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Studies of Methemoglobin Concentrations of Three Human Erythrocyte Genotypes (Hb AA, Hb AS, and Hb SS) in the Presence of Five Anti-malarial Drugs

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Abstract

Background: Malaria remains the world's most devastating human parasitic infection. Our goal was to assess the capacity of increasing concentrations of five antimalarial drugs (Fansidar™, Halfan™, Quinine, Coartem™ and Chloroquine phosphate) to elicit the generation of methemoglobin in three human erythrocyte genotypes (Hb AA, Hb AS and Hb SS).

Materials and Methods: Spectrophotometric method was used for determination of plasma methaemoglobin concentration in the presence of 0.2 g%, 0.4 g%, 0.6 g% and 0.8 g% (w/v) of the five antimalarial drugs.

Results: The five antimalarial drugs showed a concentration dependent variability to cause the elevation of plasma methemoglobin concentration in the three genotypes. Specifically, Coartem™, exhibited the highest propensity to elevate plasma methemoglobin concentration. However, the other four antimalarial drugs showed a statistically significant ($P<0.05$) but minimal effect to cause elevation of plasma methemoglobin concentration. For instance, with Hb AS blood sample and at drug concentration of 0.8g%, methaemoglobin concentrations (percentage) 0f 3.03 ± 1.82, 2.65 ± 0.45, 6.41 ± 1.21, and 3.02 ± 0.98 were obtained for halfan, quinine, coartem and chloroquine phosphate, respectively. The control value was 2.17 ± 1.82% of methemoglobin.

Conclusion: The oxidative potentials of these four antimalarial drugs and their metabolites in the red cells did not overwhelm the erythrocyte methemoglobin reducing capacity that could elicit the presentation *in vitro* toxic methemoglobinemia.

Keywords: Chloroquine, Coartem, Erythrocyte, Fansidar, Genotype, Halfan, Quinine, Methemoglobin.

Introduction

Malaria remains the world's most devastating human parasitic infection, affecting more than 500 million people and causing 1.7 to 2.5 million deaths each year.[1] Nearly all human malaria is caused by four species of obligate intracellular protozoa of the genus Plasmodium.[2] 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.[3] This drug combination effectively blocks two enzymes involved in the biosynthesis of folinic acid within the parasite.[4] Artemether represents a major advance for the treatment of severe, multi-drug resistant falciparum malaria.[2] The drug is a more potent derivative of artemisinin, administered in the form of artemisinin combination therapy (ACT), artemether–lumefantrine combination drug therapy (Coartem™). 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 nonenzymatic polymerization to hemozoin. The failure to inactivate haem then kills the parasite via oxidative damage to membranes, digestive proteases, and possibly other critical biomolecules of the parasite.[5,6]

Concisely, methemoglobin is formed when ferrous iron (Fe^{2+}) of deoxyhemoglobin is converted to the ferric iron (Fe^{3+}) state on exposure of erythrocytes to oxidizing agents and oxygen free radicals.[7,8] Ferric iron (Fe^{3+}) state hemoglobin does not bind reversibly with oxygen. Studies have shown that methemoglobin is formed continuously in plasma but rarely exceeds 1.5% of total plasma hemoglobin.[8,9] Basically two enzymes, Diaphorase I and diaphorase II, in synergy with red blood cell non–enzymatiic antioxidants, ascorbic acid, glutathione, and other sulfydryl derivatives serve to minimize erythrocyte methemoglobin level.[10-14] Cyanotic presentation is typically observed at methemoglobin concentration greater than 15% and is often one of the earliest clinical evident features of methemoglobinemia.[7]

Ali and Kadaru[15] (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 study seeks to ascertain methemoglobin concentration of three human erythrocyte genotypes, Hb AA, Hb AS, and Hb SS 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 hemoglobin 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 Methods

Collection of Blood Samples

Five milliliters (ml) of confirmed human red blood cell genotypes, Hb AA and Hb AS were obtained from subjects/volunteers within the age bracket of 18-35 years old. Blood samples of Hb SS 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 venipunture and stored in EDTA anti-coagulant test tube.

