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The antisickling effects of some micronutrients and antioxidant vitamins in sickle cell disease management

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ABSTRACT

The antisickling effectiveness of crude aqueous extract of three mineral elements: Copper, Zinc and Magnesium were investigated to ascertain the ability of the ions to inhibit sickle cell hemoglobin polymerization process. Also assayed with the mineral elements were two fat –soluble vitamins-(vitamins A & E and one water-soluble-vitamin C. The mineral samples were dissolved in distilled water to a final assay concentration of 1.0×10^{-1} mM. The fat-soluble vitamins were dissolved in ethanol of analytical grade to a final assay concentration of 100 IU for vitamin A and 1 mg/ml for each of vitamins C and E respectively. Zinc exhibited the highest level of relative percent inhibition of 89.69 ± 0.1 %, followed by Magnesium (48.40±0.02%), while Copper enhanced sickle cell hemoglobin polymerization with relative percent inhibition of -1921±0.2 % or an enhancement of (1921±0.2 %). Among the vitamins, vitamin C exhibited the highest level of relative percent inhibition of 38.14 ±0.2 %, followed by both vitamins A and E, with relative percent inhibitions of 30.93±0.10 % respectively. For the Fe^{2+}/Fe^{3+} ratio analysis ,the mineral elements Zinc and Mg, improved the ratio to varying levels (Zn, 16.21%; Mg,2.63 %).The ratio was highly reduced by Copper (69.11±0.1 %).The vitamins improved the ratio remarkably as shown in the sequence: vitamin C (10.00±0.0%);vitamin E (40.85±0.2%) and Vitamin A(24.48±0.2%) . The results revealed the nutritional and therapeutic relevance of these antioxidant minerals and vitamins in the management of sickle cell disease and other syndromes exhibiting similar etiology.

Key words: Antioxidant vitamins, micronutrients, sickle cell disease, hemoglobin polymerization.

INTRODUCTION

Sickle cell disease refers to a large group of hemoglobinopathies in which at least one sickle cell gene of the β-globin chain is inherited together with abnormal gene. The most prevalent of the syndromes are sickle cell disease (HbSS), hemoglobin SC disease (HbSC) and hemoglobin Sβ thalassemia (HbSβ [thal]) minor and major (Mahta et al.,2000).Patients with sickle cell disease (HbSS) suffer most severely and people of Africa descent are primarily affected. Today, the sickle hemoglobin is known to interact with diverse genes and environmental factors, producing a multisystemic disease with several phenotypes (Driss et al., 2009;

Nwaoguikpe et al., 2010). Since HbS was first described in 1910 effort has been made to develop clinical cure to ameliorate the severe pathophysiological complications accompanied by frequent hospital-izations. A gamut of substances have been directed to the treatment of sickle cell syndrome with little or no pronounced success. Hydroxyurea has been implicated in the management of HbS, but with little success, resulting from health complications. Since late 1980s, under-nutrition has been identified as a critical feature of sickle cell disease (Al.Saqladi et al., 2008; Badaloo et al., 1987; Khan et al., 2009, Reed et al., 1987), but this focus has not been adequately addressed. The first and most direct evidence of insufficient micronutrient intake demonstrated by clinical improvement following dietary intervention was reported by Heyman et al (1985). It was discovered that feeding HbSS patients with a protein rich diets (35 %) from dietary protein reduced

the circulating level of inflammatory protein, C-reactive protein and interleukin-6(IL-6). Micronutrient deficiencies have been identified in sickle cell disease. Traditionally, the dietary information available about HbSS has only addressed a variety of micronutrient deficiencies, including iron, zinc, copper, folic acid, pyridoxine and vitamin E (Reed et al.,1987). The role of these deficiencies has long been extensively studied, including their involvement in immunity and growth (Prassad, 1998; Zemel et al., 2002). Several studies have investigated the role of these micronutrients /micro-molecules in the pathogenesis of sickle cell disease by measuring static circulating levels and /or effects observed from dietary supplementation studies. In the context of sickle cell disease, far more attention has been focused on Zinc than any other mineral. Many health consequences of Zinc deficiency have been reported, including immune dysfunction, abnormal or delayed sexual maturation, abnormal growth pattern, poor wound healing, decreased level and activity of Zinc metalloproteins (Prasad, 2008; Bao et al., 2008). Zinc supplementation in patients with SCD resulted in significant improvements in secondary sexual characteristics in the normalization of plasma ammonia concentrations and in the reversal of abnormalities of dark adaptation (Prasad et al.,1975). In patients with SCD, Zinc supplementation corrected energy, increased natural killer cells activity, increased the ratio of CD 4+ to CD8+ and increased the activity of serum thymulin.

