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ORIGINAL RESEARCH

Oxidative stress markers and disease severity among children with Sickle Cell Anaemia

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Abstract

Background: Sickle cell anaemia has been associated with oxidative stress. Total Antioxidant Capacity (TAC), Total Oxidant Status (TOS) and Oxidative Stress Index (OSI) are cumulative markers of oxidative stress.
Objective: To evaluate the serum levels of oxidative stress markers in children with sickle cell anaemia (SCA) and determine the relationship between these markers and disease severity.
Method: One hundred and fifty-six children, comprising 78 with SCA, aged 1 - 15 years and 78 age- and sex-matched Haemoglobin AA controls were studied. Serum TOS, OSI, and TAC were determined using ELISA kits. The severity of the SCA was determined using clinical and laboratory parameters.
Result: Children with SCA had lower mean serum TAC (0.83±0.31UAE) than controls (1.19±0.24UAE) with p<0.001. However, the mean serum TOS and OSI of children with SCA was higher than among the controls (13.33±4.64U/ml vs. 9.70±2.72U/ml and 20.95±16.75 vs. 8.68±3.76 respectively) with p<0.001. SCA subjects with mild disease had higher mean serum TAC (0.91 ± 0.27UAE) than those with moderate disease (0.54±0.27UAE) (p<0.001). On the other hand, the mean TOS and OSI were lower in children with mild disease compared to those with moderate disease (12.64±4.32U/ml vs. 15.63±5.07U/ml, p = 0.016 and 16.26±10.25 vs. 36.61±23.89 p<0.001 respectively). Sickle cell disease severity score had negative correlation with TAC (r = -0.60, p < 0.001) but positive correlation with TOS (r = 0.3, p = 0.008) and OSI (r = 0.6, p < 0.001).
Conclusion: Children with SCA had lower TAC but higher TOS and OSI than matched controls. Oxidative stress markers had a significant relationship with SCD severity.

Keywords: Children, Oxidative Stress Index, Severity Score, Sickle Cell Anaemia, Total Antioxidant Capacity, Total Oxidant Status.

Introduction

Sickle cell anaemia (SCA) is the most common haematological disorder globally and it is found more frequently in sub-Saharan Africa. [1] It is a homozygous genetic disorder with approximately 150,000 to 300,000 individuals born with the disease every year in Africa and

Nigeria has one of the highest burdens of SCA with a prevalence of 2-3%. [2] Vaso-occlusive crisis is the commonest clinical phenotypic expression in children with SCA in Nigeria and its frequency has been used as a marker of disease severity.[3] However, other devastating clinical manifestations such as acute chest syndrome, stroke, priapism, osteomyelitis, cholecystitis, leg ulcer, and socio-demographic factors have cumulative lifetime effects that determine the severity of disease in these patients. [4]

Individuals with SCA are prone to oxidative damage as a result of excessive generation of reactive oxygen species such as hydrogen peroxide, superoxide, hydroxyl radicals, and malondialdehyde. This is associated with lower levels of human antioxidant vitamins and enzyme activities leading to chronic redox imbalance in their red blood cell metabolic activities. [5-9] The resultant oxidative stress has been implicated in the pathophysiologic mechanism of SCA. [10] Individual oxidants and antioxidants usually interact in cells and body fluids of humans, leading to cumulative effects that can be measured directly in plasma, with the use of serum Total Oxidative Status (TOS) and Total Antioxidant Capacity (TAC) respectively. [11-13] These parameters measure all the biological components of plasma with oxidant and antioxidant activity at once and represent a dynamic equilibrium that occurs as a result of various synergistic interactions between individual oxidants and antioxidants, giving an insight into the delicate balance between them. [14,15] Previous workers on oxidative stress used the ratio of total oxidative status to total antioxidant capacity (oxidative stress index, OSI) as another measure of oxidative stress in their various studies. [11,12]

Although some studies have assessed the effect of oxidative stress markers on individual clinical manifestations of SCA such as vaso-occlusive crises and rate of haemolysis, [16] no report has examined the relationship of the oxidative stress markers with the overall sickle cell disease severity. In addition to evaluating the serum levels of oxidative stress markers (TOS, TAC, and OSI), this study determined the relationship between them and steady-state sickle cell disease severity using a validated scoring system. [4]

Methods

Study design and location

This study was a descriptive, cross-sectional comparative study conducted at the Children Sickle cell Disease Clinic of the Wesley Guild Hospital Ilesa, one of the two tertiary units of Obafemi Awolowo University Teaching Hospitals Complex, Ile-Ife, Nigeria.

