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Inflammatory and oxidative stress markers in multi-transfused sickle cell disease patients

Florence Ifechukwude Aboderin[1], Taofeeq Oduola[2], Glenda Mary Davison[1], Oluwafemi Omoniyi Oguntibeju[1]

[1]Cape Peninsula University of Technology, Faculty of Health and Wellness Sciences, Department of Biomedical Sciences, Bellville, South Africa
[2]Federal University of Health Sciences, Faculty of Allied Health Sciences, Department of Medical Laboratory Science, Ila Orangun, Osun State, Nigeria

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Abstract

Sickle cell anaemia (SCA) is a form of haemolytic anaemia caused by an abnormal composition of the globin chains of haemoglobin. When the mutated haemoglobin is exposed to low-oxygen concentration, it polymerizes into long crystals of red blood cells giving the cell an abnormal sickled shape with extreme fragility, and less flexible erythrocytes with a reduced lifespan and various complications including inflammation and oxidative stress which has been implicated in SCD patients. This study investigated the relationship between markers of inflammatory and oxidative stress in alloimmunized sickle cell patients. Hundred participants were involved in the study; fifty SCD aged 18 to 48 from Obafemi Awolowo University Health Centre, and a control group of fifty individuals without the disease. This is a cross-sectional study undertaken at the Department of Haematology and Immunology, Obafemi Awolowo University Teaching Hospital, Ile-Ife Osun State, Nigeria. The samples were analysed for inflammatory and oxidative biomarkers as well as liver and kidney function tests following the manufacturer's instructions. Biochemical parameters and inflammatory markers show a statistically significant increase (p=0.003) in the means of test groups compared to the means of the control groups for the following: SOD (pg/mL)=284.2, AST (µ/L)=46.94, ALT (µ/L)=37.0, CREAT (µmoI/L)=56.71, and Urea (µmoI/L)=3.4. A significant difference (p=0.003) between the means of CRP (ng/mL)=3.9 and TNF (pg/mL)=8.1 in the test groups compared to the control groups. Frequent transfusions in SCD serve to prevent and alleviate microcirculatory complications and the breakdown of red cells that incite inflammation and oxidative stress, potentially mimicking alloimmunization, and reducing the risk of organ damage. Oxidative and Inflammatory markers should be included in the proper monitoring of SCD patients.

Keywords: Inflammatory stress markers, oxidative stress markers, multiple transfusion, sickle cell disease patients

Introduction

Sickle cell disease (SCD) is a worldwide health concern ravaging the health of the African population, especially in Sub-Saharan Africa, where its prevalence is highest [1]. This hereditary genetic condition, linked to chromosome 11a, stems from alterations in the Deoxyribonucleic Acid (DNA) structure, causing the replacement of glutamic acid with valine at the sixth position of the beta-globin chain. About 20 million people are affected globally, with more than 11.5 million prevalence in Africa alone [2]. Children under the age of 5 years with SCD record a mortality rate of 85 % in Africa [3]. In Ghana, the prevalence rate is 2% birth rate annually [4]. Nigeria shares 2-3% SCD of the total populace, with a 24% prevalence of sickle cell trait and about 100,000 annual SCD infant deaths [5].

Sickle cell patients often suffer from the complications of oxidative stress and inflammation. The complexity of SCD extends beyond the genetic mutation, encompassing the intricate interplay of inflammation and oxidative stress. These complications, working synergistically, manifest either as oxidative stress due to the gradual increase of inflammatory processes or inflammation resulting from the aftermath of oxidative reactions. Oxidative stress, an additional pathophysiological phenomenon in SCD, results from an imbalance between the release of reactive oxygen species (ROS) and the body's defence capacity. These molecules are generated during regular metabolic processes and play essential roles in signalling, receptor activation, nuclear transduction, and gene expression. However, when there's an imbalance between ROS production (including ROS and reactive nitrogen species,

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Corresponding Author: Oluwafemi Omoniyi Oguntibeju, Cape Peninsula University of Technology, Faculty of Health and Wellness Sciences, Department of Biomedical Sciences, Bellville, South Africa
Email: oguntibejuo@cput.ac.za

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RNS) and the body's antioxidant defences, oxidative stress occurs, leading to cellular damage [6]. It is intimately linked to inflammation and endothelial destruction, exacerbated by chronic inflammation and intravascular haemolysis [6].