Copper is another micronutrient that has been found at increased levels in the plasma of HbSS patients. Erythrocyte copper is either normal or elevated (Behera et al., 1981; Pellagrini et al., 1995; Alkenami et al., 1999). The clinical significance is unclear, but it has been reported to occur in the event of decreased plasma Zinc levels. (Behera et al., 1981).A high Zinc intake sustained over weeks is reported to induce intestinal synthesis of metalothionein, a copper binding protein that traps copper within intestinal cells, blocking its absorption. Excess copper may contribute to free radical production and oxidative damage in HbSS (Nathan et al., 1992). Reports on the level of Magnesium in sickle cell disease have been increasing with variable results. Low levels of total magnesium in sickle erythrocytes have been associated with increased sickling due to red cell dehydration and hence, increased polymerization (deFranceschi et al., 1997).It has also been demonstrated that the dehydration is due to abnormally high red cell permeability and loss of potassium ions. There has been substantive evidence on the role of magnesium on sickle cell disease management. Some workers have reported of decrease on the length of hospitalization of patients administered intravenous magnesium (Brousseau et al., 2004; Hankins et al., 2008; Rinchart

et al., 2010).

Antioxidants are compounds that inhibit or scavenge free radicals released during peroxidation reactions. There has been an evidence of low circulating levels of antioxidant vitamins eg vitamin A, C and E in the plasma measured in HbSS patients. However, no definitive proof of clinical benefit in the supplementation of these antioxidants. Many researchers have reported on the administration of vitamin C to human sickle cell red cells *in vitro*, which inhibited the formation of dense cells (Adewoye et al., 2008). Other reports in literature proposed that vitamin C prevents *in vitro* Heinz body formation (denatured hemoglobin) in adult sickle red cells and normalizes the hemodynamic changes associated with positive adjustments (Lachant and Kouichi, 1986; Jaja et al., 2008). Of the antioxidant vitamins, vitamin E has been investigated most in sickle cell disease. There are many reports on the low circulating level of vitamin E in HbSS patients (Broxson et al., 1989; Nang et al., 2006). Both an ex-*in vivo* study on a pilot clinical trial demonstrated that a cocktail consisting of daily doses of 6g of aged garlic extract, 4-6g of vitamin C and 800-1200 IU of vitamin E ,may indeed be helpful in HbSS management (Ohnishi et al ., 2000.The main objective of this work is to demonstrate in practical terms ,the antisickling effectiveness of some micronutrients and three antioxidant vitamins in inhibiting sickle cell hemoglobin polymerization and improvement of the Fe^{2+}/Fe^{3+} ratio of sickle hemoglobin.

MATERIALS AND METHODS

Materials

The materials used in the work include: Sodium metabisulphite (2% solution). Unicam Spectronic 20, Vitamin A capsules of 100,000 IU each purchased from Health Care Drwate LTD, Kancheepuram, India. Vitamin C tablets, 100 mg each, were purchased from Emzor Pharmaceutical Company LTD, Lagos, Nigeria. Vitamin E capsules, 1000mg each were purchased from Pharco Pharmaceuticals, Alexandria, Egypt. The mineral elements were supplied as 1g each of soluble salts, by the Chief Technologist of Industrial Chemistry Department of the Federal University of Technology, Owerri, Imo State, Nigeria.

Methods

One gram (1 g) of each of soluble chloride salts of zinc, copper and magnesium were prepared to give the gravimetric equivalent of 0.1mM (1.0×10^{-1} mM) final assay concentration of each metallic ion.

