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Respiratory Burst Enzymes and Oxidant-antioxidant Status in Nigerian Children with Sickle Cell Disease

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Authors' contributions

This work was carried out in collaboration between all authors. Author GA wrote the protocol and coordinated the study. Author AA wrote the first draft of the manuscript. Authors GA and AA carried out the sample analyses. Author TO performed the statistical analysis. Author OA carried out the literature searches. Author ED assessed the subjects clinically. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aim: To measure respiratory burst enzymes, pro-oxidants, antioxidants and red cell indices in Nigerian children with sickle cell disease (HbSS) below five years of age and compared with apparently healthy children with normal haemoglobin (HbAA).
Method: A total of 45 subjects were recruited which included 23 children (age range 10 – 48 months) with HbSS and 22 children (age- and sex- matched) with HbAA. Blood samples were collected and red cell indices were determined using automated haematology analyser while serum superoxide dismutase (SOD), glutathione peroxidise (GSH-Px) and myeloperoxidase (MPO) activities were measured using ELISA kits. Serum malondialdehyde (MDA), hydrogen peroxide (H_2O_2), glutathione S transferase (GST), catalase (Cat), xanthine oxidase (XO) and glutathione (GSH) were measured with colorimetric techniques. MPO, SOD and Cat represented respiratory burst enzymes; MDA, H_2O_2 and XO were measured as pro-oxidants while GSH, GST and GSH-Px were

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the measured antioxidants.
Results: Mean concentration of malondialdehyde was significantly reduced (5.56±1.12nmol/L vs. 6.46±1.11nmol/L, *P*=.04) in HbSS children compared with HbAA children. Similarly, mean serum activity of myeloperoxidase in HbSS children was significantly reduced compared with HbAA children (66.12±13.34U/mL vs 77.02±13.54U/mL, *P*=.03). However, there were no significant differences in mean concentration of serum glutathione, hydrogen peroxide; serum activities of glutathione peroxidase, catalase, superoxide dismutase, xanthine oxidase and glutathione S transferase in HbSS children compared with HbAA children
Conclusion: HbSS children in this population did not demonstrate raised oxidative stress.

Keywords: Antioxidants; free radicals; respiratory burst; sickle cell disease; children.

1. INTRODUCTION

Sickle cell disease (SCD) is an autosomal recessive disorder characterised by synthesis of abnormal haemoglobin (HbS), which results in red blood cell (RBC) sickling. Sickling damages the cell membrane, leading to haemolysis and anaemia and decreases the cell's elasticity, leading to vessel occlusion and ischaemia [1]. Millions of people are affected worldwide, particularly among people of sub-Saharan African ancestory; South America, the Caribbean and Central America; Saudi Arabia; India and Mediterranean countries.

During the re-oxygenation of RBC, electron is transferred from haeme iron to oxygen which generates significant amounts of superoxide [2,3]. Increased autooxidation has been reported in HbS [4,5], the effect of which is expected to be minimised by presence of sufficient antioxidants which reacts to form harmless byproducts. A high production rate of Reactive Oxygen Species (ROS) in sickle cell disease is caused by factors such as increased intravascular haemolysis, ischaemia-reperfusion injury and chronic inflammation [5,6,7]. ROS are produced as the result of intracellular catabolism that requires oxygen as a terminal electron acceptor [8]. During this process, ROS such as O^{-}_{2}, H_2O_2 and OH^{-} radicals are produced as intermediates, even in healthy individuals [9,10].

