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Full Length Research Paper

Studies of human HbAA erythrocyte osmotic fragility index of non malarious blood in the presence of five antimalarial drugs

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The capacity of human HbAA erythrocytes of non malarious blood to withstand osmotic stress in the presence of five antimalarial drugs, Chloroquine phosphate, Quinine, Fansidar™, Coartem™ and Halfan™ was studied by *in vitro* investigations. Aqueous solutions of four increasing concentrations of 0.2, 0.4, 0.6 and 0.8% (w/v) of the drugs were used in this investigation. The spectrophotometric method was employed in ascertaining the osmotic fragility index of the erythrocytes. The mean corpuscular fragility (MCF) index (X ± S.D) of the control sample was 0.351 ± 0.06 g/100 ml. The MCF values of the control and test samples were compared ($p < 0.05$). The results showed that Chloroquine phosphate and Fansidar™ exhibited diminishing capacity to stabilize the red blood cell membrane in a concentration dependent manner while Halfan™, Coartem™ and Quinine elicited increasing propensity to disrupt erythrocyte membrane integrity.

Key words: Erythrocyte osmotic fragility, quinine, fansidar™, chloroquine phosphate, coartem™, halfan™, mean corpuscular fragility (MCF).

INTRODUCTION

The red blood cells or erythrocytes are by far the most numerous of the blood cells. A cell measures approximately 8 mm in diameter, appears as a biconcave disc with no nucleus and a life span of 120 days (Murray, 2003). The erythrocyte is a highly dynamic functional unit although relatively simple when compared with other somatic cells. It is well organized for the biosynthesis of over 400 million molecules of haemoglobin that ultimately make up to 95% of its dry weight to fulfill oxygen transporting function (Martin, 1983). There is a required chemical structure of the membrane that is thermodynamically stable and metabolically active for selective permeability of materials and information (Singer and Nicolson, 1972; Lehniger, 1993).

A measure of the capacity of red blood cells to withstand osmotic stress defines its osmotic fragility index (Oyewale and Ajibade, 1990). The test is clinically useful for diagnosis of hereditary spherocytosis (Kumar, 2002) and to ascertaining the stability and functionality of erythrocyte plasma membrane (Dacie, 1985; Krogmeiger et al., 1993). The relative composition of fatty acid, phospholipids and cholesterol of red blood cells plasma membrane is inextricably connected with membrane stability and function (Cooper, 1969; Csordes and Schaufenstein, 1984; Sako et al., 1989). Recently, Aldrich et al. (2006) reported that the morphology, size and population of nucleated red blood cells are other physiologic factors that can significantly alter erythrocyte membrane permeability and stability.

Furthermore, certain xenobiotics such as primaquine and fava beans extracts have been reported as agents that can interfere with the redox status of red blood cells especially in those individuals with impaired glucose-6-phosphate dehydrogenase activity (Mayes, 1983; Champe et al., 2005; Ojo et al., 2006). The red blood cells of such individuals have compromised capacity to withstand osmotic stress. In addition, the malarial parasites, *Plasmoduim spp* are biological agents of red blood cell haemolysis (Ajayi et al., 2003). Some compounds with consi-

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derable influence on membrane integrity or destabilization affect their actions by direct chemical contact with biomolecules that constitute the architechtural structure of plasma membrane (Champe et al., 2005). Other compounds act in such a way that the activity of certain erythrocyte redox enzymes, such as glutathione reduce-tase (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.

This research work was designed to investigate the effect of commonly used antimalarials comprising the quinolines (Chloroquine, Phosphate, Quinine and halofantrin (Halfan^{TM}), artemether (Coartem^{TM}) and sulphadoxine – pyrimethamine (SP) Fansidar^{TM} on membrane integrity of red blood cells obtained from non-malarious blood. The findings of this study will help establish the contributions of these anti-malarials to promoting or diminishing erythrocyte membrane integrity in the absence of the malarial parasite.

