Original Article

Decreased total and ionized calcium levels and haematological indices in occupational lead exposure as evidence of the endocrine disruptive effect of lead

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Abstract

The multisystem and prime environmental and occupational toxin, lead (Pb) is seldom included in the list of endocrine disruptors group like bisphenols A, B and F, nonylphenol, benzoquine, equiline etc.

One hundred and thirty-seven subjects consisting of 86 lead workers and 51 unexposed individuals (as controls) participated in the study. Dietary intake including dairy products and micronutrients as assessed by 24-hour dietary recall was similar between lead workers and controls. Calcium homeostasis and haematological indices were evaluated in all subjects.

Blood lead level was significantly higher in the lead workers than in controls (P<0.001). Total and ionized calcium levels were in contrast significantly decreased in lead workers compared with controls (P<0.01, P<0.001 respectively). Inorganic phosphate level though slightly raised compared to controls did not reach statistical significance (P>0.05).

The haematological indices, haemoglobin, haematocrit, and mean cell haemoglobin concentration like calcium levels were all significantly reduced (P<0.001) in all cases.

Semi-quantitative assessment of erythrocyte protoporphyrin was trace (±) in both lead workers and controls (i.e. similar). Serum copper level was significantly higher in Pb workers than in controls (P<0.005).

These decreases are consistent with a repression of the endocrine systems regulating both erythropoiesis and calcium homeostasis resident in the proximal convoluted tubule(PCT) of the kidney; the most vulnerable site to Pb damage.

Our findings therefore, appear to provide evidence or a reminder that Pb satifies the conditions defining EDCs and should be recognized as one, especially in developing countries where high environmental Pb and malnutrition co-exist and may magnify this effect.

Key Words: Bloodlead, endocrine disruptors, 1, 25-dihydroxycholecalciferol, serum calcium, haematological indices, erythropoietin

INTRODUCTION

Global concerns have been raised in recent years over the potential adverse endocrine effects that may arise from exposure to chemicals in the environment or work place. These chemicals have the potential to interfere with or alter the endocrine system in humans and wildlife.[1,2] Lead is an environmental chemical and one of the oldest occupational and environmental toxins known.[3-5] Lead in contrast to calcium is not an essential nutrient and has no established toxicity threshold concentration.[6] As a biochemical analogue of calcium, lead interferes with calcium metabolism and many of its physiological functions.[7-9] The principal mechanism of the perturbation of calcium metabolism by lead lies in the metabolic activation of the hormone 1, 25-dihydroxychole calciferol (calcitriol).[10] Recent studies indicate that low levels of lead exposure are correlated with kidney dysfunction among others.[6,11]

The kidney is also an endocrine organ producing erythropoietin (EPO), a glycoprotein (hormone) which regulates both steady-state and accelerated erythrocyte production in the proximal renal tubule (PRT)[12,13] where lead accumulates.[14] Others are rennin from the juxta glomerular apparatus (JGA). The kidney also elaborates l, a-hydroxylase enzyme also from the proximal convoluted tubule for the metabolism of the prohormone

25-OH cholecalciferol from the liver to the active hormone 1, 25-$(OH)_2$-cholecalciferol.[15] The cells lining the proximal tubules appear to be the tissue in the kidney most highly sensitive to Pb.[10] Anaemia is one of the most recognized clinical signs and symptoms of lead poisoning though the mechanism is multifactoral and the mechanism of which is not entirely clear,[16-18] surprisingly this ancient toxin (Pb) is often left out when endocrine disruptive chemicals (EDCs) such as bisphenol A, bisphenol B, bispherol F, benzophenone equilin, nonyphenol mixture etc and methods of their assays are discussed.[19,20] This is probably because lead has assumed a lower priority status in the developed countries[21] in contrast to the situation in most developing countries[22] where environmental lead is still a significant public health problem.[23] Moreover, lead is sufficiently prevalent at low level (no threshold value) to cause a number of metabolic aberrations including endocrine disruption. This study, a part of a larger study on the evaluation of the nutritional, metabolic and immune status presents evidence that lead is an endocrine disruptive substance which to the knowledge of the investigators has not been documented and appears relevant for the recognition of the continuing scientific and medical rationale for ameliorating plumbism.

MATERIALS AND METHODS

Chemicals and reagents

(a) Ammonium pyrollidinedithirocarbamate (APDC) and Methyllsobutylketone (MIBK) were obtained from Wako chemicals. (Wako Pure Chemicals Industries, Osaka, Japan). (b) Ethylacetate, American Chemicals Society (ACS) certified was obtained from Fisher Scientific (Fisher Scientific Fairlawn, NJ, USA). Methyllsobutylketone (MIBK) was obtained from Wako Chemicals (Wako Pure Chemicals Industries, Osaka, Japan/ (c) Triton-X100 was obtained from British Drug Houses (BDH) (BDH Chemicals Ltd, Poole, England).

