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Studies of Methaemoglobin Concentrations of three Human Erythrocyte genotypes (HbAA, HbAS and HbSS) in the presence of five anti malarial drugs.

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ABSTRACT: *In vitro* studies were carried out to ascertain the capacity of increasing concentrations of five antimalarial drugs (Fansidar™, Halfan™, Quinine, Coartem™ and Chloroquine phosphate) to elicit the generation of methaemoglobin in three human erythrocyte genotypes (HbAA, HbAS and HbSS). Spectrophotometric method was used for determination of plasma methaemoglobin concentration in the presence of 0.2%, 0.4%, 0.6% and 0.8% (w/v) of the five antimalarial drugs. The five antimalarial drugs showed a concentration dependent variability to cause the elevation of plasma methaemoglobin concentration in the three genotypes. Specifically, Coartem™, exhibited the highest propensity of elevate plasma methaemoglobin concentration. However, the other four antimalarial drugs showed a statistically significant ($P<0.05$) but minimal effect to cause elevation of plasma methaemoglobin concentration. The implications of these findings with respect to in vitro blood processing exercise were discussed.

KEY WORDS: Antimalarial drugs, methaemoglobin, erythrocyte, genotypes, Spectrophotometric method.

INTRODUCTION

Malaria remains the world's most devastating human parasitic infection, affecting more than 500million people and causing from 1.7million to 2.5million deaths each year (WHO, 1995). Four species of obligate intracellular protozoa of the genus *Plasmodium* cause nearly all human malaria (Tracy and Webster, 2001). Antimalarial drugs are categorized by the stage of parasite they affect and the clinical indications for their use.

Fansidar™ is a combination of pyrimethamine (250mg) and sulphadoxine (50mg) commonly used for prophylaxis and treatment of certain strains of *Plasmodium falciparum* that are resistant to chloroquine (Bray *et al.*, 1998). This drug combination effectively block two enzymes involved in the biosynthesis of folinic acid within the parasite (Mithous *et al.,* 1985). Artemether represents a major advance for the treatment of severe, multi-drug resistant falciparum malaria (Tracy and Webster, 2001). The drug is a more potent derivative of artemisinin, administered in the form of artemisinin combination therapy (ACT), artemether lumefantrine combination drug therapy (Coartem™) (Artemisinin, 2001). Other regimens for malarial chemoprophylaxis are the aminoquinolines such as chloroquine phosphate and its analogs. Quinoline blood schizontocides behave as weak bases concentrated in food vacuoles of susceptible *Plasmodia* where they increase pH, inhibit the peroxidase activity of haem and disrupt its non-enzymatic polymerization to haemozoin. The failure to inactivate haem then kills the parasite via oxidative damage to membranes, digestive proteases and possibly other critical biomolecules of the parasite. (Bates *et al.,* 1990; Ducharme and Farinotti, 1996)

Concisely, methaemoglobin is formed when ferrous iron (Fe^{2+}) of deoxyhaemoglobin is converted to the ferric iron (Fe^{3+}) state on exposure of erythrocytes to oxidizing agents and oxygen free radical (Hopkins, 1998; Callister, 2003). Ferric iron (Fe^{3+}) state haemoglobin does not bind reversibly with oxygen. Studies have shown that methaemoglobin is formed continuously in plasma but rarely exceeds 1.5% of total plasma haemoglobin (Tietz, 1976; Callister, 2003). Basically, two enzymes, Diaphorase I (Gibson, 1984, Kuma, 1981; Breaking *et al.,* 1957) and diaphorase II (Yubisui *et al.,* 1980) in synergy with red cell non enzymic anti oxidants, ascorbic acid, glutathione and other sulphydryl derivatives serve to minimize erythrocyte methaemoglobin level (Jaffe and Neuman, 1985). Cyanotic presentation is typically observed at methaemoglobin concentration greater than 15% and is often one of the earliest clinical evident features of methaemoglobinemia (Hopkins, 2000).

Ali and Kadaru, (2005) described *in vitro* processing of donor blood with sulphadoxine/pyrimethamine drugs combination for the eradication and prevention of transfusion induced malaria. In furtherance of their reports, our present studies seek to ascertain methaemoglobin concentration of three human erythrocyte genotypes, HbAA, HbAS, and HbSS in the presence of five commonly prescribed antimalarial drugs. The present research findings will provide an insight into the capacity of these five antimalarial drugs to interfere and alter the redox status of haemoglobin molecule. Therefore, our results may provide a subset of preliminary data for effective, successful and safe utilization of these antimalarial drugs for *in vitro* blood processing exercise.

