

Antioxidant Enzymes and Acute Phase Proteins Correlate with Marker of Lipid Peroxide in Adult Nigerian Sickle Cell Disease Patients

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Abstract

Objective(s)

Sickle cell disease is a genetic disorder characterized by chronic haemolytic anaemia. Haemoglobin S containing red blood cells may be susceptible to oxidative stress due to imbalance between production of reactive oxygen species and the countering effect of the various antioxidants present in the body.

Materials and Methods

We evaluated some antioxidant enzymes which include glutathione peroxidase, superoxide dismutase, and catalase. We also determined malondialdehyde, C- reactive protein and fibrinogen using commercial kits in 144 adult sickle cell disease patients (68 males and 76 females) in steady state and 80 apparently healthy age/sex matched controls; 40 sickle cell trait (20 males/20 females) and 40 normal haemoglobin (20 males/20 females).

Results

The result showed that serum glutathione peroxidase, superoxide dismutase and catalase were lower in sickle cell disease patients compared with controls. Malondialdehyde, C-reactive protein and fibrinogen were significantly increased in sickle cell disease patients compared to the controls in both sexes. Malondialdehyde correlated negatively with superoxide dismutase ($P < 0.01$), glutathione peroxidase ($P < 0.05$), and catalase ($P < 0.05$) and positively ($P < 0.05$) with C - reactive protein and fibrinogen.

Conclusion

This study shows that malondialdehyde correlated negatively with antioxidant enzymes and positively with acute phase proteins in sickle cell anaemia patients in steady state.

Keywords: Acute phase protein, Antioxidant enzymes, Malondialdehyde, Sickle cell disease

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Introduction

Sickle cell disease (SCD) is a genetic disorder characterized by chronic haemolytic anaemia due to adverse effects of oxygen transport by the red blood cells. This leads to a decrease in oxygen supply to peripheral tissues. Because of the reduced oxygen tension, the red blood cells become sickle shape and sticky under condition of hypoxia, dehydration or acidosis (1). These sickled red blood cells clump inside large and small blood vessels leading to ischemia, pain and infarction (2).

The pathogenesis of SCD is due mainly to polymerization of sickle red blood cells causing chronic haemolytic anaemia, vaso-occlusive crisis and intravascular haemolysis. Sickle cell disease patients are susceptible to increased oxidative stress (3, 4), because mutant red blood cells which contain haemoglobin is one of the powerful catalyst for initiation of peroxidative reaction. Although free radicals are formed by a wide range of normal biochemical processes, they are potentially harmful, and several host defense mechanisms are in place to neutralize their effects (5). Several defense mechanisms include antioxidant enzymes such as superoxide dismutase (cu/ZnSOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reduction and glucose 6-phosphate dehydrogenase (G6PD) which are considered as the primary defensive system of the cell (6, 7). Oxidative stress occurs as the result of the imbalance between enhanced generation of reactive oxygen species and low cellular content of antioxidants (8). Lipid peroxidation refers to auto-oxidation of polyunsaturated fatty acids initiated by free radicals. Lipid peroxidation is a chain reaction producing a continuous supply of free radicals that initiate further peroxidation. It is usually assessed in humans by measuring malondialdehyde (MDA) which is one of the end products of lipid peroxidation and is formed by fatty acids with two or more double bonds.

Acute phase proteins serve as useful laboratory markers of systematic inflammatory disease. Total antioxidant status has been studied in Nigerian sickle cell disease patients (9, 10), but no study evaluated antioxidant

enzymes and acute phase protein in adult sickle cell disease patients. The objective of the current study therefore was to evaluate serum cu/ZnSOD, CAT, GPX, MDA, C-reactive protein (CRP) and fibrinogen in adult Nigerian sickle cell disease patients in Kano, Nigeria and to correlate antioxidant enzymes and acute phase proteins with makers of lipid peroxidation in these subjects.

