Effect of graded concentrations of Ca on nephrotic cells of Cd and Pb co-intoxicated rats

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ABSTRACT

This work aimed at determining the effect of co-administration of graded concentrations of Ca on Cd$^{2+}$, Pb$^{2+}$, Ca$^{2+}$ and Mg$^{2+}$ ions urinary excretion and kidney histochemistry of Cd and Pb co-intoxicated rats. Rats were divided into five groups of four rats per group in metabolic cages. Group one was fed with tap water only, group two was fed with the combination of 0.327mg/L Pb and 0.079 mg/L Cd, while group three to five were fed with the combination of 0.327mg/L Pb and 0.079 mg/L Cd concurrently with graded concentrations of Ca. A twenty-four hour (24h) urine sample from the rats in their respective groups, in the urine collector of the metabolic cages, were collected daily for fourteen days and kept frozen. After the termination of the experiments, the rats were humanely sacrificed under anaesthesia, the kidney was removed and hepatic [5,6] damage. The foods we eat. This cause renal toxicity (at the proximal tubule [20,2]) and essential metals including Ca and Mg [14,15], and the oxidative stress caused by Cd/Pb exposure [16,17]. To some extent these two mechanisms are still interrelated because the metabolic disorder of essential metals such as zinc and selenium also induces adverse effects in the oxidative and anti-oxidative systems [18, 19].

The most commonly used therapeutic strategy for heavy metal poisoning is chelation therapy to promote metal excretion. However, chelators for Cd and Pb toxicity are themselves reported to have a number of different routes by which Cd and Pb cause toxicities have been reported, the underlying basic mechanisms can be summarized as the interactions between the non-essential (Cd/Pb) and essential metals including Ca and Mg [14,15], and the oxidative stress caused by Cd/Pb exposure [16,17]. To some extent these two mechanisms are still interrelated because the metabolic disorder of essential metals such as zinc and selenium also induces adverse effects in the oxidative and anti-oxidative systems [18, 19].

The most commonly used therapeutic strategy for heavy metal poisoning is chelation therapy to promote metal excretion. However, chelators for Cd and Pb toxicity are themselves reported to have a number of different safety and efficacy concerns. None of the chelation therapies for Cd toxicity have yet been approved for clinical use thus far [20,2]. However, CaNa2EDTA can cause renal toxicity (at the proximal tubule particularly), especially during repeated high

1. INTRODUCTION

Heavy metal toxicity is one of the oldest environmental problems and remains a serious health concern today. Cadmium (Cd) and lead (Pb) are common toxic heavy metals in the environment [1]. Human exposure to Cd and Pb could be as a result of the air we breathe, the water we drink, and the foods we eat. This exposure could result as a result of anthropogenic activities via the food chain [2]. Cd and Pb exposure could cause a broad range of adverse health effects in humans and animals. Cd and Pb toxicities are associated with renal [3,4] and hepatic [5,6] damage. The International Agency for Research on Cancer classified Cd as a group 1 human carcinogen [7]. Exposure to Pb leads to neurologic and haematological dysfunctions [8,9], renal and hepatic damage [10], as well as reproductive disorders [11] in the human body. Children are especially at greater risk because they have higher intestinal Pb absorption and more vulnerable nervous systems which are still under development [12,13]. Although a number of different routes by which Cd and Pb cause toxicities have been reported, the underlying basic mechanisms can be summarized as the interactions between the non-essential (Cd/Pb) and essential metals including Ca and Mg [14,15], and the oxidative stress caused by Cd/Pb exposure [16,17]. To some extent these two mechanisms are still interrelated because the metabolic disorder of essential metals such as zinc and selenium also induces adverse effects in the oxidative and anti-oxidative systems [18, 19].