Other common chemicals and acids such as hydrochloric acid, (HCl), nitric acid (HNO_3), trichloroacetic acid (TCA) acetic acid (CH_3 COOH) all of "analar" grade were obtained from BDH (BDH Chemicals Ltd, Poole, England).

Reagents and Diagnostic Kits

Total calcium and inorganic phosphate kits were obtained from Medical Analysis System (MAS) (MAS, Inc., Camarillo, USA).

Total bilirubin Kits was obtained from Miles Laboratories (Miles Inc., Diagnostic Division, Elkhart, Indiana, USA).

Total protein and albumin kits were obtained from Sigma Diagnostic (Sigma Diagnostic, St. Louis, Missouri, USA).

Hemoglobin kit was also obtained from Miles Laboratories (Miles Inc., Diagnostic Division, Elkhart, Indiana, USA).

Lead Standard Solution (spectrosol) was obtained from BDH (BDH Laboratory Supplies, Poole, England). Microhaematocrit tubes (heparinized) were obtained from Becton-Dickins on (Becton-Dickinson, NJ, USA).

Subjects

The subjects for this study are as previously described in our earlier report.[24] The subjects were all resident at the same altitude of which the current study comprises a component, but is briefly summarized here. One hundred and thirty-seven subjects involved in common lead-based occupations and unexposed individuals were selected for the study. Eighty-six (86) subjects were lead workers while 51 individuals who were not known to be either occupationally exposed nor involved in any lead dependent hobby served as controls. The subjects were all males and age and sex matched with Pb workers, nutrient intake including dairy fruit and vegetables intake was similar between both populations. This was ascertained by 24-hour dietary recall.

Samples collection

At least 15 ml of venous blood was collected from each subject at the antecubital fossa with minimal stasis using pyrogen free disposable needles and syringes (Becton-Dickinson, Dublin). Five mililitres of the venous blood was dispensed into hepanin vacutainer tubes (Becton-Dickinson, NJ, USA) for Pb assay while 2 mls were dispensed into EDTA tubes (Becton-Dickinson, NJ, USA) for haematological studies. The rest were dispensed into anticoagulant free vacutainer tubers to yield serum for other biochemical investigations. The sera were frozen at – 20° C until time for analysis.

Blood Lead Assay

Assay was performed according to the modified method previously described.[25] Briefly the method is based upon chellation technique, blood is haemolysed with 5% Triton X100 solution, the Pb is chellated with 2% APDC and extracted with MIBK. The organic solution containing Pb is analysed by flame atomic absorption spectrophotometry (AAS) AGW AES Model 200A (Analysengerate GMbH, Germany) at 283.3 nm.

There was strict adherence to clean trace element handling procedures. Quality control was ensured by recovery studies, use of pooled samples and duplicate analysis.

Assessment of Calcium homeostasis

The colourimetric method using cresophthalein complexone (CPC) employed in this study is based on the modified method of Baginsky et al[26] using the MAS kit (MAS, Inc. Camarillo, USA). The complex formed between calcium and cresolphthalein complexone in alkaline medium was measured spectrophotometrically in a spectronic 21D (uv-vis) (Milton Roy, Analytical products Division, Rochester, NY, USA). 8-hydroxyquinoline was added to the solution to minimize interference by other metallic ions especially magnesium.

Estimation of ionized calcium

Serum ionized calcium was estimated by employing the method of Mclean and Hastings[27] as adopted by Beeler and Catrou[28] using the following formula. Ionized calcium (mg/dl) =

$$\frac{\text{SCa (mg/dl) X 6} - \text{SPr (g/dl) X 0.33}}{\text{SPr (g/dl)} + 6}$$

were SCa = Serum calcium and
SPr = Serum protein

Inorganic Phosphate Assay

The method of Fiske and Subarrow[29] in a kit supplied by Sigma (Sigma Diagnostics, St. Louis Mo, USA) in turn modified by Martinek[30] was used. The modification by Martinek has improved the sensitivity and specificity of this method. The assay was performed as instructed by the manufacturers. Briefly supernatant fluid is obtained with TCA by removing protein and lipid phosphorus. The supernatant fluid reacts with ammonium molybdate in an acid solution to form phosphomolybdate. A mixture of sodium bisulphate, sodium sulphite and 1-amino-2-naphthol-4-sulphonic acid reduces the phosphomylybdenum blue complex.