MATERIALS AND METHOD

Collection of Blood Samples:

Five milliliters (5 mls) of confirmed human red blood cell genotypes, HbAA and HbAS were obtained from subjects/volunteer within the age bracket of 18-35 years old. Blood samples of HbSS genotype were collected from patients attending clinic at the Federal Medical Centre (FMC), Owerri and Imo State University Teaching Hospital (IMSUTH), Orlu, Imo State, Nigeria. All Blood samples were obtained by venepunture and stored in EDTA anti-coagulant test tube.

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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).

Five percent (5.0%, w/v) stock solutions of the five antimalarial drugs were prepared by dissolving 2.5grams of each drug in 50ml of distilled water. Serial dilutions were made to obtain corresponding concentrations in the order; 0.8%, 0.6%, 0.4% and 0.2% (w/v).

Determination of Plasma Methaemoglobin Concentration:

The analysis of plasma methaemoglobin concentration was carried out within 60minutes of collecting the blood samples. The principle of this determination is because haemoglobin and methaemoglobin absorb light at different wavelengths, at 540nm and 630nm as their respective peak absorbance (Tietz, 1976). The approach employed the establishment procedure of lysing whole blood in distilled water.

Control:

In a test tube containing 5.0ml of distilled water, 0.02ml of whole blood was added. The mixture was allowed to stand for 60mintues at room temperature, and the absorbance was read at two different wavelength maximum (max), 540nm and 630nm using a spectrophotometer (Model 6400, Jenway).

Test:

The effect of each of the five antimalarial drugs on plasma methaemoglobin concentration was carried out by introducing 0.02ml of the specified concentrations (0.2 0.8% w/v) of each drug solution into separate test tubes. This was followed by the addition of 5ml of distilled water and 0.02ml of whole blood sample. The mixture was allowed to stand for 60mintues at room temperature, after which, the absorbance was read at 540nm and 630nm using a spectrophotometer (Model 6400, Jenway). The percentage plasma methaemoglobin was obtained with the formula.

Percentage Methaemoglobin

$$(Fe^{3+}) = \frac{(A630)2}{(A\,540)^2 + (A\,630)^2} \times \frac{100}{1}$$

(Tietz, 1976);

Where: A540 and A630 are absorbance at max of 540nm and 630nm respectively.

Statistical Analysis:

The experiment was 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.

RESULTS

The percentage of plasma methaemoglobin of total haemoglobin concentration in the presence of increasing experimental concentrations of the five antimalarial drugs is presented in Table 1 below.

In the absence of the five antimalarial drugs (control sample), the mean S.D of plasma methaemoglobin concentration (percentage) showed a genotype dependent variability. However, there was no statistically significant difference ($P<0.05$) of plasma methaemoglobin concentration between HbAA and HbAS genotype.

Quinine at 0.2% concentration exhibited the lowest capacity to generate methaemoglobin in human HbAA erythrocyte genotype with value at 2.17 0.21 percent, which however was not significantly different ($P<0.05$) from the control sample of the same genotype. Observation showed that 0.2% of another quinoline derivative, Halfan™ generated 3.72 2.48 percent of methaemoglobin in HbSS erythrocyte, being the lowest methaemoglobin concentration in this class of human erythrocyte genotype in the presence of the antimalarial drug. In a similar vein, the heterozygous genotype HbAS presented 2.20 2.10 percent methaemoglobin when 0.2% of Halfan™ was added to the blood sample. Although this value represented an increase in methaemoglobin concentration in the genotype (HbAS), it was not significantly different ($P<0.05$) from the control samples.

An overview of the results presented in Table 1 showed a general tendency of the five antimalarial drugs to elevate plasma methaemoglobin concentration in the three human erythrocyte genotypes in a concentration dependant manner.

It is worthwhile to note that Coartem™ amongst the five antimalarial drugs should the highest propensity to elicit increased plasma methaemoglobin concentration in the three genotypes. Specifically, at 0.8% concentration of Coartem™, plasma methaemoglobin concentrations in the three human erythrocyte genotypes were significantly different ($P< 0.05$) when statistically compared with the control sample of the three genotypes.

DISCUSSION

The pattern of variability of basal plasma methaemoglobin concentrations of the control samples amongst the three human genotypes, which was in the order: HbSS> HbAS > HbAA (Table1), conformed with earlier reports by Van Kiujk *et al.,* (1987) and Kirshner Zilber *et al.,* (1982). They noted that the primary reason for the relatively raised concentration of oxidized haemoglobin (methaemoglobin) in HbSS erythrocytes was higher production of superoxide ion by these erythrocytes compared to those of HbAA and HbAS erythrocytes. Furthermore, Orjih *et al.,* (1985) and Uwakwe, (1991) reported higher than normal level of erythrocyte endogenous oxidant (haemin) in HbSS genotype.

Haemin has a profound capacity to activate certain erythrocyte redox enzymes e.g NADH methaemoglobin reductase (Uwakwe, 1991) and its

presence at high concentration is attributable to the high level of haemolytic phenomenon peculiar to this haemoglobin variant cell (Orjih *et al.,* 1985). There is also the case of certain methaemoglobinopathies found in association with HbSS erythrocytes.

