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Multiply Biomarker in the Analysis of Zinc and Iron in Children in Ceres District of South Africa

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Abstract: The use of various non-invasive and invasive biomarkers has made trace elements analysis more acceptable in humans. The aim of this study was to evaluate the presence of trace elements (zinc and iron) in children using biomarkers; blood, hair and saliva. Trace element status of first grade learners was assessed in N256 children age 7-9 years, attending six primary schools in Ceres district. The influence of socioeconomic status of parent and growth indexes and other factors like; trace elements in commonly consumed foods, drinkable water and locally cultivated vegetables were also considered. Trace elements been investigated include iron and zinc. There was a significant (p<0.01) presence of trace elements in biomarkers considered; (hair, saliva and blood). The following results were obtained 95% of Hr_Zn 172 mg kg^{-1} are within reference (150-250 mg kg^{-1}), 85% of Hr_Fe 8.47 mg kg^{-1} are within reference (6-15 g kg^{-1}), also 52% of Sl_Zn 0.64 mg L^{-1} is within reference of 0.5-1.20 mg L^{-1} and 94% of Sl_Fe 1.06 gm L^{-1} is below reference of 1.52-5.72 mg L^{-1}). Similar results were observed in blood analysis were Sr_Fe in all parameters was 75% within standard reference and 63% of Sr_Zn are within standard reference. There was no significant correlation between both biomarkers. Comparing both trace elements, there was significant high percentage of both trace element in all parameters (p<0.01), except for Sl_Zn were there was a significant low concentration. The study has shown the presence of these trace elements (iron, zinc) in children hair, saliva and blood and not been influenced by either the socioeconomic status or the Anthropometric data as observed in this survey.

Key words: Zinc, iron, hair, saliva, serum, children, ceres

INTRODUCTION

Trace elements are components of food that are needed for growth, good health, blood synthesis, hormone structure, vitamin synthesis, enzyme formation, immune system development, strong resistance as well as their learning ability at school (Elmadfa and Meyer, 2010; Ramakrishnan et al., 2009; Krauss et al., 2000). However, not receiving sufficient amount of any could course biological disorder, resulting in certain degenerative conditions such as heart disease, tooth damage, obesity, high blood pressure, osteoporosis, scurvy, pellagra, rickets and low retentive memories in later life have been linked with poor or restricted intake of nutrients in early life and consuming excess of same can be toxic to the human system, as some can accumulate in the human body throughout the life time (Labadarios et al., 2001; Yaman, 2005; Litt et al., 2005). Any changes from normal can also be considered an index of excess or deficiency of these specific nutrients in the diet or as an indication of absorption contamination from the environment (Alberti-Fidanza et al., 2002).

Evaluation of trace elements in children using a biomarker is very importance due the inability of children to synthesis trace elements and they are needed for major biochemical activities to occur in the body (Loukopoulos et al., 2003a, b). Trace elements concentration and the acceptable quantity needed for normal biochemical activities are relative to body need (Zhang and Gladyshev, 2009) and any abnormality that will arise on the course of high nutritional demand resulting from consistent growth and immune response (Barany et al., 2002) need to be investigated.

The need for accurate evaluation of these elements in different organs is essential if good health is to be achieved. Techniques for evaluating these levels include invasive and non-invasive methods. Invasive methods mostly requiring blood and fluid extraction are deemed most accurate reflection of status by the scientific community. Non-invasive methods include analyses of hair, urine or saliva samples. Although, the latter methods have definite advantages such as cost and viability, it still lacks adequate evidence towards validity and reliability as a method to assess trace element status (Gmoshinskii et al., 2006; Romero et al., 2002).

The use of single technique in ascertaining the status of zinc and iron in children is considered inadequate (Alonso *et al.*, 2004; Muthayya *et al.*, 2009; Al-Samarrai *et al.*, 2008) and this study intend to investigate both invasive and non-invasive techniques and the correlation between zinc and iron in both methods. Other factors that might influence the concentration of zinc and iron in the children were also investigated such as food, water (Abulude *et al.*, 2006) and cultivated vegetable (Edeogu *et al.*, 2007) and also investigated was anthropometric status of the children, socioeconomic status.

