented in Table 1 showed that human red blood cell of HbSS genotype exhibited the least stability that was in the order HbAA>HbAS>HbSS. In agreement with these results, Dewey et al. (1982) asserted that differences in erythrocyte osmotic fragility are under the control of the individual genotype of the red blood cells. From a similar perspective, it is probable that variations in some physicochemical properties and oxidant levels of the three red blood cell genotypes contributed to the differences in mechanical stabilities and capacities of the red

blood cells to withstand osmotic stress (Senturk et al., 2001; Richards et al., 2007; Chikezie et al., 2008). Increasing evidence suggest that *in vivo* lipid peroxidation may be an important factor in sickle cell anemia (Stone et al., 1990). Sickle erythrocytes and their membranes are susceptible to endogenous free radical-mediated oxidative damage that correlates with the proportion of irreversibly sickled cell (Rice-Evans et al., 1986). In agreement with these lines of reasoning, Tamer et al. (2000) reported that higher superoxide generation in human HbSS erythrocytes was associated with increased tendency of diminished mechanical and osmotic stability compared with human HbAA erythrocytes. Furthermore,

red blood cells generate superoxide species under normal physiological conditions, but drastically increase in sickle cell disease. Unstable hemoglobin produced under this condition generates free radicals and further induce red blood cell hemolysis (Chan, 1996). Therefore, accumulation of oxidant contributes to accelerated damage of sickle erythrocyte membranes and senescence of these cells. From another perspective, comparative osmotic stability of human erythrocytes showed connection with the relative tendency of the cells to retain more sodium ion (Na^+) intracellularly with a concomitant loss of potassium ion (K^+) (Dunham and Hoffmann, 1971). Pretreatment of red blood cells with Halofantrine inhibited Na^+/K^+ ATPase pump (Bloom and Fawceth, 1975) and Chloroquine administered to rats caused renal disturbance of Na^+/K^+ ATPase systems (Nwanjo et al., 2007).

The present investigations have also demonstrated that the osmotic fragility index of parasitized cells in *P. falciparum* infections was significantly increased. These observations also, were in agreement with the reports of Fogel et al. (1966). They demonstrated that the osmotic fragility of parasitized cells in *Plasmodium berghei*, *P. knowlesi*, *P. gallinaceum* and *P. falciparum* infections was significantly increased. In addition, their findings showed that the increased fragility of erythrocytes was not solely limited to the parasite-containing cells. In a more recent study, Clark et al. (1991), proposed that nitric oxide production increase in any generalized infection particularly in acute malaria. Higher levels of nitric oxide produce poor deformability of red blood cells by inhibiting Na^+/K^+ ATPase in the red blood cell membrane and oxidizing the membrane lipids through generation of peroxynitrate. In a similar perspective, Neupane and co-researchers (2008), mentioned that patients suffering from parasitic infections, e.g. visceral Leishmaniasis, are under oxidative stress. Parasite invading the macrophages caused respiratory burst releasing different reactive oxidative species as a host defense mechanism. Increased reactive oxygen species (ROS) did not only kill the parasites, but also damaged the red blood cells and release malondialdehyde (MDA) as a secondary marker of tissue damage.

Many authors have cited a large number of drugs that cause alterations on the shape and physiology of the red blood cells (Ammus and Yunis, 1989; Braga et al., 2000). Evidence that drugs can interfere with osmotic resilience of red blood cells have been demonstrated with various natural products (Chikezie and Ibegbulem, 2004; Chikezie, 2007; de Souza Fontes, 2007). From observations of the present *in vivo* studies, the five anti-malarial drugs were agents of red blood cell destabilization. However, the pattern of red blood cell levels of destabilization, as consequence of the administered drugs, exhibited recovery. This implied the erythrocyte relative stabilization tended towards the control values as the experimental time progressed. In the same vein,

Iwalokun et al. (2004), reported that ten day after schizonticidal intervention, osmotic MCFs of malarial children fell within the normal range and were not significantly different ($p > 0.05$) from that of healthy children. Probably, connections existed between the redox status of these human red blood cells and plasma concentrations of the five antimalarial drugs and its metabolites. Reports have shown that oxidative damage of erythrocyte membrane is the primary cause of reduced capacity of the red blood cells to withstand mechanical and osmotic stress (Laurence et al., 1997; Mayes, 1983).

Destabilization of the red blood cells by the five antimalarial drugs could be because of the following metabolic consequence of the administered drugs:

- a) Rapid production and accumulation of ROS overwhelmed antioxidant defense capacities to maintain and sustain membrane integrity of the human erythrocytes.
- b) Depletion of erythrocyte glutathione concentration.

Therefore, the diminishing plasma concentrations with time of the antimalarial metabolites and positive activation of associated redox enzymes (Anosike et al., 1991) might have contributed to diminishing levels of destabilization of human erythrocytes. Furthermore, the replenishment of free radical scavengers, e.g. glutathione, redox enzymes etc engendered the relative stability of the red blood cell to approach control values and normalcy over time.

Therefore, the findings of the present *in vivo* study suggest that substances implicated to have compromised/distorted the redox equilibrium of the red blood cells are agents of membrane destabilization.

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