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Glutathione and Bilirubin Concentrations as Markers of Oxidative Stress Measured among Sickle Cell Anaemia Subjects Attending University of Calabar Teaching Hospital, Calabar Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. Authors EEO and ECA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors JOA and AOO managed the analyses of the study. Author AEO managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study aimed at examining the oxidative stress level of sickle cell anaemia subjects using glutathione and bilirubin levels as markers as well as the red cell parameters.
Study Design: Case-control study.
Place and Duration of Study: University of Calabar Teaching Hospital, Calabar-Nigeria, between August 2018 and July 2019.
Methodology: Subjects comprised 45 SCA patients (27 females, 18 males; age range 10-45 years) attending clinic at University of Calabar Teaching Hospital Calabar, Nigeria and equal number of age and sex-matched control subjects with Hb AA. Blood samples were collected and analyzed by

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standard methods. The red cell parameters were analysed by automation using FY-Smart-1 auto haematology analyzer. Bilirubin assay was performed using the colorimetric method, while glutathione was performed by enzyme-linked immunosorbent assay technique.
Results: The red blood cell count, haemoglobin concentration, and haematocrit values of SCA subjects were significantly lower (p=0.001) compared to values from control subjects, while the red cell indices and red cell distribution width values were increased in SCA subjects (p=0.001). The Total bilirubin, conjugated bilirubin and unconjugated bilirubin were significantly increased (p=0.001) among SCA subjects, while the glutathione concentration values were reduced (p=0.001) when compared to values obtained from control subjects.
Conclusion: SCA subjects have marked red cell size variation, lymphocytosis and thrombocytosis. The haemolytic events that occur in sickle cell anaemia results in glutathione depletion.

Keywords: Sickle cell anaemia; oxidative stress; haemolysis; hyperbilirubinaemia.

1. INTRODUCTION

Sickle cell anaemia, the most severe form of sickle cell disorder has been labelled a major public health challenge by the World Health Organization. It is mainly common among individuals with ancestors from sub- Saharan Africa, India, Saudi Arabia and Mediterranaen countries. In sub- Saharan Africa where Nigeria is located, sickle cell anaemia remains the most important haemoglobinopathy affecting about 2% of children mainly due to lack of knowledge on the part of uninformed couples [1,2,3]. The sickling of red blood cells results in further complications of the disease which include episodes of severe pain and residual organ damage [4]. Access to adequate healthcare greatly improves survival of affected individuals into adult life. Expert counseling, provision of prompt access to needed care and commitment and dedication of medical staff among other factors contributes significantly to improved survival and quality of life [5].

Sickle cell anaemia primarily affects red blood cells which are a significant source of free radicals in biological systems. Red blood cells are rich in oxygen supply and are densely packed with redox-active haemoglobin residues. The bonding interaction between heme iron and oxygen in oxygenated haemoglobin is associated with electron transfer. In response, there is an integrated network of the antioxidant system which helps to mitigate oxidative stress and injury to the red blood cells and tissues. Congenital haemoglobin mutations may alter this balance, thus contributing to the pathophysiology of sickle cell anaemia [6].

Oxidative stress occurs as a result of an imbalance between enhanced production of reactive oxygen species and low cellular content of antioxidants. Increased oxidative stress can damage lipids, proteins, macromolecules and subsequently lead to premature cell death [7,8]. Thus, oxidative stress plays a major role in endothelial dysfunction, inflammation and multiple organ damage [9]. The present study therefore investigated reduced glutathione and bilirubin concentrations as markers of oxidative stress alongside red cell parameters of sickle cell anaemia patients attending University of Calabar Teaching Hospital.

2. MATERIALS AND METHODS

This study was carried out among consenting steady-state sickle cell anaemia subjects (SCA) attending clinic at University of Calabar Teaching Hospital Calabar, Nigeria. The subjects had been previously diagnosed as SCA patients with Hb SS using Cellulose Acetate Method at Alkaline pH. The study subjects constituted 27 females and 18 male sickle cell anaemia patients with equal number of age and sex-matched control subjects with Hb AA. Ethical approval was obtained from Ethical Committee of University of Calabar Teaching Hospital, while informed written consent was obtained from each participant. Parental consent was obtained for participants below eighteen years of age. Biodata including age and gender were retrieved from patients' folders. Venous blood samples were aseptically collected into ethylenediaminetetraacetic acid bottles for analysis of red cell parameters, and plain bottles to obtain serum for bilirubin and glutathione assays. The red cell parameters were analysed by automation using FY-Smart-1 auto haematology analyzer. Bilirubin assay was performed using the colorimetric method, while reduced glutathione was performed by enzyme-linked immunosorbent assay technique. The population had a normal curve and statistical

analysis of data (student t-test and one-way analysis of variance) was done using SPSS 22.0. A p-value of ≤ 0.05 was considered indicative of a statistically significant difference.

