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Studies of Human HbAA Erythrocyte Osmotic Fragility Index of Non-Malarious Blood in the Presence of Five Anti-malarial Drugs

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Abstract

Background: The capacity of human HbAA erythrocytes of non-malarious blood to withstand osmotic stress in the presence of five antimalarial drugs, Chloroquine phosphate, Quinine, FansidarTM, CoartemTM and HalfanTM was studied in vitro.

Materials and Methods: Aqueous solutions of four increasing concentrations of the drugs used in this investigation were in the order: 0.2%, 0.4%, 0.6% and 0.8% (w/v). Spectrophotometric method was employed to ascertain 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/100ml. The MCF values of the control and test samples were statistically compared (p value = 0.05).

Results: At the drug concentration of 0.8g, MCF values (g) of 0.401±0.005, 0.391±0.003, 0.364±0.02, 0.344±0.02 and 0.338±0.04 were obtained for halfan, coartem, quinine, chloroquine phosphate and fansidar, respectively.

Conclusion: An overview of the results showed that in a concentration dependent manner, Choroquine phosphate and FansidarTM exhibited a diminishing capacity to stabilize red blood cell membrane while HalfanTM CoartemTM and Quinine elicited an increasing propensity to disrupt erythrocyte membrane integrity.

Keywords: osmotic fragility, quinine, fansider™, chlonquine phosphate, coartem™, halfan™

Introduction

Red blood cells or erythrocytes are by far the most numerous of the blood cells. A red blood cell measures approximately 8µm in diameter, appears as a biconcave disc with no nucleus and a life span of 120 days.[1] 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 hemoglobin that ultimately make up to 95% of its dry weight to fulfill oxygen transporting function.[2] There is a required chemical structure of the membrane that is thermodynamically stable and metabolically active for selective permeability of materials and information.[3,4]

The capacity of red blood cells to withstand osmotic stress defines its osmotic fragility index.[5] The test is clinically useful for the diagnosis of hereditary spherocytosis[6] and to ascertain the stability and functionality of erythrocyte plasma membrane.[7,8]

The relative composition of fatty acid, phospholipids and cholesterol of red blood cells plasma membrane is inextricably connected with membrane stability and function.[9,10,11] 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.[12]

Furthermore, certain xenobiotics such as primaquine and fava beans extracts have been

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reported as agents that can interfere with the redox status of red blood cells especially in those individuals with an impaired glucose-6-phosphate dehydrogenase activity.[13,14,15] The red blood cells of such individuals have a compromised capacity to withstand osmotic stress. Also, the malarial parasites, Plasmoduim spp, are biological agents that cause red blood cell haemolysis.[16] Some compounds, with a considerable influence on membrane integrity or destabilization, exert their actions through a direct chemical contact with biomolecules that constitute the architechtural structure of the plasma membrane.[14] Other compounds act in such a way that the activity of certain erythrocyte redox enzymes, such as glutathione reductase,[17,18] glutathione peroxidise,[13] and glucose–6-phosphate dehydrogenase[13-15] required for membrane integrity are compromised.

This research work investigated the capacity of commonly used antimalarials comprising the quinolines (Chloroquine Phosphate, Quinine and halofantrin (HalfanTM) artemether (CoartemTM) and sulphadoxine – pyrimethamine (SP) [FansidarTM] to distort or interfere with membrane integrity of red blood cells obtained from non-malarious blood. This finding will establish exclusive contributions of these antimalarials to promote or diminish membrane integrity in the absence of malarial parasites.

Materials and Methods

Collection and Preparation of Blood Samples

A total of twenty-one[21] blood samples of human HbAA genotype were collected by venipuncture from apparently healthy and non-malarious subjects/volunteers within the age bracket of 18-35 years old.

A blood volume of 5 ml was obtained from donors who had been screened for glucose-6-phosphate dehydrogenase deficiency and was then stored in EDTA anti-coagulant test tubes. One ml of each blood sample was introduced into a 10 ml test tube and 5ml of physiological saline solution (0.9% w/v) was added. The red blood cell suspension was mixed and centrifuged at 3500 r.p.m. for 5 minutes. The supernatant was removed by Pasteur pipette while the sediment which was formed by red blood cells was resuspended in 5ml of physiological saline solution. The procedure was repeated twice and then the red blood cells were finally suspended in 2ml of physiological saline solution and used for analysis within 6 hours of collection and preparation.

Anti-Malaria Drugs

Five[5] antimalarial drugs were used in this study. FansidarTM (Swiss Swipha) Pharmaceutical Nigeria Ltd), CoartemTM (Beijing Norvatis Pharmaceutical Company, Beijing, China), Chloroquine phosphate (May and Baker, Pharmaceutical Company Nigeria, Plc), HalfanTM (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 2.5 grams of each drug in 50 ml of distilled water. Serial dilutions were made to obtain corresponding concentrations (g) in the order: 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,[19] with minor modifications as reported by Chikezie, 2007.[20] The fraction of red blood cells lysed when suspended in saline solution of varying concentrations was investigated through spectrophotometry.

