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Susceptibility of Hemoglobin Variants to Descending Grades of Hypotonic Saline is Inversely Related to Degree of Clinical Morbidity

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Abstract
This study was designed to determine the degree of resistance to hypotonic saline for different variants of blood hemoglobin that are found locally, relative to normal adult red cell hemoglobin and to one another. Blood specimens from 25 individuals, five for each type of hemoglobin variant, were exposed to varying degrees of hypotonicity at room temperature and the optical density (OD) was read after incubation. The pattern of hemolysis was consistent in all samples for each hemoglobin variant. HbAA was found to be most susceptible to saline hypotonicity, followed by HbAC and HbAS while HbSC and HbSS were highly resistant to lysis when exposed to varying degrees of hypotonicity. The three variants containing HbA, that is HbAA, HbAC, HbAS had close similarity in the pattern of hemolysis. Similarly, HbSC and HbSS exhibited a close pattern as well. We suggest that the pattern observed in the two categories may be as a result of the presence of the adult hemoglobin gene (A) in the former group and the presence of the sickle hemoglobin gene (S) in the latter, respectively. We observed that the pattern of susceptibility to saline hypotonicity found in these variants has an inverse relationship to the severity of clinical manifestation commonly observed in individuals having these different hemoglobin variants.
Keywords: hemoglobin, morbidity, genotype, osmotic fragility, electrophoresis.

INTRODUCTION

Hemoglobinopathies are among the most common genetic disorders worldwide (Arulkumaran, 2004). There is some evidence that hemoglobinopathies had been recognized in Africa before descriptions in medical literature. The high prevalence of these hemoglobin diseases in this geographical region is partly as a result of selective pressure conferred by their relative resistance to malaria infection (Hanneman *et al*., 2011). Abnormalities in hemoglobin structure are majorly due to mutations in the globin portion of the gene (Ashley-Koch *et al*., 2000). Inheritance of hemoglobin types are by simple Mendelian pattern.

The degree of resistance of red blood cells (RBC) to hemolysis as a result of a decrease in the osmotic concentration of the suspension medium is the basis of the osmotic fragility test. The osmotic fragility test is useful for the diagnosis of certain hematological diseases like hemolytic anemia, hereditary spherocytosis and elliptocytosis, glucose-6-phosphate dehydrogenase deficiency, and sickle cell anemia, as well as for RBCs from uremic or diabetic patients (Bartosz, 1990; Massaldi *et al*., 1998). Indeed, this test has utility in conditions in which hemolysis is a potential consideration. Abnormalities in osmotic fragility have been reported in hereditary spherocytosis, hereditary elliptocytosis, pyruvate kinase deficiency, thalassemia and iron-deficient cells (Dacie and Lewis, 1991). Low osmotic resistance may lead to intravascular hemolysis, which causes a reduction in the RBC life span (Bartosz, 1990; Cordero *et al*., 2004). The osmotic fragility curve of red cells not only reflects the average membrane and cytoplasmic properties, but may also provide information on the distribution of those properties within the sample (Troiano *et al*., 2000).

Hemoglobinopathies are a group of red blood cell disorders with a characteristic of altering the membrane permeability of red blood cells, in addition to distorting the shape of the cells. The homozygote sickle cell gene variant (HbSS) is the most prominent of this group because of the severe pathology among sufferers. HbSS erythrocytes have a low osmotic fragility and a higher resistance to hypotonic solutions than normal erythrocytes (Vitoux *et al*., 1999).

There is a link between red cell membrane permeability and pathology of hemoglobin variants. Berkowitz and Orringer, (1985) suggested that membrane permeability in hemoglobinopathies may be due to inherent membrane disorders. Dalibalta *et al*., (2010) found similarities in permeability characteristics of HbSS and HbSC while also observing differences which may be important in pathogenesis. Red blood cells possessing the S hemoglobin have a tendency to polymerize and sickle when exposed to conditions of low oxygen tension, high altitude, low pH, hyperconcentration of hemoglobin and dehydration. The greater the proportion of

hemoglobin S in the cell, the greater is the propensity to sickle. The heterozygous carrier state or sickle cell trait results in the production of both hemoglobin A and S (usually 30–40% HbS content), which has a predominantly benign clinical picture as the cells only sickle under extraordinary physiological conditions (Wilson *et al*., 2010; Ashley-Koch *et al*., 2000), whereas mild to moderate stress may provoke sickling of red cells in affected HbSS individuals.

This study has the aim of investigating the relationship between the osmotic resistance of RBCs in patients with hemoglobinopathies and the pathology commonly observed among sufferers of the many variants.

MATERIALS AND METHODS

This study was carried out among patients attending Olabisi Onabanjo University Teaching Hospital, Sagamu, Ogun State, Nigeria. Ethical approval was obtained from the Babcock University Research Ethical Committee. To ensure adequate uniformity of treatment, samples were collected and assayed only on days when adequate number of specimens for all desired hemoglobin variants was available. 5mls of blood specimen was collected from each patient, primarily for hemoglobin (Hb) electrophoresis test.