3. RESULTS AND DISCUSSION

3.1 Results

The age group with the highest number of participants was 20-29 years, while the least number of participants were those above 30 years of age. There were more females than males who participated in the study (Table 1).

The red blood cell count, haemoglobin concentration and haematocrit values of the sickle cell subjects were found to be significantly lower compared to values from control subjects, while mean cell haemoglobin, mean cell haemoglobin concentration and red cell distribution width values were significantly higher in sickle cell subjects. The glutathione level was significantly reduced in sickle cell subjects compared to control subjects, while the bilirubin values (total, conjugated and unconjugated) were higher in the sickle cell subjects (Table 2).

All test subjects were grouped into three based on their ages; 10-19 years, 20-29 years and above 30 years. The analysis of variance on the obtained data showed that MCV, MCH, RDW-SD and RDW-CV were significantly lower among subjects within 10-19 years of age compared to those within 20-29 years and above 30 years' group. Glutathione value was significantly higher among subjects within 20-29 years of age compared to those within 10-19 years and above 30 years' group, while the unconjugated bilirubin value was significantly lower among sickle cell subjects within 20-29 years of age compared to the other two groups (Table 3).

3.2 Discussion

The highest percentage of participants in the present study came from the age group 20-29 years, closely followed by those within the 10-19

Table 1. Demographic parameters of the sickle cell anaemia patients and the controls

Parameter	SCA subjects number (%)	Control subjects number (%)
Age		
10-19 years	15 (33.3)	14 (31.1)
20-29 years	22 (48.9)	23 (51.1)
30years and greater	8 (17.8)	8 (17.8)
Total	**45 (100)**	**45 (100)**
Gender		
Females	27 (60)	27 (60)
Males	18 (40)	18 (40)
Total	**45 (100)**	**45 (100)**

Table 2. Red cell parameters, glutathione and bilirubin of sickle cell anaemia patients and controls

Parameters	Control subjects (n=45)	SCA subjects (n=45)	p-value
RBC ($\times 10^{12}$/l)	5.45 ± 0.54	3.42 ± 0.51*	0.001
Hb (g/l)	149.80 ± 16.27	95.71± 10.53*	0.001
HCT (l/l)	0.43 ± 0.04	0.27 ± 0.03*	0.001
MCV (fl)	78.32 ± 3.83	79.60 ± 7.68	0.322
MCH (pg)	27.48 ± 1.22	28.37 ± 2.06*	0.014
MCHC (g/l)	35.09 ± 0.93	35.89 ± 1.39*	0.002
RDW-SD (fl)	32.21 ± 1.76	36.29 ± 5.06*	0.001
RDW-CV (%)	13.09 ± 0.34	14.42 ± 0.74*	0.001
GSH (ng/ml)	6.09 ± 4.35	1.50 ± 0.46*	0.001
TB (µmol/l)	16.58 ± 2.66	33.13 ± 7.46*	0.001
CB (µmol/l)	8.25 ± 1.63	14.01 ± 5.07*	0.001
UB (µmol/l)	8.34 ± 1.97	19.12 ± 4.70*	0.001

Key: RBC=Red blood cell, Hb=Haemoglobin, HCT=Haematocrit, MCV=Mean cell volume, MCH=Mean cell haemoglobin, MCHC=Mean cell haemoglobin concentration, RDW-SD=Red cell distribution width (As standard deviation), RDW-CV=Red cell distribution width (As coefficient of variation), GSH=Glutathione. TB=Total bilirubin, CB=Conjugated bilirubin, UB=Unconjugated bilirubin.

**Statistically significant*

Table 3. Red cell parameters, glutathione and bilirubin of sickle cell anemia patients based on age

Parameters	10-19 years (n=15)	20-29 years(n=22)	30years and above (n=8)	p-value
RBC ($\times 10^{12}$/l)	3.50 ± 0.63	3.36 ± 0.39	3.44 ± 0.60	0.702
Hb (g/l)	92.93 ± 12.30	97.05 ± 9.72	97.25 ± 9.30	0.467
HCT (l/l)	0.26 ± 0.04	0.27 ± 0.02	0.28 ± 0.03	0.321
MCV (fl)	74.77 ± 5.81[ab]	81.24 ± 7.33	84.14 ± 7.71	0.005
MCH (pg)	27.06 ± 1.73[ab]	29.01 ± 1.71	29.09 ± 2.51	0.007
MCHC (g/l)	36.27 ± 1.03	35.86 ± 1.58	35.25 ± 1.28	0.248
RDW-SD (fl)	32.98 ± 4.47[ab]	37.16 ± 4.22	40.13 ± 4.98	0.002
RDW-CV (%)	13.99 ± 0.90[ab]	14.54 ± 0.55	14.91 ± 0.45	0.008
GSH (ng/ml)	1.25 ± 0.36	1.78 ± 0.41	1.23 ± 0.31	0.001
TB (µmol/l)	35.23 ± 7.37	30.52 ± 6.77	36.36 ± 7.80	0.065
CB (µmol/l)	14.71 ± 5.72	13.74 ± 4.98	13.46 ± 4.51	0.810
UB (µmol/l)	20.52 ± 3.70	16.78 ± 3.75[c]	22.90 ±5.64	0.001