Study Population

The haemoglobin genotype of each participant was confirmed using alkaline hemoglobin electrophoresis on cellulose acetate, before recruitment into the study. Steady-state in SCA was defined as a period without acute illness, pain, and infection for at least four weeks and no blood transfusion in the preceding three months. [17] Children whose parents failed to give consent, those on antioxidants and hydroxyurea were excluded from the study since these agents could modify the severity of sickle cell anaemia. [1]

Data collection

Socio-demographic and clinical information such as age, gender, frequency of vaso-occlusive crises and history of blood transfusion, hospitalization and lifetime complications were obtained from the study participants and their parents/guardians using a well-structured proforma and confirmed in the medical records. Socio-economic classification of families was described according to the method

recommended by Oyedeji, based on the assessment of the occupational and educational status of parents. Five socio-economic classes were initially generated (I to V) and these were subsequently reclassified into three major social classes: namely upper (I and II), middle (III), and lower (IV and V) classes. [18] The results of relevant clinical examinations which include determination of splenic size and liver size were also recorded.

The severity of SCD was assessed using the method proposed by Adegoke *et al*. [4] The following parameters were assessed: frequency of significant painful episodes, number of blood transfusions in the previous 12 months and number of hospitalization in the previous 12 months, splenic and liver size at the time of recruitment into the study, current haematocrit and total white cell count and the presence of lifetime cumulative incidence of complications such as cerebrovascular disease (CVD), acute chest syndrome (ACS), avascular necrosis of femoral or humeral head (AVN), pneumococcal meningitis, gall stone, osteomyelitis, chronic leg ulcer, and priapism. The disease severity was subsequently classified into mild, moderate and severe disease. Out of a possible score of 34, individuals with a score of < 8 were classified as having mild disease, 8 to 17 as moderate disease and those with scores ≥18 as severe disease. [4]

Determination of oxidative stress markers

Four millilitres (ml) of venous blood was dispensed into a plain bottle and was centrifuged at 2,000 revolutions per minute for 10minutes after allowing for clot retraction. The resulting supernatant serum was separated into another plain bottle and subsequently stored at -70^{o}C for analyses of TOS and TAC in batches within three months of sample collection. Both TAC and TOS were measured using ELISA kits (Human Total Antioxidant Capacity ELISA Kit, catalogue number 360 by Cell Biolabs, Inc. 7758 Arjons Drive, San Diego, CA 92126 and Human Total Oxidant Status ELISA kit, catalogue number UK-0512, manufactured by SPAN Biotech Ltd, Unit 5, Building 3, Sunshine Aloha, Bantian Industrial Park, Banxuegang Road, Loggang Shenzhen 518129, China). The TAC assay is based on the reduction of copper (II) to copper (I) by antioxidant uric acid standards and results were reported in Uric Acid Equivalent (UAE) with a normal reference interval of 0.95 – 1.50 UAE. Results of TOS were expressed in Unit/millilitre (U/mL) with a reference range of 4.2 - 15.0 U/mL. Oxidative Stress Index (OSI) was calculated as a ratio of TOS to TAC. [11]

Determination of haematological parameters

Haematological parameters were determined using an auto-haemoanalyser Pentra 60, Horiba® at the Institute of Virology of the hospital within 30 minutes of blood sample collection.

Ethical consideration

Ethical approval for this study was obtained from the Ethics and Research Committee of the Obafemi Awolowo University Teaching Hospitals Complex, Ile-Ife, Nigeria (ERC/2015/04/10). Written informed consents, as well as assent, were obtained from the parents/caregivers and children above seven years of age respectively.