Hb. electrophoresis: One hundred and twenty seven patients suspected of having one type of hemoglobin disease or the other were screened for Hb genotype using the Tris/EDTA/Borate (TEB) buffer (at pH 8.6) method over a period of 10 days. After centrifugation of samples, 20ul of packed red cells were diluted with 150ul of hemolysing agent, mixed gently and left for five minutes to allow for proper hemolysis. The electrophoresis tank was prepared by placing equal volume of TEB in each of the outer compartments. The cellulose acetate paper was soaked in a reservoir of buffer for five minutes before use. Sample well plate was filled with 5ul each of diluted sample and covered with coverslip to prevent evaporation. Cellulose acetate paper was removed from buffer and blotted but not allowed to dry. The applicator was loaded and samples were applied on the cellulose acetate paper. Cellulose acetate paper was placed across the bridges in the machine with the plastic side on top. The machine was covered and electrophoresis was carried out at 360V for 15 minutes. Confirmed HbAA and HbSC control samples were run along with the test samples.

Osmotic fragility test: A total of 25 subjects were selected after Hb. electrophoresis screening. Five freshly obtained samples for each Hb variant i.e. Hb type SS, AS, AC, SC and AA from consenting donor-patients were used in the study. Individuals with recent history of blood transfusion were excluded from the study. A stock solution of 1.0g/dl sodium chloride was made, out of which varying descending grades of dilutions were made to include the following saline concentrations: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0g/100ml. To 5ml of each dilution was added 0.02ml of blood, left to stand for 30 minutes before being spun in the centrifuge. The optical density (O.D.) of the supernatant was then read. The O.D. readings were taken twice and the mean results tabulated as depicted in the graph.

RESULTS

Mean Absorbance

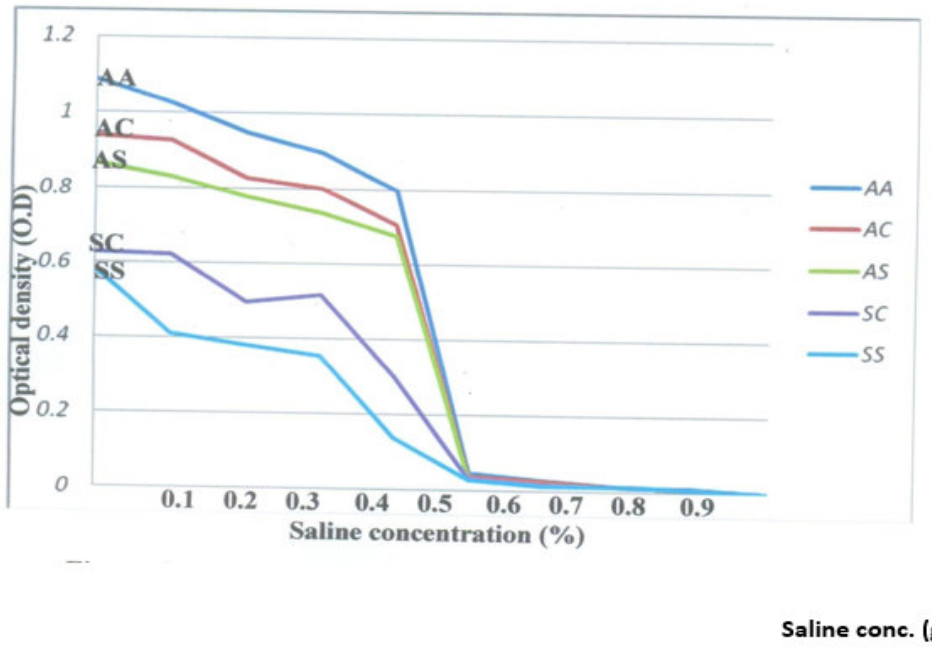

Fig. 1 Pattern of susceptibility to hypotonic saline for different Hb variants

For all variants, marked hemolysis was not observed at saline concentrations below 0.4g/100ml,

although HbAA showed clear signs of early lysis than other Hb types. The pattern of hemolysis as seen in this experiment shows individual differences but is similar for HbAA, HbAC and HbAS on one side, while the pattern for HbSC and HbSS is similar on the other. At saline concentration of 0.4g/100ml, the OD readings of HbAA, HbAC and HbAS were 0.80, 0.71 and 0.68 respectively, whereas those of HbSC and HbSS were 0.30 and 0.14 respectively, showing a marked departure from the HbA-containing group. Again, whereas HbAA, HbAC and HbAS had sharp drop in their resistance, the other two variants exhibited only a gradual decline in resistance to hemolysis. HbAA had the lowest resistance to hemolysis, whereas HbSS had the greatest resistance.