Total protein and albumin assay

Total serum protein was determined by the classical biuret reaction using the Sigma Diagnostics total protein reagent kit (Sigma Diagnostic Co. St. Louis, Mo, USA). The assay was performed according to the method of Doumas et al[31] and read at 540nm in a 21 UVD Spectrophotometer (Milton Roy, Analytical products Division, Rochester, USA).

Albumin Assay

This was determined by using the Sigma bromocresol purple (BCP) reaction according to the modified method previously described by Pinnell and Northern.[32] The principal reaction being that serum albumin reacts with BCP to form a stable – blue – purple colour complex with an absorption maximum at 600nm and read in the same spectrophotometer as for total protein.

Determination of Haematological Indices

Haemoglobin (Hgb) was determined with a solid phase test strip impregnated with potassium ferricyanide using seralyser reflectance photometry, as employed in the Ames Seralyser Reflectance Photometer (Ames Coporation, Elkhart, USA). Essentially the reaction is based on the quantitation of Hgb in the form of methaemoglobin; oxidation of Fe^{2+} in Hgb to Fe^{3+} by potassium ferricyanide (K_3 Fe $_{(CN)}6$).

Determination of Haematocrit (HCt), Mean cell haemoglobin concentration (MCHC) and red cell morphology. These were performed according to standard procedures, as recommended by the British Committee for standards in Haematology.[33]

Erythrocyte protoporphyrin (EPP) level was semiqualitatively evaluated by the method of Varley et al.[34] Essentially 2 drops of whole boold from EDTA tubes were added to 2.5 ml – ether-ethyl acetate mixture (5:1 v/v) in a 10 x 160 mm glass test tube. This was thoroughly mixed and decanted. To the supernatant was added 0.5 ml, 3M HCl. This was examined under UV light, using model UVG-54 UV lamp (Ultraviolet products Inc., San Gabriel, Carlifornia, USA).

Total bilirubin determination

This was assayed by a modification of the classical Vanden Bergh reaction[35] as used by Rand and Pasqua.[36]

The test was performed with the seralyser (Miles, Diagnostic Division, Elkhart. The Strip is based on the reaction between bilirubin and a diasonium salt in the presence of dyphyline and p-toluene sulphonic acid. Resulting in azobilirubin, a red-purple substance. The test was performed as instructed in the Ames Technical Mannual (Miles, Diagnostics Division, Elkhart, Indiana, USA).

Detemination of Serum Copper

Copper was determined by the method of Osheim et al[37] by AAS using the same equipment as for Pb. Serum was diluted using ultra pure water (ASL, IITA, Ibadan, Nigeria), The solution was thoroughly mixed and analysed for Cu at 324.7nm. The principle of the reaction is essentially as described for Pb.

A commercial standard obtained from BDH Chemicals (BDH Chemicals Ltd, Pool,e, England) was prepared in 10% glycerol to ensure similar viscosity between serum and the aqueous standard used thus ensuring similar aspiration rate into the AAS flame.

Statistics

All data were initially analysed with the students t-test to determine if there were differences between exposed and the referent (control) groups. When significant difference was evident ($P<0.05$) the person's product moment correlation test was employed to determine correlation between lead and other variables. Further analyses were carried out for multiple comparisons between groups with multiple regression analysis.

Finally, the principal component analysis was used to assess for the degree of interaction between lead and other variables. Results were expressed as mean ± SEM.

RESULTS

Blood lead assay

This was significantly higher in the Pb workers than in controls ($P<0.001$) (Table 1). The levels in the control (occupa-

tionally unexposed) group was however much higher than currently accepted levels in unpolluted environments. The Pb level in the exposed group did not vary with duration of exposure. Neither did it vary with severity of exposure (see exposure category).

The haematological indices assessed in this study are shown in Table 2. Haematocrit, haemoglobin and mean cell haemoglobin concentration, MCHC were all significantly lower in lead workers than in controls ($P<0.001$) in all cases.

Erythrocyte morphology was similar in both Pb workers and controls (no hyperchromasia evident) (Table 2).

Erythrocyte protoporphyrin,EPP assessment was also similar in Pb workers and controls, present in trace amounts in both groups (Table 2).

Serum copper level, an important factor in erytropoiesis was sinificantly higher in Pb workers than in controls ($P<0.005$) (Table 2). Total bilirubin, a degradative product of protoporphyrin was significantly lower in Pb workers than in controls ($P<0.001$) (Table 2).

