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Original Article

Micronutrient levels and antioxidant status in pediatric cerebral palsy patients

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ABSTRACT

Background: Micronutrients such as coper, zinc and selenium are essential to the functioning of the nervous system and their deficiencies are a critical concern for cerebral palsy children. The aim of this study was to investigate micronutrient levels and antioxidant status in pediatric cerebral palsy patients and compare them to neurologically normal children in Accra, Ghana. **Method:** Twenty-nine patients with cerebral palsy (0-14 years) and 17 age-bracket matched neurological normal controls were recruited. Three milliliters of venous blood was drawn from each participant into an EDTA tube and transferred on ice to the laboratory. Activity of the antioxidant enzyme superoxide dismutase (SOD) as well as micronutrient status for copper, zinc and selenium were analyzed. **Results:** About 86.2% of cases (n = 25/29) compared to 11.7% of controls (n= 2/17) had copper concentrations below the normal physiologic range; mean copper concentration was significantly lower in cases than in control. Serum zinc levels were normal in only 20.1% (n = 6/29) of cases, but in 59% (n = 10/17) of controls. Selenium concentration below normal physiologic range was recorded to be about 94% in both cases (n = 27/29) and controls (n = 16/17); but the mean selenium concentration was significantly higher in cases compared to control. Also, the mean SOD activity was significantly lower in cases than in controls. Moreover, negative correlation was observed between the age of children with cerebral palsy and SOD levels. **Conclusion:** Copper, zinc and selenium deficiencies contributed disproportionately to oxidative stress and this may be a common feature in Ghanaian children with cerebral palsy. There is the need for micronutrient supplementation.

KEY WORDS: Cerebral palsy, micronutrients, superoxide dismutase, trace elements

INTRODUCTION

Cerebral palsy (CP) is one of the most common life-long developmental disabilities and affects approximately 1 per 500 live births [1, 2]. Children with cerebral palsy have difficulties with feeding due to excessive spillage, gastro-esophageal reflux, delayed gastric emptying and oral motor dysfunction resulting in poor nutritional status [3-6]. Suboptimal nutritional status could significantly affect increased cellular activity, causing intensified metabolic demand for nutrients and oxygen, and leading to the production of free radicals often in the form of reactive oxygen species (ROS). High levels of ROS play a prominent role in tissue damage which is deleterious when uncontrolled. Antioxidants are required for scavenging of ROS [7, 8]. Decreased levels of antioxidants and increased production of ROS results in oxidative stress [9].

The assessment of nutrient status in CP children often relies on the quantification of mineral element concentrations in blood. These micronutrients including copper (Cu), zinc (Zn) and selenium (Se), also known as trace elements, are vital as cofactors for enzyme antioxidants such as superoxide dismutase (SOD) in the defense against ROS [10-12]. They are essential to the functioning of the nervous system and their deficiencies is a concern for cerebral palsy children. In literature, only few workers have studied the micronutrient and oxidative stress status of children with cerebral palsy. To our knowledge none of the studies in Ghana and West Africa have determined oxidative stress and micronutrient levels in patients with cerebral palsy vs neurologically normal children. We conducted this study to investigate oxidative stress as determined by SOD activity, and the status of Cu, Zn and Se micronutrients in a group of children with cerebral palsy and a control cohort without the disease condition.

MATERIALS AND METHODS

Study site

The study was conducted at the Neurology and Development Clinic, Department of Child Health, Korle-Bu Teaching Hospital; and laboratory analysis were carried out at the School of Biomedical and Allied Health Sciences Laboratory, College of Health Sciences, Accra, Ghana. Ethical clearance was granted by the Ethics and Protocol Review Committee, University of Ghana School of Biomedical and Allied Health Sciences. A written informed consent was obtained from all parents/guardians of study participants.

Participants

The study included 29 prospectively enrolled cerebral palsy patients (0-14 years) [1]. These were children with known diagnoses of cerebral palsy attending the Neurology and Development Clinic. Permission were sought from patients and guardians to include their wards in the study. After provision of informed consent, physician-assisted review of medical records of the cerebral palsy patients were conducted to retain those not selected by the exclusion criteria. The control cohort comprised 17 neurological normal children of similar age-brackets with cases and were selected based on similar nutritional status, i.e. weight within 10% of any case patient. Controls were healthy children visiting the clinic with parents or guardians.

