

# Susceptibility of Haemoglobin 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 haemoglobin that are found locally, relative to normal adult red cell haemoglobin and to one another. Blood specimens from 25 individuals, five for each type of haemoglobin variant, were exposed to varying degrees of hypotonicity at room temperature and the optical density (OD) was read after incubation. The pattern of haemolysis was consistent in all samples for each haemoglobin 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 haemolysis. 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 haemoglobin gene (A) in the former group and the presence of the sickle haemoglobin 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 haemoglobin variants.

## INTRODUCTION

Haemoglobinopathies 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 haemoglobin 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).

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 haematological diseases, for example, haemolytic anaemia, hereditary spherocytosis, and elliptocytosis, glucose-6-phosphate dehydrogenase deficiency, and sickle cell anaemia, as well as for RBCs from uremic or diabetic patients (Bartosz, 1990; Massaldi *et al.*, 1998). 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 haemolysis, 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). Haemoglobinopathies alter the membrane permeability of red blood cells, in addition to distorting the shape of the cells. Thus, HbSS 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 haemoglobin variants. Berkowitz and Orringer, (1985) suggested that membrane permeability in haemoglobinopathies 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 haemoglobin 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 haemoglobin A and S (usually 30–40% HbS), which has a predominantly benign clinical picture as the cells only sickle under extraordinary physiological conditions (Wilson *et al.*, 2009), whereas mild to moderate stress may provoke sickling of red cells in affected HbSS individuals.

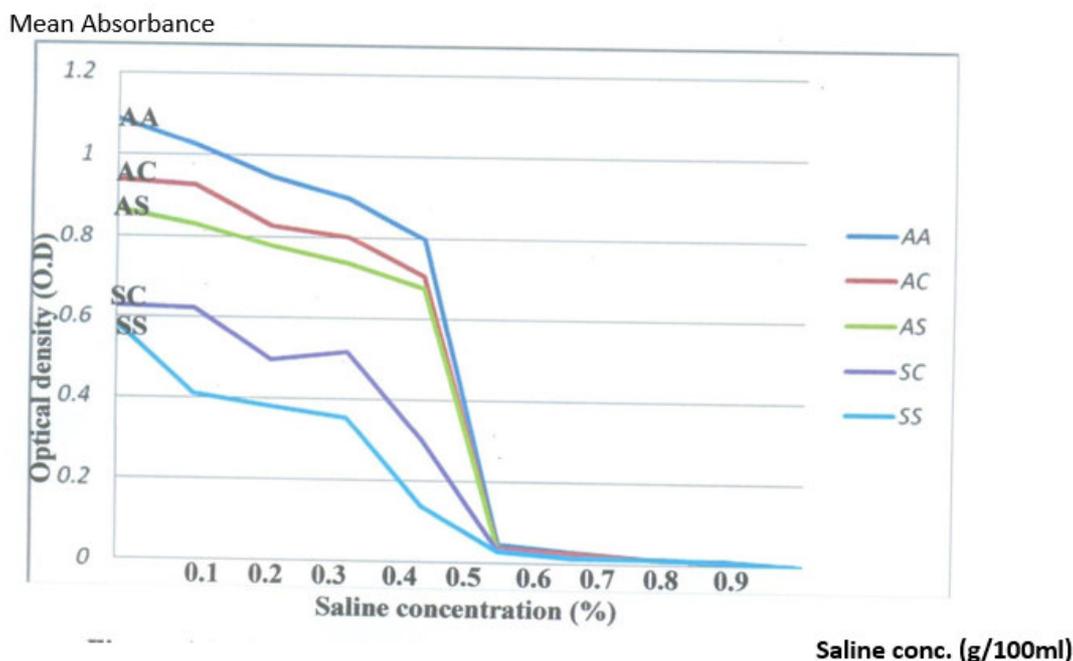
## MATERIALS AND METHODS

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 Hb variants was available. 5mls of blood specimen was collected from each patient, primarily for haemoglobin electrophoresis test.

**Hb. electrophoresis:** One hundred and twenty seven patients suspected of having one type of haemoglobin 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. 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 of each 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



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

For all variants, marked haemolysis 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 haemolysis 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 haemolysis. HbAA had the lowest resistance to haemolysis, 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.

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 haemolysis. 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 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 haemolysis 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 (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 haemoglobin gene (A) in the former category and the presence of the sickle haemoglobin gene (S) in the latter (Wilson *et al.*, 2010). Hanneman *et al.*, (2011) claimed that increase in membrane permeability of red blood cells having HbS promotes Ca<sup>++</sup> entry as well as loss of KCl and water, thus inducing dehydration and consequent pathogenesis. Hyperconcentration of the sickle haemoglobin is thus associated with membrane permeability and consequently, resistance to hypotonic haemolysis. HbAS and HbSC with lower concentrations of the S haemoglobin than HbSS are therefore justifiably less resistant to hypotonic saline.

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 haemoglobins as shown by the permeability of haemoglobin variants studied in this work. This suggestion is supported by Akram and Mahboob, (2004) who worked on thalassaemias. Permeability to hypotonic solutions may be beneficial for sickle cells and possibly other defective hemoglobin variants. Clark and Shohet, (1981) had postulated that there are permeability defects in haemoglobinopathies and it is clear from the results in this study that the more severe the clinical presentation of a haemoglobin disease, the more likely that its red cell will resist haemolysis 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. This suggestion is based on the patterns of resistance to hemolysis observed in this study.

In conclusion, it appears that among the haemoglobin 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 haemolysis in hypotonic solution.

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