RESEARCH ARTICLE

Delta-Aminolevulinic acid dehydratase enzyme activity and susceptibility to lead toxicity in Uganda’s urban children

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Abstract

Background: Rapid industrialization, urbanization, and population explosion in sub-Saharan Africa escalate environmental lead levels and subsequently blood lead levels in children. Its levels in one's environment account for their blood lead levels. One's susceptibility to lead toxicity is governed by nutrition status, age and genetics. This study aimed at expounding susceptibility to lead toxicity by relating blood lead levels, delta-aminolevulinic acid dehydratase (ALAD) enzyme activity, and genetic variations of proteins that code for ALAD in urban children of Uganda.

Methods: A total of 198 blood samples were spectrophotometrically analysed for blood lead levels (BLL), hemoglobin (Hb) levels, and ALAD enzyme activity before DNA extraction, polymerase chain reaction, and restriction fragment length digestion for ALAD polymorphism.

Results: Up to 99.5% of the total samples analyzed coded for ALAD1 allele compared to 0.05% that coded for ALAD2. There was a significant relationship between BLL, Hb status and ALAD enzyme activity in the three isozymes (ALAD1-1, ALAD1-2 and ALAD2-2) in strength of ALAD1-1 (r = 0.42, p-value = 0.02) < ALAD1-2 (r = 0.62, p-value = < 0.001) < ALAD2-2 (r = 0.67, p-value = < 0.001).

Conclusions: Majority of children in Uganda code for the ALAD1 allele, which is important for blood lead ions hoarding during lead toxicity. Hoarding of blood lead not only delays exposure effects but also accumulates its levels in deposit tissues and this poses adverse effects later in their lives.

Keywords

Blood Lead levels, Lead toxicity susceptibility, d-aminolevulinic acid dehydratase enzyme activity, d-aminolevulinic acid dehydratase gene polymorphism.
Introduction

Uganda, like many other African countries, is faced with numerous simultaneous transitions that include economic development, industrialization, population explosion, and urbanization.

These transitions are coming with both environmental and health changes. Population explosion is putting pressure on the environment through increased anthropogenic activities, elevated volumes of electronic wastes and this has resulted in increased volumes of toxic pollutants like lead in both air and water bodies. Because lead is an accumulative toxin, its increased concentration in the environment continues to cause health challenges, especially to the children.\textsuperscript{1,2} Elevated environmental lead levels correlate with the blood lead levels in exposed individuals.\textsuperscript{3} Childhood lead exposure is associated with various health challenges that include lung, stomach, and bladder cancers, anemia, neurocognitive disorders, intelligent quotient (IQ) lowering, and stunted growth.\textsuperscript{4,5} Although environmental lead pollution is preventable, little attention is accorded to this preventable problem in many African countries including Uganda. Recent studies conducted in different parts of Kampala slums report elevated blood lead levels, especially among children.\textsuperscript{3,6} One’s susceptibility to lead toxicity is modulated by age, genetics, nutritional and malaria infection status.\textsuperscript{3,7} The rate of lead ion absorption, especially in the intestines, is further shown to increase with a decrease in hemoglobin levels. Following its absorption, lead sinks in red blood cells (RBCs) where it specifically binds the delta-aminolevulinic acid dehydratase (ALAD) enzyme. This enzyme (ALAD) is second and important in the heme biosynthetic pathway and it is involved in the condensation of glycine and succinyl CoA, decarboxylation into delta-aminolevulinic acid (ALA).

The enzyme ALAD is rich with thiol groups and zinc ions, that have a high affinity for lead ions and this renders the enzyme more sensitive to attack by circulating lead ions.\textsuperscript{8,9} It is a tetramer homodimer with eight identical subunits and is located in the cytoplasm. In each of its subunits, it binds eight zinc atoms, where four zinc molecules act as catalysts, whereas the remainder serve as tertiary structural stabilizers. In times of lead burden, lead ions displace zinc from the enzyme’s active site and inhibit its activity, resulting in the accumulation of ALA.\textsuperscript{10} Accumulated levels of ALA trigger the production of reactive oxygen species (ROS), which are associated with oxidative stress.

Several studies from different regions indicate varying blood lead levels, biological markers, and even symptoms among people in the same locality. This observation is attributed to the polymorphic nature of the gene that codes for the ALAD enzyme. Polymorphism of the ALAD gene is reported to modulate one’s susceptibility to lead toxicity.\textsuperscript{11,12} The ALAD enzyme is encoded by a single gene on chromosome 9q34 region.\textsuperscript{13} This gene codes for two alleles i.e., ALAD-1 and ALAD-2,\textsuperscript{14} which are codominant (Single Nucleotide Polymorphism database (dbSNP) ID: rs1800435 [http://www.ncbi.nlm.nih.gov/SNP/index.html]). Their expression results in a polymorphic enzyme system consisting of three different isozymes: ALAD1-1, ALAD1-2, and ALAD2-2. Individuals dominantly expressing ALAD1-2 and ALAD2-2 have a higher susceptibility to lead toxicity than those expressing the ALAD1-1 isozyme. The prevalence of the ALAD-2 allele is race-specific and usually ranges from 0 to 20 percent.\textsuperscript{15} Therefore, the ALAD polymorphism affects and modifies lead metabolism and delivery to target organs.\textsuperscript{16} To date, no study regarding ALAD enzyme activity and polymorphism distribution in the Ugandan population has been conducted. The present study, therefore, aimed at expounding on the ALAD enzyme activity, and the distribution of ALAD genotypes in relation to lead exposure susceptibility in Ugandan children. Thus, this is the first study to address lead exposure susceptibility, ALAD enzyme activity, and polymorphism in Ugandan children.

