Serum levels of copper, zinc and disease severity scores in sickle cell disease patients in Benin City, Nigeria

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Abstract

Background: Micronutrient deficiency is recognized in sickle cell anaemia (SCA) but it is not known for certain whether changes in zinc, copper and copper-to-zinc ratio are associated with Sickle cell disease severity scores.

Objective: To compare serum levels of copper, zinc and copper-to-zinc ratio in SCA subjects with control group and correlate the variables with objective disease severity scores.

Methods: Serum copper and zinc were determined in 100 SCA patients and 50 controls using kits supplied by Centronic, Germany. Unpaired Students’ t-test was used to compare the variables between SCA patients in steady clinical state, vaso-occlusive crisis and controls, while Spearman correlation coefficient was used to associate the parameters with disease severity scores.

Results: Serum copper level was higher (P=0.008) in SCA patients than controls, while serum zinc level was lower (P<0.001) in SCA patients than controls. The copper/zinc ratio was higher (P<0.001) in SCA patients than controls. Significantly higher (P<0.001) copper and lower (P<0.001) zinc levels were observed in patients in vaso-occlusive crisis than in steady clinical state. Zinc correlated inversely (r=-0.2743; P=0.006) while copper-to-zinc ratio correlated positively with disease severity scores.

Conclusion: Copper-to-zinc ratio may be an indicator of disease severity in SCA patients.

Keywords: Copper/zinc ratio, disease severity score, sickle cell anaemia.

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Introduction

Sickle cell disease (SCD) is an inherited disorder of major health challenge in sub-Saharan African including Nigeria. The condition is characterized by haemolytic anaemia and periodic painful crisis as a result of occlusion of small blood vessels due to spontaneous intravascular red blood cells polymerization at reduced oxygen tension. The associated complications of SCD include growth retardation, impaired immune functions, acute chest syndrome, abdominal pain, proteinuria, increased oxidative stress and damage to cell membranes. Some authors have documented scoring indices with which to assess disease severity in subjects with Sickle cell anaemia (SCA), using parameters such as anaemia score, white blood cell count score, complication score and blood transfusion score. Zinc is involved in several cellular metabolism, plays a role in immune function, wound healing, protein DNA synthesis as well as cell division. It also plays a role in the maintenance of proper sense of taste and smell, supports growth and development. Zinc possesses anti-oxidant and anti-microbial properties and confers protection against accelerated ageing.

Copper is also an important micro-nutrient and is essential for maintaining the strength of the skin, blood vessel, epithelial and connective tissues. It plays a role in the
production of haemoglobin, myelin, melanin and proper functioning of thyroid gland. It also acts as both antioxidant and pro-oxidant. Both copper and zinc form part of the superoxide dismutase (Cu-Zn-SOD) antioxidant system. The enzyme plays important role of scavenging free radicals generated during the course of normal body metabolism. It was also observed that calcium binding to red blood cells membrane may be responsible for the formation of irreversible sickled cells and zinc acts as anti-sickling ion because it is able to antagonize calcium binding to red blood cell membrane. Sickle cell disease is a disorder that is characterized by increased oxidative stress and lipid peroxidation. Low antioxidant status may predisposes the subjects to vaso-occlusive crisis. We previously reported lower levels of serum copper and zinc in SCA patients which were associated with higher demand of antioxidants for tissue repair as a result of proteinuria, inflammation. Okocha et al had evaluated the levels of some micronutrients and disease severity score in patients with SCD in Eastern Nigeria. Other authors also reported on the relationship between painful crisis and serum zinc level in subjects with SCA. The authors observed that it is not known for certain if zinc deficiency in SCA is associated with Sickle cell disease severity score. An insignificant relationship between serum zinc level and disease severity was reported from University College Hospital, Ibadan, Nigeria. The authors reported that even though the level of zinc in the patients was significantly lower than controls, no correlation with disease severity score was observed. The limitation of the previous studies was small samples which makes it difficult to draw concrete inference. This study was designed to compare serum levels of copper, zinc and copper-to-zinc ratio in SCA subjects with control group and to correlate the variables with disease severity score.