Data analysis

Data collected was analysed using descriptive and inferential statistics as provided for in the IBM Statistical Program for Service Solutions (SPSS), version 20.0 (SPSS Chicago Inc., IL, U.S.A). Means and standard deviations (SD) were calculated for continuous variables and were compared using the Independent Sample *t*-test. Also, the proportions/percentages of categorical variables were calculated and compared using Chi-square tests. The degrees of correlations between serum TOS, TAC, OSI, and

SCD severity scores were determined using Pearson's Correlation analysis. Statistical significance was established with a probability value of < 0.05 at 95% confidence interval.

Results

Socio-demographic and haematological characteristics of the study population

A total of 156 children, aged 1 – 15 years, comprising 78 with SCA and 78 age and gender-matched apparently healthy HbAA controls, were included in the study. The mean ages of the subjects were 6.6±3.1 years and 6.3±3.1 years for the cases and controls respectively, with p = 0.646. Eighty five (54.5%) were males, with a male to female ratio of 1.2: 1 (p = 0.872). Sixty-eight (43.6%) children belonged to the middle class while 29 (18.6%) and 59 (37.8%) belonged to the upper and lower classes respectively. There was no statistically significant difference in the socio-economic class distribution of the two groups (χ^2 = 3.452, p = 0.178). Table I shows the detailed socio-demographic distribution of the subjects and the controls.

Table I: Demographic and socioeconomic characteristics of the study participants.

Variables	Cases (%)	Control (%)	Chi-Square	*P* value
Age group (years)			0.357	0.836
< 5	22 (14.1%)	25 (16.0%)		
5 – 9	42 (26.9%)	41 (26.3%)		
10 – 15	14 (9.0%)	12 (7.7%)		
Gender			0.026	0.872
Male	42 (26.9)	43 (27.6%)		
Female	36 (23.1%)	35 (22.4%)		
SEC			3.452	0.178
Upper	10 (6.4%)	19 (12.2%)		
Middle	36 (23.1%)	32 (20.5%)		
Lower	32 (20.5%)	27 (17.3%)		
Total	78 (50%)	78 (50%)		

SEC- Socioeconomic class

Haematological parameters and serum oxidative markers (TOS, TAC, and OSI) in children with SCA and HbAA controls

The haematocrit of the children with SCA was significantly lower than their age- and sex-matched controls (p<0.001) while their total leucocyte and platelet counts were significantly higher than for the controls, p<0.001 and p < 0.001 respectively. However, there was no significant difference between the MCV, MCH, and MCHC of the cases and controls; p = 0.184, 0.242 and 0.972 respectively as shown in Table II.

The mean serum levels of TAC (0.83±0.31 UAE) was significantly lower in children with SCA compared to the controls (1.19±0.24 UAE), t = -8.130, p<0.001. However, the mean serum TOS of children with SCA (13.33±4.64 U/ml) was significantly higher than 9.70±2.72 U/ml for the controls, p<0.001. Also, the Oxidative Stress Index (OSI) was significantly higher among the cases (20.95±16.75) compared to 8.68±3.76 for the controls, p<0.001 (Table II).

Table II: Oxidative stress and haematological parameters of study subjects

Variable	Cases Mean ± SD	Control Mean ± SD	t- test	*P* value
Haematocrits (%)	23.79±3.58	35.55±3.06	-22.06	< 0.001
Leucocyte (x10⁶/ml)	14.62±5.41	6.14±2.10	12.91	<0.001
Platelet (x 10³/ml)	344.99±149.62	257.79±93.78	4.361	< 0.001
MCV (fl)	79.26±8.68	77.61±6.55	1.334	0.184
MCH (pg)	26.18±3.33	25.63±2.45	1.173	0.242
MCHC (g/dl)	32.99±1.53	33.00±1.22	-0.035	0.972
TOS (U/ml)	13.33±4.64	9.70±2.72	5.967	< 0.001
TAC (UAE)	0.83±0.31	1.19±0.24	-8.13	< 0.001
OSI	20.95±16.75	8.68±3.76	6.308	< 0.001

MCV - Mean Corpuscular Volume, MCH- Mean Corpuscular Haemoglobin, MCHC- Mean Corpuscular Haemoglobin Concentration. TAC- Total Antioxidant Capacity, TOS- Total Oxidative Status, OSI- Oxidative Stress Index, SD- Standard deviation; UAE- Uric Acid Equivalent.