MATERIALS AND METHODS

Collection and preparation of blood samples

A total of twenty-one (21) blood samples of human HbAA genotype were collected via venepuncture from apparently healthy and non-malarious human volunteers between the ages of 18 and 35 years.

Blood (5 ml) was collected from each donor who had been screened for glucose-6-phosphate dehydrogenase deficiency and stored in EDTA tubes. One milliliter of each blood sample was introduced into a 10 ml test tube and 5 ml of physiological saline solution (PSS) (0.9% w/v) was added. The red blood cell suspendsion was mixed and centrifuged at 3500 rpm for 5 min. The supernatant was aspirated using a Pasteur pipette while the sediment which constituted the red blood cells was re-suspended in 5 ml of PSS. The procedure was repeated twice after which the red blood cells were finally suspended in 2 ml of PSS and used for analysis within 6 h of collection.

Anti-malarial drugs

Five (5) antimalarial drugs were used in this study: Fansidar^{TM} (Swiss SWIPHA) Pharmaceutical Nigeria Ltd), Coartem^{TM} (Beijing NORVATIS Pharmaceutical Company, Beijing, China), Chloroquine phosphate (MAY and BAKER, Pharmaceutical Company Nigeria, Plc), Halfan^{TM} (SMITHKLINE BEECHAM Laboratories Pharmaceutical Company, France) and Quinine (BDH, UK).

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

Erythrocyte osmotic fragility tests

Determination of red blood cells osmotic fragility was carried out based on the method described by Dewey et al. (1982) with minor modifications as reported by Chikezie (2007). The fraction of red blood cells lysed when suspended in saline solution of varying concentrations was investigated by spectrophotometric method. A stock solution of buffered sodium chloride, osmotically equivalent to 100 g/L NaCl was prepared as follows: NaCl (90 g), $Na_2HPO_4.2H_2O$ (17.1 g) and $NaH_2\ PO_4.\ 2H_2O$ (2.43 g) were dissolved in 1 litre of distilled water. Dilution equivalents of 9.0, 7.0, 5.0, 4.0 and 3.0 g/l NaCl respectively were prepared. Each dilution had a final volume of 50 ml (Chikezie and Ibegbulem, 2004).

Five milliliter of each saline solution (9.0 – 3.0 g/l NaCl) was introduced into corresponding test tubes while 5 ml of distilled water was added to the sixth test tube. A 0.5 ml of each antimalarial drug solution of varying concentrations as specified was delivered into each of the given set of test tubes (1 – 6). To each test tube, 0.05 ml of the red blood cell suspension was added and mixed thoroughly by inverting the tubes several times. For the control experiment, the same procedure was repeated but devoid of antimalarial drug solution. The suspensions were allowed to stand for 30 min at room temperature after which the contents were centrifuged at 1200 rpm for 5 min. The relative amount of haemoglobin released into the supernatant was determined with the use of a spectrophotometer (SPECTRONIC 20, Labtech – Digital Blood Analyzer®) at maximum wavelength (λmax) of 540 nm. The PSS and distilled water served as blank and 100% lysis point, respectively.

Evaluation of percentage haemolysis and stabilization of red blood cells

The quotient of absorbance of the content of individual corresponding test tubes (1 – 5) and the sixth test tube were obtained and multiplied by a factor of 100. The range of values represented the percentage of erythrocyte lysis at each saline concentration (9.0 – 3.0 g/l NaCl), respectively.

The corresponding concentration of saline solution (NaClg/l) that caused 50% lysis of red blood cells was the mean corpuscular fragility (MCF) index (Dewey et al., 1982; Krogmerer et al., 1993). The MCF values were interpolated from the cumulative erythrocyte osmotic fragility curves obtained by plotting the percentage lysis against saline concentrations (not shown).

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

$$\% \text{ Stability} = \frac{\text{MCFTest} - \text{MCFcontrol}}{\text{MCFcontrol}} \times 100$$

Statistical analysis

The data were analyzed by Student's t- test as described by Pearson and Hartley (1966). Mean $p<0.05$ was considered significant.