Patients and methods
Selection of study participants
This is a case control study of SCA patients conducted at Specialist Hospital, Benin City, Nigeria. One hundred (100) confirmed SCA patients (74 in steady clinical state and 26 with painful crisis) were consecutively recruited while 50 age and sex matched individuals with normal haemoglobin (HbAA) were used as controls. In this study, SCA patients on steady clinical state were defined as subjects who were apparently well without evidence of recent infection, bone pain or other illnesses for at least 4 weeks and have not had blood transfusion within the last 3 months while painful crisis referred to subjects who had pains at the time of enrolment or within 48 hours before enrolment in any of the limbs. The study participants were aged 4-20 years, 53 males and 47 females while the control group was aged 4-21 years made up of 25 males and 25 females. Severity Scoring Protocol was determined using the combination of anaemia, complications, white blood cell count and transfusion scores to generate an objective score of disease severity as follows:

### Anaemia score:
- Hb ≥ 10g/dl → 0
- Hb ≥ 8g/d < 10g/dl → 1
- Hb ≥ 6 < 8g/dl → 2
- Hb ≥ 4 < 6g/dl → 3
- Hb < 4g/dl → 4

### Complications score:
- Each complication was scored 1 except
  - Nephropathy → 2
  - Stroke → 2

### White Cell Count score:
- Count < 9 × 10^3 → 0
- Count ≥ 9 < 11 × 10^3 → 1
- Count ≥ 11 < 15 × 10^3 → 2
- Count ≥ 15 × 10^3 → 3

### Transfusion score:
- Life Transfusion Rate = Total Number of pints of Blood / Age
- Transfusion rate was approximated to the nearest whole number.

Then the mean score of a combination of various scores represents the severity score. Disease severity was scored as:
- Mild (< 3)
- Moderate (≥ 3 ≤ 5)
- Severe (≥ 5)

The minimum and maximum disease severity scores were 2 and 6.

Inclusion criteria: The SCA subjects who were not on any zinc and copper containing medications, had not been transfused with blood or blood products within the last 3 months, none-had any symptom or signs of infec-
tions such as fever, acute respiratory infection and diarrhea were included. The control subjects had no evidence of chronic diseases including protein energy malnutrition. The haemoglobin phenotype of all subjects was determined by haemoglobin electrophoresis using cellulose acetate paper and Tris buffer.

**Exclusion criteria:** Subjects excluded were those on zinc containing medications or transfused with blood or blood products in the previous 3 months before recruitment into the study.

**Ethical consideration:** The study protocol was reviewed and approved by the ethics committee of the Edo state Ministry of Health with code HM.1208/II/160; dated 27th March 2017) and all participants gave informed consent before blood specimen was collected.

**Specimen collection and analytical methods:**
Five millilitres (mL) of venous blood was obtained aseptically and 2mL dispensed into EDTA containing tube for full blood count while 3mL was dispensed into a zinc and copper free plain container which was previously washed after soaking in 10% nitric acid for 24 hours and rinsed three times with deionized water. The blood was allowed to clot at room temperature and centrifuged at 3000rpm for 5 minutes. The serum was separated into another copper/zinc free tube. The serum was stored at -200C (for a maximum of one month) until analyzed. Serum copper, zinc were assayed by colorimetric method using kits supplied by Centronic, Germany while copper/zinc ratio was calculated by dividing the value of copper with zinc value. Commercially available control sera were included in the assay to ensure accuracy of analyses.

**Statistical analysis:** The results obtained are presented as mean± standard error of the mean (SEM) and were analyzed using a statistical software package (SPSS version 20, IBM, IL, USA). Students’ t-test was used to compare the levels of copper, zinc and copper/zinc ratio between SCD patients in steady clinical state, vaso-occlusive crisis and controls. The measured parameters were correlated with disease severity scores using Spearman’s correlation coefficient. A p≤0.05 was considered as significant.

**Results**
Table 1 shows the comparison of measured variables between sickle cell disease patients (Hb SS) and controls (Hb AA). Serum copper level was significantly higher (P=0.008) in sickle cell disease patients than controls, while serum zinc levels was significantly lower (P< 0.001) in sickle cell disease patients than controls. Similarly copper/zinc ratio was significantly higher (P< 0.001) in sickle cell disease patients than controls.

**Table 1:** Comparison of measured variables between sickle cell disease patients (Hb SS) and control subjects with normal haemoglobin (Hb AA) (Mean± SEM)

| Measured variables       | Sickle cell anaemia (HbSS) n=100 | Control subjects (HbA A) n=50 | P-value |
|---------------------------|---------------------------------|-------------------------------|---------|
| Number of males           | 53                              | 25                            |         |
| Number of females         | 47                              | 25                            |         |
| Age (Years)               | 18.60±0.5                       | 19.00±0.6                     | NS      |
| Serum copper (µg/dL)      | 112.10±2.36                     | 102.60±1.58                   | 0.008   |
| Serum zinc (µg/dL)        | 40.50±1.78                      | 54.60±1.23                    | 0.001   |
| Copper/zinc ratio         | 3.35±0.16                       | 1.93±0.05                     | 0.001   |

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Table 2 shows the comparison of measured variables between sickle cell disease patients in clinical steady state (HbSS) and controls (HbAA). Serum copper level was insignificantly higher (P=0.621) in sickle cell disease patients in clinical steady state than controls, while serum zinc level was significantly lower (P=0.002) in sickle cell disease patients in clinical steady state than controls. Similarly copper/zinc ratio was significantly higher (P<0.001) in sickle cell disease patients in clinical steady state than controls.