Exclusion criteria

Cerebral palsy children with chronic liver disease or kidney disease were excluded from the study. Patients with neurological disorders including degenerative brain disease, encephalopathy due to well defined causes, inflammatory brain disease as well as those with intracranial space occupying lesions were excluded. Other cases exempted from the study were those with inflammatory conditions such as infections, surgical trauma, tissue infarctions and malignancies. Among the controls exclusion criteria included metabolic disorders and any chronic disease such as renal or gastric resection which may otherwise confound results. Clinical characteristics and demographic data were recorded.

Blood sampling

Three milliliters of venous blood was drawn from each participant into an EDTA (ethylenediaminetetraacetic acid) tube. The blood samples obtained were then transported on ice to the laboratory immediately for further evaluation. Blood samples were centrifuged at 4000 rpm for 10 min. The serum and plasma generated were pipetted into 1.5 ml microfuge tubes appropriately. The plasma was stored at -20°C until required. The serum was analyzed for Zn, Se and Cu concentrations for both the controls and the cases using a flame spectrophotometer. Table 1 shows the working conditions of the atomic absorption flame spectrophotometer.

Determination of micronutrient levels

Atomic absorption flame spectrophotometer is an instrument which quantitatively measures the concentrations of elements present in a liquid sample. It utilizes the principle that elements in the gaseous phase absorb light at very specific wavelengths. This gives the technique excellent specificity and detection limits. The sample is drawn into a flame where it is ionized in the gaseous phase. Light of a specific wavelength is shone through the flame, and the absorption of this light is proportional to the concentration of the elements. The analytical conditions of the assays are listed in Table 1.

Sample preparation prior to atomic absorption spectrometry and assay digestion of the samples was carried out to get rid of the organic component leaving only trace elements to be analyzed. One milliliter of serum samples was accurately weighed into the teflon vessels of a microwave digester (Milestone ETHOS 900; Shelton, CT, USA). Three milliliters of 69% HNO_3 and 0.5 ml of 40% H_2O_2 were added to each vessel containing the sample. The vessels were swirled gently to mix well and fitted vertically into the microwave digester and digested for 25 min. Once digestion was completed, the solution containing the samples was cooled down in a water bath for 20 min to reduce the high temperature and pressure build up within the vessels. The digestate was then transferred quantitatively into a volumetric flask and diluted with 20 ml deionized water. A blank was prepared in a similar fashion but without the analyte. All the samples were analyzed using Varian 240FS (Agilent Technologies; Santa Clara, CA, USA) flame atomic absorption spectrometer. To ensure reliability during digestion and sample preparation, blank samples were also digested along with each set of samples and subsequently analyzed for appropriate elements through the same procedure. Standards of high purity metals for atomic spectroscopy for the various assays were prepared from Spectrascan® single-element standards (Teknolab AS, Kungsbacka, Sweden).

Table 1. Technical data of Varian 240FS flame atomic absorption spectrometer

Element	Serum physiologic range (mg/l)*	Wavelength (nm)	Lamp current (ma)	Slit width (nm)	Fuel gas	Oxidant support
Zinc	7 -12	213.9	5	1	Acetylene	Air
Copper	0.7 - 1.5	324.7	4	0.5	Acetylene	Air
Selenium	7 -15	196	10	1	Acetylene	Nitrous oxide

Physiologic normal ranges according to Kratz *et al* [15]

Determination of superoxide dismutase activity

The red cells obtained were used to determine activity of the antioxidant enzyme SOD in the samples using a commercial assay kit. The manufacturer's instruction was followed. Briefly, the red cells were obtained and diluted in five dilutions with icy deionized water which lysed the red cells after being spun at 6000 rpm for 20 min. Five-hundred microliters of the supernatant was pipetted into 1.5 ml eppendorf tubes and was stored in -20°C being analyzed. The sample buffer was prepared by obtaining 2 ml of buffer provided in the kit against 18 ml of deionized water for 96 wells and was stored at 4°C until use. The assay buffer was prepared by diluting 3 ml of buffer provided in the kit against 27 ml of deionized water for 96 wells and stored at 4°C until use. The SOD standard wells contained 200 μl of diluted radical detector and 10 μl of standard provided in the kit per well. Sample (1.5 μl) was pipetted against 150 μl to make 1 in 100 dilutions with sample buffer and this dilution was used in the preceding steps. The diluted radical detector (200 μl) was pipetted into the sample wells and 10 μl of the diluted sample was added. The reaction was then initiated by adding xanthine oxidase to all the wells of both standard and sample. The precise time of starting the reaction was noted. The 96-well plate was shaken slightly for a few seconds to mix and was sealed and incubated for 20 min. The absorbance of the samples were read at 450 nm using a plate reader.