RESULTS

The contribution and capacity of the five antimalarial drugs to distort or stabilize erythrocyte membrane is represented and interpreted based on the MCF values presented in Table 1. According to Dewey et al. (1983), when MCF values of the test sample are greater in numerical value than the reference or control sample, it generally connotes enhanced erythrocyte fragility.

MCF values are means of 3 determinations ± S.D:

a: Difference in MCF values are significant ($p<0.05$)
d: Percentage of membrane destabilization
s: Percentage of membrane stabilization

Table 1. Human HbAA erythrocyte mean corpuscular fragility and stability in the presence of Halfan™, Coartem™ and Quinine.

Drug concentration (%)	Halfan™		Coartem™		Quinine	
	MCF (g/100 ml)	Stability	MCF (g/100 ml)	Stability	MCF (g/100 ml)	Stability
0.0(control)	0.351±0.06	0.00	0.351±0.06	0.000	0.351±0.006	0.00
0.2	0.362±0.04a	3.13d	0.349±0.06	0.57s	0.343±0.05a	2.28s
0.4	0.374±0.07a	6.55d	0.388±0.03a	10.54d	0.351±0.04	0.00
0.6	0.393±0.05a	11.97d	0.389±0.04	10.83d	0.354±0.12	1.71d
0.8	0.401±0.05a	14.25d	0.391±0.03a	11.40d	0.364±0.02a	3.70d

Table 2. Human HbAA erythrocyte mean corupscular fragility and stability in the presence of Chloroquine phosphate and Fansidar™.

Drug concentration (%)	Chloroquine	Phosphate	Fansidar™	
	MCF (g/100 ml)	Stability (%)	MCF (g/100 ml)	Stability (%)
0.0(control)	0.351±0.06	0.00	0.351±0.06	0.000
0.2	0.307±0.03	12.54s	0.331±0.08	5.70s
0.4	0.325±0.04	7.41s	0.332±0.03	5.41s
0.6	0.332±0.08	5.41s	0.335±0.06	4.56s
0.8	0.344±0.02	1.99s	0.338±0.04	3.70s

MCF values are means of 3 determinations ± S. D.

Therefore, within the concentration range of (0.2-0.8%), (0.4-0.8%) and (0.6-0.8%) of Halfan™, Coartem™ and Quinine respectively, these three drugs promoted red blood cell membrane destabilization. However, at relatively low concentrations, specifically at 0.2%, Coartem™ and Quinine exhibited membrane stabilizing effect [Coartem™] = 0.2%, MCF = 0.349+0.06g/100 ml; percentage stability = 0.57%: $p>0.05$), ([Quinine] = 0.2%; MCF = 0.343+0.05 g/100 ml; percentage stability = 2.28%; $p<0.05$). A cursory look at Table 1 showed that the capacity of the three drugs to destabilize red blood cell membrane was in the order: Halfan™ >Coartem™> Quinine.

The results in Table 2 showed Chloroquine phosphate and FansidarTM as agents of red blood cell stabilization. Within the limits of experimental concentrations of the two drugs, chloroquine phosphate at 0.2% apparently exhibitted the highest capacity to promote membrane stability (MCF = 0.307+ 0.03 g/100ml; percentage stability = 12.54%. $p<0.05$). However, the contributions and capacities of these two drugs to stabilize the red blood cell membrane diminished in a concentration dependent manner.