**Table 2:** Comparison of measured variables between sickle cell disease patients (HbSS) in steady clinical state and control subjects with normal haemoglobin (HbAA) (Mean± SEM).

| Parameters                  | Sickle cell disease subjects in steady clinical state (n=74) | Control subjects (n=50) | P-value |
|-----------------------------|---------------------------------------------------------------|-------------------------|---------|
| Serum copper (μg/dL)        | 105.80 ± 2.46                                                 | 102.60 ± 1.585          | 0.621   |
| Serum zinc (μg/dL)          | 46.26 ± 1.986                                                 | 54.60 ± 1.237           | 0.002   |
| Copper/Zinc ratio           | 2.57 ± 0.1069                                                 | 1.94 ± 0.0516           | 0.001   |

Table 3 shows the comparison of measured variables between sickle cell disease patients in clinical steady state (HbSS) and vaso-occlusive crisis. Serum copper level was significantly lower (P<0.001) in sickle cell disease patients in clinical steady state than vaso-occlusive crisis, while serum zinc levels was significantly higher (P<0.001) in sickle cell disease patients in clinical steady state than vaso-occlusive crisis. Similarly copper/zinc ratio was significantly lower (P<0.001) in sickle cell disease patients in clinical steady state than vaso-occlusive crisis.

**Table 3:** Comparison of measured variables between sickle cell disease patients in steady clinical state and vaso-occlusive crisis (Mean± SEM)

| Measured Parameters          | Sickle cell disease patients in steady clinical state (n=74) | Sickle cell disease patients in painful crisis (n=26) | P-value |
|------------------------------|---------------------------------------------------------------|------------------------------------------------------|---------|
| Serum copper (μg/dL)         | 105.80 ± 2.46                                                 | 131.10 ± 4.251                                      | 0.001   |
| Serum zinc (μg/dL)           | 46.26 ± 1.986                                                 | 24.15 ± 0.943                                       | 0.001   |
| Copper/Zinc Ratio            | 2.57 ± 0.1069                                                 | 5.59 ± 0.249                                        | 0.001   |

Serum zinc levels correlated negatively (r= -0.274; p=0.006) while copper-to-zinc ratio correlated positively(r=0.235; p=0.019) with disease severity score in SCD patients. There was however no significant correlation between serum copper levels with disease severity score.

Table 5 shows the proportion of SCD patients with mild, moderate and severe disease severity scores and the concentrations of the measured variables. The distribution indicates 20 mild, 05 moderate and 03 severe based on the scoring protocol.
Table 4: Correlation of measured variables against disease severity scores in sickle cell disease patients (Mean± SEM)

| Correlation                          | R-value | P-value |
|--------------------------------------|---------|---------|
| Serum copper and Disease severity score | 0.062   | 0.537   |
| Serum zinc and Disease severity score  | -0.274  | 0.006   |
| Copper/zinc ratio and Disease severity score | 0.235   | 0.019   |

Table 5: Sickle cell disease severity score categories

| Severity Scores | Number of subjects | Serum Copper (μg/dL) levels | Serum Zinc(μg/dL) levels | Copper/Zinc ratio |
|-----------------|--------------------|-----------------------------|--------------------------|-------------------|
| Mild (< 3)      | 20                 | 118.01±1.20                 | 32.80±0.91                | 3.59±0.31         |
| Moderate (>3 ≤ 5) | 05                | 130.02±0.30                 | 28.10±0.30                | 4.62±0.23         |
| Severe(> 5)     | 03                 | 135.01±0.10                 | 22.20±0.20                | 6.00±0.05         |

Discussion
Sickle cell anaemia is a disorder characterized by haemolysis, subnormal immune status, inflammation and increased susceptibility to infections and vaso-occlusive crisis. Available evidence suggests that zinc supplementation could ameliorates vaso-occlusive crisis but it is not clear whether zinc and copper levels are associated with sickle cell disease severity scores. This study was therefore conducted to correlate serum levels of zinc, copper and copper/zinc ratio with sickle cell disease severity scores in sickle cell disease patients in both steady clinical state and vaso-occlusive crisis.

Serum copper level was significantly higher (P=0.008) in sickle cell disease patients than controls, while serum zinc level was significantly lower (P<0.001) in sickle cell disease patients than control. Hence, copper/zinc ratio was significantly higher (P<0.001) in sickle cell disease patients than controls. This study also shows a significantly higher (P<0.001) copper level in patients with vaso-occlusive crisis than those in steady clinical state of sickle cell disease. The zinc level was significantly lower (P<0.001) leading to a significantly higher (P<0.001) copper/zinc ratio in vaso-occlusive crisis than steady clinical state of sickle cell disease patients. Zinc was inversely associated (r=-0.2743; P=0.006) with disease severity scores, while copper-to-zinc ratio correlated positively with disease severity score.