Relationship between socio-demographic characteristics and sickle cell disease severity

The minimum and maximum sickle cell disease severity scores (SCDSS) were 0 and 15 respectively with an overall mean score of 5.18 ± 3.18. Sixty (76.9%) of the 78 children with SCA had mild disease, 18 (23.1%) had moderate disease while none had severe disease. Age is associated with worsening disease severity. A significantly higher proportion (30.4%) of the cases aged ≥ 5 years had a moderate disease compared to 4.5% of those younger than 5 years of age. (p = 0.015). As shown in Table III, gender and socioeconomic class did not significantly influence disease severity (p = 0.213 and 0.377 respectively).

Relationship between laboratory indices (haematological and oxidative stress markers) and SCD severity

The mean serum TAC in children with mild disease (0.91±0.27 UAE) was significantly higher compared to that in children with moderate disease (0.54±0.27 UAE) (p<0.001). However, the mean serum level of TOS and OSI were significantly lower in those with mild than in moderate disease (12.64±4.32 U/ml vs. 15.63±5.07 U/ml, p = 0.016, and 16.26±10.25 vs. 36.61±23.89 p<0.001 respectively) (Table IV). There was a strong negative correlation between SCDSS and TAC (r = -0.60, p<0.001). In contrast, there was mild positive significant correlation between SCDSS and TOS (r = 0.3, p = 0.008) and strong positive significant correlation with OSI (r = 0.6, p<0.001) (Figures 1 and 2). As expected, the mean haematocrit of subjects with the mild disease was significantly higher than for moderate disease (p<0.001). The mean total leucocyte and platelet counts were significantly lower in those with mild disease p<0.001 and 0.049 respectively. (Table IV). There was no significant difference between the MCV, MCH, and MCHC of children with mild disease and those with moderate disease (p = 0.328, 0.198 and 0.219 respectively).

Discussion

Oxidative stress is one of the pathophysiologic mechanisms associated with the severity of clinical manifestations in patients with SCA. [19] Although several efforts have been made to determine the levels of individual oxidants and antioxidants in SCA, data on cumulative or total oxidative stress markers such as TOS, TAC, and OSI are scanty. Also, studies on the influence of these markers on SCD severity are limited.

Table III: Relationship between socio-demographic factors and the severity of the disease

Socio-demographic characteristics	Mild disease	Moderate disease	χ^2	*P* value
Age			5.928	0.015
Under 5	21 (26.9%)	1 (1.3%)		
5 and above	39 (50.0%)	17 (21.8%)		
Gender			1.548	0.213
Male	30 (38.5%)	12 (17.4%)		
Female	30 (38.5%)	6 (7.7%)		
SEC			0.780	0.677
Upper	8 (10.3%)	2 (2.6%)		
Middle	29 (37.2%)	7 (9%)		
Lower	23 (29.5%)	9 (11.5%)		
Total	60 (76.8%)	18 (23.1%)		

SEC- Socioeconomic class

Table IV: Relationship between haematological parameters and serum levels of oxidative stress markers, and sickle cell disease severity

Laboratory markers	Mild disease (Mean±SD)	Moderate disease (Mean ± SD)	t- test	*P* value
Haematocrits	24.76±3.08	20.54±3.27	5.032	< 0.001
Leucocyte count	13.49±4.57	18.41±6.37	-3.643	<0.001
Platelet count	326.78±142.79	405.67±159.86	-2.000	0.049
MCV	79.79±9.34	77.49±5.85	0.985	0.328
MCH	26.45±3.52	25.60±2.48	1.299	0.198
MCHC	33.11±1.56	32.60±1.38	1.240	0.219
TAC (UAE)	0.91±0.27	0.54±0.27	5.055	< 0.001
TOS (U/ml)	12.64±4.32	15.63±5.07	-2.468	0.016
OSI	16.26±10.25	36.61±23.89	-5.234	< 0.001

MCV- Mean Corpuscular Volume, MCH- Mean Corpuscular Haemoglobin, MCHC- Mean Corpuscular Haemoglobin Concentration. TAC- Total Antioxidant Capacity, TOS- Total Oxidative Status, OSI- Oxidative Stress Index. SD - Standard deviation; UAE- Uric Acid Equivalent.