Table 1 .The effect of micronutrients on the rate of Polymerization, the relative % polymerization and the relative % inhibition of sickle cell hemoglobin

Sample	Final Assay (conc. mM)	Rate of polymerization	Relative % polymerization	Relative % inhibition
Control	_	0.00970 ± 0.0^b	100.0 ± 0.0^b	00.00 ± 0.0^b
Zinc	0.01	0.00110 ± 0.0^a	10.31 ± 0.1^a	89.69 ± 0.2^a
Copper	0.01	0.01960 ± 0.0^c	2021.0 ± 0.1^c	-1921.0 ± 0.0^c
Magnesium	0.01	0.00050 ± 0.0^a	51.60 ± 0.1^a	48.40 ± 0.0^a

The values in the table are the Mean±SD   from triplicate determinations. The values with the same superscript are significantly related at p≤0.05 and different from others. Values with the superscript c, show enhancement of polymerization or sickling and are significantly different at p≤0.05.

Table 2. The rates of polymerization, relative percent polymerization and the relative percent inhibition by the antioxidant vitamins

Sample	Final assay conc.	Rate of polymerization	Relative % polymerization	Relative % inhibition
Control	-----	0.0097 ± 0.0^b	100.0 ± 0.0^b	0.00 ± 0.0^b
VITAMIN A	100IU/ml	0.0067 ± 0.0^a	69.07 ± 0.1^a	30.9 ± 0.1^a
VITAMIN C	1 mg/ml	0.0060 ± 0.0^a	61.86 ± 0.0^a	38.14 ± 0.0^a
VITAMIN E	1 mg/ml	0.0067 ± 0.0^a	69.07 ± 0.1^a	30.93 ± 0.1^a

The values in the table are the Mean± SD from triplicate determinations. Values with the same superscript are significantly the same and are different from others along the rows and columns.

Preparation of Vitamin Samples

One gram (1 g) of vitamin C was dissolved in 100 ml of distilled water to give vitamin C solution of 10 mg/ml to give a final assay concentration of 1mg/ml. This solution was filtered using Whatman filter paper No.1. Vitamins A and E were dissolved in 100 ml of ethanol for each, to give a final assay concentration of 100 IU/ml for vitamin A, while vitamin E equally gave a final assay concentration of 1mg/ml. These were prepared fresh and used immediately for various assays.

Preparation of Sickle Cell Blood

Erythrocytes were isolated from whole blood by centrifuging at 1500 x g for 15 minutes. Sickle cells sedimented while the plasma was siphoned out carefully using Pasteur pipette. The erythrocytes were by repeated inversion suspended in a volume of isotonic saline equivalent to the siphoned plasma. The suspended erythrocytes were freeze thawed in a freezer to release a hemolysate, used for the hemoglobin polymerization experiment.

Sickle Cell Hemoglobin Polymerization Experiment

The original methods of Noguchi and Schechter, 1978 was used for the HbSS polymerization experiment. HbSS polymerization was assessed by the turbidity of the polymerizing mixture at 700 nm using 2% solution of Sodium metabisulphite as a reductant or deoxygenating agent (Iwu et al.,1988); 4.4 ml of 2% Sodium metabisulphite, 0.5 ml normal saline(0.9 % NaCl) and 0.1 ml of hemoglobin were pipette into a cuvette ,shaken and absorbance read in a (Spectrophotometer, Spectronic 20) at 700 nm for 30 minutes at 2 minutes Intervals. This served as blank for all assays. For the test assay, 0.5 ml normal saline was replaced with 0.5 ml antisickling agent or sample and readings taken as usual .The rates of polymerization were calculated from the formula of average change in absorbance against time in minutes (Nwaoguikpe et al., 1999)

$Rp = OD_f - OD_l /t$

$Rp = \Delta OD/t$, where Rp = rate of polymerization

OD_f = final absorbance, ODi= initial absorbance at

Table 3. *In vitro* effects of Micronutrients and antioxidant vitamins in the Fe^{2+}/Fe^{3+} ratio of Sickle cell blood.

