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E-mail: lasujms@lasucom.edu.ng

Research Article

Association between Protein C, Protein S and Cerebral blood flow velocity in Sickle cell anaemia patients with high risk of Cerebrovascular Accident

Olowoselu Festus O[1], Uche Ebele I[2], Ogunmola Jacob A[1], Oyedeji Olufemi A[1], Olowoselu Olufunke I[3], Ogunlade Abosede A[1], Ajie Obiefuna I[4], Osunkalu Vincent O[1], Oyedemi Jeremiah P[5], Akinbami Akinsegun A[2]

[1]Department of Haematology and Blood Transfusion, College of Medicine, University of Lagos, Lagos. Nigeria.
[2]Department of Haematology and Blood Transfusion, Lagos State University College of Medicine, Ikeja, Lagos, Nigeria.
[3]Department of Community Health, Lagos University Teaching Hospital, Lagos, Nigeria.
[4]Department of Chemical Pathology, College of Medicine, University of Lagos, Lagos, Nigeria.
[5]Department of Physiotherapy, College of Medicine, University of Lagos. Lagos, Nigeria.

[*]***Author for Correspondence:***
Uche E. I.
eifeyinwa2000@yahoo
Phone No: +2348033215159

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ABSTRACT

Objectives: The main trigger of thrombosis in SCA patients is a hyper-coagulable state which may due to deficiency of natural anticoagulants such as protein C and protein S, among other abnormalities of the coagulation process. This study aimed to determine the association (if any) between protein C and S vis-a-vis cerebral blood flow velocity in sickle cell disease patients with high risk of cerebrovascular accident (CVA).

Method: This was a case-control study involving 180 HbSS patients who were categorized based on their risk of cerebrovascular accidents (measured using transcranial doppler (TCD) and 50 age-matched HbAA controls. All participants 'samples were assayed for protein C, S, full blood count, prothrombin time (PT) and activated partial prothrombin time (APTT) and the association between the assayed parameters and TCD values were established.

Results: There was a statistically significant ($p<0.05$) increase in the time average mean velocity (TAMV) of the participants with sickle cell anaemia compared to the control group. Similarly, decreased levels of proteins C and S observed in the participants with sickle cell anaemia in comparison to the control, were also statistically significant ($p<0.05$). As the risk of CVA increased from standard/normal risk, conditional risk to high risk, the levels of protein C significantly ($p<0.05$) decreased. However, the observed decrease in the levels of Protein S was not statistically significant.

Conclusion: There was a significant difference ($p<0.05$) in the plasma levels of protein C and S in the HbSS patients and control subjects.

INTRODUCTION

Sickle cell disease (SCD) is a chronic disorder characterized by abnormal, rigid, sickle-shaped red blood cells. It denotes all genotypes that contain at least one sickle gene causing deformity in the red blood cell, which led to the disorder being termed sickle cell anemia.[1] The pathophysiology of this disease includes erythrocyte-leukocyte-endothelial adhesion, alteration of proper blood flow, inflammation, oxidative stress, immune response activation, chronic haemolytic anaemia which finally culminates in organ infarction.[2]

The main trigger of thrombosis in sickle cell disease patients results from hyper-coagulable state and this may be due to the deficiency of natural anticoagulant (such as protein C, protein S or Anti-thrombin) among other abnormalities of the coagulation factors.[3]

One of the major neurological complications of vaso-occlusion is stroke (also known as cerebrovascular accident), which is a serious complication of SCA, and affects 6 to 17% of children and young adults.[4,5,6,7] Other causes of stroke include intra-cerebral or subarachnoid hemorrhage which affect much older children.[5,6,8] The incidence of cerebro-vascular complications in SCD is 5 to 10% 9. In 11% of SCD patients, stroke occurred by age 20 years, with infarction mainly in the internal carotid and middle cerebral arteries.[10]

With the use of a Transcranial Doppler (TCD), relative changes in cerebral blood flow (CBF) can be measured by observing blood flow velocity (FV) in basal cerebral arteries,[11] Even though this method requires a certain degree of technical expertise, it is non-invasive, relatively inexpensive and provides real time information with high temporal resolution. TCD can be used to measure flow velocities from several vessels of the circle of Willis,[12] but most published data refer to the middle cerebral artery (MCA). This vessel has a favorable orientation and is readily

accessible to TCD and provides the most reliable flow velocity signal with a high signal to noise ratio.