These are $HbM_{Boston,}$ HbM_{Iwate}, $HbM_{Hydepark}$ and $HbM_{Hammersmith}$, which are noted to have tendency towards spontaneous oxidation *in vivo*. This structurally and functionally defective haemoglobin are resistant to enzymatic reduction and exhibit high molecular stability (Martins *et al.,* 1983).

Therefore, their presence could as well result to the generation of significantly higher concentration of methaemoglobin than what is observed in normal haemoglobin erythrocyte (White *et al.,* 1978).

Early studies have noted that certain xenobiotics are capable of eliciting the formation and elevation of erythrocyte methaemoglobin concentration, thereby distorting the normal plasma haemoglobin (Fe^{2+})/ methaemoglobin (Fe^{3+}) ratio. Callister, (2003) reported the nitrates and anilines as the most common causes of methaemoglobin toxicity in man. This physiologic dysfunctional state (methaemoglobinemia) is presented as clinical cyanosis when plasma methaemoglobin concentration exceeds 15% (Hopkins, 1998).

Our present findings showed that at the four increasing experimental concentrations of Fansidar™, plasma methaemoglobin concentration was significantly elevated in a concentration dependent manner in the three human erythrocyte genotypes (Table 1). However, the oxidative potential of Fansidar™ at the four increasing experimental concentrations (0.2% - 0.8%) were not high enough to engender the oxidation of significant quantity of ferrous state haemoglobin (Fe^{2+}) to ferric state haemoglobin (Fe^{3+}) that is diagnostic of toxic methaemoglobinemia. These results agreed with the reports of Hopkins, (1998) and Callister, (2003). They noted that sulphonamide, a component of the drug Fansidar™, was capable of elevating plasma methaemoglobin concentration. This property is probably related to the chemical characteristics of sulphadoxine. Sulphadoxine is acidic in nature and present in plasma in anionic form that gives it high oxidative potentials (Milhous *et al.,* 1985). It is worthwhile to note that for the test experiment, values of plasma methaemoglobin concentrations of the three human erythrocyte genotypes when related to the blood volume and the experimental concentration of Fansidar™ administered seem to suggest a safe drug combination to minimize *in vitro* drug induced methaemoglobinemia. Furthermore, for the fact pyrimethamine is present at relatively higher concentration in the drug combination may also suggest pyrimethamine was not profoundly associated with methaemoglobin generation as earlier described. However, animal experiment studies have shown pyrimethamine can interfere with haematopoiesis (Matindale, 1993).

Table 1 Methaemoglobin Concentrations of Three Human Erythrocyte Genotypes in the Presence of Five Antimalarial Drugs

%Methaemoglobin

Drugs %	Fansidar™			Halfan™			Quinine			Coartem™			Chloroquine Phosphate		
Genotype	AA	AS	SS	AA	AS	SS	AA	AS	SS	AA	AS	SS	AA	AS	SS
0.2	3.48±0.75^{b}	3.31±2.45^{b}	4.98±3.72^{d}	2.18±0.88^{c}	2.20±2.10^{C}	3.72±2.18^{d}	2.17±0.21^{d}	2.25±0.97^{c}	3.83±2.11^{d}	3.81±0.74^{d}	3.32±2.41^{d}	4.01±2.11^{d}	2.63±0.69^{d}	2.61±1.84^{d}	4.81±2.61^{d}
0.4	3.28±0.64^{c}	3.46±1.98^{b}	5.63±2.51^{c}	2.35±1.10^{b}	2.30±1.31^{c}	3.81±3.22^{b}	2.48±0.58^{c}	2.50±1.61^{b}	4.78±3.21^{a}	4.98±0.89c	4.62±2.33^{c}	5.63±1.88^{c}	2.65±0.35^{c}	2.89±1.44^{b}	4.82±1.66^{c}
0.6	2.55±0.61^{d}	3.76±2.01^{a,b}	5.66±3.12^{b}	2.30±0.55^{b}	2.41±2.01^{b}	3.75±1.52^{c}	2.62±0.29^{b}	2.71±1.54a	4.13±1.08^{C}	5.31±0.42^{b}	4.95±1.01^{b}	6.99±1.32^{a}	2.83±0.12^{b}	2.84±0.83^{c}	5.01±0.99^{b}
0.8	3.93±1.99^{d}	4.32±1.94^{a}	5.85±1.49^{a}	2.48±2.66^{a}	3.03±1.82^{a}	3.95±2.62^{a}	2.86±0.79^{a}	2.65±0.45^{a}	4.21±1.82^{b}	6.81±0.21^{a}	6.41±1.21^{a}	6.72±1.17^{b}	2.86±0.83^{a}	3.02±0.98^{a}	5.23±3.61^{a}
0.0	2.14±0.74^{a}	2.17±1.82^{c}	3.64±4.48^{e}	2.14±0.74^{c}	2.17±1.82^{d}	3.64±4.48^{e}	2.14±0.74^{e}	2.17±1.82^{c}	3.64±4.48^{e}	2.14±0.74^{e}	2.17±1.82^{e}	3.64±4.48^{e}	2.14±0.74^{e}	2.17±1.82^{e}	3.64±4.48^{e}