DISCUSSION

A number of disorders have been ascribed to the environmental and occupational toxicant lead. Though endocrine dysfunction has also been associated with this ubiquitous toxicant[38] such as its role in calcium homeostais through its impairment of calcitriol (1, 25-DHCC)[39] or impairment of erythropoietin synthesis[40] in the kidney, a principal target organ for Pb toxicity, Pb is seldomly referred to as an endocrine disruptive chemical. We report decreased total and ionised calcium levels as well as haematological indices as evidence of endocrine disruption in an occupational cohort exposed to Pb.

Table 1: Blood lead and indices of calcium heomeostasis

	Lead workers	Controls	t	P
Blood lead level (mmol/L)	2.72 ± 0.0.05	1.47 ± 0.07	18.91	<0.001
Total calcium (mmol/L)	2.22 ± 0.02	2.31 ± 0.02	2.6	<0.01
Ionized calcium (mmol/L)	0.88 ± 0.01	0.99 ± 0.01	6.67	<0.001
Inorganic Phos (mmol/L)	1.15 ± 0.00	1.09 ± 03	1.5	>0.05
Total protein (g/L)	82.0 ± 5.6	75.0 ± 0.09	1.05	>0.05
Albumins (g/l)	44 ± 0.40	44 ± 0.05	0.56	>0.05
Per cent (%) of subjects with total serum calcium level below 8 mg (2.0 mmol/L)	19.0	4.0	-	-

Table 2: Haematological indices in lead workers and controls

	Lead workers	Controls	t	P
Haematocrt (%/L)	41.0±0.33	43.0±0.41	3.77	<0.001
Haemoglobin Hg (g/L)	135.7±0.90	144.0±1.3	4.99	<0.001
MCHC (%)	33.0±0.33	34.0±0.18	4.53	<0.001
Erythrocyte Morphology	NO hyperchromasia seen	NO hyperchromasia seen	-	-
Erythrocyte protoporphyrin (EPP) Assessment	±(trace)	±(trace)	-	-
Serum Copper umol/L	118±3.40	104.0±3.07	3.06	<0.005
Total bilirubin level (umol/l)	13.26±0.51	17±1.02	3.35	.001

Classification according to exposure categorise (dose response) did not reveal any change.

The significantly elevated Pb level in the Pb workers was associated with decreases in total and ionized calcium levels. This decreased calcium level confirms earlier experimental and clinical reports and reflects perturbation of calcium metabolism.[15,41] Lead is a biochemical analogue of calcium, thus it interferes with calcium in several metabolic pathways. Of the several mechanisms that may lead to Pb-induced decreases in calcium levels the most widely accepted is the interference of Pb with the final metabolism of vitamin D to the active metabolite, calcitriol (1,25-DHCC), a hormone required for adequate calcium absorption.[10,24,42] The 19% compared with 4% of the Pb workers and controls respectively with serum calcium levels with 2.0 mm ul/L (8 mg/dl) appears to indicate the magnitude of impairment of calcium metabolism in Pb workers.

Though the mechanism of the reduction of ionized calcium is still not clearly understood it is not unlikely that parathyroid hormone (PTH) which has a more direct effect on ionized calcium was also perturbed by the elevated Pb level. Similarly, although the elevation of inorganic phosphate level is not statistically significant it may be considered as part evidence for a corresponding slightly depressed PTH level. Hyperparathyroidism is associated with phosphaturia resulting in reduced serum phosphate level (hypophosphataemia). Thus the level seen here may represent a case of early or borderline pathology of this edocrine system. Our results therefore, appear to indicate that Pb is an endocrine modulator and thus a candidate for the endocrine disruptors group. Other factors that could have contributed to altered calcium metabolism such as total protein and albumin were not significantly different between Pb workers and controls; excluding these in the pathogenesis of the disorder.

The haematological indices that were decreased in these workers suggesting subclinical or preclinical anaemia is consistent with earlier reports.[14,16-18] Though the mechanisms involved are not completely elucidated, elevated blood lead levels are associated with impaired haem synthesis[43-45] this is unlikely to be the explanation in this study as erythrocyte protoporphyrin activity was similar between Pb workers and controls. Additionally, we previously reported in our observations on the haemopoietic system in this cohort[42] that the haembiosynthetic pathway was uninhibited.