The most commonly used therapeutic strategy for heavy metal poisoning is chelation therapy to promote metal excretion. However, chelators for Cd and Pb toxicity are themselves reported to have a number of different safety and efficacy concerns. None of the chelation therapies for Cd toxicity have yet been approved for clinical use thus far [20,2]. However, CaNa2EDTA can cause renal toxicity (at the proximal tubule particularly), especially during repeated high

Keywords: calcium, urinary excretion, cadmium, lead, co-intoxicated, environmental pollution

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doses treatment (above 75 mg/kg) and in subjects with previous history of kidney damage [21]. Because of its relative lack of specificity, other essential metals such as zinc, iron and manganese are also reported to be excreted and depleted following CaNa2EDTA therapy [22]. Dimercaptosuccinic acid (DMSA) also has side effects such as appetite loss, nausea and diarrhea [23]. A study of children being treated with DMSA showed that 12% had mild gastrointestinal symptoms and 5% experienced general malaise [24].

The development of safe and efficient strategies against Cd and Pb toxicities is an area of ongoing research which requires much attention as chelation therapy has grave consequences to the liver and kidney as outlined above. Dietary supplements have been reported to play important roles in the alleviation or prevention of Cd and Pb toxicities [25, 26]. Therefore, dietary strategies are advantageous, as nutritional ingredients can easily and affordably be added to the diet and can overcome the negative side effects of the chelation therapy, by an average individual in a population that is exposed to the danger of Cd and Pb toxicities through anthropogenic activities or natural occurrences. In our previous works, which some have been quoted above, the potential dietary strategies for Cd and Pb toxicities of Ca and Mg were demonstrated [27, 28]. Ca and Mg have similar chemical and physical properties to Cd and Pb, and they compete for the binding sites of metal absorptive and enzymatic proteins [29], thereby reducing Pb/Cd intestinal absorption and prevent heavy metal induced tissue damage by competitive binding to active sites of the enzymes [30]. Diet associated essential metal supplementation should be regarded as important for essential metal-deficient people, such as children and pregnant women. It should also be noted that Cd and Pb exposure cause the loss of essential metals, which leads to complications such as iron-deficiency anaemia and osteoporosis [31, 32]. Appropriate concentrations of essential metal supplementation is therefore also beneficial for preventing these complications.

From the foregoing, this work aimed at determining the effect of co-administration of graded concentrations of Ca on urinary excretion of Cd²⁺, Pb²⁺, Ca²⁺ and Mg²⁺ ions and kidney histochemistry of Cd and Pb co-intoxicated rats. This knowledge would give an idea of dietary interventions with Ca especially for people exposed to the dangers of heavy metals toxicities, such as people living in mining areas of Plateau State, who use the mining pond waters for irrigation as well as for domestic purposes.

2. MATERIAL AND METHODS

2.1 Materials

2.1.1 Experimental animals

Ethical Clearance was obtained from The University of Jos Committee on Care and Use of Laboratory Animals before the commencement of this work. Twenty (20) adult male Wistar strain rats weighing 178g on the average were obtained from the University of Jos Animal House. Commercial feed produced by Grand Cereal and Oil Mill Limited, Jos, Nigeria, was used to feed the animals. The animal House of Pharmacology Department, Anatomy and Biochemistry laboratories, University of Jos, Nigeria, were used for treatments, histochemical and biochemical analyses respectively, between December 2014 and April 2015.

2.1.2 Chemicals

Lead acetate and magnesium sulphate, both analar, were products of British Drug House (BDH), Poole, England. Bovine Serum Albumin (BSA) was a product of Sigma Chemicals. Cadmium chloride and calcium sulphate were products of May and Baker (M & B) Limited, Dagenham, England. All other chemicals used were of analytical grade purchased by the Department of Biochemistry, University of Jos, from reputable chemical companies in Jos, Plateau State, Nigeria.