The important of developing effective biomarker to monitor the levels of zinc and iron in these children is synonymous with the role they play for instance; iron plays a significant role in assisting the human body in combating infections. Iron deficiency is the most common nutritional deficiency in Africa, Asia and most part of the developing world; affecting as much as 66-80% of the world's population (Andang'o *et al.*, 2007; Nwafia *et al.*, 2006). Iron deficiency is the leading nutritional cause of anemia (Allen and Casterline-Sabel, 2001). Those particularly at risk has always been children, pregnant women, people with mal-absorption problems and infants and young children who are especially vulnerable to iron deficiency because of their rapid growth and increased physiological demands for iron (Eneroth *et al.*, 2011; Quihui-Cota *et al.*, 2010; Andang'o *et al.*, 2007).

Zinc is an essential trace element involved in a variety of biological functions (Florescu *et al.*, 2009) responsible for more than 300 metalloenzyme, such as lactate dehydrogenase, alkaline phosphatase and alcohol dehydrogenase (Liuzzi *et al.*, 2001; Pfaffl and Windisch, 2003). The highly proliferative immune system is reliant on Zn-dependant proteins involve in general cellular functions such as replication, transcription, signal transduction and useful in wound healing, fighting skin problems such as acne, boils and sore throats (Wellinghausen *et al.*, 1997). Zinc is involved in the production, storage and secretion of insulin and is necessary for growth hormone. The importance of Zn in cell physiology is related mainly to its intracellular involvement into enzyme catalysis, protein structure, protein-protein interactions and protein oligonucleotide interactions (Lichten and Cousins, 2009). It maintains the body's alkaline balance and helps in normal tissue function. Zinc also aids in the digestion and metabolism of phosphorus.

This analytical survey is a baseline research designed to evaluate the presence of trace elements (zinc and iron) in children using both invasive and non-invasive parameters; blood, hair and saliva as to ascertain the usefulness of multiple biomarkers in trace elements analysis. Also the ability to have a biomarker that will best identify this abnormality as they arise due to poverty, poor hygiene, malaria, kwashiorkor and malnutrition, to enhance the life expectance of this children (Ahluwalia, 2002).

MATERIALS AND METHODS

Study design: Present study is designed to assess the levels of iron and zinc in hair, saliva (noninvasive) and blood (invasive). These techniques are widely used for the determination of trace elements in matrices, especially biological materials. The analysis of all trace elements in grade one learners in primary schools in Ceres district of the Western Cape, took place within two weeks and over two academic years of 2003 and 2004.

Prior to data collection, parents of the children under investigation were asked through questioner, if they had any direct contact with objects that might increase their venerability to trace elements contamination. Where, the answer was a yes, the samples collected were discarded and not included in the analyses.

Study population and data collection: The study sample was selected randomly from each school, using random number tables, with proportionate representation of each school. A total number of 265 learners were included in this study. Following informed consent, 62% of those asked to participate, signed consent forms.

Sample and data collection took place during school hours over a period of one week towards the end of each year. Samples were prepared for analyses within one week of sample collection.

Determination of iron and zinc in hair and saliva samples by conventional Aqua Regia digestion method: The determination of iron, zinc in hair and saliva, using the conventional aqua regia digestion procedure, consists of dissolving of samples in a 3:1 mixture of HCl and HNO$_3$ and digested on a hotplate for 3 h (Nieuwenhuize *et al.*, 1991). A photometric method was used in analyzing the digested samples using an atomic absorption spectrophotometer (AAS) (Unicam AAS Type solar) (Vercoutere *et al.*, 1995; Abramovitch *et al.*, 2003).

Hair: Approximately 0.5 g of hair, from the back of the head close to the neck, was obtained from each learner using a sterilized stainless steel scissors. The scissors were cleaned with ethanol after each hair collection.

