Original Research Article

Urinary calcium: a promising predictive biomarker for early recognition of environmental lead exposure in children

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ABSTRACT

Background: In the continuous search for accessible, reliable and sensitive biomarkers for early detection of environmental lead exposure, authors determined the interaction between blood lead level (BLL), the conventional marker of lead exposure, and the indices of calcium and bone metabolism in children.

Methods: This cross-sectional study involved 309 apparently healthy children from eight public primary schools in Ibadan, Nigeria who were classified as Elevated BLL (EBLL) and control based on standard cut-off for childhood BLL. BLL, serum Ca (iCa), phosphate, magnesium (Mg), 25-hydroxy-Vitamin D, alkaline phosphatase (ALP), urinary calcium (uCa) and urinary deoxypyridinoline (uDPD) were determined using AAS, HPLC and ELISA as appropriate. Bone-specific ALP (B-ALP) and ionized calcium (iCa) were calculated using standard formulae. Data analyses involved Student’s t-test, Pearson correlation and multivariate regression analysis. p<0.05 was considered statistically significant.

Results: BLL and 25-OH-Vitamin D levels were increased in EBLL (0.4±0.1 μmol/L and 60.1±10.7 mmol/L) compared with control (0.2±0.0 μmol/L and 55.1±14.3 mmol/L) p<0.05. No significant differences existed in the levels of ALP, B-ALP, uCa, uDPD, iCa, iCa, phosphate and Mg in both groups (p>0.05). BLL had significant positive correlation with uCa (r=0.176, p=0.002) (p<0.05) but no significant correlation with uDPD, ALP, B-ALP, iCa, iCa, phosphate, Mg and 25-OH-Vitamin D (p>0.05). BLL could be accounted for by uCa by applying the equation, BLL=0.329+0.324uCa.

Conclusions: Urinary calcium could be a promising predictive biomarker for early recognition of significant environmental lead exposure in children.

Keywords: Bone metabolism, Calcium metabolism, Environmental lead exposure

INTRODUCTION

Lead (Pb) is a versatile toxic metal which has become widely distributed and mobilized in the environment because of uncontrolled use. The resultant increased human exposure to this metal remains a serious problem particularly in developing countries and children are the population at greatest risk owing to frequent exposure and higher risk of nutritional deficiencies that may lead to increased lead absorption. Literature has shown that this
exposure constitutes 7% to 25% of the burden of diseases in Nigerian children as well as impacts heavy costs on both the health and educational sectors of the Nigerian economy.3

Lead enters the body of children mainly through food and fluid intake and less commonly, through inhalation and dermatological contact.5 Following its absorption into the blood stream, this metal is distributed to different organs and tissues where it has no known biological function.4

Blood Lead Level (BLL) is a frequently used biomarker of lead exposure but there has been controversy over its use and interpretation in early detection of lead toxicity.5 This therefore, necessitates a continuous search for a simple, accessible and reliable biomarker for early detection of lead exposure and its toxicity particularly in the paediatric population in chemical-laden, nutritional-deficit environments.

Nutrition is recognized as a very important factor that can modulate lead exposure or the risk of its toxic effects when exposure is held constant.5 Unlike lead, calcium (Ca) is a macronutrient with numerous physiological importance which include skeletal mineralization, muscle contraction, nerve impulse transmission among others.6 Serum calcium level is maintained under tight homeostatic control and directly and indirectly involves the influence of diverse roles played by parathyroid hormone (PTH), vitamin D and phosphate.7 Magnesium (Mg), may also be added to the group because it remotely plays a fundamental role in the release and action of PTH and controls many calcium channels, making it indispensable in Ca metabolism.8

The close and complex association between Pb and Ca metabolism has been recognized for years. Both elements have similar metabolic characteristics because of their similar biochemical nature as divalent cations.9 However, compared to Ca, Pb has larger ionic radius, greater electronegativity and irregular charge distribution in the electron cloud which allow it bind with greater affinity than Ca to the protein binding sites resulting in impairment of physiological regulatory function of Ca.9