Transfusion therapy has been shown to prevent the development of stroke by 92%[13] but unfortunately this procedure has numerous side effects such as alloimmunization and iron overload. Identifying sickle cell patients at high risk is crucial in the selection of patients that would most benefit from this prevention intervention. Various studies have shown that patients developing cerebral vasculopathy can be detected using TCD.[13] Elevated cerebral blood flow (CBF) velocity (≥200 cm/s), measured by transcranial Doppler (TCD), has been identified as a risk factor for stroke in SCD.[14] In their study on the pattern of cerebral blood flow among children with SCD in Lagos, Nigeria, Adekunle and colleagues found the prevalence of stroke to be 10.8%.[15]

Protein C is a vitamin K–dependent plasma protein that, when activated by the thrombin-thrombomodulin complex to activated protein C (APC), inactivates factors Va and VIIIa to inhibit coagulation 16. Inherited deficiency of protein C and its association with thrombosis was first described by Griffin and co-workers in 1981.[17]

Protein S is a vitamin K–dependent plasma protein that facilitates the anticoagulant activity of activated protein C and its deficiency is linked to the development of thrombosis. A hyper-coagulable state which is commonly seen in patients with SCA may be due to deficiency of natural anticoagulants such as protein C and protein S.

The aim of this study was to determine the association (if any) between protein C and S vis-a-vis cerebral blood flow velocity in sickle cell disease patients with high risk of cerebrovascular accident (CVA)

MATERIALS AND METHODS

Study Area and Population

One hundred and fifty (150) homozygous sickle cell disease patients (both males and females) from the Sickle Cell Centre, Lagos Nigeria, were recruited for this study and eighty (80) apparently healthy age and sex matched Haemoglobin AA individuals were recruited as control.

Study Design

This was a cross sectional comparative study which involved patients with SCA and apparently age matched HbAA controls.

Study Period

The study was done between April 2018 and April 2019

Inclusión Criteria

All Sickle cell anaemia patients already diagnosed with the use of haemoglobin electrophoresis in steady state, whose parents/legal guardians gave informed consent. Steady State is defined as the absence of infection or acute clinical symptoms or crisis for at least 3 months.

Exclusion Criteria

1. Patients on anticoagulants, oral contraceptives or aspirin
2. Recently transfused SCA patients
3. Individuals with a past medical history of thrombosis and/or other coagulation disorders

Sampling Technique and Sample size Determination

Participants who met the inclusion criteria were recruited consecutively (following informed consent and filling of questionnaire) until the required sample size was obtained.

Sample size estimation was done using[18]

$$(n) = Z_1 - \alpha /2^2 \frac{\mathbf{SD^2}}{\mathbf{d^2}}$$

Where $Z_1 - \alpha /2$

SD= Standard deviation of variable (Protein C/S) in a previously reported research

d = Degree of error (5%).

A minimum sample size of 50 per group was derived for HbSS study group and 80 participants for HbAA control group.

Ethical Consideration

Ethical approval was obtained from the Ethics Committee of Lagos University Teaching Hospital, Lagos State. Ethics Number ADM/DCST/HREC/APP 2119

Subjects Classification:

Homozygous sickle cell disease subjects (HbSS) were grouped into 3 categories (group I to III) according to their degree of risk of cerebrovascular accident using their time average of maximum velocity (TAMV) which was assessed by TCD, while the HbAA subjects served as control group (group IV).

Group I: HbSS subjects with high/ increased risk (TAMV ≥ 200 cm/s)

Group II: HbSS subjects with conditional risk (TAMV 171-199 cm/s)

Group III: HbSS subjects with normal velocity (TAMV <170 cm/s)

Group IV: Age and sex matched apparently healthy Hb AA individuals with no previous history of thrombosis or other coagulation disorders.

Sample Collection

From each participant, 2.5ml of venous blood was collected under aseptic conditions and dispensed into trisodium citrate bottle containing 0.28ml of trisodium citrate (3.2g/dl) anticoagulant.