Moreover it has also been observed by earlier investigators that in Pb intoxication the absolute depression in haem synthesis is inconsequential and cannot explain the fall in a major haematological index, Hgb.[44,45] One of the most likely contributors to this decrement, and the currently most accepted major contributor to this reduction in haematological indices is impaired synthesis of erythropoietin.[14] This glycoprotein hormone regulates both the equilibrium and synthesis of 90% of erythrocytes. It is produced in the proximal renal tubule (PRT)[12,13] where lead accumulates.[10] This site coincidentally is also where the important calcium regulating hormone calcitriol (1, 25-$(OH)_2O_3$) is synhthesized.[46] A more recent report has demonstrated that EPO is significantly depressed among pregnant women with moderately raised blood lead level.[40] Thus it appears more likely that the decreases in haematological indices reflect gradually decreasing ability to produce EPO. The normocytic normochromic morphology of the erythrocytes in addition to similar serum iron level in our previous report[42] suggest that iron deficiency is excluded.

The significantly raised copper level though may be suggestive of oxidative stress[23,47-49] appears to corroborate that iron deficiency anaemia is unlikely, as it rules out inadequate feroxidase activity of copper a contributor to refractory microcytic hyprochromic anaemia as a factor. The significantly lower level of the protoporphyrin degradative product, bilirubin and absence of hyperchromasia in the erythrocyte morphology (which may arise from increased erythropoietic activity, active bone marrow activity or bone marrow hyperplasia) together rule out increased haemolysis as a factor in the decrement of haematological indices. The reduced bilirubin level may however, corroborate the existence of oxidative stress in these lead workers.[50]

It is interesting to note that the bone of which the major mineral is calcium is the major repository of lead, over 90% of the body lead burden is in the bone.[51] Bone metabolism is closely regulated by the combined endocrine activities of calcitoriol and PTH. It has been suggested that bone lead may be a major important biomarker of continuing Pb toxicity than blood lead level.[14] By corollary, an elevated blood lead level suggest a high bone burden. It has also been suggested that the observed association between bone Pb and Pb toxicity might be a reflection of inhibition of haematopoiesis through depression of erythropoietin with bone serving as a proxy for kidney lead.[52] It should also be recalled that though EPO is elaborated by the kidneys it acts in the bone marrow. These observations all point to Pb as an endocrine modulator or endocrine disrupting chemical (EDC) not only of calcium homeostasis but also that regulating both steady – state and increased production of erythropoietin and is consistent with an earlier observation that in acute toxicity, there is rapid red cell destruction followed by active marrow hyperplasia[53] indicating active EPO synthesis (inretrospect). In chronic Pb toxicity on the otherhand, as in occupational exposure in this cohort, EPO is depressed due to chronic renal impairment[42] Consistent with Marrow hypoactivity arising from a gradually decreasing ability of the kidneys to produce EPO. Indeed one of the haematological hallmarks of chronic renal impairment is normocytic normochromia. Thus though EPO was not measured in this study a previous study has shown that serum EPO concentration is depressed at blood Pb level substantially lower than the level in this study.[40] Secondly Hgb is a strong determinant of EPO concentration.[13] High BPb level in this country derives mainly from the hitherto high Pb content of Nigeria's gasoline.[54]

It is worthy of note that high environmental heavy metal concentration including Pb and Cd leads to high PBb in the general population and gradual impairment of calcium metabolism[55] and induces anaemia.[16-18,42] These processes may insidiously develop in unexposed populations. Thus the high Pb level compared to currently acceptable level[42,56] may also be associated with subtle endocrinopathies in the general population.

Though most of the reported studies on endocrine disrupting chemicals appear to have concentrated on direct endocrine effects of EDCs, some other mechanisms are recognized. [The mechanism or mode of action of EDCs is not limited to those agents that interact directly with hormone receptors]. Other mechanisms of interest include hormone synthesis, transport, or metabolism and activation of receptors through processes such as receptor phosphorylation or the release of cellular complexes for hormone action.[1,2,57] Another feature of EDSs is that their concentrations are magnified through bioaccumulation increasing their potency. The mechanism of disruption of the homones 1, 25$(OH)_2D_3$ and erythropoietin are consistent with some of those of established EDCs. From the foregoing Pb at least in part qualifies to be included in the list of endocrine disruptive substances.

To our knowledge, attention has not been drawn to the endocrine disruptive effect of lead. This study has provided evidence or a reminder that the ancient toxin, Pb is an endocrine disruptive chemical. This is principally through its inhibition of calcitriol and erythropoietin synthesis as a result of the pathological effects of Pb on the proximal renal tabular cells. This leads to a decrease in both calcium levels and haematological indices. parathyroid hormone may also be slightly depressed in these subjects. Though great efforts have been expended in reducing the lead level in the environment this goal is yet to be achieved in most developing countries.[22] Thus for these countries excessive Pb in the environment probably combined with hydrocarbons (HCs),[42,58] may be among the most important endocrine disruptors; probably not the bisphenols that need focus.

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