Previous studies suggested that an imbalance between the levels of TAC and TOS are responsible for the alterations in various physiological and biochemical processes leading to various stress-induced disorders in human. [20-22] In this study, we observed that patients with SCA in the steady-state had significantly higher levels of TOS and OSI but lower levels of TAC compared to controls. This is consistent with the data among Egyptian and Nigerian children. [16,23,24] A previous study in which SCA cases were supplemented with low molecular weight antioxidants such as vitamins C and E witnessed a significant increase in the levels of TAC. This suggests that the lower levels of TAC observed among SCA patients may be attributed to increased utilization of TAC and depletion of the low molecular weight antioxidants in the body. [25] Also, HbS-containing red cells auto-oxidize faster, generating greater extent of oxidants such as superoxide, hydrogen peroxide, hydroxyl radical and lipid peroxidation products when compared with HbA–containing red cells.

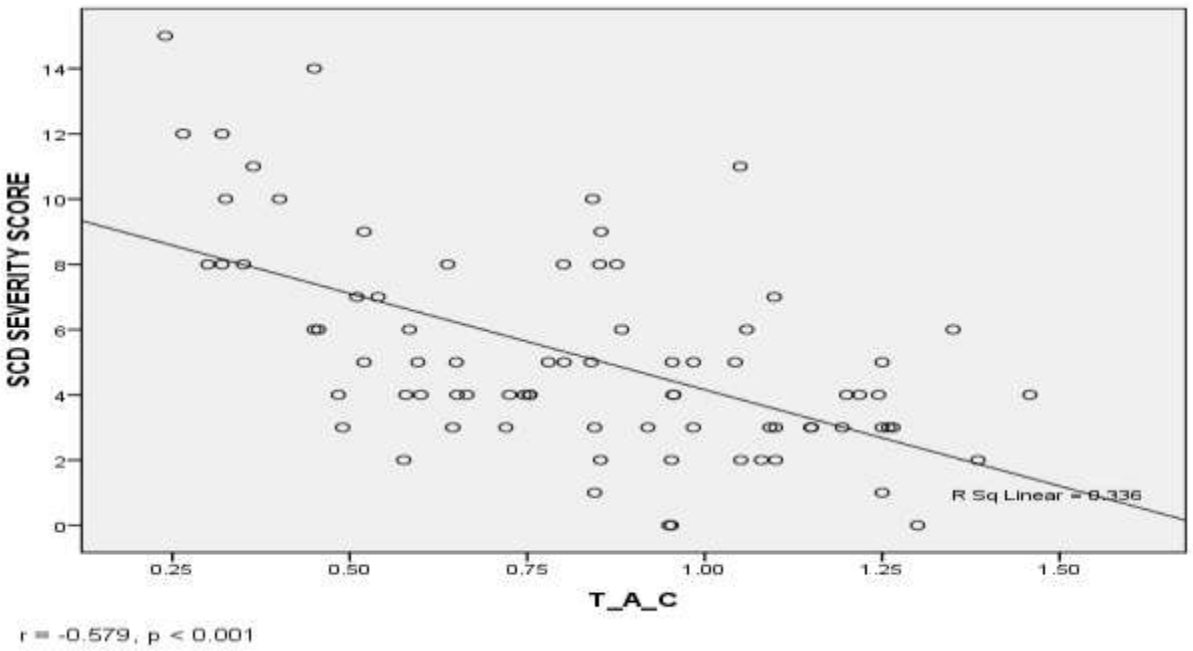

Figure 1: Scatter plot showing the correlation between SCD severity score and Total antioxidant capacity.