Opsonophagocytosis requires respiratory burst enzymes and is involved in the clearing of infections caused by *Streptococcus pneumoniae* and *Haemophilus influenzae*. The respiratory burst by polymorphonuclear neutrophils (PMNs), which involves the production of reactive oxygen species (ROS), is an important part of the innate immune response in the killing of engulfed pathogens [11]. The chronically elevated production of ROS in PMNs of sickle cell patients lead to an exhausted respiratory burst capacity of these cells resulting in less effective eradication of engulfed pathogens [12]. Generation of ROS also result from the activation of enzymes such as xanthine oxidase (XO), NADPH oxidase, nitric oxide synthase (NOS), cytochrome P450, cyclo-oxygenase and lipoxygenase; which are activated during repeated cycles of hypoxia/re-oxygenation or ischemia/reperfusion [13,14]. Reactive oxygen species (ROS) such as superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) induce oxidative damage to the cell and form a very stable structure by extracting electrons from other sources. They are also able to generate other forms of ROS [15]. Furthermore, ROS degrade polyunsaturated lipids forming malondialdehyde (MDA) [16] that cause toxic stress in cells [17].

Under normal condition, there is a balance in ROS production and antioxidant generation. However, oxidative stress is the result of an imbalance between the production of ROS and antioxidants [18]. Major ROS defense mechanisms include enzymatic and non-enzymatic systems. These protective mechanisms include the enzymatic antioxidants: superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and the non-enzymatic antioxidants tocopherols, reduced glutathione (GSH), nitric oxide (NO), carotenoids, ascorbic acid (A-HOOH), lipoic acid, ubiquinols, selenium, riboflavin, zinc, carotenoids and uric acid as well as metal-binding proteins [19].

Previous studies measured oxidants and antioxidants in HbSS patients but without age stratification and not in children [20-23]. Also, defective humoral immune responses were reported in HbSS subjects [24]. However, there is gap in knowledge on the effectors of intracellular killing (respiratory burst enzymes) in HbSS children below five years. This study is therefore designed to compare respiratory burst enzymes and oxidant – antioxidant status in HbSS patients below age five, with HbAA controls and to correlate these findings with their haematological parameters.

2. MATERIALS AND METHODS

2.1 Subjects

A total of 45 subjects were recruited which included 23 HbSS children, aged 10 to 48 months, from Paediatrics Out-patient Clinic of Lagos State University Teaching Hospital, Lagos, Nigeria and 22 age and sex matched apparently healthy HbAA subjects who served as controls. Institutional ethical clearance was obtained and assent was obtained from parents as children were too young to give consent. HbSS children were ensured to be in a steady state and all subjects belonged to the same socio-economic class of the society.

2.2 Sample Collection and Analysis

Blood sample (5mL) was collected as follows; 2mL was collected in EDTA bottle to determine red cell indices which includes red cell count (RBC), haematocrit (hct), haemoglobin concentration (Hb), mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC). The remaining 3mL was spun for serum and was used to measure the serum concentration of hydrogen peroxide (H_2O_2), glutathione (GSH), malonyldialdehyde (MDA), as well as serum activities of myeloperoxidase (MPO), catalase (Cat), glutathione peroxidise (GSH-Px), glutathione S transferase (GST), superoxide dismutase (SOD) and xanthine oxidase (XO).

Red cell indices were determined using automated haematology analyzer (SYSMEX SF-3000, Japan). Serum activity of SOD and GSH-Px were measured in serum using ELISA technique, with kits supplied by NORTHWEST LIFE SCIENCE SPECIALTIES, Vancouver, Canada while Catalase was estimated using a kit by SIGMA, Missouri, USA. Myeloperoxidase activity was measured using ELISA technique with the kit supplied by OXFORD BIOMEDICAL RESEARCH, MI, USA; glutathione-S-transferase activity and hydrogen peroxide concentration were measured colorimetrically using reagent manufactured by ARBOR ASSAYS, MI, USA; MDA was measured with a method based on the thiobarbituric acid reactive substance (TBARS) assay, a colorimetric method described by Badcock et al. [25]; xanthine oxidase activity was determined using the method described by Bergmeyer et al. [26]. The Statistical package for social sciences (SPSS) version 17.0 for

Windows was used for statistical analysis. Results were expressed as Mean±S.D. Student's test was used to compare the differences between the mean values of parameters of HbSS subjects and controls, while Pearson's correlation coefficient was used to test for association between haematological indices and biochemical parameters. Values of *P* less than .05 were considered to be statistically significant.

