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Evaluation of Testosterone and Mineral Elements (Cu, Zn And Mg) Levels In Male Sickle Cell Patients In Benin City, Edo State.

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Abstract
Sickle cell diseases (SCD) are groups of blood disorders that are inherited from both parents. Sickle cell anaemia is the most well-known among them. SCD affects 2 – 3 % of Nigeria□s population. The evaluation of testosterone and mineral elements (Cu, Zn and Mg), which promotes secondary male characteristics and red blood cell integrity, places emphasis on sickle cell disease management. This study was aimed to determine testosterone and mineral elements (Cu, Zn and Mg) in sickle cell patients in Benin City, Edo state and to correlate the findings between those in steady state and vaso-occlussive crisis. Serum testosterone, copper, zinc and magnesium were determined in 100 male sickle cell patients attending sickle cell centre in Benin City, and in 50 male apparently healthy controls. A detailed and structured questionnaire was used to obtain information about their, age, gender, occupation, frequency of hospital visit, and history of blood transfusion. The level of serum testosterone was measured using the microwell enzyme linked immunosorbent assay (ELISA) technique, while the levels of serum copper, zinc and magnesium were measured using atomic absorption spectrophotometer (AAS). The mean value was obtained using paired Student's T-test and Pearson's correlation. The mean levels of testosterone (2.52±0.153 ng/ml), copper (72.6±3.06 µg/dl) and zinc (73.0±1.96 µg/dl) were significantly lower ($p< 0.001$) in sickle cell disease than controls. The mean level of magnesium (1.63±0.187 mg/dl) was significantly lower ($p < 0.007$) than controls. The mean copper to zinc ratio (0.999±0.0326 µg/dl) was also significantly lower ($p < 0.001$) than controls. The testosterone ($p < 0.746$), copper ($p < 0.48$), zinc ($p < 0.743$), magnesium ($p < 0.566$) and copper to zinc ratio ($p< 0.517$) were independent of the age groups of male sickle cell disease patients used for this study. Testosterone level in male sickle cell disease patients showed positive significant correlation with copper ($r=0.71$; $p < 0.001$), zinc ($r=0.50$; $p < 0.001$), copper to zinc ratio ($r=0.45$; $p < 0.001$) and on magnesium ($r=0.19$; $p < 0.05$). The study showed that serum testosterone and mineral elements (Cu, Zn and Mg) levels in male sickle cell patients were significantly low and could be responsible for infertility and severe anaemia in male SCD patients.

Keywords: Sickle cell disease, Testosterone, Mineral elements.

Introduction

Sickle cell disease (SCD) and its variants are genetic disorders resulting from the presence of a mutated form of haemoglobin, haemoglobin S (HbS) (Strouse, 2016). The gene defect is a single nucleotide mutation of the β-globin gene, which results in glutamic acid (E/Glu) being substituted by valine (V/Val) at position 6 (Suzanne, 2008). Haemoglobin S with this mutation is referred to as HbS, as opposed to the normal adult HbA. This is normally a benign mutation, causing no apparent effects on the secondary, tertiary, or quaternary structures of haemoglobin in conditions of normal oxygen concentration. The prevalence of sickle cell trait ranges between 10 and 45% in various parts of sub-Saharan Africa (Okwi *et al.,* 2010). In Nigeria carrier prevalence is about 20-30% (Ademola, 2015). SCD affects about 2 to 3% of the Nigerian population of more than 160 million. Recent estimate from a large retrospective study in Benin City, South-South Nigeria revealed in SCD prevalence of 2.39% and carrier rate of about 23% (Nwogoh *et al.*, 2012).

Morbidity, frequency of crisis, degree of anaemia and the organ system involved vary considerably from individual to individual (Joseph, 2020). Healthy red blood cells are round, and they move through small blood vessels to carry oxygen to all parts of the body. In someone who has SCD, the red blood cells become hard and sticky and look like a C-shaped farm tool called a 'Sickle'. The sickle cells die early, which causes a constant shortage of red blood cells. Also, when they pass through small blood vessels, they get stuck and clog the blood flow. This can cause pain and other serious problems such as infection, acute chest syndrome and stroke. HbSS People who have this form of SCD inherit two sickle cell gene ('S'), one from each parent. This is commonly called sickle cell anaemia and is usually the most severe form of the disease (CDC, 2017).

Sickle cell anaemia is a public health problem in Africa than any other continent in the world and it affects about 2% of Nigeria population (Garba *et al*., 2016).