Statistical analysis

Data obtained was cleaned and imported from Microsoft excel to STATA (version 11.0) software. Data was summarized as means ± standard deviations for continuous variables. After checking for normality using the Shapiro-Wilk test, the independent t-test was used in determining significant differences in the mean levels of Cu, Se and Zn between controls and cases. Pearson correlations were also performed for associations. Chi-square was used to compare proportions between 2 categorical variables. All tests were two-sided and a P value less than 0.05 was interpreted as significant.

RESULTS

A total of 46 children were recruited and evaluated, including 29 cerebral palsy cases and 17 control subjects. Females were 51.7% and males 48.3%. The mean age of cerebral palsy cases was 8.3 ± 5.2 (SD, standard deviation) and those of controls was 9.2 ± 4.6 years (range, 0-14 years for both groups). Age and sex distribution differed in both groups: most cases belonged to the age group of 0-5 years and majority were females, controls comprised largely of children aged 6-8 years and were mostly males.

Table 2. Comparison of mean levels of copper, selenium and zinc among cases and controls

Age/years		n	Mean	P-value	95% CI
Copper (mg/l)					
0-5	Cases	23	0.57 ± 0.82	0.31	-0.807 – 0.687
	Control	2	1.13 ± 0.78		
6-8	Cases	3	0.16 ± 0.28	0.038	-1.039 – -0.040
	Control	14	0.7 ± 0.38		
>9	Cases	3	0.76 ± 1.06	-	-
	Control	1	1.49 ± *inf*		
Overall	Cases	29	0.49 ± 0.72	0.0042	
	Controls	17	1.11 ± 0.58		
Zinc (mg/l)					
0-5	Cases	23	3.3 ± 1.09	0.006	-4.747 - -1.492
	Control	2	6.42 ± 0.22		
6-8	Cases	3	6.73 ± 2.78	0.381	-1.841 – 4.541
	Control	14	5.38 ± 2.28		
>9	Cases	3	5.88 ± 3.32	-	-
	Control	1	6.99 ± *inf*		
Overall	Cases	29	5.33 ± 2.39	0.144	
	Controls	17	6.26 ± 1.25		
Selenium (mg/l)					
0-5	Cases	23	3.31 ± 1.09	0.99	-1.262 – 1.626
	Control	2	3.31 ± 0.2		
6-8	Cases	3	4.06 ± 1.2	0.021	0.275 – 2.885
	Control	14	2.48 ± 0.92		
>9	Cases	3	4.89 ± 0.59	-	-
	Control	1	3.44 ± *inf*		
Overall	Cases	29	4.09 ± 0.96	0.0022	
	Controls	17	3.08 ± 1.12		

All P values were determined by t-test; **CI**, confidence interval; ***inf***, indeterminate.

Micronutrient levels

Table 2 compares the trace element levels among cerebral palsy patients and controls. In brief, the overall mean concentration of Cu was lower in cases than in controls (P = 0.0042) but that of Se was higher in cases compared to the control cohorts (P = 0.0022). However, the overall mean Zn concentration was comparable for both groups (P = 0.144).

We compared the data across age groups 0-5, 6-8 and > 9 years. Among children aged 6-8 years, mean Cu concentration was significantly lower (P = 0.038) in cerebral palsy cases than controls. Similarly, Zn level amongst cerebral palsy patients 0-5 years was lower (P = 0.006) than their controls. In contrast, serum concentration of Se was higher (P = 0.021) in 6-8 year cases than their controls.

There was no correlation between the age of cerebral palsy children and serum concentration of Cu (r = 0.416, P = 0.218), Zn (r = 0.599, P = 0.331) or Se (r = 0.274, P = 0.428). Likewise, no correlation was observed in the control cohort between age and the concentration of Cu (r = 0.831, P = 0.419), Zn (r = -0.327, P = 0.414) or Se (r = 0.216, P = 0.336). The results show that 86.2% of cases (n = 25 of 29) had Cu concentration below the normal physiologic range compared to controls (11.7%, n = 2 of 17, P = 0.001). Zinc levels were normal in only 20.1% (n = 6 of 29) of cerebral palsy patients, but in 59% (n = 10 of 17) of controls (P = 0.008). On the other hand, Se levels were below the normal physiological range in 94% of both cerebral palsy cases (n = 27 of 29) and controls (n = 16 of 17) (P = 0.186).

SOD activity levels

Table 3 shows the mean levels of SOD activity for patients with and without cerebral palsy. The overall mean SOD activity was significantly lower (P = 0.001) in cases than in controls. When SOD activity was compared across different age groups, it appeared to be lower in cases compared to controls for each category but the reductions did not achieve statistical significance (P > 0.05 for all comparisons). Meanwhile, there was significant negative correlation between the age of children with cerebral palsy and SOD levels (r = -0.361, P = 0.0194). However, no such correlation was observed between the age of the control cohorts and SOD activity (r = 0.0651, P = 0.0879).