Bone stores about 99% of the body’s calcium in the form of hydroxyapatite and experiences the greatest deposition of Pb for extended period of time.10,11 Lead has been reported to interfere with bone metabolism through multiple mechanisms that may include interferences with bone formation and bone resorption.12 Biomarkers of osteoblastic bone formation such as total alkaline phosphatase (ALP) and bone-specific alkaline phosphatase (B-ALP) and biomarkers of osteoclastic bone resorption which include urinary calcium (uCa) and urinary deoxypyridinoline (uDPD), have been reported to provide clinically useful evidence of the normal and pathological processes that reflect bone cell activities and can predict changes in bone-Pb dynamics long before changes in bone density are detected in children.13-16 In the continuous search for indices of early recognition of environmental lead exposure, the interaction between blood lead level and indices of calcium and bone metabolism has not been given serious considerations in developing countries, particularly, in children who experience constant bone re-modelling for continuous skeletal growth and higher risk of nutritional deficiency that might predispose to increased lead exposure.4 This study was therefore, designed to investigate the interaction between blood lead level and indices of calcium and bone metabolism and their possible use in the early recognition of environmental lead exposure and toxicity in children.

METHODS

Study area, study design and study population

This cross-sectional study involved 309 apparently healthy children (aged 8-10 years) selected from 8 public primary schools in Ibadan, south-west, Nigeria. The selection of the schools was based on multistage random sampling technique while the recruitment of the children from the schools was based on parental consent, child’s assent, physical presence at the day of sampling and residence at Ibadan for ≥5 years. Exclusion criteria included history of lead exposure requiring chelation therapy, evidence of sexual maturation (Tanner stage ≥2), anaemia, malnutrition, renal disease, liver disease, metabolic bone disease, intake of mineral supplements or medications that affect bone metabolism such as corticosteroids, anticonvulsants and diuretics.

The children were classified into two groups children with Elevated blood lead level (EBLL) (n=169, 83 boys and 86 girls) and children with acceptable blood lead level who also served as control (n= = 140, 71 boys and 69 girls). Elevated blood lead level was defined as BLL >5 µg/dL (>0.2415 µmol/L) while acceptable blood lead level was defined as ≤5 µg/dL (≤0.2415 µmol/L) based on the recommendation of Centre for Disease Control and Prevention cut-off for childhood blood lead level.17

Sampling

Data collection, blood and urine sampling of the participants were carried out on site during regular school days before the beginning of classes between 8 a.m and 10 a.m in the months of January and February to account for possible seasonal effects on lead levels and for other exposure and blood variables. A short-structured questionnaire was used to obtain information on calcium intake per day and hours of play/day.

Anthropometric indices such as height, body weight and body mass index (BMI) were carried out using standard procedures. Complete clinical assessment was carried out by a pediatrician using standard procedures to exclude pathology such as pallor, jaundice, cyanosis, peripheral oedema, peripheral lymphadenopathy, fluffy hair, angular
stomatitis, hepatomegaly, splenomegaly, enlarged kidneys, palpable masses, ascites, hernia, inconsistent of heart sound and unclear respiratory system.

Venous blood samples (5 mL) were collected from each child by a trained paediatric phlebotomist using pyrogen free graduated syringes. Two millilitre of the blood was dispensed into lithium heparin containing sample bottles for blood lead determination while 3 mL was dispensed into plain sample bottles to obtain serum for determinations of levels of 25-OH-Vitamin D, total Ca (tCa), phosphate, Mg, total protein, albumin and activities of ALP, Aspatate transaminase (AST), Alanine transaminase (ALT) and gamma glutamyl transaminase (GGT).

On the same day and time of blood collection, about 5 ml of mid-stream spot urine sample was collected from each child in clean polyethylene universal bottles for uDPD and uCa determinations. The obtained blood and urine samples were stored on ice and transferred from the point of collection to the laboratory either for immediate analysis and/or storage at -20°C. All urine samples were standardized with urinary creatinine same day of collection.