Anti-malarial Drugs

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

Determination of Plasma Methaemoglobin Concentration

The analysis of plasma methemoglobin concentration was carried out using the spectrophotometric method described by Tietz (1976).[9] The analysis was done within 60 minutes of collecting the blood samples. The principle of this determination is based on the fact that hemoglobin and methemoglobin absorb light at different wavelengths, at 540 nm and 630 nm as their respective peak absorbance.[9] The approach employed the establishment procedure of lysing whole blood in distilled water.

Control

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

Test

The effect of each of the five antimalarial drugs on plasma methemoglobin concentration was carried out by introducing 0.02 ml of the specified concentrations (0.2-0.8 g% w/v) of each drug

Table 1. *The percentage of plasma methemoglobin of total hemoglobin concentration in the presence of increasing experimental concentrations of the five antimalarial drugs in three human erythrocyte genotypes.*

Drugs (g %)	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. AA, AS, and SS are the different blood hemoglobin genotypes

solution into separate test tubes. This was followed by the addition of 5 ml of distilled water and 0.02 ml of whole blood sample. The mixture was allowed to stand for 60 mintues at room temperature, after which, the absorbance was read at 540 nm and 630 nm using a spectrophotometer (Model 6400, Jenway). The percentage plasma methemoglobin was obtained with the formula:

$$\text{Percentage Methaemoglobin } (Fe^{3+}) = \frac{(A630)^2}{(A540)^2 + (A630)^2} \times \frac{100}{1}$$

where A540 and A630 are absorbance at λmax of 540 nm and 630 nm, respectively (Tietz, 1976).

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 methemoglobin of total hemoglobin concentration in the presence of increasing experimental concentrations of the five antimalarial drugs is presented in table 1. In the absence of the five antimalarial drugs (control sample) the mean ± S.D of plasma methemoglobin concentration (%) showed a genotype dependent variability. However, there was no statistically significant difference (P<0.05) in plasma methemoglobin concentration between Hb AA and Hb AS genotypes.

Quinine at 0.2% concentration exhibited the lowest capacity to generate methemoglobin in human Hb AA enthrocyte 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 methemoglobin in Hb SS erythrocyte, being the lowest methemoglobin concentration in this class of human erythrocyte genotype in the presence of the antimalarial drug. In a similar way, the heterozygous genotype Hb AS presented 2.20 ± 2.10 percent methemoglobin when 0.2% of Halfan™ was added to the blood sample. Although this value represented an increase in methemoglobin concentration in the genotype (Hb AS), it was not significantly different (P<0.05) from the control samples.

An overview of the results presented in table 1 shows a general tendency of the five antimalarial drugs to elevate plasma methemoglobin concentration in all three human erythrocyte genotypes in a concentration dependant manner. It is worthwhile to note that Coartem™ amongst the five antimalarial drugs showed the highest propensity to elicit increased plasma methemoglobin concentration in the three genotypes. Specifically, at 0.8% concentration of Coartem™, plasma methemoglobin 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 methemoglobin concentrations of the control samples amongst the three human genotypes, which was in the order: HbSS> HbAS > HbAA (table1), was in concordance with earlier reports.[16] They noted that the primary reason for the relatively raised concentration of oxidized hemoglobin (methemoglobin) in Hb SS erythrocytes was the higher production of superoxide ion by these erythrocytes compared to those of Hb AA and Hb AS erythrocytes. Furthermore, Orjih et al[17] (1985) and Uwakwe[18] (1991) reported higher than normal level of erythrocyte endogenous oxidant (haemin) in Hb SS genotype. Haemin has a profound capacity to activate certain erythrocyte redox enzymes e.g NADH methemoglobin reductase,[18] and its presence at high concentration is attributable to the high level of haemolytic phenomenon peculiar to this hemoglobin variant cells.[17] There is also the case of certain methemoglobinopathies found in association with Hb SS erythrocytes.