A stock solution of buffered sodium chloride, osmotically equivalent to 100g/L NaCl, was prepared as follows: NaCl (90 g), Na2HPO4.2H2O (17.1g), and NaH2PO4.2H2O (2.43g) 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 50ml.[21]

Five milliliter (5.0 ml) of each saline solution (9.0 – 3.0 g/l Nacl) was introduced into corresponding test tubes while 5.0ml of distilled water was added to the 6th test tube. A 0.5ml of each anti-malaria drug solution of varying concentrations as specified was delivered into each of the given sets of test tubes (1st – 6th). To each test tube, 0.05ml

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of red blood cells suspension was added and mixed thoroughly by inverting the tubes several times. For the control experiment, the same procedure was repeated but devoid of anti-malaria drug solution. The suspensions were allowed to stand for 30 minutes at room temperature after which the content was centrifuged for 5 minutes at 1200r.p.m. The relative amount of hemoglobin released into the supernatant was determined with the use of a spectrophotometer (Spectronic20,Labtech– Digital Blood Analyzer) at the maximum wavelength (λmax) of 540 nm. The physiological saline solution and distilled water served as blank and 100% lysis point, respectively.

Evaluation of the Percentage of s- Hemolysis and Stabilization of Red Blood Cells

The quotient of absorbance of the content of individual corresponding test tubes (1st – 5th) and the 6th 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 (NaCl g/l) that caused 50% lysis of red blood cells was the mean corpuscular fragility (MCF) index.[8,19] The MCF values were interpolated from the cumulative erythrocyte osmotic fragility curves obtained by plotting the percentage of lysis against saline concentrations (not shown).

The relative capacity of the five anti-malaria 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.[22] Thus, % lysis= MCFTest – MCFcontrol x 100/ MCFcontrol

Table 1*: Human HbbA Erythrocyte Mean Corpuscular Fragility and Stability in the Presence of Halfan™, Coartem™ and Quinine.*

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

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 2: *Human HbAA Erythrocyte Mean Corupscular Fragility and Stability in the Presence of Chloroquine phosphate and Fansidar™.*

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

MCF values are means of 3 determinations + S.D

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Statistical Analysis

The data were analyzed using student's t-distribution test of significance.

Results

The contribution and capacity of the five anti-malaria 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,[19] when MCF values of the test sample is greater in numerical value than the reference or control sample, it generally connotes enhanced erythrocyte fragility.

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

The results in table 2 show that Chloroquine phosphate and Fansidar^{TM} are agents of red blood cell stabilization. Within the limits of experimental concentrations of the two drugs, chloroquine phosphate at 0.2% apparently exhibited the highest capacity to promote membrane stability (MCF = 0.307$\pm$0.03g/100ml; %stability = 12.54. $p<0.05$). However, the contributions and capacities of these two drugs to stabilize red blood cell membrane diminished in a concentration dependent manner.

Discussion

In this study, the pattern of lysis of human red blood cells when suspended in varying concentrations of saline solution conformed to previous findings as reported by Wegrzynowicz *et al.,* 1972, Dewey *et al.,* 1982, and Aldrich *et al.,* 2006.[12,19,22] The present report showed that the MCF value of human read blood cells suspension obtained from venous blood was 0.351$\pm$0.06 g/100 ml. In the same way, Wegrzynowieze *et al.,* 1972, reported 0.350 g/100ml as the MCF value of erythrocytes from bream *Abramis Brama* (L).[23] In contrast to our findings, Dewey *et al.,* 1982 reported 0.465g/100ml and 0.415g/100ml as MCF values of heparinized blood obtained from two strains of allophenic mice (19). The difference in MCF values may not be unconnected with the observations of Kafka and Yermiahu, 1998, who noted a significant increase in the osmotic resistance of erythrocytes obtained from blood samples which were stored in EDTA anti-coagulant test tubes compared to heparinized blood.[24] Likewise, Aldrich *et al.,* 2006, and more recently, Chikezie *et al.,* 2007, established variations in erythrocyte osmotic resistance amongst animal kingdoms and strains.[12, 20] Furthermore, red blood cell osmotic fragility index is known to be influenced by certain environments,[11,23] physiological[1, 19, 25] and pathologic[15, 26] factors. Therefore, every laboratory should determine its own reference value, which would reflect 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.[27-30] In accordance with these reports, our present study showed that the five antimalarial drugs interfered with red blood cell membrane stability. The destabilizing effect on red blood cell membrane by aqueous solutions of Halfan^{TM}, Coartem^{TM} and Quinine increased proportionately with increasing concentrations of the three drugs (Table1). However, seemingly, the capacity of Chloroquine phosphate and Fansidar^{TM} to stabilize red blood cell membrane dwindled with increasing the concentration of the two drugs (Table2). These observations were obvious indications that the five anti-malarial drugs promoted red blood cell membrane disintegration proportionate to the concentrations of the experimental drugs which were administered to the suspension of red blood cells. In agreement with our results, Ali and Kadaru, 2005 reported that sulphadoxine –

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pyrimethamine (SP) (Fansidar™) treated blood sample, incubated for 48 hours, did not cause appreciable haemolysis except for samples with a final SP concentration of greater than 300 μg/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 the MCF value of red blood cells in the presence of artemether (Coartem™) and halofantrine (Halfan™) could be described from what has been established about their biochemical transformation in red blood cells. Artemether has a perioxide group in its structure. When the peroxide comes in contact with high iron concentrations, 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.

Most drugs are metabolically inert before they are transformed to biologically active compounds.[31] Therefore, the interference of these five anti-malaria drugs with erythrocyte membrane stability as reported in our research is consequences of their metabolic fate in red blood cells. Some of these xenobiotics exert their membrane destabilizing properties by generating high levels of free radicals proportionate 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 antimalaria 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 through modifying certain protozoan proteins.[32]

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

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