**Biochemical analysis**

Blood lead was analysed based on the method of Miller et al, using a graphite furnace atomic absorption spectrometer Perkin-Elmer A Analyst 800 with Zeeman-effect background correction (Norwalk, U.S.A.). Sample preparation involved simple dilution (1+9) with a matrix modifier which contained 0.5 %V/V Triton X-100, 0.2 %V/V 16 M nitric acid and 0.2 %m/V dibasic ammonium phosphate.

Urinary and serum calcium (tCa) was determined using the colorimetric method described by Sarkar and Chauhan (1967). Ionized calcium (iCa) was calculated by standardized, validated equation of Beelar MF et al.

iCa in mg/dL = S Ca (mg/dL) x 6 - S Pr (g/dL) x 1.2/S Pr (g/dL)+6.

Inorganic phosphate was determined based on colorimetric method of Henry RJ. Magnesium was estimated based on colorimetric end-point-xylidyl-blue method as described by Samie et al. 25-Hydroxy Vitamin D, the major representative of vitamin D status, was estimated by the method of Neyestani et al, as adapted in 25-OH Vitamin D3 HPLC assay kit (Eagle Bioscience Inc, Nashua, NH 3063, USA), using Waters 616/626 LC System (Young Lin, Seoul, South Korea). Total ALP activity was determined by the kinetic method of Hausamen et al. Bone-ALP was determined by the heat inactivation method of FitzGerald MX et al. Urinary DPD was determined using a solid phase ELISA kit supplied by Qayee-Bio, Shangai, China.

Haemoglobin concentration was estimated cyanmethemoglobin method of Drabkin DL et al. Total protein was estimated by the Biuret method of Reinhold JG. Albumin was estimated by the Bromocresol Green method of Doumas et al. Globulin was calculated as described by Jolles et al. Different colorimetric methods were used for the assessments of serum AST, ALT, GGT, creatinine and urea.

All reagents and standards used were of analytical grade. The samples were all analysed in one day in batches of 20. Results were only acceptable when data obtained fell within expected control limits (X±2SD). A mean recovery rate of >95% was obtained for each analyte after two determinations.

Data were using SPSS statistical software programme version 21.0 (SPSS Inc, Chicago, IL). Results were expressed as Mean±SD. Independent sample t-test was used to determine differences between EBLL group and control. Pearson’s product moment correlation analysis was used to evaluate the relationship between BLL (independent variable) and tCa, iCa, phosphate, Mg, 25-OH Vitamin D, uCa, uDPD, ALP, B-ALP as dependent variables.

Stepwise Multiple Regression Analysis was used to model cause-effect relationship between BLL (independent variable) and tCa, iCa, phosphate, Mg and 25-OH vitamin D, uCa, uDPD, ALP, B-ALP (dependent variables). P <0.05 was considered as statistically significant (two-tailed analysis).

**RESULTS**

There were no significant differences between the children with elevated blood lead level (EBLL) and control in terms of age, anthropometric indices, calcium intake per day and number of hours of both active and inactive play per day that could be confounders (p>0.05) (Table 1).

No significant differences also existed between both groups in terms of haemoglobin levels, serum protein levels, indices of liver and kidney functions that could also be confounders (p>0.05) (Table 2). Tables 1 and 2 suggest that children with EBLL and control were properly matched and therefore comparable.

The levels of 25-OH-vitamin D was significantly higher in children with EBLL compared with the control (p<0.05). However, the mean levels of total Ca, ionised Ca, inorganic phosphate, Mg, uCa, uDPD, ALP and B-ALP did not show any significant difference between children with EBLL and the control (p>0.05) (Table 3).

Blood lead level had significant positive correlation with uCa (r=0.176, p=0.002) (p<0.05) but did not show any significant correlation with uDPD, ALP, B-ALP, iCa,
Mg, and 25-OH-vitamin D (p>0.05) (Table 4).

Multiple regression analysis between blood lead level (independent variable) and Ca, iCa, phosphate, Mg and 25-OH-vitamin D, uCa, uDPD, ALP and B-ALP (dependent variables) showed that there was a significant positive relationship between BLL and uCa (Model Coefficient: (B=0.329, R=0.0162, β=0.324, p<0.001) (Table 5). However, BLL had no significant relationship with ionized Ca, total Ca, phosphate, Mg, 25-OH-vitamin D, ALP, B-ALP, uDPD (p>0.05) (Table 5).

