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Original Research Article

Assessment of Lipid Peroxidation and Antioxidant Status in Common Haemoglobin Phenotypes in Osun State, Southwestern Nigeria

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Abstract

Background: Generally speaking, erythrocyte haemoglobin (Hb) is pro-oxidant in nature constantly generating superoxide ($O_2^{\cdot-}$) species in biological system of all Hb phenotypes. Sometimes, this reactive oxygen species (ROS) triggers lipid peroxidation with eventual rise in oxidative stress, particularly when the supposed self-sustaining antioxidant defence system is overwhelmed. Worse still, abnormal Hb generates multiple of $O_2^{\cdot-}$ specie. Recent evidence has linked abnormal haemoglobins S and C in sickle cell diseases (SCD) to various complications with multiple pro-oxidant processes; however, similar studies in relation with abnormal haemoglobin traits are sparse. More so, reports on rate of activities of various antioxidant enzymes in SCDs are still contradictory. This study aimed to assess interplay between lipid peroxidation and antioxidant defence capacity in various haemoglobin variants.

Method: A total number of 150 participants with different haemoglobin variants were recruited. Plasma levels of malondialdehyde (MDA), total antioxidant status (TAS), superoxide dismutase (SOD) and glutathione peroxidase (GPx) enzymes were assayed using spectrophotometric method.

Result: The results showed that plasma levels of MDA and erythrocytic SOD were significantly increased ($P<0.05$) in abnormal haemoglobins in that order of HbSS >HbSC >HbAC >HbAS, when compared with controls (HbAA). Conversely, plasma levels of GPx and TAS showed significant reductions ($p<0.05$), respectively.

Conclusion: The results suggest that both sickle cell diseases and the carriers were relatively more vulnerable to systemic oxidative stress (in that order of HbSS> HbSC > HbAC >HbAS) contrary to normal haemoglobin phenotype, possibly owing to ineffective antioxidant mechanisms needed for keeping spontaneous generation of free radicals in control.

Keywords: Glutathione peroxidase; lipid peroxidation; malondialdehyde; sickle cell disease; superoxide dismutase.

1. Introduction

Erythrocytes in biological systems are associated with uninterrupted pro-oxidant generation attributed to superoxide anion (autoxidation of haemoglobin, Hb) [1]. This autocatalytic reaction taking place involves the nitrite and oxy forms of globin naturally thus giving it potential to generate free radicals [2]. Reports have shown that sickle red cells generate greater amounts of $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$ compared with healthy hemoglobin phenotypes [3]. On the other hand, antioxidant molecules, both enzymatic and non-enzymatic, form the defensive system in preventing erythrocyte membrane attack [4]. Despite the function of antioxidant system, reports have indicated rising and continuous pro-oxidant generation in SCD prompting consumption of antioxidant reserve very rapidly, and consequently results in antioxidant insufficiency, thus building up oxidative stress and possible complications [5]. Meanwhile, recent suggestions have exclusively linked abnormal haemoglobins S and C to many complications with multiple pro-oxidant processes [6].

However, in spite of the associated complications in majority of these common Hb traits, very few studies focussed attention on pro- and anti-oxidant system interaction in sickle cell and HbC carriers. This is in opposite to the ample of data on SCD, although without resolute agreement, currently. More so, there are still contradictive reports on reduced or increased activities of various antioxidant enzymes such as SOD, CAT, and GPx [7, 8]. in sickle RBCs [1]. The aim of this study was to assess interplay between erythrocyte lipid peroxidation and antioxidant defence system in all common haemoglobin variants- both sickle cell diseases and the carriers.

2. Materials and methods

2.1 Subjects and Participant Selection

Participants were recruited at Obafemi Awolowo University Teaching Hospital, Ile-Ife and Ladoke Akintola University of Technology Teaching Hospital Osogbo, both the only tertiary hospitals in Osun State, Nigeria. The study protocol was carried out in accordance with Helsinki decleration as revised in 2000. The study was also approved by Ethics Committees of the two study centers. The participants were informed of the purpose and procedures of the study, thus voluntarily gave written consent. The study population comprised a total number of one hundred and fifty (N=150) overall participants with confirmed different haemoglobin (Hb) genotypes using haemoglobin electrophoresis. The participants included were consecutive patients with sickle cell anaemia (SCA, n=30) and HbSC haemoglobinopathy (n=30) regularly followed up in a steady state. Other participants included were subjects in the same geographical locations with abnormal haemoglobin traits (HbAS, n=30; HbAC, n=30). The control subjects were apparently healthy subjects with normal heamoglobin phenotype (HbAA, n=30), all confirmed with the same method before enrollment, respectively.