Plasma was extracted from the sodium citrate bottle by centrifuging the whole blood for 15 minutes at 3000g for Protein C, S, Prothrombin time (PT) and Activated Partial Thromboplastin Time (APTT) estimation. The PT and APTT were analyzed within an hour of collection using the automated Erba ECL 412 Mannheim Analyzer. The samples for Protein C and Protein S assays were stored at -80^{0}C

Determination of Protein C and S activities

Protein C and S were estimated using chromogenic assay method on the Erba ECL 412 Mannheim Analyser (*Erba Mannheim* Corporate Services Ltd, UK, London) according to the manufacturer's instruction. The stored/frozen plasma sample was thawed quickly at 37^{o}C prior to testing.

Data Analysis Tool

Data obtained from the study were captured on Microsoft excel for analysis. The quantitative data/variables obtained from this study were analyzed using Statistical Package for Social Sciences (SPSS) version 25.0 for PC (Armonk NY: IBM Corp. USA). Comparative statistics were expressed as number (frequency) and mean ± standard error of mean. The multiple comparisons among variables was done using ANOVA test with Turkey post-hoc analysis, and level of significance was set at $p \leq 0.05$. The risk for high TAMV TCD with the levels of plasma protein C and S were evaluated using linear logistic regression model.

RESULTS

Socio Demographics of the Sickle Cell Anaemia Patients and controls

A total of 150 HbSS participants consisting of 65 males and 85 females with a mean age of 7.79 ± 0.43 years were recruited. Also 80 age-matched Hb AA controls consisting of 42 males and 38 females with a mean age 7.14 ± 0.83 years were used. The mean age and gender of the two groups showed no statistically significant difference. (Table 1)

Time Average Mean Velocity of Participants and relationship with gender and risk of Cerebrovascular Accident.

There was a statistically significant ($p<0.05$) increase in the time average mean velocity (TAMV) of the participants with SCA in comparison to the controls (Table 2). No statistically significant difference was observed between the TAMV of male and female participants with sickle cell anaemia. On the basis of the TAMV, the participants with SCA were grouped into 3 categories including conditional risk (n=50), high risk (n=50) and standard/normal risk (n=50). Significant ($p<0.05$) difference was observed in the TAMV across the group increasing from participants with sickle cell anaemia at standard/normal risk, conditional risk to high risk (Table 2).

Plasma levels of Protein C and Protein S and the risk of Cerebrovascular Accident in the SCA patients.

Statistically significant ($p<0.05$) decreased levels of protein C and protein S were observed in the HbSS participants compared with control. However, a reciprocal observation was noted in the values of pro-thrombin time and activated partial thromboplastin time (APTT) as there was statistically significant ($p<0.05$) increase in mean time of PT and APTT in the HbSS participants compared to the control (Table 3). No statistically significant difference was observed between the protein C, protein S, PT and APTT of male and female HbSS participants. As the risk of CVA increased from standard/normal risk, conditional risk to high risk, the levels of protein C significantly ($p<0.05$) decreased. There was decrease in the level of Protein S but it was not statistically significant ($p>0.05$). Also, there was no statistically significant ($p>0.05$) difference in the rate of PT and APTT across the risk groups (Table 3).

Protein C and Protein S activity and High Cerebrovascular Accident Risk in Participants with Sickle Cell Anaemia

In Table 4, there was a statistically significant ($p<0.05$) negative regression coefficient in the high risk TAMV possibly elicited by a unit change in the levels of plasma protein C of the participants with sickle cell anaemia. Changes in protein C levels accounted for approximately 7.3% of the variations observed in the TAMV values among HbSS participants ($R2=0.073$). However, no statistically significant ($p>0.05$) association was observed between variations in TAMV and changes in the levels of plasma protein S of the participants with sickle cell anaemia.

Table 1: Demographics of the Participants with HbSS and HbAA

Characteristics	Test-HbSS (n=150)	Control-Hb AA (n=80)	p-value
Mean Age (years)	7.79 ± 0.43	7.14 ± 0.83	0.494
Gender			
Male	65 (43.33%)	42 (52.5%)	0.184
Female	85 (56.67%)	38 (47.5%)	

Table 2: Time Average Mean Velocity of Participants and relationship with gender and risk of Cerebrovascular Accident.