Hair samples were washed with non-ionic detergent and rinsed with distilled water, oven dried for 4 days at

60-70°C and stored in an airtight plastic bag. 0.25 g well-mixed dried hair was weighed into a beaker and digested in 12 mL of aqua regia ($^1/_3$ HNO_3 and $^3/_4$HCl) acid, heated in a heated Gerhardt (Trace metal digestion units, DIN 38414) digestion block. The maximum digestion temperature was 120°C and to avoid loss of materials each beaker had a glass lid. Digestion continued until a clear colourless solution was obtained. Each sample took 2-3 h to digest. The clear solution obtained was allowed to cool, filtered with Whatman No. 42 paper and diluted to a final volume of 100 mL with doubly deionised distilled water (DDW) (Moore and Chapman, 1986; Ogboko *et al.*, 2009). This solution was stored in a plastic container until analysis using AAS was performed.

Saliva: At least 5.0 mL mixed saliva was collected from the learners into a detergent washed polypropylene vial by direct collection. The samples were checked for food and blood or nasal discharge contamination and contaminated samples were discarded. A total of 4 saliva samples that did not have matching hair samples were discarded. The mixed saliva was then frozen and stored in a freezer at 0 to -4°C.

Prior to sample preparation, the saliva samples were defrosted and allowed to equilibrate to room temperature before being rechecked for any trace of contaminants. Five milliliter of saliva was then measured into a beaker and 20 mL of 2% nitric acid (HNO_3) was added. This solution was filtered with Whatman no. 42 filter paper into a volumetric flask and diluted to a final volume of 100 mL with DDW (Moore and Chapman, 1986; Ogboko *et al.*, 2009). The 100 mL solution was then stored in a plastic container until analysis with an AAS for zinc was done. The levels of zinc in hair and saliva were determined using the AAS.

Blood: Five milliliter of whole venous blood was collected in a zinc free heparinized tube. This process was carried out by a trained community staff nurse. Through the process care was taken to avoid any health risk situation that will endanger the children or the nurse through blood contamination, infections or psychological situation. All blood samples collected were adequately marked and labelled. The blood samples were then stored in coolers under ice bags at 0 to -4°C, for usually <24 h (Tietz, 1994; Bruns and Boyd, 2010). The ferrous ion is immediately complexed with the FerroZine Iron Reagent "Beckman Coulter, Inc, 1998-2006". Atomic absorption spectroscopy analysis was used for Zn concentration.

All children had data for Hb (hemoglobin), MCV (mean cell volume). Values for transferrin saturation, ferritin, TfR and body iron were available for all children.

A total of 236 infants had complete data for the measures used to determine iron status in NHANES II or III (i.e., Hb, MCV, transferrin saturation and ferritin) (Looker *et al.*, 1997; Bruns and Boyd, 2010). Missing data were due to technical problems, such as trouble obtaining sufficient blood, samples with CRPC conc. >10 mg L^{-1} and ethical exceptions.

Precautions: Blood specimen storage and stability: Tubes of blood were kept closed at all times in a vertical, stopper-up position. Serum was physically separated from contact with cells as soon as possible. A maximum limit of two hours from the time of collection is needed for the assay to expire if not used (National Committee for Clinical Laboratory Standards, 1990).

Exclusion and inclusive criteria: Children with CRP concentration greater than 10 mg L^{-1} were excluded while those with CRP concentration less than 10 mg L^{-1} were included. The CRP concentration was used to include participants as a sign of apparent healthy state and to exclude participants as a sign of inflammation and ill health. The 265 pupils met the criteria of = abnormal iron measure.

Ethical considerations: The Senate Research committee of the University of the Western Cape provided ethical approval for this study (SHD of 2004/6). The participation of learners was voluntary following informed consent by parents or guardians. The participants were free to terminate participation at their convenience. Confidentiality of the data collected and subsequent findings were assured by using only code numbers for each participant.

Statistical analysis: The data were analyzed using SAS version 8.12 (SAS, 1999). The results are presented as mean, standard deviation and Pearson Correlation Coefficient between selenium in hair and saliva, also manganese in hair and saliva. The p-values <0.01 were considered statistically significant.

RESULTS

Sixty-two percent of the study samples consented to participation. The number represents 256 of the total number of 426 grade one learners over the study period (Table 1) with a male: female ratio 1:1. The mean age was 7.72 years. The average weight and height of the children were 21.27 kg and 118.67cm, respectively. The median household income contributors were 2 persons and that of income was R250 - R999 per month (Table 2).