Means in the column with the same letter are not significantly different at p< 0.05 according to LSD

Although the introduction of quinine in the three human red blood cell genotypes caused moderate but significant increase in plasma methaemoglobin concentration, the data presented in table 1 notwithstanding, did not indicate quinine as capable of inducing toxic or drug induced methaemoglobinemia *in vitro*. This observation by implication was in concord with the reports of Laurence *et al.,* (1997). They noted that quinine antimalarial action was more specific upon plasmodia parasites than on the elements of blood. Furthermore, amongst all the 4-aminoquinolines derivatives, Ursual, (1998) through *in vivo* investigations, established primaqunine and chloroqunine, but not quinine, as two antimalarial agents that can induce toxic methaemoglobinemia. The clinical presentation is exacerbated in individuals with impaired activity or deficiency in glucose -6- phosphate dehydrogenase (Ethnasios, 2000). However, reports from *in vivo* studies showed that the administration of quinine above a critical dose resulted in the diffusion of substantial quantity of the drug into the interior of the erythrocyte and predisposed the red cells to haemolysis elicited by tissue lytic factor (Laurance *et al.,* 1997, Chikezie, 2005).

The antimalarial activity of Halfan™ (halofantrine) is similar with the quinolines in that it forms a complex with ferritoporphyrin IX that is toxic to the malarial parasites (Mungthin *et al.,*1998). In a similar vein, the results of our present studies (Table 1) showed that Halfan™ like the quinolines do not possess the capacity to profoundly elevate plasma methaemoglobin in the three human erythrocyte genotypes that is diagnostic of methaemoglobinemia and presented as clinical cyanosis.

The relative high concentration of plasma methaemoglobin in the three human erythrocyte genotypes in the presence of increasing experimental concentrations of Coartem™ (artemether) when compared to the other four antimalarial drugs may be connected with the generation of free radicals that is associated with the metabolism of this drug in the red blood cells. The interaction of artemether with haem iron caused the cleavage of the drug endoperoxide bridge that engendered the formation of metabolites (free radicals) of high oxidative potentials (Tracy and Webster, 2001). The red blood cells methaemoglobin reduction systems may have been overwhelmed by these metabolically generated oxidizing species. In contrast, toxicity of the artemether endoperoxide is well tolerated and safe in human subjects when administered up to seven days at therapeutic doses (de Vries and Dien, 1996).

This paradoxical presentation obviously implied that the red blood cells antioxidant/reduction capacity was relatively not commensurate to the level of free radicals generated, which was a consequence of the metabolism of artemether by the red blood cells *in vitro.*

CONCLUSION:

The five antimalarial drugs caused a concentration dependent increase in plasma methaemoglobin concentration in the three human erythrocyte genotypes. Coartem™ (artemether), when compared to the other four antimalarial drugs (Fansidar™, Halfan™, Quinine and Chloroquine Phosphate™) showed a profound capacity to distort and elevate plasma methaemoglobin concentration. The other four antimalarial drugs showed a significant but minimal distortion of the redox status of haemoglobin molecules in the three human erythrocyte genotypes. Therefore, we may infer that the oxidative potentials of these four antimalarial drugs and their metabolites in the red cells did not overwhelm the erythrocyte methaemoglobin reducing capacity that could elicit the presentation *in vitro* toxic methaemoglobinemia.

Based on the foregoing interpretations of our results and observations, we suggest and recommend the possible utilization of the four antimalarial drugs (Fansidar™ , Halfan™, Quinine and Chloroquine phosphate) to provide for a safe and successful *in vitro* HbAA erythrocyte donor blood processing procedure for the eradication of malarial parasites. However, our present findings and conclusion are not exhaustive. Further research should be done to ascertain the capacity of these drugs, within the specified experimental concentrations, to change other biochemical indices of blood elements and effectively eradicate the malarial parasites obtained from donor-malarious blood.

Finally, it is worthwhile to note that the capacities of these drugs to alter and distort the redox status of haemoglobin by oxidative reactions are the consequence of their metabolic fate in the red blood cells (Coleman and Coleman 1996). Therefore, animal studies with these drugs may reveal paradoxical results and observation with respect to the capacity of these antimalarials to distort and elevate plasma methaemoglobin concentrations.

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