The equation for the regression is given below;

\[ Y = B + \beta_1X_1 + \beta_2X_2 + \ldots \]

Where, \( Y = \text{BLL (independent variable)} \), \( X = \text{uCa (dependent variable)} \), \( B = \text{intercept} \), \( \beta = \text{Slope of each variable} \), \( R = \text{Slope} \), \( R^2 = \text{coefficient of determination} \).

BLL (µmol/L) = 0.329+0.324uCa

Where, \( R = 0.162, R^2 = 0.026, p < 0.001^* \).

### Table 1: Age, anthropometric indices, calcium intake and hours of play per day in children with elevated blood lead level and control.

| Indices                  | Participants with EBLL (N=169) | Controls (N=140) | t-value | P value |
|--------------------------|---------------------------------|------------------|---------|---------|
| Age (years)              | 8.6±1.6                         | 8.9±1.5          | -1.711  | 0.088   |
| Weight (kg)              | 23.8±4.8                        | 24.5±4.8         | -1.015  | 0.311   |
| Height (cm)              | 125.0±9.2                       | 126.7±10.1       | -1.285  | 0.200   |
| BMI (kg/m²)              | 15.1±1.5                        | 15.3±1.8         | -0.539  | 0.590   |
| Ca intake (mg/day)       | 775±116.0                       | 784±84.0         | -0.789  | 0.431   |
| Active play (hr/day)     | 4.8±0.4                         | 4.6±0.5          | 0.516   | 0.802   |
| Inactive play (hr/day)   | 3.0±0.3                         | 3.2±0.3          | 0.122   | 0.721   |

Results are presented as mean±standard deviation, EBLL=Elevated blood lead level, Ca=Calcium, BMI=Body Mass Index, hr=Hour.

### Table 2: Haemoglobin and serum protein levels, indices of liver and kidney function in children with elevated blood lead level and control.

| Indices                  | Participants with EBLL (N=169) | Controls (N=140) | t-value | P value |
|--------------------------|---------------------------------|------------------|---------|---------|
| Total protein (g/dL)     | 6.9±0.7                         | 7.0±0.5          | -0.768  | 0.443   |
| Albumin (g/dL)           | 4.5±0.4                         | 4.5±0.4          | -0.941  | 0.347   |
| Globulin (g/dL)          | 2.4±0.6                         | 2.4±0.5          | -0.256  | 0.799   |
| Hb (g/dL)                | 10.8±0.9                        | 10.9±1.0         | -0.830  | 0.407   |
| AST (U/L)                | 16.4±4.7                        | 11.1±4.9         | 0.745   | 0.457   |
| ALT (U/L)                | 2.2±1.3                         | 2.0±1.3          | 1.095   | 0.275   |
| GGT (U/L)                | 16.7±4.6                        | 17.4±4.5         | -1.063  | 0.289   |
| Urea (mg/dL)             | 22.5±7.5                        | 21.1±5.5         | 1.342   | 0.181   |
| Creatinine (mg/dL)       | 0.4±0.1                         | 0.4±0.3          | 0.465   | 0.643   |

Results are presented as mean±standard deviation, EBLL=Elevated blood lead level, Hb=Haemoglobin level, AST=Aspartate transaminase, ALT=Alanine transaminase, GGT=Gamma glutamine transaminase.