The origin of the chronic inflammatory state in SCD remains unpredictable, though certain factors could contribute to it such as red cell deformability, haemolysis, histamine release, thrombin generation, and complement activation collectively resulting in inflammation in SCD patients, with haemolysis playing a pivotal role. Haemolysis, particularly its impact on anti-inflammatory molecules like nitric oxide (NO) and heme oxygenase 1 (HO-1), obstructs their functionality, leading to an excessive buildup of reaction products [7-12].

Blood transfusion is important as it is a therapy for the life-saving of SCD patients, but often results in severe iron overload [8], increased biomarkers of oxidative damage [7], immune antibodies, and haemoglobin binding to the cell membrane acting as a Fenton Reagent thereby elevating the production of oxidants such as superoxide and hydroxyl radical [7,9]. The increase in the components of haemolysis such as intravascular haemoglobin and oxidants may contribute to the consumption of nitric oxide in SCD [11]. In contrast turn, a reduced level of NO, may lead to haemodynamic instability [12,13] and a reduction in antioxidant capacity [14]. Efforts to monitor oxidative damage in SCD involve measuring biomarkers such as F2-isoprostanes, 4-hydroxynonenal (4-HNE), SOD, and malondialdehyde (MDA). These stable end products of oxidative reactions provide valuable insights into oxidative stress levels and disease severity, with increased levels noted in SCD [15,16]. Advanced glycation end-products (AGE) also serve as indicators, correlating positively with haemolysis-related tissue complications in patients with SCD [17]. Other therapies used in the management of SCD complications include hydroxyurea, voxelotor, crizanlizumab and L-glutamine therapy, of which voxelotor 900mg seems to be more effective, in that the efficacy yielded elevated haemoglobin level and reduced markers of haemolysis in SCD [18]. In addition, voxelotor reduces the risk of stroke, albuminuria, pulmonary arterial hypertension and mortality [18].

Inflammatory and oxidative stress pose significant challenges for individuals with SCD who undergo frequent transfusions. The repetitive administration of blood transfusions can induce the production of allogeneic antibodies among other complications, thereby exacerbating oxidative stress levels. Moreover, chronic inflammation is the height of SCD, as the presence of damaged red cells triggers immune responses. Vaso-occlusive crises (VOCs) and iron intoxication resulting from recurrent transfusions can inflict damage on various organs, such as kidneys, lungs, pancreas and the brain [14]. Consequently, the focus of this study is to unravel the relationship between markers of inflammation and oxidative stress in alloimmunized patients with SCD.

Aim

This study aims to assess inflammatory and oxidative stress markers in multi-transfused patients with sickle cell disease to better understand their roles in the pathophysiology of SCD.

Material and Methods

This is a cross-sectional study conducted at the Haematology and Immunology Department of Obafemi Awolowo University Teaching Hospital, Nigeria. We investigated the relationship between oxidative stress and inflammatory profiles in sickle cell patients who received two or more pints of blood transfusion with a group of individuals without the disease (the control group) but received equivalent blood. A questionnaire was served to compile some information from participants.

Inclusion criteria

Inclusion criteria for this study involved SCD patients with multiple blood transfusions. The diagnosis of SCD was confirmed with the haemoglobin electrophoretic method.

Exclusion

Patients who were not diagnosed with SCD and were not between 18 and 48 years of age were excluded. Additionally, individuals experiencing crisis were also excluded.

Ethical Consideration

The study received ethical clearance from both Cape Peninsula University of Technology, (CPUT/HWS-REC2021 renewal) Bellville, South Africa (the Human Research Ethics Committee, Faculty of Health and Wellness Sciences), and the ethics committee of Obafemi Awolowo University Health Centre, Ref: (D.MHS/2023) Ile-Ife. Informed written consent was obtained from all participants involved in the research.