3. RESULTS AND DISCUSSION

3.1 Results

Table 1 below show red cell indices in children with HbSS compared with controls. Red cell count, haemoglobin concentration as well as haematocrit were significantly lower in children with HbSS compared with controls; while there were no differences in mean MCV, MCH or MCHC of children with HbSS compared with controls.

Table 1. Red cell indices of patients with HbSS as compared with controls

	HbSS children n= 23	**HbAA Controls n = 22**	***P*-value**
Red cell count	3.5±1.1	4.6±0.3	.04*
Haemoglobin concentration (g/dL)	6.9±1.6	9.2±1.1	<.001*
Haematocrit (%)	24.0±5.3	33.1±3.1	<.001*
Mean cell volume (fl)	73±16.4	72.4±6.7	.53
Mean corpuscular haemoglobin (pg)	21.7±1.3	21.0±3.1	.78
Mean cell haemoglobin concentration (g/dL)	28.6±1.2	27.8±1.3	.12

** P is significant (<.05)*

Table 2 shows the results of respiratory burst enzymes, proxidants and antioxidants in HbSS children compared with HbAA. Serum myeloperoxidase activity was significantly lower in HbSS subjects compared with controls. However, there were no differences in serum activities of superoxide dismutase and catalase in HbSS subjects compared with controls. Serum malondialdehyde concentration was significantly lower in HbSS than in controls, while there were no differences in hydrogen peroxide concentration as well as xanthine oxidase activity in HbSS children compared to controls. Serum glutathione, activities of glutathione S transferase and glutathione peroxidase were not significantly different in children with HbSS as compared to controls.

Table 2. Serum activities of respiratory burst enzymes, levels of pro-oxidants and antioxidants in children with HbSS compared with controls

	HbSS Subjects n= 23	**Controls n = 22**	***P*-value**
Myeloperoxidase (U/mL)	66.12±13.34	77.01±13.54	.03*
Superoxide dismutase (U/mL)	401.98±30.23	398.51±39.03	.71
Catalase (U/mL)	291.58±47.64	303.36±29.25	.90
Malondialdehyde (nmol/mL)	5.56±1.12	6.46±1.11	.04*
Hydrogen peroxide (µmol/L)	47.71±9.40	52.67±6.64	.13
Xanthine oxidase (U/mL)	49.25±10.10	55.90±9.82	.11
Glutathione (µmol/L)	23.71±2.09	23.04±2.26	.88
Glutathione S transferase (U/L)	374.22±23.89	360.05±35.26	.56
Glutathione peroxidase (U/mL)	424.45±27.40	412.11±40.36	.81

** P is significant (<.05)*

In HbSS patients, there was significant negative correlation between catalase with RBC (r = -.86, *P* =.03) as well as a significant negative correlation between H_2O_2 with MCV (r =-.46, *P*.04). In HbAA controls however, no association was found between haematological and biochemical parameters.

3.2 Discussion

Sickle cell disease patients have been reported to have an increased risk of infections, notably infections with encapsulated organisms e.g. *Streptococcus pneumonia* [27,28], primarily as a result of dysfunctional antibody production, defective opsonophagocytosis as well as defective splenic clearance [29]. A study showed that HbS auto-oxidize faster than HbA which is one of the causes of increased oxidative stress in individuals with SCA [4,5].

This study investigated respiratory burst enzymes (myeloperoxidase, superoxide dismutase, catalase), antioxidants (glutathione, glutathione peroxidase and glutathione S transferase), pro-oxidants (malondialdehyde, xanthine oxidase, hydrogen peroxide) and haematological indices (red cell count, haemoglobin concentration, haematocrit, mean cell volume, mean cell haemoglobin and mean cell haemoglobin concentration); in HbSS children compared with HbAA.

Oxidative free radical species are generated in the normal course of a variety of essential biochemical reactions that progress at the sub-cellular organelle level. Free radicals are also derived from exposure to environmental components such as X-rays, ozone, cigarette smoking, air pollutants and industrial chemicals. They are particularly important in the intracellular killing of engulfed pathogens [30] and result in the damage of DNA, membrane phospholipids and proteins.