Key a= 10-19 years statistically different from 20-29 years
b= 10-19 years statistically different from 30 years and above
c= 20-29 years statistically different from 10-19 years as well as 30 years and above
RBC=Red blood cell, Hb=Haemoglobin, HCT=Haematocrit, MCV=Mean cell volume, MCH=Mean cell haemoglobin, MCHC=Mean cell haemoglobin concentration, RDW-SD=Red cell distribution width (As standard deviation), RDW-CV=Red cell distribution width (As coefficient of variation), GSH=Glutathione, TB=Total bilirubin, CB=Conjugated bilirubin, UB=Unconjugated bilirubin

years' age group. The least participation was obtained from sickle cell subjects that are 30 years and above. This distribution pattern in terms of age is attributable to the high mortality rate noted among sickle cell patients particularly those in Africa and other developing regions [10]. This trend has been linked to issues related to transition from paediatric to adult care [11,12,13]. The present study also revealed a higher percentage of female participation of 60%, while male participation was 40% (Table 1). Previous reports have attributed this kind of finding to increase life expectancy among affected females compared to the males. One possible explanation is the fact that females have a relatively reduced blood viscosity as a result of reduced haemoglobin concentration and haematocrit levels which is thought to generally reduce the risk of vaso-occlusion. It has also been suggested that females may have a higher foetal haemoglobin level as a result of partial control of foetal haemoglobin by an X-linked gene [13]. Again, beyond possible gender-mediated physiological variations, females have been adjudged more responsible in managing health conditions as they are generally known to talk about issues bothering them and seek help more readily than the males [14].

The mean red blood cell count, haemoglobin and haematocrit values were significantly lower in the sickle cell patients than controls. This is expectedly so due to the fact that increased red blood cell destruction is a characteristic feature of sickle cell anaemia, and the finding of lower haemoglobin and haematocrit values in sickle cell patients has been consistently reported in previous studies [15,16]. Other red cell indices that were altered, in slightly increasing manner, among the sickle cell subjects include MCH, MCHC and RDW. The observed higher MCH and MCHC values may be a resultant effect of the reduced haematocrit seen in sickle cell patients. It has also been attributed to cellular dehydration which is characteristic of sickle cell anaemia and hereditary spherocytosis [17]. The mean red cell distribution width values (as standard deviation and coefficient of variation) were also significantly increased in the sickle cell patients than the control individuals. This is a reflection of the marked variation in red cell size resulting from influx of new red cells sequel to haemolytic episodes. Considering that sickle cell anaemia has the characteristic feature of haemolysis, red cell distribution width as a measure of red blood cell variation in size increases in sickle cell anaemia [15]. In fact, it is known that sickle haemoglobin releases high amounts of reactive oxygen species, and also has reduced antioxidant capacity; thus, leading to increased oxidative stress [18]. Increased oxidative stress has been suggested as a precursor event to the decline in the mechanical characteristics of the red blood cells resulting in impaired tissue perfusion; thus, increasing red cell distribution width levels [19,20]. The higher mean cell haemoglobin and anisocytosis as measured by the red cell distribution width values were

observed to be more pronounced with increasing age of the sickle cell anaemia subjects which is also a reflection of the duration of the condition [15]. The present study observed the lowest mean values for MCV, MCH, RDW-SD and RDW-CV among subjects within 10-19 years of age compared to those within 20-29 years and above 30 years' group.

Hyperbilirubinaemia is one of the hallmark findings in this present study. Literature consistently suggests that hyperbilirubinaemia commonly observed in sickle cell patients is a result of chronic haemolysis which is a common feature in sickle cell disease [21,22]. One of the consequences of chronic haemolysis as seen in sickle cell anaemia is the increased generation of reactive oxygen species, reduced antioxidant capacity and ultimately increased oxidative stress [15]. This line of events would have occasioned the depletion of glutathione values of the sickle cell anaemia subjects seen in this study. Glutathione is an important antioxidant, which reacts directly with reactive oxygen species, reactive nitrogen species, and other reactive species. In the present study, the glutathione concentration of the sickle cell patients depleted in the ratio of 6:1 compared to the control group, while the bilirubin concentration was increased among the sickle cell patients. It appears that the increased haemolysis results in increased glutathione depletion as observed in this study. This pattern of finding has also been earlier reported [23,24].

4. CONCLUSION

The present study supports the fact that there is anaemia with marked red cell size variation, lymphocytosis and thrombocytosis among sickle cell patients. Also, it is evident that the haemolytic events that is characteristic of sickle cell anaemia results in glutathione depletion.

CONSENT AND ETHICAL APPROVAL

Ethical approval was obtained from the University of Calabar Teaching Hospital Health Research Ethical Committee, while informed consent was given by each participant.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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