Materials and Methods

The study was conducted in Aminu Kano Teaching Hospital, Kano, Nigeria. The study was approved by the ethics committee of the hospital and informed consent was given by the individual patients. The study population was 144 confirmed sickle cell disease patients in steady state attending routine sickle cell disease clinic of the hospital. They consisted of 68 males, aged 23.2 ± 2.8 years and 76 females aged 22 ± 5 years. The control subjects consisted of apparently healthy 20 HbAS males, aged 23.2 ± 5 years, 20 HbAA males aged 25.2 ± 3 years, 20 HbAS females, aged 22.5 ± 2 years and 20 HbAA females aged 22.9 ± 4 years. Demographic and clinical examination findings were obtained using structured questionnaire. Those with oedema, severe jaundice, abnormal chest and abdominal findings were excluded from the study.

Eight milliliters of blood was collected and 3 ml was dispensed into tube containing 3.8% sodium citrate for fibrinogen estimation and the remaining 5 ml was emptied into plain specimen bottles which were allowed to clot at room temperature for 30 min. The specimens were centrifuged at 3000 rpm for 10 min to obtain sera. The sera were stored at -20°C and an analysis was done within two weeks of collection.

Malondialdehyde (MDA) was assayed using thiobarbituric acid reacting substances by Northwest Life Science Specialties, Vancouver, Canada. The assay was based on the reaction of MDA with thiobarbituric acid (TBA); forming MDA-TBA₂ adduct that absorbs strongly at 532 nm. Butylated hydroxytoluene (BHT) and EDTA were added to the sample and reaction mixture to

minimize oxidation of lipids that contribute artifactually during sample processing and the TBA reaction. The temperature of the reaction mixture was also reduced to minimize the decomposition of lipid hydroperoxides. Because much of the MDA is protein bound, mostly as a Schiff base, the pH of the reaction was optimized to facilitate hydrolysis of the MDA. Results were obtained by extrapolation from the calibration curve of log of absorbance against concentration of the various standards.

Glutathione Peroxidase (GPX) and Cu/ZnSOD were determined using reagents supplied by Northwest Life Science Specialties, Vancouver, Canada.

Glutathione peroxidase catalyzes the reduction of hydrogen peroxide, oxidizing reduced glutathione (GSH) to form oxidized glutathione (GSSG). GSSG is then reduced by glutathione reductase and β -nicotinamide adenine dinucleotide phosphate (NADPH) forming NADP^+ , resulting in decreased absorbance at 340 nm and recycling the GSH. Because GPX is limiting the decrease in absorbance at 340 nm, it is directly proportional to the GPX concentration. GPx activity is reported as units based on the definition: 1 unit of GPx= the amount of enzyme necessary to catalyze the oxidation by hydrogen peroxide of 1.0 μmole GSH to GSSG, per minute at 25 °C, pH 7.0.

Superoxide dismutase was estimated using a sandwich ELISA technique. The sandwich ELISA uses a plate bound capture antibody (anti hu Cu/ZnSOD) and a horseradish peroxide conjugated secondary tracer antibody. Subsequent addition of tetramethylbenzidine (TMB) substrate solution facilitates blue colour development directly proportional to the nitrotyrosine present in the sample. The reaction was stopped using a phosphoric acid solution causing a colour change to yellow that was read on a plate reader at 450 nm. Sample Cu/ZnSOD concentration was determined by comparing sample absorbance at 450 nm with those of a seven point standard curve created by diluting the assay calibrator supplied.

C-reactive protein was assayed using ELISA technique by Anogen, Ontario, Canada. The provided microtitre plate was pre-coated with

a monoclonal antibody specific for CRP. Standards or samples were then added to the appropriate microtitre plate wells and were incubated. CRP binds and become immobilized by the antibody pre-coated on the wells. After washing the microtitre plates to remove unbound CRP and other components of the sample, a standard preparation of horseradish (HRP) peroxidase-conjugated antibody specific for CRP was added to each well to sandwich the CRP immobilized during the second incubation. The wells were thoroughly washed to remove all unbound HRP-conjugated antibodies and a tetramethylbenzidine (TMB) substrate solution was added to each well. After incubation for 15 min, only those wells that contain CRP and enzyme-conjugated antibody will exhibit a change in colour. The reaction was stopped by the addition of a sulphuric acid solution and colour change was measured at 450 nm. The concentrations of samples were determined by comparing absorbance of sample with those of an eight point standard curve.