Parameter	Description mean (±SD)			p-value
Subject groups	**Test (n=150)**		**Control (n=80)**	
TAMV (cm/s)	184.29 ± 4.39		119.00 ± 1.42	0.000
Gender (HbSS)	Male (n=65)		Female (n=85)	
TAMV (cm/s)	185.07 ± 7.93		183.69 ± 4.91	0.882
Risk Category	Conditional	High	Standard/normal	
TAMV (cm/s)	184.5 ± 1.68	220.86 ± 5.07	147.52 ± 3.26	0.000

Table 3: Protein C and Protein S levels and the risk of Cerebrovascular Accident in the study group and control

Parameters	Protein C (%)	Protein S (%)	PT (secs)	APTT (secs)
Subject Category				
HbSS	46.21 ± 5.56	128.90 ± 3.86	15.18 ± 0.14	34.79 ± 0.38
HbAA	65.76 ± 3.87	155.13 ± 3.50	14.00 ± 0.21	33.00 ± 0.68
p-value	0.032*	0.000*	0.000*	0.029*
Gender				
Male (n=65)	58.77 ± 4.37	148.09 ± 2.48	15.20 ± 0.20	34.43 ± 0.53
Female (n=85)	53.86 ± 2.59	146.41 ± 2.39	15.17 ± 1.21	35.08 ± 0.53
p-value	0.340	0.628	0.921	0.387
TAMV risk category				
conditional	50.82 ± 2.75	142.78 ± 3.15	15.19 ± 0.29	34.77 ± 0.69
high risk	36.62 ± 8.17	94.90 ± 3.86	15.20 ± 0.25	35.46 ± 0.65
standard/normal	51.19 ± 4.94	146.72 ± 2.69	15.16 ± 0.21	34.15 ± 0.63
p- value	0.012*	0.078	0.993	0.368

Table 4: Regression analysis between Time Average Mean Velocity and the Plasma Levels of Protein C and Protein S of the Participants with Sickle Cell Anaemia

Dependent Variables	R-square	Coefficients	p-value	Remarks
Plasma Protein C (secs)	0.07305	-0.4942	0.03	S
Plasma Protein S (secs)	0.01938	132.0447	0.280	NS

NS - Not significant; S- Significant

DISCUSSION

Sickle cell disease (SCD) is a genetic disease characterized by a hypercoagulable state in which various haemostatic systems both in steady state and during vaso-occlusion are disrupted, with increased activation of the coagulation system, platelets activation, thrombin generation, and occurrence of thrombosis,[19,20,21] The pathogenesis of hypercoagulability is considered to be multifactorial. Altered components of haemostasis system in SCD have been suggested.[20, 22] These abnormalities in the haemostatic system in SCD patients lead to an increased risk of thrombosis and thrombosis has been linked to the development of stroke 3. Our study evaluated the levels of natural anti-coagulant factors (Protein C and Protein S) and their predictive roles in the assessment of the risk of CVA in patients with sickle cell anaemia.

In our study, the significantly decreased mean plasma levels of protein C and protein S in the SCA group in comparison to HbAA control is similar to the findings of several previous studies[22-25] The low circulating protein C and S levels may be due to chronic consumption in the background of increased tissue factor expression and activation of coagulation on the surface of sickled red blood cells with phosphatidyl-serine externalization.[22] Even though the degree of deficiency does not correlate with incidence of vaso-occlusive pain crisis,[24] Protein S and C activities do appear to correlate with a history of stroke in pediatric patients.[26,27] Mean baseline activity levels in patients with prior stroke have been noted to be as low as 47 % for protein S and 52 % for protein C.[26,27] Individuals with SCA, even when clinically well, have continuous haemolysis and vaso-occlusion. This is associated with evidence of an acute phase response[28] even in patients with rigorous definition of the steady state. Such patients also have low levels of factor V, factor VII, factor XIII, thrombin–antithrombin III complex, and plasminogen 29-30 and increased thrombin generation most readily explained by continued activation of coagulation and a consumption coagulopathy. In the presence of an increased tendency of thrombin generation, factors inhibiting coagulation assume particular importance. Nearly all the components of the haemostatic system (platelet function, procoagulant, anticoagulant and fibrinolytic system) are altered in favor of a procoagulant phenotype in the disease; hence SCD is frequently referred to as a "hypercoagulable state."[31]