DISCUSSION

In this study, the pattern of lysis of human red blood cells suspended in varying concentrations of saline solution conformed to previous findings (Wegrzynowicz et al., 1972; Dewey et al., 1982; Aldrich et al., 2006). The present report showed the MCF value of human red blood cells suspension obtained from venous blood was 0.351+0.06 g/100ml. In the same vein, Wegrzynowieze et al., (1972) reported an MCF value of 0.350g/100ml erythrocytes from bream Albramis brama (L). In contrast to our present findings, Dewey et al., 1982 reported MCF values of 0.465 and 0.415 g/100ml heparinized blood obtained from two strains of allophenic mice. The difference in MCF values may not be unconnected with the observations of Kafka and Yermiahu (1998) who noted a significant increase in osmotic resistance of erythrocytes obtained from blood samples stored in EDTA compared to heparinized blood. Likewise, Aldrich et al. (2006) and more recently, Chikezie et al. (2007) established variations in erythrocyte osmotic resistance amongst animal kingdoms and strains. Furthermore, red blood cell osmotic fragility index is known to be influenced by certain environmental (Wegrzynowicz et al., 1972; Sako et al., 1989), physiological (Dewey et al., 1982; Lux 1999; Murray et al., 2003) and pathologic (Sackey, 1999; Ojo et al., 2006) factors. Therefore, every laboratory should determine its own reference value which would reflect on local environmental and technical factors.

Previous studies by Soforawa (1975), Dean and Schechter (1978), (Uwakwe and Ezeh, 2000) and Ali and Kadaru (2005) reported the capability of xenobiotics to interfere with red blood cell membrane integrity and stability. In concord with these reports, our present studies have shown the five antimalarial drugs interfered with red blood cell membrane stability. The destabilizing effect on red blood cell membrane by aqueous solutions of Halfan™, Coartem™ and Quinine increased proportionnately with increasing concentrations of the three drugs (Table 1). However, the seemingly capacity of Chloroquine phosphate and Fansidar™ to stabilize red blood

cell membrane dwindled with increasing concentration of the two drugs (Table 2). These observations were obvious indications that the five antimalarial drugs promoted red blood cell membrane disintegration in proportion with the experimental drug concentrations administered to the suspension of red blood cells. In agreement with our results, Ali and Kadaru (2005) reported that sulphadoxine – pyrimethamine (SP) (Fansidar™) treated blood samples incubated for 48 h did not cause appreciable haemolysis except in samples with final SP concentration greater than 300 mg/L. The results in Tables 1 and 2 did not show the two quinolines, Chloroquine phosphate and quinine as major contributors to membrane disintegration. However, from the pattern of membrane stability under varying concentrations of the two drugs, we presumed that at relatively higher concentrations, a paradoxical property might be exhibited.

The significant increase ($p<0.05$) in MCF value of red blood cells in the presence of artemether (Coartem™) and halofantrine (Halfan™) could be described from what has been established of their biochemical transformation in red blood cells. Artemether has a peroxide group in its structure. When the peroxide is exposed to high iron concentration as found in the red blood cells, the drug molecule becomes unstable. The unstable molecule creates free radicals that are highly destructive to the non-covalent assemblies of erythrocyte plasma membrane (Artemis, 2007).

Most drugs are metabolically inert before transformed to biologically active compounds (Zakrzewski, 1983). Therefore, the interference of erythrocyte membrane stability by these five antimalarial drugs as reported in our study may be consequences of their metabolic fate in red blood cells. Some of these xenobiotics effect their membrane destabilizing property by generating high levels of free radicals in proportion to the concentration of the drug administered, which can overwhelm the capacity of the redox enzymes to maintain and sustain membrane integrity. We recommend that investigation to ascertain the relationship between membrane stability and cellular activities of these redox enzymes in the presence of these antimalarial drugs should be carried out.

Furthermore, other membrane destabilizing agents may act by direct interaction with architectural membrane proteins and enzymes, thereby modifying their structure/ function relationship that is necessary and required for membrane integrity. Chloroquine and Quinine have been described to act by modifying certain protozoan proteins (Tracy and Webster, 2001).

Finally, our present findings are not exhaustive and conclusive since these drugs may exhibit profound variations under *in vivo* studies because marked differences of metabolic fate and end products of xenobiotics exist amongst organs and tissues of animals (Klaasen, 1986).

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