2.2 Inclusion criteria

The inclusion criteria were based on;

a) Patients confirmed with sickle cell diseases (homozygous HbS and heterozygous HbS+C)
b) Subjects with other common haemoglobin variants (i.e HbAA, HbAS and HbAC).
c) All the participants were enrolled without any sign or symptom of crisis and without bacterial infections.
d) Only non-smokers were considered.

2.3 Exclusion criteria

The exclusion criteria were based on;

a) Any patient with clinical history/record of conditions such as leukaemia and renal diseases.
b) Subjects with recent blood transfusion, less than three months before the time of recruitment.
c) Subject on treatment with hydroxyl urea.
d) Pregnant subjects

2.4 Specimen Collection and storage

5 ml of blood was withdrawn aseptically from ante-cubital vein from each subject into a sterile lithium heparin bottle and centrifuged at 3000 rpm for 10minutes.The supernatant was extracted and stored in plain tube at -20 °C until the time of analyses.

2.5 Laboratory methods

Various methods were employed for estimating different analytes as itemized below;

A. Determination of Total Antioxidant Status (mmol/L) by Koracevic *et al*. [9].
B. Estimation of Malondialdehyde (µmol/L) content by Gutteridge *et al*. [10].
C. Estimation of Glutathione peroxidase activity (mu/ml) by Rotruck *et al*. [11].
D. Estimation of superoxide dismutase level (ng/ml) by Misra and Fridovich [12].

2.6 Statistical analysis

The study data were statistically analyzed using the Statistical Package for Social Science version program (SPSS program-version 10.0 – SPSS Inc., Chicago, IL. USA). The data were expressed as mean (± standard deviation, SD). Between-group comparisons were assessed for nominal variables with the $\chi 2$-square test, while Analysis of Variance (ANOVA) and post hoc for multiple comparisons were used to compare the quantitative variables for all the groups. Statistical significance was assessed at $P<0.05$. All calculated P-values were two-tailed.

3. Results

Table 1 shows comparisons of demographic and biochemical parameters of erythrocyte lipid peroxidation and antioxidant defense system in different groups of haemoglobin variants.

There were no significant differences ($P>0.05$) in the mean ages and sex ratios, both across and in-between the groups.

However, there were significant increases ($P<0.05$) both across and in-between the groups comparing the mean plasma values of malondialdehyde (MDA) in HbSS, HbSC and HbAC groups with normal HbAA control group, respectively. However, comparison of mean plasma value of MDA in HbAS group against HbAA controls showed no statistical significance ($P>0.05$), even though, increase in the value was observed.

Similarly, there were significant increases ($P<0.05$) comparing mean plasma values of superoxide dismutase (SOD) in abnormal Hb groups (of HbSS, HbSC, HbAC and HbAS phenotypes) against the normal HbAA control group, both across and in-between the groups. However, comparison of mean plasma value of SOD in HbSC group against that of HbC trait group showed no significant difference ($P>0.05$).

On the contrary, there were significant reductions (P<0.05) across the groups comparing mean plasma values of total antioxidant status (TAS) in abnormal haemoglobin HbSS, HbSC, HbAC and HbAS groups with normal HbAA control group, respectively. In the same vein, in-between group comparisons involving mean plasma values of TAS in HbSS group against the other groups (HbSC, HbAC, HbAS and HbAA) also showed significant reductions (P<0.05) par case-by-case basis. In addition, in-between comparisons involving mean plasma values of TAS in HbAC group against each group of HbAS and HbAA equally showed significant reductions (P<0.05), correspondingly. Unpredictably, there was reduction in mean plasma value of TAS in HbAS group compared with the control group with no statistical significance (P>0.05). Likewise, there was increase in mean plasma value of TAS in HbSC group against HbAC group, although no statistical significance (P>0.05) was equally observed.