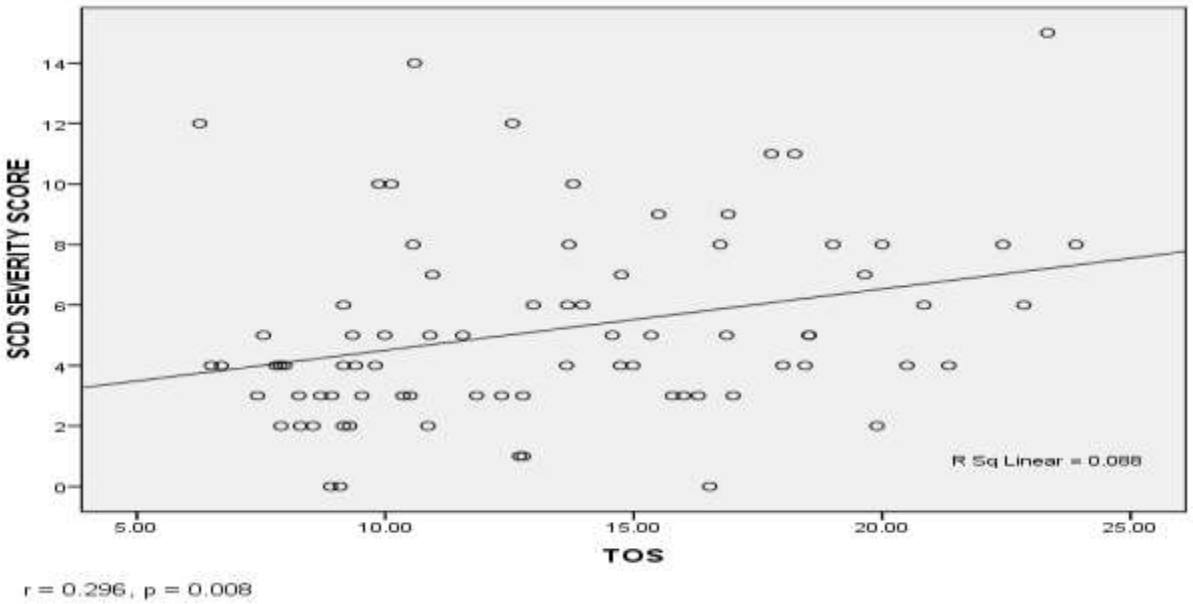

Figure 2: Scatter plot showing the correlation between SCD severity score and Total oxidative status.

This is likely responsible for the increase in TOS seen among patients with SCA. [16,26] Previous studies have related these oxidative markers to single clinical manifestations such as vaso-occlusive crises and the level of haematocrit. [16, 23] In the present study, it was observed that children with moderate disease had significantly higher TOS and OSI but lower TAC when compared to children with mild disease. A significant correlation also existed between oxidative stress markers and SCD severity score. To the best of authors' knowledge, no study has been done on this. This observation, therefore, buttresses the need to improve the oxidative milieu of patients with SCA to reduce the severity of the disease and prevent attendant complications. Although there is increasing evidence of the efficacy of HbF-inducing agents, anti-inflammatory and anti-adhesive drugs in improving disease severity in patients with SCA, antioxidants also may have an immense role to play to reduce the severity of the disease. Although more randomized controlled trials are needed to establish the influence of some proposed antioxidants such as Omega-3 Fatty acid and N-acetyl cysteine on clinical manifestations of SCA for consideration in the routine management of the disease. [27-29]

This study is subject to recall bias, especially concerning information on the lifetime incidence of complications and the frequency of significant pain episodes. We, however, did an extensive review of relevant medical charts in addition to clinical histories to limit this type of bias. Secondly, this study was limited by the inability to quantify the number of antioxidants present in the food taken by each participant to exclude the possible effects of dietary intake on the levels of oxidative stress markers. Lastly, none of our study participants had severe disease based on the scoring system. Hence, specific effects on the level of TOS, TAC, and OSI on the subset with severe disease could not be conclusively verified.

Conclusion

This study shows that children with SCA had higher TOS and OSI but lower TAC than matched controls. Oxidative stress markers appeared to be related to SCD severity. Therefore, it is recommended that individuals with SCA should be encouraged to take food supplements containing antioxidants to increase their antioxidant status and subsequently improve their clinical outcomes.[25] Also, SCA patients are encouraged to avoid factors such as infections, acidosis, dehydration, extremes of temperature and strenuous exercise, which increase the generation of reactive oxygen species.

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Authors' Contributions: SOS, ASA, and AOA conceived and designed the study. SOS, ASA, and AMA participated in data acquisition, analysis and interpretation. SOS drafted the manuscript while ASA and AOA participated in critical revision for important intellectual content. All the authors approved the final version of the manuscript.

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