2.1.3 Experimental design

The rats were randomly divided by body weight equally into five groups of four per group in metabolic cages. Group one (control) was placed on tap water only, while group two was placed on the combination of 0.327mg/L Pb and 0.079 mg/L Cd; group three was placed on 0.327mg/L Pb and 0.079 mg/L Cd with the addition of 0.193mg/L of Ca; group four was placed on 0.327mg/L Pb and 0.079 mg/L Cd with the addition of 0.221mg/L of Ca; while group five was placed on 0.327mg/L Pb and 0.079 mg/L Cd with the addition of 0.348mg/L of Ca as shown in table 1 below. The choice of Cd and Pb concentrations of (0.327mg/L Pb and 0.079 mg/L Cd) was based on the fact that the combination of the two concentrations caused the most damage to the kidney in our previous work, hence the need to test the protective effect of Ca. The mining pond waters of Plateau state contain Cd and Pb in concentrations above WHO permissible limits, and also contain Ca and Mg in high concentrations, which the inhabitants of the areas use for their domestic purposes.

Twenty-four (24) hours prior to the commencement of the experiment the rats were fasted to clear the gastrointestinal tract of any other food eaten before, as described by Rodriguez-de Fonseca et al, 2002 [33]. Their feed was mashed with the same water meant for each group. All the groups fed on the mashed vital growers’ food, and freely drank from the water for a period of fourteen (14) days.
Table 1. Experimental design

<table>
<thead>
<tr>
<th>Metals</th>
<th>Group 1 (control)</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Group 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pb</td>
<td>-</td>
<td>0.327</td>
<td>0.327</td>
<td>0.327</td>
<td>0.327</td>
</tr>
<tr>
<td>Cd</td>
<td>-</td>
<td>0.079</td>
<td>0.079</td>
<td>0.079</td>
<td>0.079</td>
</tr>
<tr>
<td>Ca</td>
<td>-</td>
<td>0.193</td>
<td>0.221</td>
<td>0.348</td>
<td></td>
</tr>
</tbody>
</table>

Concentrations in mg/L

2.2 Methods

2.2.1 Urine collection

Clean, dry, leak proof sterile containers were used in collecting a twenty-four hour (24h) urine sample from the rats at their respective groups in the urine collector of the metabolic cages, after which the urine samples were kept frozen until needed for clinical analysis [34].

2.2.2 Histopathological studies

The kidney was fixed in 10% neutral formalin solution. After a week of fixing, the liver tissues were dehydrated with a sequence of ethanol solutions, embedded in paraffin, cut into 5µm section, stained with haematoxylin eosin dye (H & E stain) and observed under a microscope at x400 magnification. Morphological changes were observed including absence of nuclei in the convoluted tubules, enlargement of tubular epithelium, vacuolated epithelial cells, necrotic tubular damage, acinic differentiation and solid cords of cells.

2.2.3 Digestion of urine samples

Digestion of urine samples was done by the Adler and Wilcox method [35]. The digestion mixture used was the combination of concentrated perchloric acid (HClO4) and concentrated nitric acid (HNO3) in the ratio of 6:1v/v. Ten millitre (10ml) portions of urine samples were transferred into 25ml pyrex beakers. To each of the samples, 5ml of the digestion mixture was added and heated on hot plates in a fume cupboard until the samples became almost dry. Another 5ml of the digestion mixture was added to each of the beakers and heated as above. This procedure was repeated until the samples turned whitish signifying that all organic component of the urine are burnt off. The white precipitate was then dissolved in 2ml of de-ionised water and made up to the initial volume of the urine digested (10ml). This was then analysed using a Hitachi atomic absorption spectrophotometer.

2.2.4 Determination of urinary Cd^{2+}, Pb^{2+}, Ca^{2+} and Mg^{2+} ions

The urine samples were analysed for the concentrations of Cd^{2+}, Pb^{2+}, Ca^{2+} and Mg^{2+} ions using a Hitachi 180-80 Polarised Zeeman atomic absorption spectrophotometer (AAS).