Furthermore, there were significant reductions (P<0.05) comparing individual case group of HbSS, HbSC, HbAC and HbAS against the control group regarding the mean plasma values of glutathione peroxidase (GPx) enzyme, both across and in-between the groups, although there were exceptions to these observations. In comparing HbSS group against HbSC group, and HbSC group against HbAS group, there were actually reductions but significant differences (p>0.05) were not observed by statistical analysis.

Figures 1-4 show plasma levels of biochemical parameters in all common haemoglobin phenotypes.Figure 1 shows elevated plasma levels of MDA in all abnormal haemoglobin phenotypes when tested against normal haemoglobinHbA controls, with different magnitude.

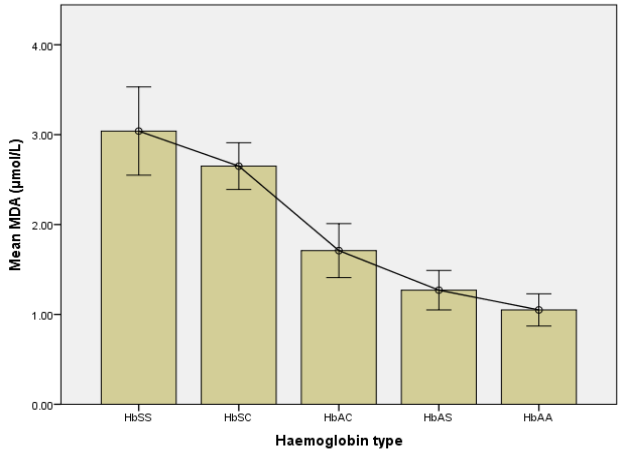

Figure 1: Plasma level of malondialdehyde in different haemoglobin variants

Figure 2 shows increased plasma levels of SOD in different abnormal haemoglobin variants relative to normal HbA controls.

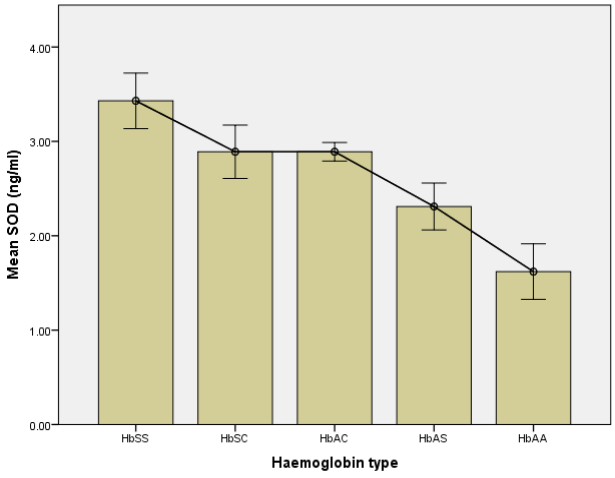

Figure 2: Plasma level of superoxide dismutase in different haemoglobin variants

However, in figure 3, the graph shows reductions in plasma levels of TAS in different abnormal haemoglobin variants relative to normal HbA controls.

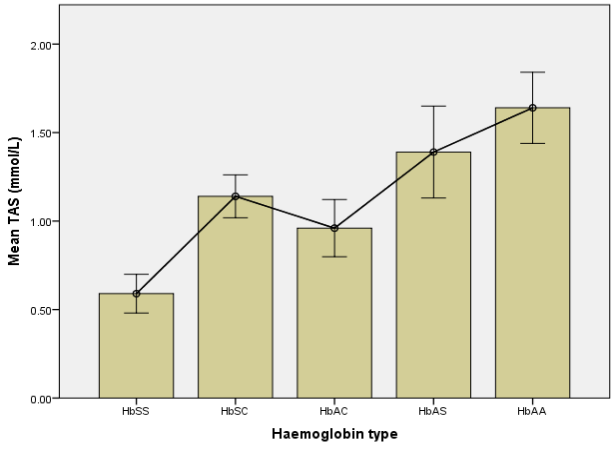

Figure 3: Plasma level of total antioxidant status in different haemoglobin variants

Similarly, figure 4 shows barely noticeable reductions in plasma levels of glutathione peroxidase in different abnormal haemoglobin variants as against the normal HbA controls.