Plasma levels of prothrombin fragments 1.2 (F 1.2) are associated with the number of circulating Phosphatidyl Serine-positive RBC.[32] Patients with SCD have increased levels of markers of thrombin generation and thrombin antithrombin (AT) complexes (F 1.2, TAT complexes) in the non-crisis state.[33] Decreased levels of natural anticoagulant proteins are also observed in SCD.[23,34] Therefore, the levels of protein C and S are decreased in the non-crisis steady state and perhaps even more so during acute pain episodes.[23, 35] These may be a consequence of the chronic consumption of proteins C and S due to increased thrombin generation, resulting from intravascular tissue factor (TF) expression and red blood cell (RBC) prothrombinase activity.[36] Increased binding of protein S by sickled RBC due to membrane PS exposure and inhibition of the binding of protein S to β2-glycoprotein 1 by anti-phospholipid antibodies result in inactivation of protein S by circulating C4b-binding protein[37,38] Significantly decreased levels of proteins C and S were reported in patients with SCD who developed thrombotic strokes,[26] as compared to neurologically normal children with SCD.

The mechanism of the lowered protein C and protein S levels remains unknown. Approximately 60% of circulating

protein S is bound to C4B binding protein, an acute-phase reactant (and a product of complement activation) involved in the complement pathway, and increased levels of C4B binding protein may reduce the potency of the protein C pathway by reducing the amount of free protein S.[39] However, the study reported by Wright *et al*., 1997 34 showed a decrease rather than increase in C4B binding protein levels which could therefore not account for the reduction in free protein S. The data presented in their study suggest that the reduced concentrations of C4B binding protein probably reflect impaired hepatic production similar to that seen in other hepatic disorders.[40] Protein C levels may be decreased as a result of a consumptive coagulopathy 17 and this mechanism can be shown by the level of D-dimer in patients' plasma.[34] The possibility that sustained tissue destruction, associated with either haemolysis or continued vaso-occlusion can explain the reduction in Protein C and S levels.

Protein C and protein S are both produced in the liver and are vitamin K dependent proteins. Impaired hepatic function with reduced synthesis of protein C and protein S might be another possible explanation for its decrease in SCA patients. The liver is frequently affected in SS disease; red cells traversing the low oxygen tension areas of the sinusoids are prone to sickling and sinusoidal obstruction by distended Kupffer cells.[41] Enzymes partially reflecting hepatic function, aspartate transferase and alkaline phosphatase were significantly elevated in SS disease and g-glutamyl transferase levels did not differ from those in controls as reported by Wright *et al*.[34]. The strongest evidence of hepatic dysfunction comes from the close correlation between prolonged PT and low factor V, factor VII and C4B binding protein which are synthesized in the liver 34 and in our study, there was a significant difference in the PT of SCA patients and controls which backed up the finding reported by Wright and colleagues 34. They also reported the absence of detectable Protein-induced vitamin K absence/antagonist (PIVKAs) and the low factor V (a vitamin K independent protein) favors hepatocellular compromise rather than vitamin K deficiency from either biliary obstruction or diet. Hepatocellular dysfunction is likely to result from transfusional iron overload or chronic hepatitis C since some of their study patients had a history of blood transfusion.

It has been proposed that the most likely cause of reduced protein C and protein S in sickle cell subjects is impaired hepatocellular function secondary to chronic intrahepatic sickling in the hepatic sinusoids.

In this study, the statistically significant ($p<0.05$) negative regression coefficient between high TAMV and protein C levels suggests that mean changes in the plasma levels of protein C may be associated with of high risk TAMV; thus, predicting risk of stroke in individuals with sickle cell anaemia. In addition, the observed statistically insignificant ($p>0.05$) high positive regression coefficient between high TAMV and protein S plasma level suggests that unit change in the levels of plasma protein S has no effect in the mean changes of high risk TAMV; thus plasma protein S might not be considered as predictor of the risk of stroke in individuals with sickle cell anaemia.

The suggested mechanisms behind the deficiency of natural anticoagulant in SCD include increased consumption due to the increased activation of coagulation proteins in SCD patients and the decreased synthesis by the liver due to hepatic dysfunction.[23,42] The increased pro-inflammatory activity in SCD patients may contribute to the consumption of the natural anti-coagulants. However, there is also a possibility of an inherent anti-coagulant abnormality due to genetic defect that can further accentuate the pro-thrombotic state of SCD; this however needs to be further investigated.

CONCLUSION

The result of this research revealed a significant difference ($p<0.05$) in the plasma level of protein C and S activities between HbSS patients and control subjects and Protein C activity was significantly associated with severity of TAMV in HbSS.

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