DISCUSSION

The present study was conducted to determine the antioxidant status as measured by SOD enzyme activity and evaluate the serum concentrations of Cu, Zn and Se in children with cerebral palsy compared to neurologically normal controls. We observed that the mean concentrations of Cu and Zn showed a tendency to be lower in cases compared to controls and the difference was statistically significant within the age groups 0-5 years and 6-8 years for Zn and Cu, respectively. The results also demonstrated a range of nutritional imbalances with most cerebral palsy children displaying particular deficiencies in Cu and Zn below their physiological concentrations.

Micronutrients such as Cu and Zn have been implicated in the functioning of the nervous system [2, 13] and their deficiencies, as widely recorded in this study, is a critical concern for cerebral palsy children in our institute. For example, reductive adaptation which occurs when micronutrients are inadequate for the body's demands could lead to the impairment of cellular functions when allowed to progress over time; and the body's standard compensatory mechanisms may no longer be able to rectify the ensuing cell damage [11, 14].

The present study showed significantly lower SOD activity in cerebral palsy patients compared to their neurologically normal controls, and this observation negatively correlated with age. This result could be explained by the fact that SOD is a metalloenzyme whose activity in the blood is Cu- and Zn-dependent [15, 16]; these micronutrients were considerably deficient in the cerebral palsy children [17, 18]. It must be mentioned however that high daily Zn intake can inhibit intestinal absorption of Cu leading to Cu deficiency in the face of elevated serum Zn levels thereby exacerbating oxidative stress [17, 18]. In this study, none of the recruited children received daily Zn or Cu supplements. It is of note that among the case patients, age increase was associated with the reduction in SOD activity which may indicate oxidative stress. An interpretation for this finding may be that at the early years of life, the management of oxidative stress is done effectively by the physiological system of children, but as age increases the system depreciates in function leading to increase in ROS generation and oxidative stress. Indeed this observation is well corroborated by Schoendorfer et al [11], Gropper et al [16] and Klotz et al [12].

Table 3. Superoxide dismutase activity values for the cases and control

Age/years		n	SOD (U/l)	P-value	95% CI
0-5	Cases	23	459.22 ± 125.77	0.645	-188.224 -63.75
	Control	2	493.81 ± 49.66		
6-8	Cases	3	355.01 ± 50.82	0.215	-260.251 – 63.752
	Control	14	453.26 ± 126.77		
>9	Cases	3	293.36 ± 71.91	-	-
	Control	1	560.51 ± *inf*		
Overall	Cases	29	369.19 ± 82.83	0.001	
	Controls	17	502.53 ± 88.23		

CI, confidence interval; ***inf***, indeterminate

Meanwhile, mean Se concentrations reported in this study were significantly higher in cases than controls despite the fact that a large majority of the children participants (97%) exhibited Se deficiencies. This results, together with the finding of significantly low SOD activity in case patients, is in line with other reports which associate high serum Se concentrations with increased oxidative stress [13, 19, 20]. Our data, however, raises critical questions on the possibility of chronic Se deficiency in our children population as well as the potential contribution of gene-environment interactions towards this phenomenon [19].

There are some potential limitations of this study that must be discussed. First, it is of note that in an acute phase response (APR) which often occurs during inflammatory tissue conditions and is characterized by fluctuating levels of several proteins, trace elements may be severely redistributed without corresponding alterations in total body content. In such situations, trace element concentrations may not effectively reflect the true micronutrient status resulting in inaccurate measurements. Excluding children with known inflammatory conditions minimizes the effect of APR on a study. This was not always the case with our study controls, since their medical records were not available to us. Exclusion was based primarily on physician physical examinations and interviews. Second, the limited sampling size is also worth mentioning. This could partly explain our findings of non-significant differences in micronutrient concentrations between cases and controls within certain age groups. A more large-scale survey is likely to be more accurate with little bias for marginal imbalances in micronutrient concentrations.

In conclusion, copper, zinc and selenium deficiencies contribute disproportionately to oxidative stress and this may be a common feature in Ghanaian children with cerebral palsy. In the light of present findings outlined in this article, a further understanding of the multi-factorial relationship between micronutrients, oxidative stress and age will be essential in the development of diagnostic and therapeutic strategies for such children. Adjuvant micronutrients therapy should be seriously considered in the management of cerebral palsy patients to obviate complications that may arise from such nutritional insufficiencies.

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Conflict of Interest: None declared