DISCUSSION AND CONCLUSION

This study was carried out to investigate how fragile different red blood cell hemoglobin variant membranes are relative to each other and to normal adult red cells. Red blood cells of the variants were exposed to different concentrations of hypotonic salt solutions and also to normal saline. The degree of osmotic fragility of the hemoglobin types was compared. Only five variants were available for testing. Samples from thalassemic individuals were excluded as sufferers of thalassemia are rare in the study population.

The osmotic fragility lysis test carried out showed a fairly consistent pattern of hemolysis for each of the five variants available and results obtained were in the following order of resistance to lysis; HbSS > HbSC >HbAS > HbAC > HbAA. It was also observed that the three variants: HbAA, HbAC and HbAS had a close similarity in their pattern of hemolysis. In like manner, HbSC and HbSS had a close pattern as well. The category that has the following: HbAA, HbAC and HbAS are generally benign with HbAA being completely normal. While not much is known about the clinical presentation of HbAC, it is generally accepted that it is clinically milder than HbAS which may occasionally present symptoms in certain conditions in which red blood cells undergo stress (Eckman, 2010).

However, HbSS was observed to show a stronger resistance to hemolysis when subjected to hypotonicity than HbSC in spite of the similarity in their membrane characteristics. This finding has support in the work of Dalibalta *et al*., (2010) who found similarities in permeability characteristics of HbSS and HbSC while also observing differences which may be important in pathogenesis. They remarked that HbSC not only has permeability characteristics like HbSS, there is also an overlap in the symptoms presented by the two, although clinical morbidity is less severe in HbSC. Incidentally, the degree of morbidity commonly observed in individuals with these genetic traits is in the following increasing order: HbAA< HbAC< HbAS< HbSC< HbSS (Ashley-Koch *et al*., 2000; Marengo-Rowe, 2006; Eckman, 2010; Hanneman *et al*., 2011). The patterns observed in the two categories may be as a result of the presence of the adult hemoglobin gene (A) in the former category and the presence of the sickle hemoglobin gene (S) in the latter (Wilson *et al*., 2010). Ashley-Koch *et al*., (2000) stated that the individual's genotype is the most important factor for disease severity. Of relevance here is the mitigating effect of the normal adult (A) gene on pathology, where there is co-existence of two hemoglobin types. Both HbAS and HbAC have a greater alignment to HbAA than to HbSC or HbSS. This feature may simply be a reflection of the dominant effect of the HbA gene on the phenotypic expression of the other variants. On the contrary, the presence of HbS with another variant, other than the normal HbA, may predict greater morbidity. Hanneman *et al*., (2011) claimed that increase in membrane permeability of red blood cells having HbS promotes Ca++ entry as well as loss of KCl and water, thus inducing dehydration and consequent pathogenesis. Hyperconcentration of the sickle hemoglobin is thus associated with membrane permeability and consequently, resistance to hypotonic hemolysis. HbAS and HbSC with lower concentrations of the S hemoglobin than HbSS are therefore justifiably less resistant to hypotonic saline, while also exhibiting less morbidity in affected patients.

It is pertinent to note that membrane permeability in this study is not deoxygenation-induced as claimed by Gibson and Ellory, (2002). Rather, it appears that membrane permeability defect is a common phenomenon in abnormal hemoglobins as shown by the permeability of hemoglobin variants studied in this work. This suggestion is supported by the findings of Akram and Mahboob, (2004) that worked on thalassemias and also found that RBCs of thalassemic patients had permeability defects. Permeability to hypotonic solutions may be beneficial for sickle cells and possibly other defective hemoglobin variants. Clark and Shohet, (1981) had earlier postulated that there are permeability defects in hemoglobinopathies; and it is clear from the results in this study that the more severe the clinical presentation of a hemoglobin disease, the more likely that its red cell will resist hemolysis to hypotonic solutions. Red cell membrane permeability defect in hemoglobinopathies may be an evolutionary mechanism to compensate for the defective hemoglobin contained within the membrane. According to Ashley-Koch *et al*., (2000), the unusually high frequency of HbAS in people of African and Mediterranean ancestry has continually been maintained because it confers selective advantage for carriers against malaria. The red cell membrane defect in hemoglobin diseases may therefore be part of the adaptation to this adverse phenomenon. This suggestion is based on the generally common but variable trend of resistance to hemolysis observed among all abnormal variants in this study.

In conclusion, it appears that among the hemoglobin disease variants, the more pathologic the clinical

condition, the greater is its red cell permeability to hypotonic saline. Thus severity of clinical symptoms to be expected for a hemoglobin type may be predicted as being inversely related to its susceptibility to hemolysis in hypotonic solution. In addition, the presence of HbA in an individual's genotype mitigates against morbidity while the presence of HbS increases the risk of a greater degree of pathology.

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