Analysis

Enzyme-linked immunosorbent assay (ELISA) KITS for Tumour Necrotic Factor (TNF), C-Reactive Protein (CRP), Interleukin 6 (IL-6), and Interleukin 1 beta (IL-1β) manufactured by Elabscience Biotechnology. USA. were used for the inflammatory analysis.

Using the micro-ELISA plate, samples were added to the wells and incubated with specific antibodies.after which antibody specific for Human IL-1β, TNF- α, IL-6, and CRP. Avidin-horseradish peroxidase (HRP) conjugate was included in all the microplate wells for incubation, after which washing took place and substrate solution was added. Those containing Human IL-1β, TNF- α, IL-6, and CRP and Avidim -HRP conjugate will appear blue, the reaction is stopped by adding stop solution and it turns yellow. The concentration of the samples is proportional to the optical density values read at a wavelength of 450±2mm. The analytes' levels in the quality control reagents of the kits were within the expected ranges [19].

Urea: (Kidney Function Test Ultraviolent (UV))

Urea is hydrolysed to produce ammonia and carbon dioxide in the presence of water and urease. The ammonia released

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combines with -oxoglutarate and reduced nicotinamide adenine dinucleotide (NADH) to yield glutamate and oxidised nicotinamide adenine dinucleotide (NAD+) in the presence of glutamate-dehydrogenase.

Creatinine: Colorimetric Method

The muscle tissues are responsible for the production of creatine and phosphate in the body. It is the source of creatinine It can be explained as a Nitrogenous byproduct. Creatinine does not store in the body, therefore it can not be reutilised but is excreted as a waste proportional to the body's muscle mass. Creatinine measurement is basically to determine kidney efficiency. It has some merits over the assessment of urea because it does not depend on any substances like protein ingestion, water intake etc. it is constant. An increased amount is indicative of kidney dysfunction. Decreased levels of plasma creatinine seem rare and not clinically significant.

Principle

Creatinine gives a coloured complex when reacts with picric acid in an alkaline solution. The amount of the compound formed is directly proportional to the creatinine concentration.

AST (Aspartate aminotransferase or glutamate oxaloacetate) Liver Function Test. UV Method

Aminotransferases are a variety of enzymes that speed up the conversions of amino acids and α- oxoacids by the transfer of amino groups. Some cells have been studied and AST is seen in cytoplasmic and mitochondria of cells. In cases of mild tissue damage e.g. Liver. AST is more domicile in the cytoplasm than mitochondria, therefore severe organ destruction results in increased mitochondria enzymes being released. Increased levels of AST indicate hepatic disease, muscular dystrophy, myocardial infarction, and organ damage.

Principle

α- oxoglutarate combinds with L- aspartate in place of AST to produce L-glutamate plus oxaloacetate. The indicator reaction utilises the oxaloacetate for a kinetic determination of NADH consumption.

ALT (Alanine Aminotransferase) UV Method

This is a maximum standard method according to the concentrations recommended by the International Federation of Clinical Chemistry (IFCC).

Statistical Analysis

Data were to undergo statistical analysis using SPSS version 24.0 statistical package and relevant statistical values were obtained. Student t-test was carried out and data were presented as mean ± standard deviation (SD), A Chi-square test was also carried out where appropriate. Values of $P<0.05$ were considered statistically significant.

Results

Demographic profile of the participants

This study comprises two different groups: the control and the test group. The control group consists of 26 (52.00%) females and 24 (48.00%) males, while the test group consists of 30 (60.00%) females and 20 (40.00%) males. The subjects were educated young adults of the black race ages 18 to 48 years. The body mass index (BMI), weight, and height were measured. The control group received less blood compared to the test group. There was no significant association between gender, age, and haemoglobin genotypic variation as shown in Table 1.