Table 1: Participants data one: Number of participant and their percentages over the two phases

Phase one	Date	Learners	Selected	Consent	Response rate (%)	Ration of girls/boys (M/f)
1	November. 2003	544	200	115	60.0	43/57
2	September 2004	688	226	150	64.5	54/46
Total		1232	426	265	62.3	49/51
						Ratio 1:1

Table 2: Demographic and Socio-economic data of learners

Phase one	Date	Age of participants years	Weight (kg)	Height (cm)	Family members contributing to household income (*avg. No. of person)	Family average wage (**income per month)
1	November 2003	7.60	20.46	118.71	(2-3)	(R250-R3000)
2	September 2004	7.84	22.48	118.62	(2-3)	(R250-R3000)
Total		7.72	21.47	118.66	(2-3)	(R250-R999)

*No. of people contributing to household income, **Average family wages per month in Rands is (R 250-R3000) per month

Table 3: Average levels of trace elements in hair and saliva, percentages and references

Trace element	No. of children (N)	Trace element (T/E)	Ref.** standard	Within	Below	Above	SD
Hr_Fe in mg kg^{-1}	193	8.47	6-15	86	10	4	0.99
Hr_Zn in mg kg^{-1}	193	172.08	150-250	3	95	2	0.44
Sl_Fe in mg L^{-1}	247	1.06	1.52-5.72	5	94	1	0.49
Sl_Zn in mg L^{-1}	249	0.64	0.5-1.20	45	52	3	0.39

*Hr_ Fe: Hair iron, Hr_Zn: Hair Zn, Sl_Fe: Saliva iron, Sl_Zn: Saliva zinc, T/E: Trace elements index concentration, N: No. of children involved in each analysis and (**)Ref range (Assaf and chung, 1984)

Table 4: Percentages of iron and zinc in serum and references

Parameters	Mean±SD	**Standard reference range for category of participants	Below	Within	Above	SEM
Serum iron (μmol L^{-1}) (n = 236)	13.45±5.19	9.5-21.3	20	75	5	0.34
sTfR (mg L^{-1}) (n = 235)	1.83±0.67	0.8-2.3	24	75	1	0.04
Ferritin (μg L^{-1}) (n = 235)	30.11±15.52	20-100	24	75	1	1.01
HB (g dL^{-1}) (n = 232)	12.53±1.10	11.5-15 5	5	95	0	0.07
MCV (ft) (n = 232)	83.10±4.42	77-95	15	85	0	0.29
Sr_Zn (mg L^{-1}) (n = 235)	18.4±0.58	7.0-22.0 μmol L^{-1}	30	63	7	0.16

sTfR: Soluble transferrin receptor, Hb: Hemoglobin, MCV: Mass cell volume, Sr_ Zn: Serum zinc, **Ref Std (Beard, 2003)

Table 3 shows the total number of samples collected from each participant, the average levels of zinc and iron in the hair and saliva samples and their reference values. The average concentration of all elements in hair include; Hr_Zn 172.08 mg kg^{-1} with a standard reference of 150-250 mg kg^{-1} and 95% of samples are below the reference, Hr_Fe is 8.47 mg kg^{-1} with the reference 6-15 mg kg^{-1} and 86% of samples are within the reference. The saliva showed a contrary result to that of hair with Sl_Fe 1.06 mg L^{-1} and the reference 1.52-5.72 mg L^{-1}, 94% of values are below the reference, similar results can be found with Sl_Zn 0.64 mg L^{-1} and reference 0.5-1.20 mg L^{-1} with 52% of samples below the reference.

When compared with the standard value of less than 1 mg kg^{-1} in hair and contrary result was also observed in saliva with most of the samples concentration are within reference value in both iron (Sl_Fe 1.06±0.03 mg L^{-1}) with the standard been Fe 1.52-5.72 mg L^{-1} and the concentration in Sl_Zn 0.64±0.39 mg L^{-1} was low when compared with standard at 0.5-1.20 mg L^{-1}.