Testosterone is necessary for normal sperm development. It activates genes in sertoli cells, which promote differentiation of spematogonia. It regulates acute HPA (hypothalamic-pituitary-adrenal axis) response under dominance challenge (Mehta *et al*., 2008). Testosterone enhances muscle growth. Testosterone also regulates the population of thromboxane A_2 receptors on megakaryocytes and platelets and hence platelet aggregation in humans. Adult testosterone effects are more clearly demonstrable in males than in females but are likely important to both genders. Some of these effects may decline as testosterone levels might decrease in the later decades of adult life (Kelsey *et al*., 2014). Testosterone deficiency in SCD is associated with retardation of physical development, infertility, bone mass loss and possibly priapism (Morrison *et al.,* 2013).

Mineral elements are important in red blood cell maintenance, body growth and development (Okpuzor and Okochi, 2009). Mineral elements are pharmacologically beneficial at low dose and toxic at high dose, thus the need for monitoring of the dosage (Burtis *et al*., 2008). People with sickle cell disease suffer from mineral elements (Copper, Zinc and Magnesium) deficiency, and higher nutrients deficiency may be due to increased needs of many nutrients in sickle cell patients (Hyacinth *et al*., 2010). There is increased turnover of haemopoietic cells due to chronic haemolysis and cell death leading to tremendous red marrow expansion. These conditions lead to hyper metabolic rates and increases in nutrients and energy demand (Hibbert *et al*., 2006). Protection of red cell membranes from free radical mediated oxidation stress is crucial to the management of sickle cell disease (SCD) (Garba *et al*., 2016). Mineral elements play an important role in maintaining red cell membrane integrity and function (Okpuzor & Okochi, 2009).

Materials and Methods:

Study Design

This is a case – control study to evaluate testosterone and mineral elements (Cu, Zn and Mg) of sickle cell patients at Sickle Cell Centre in Benin City over the period of July, 2018 to April 19, 2019. The study population was recruited from both male sickle cell patients and non-sickle cell patients (homozygous AA) from eighteen (18) to fifty-eight (58) years.

Ethical Approval/ Informed Consent

Ethical approval was sought and obtained from Ethics Review Committee of the Ministry of Health where prior to commencement of sample collection from voluntary patients in Sickle Cell Centre, Benin City with reference number (REF NO: HA. 577. 186). Informed consent was obtained from participants and utmost confidentiality was maintained throughout the process of investigation.

Sample collection

Clinical data and other relevant information were obtained from sickle cell patients involved in the study. Under aseptic conditions, three milliliters (3mls) of venous blood were collected from each subject and control participants and transferred to plain container and allowed to clot. After carefully dislodging the clotted sample, the samples were centrifuged at 3000rpm for 5 minutes and sera were separated. The separated serum was transferred into a clean sample bottle and stored at -20^{0}C.

Sample size

The sample size calculation was done using prevalence of 2.39% from previous studies done on prevalence of sickle cell disease in Benin City (Nwogoh *et al*., 2012). The sample size for this study was obtained using the Fischier fomular

$N = Z^2 pq/d^2$

N = minimum number of sample

Z = confidence level corresponding to 95%, usually set at 1.96

P = estimated prevalence (2.39%)

q = 1 - p

d = degree of accuracy, set as 0.05 (5%)

$N = 1.96^2 \times 0.02391 (1 - 0.0239)/ 0.05^2$

N = 0.0896 x 0.9761 / 0.0025

N = 0.0896/0.0025 = 35.8 Approximately 36

This formula gives a minimum of 36 sample size. However, 150 subject samples from sickle cell patients were analyzed in the study.

Method of Sample Analysis

Testosterone was analyzed by Enzyme Immunoassay technique based on the principle of Competitive Enzyme Immunoassay (Type 7), while Copper, Zinc and Magnesium were determined by Flame Atomic Absorption Spectrometry (FAAS) based on the principle of aspiration of sample into a flame and atomized.

Statistical Analysis

The data obtained were analyzed using The Statistical Package for Social Scientific (SPSS) version 16. The data were expressed as mean ± standard deviation (Mean ± SD), using paired Student's T - test and Pearson's correlation were used to compare means between sickle cell patients and non-sickle cell patients, and a P-value of 0.05 was considered significant.