2.2.5 Preparation of the working standards

The stock for each of the following elements, which was a product of Sigma-Aldrich RTC Inc. USA, was used. The elements are: Cadmium (Cd), lead (Pb), calcium (Ca) and magnesium (Mg). Each stock had a concentration of 1000 parts per million (ppm) from which different concentrations of 1ppm, 2ppm and 5ppm were prepared and used as standards to calibrate the machine.

2.2.6 Operation of the atomic absorption spectrophotometer

The machine was switched on and allowed to run for five (5) minutes to equilibrate. After that, a blank was aspirated and followed by the standards of 1ppm, 2ppm and 5ppm. This was to calibrate a standard curve so that the concentrations of the samples could be automatically read off from it. The calibration was done automatically by the machine. Samples were then aspirated one after the other and two readings were taken. The means and standard deviations were automatically determined by the machine and printed out.

2.2.7 Principles of atomic absorption spectrophotometry

Atomic absorption spectrometry (AAS) is a spectro-analytical procedure for qualitative and quantitative assessment of chemical elements based on the absorption of light (optical radiation) by free atoms in the gaseous state. Atomic absorption spectroscopy is based on standards with known analyte content to establish the relation between the measured absorbance and the analyte concentration and on the Beer-Lambert Law. The electrons in the atomizer can be placed in an excited state for a period of nanoseconds by absorbing the radiation of a given wavelength. This wavelength is specific for a particular electron transition in a particular element, so each wavelength corresponds to only one element, and the width of an absorption line is only a few picometers (pm), which gives the technique its elemental selectivity. The radiation flux, with and without a sample in the atomizer, is measured and the ratio between the two values is converted to analyte concentration or mass using the Beer-Lambert Law.

2.2.8 Statistical analysis
Tukey-Kramer multiple comparisons test at 95% level of confidence was used to test for the significant differences between the concentrations of Cd²⁺, Pb²⁺, Ca²⁺, and Mg²⁺ ions in the control group and the test groups, and results expressed as mean ± S.D. The INSTAT3 statistical software was used.

3. RESULTS

The effect of concurrent administration of a constant concentrations of Cd and Pb with graded doses of Ca on kidney biomarkers and kidney histochemistry are presented in Table 2 and Plates 1-5. When the combination of Cd and Pb were co-administered without the addition of Ca, the urinary excretion of Ca and Mg was greatly increased, while the histochemistry of the group showed that the kidney section had significant loss of nuclei in the convoluted tubules but there was well defined membranes and clear cytoplasm. There was also urinary excretion of Cd and Pb. When the same concentrations of Cd and Pb were co-administered with graded concentrations of Mg, the urinary excretion of Cd and Pb increased as the concentration of Mg was elevated, while that of Ca and Mg decreased. The histochemistry of these groups showed that as the concentration of Mg was elevated, the presence of nuclei were increased in the kidney section, but there was presence of enlarged epithelial cells, vacuolated epithelial cells, and acinic differentiations. There was significant difference (P < .05) between the urinary excretion of Cd and Pb of all the groups as compared with control. The histochemistry shows that there was significant difference (P < .05) between the kidney integrity of control and the test groups.

Table 2. Effect of Concurrent Administering of Cd and Pb, Cd and Pb with the addition of graded concentration of Ca on the urinary excretion of cadmium and lead in rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Ca²⁺ (mg/l)</th>
<th>Mg²⁺ (mg/l)</th>
<th>Cd²⁺ (mg/l)</th>
<th>Pb²⁺ (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Control</td>
<td>82.33±0.31</td>
<td>4.6±0.02</td>
<td>0.000±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>2.</td>
<td>Cd+Pb</td>
<td>108.3±0.21</td>
<td>6.57±0.02</td>
<td>0.006±0.00*</td>
<td>0.47±0.04*</td>
</tr>
<tr>
<td>3.</td>
<td>Cd+Pb+Ca</td>
<td>229.1±1.46</td>
<td>13.15±0.09</td>
<td>0.009±0.00*</td>
<td>0.55±0.02*</td>
</tr>
<tr>
<td>4.</td>
<td>Cd+Pb+Ca</td>
<td>350.9±0.87</td>
<td>26.57±1.17</td>
<td>0.009±0.00*</td>
<td>0.64±0.00*</td>
</tr>
<tr>
<td>5.</td>
<td>Cd+Pb+Ca</td>
<td>371.7±1.58</td>
<td>37.1±0.06</td>
<td>0.012±0.00*</td>
<td>3.55±0.04*</td>
</tr>
</tbody>
</table>