### Table 3: Indices of calcium and bone metabolism in children with elevated blood lead level and control.

| Indices                  | Participants with EBLL (N = 169) | Control (N = 140) | t-value | P value |
|--------------------------|----------------------------------|------------------|---------|---------|
| Total Ca (nmol/L)        | 2.4±0.2                          | 2.5±0.2          | -0.553  | 0.595   |
| Ionized Ca (mmol/L)      | 1.1±0.2                          | 1.1±0.1          | -0.334  | 0.739   |
| Phosphate (mmol/L)       | 1.6±2.4                          | 1.7±0.2          | -0.096  | 0.924   |
| Mg (mmol/L)              | 0.8±0.1                          | 0.8±0.3          | -1.553  | 0.122   |
| 25-OH-vit D (nmol/L)     | 60.1±16.7                        | 55.1±14.3        | 2.170   | 0.031*  |
| Urinary Ca               | 0.05±0.2                         | 0.04±0.1         | 1.000   | 0.318   |
| uDPD (µmol/mo/cr)        | 21.6±1.5                         | 20.2±1.1         | 0.516   | 0.606   |
| ALP (U/L)                | 188.9±47.8                       | 188.1±40.7       | 0.120   | 0.904   |
| B-ALP (U/L)              | 175.0±48.4                       | 174.0±40.9       | 0.155   | 0.877   |

Results are presented as Mean±standard deviation, EBLL = Elevated blood lead level,* = significant at p<0.05, Ca = Calcium, Mg = Magnesium, 25-OH-vitamin D = 25-Hydroxy-vitamin D, Ca = Calcium, uDPD = Urinary Deoxypyridinoline, ALP = Total Alkaline phosphate, B-ALP = bone-specific Alkaline phosphatase.
**DISCUSSION**

Environmental lead exposure and its toxicity remains a topic of substantial concern and interest particularly in developing countries.\(^1\) In the continuous search for indices of early recognition of environmental lead exposure and toxicity, the interaction between blood lead level (BLL) and indices of calcium and bone metabolism has not been given serious considerations particularly in children who experience constant bone re-modelling for continuous skeletal growth and higher risk of increased lead exposure.\(^4\)

This study tried to investigate the possible role of the combination of BLL, indices of calcium and bone metabolism in the early detection of lead exposure and toxicity in Children.\(^5\)

Out of the 309 participants in this study, 169 (54.7%) had elevated blood lead levels (EBLL) depicting BLL greater than the current CDC cut-off for acceptable limit for children.\(^5\) Since, BLL is a conventional marker of lead exposure, our observation demonstrated a high prevalence of increased lead exposure in the children in this environment. This increase in BLL also implies increased lead bioavailability and concomitant increase in lead deposition in the bone since lead substitutes for calcium in the hydroxyapatite.\(^12\)

The interactions between lead, calcium and vitamin D are complex.\(^35\) In this study, authors observed an increased vitamin D levels in children with EBLL compared with control but no difference in the serum concentrations of ionised calcium in children with EBLL and control.

The present finding of similar serum concentrations of ionised calcium in the children with EBLL and control probably reflects the tight regulation of this mineral and suggests that the observed increased vitamin D levels in children with EBLL is likely to be a counteracting outcome of a tendency to systemic hypocalcaemia which reflected as a substantial compensatory increase in vitamin D levels to keep serum calcium levels within normal range.

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**Table 4: Correlation of blood lead level with indices of calcium and bone metabolism in study participants.**

| Variables                  | Correlating Pair BLL (µmol/L) | r-value | P Value  |
|---------------------------|-------------------------------|---------|----------|
| Urinary Ca                |                               | 0.176   | 0.002**  |
| uDPD (µmol/mol/cr)       |                               | 0.088   | 0.128    |
| ALP (U/L)                 |                               | 0.053   | 0.356    |
| B-ALP (U/L)               |                               | 0.055   | 0.339    |
| Ionized Ca (mmol/L)      |                               | -0.009  | 0.877    |
| Total Ca (mmol/L)        |                               | 0.017   | 0.763    |
| Phosphate (mmol/L)       |                               | -0.016  | 0.785    |
| Mg (mmol/L)              |                               | -0.060  | 0.295    |
| 25-OH- Vit D (mmol/L)    |                               | 0.043   | 0.451    |

* = Significant at p < 0.05, ** = Significant at p < 0.01, BLL = blood lead level, Ca = Calcium, Mg = magnesium, 25-OH-VitD = 25-Hydroxy-vitamin D, ALP = Total Alkaline phosphatase, DPD = Deoxypyridinoline, ALP = Alkaline phosphate, B-ALP = Bone-specific Alkaline phosphatase.