These are Hb M_{Boston}, Hb M_{Iwate}, $HbM_{Hydepark}$, and $HbM_{Hammersmith}$ which are noted to have tendency towards spontaneous oxidation in vivo. Basically, these structurally and functionally defective hemoglobins are resistant to enzymatic reduction and exhibit high molecular stability.[19] Therefore,

their presence could as well result to the generation of significantly higher concentration of methemoglobin than what is observed in normal hemoglobin erythrocyte.[20]

Early studies have noted that certain xenobiotics are capable to elicit the formation and elevation of erythrocyte methemoglobin concentration, thereby distorting the normal plasma hemoglobin (Fe^{2+})/ methemoglobin (Fe^{3+}) ratio. Callister (2003) reported the nitrates and anilines as the most common causes of methemoglobin toxicity in humans.[8] This physiologic dysfunctional state (methemoglobinemia) is presented as clinical cyanosis when plasma methemoglobin concentration exceeds 15%.[7]

Our present findings showed that at the four increasing experimental concentrations of Fansidar™, plasma methemoglobin 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 a significant quantity of ferrous state hemoglobin (Fe^{2+}) to ferric state hemoglobin (Fe^{3+}) that is diagnostic of toxic methemoglobinemia. These results agreed with the reports of Ursula (1998) and Callister (2003).[7,8] They noted that sulfonamide, a component of the drug Fansidar™, was capable of elevating plasma methemoglobin concentration. This property is probably related to the chemical characteristics of sulfadoxine. Sulfadoxine is acidic in nature and present in plasma in anionic form which gives it high oxidative potentials.[4] It is worthwhile to note that for the test experiment, values of plasma methemoglobin 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 methemoglobinemia. Furthermore, the fact that pyrimethamine is present at a relatively higher concentration in the drug combination may also suggest pyrimethamine was not profoundly associated with methemoglobin generation as earlier described. However, animal experiment studies have shown pyrimethamine can interfere with hematopoiesis.[21]

Although the introduction of quinine in the three human red blood cell genotypes caused moderate but significant increase in plasma methemoglobin concentration, the data presented in table 1 did not indicate quinine to be capable of inducing toxic or drug–induced methemoglobinemia in vitro. This observation, by implication, was in concordance with the reports of Laurence et al (1997).[22] They noted that quinine antimalarial action was more specific upon plasmodia parasites than on the elements of blood. Furthermore, amongst all the 4-aminoquinoline derivatives, Ursula (1998) through in vivo investigations, established primaquinine and chloroquinine but not quinine as two antimalarial agents that can induce toxic methemoglobinemia.[7] The clinical presentation is exacerbated in individuals with impaired activity or deficiency of glucose-6-phosphate dehydrogenase.[24] 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 hemolysis elicited by tissue lytic factor.[23]

The antimalarial activity of Halfan™ (halofantrine) is similar to that of the quinolines, since it forms a complex with ferritoporphyrin IX which is toxic to the malarial parasites.[24] In a similar way, the results of our present study (table 1) showed that Halfan™ like the quinolines does not possess the capacity to profoundly elevate plasma methemoglobin in the three human erythrocyte genotypes that is diagnostic of methemoglobinemia and presented as clinical cyanosis.

The relative high concentration of plasma methemoglobin 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 not be unconnected with the generation of free radicals which 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 which engendered the formation of metabolites (free radicals) of high oxidative potentials.[2] The red blood cells methemoglobin 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.[25]

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 methemoglobin 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 methemoglobin concentration. The other four antimalarial drugs showed a significant but minimal distortion of the redox status of hemoglobin molecules in the three human erythrocyte genotypes. Therefore, we may conclude that the oxidative potentials of these four antimalarial drugs and their metabolites in the red cells did not overwhelm the erythrocyte methemoglobin reducing capacity that could elicit the presentation in vitro toxic methemoglobinemia.

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) for a safe and successful in vitro Hb AA 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 malarious donor blood.

Finally, it is worthwhile to note that the capacities of these drugs to alter and distort the redox status of hemoglobin by oxidative reactions are the consequence of their metabolic fate in the red blood cells.[26] 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 methemoglobin concentrations.

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