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Nitric oxide and trace metals in relation to haemoglobin F concentration in Nigerian sickle cell disease patients

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Aim: Sickle cell disease (SCD) is an inherited haemoglobin (Hb) disorder associated with vaso-occlusive events, oxidative stress, high energy demand, and endothelial dysfunction. The endothelium plays a central role in the regulation of vascular homeostasis by releasing nitric oxide (NO) among other factors. Therefore, the objective of this study was to measure plasma levels of NO, trace metals, and HbF in SCD patients.

Materials and methods: Plasma levels of NO, Zn, Mg, Mn, Se, Cu, Fe, Haemoglobin F (HbF) were measured in 59 steady state SCD patients referred to the Department of Haematology, University College Hospital, Ibadan, Nigeria and 35 age/sex matched controls using Griess reagents and spectrophotometry.

Results: Mean levels of HbF, NO, and Zn were significantly elevated while the levels of Fe, Cr, and Se were significantly reduced in SCD patients compared with the controls. In SCD patients, HbF showed significant negative correlations with Fe, Mn, Cr, and Se. Also in SCD patients, HbF showed significant positive correlation with NO.

Conclusion: Nutritional supplements that will increase certain essential trace metals but will reduce NO and oxidants may be tested in the management of SCD patients.

Key words: Sickle cell disease, trace metals, haemoglobin F, oxidants

Introduction

Patients with sickle cell disease (SCD) have a single amino acid substitution in the beta haemoglobin chain (1). This leads to altered shape of red blood cells (RBCs), lack of ability of sickled RBCs to flow through the microvasculature causing infarct in organ systems [(2)] and production of reactive oxygen species (ROS), namely superoxide, hydroxyl, and peroxide radicals within and outside RBCs (1). Nitric oxide (NO) is a by-product of the oxidative reaction catalysed by nitric oxide synthase (NOS) that converts L-arginine to citrulline. Nitric oxide synthase has 3 major isoforms; neuronal (nNOS) and endothelial (eNOS) being constitutive, and inducible (iNOS) is expressed only following the induction by the inflammatory mediators, such as lipopolysaccharid (LPS), interleukins (IL-1, IL-11), and tumour necrosis factor (TNF-alpha) (1, 2).

The effect of overproduced reactive oxygen species leads to consumption of antioxidants that are necessary for normal oxygen detoxification. Reduced level of Vitamin E, Vitamin C, carotenoids, glutathione peroxidase, glutathione reductase, and Zn were reported in SCD patients (3). This depletion of free radical quenchers suggest excessive on-going generation of free radicals.

Nutritionally essential trace metals (Zn, Fe , Se, Cu, Mn, and Mg) are not antioxidants on their own, but are integral parts and are necessary for the proper functions of antioxidant enzymes like

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catalase, glutathione peroxidase and reductase, and superoxide dismutase (4). Glutathione peroxidase and reductase were found to be low in SCD patients (3). Thus, it is likely that the levels of these nutritionally essential trace metals, which are important to these antioxidant enzymes, might be low in SCD patients.

Nitric oxide (NO) has a dual effect depending on the concentration. At an elevated level, it is an oxidant while at normal level it has tremendous effects on almost all systems in the body, especially the blood circulatory system (1).

A study showed that selective inhibition of endothelium dependent vaso-relaxation by sickled red blood cell (HbSS- RBC) membrane suggests the possibility of dysregulation of NO homeostasis (5). Most of the studies in Nigerian SCD patients were not related to the severity index of sickling (6). Thus, the objective of this study was to have a knowledge about the levels of trace metals (Mg, Mn, Zn, Fe, Se, Cu, and Cr) and nitric oxide (NO) in relation to the index of severity of sickling, such as HbF. The hypothesis was that HbSS is associated with oxidative stress and endothelial dysfunction; therefore, a reduction in the levels of certain antioxidant essential micronutrients and deranged level of NO are expected in SCD patients.

Therefore, the aim of this study is to measure plasma levels of NO, trace metals, and HbF in SCD patients compared with age and sex matched Nigerians with HbAA genotype.