Sample	%Hb	% mHb	Fe^{2+}/Fe^{3+}	% reduction/Increase
HbSS blood (Control)	92.86 ± 0.0^d	07.14 ± 0.2^d	12.46 ± 0.0^d	00.00 ± 0.0^d
Copper	53.21 ± 0.0^c	46.78 ± 0.1^c	01.14 ± 0.1^c	-85.02 ± 0.2^c
Magnesium	93.00 ± 0.0^a	07.00 ± 0.0^a	13.29 ± 0.1^a	02.63 ± 0.1^b
Zinc	94.00 ± 0.0^a	06.00 ± 0.0^a	15.80 ± 0.1^a	22.00 ± 0.0^a
Vitamin A	93.40 ± 0.0^a	06.60 ± 0.0^a	14.24 ± 0.0^a	09.96 ± 0.0^b
Vitamin C	94.16 ± 0.2^a	05.84 ± 0.0^a	16.12 ± 0.1^a	24.48 ± 0.0^a
Vitamin E	95.07 ± 0.1^a	04.93 ± 0.1^a	19.28 ± 0.2^a	48.88 ± 0.2^a

The values are the Mean ± SD from triplicate determinations. The values with the same superscript are significantly related at p≤0.05 along the rows and columns and different from others. The values with the superscript c , showed reduction in the Fe^{2+}/Fe^{3+} ratio
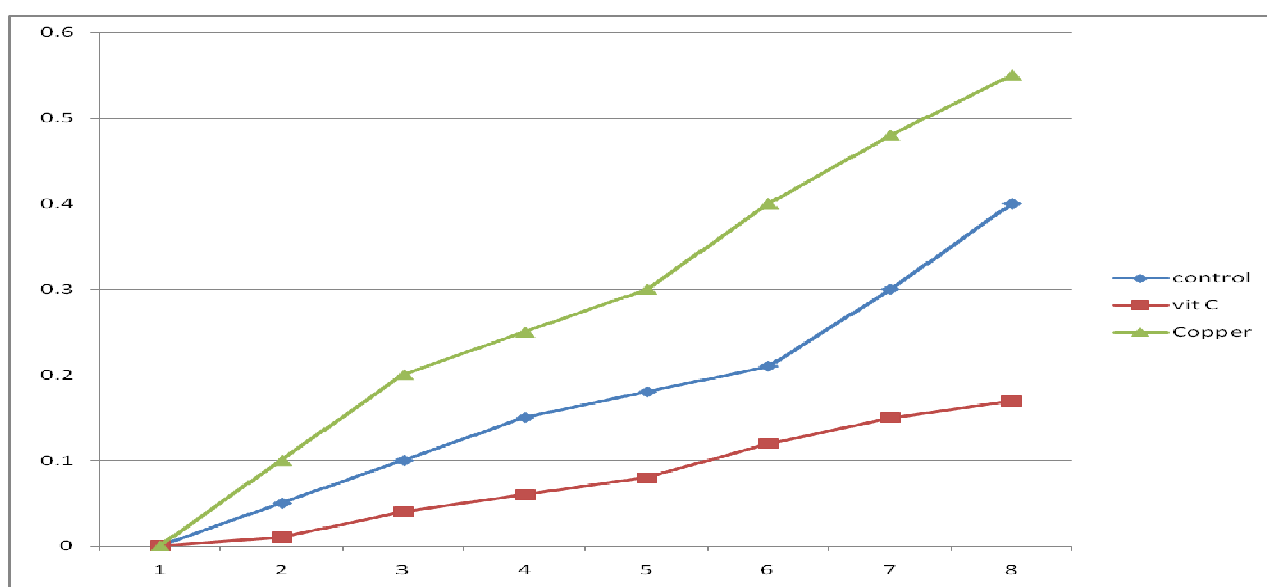
Figure 1. A plot of average change in absorbance (ΔO) at 700 nm against (t) time in minutes for Copper and Vitamin C, compared with control. It shows hemoglobin polymerization enhancement action of copper ion compared with Vitamin C, a standard antisickling agent.

time zero

ΔOD = change in optical density,   t= time of reaction in minutes

Determination of Fe^{2+}/Fe^{3+} Ratio

The Fe^{2+}/Fe^{3+} ratio was determined by the methods of Davidson and Henry, 1974. The oxygen affinity of hemoglobin and methemoglobin were measured at 540 nm and 630nm respectively. The approach employs lyzing 0.02 ml whole blood in 5.0 ml distilled water and 0.02 ml normal control. To determine the Fe^{2+}/Fe^{3+} ratio, 0.02 ml of the antisickling agent was added to 5.0 ml distilled water and 0.02 ml of blood and incubated for 1 hr in a test tube. The absorbances of Hb and metHb were measured according to the method above.