Concentrations of cadmium and lead are 0.079 and 0.327mg/l respectively and the varied concentrations of Ca are 0.193, 0.221 and 0.348 respectively. *Significant difference (p<0.05) between the control and the treatments.

Plate 1. Representative kidney section of the rats fed vital feed without the addition of any metal (control) showing normal convoluted tubule (arrow) with normal nuclei arrangement within the cell.
Plate 2. Representative kidney section of the rats fed with the combination of 0.023 mg/L of Cd and 0.077 mg/L of Pb, showing portions of the convoluted tubules that have well defined membranes but with limited nuclei (arrows).

Plate 3. Representative renal section of the rats fed with the combination of 0.023 mg/L of Cd and 0.077 mg/L of Pb with the addition of 0.193 mg/L of Ca, showing enlargement of tubular epithelium (arrow). There is improvement in the presence of nuclei in the convoluted tubules.
Plate 4. Representative renal section of the rats fed with the addition of the combination of 0.023 mg/L of Cd and 0.077 mg/L of Pb and 0.221 mg/L of Ca, showing acinic differentiation and solid cords of cells (arrow), but most portions are relatively normal.

Plate 5. Representative renal section of the rats fed with the addition of the combination of 0.023 mg/L of Cd and 0.077 mg/L of Pb and 0.348 mg/L of Ca, showing acinic differentiation and solid cords of cells (arrow). Most portions of the kidney sections show normal nephrocytes.
4. DISCUSSION

From the results, when 0.327mg/L of Pb and 0.079mg/L of Cd concentrations were administered without the addition of Ca, there was significant difference between the kidney of control group with this group as indicated by the urinary excretion of Ca and Mg, and the histochemistry of the kidney. The histochemistry show a large portion of the convoluted tubule that have well defined membranes but with limited nuclei. This indicates that there was marked compromise of the kidney integrity of this group. This is in agreement with the fact that when Cd and Pb are ingested either in food, water, or breathe in the air beyond the WHO admissible limits, kidney toxicity occurs [36, 6, 10]. Cadmium is known to competitively interfere with the physiologic action of Ca or Mg [36], as shown in the result of this work, more Mg and Ca were excreted in the urine of the rats in this group even though no additional Ca was added. Other pathologic mechanisms include: induce tissue injury through creating oxidative stress [37], epigenetic changes in DNA expression [38], inhibition or upregulation of transport pathways [39], particularly in the proximal S1 segment of the kidney tubule [40], inhibition of haeme synthesis [41], and impairment of mitochondrial function potentially inducing apoptosis [42]. Depletion of glutathione and structural distortion of proteins is said to also occur due to Cd binding to sulfhydryl groups. These effects are magnified by interaction with other toxic metals such as Pb and As [43], and possibly ameliorated by Zn or Se [44].