Myeloperoxidase is important in oxygen dependent intracellular killing which involves the generation of hypochlorite, a toxic compound, from hydrogen peroxide (H_2O_2) and chloride ions [31]. Plasma myeloperoxidase activity has been proposed as a useful marker of neutrophil proliferation and severity of inflammation [32]. Also, infectious diseases, especially with species of *Candida* have been observed predominantly in MPO-deficient patients [33]. In this study, plasma MPO activity was observed to be significantly lower in HbSS patients compared with controls, this might explain the susceptibility of HbSS subjects to infections caused by intracellular pathogens. Myeloperoxidase-deficient neutrophils were reported not to convert H_2O_2 to HOCl [34], thus, neutrophil killing of ingested organisms is diminished. It has also been reported that a G-463A MPO gene polymorphism may reduce MPO release by neutrophils thus increasing susceptibility to infections in children with HbSS [35]. This might explain the lower levels of MPO observed in our HbSS children.

Xanthine oxidase (XO) catalyses the generation of superoxide radical from hypoxanthine. XO production has been shown to be considerably increased in sickle transgenic mice following hypoxia and its deleterious effect after reperfusion [36]. It has been reported that HbSS patients are more prone to hypoxia-reoxygenation injury [36,37]. Slightly reduced XO in HbSS subjects might explain slightly reduced H_2O_2 in them.

ROS can cause damage to biological macromolecules [38] and membrane lipids readily react and undergo peroxidation. The peroxidative process yields lipid peroxides, lipids alcohol and aldehydic products and malondialdehyde [39]. Accumulation of MDA disturbs the organization of phospholipids in the human erythrocyte membrane bi-layer leading to membrane damage considered as an important factor contributing towards pathophysiology

due to the formation of irreversible sickle cells (ISC) [40]. RBCs are known to be particularly susceptible to peroxidative damage because they contain hemoglobin, one of the most powerful catalysts for initiation of peroxidative reaction [41]. It is also stated that excess quantity of malondialdehyde can promote erythrophagocytosis [42]. Our result of low MDA in HbSS children might indicate that RBC damage is reduced in them. Emokpae et al. [43] reported an increase in MDA in Nigerian adults with sickle cell disease, which correlates positively with acute phase proteins and prooxidants. Therefore, the reduced MDA observed in this study may be a consequence of slightly reduced production of H_2O_2, a ROS, in our population of HbSS children.

A decrease in erythrocyte glutathione (GSH) concentration of individuals with sickle cell disease has been reported [44]. Alsultan et al. [45] reported a decreased erythrocyte superoxide dismutase, catalase and glutathione peroxidase. Furthermore, a number of authors reported significant reduction in antioxidant levels of individuals with sickle cell disease [20-22,46,47]. The present study however observed no significant differences in antioxidants (glutathione, glutathione peroxidase, glutathione S transferase, superoxide dismutase and catalase) measured in children with sickle cell disease. This could be due to age differences of study population. Moreso, potentially higher HbF levels in SCD children (particularly young children) may be one of the reasons to explain reduced oxidative stress in them. Many of the previous studies focused on adults with sickle cell disease as well as children above five years of age [20-22]. This present study however studied children below five years of age.

Our study observed a significantly lower red blood cell count and haemoglobin concentration in children with sickle cell disease compared with controls, and also, there was a significant negative correlation between red blood cell count and serum catalase activity in children with sickle cell disease. This could be as a result of depletion of red cell catalase consequent to oxidative processes and red cell destruction.

4. CONCLUSION

The finding from this study shows that HbSS children in this population did not demonstrate increased oxidative stress, but may be more susceptible to infections caused by intracellular pathogens.

CONSENT

Subjects were too young to give consent; therefore, assent was obtained from each infant's parent. All authors declare that written assent was obtained from each subject before being enrolled into the study.

ETHICAL APPROVAL

The authors declare that this study was approved by UI/UCH ethical review committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

The authors declared there were no competing interests exist.

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