The results of blood showed some similarities, in Iron the mean soluble transferrin receptor (mg L^{-1}) and mean serum ferritin of the participating children were within the range of the standard reference. However, 24, 75 and 1% of the children presented with values below, within and above the standard reference range in each case. This value did not show any significant drop in soluble transferrin value (p>0.05) (Table 4).

The mean haemoglobin (g dL^{-1}) was within the range of the standard reference but 5, 95% and none of the children presented with values below, within and above the standard reference range. There was no significant drop in haemoglobin (p>0.05) Table 4. The mean mass cell volume (ft) of the participating children was also within the range of the book reference, however, 15 and 85% of the children presented with values below and within the book reference range. There was no significant drop in MCV (p>0.05) (Table 4).

In comparing each biochemical indicator against serum iron using SAS (Pearson correlation coefficient),
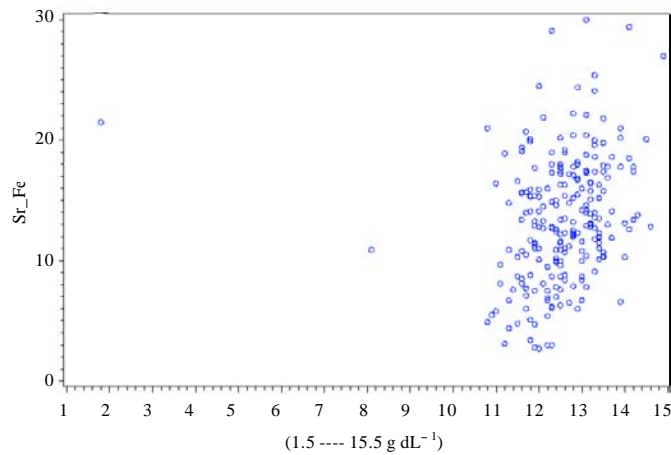
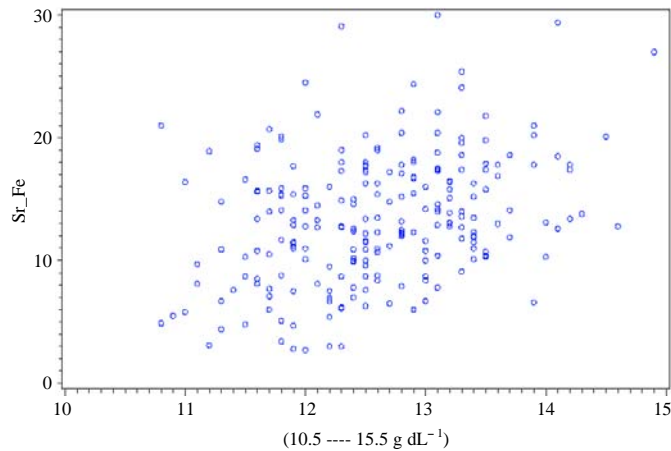Fig. 1: Variables showing some correlations between Sr_Fe and HB

Fig. 2: Correlation between Sr_Fe and HB (two outliers dropped)

there was a significant ($p<0.01$) correlation between serum iron and hemoglobin, as shown in Fig. 1 with few out-layers. Removing all out-layers (Fig. 2) a strong correlation could be seen, indicating marginal serum iron decrease in children investigated and the lack of consistence of serum iron decrease was also observed. Only one child in this study had a value above this cut off MCV and showed a corresponding level of low iron deficiency.

The results of serum Zn showed high percentage of 63% within reference mean 7% above and less than 30% below the reference mean. Comprehensively the level of serum Zn is considered high, meaning the children not deficient in Zn. There is no correlation between both parameters as shown in hair, saliva and blood serum, an indication that Zn and Fe can be identified using both biomarkers and also validate the finding that trace elements concentration differs in individual organs.

In other results there were high levels of zinc and iron found in other samples; water, cultivated vegetable and most commonly eaten foods) analysed, although these values were considered high but were within the book reference values (RDA, 1989). Other factors investigated are gender, weight and height, there were no significant difference within anthropometric data, as there were no difference between genders although there was a significant difference in the socio-economic status of children but these did not influence the levels of these elements; zinc and iron in all parameter; hair, saliva and blood of learners investigated.