Catalase was determined colorimetrically using kit supplied by Sigma, Missouri, USA. The assay is based on measurement of the hydrogen peroxide substrate remaining after the action of catalase. First, the catalase converts hydrogen peroxide to water and oxygen and then this enzymatic reaction was stopped with sodium azide. An aliquot of the reaction mixture was then assayed for the amount of hydrogen peroxide remaining by a colorimetric method. The colorimetric method uses a substituted phenol (3, 5-dichlo-2-hydroxybenzene-sulfonic acid), which couples oxidatively to 4-aminoantipyrine in the presence of hydrogen peroxide and horseradish peroxidase to give a red colour that absorbs at 520 nm. The results were read from standard curve plotted from a series of standard solutions of hydrogen peroxide.

Fibrinogen was assayed using rapid method for the determination of plasma fibrinogen by Burmester *et al* (11). When calcium-thrombin reagent is added to citrated plasma, the thrombin reagent polymerizes fibrinogen and the produced turbidity was measured spectrophotometrically at 300 nm. A

calibration curve was prepared from fibrinogen standards and results were extrapolated from the curve.

Statistics

Results were expressed as mean \pm SD and were analyzed by unpaired Student's t-test. Values of $P < 0.05$ were considered statistically significant and Pearson correlation coefficient was calculated to determine the association of antioxidant enzymes, acute phase protein and marker of lipid peroxide.

Results

The results are as shown in tables 1 and 2. There was a significant decrease in the mean level of GPX in HbSS males when compared with HbAS ($P < 0.01$) and HbAA ($P < 0.001$). Similarly, the mean level in the female HbSS was lower than HbAS ($P > 0.05$) and HbAA ($P < 0.001$). The mean Cu/ZnSOD in the male HbSS was lower than HbAS ($P > 0.05$) and HbAA ($P < 0.001$). For the female HbSS, the level was lower than HbAS ($P > 0.05$) and

HbAA ($P < 0.001$). The mean level of CAT in the male HbSS was lower than HbAS ($P < 0.01$) and HbAA ($P < 0.001$), while in the female HbSS the level was lower than HbAS ($P < 0.001$) and HbAA ($P < 0.001$). The mean MDA level in male HbSS was higher than those of HbAS ($P < 0.05$) and HbAA ($P < 0.001$). For the female HbSS, the level was higher than those of HbAS ($P < 0.001$) and HbAA ($P < 0.001$). The mean level of CRP in HbSS was higher than those of HbAS ($P < 0.001$) and HbAA ($P < 0.001$) for both males and females respectively. The mean fibrinogen level of HbSS was also higher than those of HbAS ($P < 0.01$) and HbAA ($P < 0.001$) for the males while the mean value in females was also higher than those of HbAS ($P > 0.05$) and HbAA ($P < 0.001$). MDA correlated negatively with GPX ($P < 0.05$), Cu/ZnSOD ($P < 0.01$) and CAT ($P < 0.05$) using Pearson correlation coefficient in both male and female SCD patients. Conversely, MDA correlated positively with CRP ($P < 0.05$) and fibrinogen ($P < 0.01$).

Table 1. Changes in some antioxidant enzymes, malondialdehyde, C-reactive protein and fibrinogen in male sickle cell disease patients and male controls sickle cell trait and normal haemoglobin.

	HbSS Males	HbAS males	P- value	HbAA males	P- value
No of subjects	68	20		20	
Glutathione peroxidase (mU/ml)	9.8 \pm 0.9	10.1 \pm 1.8	$P < 0.01$	10.6 \pm 2.2	$P < 0.001$
Superoxide dismutase (ng/ml)	32.6 \pm 5.1	33.4 \pm 1.2	$P > 0.05$	35.5 \pm 1.8	$P < 0.001$
Catalase (μ mol/min/l)	156 \pm 7.9	159 \pm 5.2	$P < 0.01$	162 \pm 6.2	$P < 0.001$
Malondialdehyde (μ mol/l)	2.6 \pm 0.4	2.5 \pm 0.3	$P < 0.05$	2.4 \pm 0.4	$P < 0.001$
C-reactive protein (μ g/ml)	1.12 \pm 0.02	1.06 \pm 0.6	$P < 0.001$	0.81 \pm 0.92	$P < 0.001$
Fibrinogen (mg/dl)	297 \pm 15.2	292 \pm 18	$P < 0.01$	290 \pm 30.5	$P < 0.001$

Table 2. Changes in some antioxidant enzymes, malondialdehyde, C-reactive protein and fibrinogen in female sickle cell disease patients and female controls sickle cell trait and normal haemoglobin.