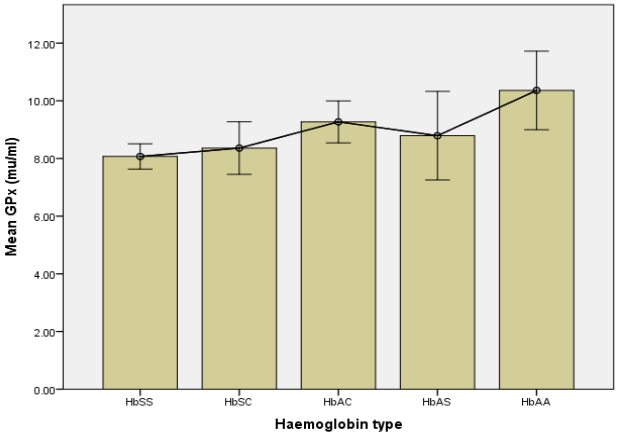

Figure 4: Plasma level of glutathione peroxidase in different haemoglobin variants

Table 1: Demographic and biochemical parameters of erythrocyte lipid peroxidation and antioxidant defense system in different groups of heamoglobin variants

Parameters	HbSS (n = 30)	HbS (n = 30)	HbAC (n = 30)	HbAS (n = 30)	HbAA (n=30)	P-value
Age (years)	23 ± 5	22 ± 4	23 ± 5	23 ± 5	23 ± 4	0.891
Sex ratio (M/F)	13/17	13/17	14/16	15/15	15/15	0.796
MDA(µmol/L)	3.04 ± 0.85^{a,b,c}	2.65 ±0.45^{a,b,c}	1.71 ± 0.52^{a,b}	1.27 ± 0.38	1.05 ± 0.31	0.003*
SOD(ng/ml)	3.43 ± 0.51^{a,b,c,d}	2.89 ± 0.49^{a,b}	2.89 ±0.17^{a,b}	2.31 ± 0.43^{a}	1.62 ± 0.51	<0.001*
TAS(mmol/L)	0.59 ± 0.19^{a,b,c,d}	1.14 ± 0.21^{a}	0.96 ± 0.28^{a,b}	1.39 ± 0.45	1.69 ± 0.42	0.001*
GPx(mu/ml)	8.07 ± 0.38^{a,b,c}	8.36 ± 0.79^{a,c}	9.27±0.63^{a,b}	8.79 ± 1.33^{a}	10.36± 1.18	<0.001*

a= significantly different from HbAA controls (Post Hoc); **b**= significantly different from HbS trait (Post Hoc); **c**= significantly different from HbC trait (Post Hoc); **d**= significantly different from HbSChaemoglobinopathy (Post Hoc); **M:** Male; **F:** Female; **MDA**:Malondialdehyde; **SOD**: Superoxide dismutase; **TAS**: Total antioxidant status; **GPx**: Glutathione peroxidase
HbSS: Haemoglobin SS for sickle cell disease; **HbSC**: Haemoglobin SC for sickle cell disease; **HbAC:** Haemoglobin C trait; **HbAS**: Haemoglobin S trait; **HbAA**: Haemoglobin A for normal subjects (controls); *= statistically significant ($p \leq 0.05$).

4. Discussion

Sickle Cell Diseases (SCD) are hereditary disorders with higher potential for oxidative damage due to chronic redox imbalance in red cells that often results in clinical manifestation; from mild to severe haemolytic anaemia. Previous reports showed that under ambient oxygen tensions, sickle cells spontaneously generate $O_2^{\bullet-}$, H_2O_2 and $OH^{\bullet-}$ approximately two times more when compared to normal RBCs [1]. Today, there are reports from various studies that many complications are currently attributed to sickle cell trait (SCT) and other abnormal haemoglobin trait [6]. However, considering pro- and antioxidant interactions among various abnormal haemoglobin traits, causal relationship has not been substantially proved.