Table 1. Demographic distribution of SCD and control subjects with the various diagnoses

		Control n=50 (%)	SCD n=50 (%)	Total n=100 (%)	Statistical indices
Age (years)	**<=20**	08 (16.00)	08 (16.00)	16 (16.00)	X^2=0.1926, df=3 P-value=0.979
	21–30	19 (38.00)	21 (42.00)	21 (11.73)	
	31–40	13 (26.00)	12 (24.00)	54 (30.17)	
	41+	10 (20.00)	09 (18.00)	27 (9.50)	
Gender	**Female**	26 (52.00)	30 (60.00)	56 (28.00)	X^2=0.6494, df=1 P-value=0.4203
	Male	24 (48.00)	20 (40.00)	44 (22.00)	
Diagnosis	**Anaemia**	21 (42.00)	0 (0.00)	21 (21.00)	X^2=100.00, df=7 P-value<0.0001*+
	CML	04 (8.00)	0 (0.00)	04 (4.00)	
	HB SS	0 (0.00)	50 (100.00)	50 (50.00)	
	MM	01 (2.00)	0 (0.00)	01 (0.50)	
	Sepsis	04 (8.00)	0 (0.00)	04 (4.00)	
	Stomach pain	01 (2.00)	0 (0.00)	01 (0.50)	
	Surgery	15 (30.00)	0 (0.00)	15 (15.00)	
	Transfusion	04 (08.00)	0 (0.00)	04 (2.00)	

+ Significant p value, * chi square test

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Oxidative stress markers are represented in Table 2. Superoxide dismutase (SOD) showed a significant difference with P=0.008 in the test group compared to the control group, whereas catalase showed no significant difference (P=0.079) in both the test and the control groups.

Table 2. Shows the means and standard deviation of biochemical enzymes of participants

	M	SD	P-value
AST (µ/L)	46.9	38.70	0.036*
ALT (µ/L)	37.0	37.44	0.001*
Creatinine (µmol/L)	56.7	23.98	0.005
Urea (µmol/L)	3.4	1.68	0.004
Catalase (µ/mL)	139.6	73.82	0.079
SOD (ng/dL)	271.8	47.52	0.008

M: mean, SD: standard deviation

Table 3, Represented markers of inflammation. CRP and TNF were significantly higher in the means of the test group compared to the means of the control group. Whereas, IL-6 and IL-1β showed no significant difference between the means of the test and control groups. The biomarkers such as creatinine (CR) and urea (U) showed a significant increase in the means of the control groups compared with the test groups, as represented in Table 4. Additionally, the enzymes AST and ALP showed a significant increase (P=0.036 and P=0.001 respectively) in the test groups compared with the control groups. However, Table 5. Shows a notable positive correlation (P<0.05 r=) between CRP and IL-6, TNF and Catalase. However, a significant negative correlation (P<0.05 r=) with AST and ALT, IL-1β, SOD, creatinine and Urea.

Table 3. Inflammatory parameters of participants by study group

	Group						
	Control		Test				
	M	SD	M	SD	t	Df	p
CRP	1.2	2.78	3.9	5.62	-3.099	97	0.003*
IL-6	84.3	101.96	58.1	86.10	1.380	97	0.171
TNF	2.8	5.76	8.1	14.31	-2.449	97	0.016*
IL-1β	2.5	.38	4.1	6.73	-1.710	97	0.090

Table 4. Shows the means and standard deviation of biochemical enzymes of participants by study group

	Group						
	Control		Test				
	M	SD	M	SD	T	Df	p
AST (µ/L)	29.94	40.51	46.94	38.90	2.130	97	0.036*
ALT (µ/L)	14.90	13.65	37.00	37.63	3.866	97	0.001*
CREATININE (µmol/L)	140.82	203.72	56.71	24.98	2.899	97	0.005
UREA (µmol/L)	6.886	8.31	3.4	1.68	2.918	97	0.004
CATALASE (µ/mL)	152.7	77.92	126.7	67.87	1.778	97	0.079
SOD (ng/dL)	259.1	41.91	284.2	49.77	-2.716	97	0.008*

P<0.05 indicates a significant difference.