Results:

Table 1 shows that the Testosterone, copper (Cu), and zinc (Zn) was significantly lower ($p<0.001$) in male sickle cell patients compared to control. Also, copper to zinc ratio (Cu/Zn) were significantly lower $p<0.001$) in male sickle cell patients compared to control subjects. Magnesium in male sickle cell patients were also significantly lower ($p < 0.007$), when compared with controls. Table 2 shows an independent association of testosterone, copper, zinc and magnesium with the age of sickle cell patients. Table 3 shows the relationship between testosterone, copper, zinc and magnesium. Testosterone showed a positive correlation with copper ($r=0.71$; $p <0.001$), zinc ($r=0.50$; $p <0.001$) and copper to zinc ratio ($r=0.45$; $p <0.001$) in sickle cell patient. Table 4 shows the comparison of sickle cell patients in steady state and vaso-occlusive crisis. Testosterone was significantly lower ($p < 0.001$) in vaso- occlusive crisis than those in steady state.

Table 1: Comparison of testosterone and mineral element levels in male sickle cell patients and male controls

Parameters	Control Subject (n = 50)	Test Subjects (n = 100)	t value	p value
Testosterone (ng/ml)	8.03±0.450	2.52±0.153	14.4	0.001
Cu (µg/dl)	116±2.58	72.6±3.06	9.29	0.001
Zn (µg/dl)	95.1±2.88	73.0±1.96	6.41	0.001
Cu/Zn ratio	1.29±0.0540	0.999±0.0326	4.86	0.001
Mg (mg/dl)	2.35±0.0316	1.63±0.187	2.71	0.007

Table 2: Comparison of testosterone and mineral elements in sickle cell Patients when stratified according to age (10yrs interval)

	Age Range						
Parameters	**18 - 28 years**	**29 - 38 years**	**39 - 48 years**	**49 - 58 years**	**Fvalue**	**P-value**	**Summary**
Testosterone (ng/ml)	2.57±0.234	2.45±0.270	2.7±0.352	1.97±0.495	0.41	0.746	ns
Cu (µg/dl)	74.6±4.36	67.8±5.45	78.9±8.91	61.1±9.13	0.831	0.48	ns
Zn (µg/dl)	73.8±2.64	73.2±3.43	73.6±6.01	65±9.29	0.415	0.743	ns
Cu/Zn ratio	1.01±0.0450	0.931±0.0600	1.08±0.0996	0.958±0.0976	0.763	0.517	ns
Mg (mg/dl)	1.43±0.0646	2.03±0.685	1.74±0.309	1.3±0.141	0.68	0.566	ns

Table 3: Correlation of testosterone, copper, zinc and magnesium in sickle cell patients

Correlation	**r value**	**p value**
Testosterone (ng/ml) vs Cu (µg/dl)	0.71	0.001
Testosterone (ng/ml) vs Zn (µg/dl)	0.5	0.001
Testosterone (ng/ml) vs Cu/Zn ratio	0.45	0.001
Testosterone (ng/ml) vs Mg (mg/dl)	0.19	0.05
Cu (µg/dl) vs Zn (µg/dl)	0.66	0.001
Cu (µg/dl) vs Cu/Zn ratio	0.69	0.001
Cu (µg/dl) vs Mg (mg/dl)	0.26	0.0097
Zn (µg/dl) vs Cu/Zn ratio	-0.05	0.621
Zn (µg/dl) vs Mg (mg/dl)	0.1	0.319
Cu/Zn ratio vs Mg (mg/dl)	0.23	0.023

Table 4: Comparison of sickle cell patients in steady state and vaso-occlusive crisis.

Parameters	**SCD subjects (No Crisis) (A) n = 72**	**SCD Subjects (On Crisis) (B) n = 28**	**Control Subjects (C) n = 50**	**F-value**	**p - value**	**Summary**
Testosterone (mg/dl)	2.978±0.1687	1.375±0.2051[a]	8.176±0.4791[ab]	110.8	0.001	significant
Cu (µg/dl)	152.2±9.591	242.1±19.99[a]	261.3±15.74a	21.22	0.001	significant
Zn (µg/dl)	216.5±17.23	224.7±21.49	244.9±16.10	0.7094	0.494	NS
Cu/Zn ratio	0.9099±0.07111	1.156±0.05745	2.207±0.9785	1.595	0.206	NS
Mg (mg/dl)	0.4864±0.02979	0.3779±0.05242	0.3323±0.01492[b]	7.676	0.001	significant

Superscript a = significant with SCD subjects (No Crisis), b = significant with SCD subjects (On Crisis). NS = Not Significant, P<0.05 was significant

Figure 1 shows that there is an association between serum zinc and copper. There is a significant regression of zinc on copper (r=0.66; p <0.001). Figure 2 revealed that there is an association between serum copper to zinc ratio and copper. And it showed a significant regression of copper to zinc ratio, on copper (r=0.69; p <0.001). Figure 3, shows an association exist between serum magnesium and copper in sickle cell patients, and a significant regression of magnesium on copper (r=0.26; p < 0.0097) exist.