DISCUSSION

Present study has investigated the possibility of using both biomarker (invasive and noninvasive) in analysing the presence of Zn and Fe in children. This can

be observed in research carried out by both Mark (2003) and Marlowe *et al.* (1983) in validating the use of both parameters for trace elements analysis. Most parameters provided relatively more information on levels of each trace element either its exposure or an implication of lack or excess of one trace element being able to interfere with the metabolic utilization of another element present as observed in the case of excess copper lowering zinc levels and can result in hair loss, insomnia, depression and schizophrenia and this can be found in different correlation factors found with and between parameters (Nielsen, 2000; Moses and Prabakaran, 2011).

Similar results can be observed in experiments on saliva, were more laboratories and clinic are relying on saliva for diagnosis and treatment of different diseases, due to the relative easy with which samples can be collected and analyzed and the relationship between salivary trace element concentrations and dietary intake can be observed in levels of elements found in both saliva and food analyzed and as reported in other literatures (Chavez, 1998; Watanabe *et al.*, 1994).

The cost-effectiveness of this method when compared to blood analysis in disadvantage communities has sparked our interest in the potential evaluation of zinc, iron in hair and saliva as an assessment index for trace elements (Barton, 2010) and using blood results as reference point.

The results of blood serum has shown a considerable high amount of Fe and Zn in samples and are within standard, they have complemented hair and saliva and can be related to other elements. Contrary to the potential Fe deficiency during childhood observed in most developing countries although not observed in this survey has multiple consequences like neurochemistry disorder; alteration of dopamine receptor (Beard, 2003) and decreased monoamine oxidize activities (Prpic-Majie *et al.*, 2003). This study has linked iron deficiency with lead toxicity and has provided evidence that these two conditions are related (Wright *et al.*, 2003; Kim *et al.*, 2003) thus the prevention of iron deficiency may represent a potential public health intervention for reducing lead exposure in human (Centres for Disease Control and Prevention, 2002) and this can be observed in levels of iron found in all parameters investigated.

Studies like these will enable researchers pre-empt the possible out come of subsequent findings but caution most be taken as concentration of an element in one parameter might not indicate the same with others (saliva, blood and hair), as can be noticed in this experiment and the one carried out by Wadaan and Mubarak (2009). Also, they might exhibit different concentrations even though the samples are collected at the same time (Ogboko *et al.*,

2009). The lack of standard procedure for hair sample collection, washing, treatment and analytical quality control protocol arouses considerable resistance towards the reliability of noninvasive techniques (Oxley *et al.*, 2008), although this has been complemented with results from saliva and blood. Hence, the multiple analyses like the use of hair, saliva and blood is required especially in non-urban communities like Ceres.

CONCLUSIONS

The reliability of single biomarker as indices of trace element status in children can be significantly enhanced when combined with other related biomarkers measured simultaneously (hair, saliva and blood). Hence, the use of both parameters in this experiment was encouraged. The interaction of trace elements in the body may have a dramatic impact on the utilization of other nutrients, the information on the levels of trace elements in biological tissues (saliva and hair) are scarce and some times inaccurate especially in essential elements and acceptable recommended levels varies with organs and in most times lacking in children.

However, careful observation and diagnostic examination of cases with abnormal results will establish the presence or evidence of some form of diseases such as inflammation, infection and malignancy undermining the health status of the children and this can be triggered by low nutrient intake resulting from feeding habit, poverty, poor nutrient intake or contamination from the environment. Trace element can be best evaluated through a simultaneous comparison of a variety of biochemical and physiological parameters of trace elements, Anorexia, short stature and low nutrient levels.

In most part of the world trace element deficient has been a major source of problem in children and critical evaluation of these elements will assist in diagnosing most food related illness but not finding easier and less expensive technique will hinders this process, especially in rural communities were poverty is high. The use of both invasive/noninvasive parameters as biomarkers of trace element evaluation as shown in this study will ameliorates this problem.

Relationship between invasive and noninvasive levels of trace element could be enhanced if there is equilibrium between the two components. However much is not known in terms of the relationship between both and trace element utilization in children. This could be made possible when more research is conducted using both parameters. However, there are needs for more studies to be carried out in this area as to validate this finding.

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