Table 5. Shows the correlation between the study parameters.

CRP	AST (µ/L)	ALT (µ/L)	IL-6	TNF	IL-1β	Catalase (µ/mL)	SOD (ng/dL)	Creatinine (µmol/L)	Urea (mmol/L)
--	-.094	-.096	-.210*	.210*	.018	-.222*	-.079	-.109	-.112

*Indicates a significant correlation between CRP and study parameters

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Discussion

Findings from this research revealed that the inflammatory parameters of participants showed significantly increased levels in the means of the test group for C-reactive protein (CRP) (ng/mL)=3.9; p=0.003 and Tumor necrosis factor (TNF) (pg/mL)=8.1; p=0.016 compared to the means of the control group. Using T-test with SPSS statistical package version 24. These results are consistent with the earlier studies by [20] which reported higher CRP and TNF levels in SCD compared to controls. Clinically increased CRP in SCD patients denotes the presence of a serious health condition that causes acute inflammation. Repeated CRP levels are useful in monitoring patients with inflammatory challenges like SCD, especially in the first week of follow-up of antibiotic treatment of pneumonia. Prolonged delay in the reduction of CRP levels could be associated with an increased risk of inappropriately treatment with antibiotics [21]. CRP modulates inflammation, the increase in CRP is indicative of inflammation, infeclltion, or injury in the body. It is likened to vaso-occlusion hence hindering the smooth flow of blood to the tissues [22]. It is evidenced that patients with SCD have moderately increased CRP during steady state and significantly increased during painful vaso-occlusive crisis and haemolysis.

TNF was significantly increased in SCD patients than in controls, this concurs with the report of [23]. TNF-alpha is a pro-inflammatory cytokine an early indicator of inflammation which is formed by macrophages/ monocytes during acute inflammation and is accountable for a huge range of signaling events that lead to apoptosis and necrosis. This protein is also necessary for the inhibition of infection and cancers [24].

Additionally, from the study, SOD showed a significantly increased activity in the test group than the control, this agrees with Gizi, Papassotiriou [25] and Younus [26]. SOD is an essential antioxidant defence against oxidative stress in the body. It acts both as an enzyme and curative agent ROS. Charles Antwi- Boasiako et al., [27], reported low SOD, and Repka and Hebbel, [9], recorded an increase in biomarkers of oxidative damage both in SCD and thalassaemia. Some other authors like Lazzaretti et al., [28] and, Schater et al., [29] reported low activity as well. In this study, SOD was significantly increased, this might be because our subjects had been on a series of blood transfusions which in a way to ameliorate hypoxia, a state of insufficient amount of oxygen to the tissues to maintain adequate homeostasis; this can result from inadequate oxygen delivery to the tissues either due to low blood supply or low oxygen content in the blood (hypoxemia). The cause of inadequate oxygen in SCD patients is the sickled shape of the erythrocytes, which deprives and short-changes the normal amount of oxygen to the organs of the body (Ischaemia) this is one of the challenges of SCD that regular blood transfusion tends to solve though some risks are involved such as increasing chances of foreign antigens and the risks of producing alloantibodies that could cause DHTRs. This usually poses a challenge of suitability [30]. Conrath [31], and Khatun [15] reported that multiple blood transfusions (MBT) can lead to transfusion-transmitted infections iron overload, and inflammation El Chaer et al., [16,17]. These factors trigger the pathogenesis and emergence of SCD; however, the outcome of a dysfunctional immune response is complicated and further investigation on alloantibody and immune response is required. The properties of haemolysis breakdown (haem and free haemoglobin) have been implicated in the activation of innate and adaptive immune responses. Allalli et al., [32] report suggests that patients with high haemolysis rates are at greater risk of early mortality [31]. The continual breakdown and destruction of erythrocytes result in sustained activation of innate immune cells resulting in a chronic inflammatory state [33-35].