In this present study, we observed elevations in blood levels of malondialdehyde (MDA) in HbSS, HbSC, HbAC and HbAS phenotypes in that order when evaluated against the normal HbA controls. The degree of rises in MDA blood levels may be attributed to vulnerability of lipid peroxidation which agrees with earlier studies [13, 14]. MDA is a major aldehyde and end product of lipid peroxidation of erythrocytes and reveals damage level to their membrane lipids [15].

It was also noted that there was no considerable difference in plasma values between HbSC and HbSS regarding MDA accumulation, even though there was a remarkable elevation in HbSS patients, which may possibly be an indication that HbSS patients are more vulnerable than HbSC, albeit both are structurally sickled.

Again, accumulation of MDA in the blood system is another cardinal factor in disrupting organization of phospholipid bi-layer present in erythrocyte membrane. This membrane damage is therefore a notable feature contributing to disease pathophysiology in the formation of irreversible sickle cells (ISC) as stated by [16]. Peroxidative reactions have long been recognized as a potential factor that contributes towards many degenerative cellular processes in the body. Furthermore, the increase of MDA in the same group has been attributed to auto-oxidation of iron seen in these patients [17]. Besides, the excess production of MDA poses additional deleterious effects which may result in modifications of the proteins and amino-acid side chain, and lipids structure. These modifications may ultimately result in loss of protein functionality including antioxidant enzymes, protein receptors [8] and externalize the phosphatidylserine component of red cell membranes thus enhancing complement activation and eventually result in hemolysis which has been indicated by [18].

More so, subjects with sickle cell trait (SCT) and haemoglobin C trait are identified as carriers and they are usually considered to be benign and asymptomatic conditions because of level of MDA in their blood system [19]. Again, one report suggests that these carrier groups should be reclassified as disease states [20]. with a number of associated clinical conditions, including exercise-related deaths, pregnancy-related complications, splenic infarction, complicated hyphema, hyposthenuria, hematuria, acute chest syndrome, thrombo-embolic disease, and many renal associated problems such as renal medullary carcinoma and renal papillary necrosis [21].

In fact, currently, SCT has been linked with various complications at rest and during exercise, particularly in hypoxic conditions as a cofactor for morbidity and mortality. This is attributed to increased auto-oxidation and exacerbated by reduced antioxidant capacity in those carriers [22].

There are many studies on the activities and functions of superoxide dismutase (SOD) with conflicting reports. The present study, however, assessed the relative functional status of SOD in red cells from five most common phenotypes. The comparative assessment from this study (table 1) showed that erythrocyte SOD activities exhibited in the five common haemoglobin classes have increased variable activities from normal haemoglobin to abnormal phenotypes which was in line with earlier studies [17, 23]. Conversely, this was in contrary to some reports with decreased activity of SOD in the homozygous (HbSS) patients as opposed to heterozygous (HbAS) and normal haemoglobin subjects [24].

In addition, there was no substantial difference in SOD levels between HbAC and HbSC subjects; they have the same mean values (figure 2). This may be due to related genetic expression pattern [25] of haemoglobin C. Again, the elevated plasma level of SOD as revealed in our findings is one important outcome that signifies an imbalance in the cellular antioxidant defence, resulting in accelerated generation of H_2O_2 which is another oxidative product of dismutation involving $O_2^{\bullet-}$ specie. Therefore as a consequence, this may exacerbate the effect of complications associated with oxidative stress. Meanwhile, enhanced activity or increased plasma level of SOD does not minimize the intensity of oxidative damage, rather, it increases the concentration of H_2O_2 which may also inhibit the activity of erythrocytic catalase enzyme, thereby leading to denaturation of haemoglobin and the formation of Heinz bodies, consequently contributing to sickling and/or haemolytic effects as earlier reported by Scott *et al*. [26].

Moreover, considerable reductions in total antioxidant status (TAS) in patients with HbSS and HbSC, as well as subjects with HbC and HbS traits, as observed in this present study are in agreement with previous findings [27, 28] where low level of TAS is associated with manifestations of sickle cell disease such as- increased susceptibility to infections, acute chest syndrome and chronic haemolytic anaemia.

