

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

Paul Chikezie MSc¹, Augustine Uwakwe PhD², Comfort Monago PhD²

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

2. Department of Biochemistry, University of Port-Harcourt, Port-Harcourt, Nigeria

Corresponding Author: Augustine Uwakwe PhD in Biochemistry, Department of Biochemistry, University of Port-Harcourt, Port-Harcourt, Nigeria, Tel: +23408055404143, Email: uwadik@gmail.com

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, Chloroquine 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, fansidarTM, chloroquine phosphate, coartemTM, halfanTM

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 spherocytosis⁶ 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

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, *Plasmodium* 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 architectural 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 peroxidase,¹³ and glucose-6-phosphate dehydrogenase¹³⁻¹⁵ 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-one²¹ 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

Five⁵ antimalarial drugs were used in this study. FansidarTM (Swiss Swipha) Pharmaceutical Nigeria Ltd), CoartemTM (Beijing Novartis 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

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 (λ_{max}) 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= $\frac{MCF_{Test} - MCF_{control}}{MCF_{control}} \times 100$

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

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 HalfanTM, CoartemTM and Quinine, respectively, these three drugs promoted red blood cell membrane destabilization. However, at a relatively low concentration, specifically at 0.2%, CoartemTM and Quinine exhibited a membrane stabilizing effect [CoartemTM] = 0.2%, MCF = 0.349±0.06g/100ml; % stability = 0.57: p>0.05) ([Quinine] = 0.2%; MCF = 0.343±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: HalfanTM >CoartemTM > Quinine.

The results in table 2 show that Chloroquine phosphate and FansidarTM 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±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 red blood cells suspension

obtained from venous blood was 0.351±0.06 g/100 ml. In the same way, Wegrzynowicz 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 HalfanTM, CoartemTM and Quinine increased proportionately with increasing concentrations of the three drugs (Table1). However, seemingly, the capacity of Chloroquine phosphate and FansidarTM 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 –

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 peroxide 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.

References

1. Murray RK. Red and white blood cells. In: Harper's Illustrated Biochemistry. 26th ed. Murray RK, Granner DK, Mayes PA and Rodwell VN eds. California (CA): Lange Medical Publication; 2003
2. Martin DW. Structure and function of protein haemoglobin. In: Harper's Review of Biochemistry. 19th ed. Martin DW, Mayes PA and Rodwell VN eds. California (CA): Lange medical Publications; 1983
3. Lehninger AL. Principles of Biochemistry. 2nd ed. New York (NY): New York Publishers; 1993.
4. Singer SJ, Nicolson GL. The fluid mosaic model of the structure of all membranes. *Science*; 1972. 175: 720-731.
5. Oyewale JO, Ajibade HA. The osmotic fragility of erythrocyte of turkey. *Vet. Archi*; 1990. 60: 91-1550.
6. Kumar S. An analogy for explaining erythrocyte fragility: concepts made easy. *Advan. Physiol. Edu*; 2002. 26: 134-5.
7. Dacie JV. The Haemolytic anemias. 3rd ed. Churchill Livingstone; 1962.
8. Krogmeier DE, Mao IL, Bergen WG. Genetic and non-genetic effects on erythrocyte osmotic fragility in lactating Holstein cow and its association with yield traits. *J. Dairy Sci*; 1993. 76: 1994-2000.
9. Cooper RA. Influence of membrane lipid alteration on red cell function: Red Cell Membrane: Structure and Function. Jamieson GA and Granwalt TJ ed. Toronto, ON, Canada: Lippincott J.B. CO.; 1969.
10. Csordas A, Shaufenstein K. Structure and configuration – dependent effects of C18 unsaturated fatty acids on the chicken and sheep erythrocyte membrane. *Biochem. Biophys. Acta*; 1984. 769: 571.
11. Sako T, Takeda S, Shibuya M, Koyama H, Uchino T, and Motoyoshi S. Seasonal changes in phospholipids and fatty acid composition of the bovine erythrocyte membrane. *Jon. J. Vet. Sci*; 1989. 51: 1243.
12. Aldrich KJ, Saunders DK, Sievert LM, Sievert G. Comparism of erythrocyte osmotic fragility among amphibians, reptiles, birds and mammals. *Transactions of Kansa Academy of Science*; 2006. 109: 149-158.