A significant difference was recorded for aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the means of test compared with control. This concurs with the findings of Taiwo et al., [36], stating that there is dysfunction of the liver in the steady SCD, also according to Kotila et al., [36] extreme haemolysis causes hyper bilirubinaemia leading to increased alkaline phosphatase (ALP) in SCD patients. In addition, haemolysis raises AST and ALT levels in SCD. Emokpae and Umeadi [37] reported a high level in the means of test patients for AST and ALT compared to the control, again this finding tallies with our report.

Considering the Creatinine (Cr) and urea (U) as markers of renal function test, Cr showed a significant increase in the means of the control group compared with the test group which affirms with [38]. In this study, serum creatinine was reportedly low in SCD patients which contradicts that of [38]. The discrepancies might be due to the series of precautionary measures prescribed for their patients before the test was carried out. They were not allowed to take medications, block the renal pathway to protein or creatinine load, alter renal haemodynamics and many restrictions melted to the patients. However, in this study, there were no restrictions related to renal function pathways that was observed because there was no need of such since our subjects were mainly Multi- transfused patients.

Serum creatinine is a biochemical substance that usually should be filtered out of the body through a healthy kidney but accumulation and increased levels of creatinine jeopardize and render the kidney dysfunctional and put the patient on a danger list. Furthermore, when sickled red blood cells lack sufficient oxygen (hypoxia), it results in dysfunction of the heme and triggers hemolysis (due to antigen-antibody incompatibility), accompanied by a sickle cell pain crisis Vaso-occlusion (VOC), microvascular occlusion and tissue Ischaemia brings about the pathophysiology of SCD which eventually prompt renal failure and results into death. Consequently, Urea showed a significant difference in the means of the control compared to the test group this agrees with [39,40] where Urea was reported lower in children with Hb-SS than in Hb-AA subjects. Normally proteins are broken down to urea on a normal day when the kidney is working fine, but where the kidney is malfunctioning, the urea nitrogen stays in the blood if the level exceeds the normal range or is too low (7-20mg/dl), it means the kidney is not working fine.

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Conclusion

In summary, our findings revealed a significant increase in inflammatory markers, such as CRP and TNF, among sickle cell patients. Moreover, there was a notable increase in superoxide dismutase (SOD) levels, alongside some enzymes including AST and ALT. Furthermore, sickle cell patients demonstrated lower urea levels compared to the control group, These results underscore the facts that haemolysis could trigger inflammation and pro-inflammatory cytokines as well as induce oxidative stress in sickle cell disease patients, ultimately posing challenges to the efficacy of the immune system in the long term. The evidence of this is prominently displayed in the results. It is advisable to include markers of inflammation and oxidative stress in routine examinations to gain a better understanding of their roles in the pathophysiology and appropriate monitoring and management of SCD patients.

Recommendation

The primary strength of this study is that it is the first to be conducted in southwest Nigeria. It provides a better understanding of the relationship between inflammation and oxidative stress biomarkers in a multiple transfusion in patients with sickle cell disease. The management of SCD should be supported with blood transfusions on the condition that the patients need it and would improve the quality of life and longevity in the SCD patients. Future research should explore specific immunological mechanisms to provide deeper insights.

Limitation

This study faced certain limitations. Initially, we intended to work with a larger sample size, however, logistical challenges and some individuals' unwillingness to participate prevented us from achieving this goal. A larger sample size could have provided more robust data.

Conflict of Interests
The authors declare that there is no conflict of interest in the study.

Financial Disclosure
This research was funded by Grant RJ23-Cape Peninsula University of Technology, (CPUT).

Ethical Approval
The study received ethical clearance from both Cape Peninsula University of Technology, (CPUT/HWS-REC2021 renewal) Bellville, South Africa (the Human Research Ethics Committee, Faculty of Health and Wellness Sciences), and the ethics committee of Obafemi Awolowo University Health Centre, Ref: (D.MHS/2023) Ile-Ife. Informed written consent was obtained from all participants involved in the research.

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