RESULTS

Results of all assays are shown in tables 1-3 and figures 1 and 2 respectively. Table1 shows the rates of polymerization, the relative percent (%) polymerization and the relative percent % inhibition of HbSS at a final assay concentration of 0.01mM of zinc, copper and magnesium ions. Table 2 depicts the rate of polymerization, the relative percent polymerization and the relative % inhibition by the antioxidant vitamins. Table 3 shows the *in vitro* effects of the micronutrients and vitamins on the Fe^{2+}/Fe^{3+} ratio of sickle cell blood. Fig 1 represents a plot of average change in optical density against time in minutes for the micronutrients. Figure 2 represents the same plot
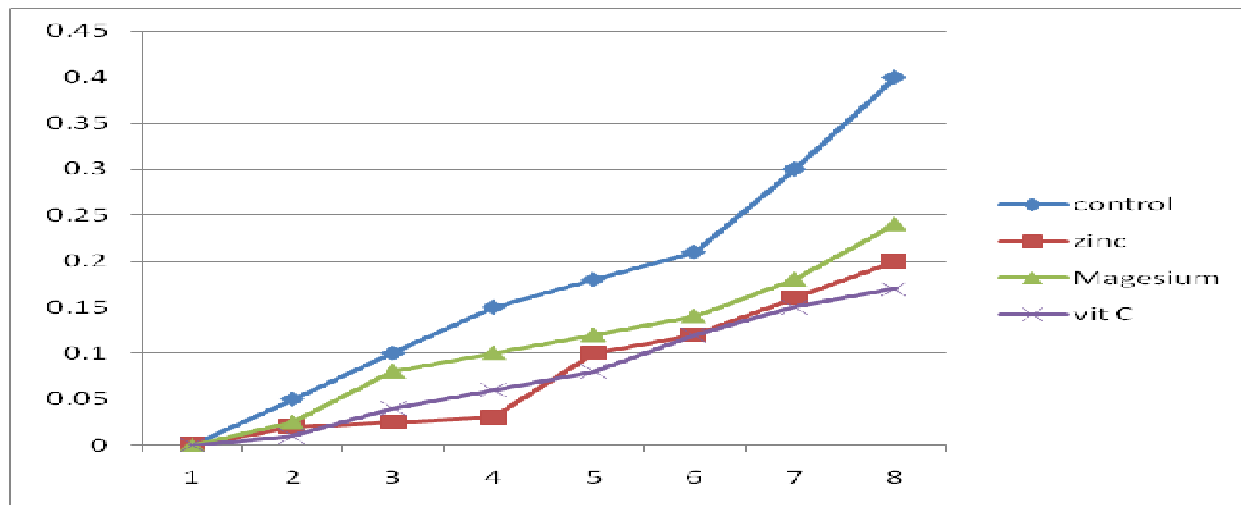
Figure 2. shows a plot of average change in absorbance (ΔOD) at 700 nm against time (t) in minutes. It also shows the rate of sickle cell hemoglobin polymerization inhibitions by Zinc, Magnesium and Vitamin C respectively, compared with the control.

against time for the antioxidant vitamins.

The values in the table are the Mean±SD from triplicate determinations. The values with the same superscript are significantly related at p≤0.05 and different from others. Values with the superscript c, show enhancement of polymerization or sickling and are significantly different at p≤0.05.

DISCUSSION

The nutritional approach to the management of sickle cell disease has been the most modern and most effective protocol adopted in the management of the syndrome. Many studies have provided humanity with reliable data on the deficiencies of various nutrients some of which are exacerbated by the sickling episode. Some of these deficient nutrients include Zinc, Magnesium, Copper, vitamin A, vitamin C and vitamin E, resulting in many pathophysiological complications of the syndrome (Hyacinth et al., 2010; Almeida and Roberts, 2005). Vitamin D had also been found to be very low in children suffering from sickle cell disease (SCD). Some researchers have equally reported on low level of serum 25-hydroxyvitamin D (25-OHD) in children with sickle cell disease when compared with their age and racially related peers (Buison et al., 2004; Rovner et al., 2008). We have demonstrated experimentally, the antisickling roles of both the micronutrients and vitamins under survey, their antisickling roles by assaying their ability to inhibit sickle cell hemoglobin polymerization.