13. Mayes PA. Biological oxidation: Harper's Illustrated biochemistry. 26th ed. (Murray, RK, Granner DK, Mayes PA and Rodwell VW (eds). California: Lange Medical Publications; 2003.
14. Chempe PC, Harvey RA and Ferrier DR. Lippincott's Illustrated Reviews: Biochemistry. Jaypce Brother Medical Publisher (P) Ltd; 2005.
15. Ojo OO, Kabutu FR, Bell M, Babayo U. Inhibition of paracetamol induced oxidative stress in rats by extract of cymbopogon citracus (green tea). African J. of biotech; 2006. P. 5-12.
16. Ajayi OI, Famodu AA, Halim NKD. Haemostatic evidence of increased thrombotic risk in Nigeria parents. Nig. Soc. Haematol. B/d. Trans; 2003. 31: P. 13.
17. Becker K, Gui M, Schirmer RH. Inhibition of human glutathione reductase by S-nitrosoglutathione. Journal of Biochemistry; 1995. 234: 472-8.
18. Forchetti O, Maf frand C, Vissio C, Boaglio C, Cufre G. Hipofosfatemia y Fragilidad osmotical eritroica en cabras. Revista Electronica de veterinaria REDVET®; 2006. 7: 1.
19. Dewey MJ, Brown JL, Nallaseth FS. Genetic differences in ted cell osmotic fragility: Analysis in allophonic mice. Blood; 1982. 59: 986-9.
20. Chikezie PC. Osmotic fragility index of HbAA red blood cells in the presence aqueous extracts of three medicinal plants (Aframomum melegueta, Garcina Kola, and Cymbopogon citracus). Global Journal of Pure and Applied sciences; 2007. 13: 497-9.
21. Chikezie PC, Ibegbulem CO. Effect of quinine on osmotic fragility of HbAA red blood cells of guinea pig. J. of innov life Sci; 2004. 8: 5-8.
22. Chikezies PC, Chikezie CM, Nwanegwo CO. Osmotic fragility index of chicken (Gallus gallus) and human HbAA erythrocytes. International Science Research Journal; 2007. 1: 1-5.
23. Wegrzynowicz R, Marczuk K, Klyszejko B. Osmotic fragility of erythrocytes of bream Abramis Brama (L) from Zalew szczecinski. Acta. Ichthyologica et Piscatoria; 1972. 2: 95-9.
24. Kafka M. and Yermiahu T. The effect of EDTA as an anti-coagulant on the osmotic fragility of erythrocytes. Clinical Lab. Of Haematology; 1998. 20: 213-6.
25. Lux. Dissecting the red cell membrane skeleton. Nature; 1999. 281: 428-9.
26. Sackey K. Hemolysis as an interference factor in clinical chemistry. J. Clinical Biochemistry; 1999. P. 127-38.
27. Soforawa EA. Isolation and Characterization of anti-sickling agent from fagara zanthoxybids roots. Lloydia; 1975. 38: 169.
28. Dean J, Schectiter AN. Sickle cell anemia: molecular and cellular basis of therapeutic approach. The New England J. of Medicine; 1978. 229: 755.
29. Uwakwe AA, Ezeh SO. Effect of aqueous extracts of some medicinal herbs on hemoglobin S gelation, erythrocyte sickling and osmotic fragility: International conference on sickle cell disease and Environmental sickling Agents. Sickle Cell Research and Awareness Group (SCRAG Incorporated); 2000. Pp. 32.
30. Ali MSM, Kadaru AGM. In vitro processing of donor blood with sulphadoxine-pyrimethamine for eradication of transfusion-induced malaria. Am. J. Trop. Med. Hyg; 2005. 73(6): 1119-23.
31. Zakrzeroski SF. Principles of Environmental Toxicology. Washingto, D.C.: ACS Professional Reference Book American Chemical Society; 1983.
32. Tracy JW, Webster LT. Drugs used in the chemotherapy of protozoal infections. 10th ed. Adam, J G Limbird LE and Gilman AG, eds. McGraw-Hill, U.S.: Goodman and Filman's Pharmacological Basis of Therapeutics; 2001.
33. Kalasen CD. In Cassavett and Doull's Toxicology. Klaasen CD, Amdur MO, Doull J, eds. New York (NY): Macmillian Publishing; 1986.