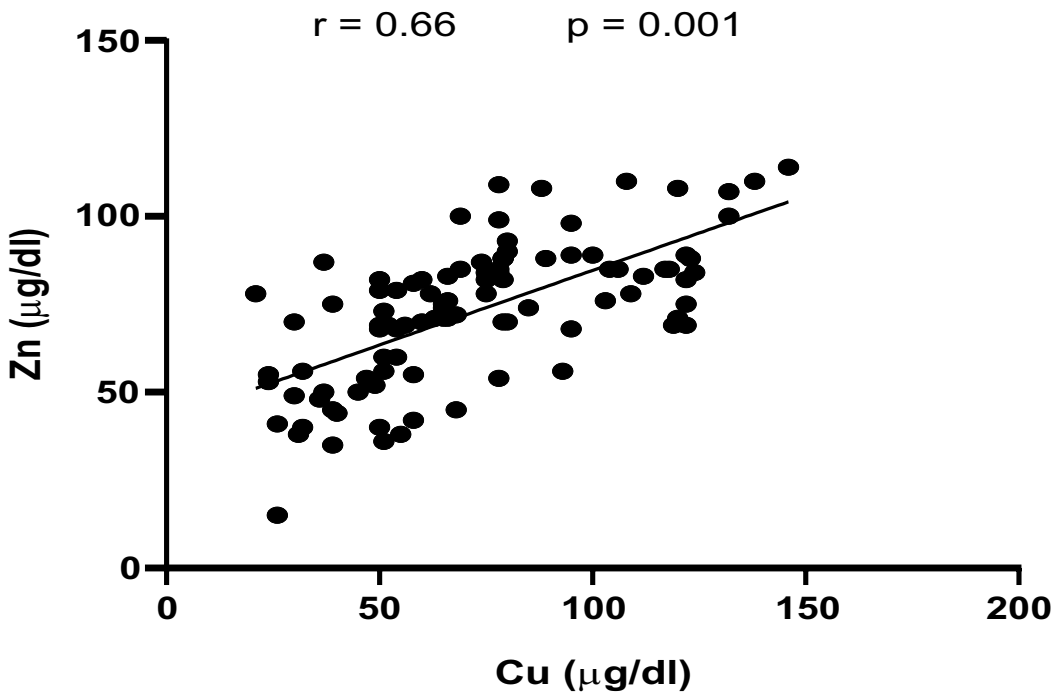

Figure 1: Scatter plot showing an association between copper and zinc

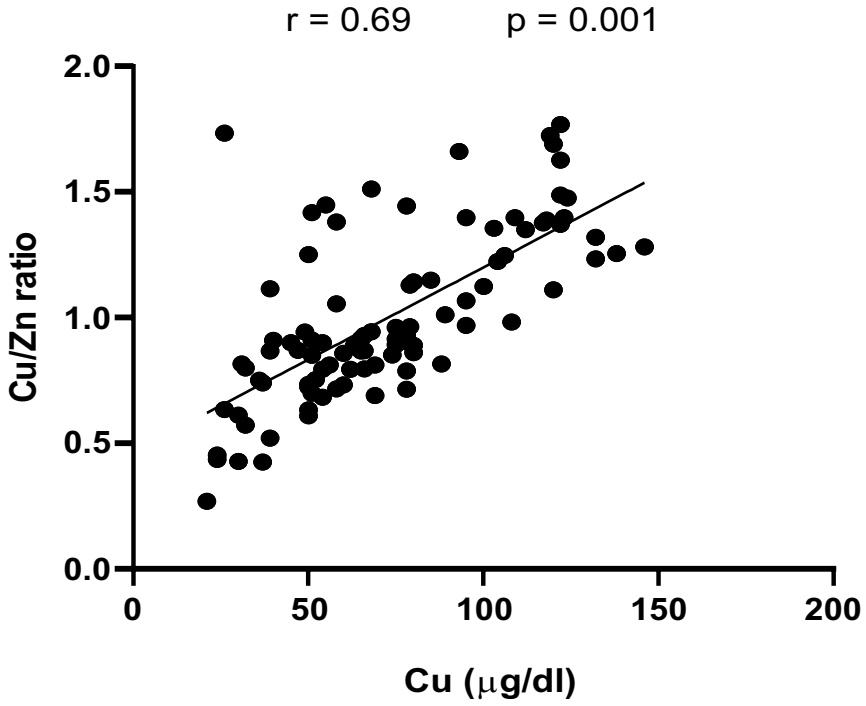

Figure 2: Scatter plot showing