When the Cd and Pb concentrations above were administered concurrently with 0.193 mg/L of Ca, there was increased urinary excretion of Pb than Cd as compared with the test control. This could be as the result of Ca being present in higher concentration and therefore could compete more favourably with Cd and Pb for binding sites on transport systems and sulfhydryl groups of proteins. This is in agreement with the fact that divalent metal ions are all transported into the cell based on their relative presence in the gastrointestinal tract [35]. The histochemistry of this group show that there were enlargement of tubular epithelial cells, but there was presence of nuclei in most portions of the kidney section. This shows an improvement in the kidney integrity and the extent of damage had been ameliorated but not obliterated. Increasing the availability of essential micronutrients had proven in various studies to decrease the toxicity of toxic heavy metals. Zinc can increase synthesis of Metallothionine (MT), a thiol-rich protein that sequester cadmium and prevent acute hepatotoxicity, which will lead to chronic kidney toxicity as Cadmium-MT is excreted from the liver and absorbed by the kidney. Gastrointestinal lead absorption and retention constitutes the major pathway of lead intake and depends on the micronutrients status of the individual. Adults are said to absorb approximately 10% of ingested lead and small children absorb approximately 50% of ingested lead. From this studies, Calcium decreased the susceptibility of cadmium and lead intoxication in rats. This could be as a result of decrease intestinal absorption of cadmium and lead as a result of competition for similar binding sites on intestinal proteins which are important in the absorptive process. These shared binding sites on absorptive proteins could explain why sufficient dietary calcium could decrease lead and cadmium absorption [44, 15, 1].

When the concentration of Ca was increased to 0.221 mg/L and concurrently administered with Cd and Pb concentrations above, there was increased urinary excretion of Cd and Pb. There was also improvement in the histochemistry of the kidney, which showed acinic differentiation and solid cords of cells (arrow), but most portions are relatively normal. The improvement in the histochemistry did not completely remove the damaging effects of Cd and Pb but merely ameliorated it; the improvement in the histochemistry was better than the result we got when we used Mg [45]. As stated above, the improvement could be as a result of decrease intestinal absorption of cadmium and lead as a result of a more effective competition for similar binding sites on intestinal proteins by calcium, which are important in the absorptive process. These shared binding sites on absorptive proteins could explain why sufficient dietary calcium could decrease lead and cadmium absorption [44, 15, 1].

A further increase in Ca concentration to 0.348 mg/L concurrently administered with the Cd and Pb concentrations above, there was a further increase in the urinary excretion of Cd and Pb, which were significant different as compared with the test control. The histochemistry of this group showed relatively normal kidney section, but with some acinic differentiations and solid cords of cells were observed. On the overall, there was no significant difference between the histochemistry of this group and control, but there was significant difference between it the test control and the other test groups. This suggests that as the concentration of Ca was elevated, the nephroprotective effect of Ca against Cd and Pb co-intoxicated rats was increasing. This could be as a result of the fact that Cd and Pb have no specific transport proteins, but rather rely on their similarities in chemical and physical properties to the essential metal Ca for their transport and uptake into the cells by a process referred to as “ionic and molecular mimicry” [46]. Some studies have demonstrated that Cd and Pb ions are taken up by the divalent metal transporter 1 (DMT1), and the metal transporter protein 1 (MTP1), which are located in the basolateral and the apical membranes of the enterocytes respectively [47, 48, 49]. The result of this work therefore points to the fact that disruption of the homeostasis of Ca ions in the cell can lead to kidney diseases.
and adequate intake of Ca can mitigate the toxicities of non-essential metals (Cd and Pb).

5. CONCLUSION
This study shows that disruption of the homeostasis of Ca ions in the cell can lead to kidney diseases and adequate intake of Ca can mitigate Cd and Pb nephrotoxicities in rats. Based on these results, we recommend that people around the world who are at risk of exposure to toxic metals Cd and Pb should ensure a sufficient intake of Ca through enhanced consumption of vegetables, fruits and foods which are known to be high in Ca. This metal acting alone could be an important natural antagonist to Cd and Pb toxicities and should be consumed on a regular basis. Providing livestock and farmed fish with the above-mentioned food interventions may also be helpful to reduce Cd and Pb exposure to humans through the food chain.

AUTHOR CONTRIBUTIONS
This work was carried out in collaboration between all authors. JDD and GAU designed the study. JDD performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. SYG and GAU managed the analyses of the study. Authors JDD and SYG managed the literature searches.

ETHICAL APPROVAL
All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

REFERENCES