	HbSS females	HbAS females	P- value	HbAA females	P- value
No of subjects	76	20		20	
Glutathione peroxidase (mU/ml)	9.8 \pm 1.6	10.1 \pm 1.2	$P > 0.05$	10.5 \pm 1.8	$P < 0.001$
Superoxide dismutase (ng/ml)	32.8 \pm 3.8	33 \pm 2.6	$P > 0.05$	36.2 \pm 2.1	$P < 0.001$
Catalase (μ mol/min/l)	157 \pm 4.6	161 \pm 10.2	$P < 0.001$	165 \pm 12.2	$P < 0.001$
Malondialdehyde (μ mol/l)	2.6 \pm 0.3	2.40 \pm 0.6	$P < 0.001$	2.49 \pm 0.4	$P < 0.001$
C-reactive protein (μ g/ml)	1.13 \pm 0.02	1.10 \pm 0.7	$P < 0.001$	1.06 \pm 0.97	$P < 0.001$
Fibrinogen (mg/dl)	298 \pm 15.1	295 \pm 25	$P > 0.05$	292 \pm 31.2	$P < 0.001$

Discussion

There was a significant reduction in the activity levels of antioxidant enzymes in the serum of SCD patients compared with controls sickle cell trait and normal haemoglobin in both male and female patients ($P < 0.05$). This observation is consistent with other authors (8, 9, 12, 13). This is an indication that SCA patients produced greater quantities of reactive oxygen species than control HbAS and HbAA. HbS containing red blood cells auto-oxidize faster thereby generating more superoxide, hydrogen peroxide, hydroxyl radicals and lipid peroxides than HbAA containing red blood cells (14). Oxidative damage is due to imbalance between the production of reactive oxygen species and the countering effect of the various antioxidants present in the body.

In SCD, the production of reactive oxygen species can be grossly amplified in response to variety of pathophysiological conditions such as inflammation immunologic disorders, hypoxia, metabolism of drugs or alcohol and deficiency in antioxidant enzymes (15). Sick cell anemia patients in this study showed low activity levels of Cu/ZnSOD ($P < 0.001$), GPX ($P < 0.001$) and CAT ($P < 0.001$) in serum compared with HbAA controls. This may be due to the consumption of these substances by pro-oxidants in SCA. This therefore place SCA patients at increase risk of oxidative stress and injury, even with HbAS controls, the GPX and CAT activities were significantly lower ($P < 0.001$) than those of HbAA counterpart. The oxidative stress may contribute to the sickling process with formation of dense cells, the development of vaso-occlusion and shortened red blood cells survival (8, 16).

Reactive oxygen species can cause damage

to biological macromolecules (15) and membrane lipids readily react and undergo peroxidation. The peroxidative process yields lipid peroxides, lipids alcohol and aldehydic products and MDA (17). In this study we observed an increase level of MDA in SCD patients ($P < 0.001$) compared with HbAS and HbAA controls. This is also consistent with other authors (15, 18). Malondialdehyde correlated negatively with antioxidant enzymes and positively with acute phase proteins.

This study demonstrated increased serum levels of some acute phase proteins in patients during steady state of sickle cell disease. The increased in acute phase protein in SCD may be as a result of subclinical vaso-occlusion which in turn lead to a hidden inflammatory response. Cytokine and in particular, interleukine 6 (IL-6) produced after this response seem to be responsible for the high levels of acute phase proteins in the steady state SCD (19). These acute phase proteins in this study were significantly different in SCD patients compared with HbAS and HbAA controls. Svarch *et al* also observed increase CRP level in majority of 83 patients with SCA in the steady state (20).

Conclusion

Based on the results of this study, increased level of MDA is accompanied with significantly lower activity level of antioxidant enzymes, and increased acute phase proteins.

Acknowledgement

The work was partly sponsored by the research grant committee, Aminu Kano Teaching hospital, Kano, Nigeria.

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