Materials and methods

Patient recruitment

Ninety four subjects were recruited for the study. They were 59 SCD patients in steady state (without crisis 3 months to recruitment) from the Department of Haematology, University College Hospital, Ibadan, Nigeria and 35 HbAA controls selected among the blood donors and staff of the University College Hospital, Ibadan, Nigeria. They were aged between 26 and 55 years. Ethical clearance was obtained from the Institutional Ethical Review Committee and informed consent was obtained from the subjects before sample collection.

Exclusion criteria were those with history of malignant diseases, metabolic disorders, and apparent respiratory dysfunctions/diseases. Others excluded were those with high parasite densities, abnormal liver functions, pathogenic infections, and abnormal renal functions as observed in laboratory investigations. The strict selection criteria lead to low number of subjects.

Blood samples (10 mL) were collected by venipuncture from each participant; 4 mL of which was put in bottles containing E.D.T.A (ethylene diamine tetra-acetic acid) for haemoglobin electrophoresis and estimation of HbF using Betkes Method (7); while the remaining 6 mL was put in bottles containing lithium heparin for the determination of NO and trace metals using Griess reagent (8) and atomic absorption spectrophotometer, (9) respectively.

Statistical methods

Statistical analysis was performed using SPSS 15.0. Results were expressed as mean ± SD. The difference between mean and standard deviation was compared using the Student's t test. Pearson's correlation coefficient was used to establish relation between NO, trace metals, and HbF. Values of $P < 0.05$ were considered statistically significant.

Results

The levels of HbF, Zn, and NO were significantly elevated while the levels of Fe, Mn, Cr, and Se were significantly reduced in SCD patients compared with the controls (Table 1). NO showed positive correlation with HbF while Fe, Mn, Cr, and Se showed negative correlations with HbF in SCD patients (Table 2). In HbAA Nigerians, only Se showed significant positive correlation with HbF values (Table 3).

Discussion

Sickle cell disease is a heritable disease for which no cure has been found. It is characterized by inflammation, vaso-occlusion, anaemia, and increased energy demand among others (3). However, our understanding of the full mechanism of the disease is incomplete (10). Haemolysis leads to loss of haemoglobin, which in turn, leads to

Table 1. The levels of HbF, NO, and trace metals in SCD patients compared with the control (HbAA subjects).

	HbAA (n = 35)	HbSS (n = 59)	t-, P-
HbF (%)	1.03 ± 0.44	5.16 ± 4.04	7.25,<0.01
Mg (mg/L)	9.15 ± 2.0	9.45 ± 2.1	0.71, >0.2
Zn (mg/L)	11.7 ± 2.0	13.2 ± 2.3	3.41, <0.01
Fe (ug/dL)	80.6 ± 9.5	69.5 ± 10.3	5.29, <0.01
Mn (ng/dL)	76.2 ± 8.5	73 ± 8.1	1.88, >0.05
Se (ug/dL)	78.5 ± 9.5	68 ± 10.3	5.00, <0.01
Cu (ug/dL)	68.5 ± 10	67 ± 10.1	0.71, >0.2
Cr (ug/dL)	74.1 ± 9.5	66 ± 8.5	5.40, <0.01
NO (uM/L)	41.8 ± 7.5	78.5 ± 10.3	20.38, <0.01

Table 2. Correlation of HbF with NO and trace metals in SCD patients.

	HbF (n = 59)	
	r	P
Mg	- 0.11	0.39
Zn	- 0.12	0.58
Fe	- 0.26	0.04(s)
Mn	- 0.26	0.04(s)
Cr	- 0.27	0.03(s)
Se	- 0.25	0.05(s)
Cu	- 0.06	0.96
NO	+ 0.49	0.02(s)

Table 3. Correlation of HbF with NO and trace metals in HbAA subjects.

	HbF (n = 35)	
	r	P
Mg	- 0.23	0.25
Zn	- 0.05	0.76
Fe	- 0.23	0.25
Mn	- 0.25	0.15
Cr	- 0.20	0.26
Se	- 0.41	0.02 (s)
Cu	- 0.20	0.26
NO	0.19	0.24

depletion of essential nutrients and micronutrients required for proper cell function. Fe is important in the synthesis of Hb while Cu and Zn play very important roles in Fe metabolism (3,4). Therefore, continuous use of Fe in haemoglobin synthesis in SCD patients may explain low plasma Fe in these patients. This low level of plasma Fe might have resulted in raised level of Zn since little Fe will be available for Zn metabolism.