Zinc has been found to inhibit polymerization by 89.69 % and Magnesium 48.40%. Contrarily, copper elevated the process by 1921 %, hence, it exhibited

sickling effect. Zinc deficiency has been associated with a myriad of disease states (Krasovec and Frank, 1996; Hambadge and Krebs, 2007).it has been reported that sickle cell disease children have high level of Copper in their serum and that zinc therapy leads to a state of hypocupremia. The main mechanism of the action of zinc in blood and in general metabolism has been to antagonize calcium retention and moreover, stabilizing the erythrocyte, thereby preventing hemolysis. (Dean and Schechter, 1978; Sandstead, 1994; Solomon, 1998) . Zinc has equally been found as a strong antioxidant, since it's a component of many antioxidant enzymes like catalase, Glutathionine -S-transferase and Alkaline phosphatase and over 200 other enzymes that are zinc dependent (Sandstead,1994; Maret and Sandstead, 2006). Zinc is also an inhibitor of Calmodulin. Other inhibitors of Calmodulin like the Phenothiazines and Cetidel, which possess antisickling properties (Brewer, 1981). The antioxidant effect of zinc was reflected in our result especially. In the Fe^{2+}/Fe^{3+} ratio. Zinc improved the ratio by 16.21%, Magnesium by 2.63%, while copper reduced the ratio. It has been reported by many workers that copper antagonizes zinc action in the plasma (Bao et al., 2008; Behera et al.,1981; Underwood,1977). This action of Zinc and Magnesium in improving the Fe^{2+}/Fe^{3+} ratio is likened to reversing the functional state of sickle erythrocytes to their original biconcave structure, thereby increasing the oxygen affinity of the drepanocytes .

The antioxidant vitamins, A. C and E were assayed and found to be potent inhibitors of sickle cell hemoglobin polymerization, and equally improved the oxidant status of sickle erythrocytes. Vitamin A inhibited the polymerization process by 30.93 %, which is equally the same value obtained for vitamin E. Vitamin C, a

known antisickling agent, inhibited the process by 38.14% .There is no significant difference in the antisickling properties of these vitamins at p≤0.05. In terms of improvement in the Fe^{2+}/Fe^{3+} ratio of sickle cell blood; the vitamins improved the ratio by 10.00% for vitamin A; 24.48% for vitamin C and 48.88% for vitamin E respectively. In the past, vitamin E was promoted as a patent medicine, capable of curing many diseases. Vitamin E deficiency leads to red cell hemolysis and supplemental vitamin E has been shown to improve red cell survival in some patients with glucose 6-phosphate dehydrogenase deficiency, β-thalassemia and sickle cell anemia (Delvin,1986). Naturally, vitamin E is present in many fruits and various foods such as tomatoes, apples, bananas, carrots ,orange juice, potatoes, etc (Wilson et al.,1975).Vitamin E has been proposed to prevent in vitro formation of Heinz bodies (denatured Hb) and normalizes blunted hemodynamic changes associated with posture adjustments. Many workers have suggested that antioxidant vitamins should be given to sickle cell patients as a cocktail rather than individually (Ohnishi et al., 2000).   Like many proven antisickling agents, the most probable site for binding is the heme pocket and the erythrocyte membrane. It is therefore legitimate to assume that these metallic ions diffuse readily into the heme pocket interfering with protein-protein interaction necessary for fiber formation. It is therefore necessary to propose that according to the federal government 2010 dietary guidelines for Americans, that"nutrients should come primarily from foods". Foods that are nutrient dense, mostly intact forms that contain not only the essential vitamins and minerals that are often contained in nutrient supplements, but also in fiber and other naturally occurring substances that may have positive health effects (Evan,2006). There is no doubt that genetic abnormality can be managed successfully only by nutrition alone in the near future as the knowledge of the pathophysiology of the disease become more apprehensive to nutritionists and other scientists. The introduction of an antisickling nutrient "Ciklervit™". In Nigeria, It is a step forward. It contains natural food extracts, amino acids, vitamins and mineral nutrients. This nutrient normalizes most complications of the syndrome and patients on this nutrient rarely experience crises (Research review, 2007).

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