**Table 5: Multiple regression between lead and indices of calcium and bone metabolism in study participants.**

| Variables                  | B     | T     | P      |
|---------------------------|-------|-------|--------|
| Constant                  | 0.329 | 38.095| <0.001*|
| Urinary Ca                | 0.324 | 2.825 | 0.005* |

Excluded variable

| Variables                  | B     | T     | P      |
|---------------------------|-------|-------|--------|
| uDPD (umol/molcr)         | 0.027 | 0.663 | 0.082  |
| Total ALP (U/L)           | 0.055 | 0.954 | 0.341  |
| Bone ALP (U/L)            | 0.058 | 1.001 | 0.318  |
| Ionised Ca (mmol/L)       | -0.002| -0.039| 0.969  |
| Total Ca (mmol/L)         | -0.002| -0.039| 0.969  |
| Phosphate (mmol/L)        | -0.047| -0.809| 0.419  |
| Mg (mmol/L)               | -0.088| -1.531| 0.127  |
| 25-OH-vit D (mmol/L)      | 0.063 | 1.084 | 0.279  |

* = Significance at p < 0.05, uCa = Urinary calcium, uDPD = Urinary Deoxypyridinoline, ALP = Alkaline phosphate, uCa = Urinary calcium creatinine ratio, Ca = Calcium, Mg = magnesium, 25-OH-vit D = 25-OH-vitamin D.
Present study result may further be explained by the similarities in intracellular transport between lead and calcium and the ability of lead to cause interferences in intracellular calcium metabolism independent of the physiological regulators of systemic calcium metabolism. Lead can enhance the abnormal activation of many calcium-binding proteins. Thus, the observed increased vitamin D levels may at least in part, have led to the synthesis of Calbindin-D, a calcium binding protein which binds lead with a greater affinity than calcium. The Pb-binding properties of Calbindin-D may also have resulted in vitamin D enhancement of Pb absorption leading to increased blood lead level, further toxicity and subsequent increased deposition in the bone. This may result to disruption of mineralization and increased bone resorption and possibly the increased Pb-induced Ca release from the storage in the bones. This observation possibly reflected in the urine due to the tight serum regulation of calcium. The significant strong positive relationship observed in this study between BLL and urinary calcium (uCa) may be based on the fact that both calcium and lead are reabsorbed in the same tubule segments and lead may compete with calcium for reabsorption. Lead has been reported to reduce the Ca-pump activity. Since lead lowers renal sodium-potassium-ATPase activity and much calcium reabsorption is sodium dependent, renal calcium reabsorption may be impaired with lead exposure resulting to hypercalcicuria as observed in this study. The significant linear relationship between BLL and uCa as indicated in the derived equation with the slope of 0.162, may suggests that 16.2% of the observed variation in BLL could be accounted for by uCa using the linear equation (BLL (μmol/L)=0.329+0.324uCa). This strong positive linear relationship between BLL and uCa from the derived equation appears to suggest that uCa, if measured regularly in children exposed to similar kind of lead toxicity, might act as a surrogate biomarker for the duration and level of lead exposure. Hence, uCa may be a promising early predictive biomarker of lead exposure and could be used as a rapid assessment tool for early detection of significant environmental lead exposure and toxicity in children.

The relationship between BLL and uCa level may also be an indication that children exposed to lead beyond the acceptable limit are at increased risk of bone resorption and demineralization which if sustained, may have some clinical consequences such as increased risk of bone disorders, later in life.

This understanding could provide a rationale for the development of more effective therapeutic approaches for the treatment of lead toxicity, a concerted effort to limit lead exposure as much as possible and a more proactive, implementable policy to curb this high level of lead exposure in Nigeria. These steps may avert future devastating consequences of lead exposure in Nigerian children as well as relieve the huge economic burden of this menace.

CONCLUSION

In conclusion, urinary calcium may be a promising early predictive biomarker of lead exposure and toxicity in children and could be used as a rapid assessment tool for the early detection of significant environmental lead exposure and toxicity in the paediatric population. Present study findings might have important implications for environmental policies, especially those designed to protect children’s health and prevent factors that are operative early in life.

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