Zn is known to inhibit the activity of calmodulin, which controls the calcium pump of the RBCs (11). During sickling, there is influx of calcium into RBC due to over activity of calmodulin, which destroys RBC membranes. It may therefore be suggested that raised level of Zn in SCD patients is a natural mechanism to suppress the activity of calmodulin whose over activity causes the destruction of RBC membranes.

Cu is essential for proper functioning of different metalloenzymes including caeruloplasmin, which is required for Fe metabolism. Caeruloplasmin mobilizes stored Fe in the liver, which makes Fe available for haemoglobin synthesis (11) and prevents Fe overload (haemosiderosis). Thus, explaining reduced level of Fe and Cu in SCD patients as shown in this study.

Sickled RBCs are fragile and dehydrated (12). It has been shown that magnesium is not only useful in reducing the painful episode in SCD patients but also affects the hydration of the RBC. Our result of raised plasma magnesium in SCD patients is therefore advantageous to SCD patients.

The involvement of Cr in SCD patients is not clearly defined but Cr is known to potentiate insulin action by acting as a cofactor in the initial reaction of insulin with the receptor sites of insulin sensitive cell membrane (13). In SCD patients, there is an increased energy demand, which activates more Cr for carbohydrate metabolism. This will lead to low plasma concentration of Cr in SCD patients as reported in this study.

Mn and Se are antioxidants as they are incorporated into antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase (14). Significantly reduced levels of Mn and Se in SCD patients could be due to consumption as antioxidants to ameliorate oxidative stress, which follows free radical load in SCD patients.

An earlier study has shown higher (10-30 folds) production of reactive oxygen species (ROS) and 20%-50% lower glutathione content in RBC of SCD patients compared with controls (4). Lower cellular content of anti-oxidants and ROS have been found to play an important role in the pathophysiology of SCD (1,16). This is supported by the present study where elevated levels of NO and reduced levels of antioxidant trace metals (Se, Fe, and Mn) were found in SCD patients.

Biomarkers of oxidative damage are increased in SCD. These include superoxide, hydroxyl radical, and oxides of nitrogen and xanthine oxidase (15). Sickle cell anaemia is characterized by recurrent vaso-occlusive manifestations due to plugging of micro-circulation by deformed sickled RBCs (2,17). Evidence suggests that sickled RBCs may also induce changes in the vascular wall that contribute to the occlusive process (17,18). The endothelium plays a central role in the regulation of vascular homeostasis by releasing a number of factors, including NO (19). Loss of biological activity of NO could predispose to vaso-occlusion promoting RBC adhesion and impairing the regional regulation of blood flow (19). Several lines of evidence suggest that there is vascular dysfunction and impaired NO bioactivity in SCD patients. In this study significantly elevated level of NO was observed. This may be due to increased shear stress and tissue hypoxaemia in SCD patients. This is supported by a report that haemodynamic consequences of anaemia in HBSS include increased cardiac output and blood flow, which stimulates the NO production (20).

Our finding of increased level of NO in SCD patients is further supported by a previous study reporting accelerated antioxidation, enhanced oxidative stress, increased susceptibility to lipid peroxidation, and increased generation of ROS in SCD patients (1, 3,16).

Clinical observations and experimental studies have long established the fact that a high level of foetal haemoglobin (15, 21) in adult sickle cell anaemia [SCA] patient has a protective effect on the polymerization of haemoglobin S in deoxygenated state thereby ameliorating clinical disease severity. It is therefore not surprising that HbF values were higher in SCD patients compared with the control (HbAA subjects). Polymerization of HbS in deoxygenated state is the watershed leading to vaso-occlusion. Other factors that play a crucial role in the process include factors that retard transit time of sickle erythrocyte in the micro-vascular circulation, chronic intense inflammatory state, and leukocyte adhesion to vascular endothelium (9, 10). It may be concluded from this study that antioxidant supplement therapy might be useful in SCD patients and further work is required to elucidate mechanisms underlying these observations.

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