Conversely, we noted substantial reduction in the TAS level in subjects with HbC trait than in HbS trait contrary to previous study [23]. This signifies that HbC carriers are more vulnerable to lipid peroxidation as a result of low TAS and therefore suggestive of increase and overburden of reactive oxyegen species (ROS) more than HbAS. Also, assessment between HbAC and HbSC groups revealed that there was no remarkable difference in the mean values of TAS, even though there was slight reduction in HbAC heterozygozity than in HbSC disease. What contributed to the reduced TAS in HbC trait relative to HbSC sickle cell disease is currently unknown.

However, there could be possibility of increased $O_2^{\bullet-}$ specie and consequent generation of reactive metabolites. Therefore, this may possibly have causal relationship with TAS depletion in all haemoglobin carriers especially SCT and HbC trait, hitherto unidentified. Again, the defined basis for haemolysis in some HbC haemoglobin is presently unclear; however this has been hesitantly associated with either intra-erythroid crystal formation or cellular dehydration attributed to raise potassium efflux [29]. Other reports also link elevated MDA production with alteration of proteins and lipid structures that may ultimately result in loss of functionality in antioxidant enzymes and protein receptors [8] thereby exposing the phosphatidylserine in red cell membranes and consequently enhance the complement activation resulting in haemolysis [18].

During haemolysis, circulating haemoglobin and haeme characterize erythrocytic danger-associated molecular pattern (eDAMP) molecules [30]. Experimental studies show that eDAMP molecules are capable of promoting sickle vaso-occlusion. However, reports on humans have shown that intravascular haemolysis can impair nitric oxide (NO) bioavailability and consequentially result in oxidative stress thereby prompt redox imbalance and indirectly cause inflammation, angiogenesis, thrombotic and haemostatic complications [1, 30].

However, currently, chronic hemolytic anemia have been reported in sickle-cell disease (SCD) and HbSC haemoglobinopathy [25] while in HbC trait, complications such as microcytosis have been reported and associated most times with crystallisation, and more rigid compared with normal RBCs. Therefore, they form rod-like crystals in situations with hypoxia. More so, owing to their rigid shape, small blood vessels may be blocked, thus resulting in ischemia and endothelial cell damage [29]. Consequently, this may further be disposed to worsening oxidative stress by generation of more ROS.

Furthermore, our finding revealed that glutathione peroxidase, one symbolic antioxidant enzyme, was considerably reduced in HbSS, HbSC, and HbAC subjects by matching with that of normal HbAA subjects. This observation was consistent with some previous studies [14, 17]. Meanwhile, there was no substantial difference in the mean value of glutathione peroxidase antioxidant enzyme between HbAC and HbAS, although there was a slight increase in HbAC compared with HbAS.

In view of the assessments above, abnormal haemoglobins involving both homozygous and heterozygous classes are relatively more vulnerable to systemic oxidative stress (although in that order of HbSS> HbSC > HbAC >HbAS) in contrast to normal haemoglobin phenotype, probably as a result of deficient antioxidant mechanisms required to counteract the spontaneous generation of free radicals.

5. Conclusion and recommendation

It is evident that both homozygous and heterozygous abnormal haemoglobin variants may be predisposed to enhanced oxidative stress compared with homozygous normal haemoglobin phenotype, even so at varying degrees. In other words, our findings revealed that abnormal HbSS, HbSC, HbAC, and HbAS subjects were more at risk of lipid peroxidation attributable to overburden of reactive oxygen metabolites than in normal HbAA subjects. More so, deficient anti-oxidant system in these subjects may probably serve the reason for ineffective counteracting the augmented oxidative stress. At this instant, further study may therefore be required to suggest whether oral intake of anti-oxidant supplements could be of

help in ameliorating these shortfalls, most especially in various forms of related clinical outcomes.

Declarations

Ethics Approval and Consent: This study was conducted following Helsinki declaration on human study guideline and approved by the ethical committees at the two study centers; Obafemi Awolowo University Teaching Hospital, Ile-Ife and Ladoke Akintola University of Technology Teaching Hospital Osogbo, in Osun State, Nigeria.

Consent for publication: All authors have contributed to the information or material submitted for publication, and have read and approved the manuscript. The authors have no direct or indirect commercial financial incentive or individual person's data in any form associated with publishing the manuscript.

Availability of data and material: For all datasets, on which the outcomes of the manuscript rely, please contact authors for request.

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