The observed lower level of serum zinc and higher level of copper in this study is partially consistent with previous studies. Zinc and copper are co-factors of copper/zinc superoxide dismutase (Cu/Zn-SOD) and important antioxidant enzyme that catalyze the breakdown (dismutase) of superoxide radicals into molecular oxygen or hydrogen peroxide. This helps to prevent or repair the damage caused by free radicals and also to regulate redox-sensitive signaling pathways. Deficiency of any of the two metals has been associated with reduced antioxidant activity and may potentially subject the SCD patients to increased risk of acquiring associated complications and growth retardation.

Some authors who previously reported low serum zinc levels in SCD patients had attributed such low levels to increased urinary loss, adverse effects of hydroxyurea used in the management of the patients and increased demands. Okocha et al observed that serum copper and zinc levels were lower in both SCD patients and control subjects with normal haemoglobin. Our report however shows that the mean level of zinc in the control subjects was within normal reference range (50-150µg/dL). We observed a higher level of serum copper and an insignificant correlation between serum copper and disease severity score which contradicts that reported by Okocha et al, the authors observed that serum copper was significantly associated with disease severity score in a group of SCD patients. The observed higher level of se-
rum copper in our study was supported by some authors who suggested that high intracellular copper may potentially induce red cell haemolysis by its ability to produce superoxide radicals in the presence of thiol group. Intracellular haemolysis releases free haemoglobin and the free haemoglobin mop-up and depletes nitric oxide (a potent vasodilator) and plays a role in the dysregulation of arginine metabolism. This may lead to several associated complications such as vaso-occlusive crisis, sickle cell nephropathy and acute chest syndrome. A contradicting report from Saudi Arabia states that serum copper and zinc levels were normal and not different from those with normal haemoglobin individuals. The lower level of serum zinc observed in SCA subjects than control group may be attributed to several causes such as chronic haemolysis leading to loss of zinc from red blood cells, an important storage site for zinc, excessive urinary loss and increased consumption due to increased oxidative stress and redox imbalance in SCA. The observed association between serum zinc and disease severity did not agree with previous studies. The authors observed that serum zinc level in SCA patients, although significantly lower than control subjects with normal haemoglobin did not correlate with various disease severity scores. The authors stated that small sample size used in that study may be a limitation to drawing concrete inference and therefore suggested further studies that involve larger population size. The further lowering of serum zinc in SCD patients with vaso-occlusive crisis is consistent with previous studies. It was suggested that SCA subjects who are severely zinc deficient may be more susceptible to vaso-occlusive crisis. In addition, SCA patients with frequent vaso-occlusive crisis may not feed well thereby making nutritional deficiencies worse. The observed association between serum zinc and disease severity score is inconsistent with that reported by Garba et al. The authors observed no significant difference in the levels of serum copper and zinc between SCA patients in steady clinical state and vaso-occlusive crisis. But in our study, the distribution of some participants spanned across mild, moderate to severe with decreasing concentration of zinc and increasing copper/zinc ratio.

Trace metals play vital role in several biological systems through their actions as activators or inhibitors, thus competing with other biomolecules for binding site, influencing the permeability of membrane. It was suggested that copper-to-zinc ratio may be more valuable indicator of state of disease in affected individuals. In SCD, serum copper level is inversely proportional to zinc and this inverse relationship precipitates the generation of reactive oxygen radicals thus exacerbating associated complications. The low zinc and high copper levels are responsible for the higher copper-to-zinc ratio which correlated with disease severity score in SCA patients. In conclusion, low serum zinc, high copper and copper-to-zinc ratio were observed in SCA patients than control. The further lowering and increases in these variables were observed in patients with vaso-occlusive crisis. Serum zinc correlated negatively while copper-to-zinc ratio correlated positively with disease severity scores while copper shows no significant correlation with disease severity scores. This finding indicates that demand for zinc utilization in SCD patient increases with disease severity. Evaluation of copper/zinc ratio in the management of SCD patient is suggested and may serve as reference for diagnosing deficiencies and supplementation at a daily dose of zinc has been advocated. Copper-to-zinc ratio may be used as an indicator of disease severity in SCA patients.

Limitations: The inability to determine the dietary intake because of difficulty of the subjects to recall accurately and quantity of meals consumed as well as unavailability of socioeconomic status, urinary zinc level which is a better assessment of zinc loses are limitations in this study.

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Conflict of interest
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