an association between copper/zinc ratio and copper

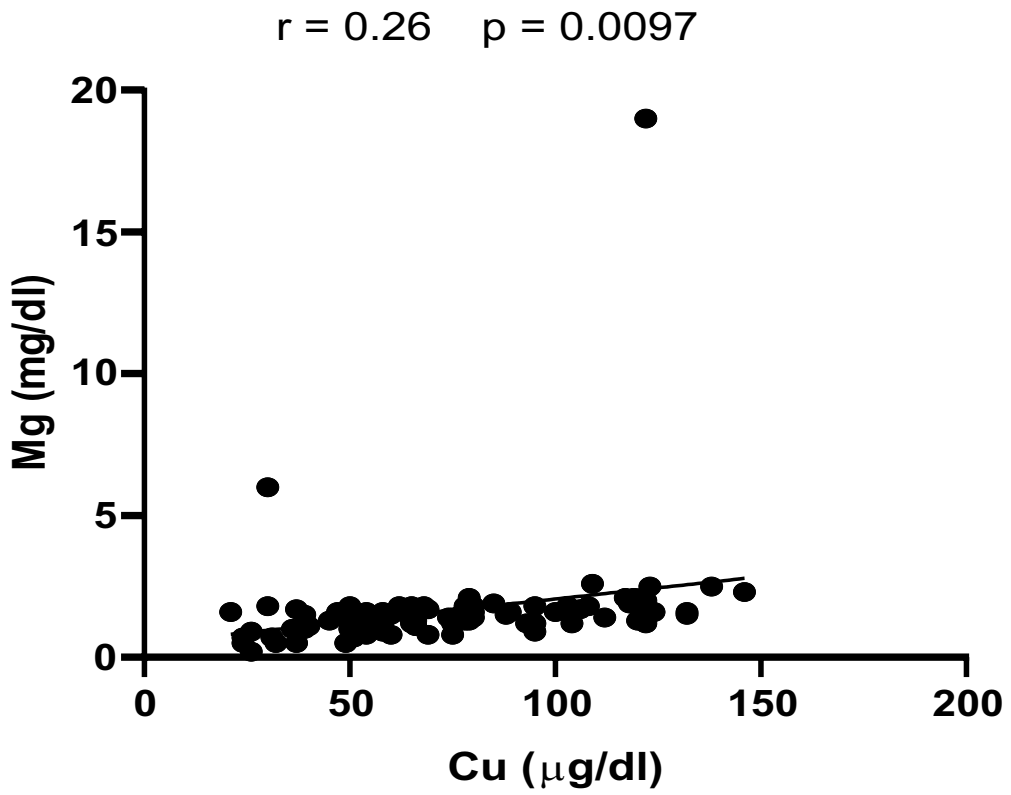

Figure 3: Scatter plot showing an association between magnesium and copper

Discussion:

This study showed that serum testosterone levels are significantly lower (P<0.001) in male sickle cell patients compared to male controls and was even significantly lower (p<0.001) in vaso occlusive crisis, when sickle cell patients in steady state and vaso occlusive crisis were compared. This finding is similar to work done by Parshed *et al*. (1994) which indicated a significantly lower testosterone levels in male patients with homozygous sickle cell (SS) disease attributable to either abnormalities of the hypothalamus - pituitary axis or primary testicular failure. The low-level serum testosterone may reflect hypoganadism secondary to hypopituitarism (Davis *et al*., 1989). Hypopituitarism in HbSS disease may result from intravascular thrombosis and pituitary infarction. This study showed that serum testosterone in 100 sickle cell patients assayed were non-significant (p <0.746) of the ages of the patient and is similar to the work done by Abudu *et al*. (2011), were there is no statistically significant difference in the serum testosterone level when the data were stratified by age group. Also, this study also showed that the serum copper, zinc and magnesium levels based on age of the sickle cell patients were non-significant (Cu = p < 0.48, Zn = p < 0.743; Mg = p < 0.566) of the respective age of the patients. Most likely because they are affected by several pathophysiological factors in HbSS patients irrespective of age. Copper to zinc ratio was also statistically non-significant (p < 0.517) of the ages of HbSS patients. In this study, serum copper was significantly lower (p < 0.001) compared to controls. And it is similar to the study carried out by Garba *et al*. (2016) and Arinola *et al*. (2008) where low level of copper has been reported in sickle cell anaemia patients. However, when sickle cell patients in steady state and vaso occlusive crisis was evaluated, copper was significantly higher (p < 0.001) in vaso- occlusive crisis, thus, agreeing with the study carried out by Bot *et al*. (2013) and Nnodim *et al*. (2014) which reported a significantly elevated level of copper in sickle cell patients. A study carried out by Emokpae *et*

*al. (*2019) also similarly revealed that serum copper is significantly elevated in sickle cell patients with vaso-occlusive crisis than those in steady clinical state of sickle cell disease. When sickle cell patients in steady state and vaso occlusive crisis was evaluated, zinc was non-significant ($p < 0.494$). This agrees with the work done by Garba *et al. (*2016), which indicated that sickle cell anaemia patients present with significantly lower serum zinc levels than control group. This study is also in agreement with a study conducted by Emokpae *et al*. (2019), carried out on sickle cell patients, revealed that the low serum zinc levels in SCD patients is attributed to increased urinary zinc loss, adverse effects of hydroxyurea used in management of the patients (Idonije *et al*., 2011) and increased demands. Chronic haemolysis leads to loss of Zinc from red blood cells, an important storage site for zinc and increased consumption due to increased oxidative stress and redox imbalance in SCA (Temiye *et al*., 2011). In this study, the serum copper to zinc ratio in sickle cell patients were significantly lower ($p < 0.001$) than that of controls and showed non-significance ($p < 0.206$) when sickle cell patients in steady state and vaso- occlusive crisis were evaluated. However, it disagrees with a study carried out by Antwi – Boasiako *et al*. (2019) and Canellas *et al*. (2012), which reported that copper to zinc ratio are significantly higher in sickle cell patients. The lower levels of serum zinc in sickle cell patients accounted for this high ratio. They stated that the ratio was even higher in patients with HbSS in the vaso-occlusive crises. This may partly be a result of frequent inflammation and associated intravascular haemolysis in these patients. Thus, the serum copper to zinc ratio could give a better predictive diagnosis of oxidative stress in these patients than copper or zinc status alone. In this study, serum magnesium level of sickle cell anaemia patients was significantly lower ($p < 0.007$) than that of controls and was also significantly lower ($p < 0.001$) in patient on vaso occlusive crisis, when sickle cell patients on steady state and vaso- occlusive crisis were evaluated. Our finding agrees with the study done by Garba *et al*. (2016), that there was a significantly low level of serum magnesium in sickle cell anaemia. And it also agrees with the study done by Zehtabchi *et al*. (2004), that low levels of magnesium were reported in a group of 74 SCD patients compared to levels observed in 61 subjects with normal haemoglobin. They reported that the participant with HbSS had significantly lower levels of serum magnesium compared with healthy ethnicity matched and Caucasian controls.

Conclusion:

The outcome of this study shows that SCD is aggravated in male sickle cell patients due to low levels of testosterone, copper, zinc and magnesium. The low testosterone level is likely responsible for delayed onset puberty and impairment of sexual development and activities, observed in male sickle cell patients. Low levels of mineral elements observed could be responsible for severe cases of anaemia, frail appearance and possible fatality experienced in sickle cell clinics in Benin City. Copper to zinc ratio in male sickle cell patients was low, and maybe accountable for the low RBC oxidative stress in these patients.

Recommendation:

From the data obtained from this study the following recommendations are made:

1. There is a need to evaluate mineral elements regularly in the course of management of SCD patients.
2. Testosterone levels should be monitored regularly in the management of SCD patients.
3. Large sample size is recommended for future studies since the disease is on the increase.
4. Public enlightenment of SCD patients to purchase blood medications containing mineral elements.

Conflict of Interest Declaration: The data from this study has not been submitted to any pair-review journal and all the authors contributed significantly to the outcome of this work.

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