Methods

Ethical considerations

This study was approved by Gulu University Research Ethics Committee No. (GUREC-048) dated 31/05/2019. Intentions of the study were first clearly explained in both English and a local language to the participant’s parents/guardians before signing informed consent forms.

Study design

This was a cross-sectional study that involved randomly selected children that resided in Katanga slum of Kampala Uganda (00°18'49"N 32°34'52"E, coordinates) for at least a year. The area has approximately 7000 inhabitants and 15.2% of these are under 5 years of age [http://www.askyourgov.ug].
The sample size for the study was derived from the expression

\[
\text{Margin of error}^2 = \frac{Z - \text{Score}^2 \times d(1 - d)}{\text{confidence of } 95\%}
\]

where \(d\) = the prevalence in percentage of study group of interest.

Through their local leaders, homes of study participants were visited and explained the purpose of the study prior to signing of consent forms by their parents/guardians. Visibly malnourished children and those with a history of blood transfusion were excluded from this study. Duplicate samples of venous blood (5 ml; \(n = 198\)) were drawn from each study participants by qualified nurses and technicians. One tube contained heparin and this was used for hematocrit determination, while the other tube containing ethylenediamine tetra acetic acid (EDTA) was used for other assays. The samples were transported on ice to Makerere University Biochemistry Department laboratory for analysis.

**Assay for blood lead using atomic absorption spectrophotometer**

Blood lead levels were determined on an atomic absorption spectrophotometer (Agilent MY17180002 200 series) equipped with a graphite tube atomizer (GTA 120), a hollow-cathode lead lamp with a working current of 5 mA, 283.3 nm spectral line, and 0.5 nm bandwidth as described elsewhere. \(^1\) Five hundred microliter (500 \(\mu\)l) aliquots of blood samples were mixed with 1.2 ml of a solution that was prepared by mixing equal volumes of 0.5% Triton X-100 and 1% di-ammonium phosphate ((NH\(_4\))\(_2\)HPO\(_4\)). A total volume of 1.8 ml of deionized water was added to each sample in the tube and followed by the addition of 1.5 ml of 20% trichloroacetic acid (TCA) before vortex mixing. The samples were centrifuged at 5000 rpm for 20 min and 10 \(\mu\)l of the supernatant from each was collected and injected into the graphite tube. Lead standard concentrations ranged from 2 \(\mu\)g/dL to 50 \(\mu\)g/dL. Samples were analyzed in duplicate, and their mean values were determined with occasional blanking with deionized/distilled water. The equipment had a detection limit of 2 \(\mu\)g/dL.

**Colorimetric determination of hemoglobin levels by blood cyanmethemoglobin reaction method**

Hemoglobin levels were determined following a cyanmethemoglobin reaction method described elsewhere. \(^1\) Blood samples were processed and analyzed as described in our previous study. \(^5\) Briefly, 100 \(\mu\)l of each sample were reacted with cyanide reagent and incubated at room temperature for 15 min. Hemoglobin concentration was then determined using Jen way 6051 colorimeter at 540 nm against a reagent blank.

**Determination of hematocrit levels of the study blood samples**

The hematocrit levels of the study blood samples were assayed as described elsewhere. \(^2\) Whole blood samples in heparinized tubes were forced into narrow diameter glass capillary tubes to two-third levels. The capillary tubes had a self-sealing compound from one end. The capillaries together with the blood were loaded onto a microhematocrit centrifuge and ran at a relative centrifugal force of 14,000 \(\times g\) for five minutes. Following centrifugation, hematocrit levels of each sample were measured within 10 min while the tubes were kept in a horizontal position to avoid merging of the layers. Hematocrit levels were estimated by calculating the ratio of the column of packed erythrocytes to the total length of the sample in the capillary tube.

**Determination of delta-aminolevulinic acid dehydratase (ALAD) enzyme activity**

The blood \(\delta-\text{ALAD}\) enzyme activity in all the samples collected was measured following a method described by. \(^2\) The \(\text{ALAD}\) enzyme activity of each sample in duplicate was determined by incubating 0.20 ml of the sample with 1.30 ml of Triton X-100 reagent in disposable plastic tubes and thereafter adding 1 ml of buffered ALA substrate (0.01M). The buffered ALA substrate was prepared by dissolving 0.1676 g of ALA-HCL in 100 ml of phosphate-citrate buffer pH 6.65. The buffer was previously prepared by dissolving 6.703 g/dL Na\(_2\)HPO\(_4\) (0.25 M) and citric acid 5.25 g/dL (0.25 M). Aliquots equivalent to 1 ml of Trichloroacetic acid (TCA) reagent were added to each sample and the blank (plain distilled water).

To both test and blank aliquots, 1.0 ml of the modified Ehrlich’s reagent was added. This was previously prepared by dissolving 10 g of p-dimethylaminobenzaldehyde (DMBA) in 420 ml of acetic acid and diluted to 1 L with distilled water. Before storing the reagent at 40°C a working solution was prepared by mixing 50 ml of DMBA-acetic acid with 8 ml of 70% perchloric acid. Following the addition of the modified Ehrlich’s working reagent, the mixtures were allowed to stand for 13 min for color development before measurement at 555 nm on a spectrophotometer.
The corrected absorbance $A = (\text{Test absorbance} - \text{the blank absorbance})$ was used to calculate the activity of the enzyme.

$$\frac{\text{Corrected Absorbance} \times 12500}{\text{Hematocrit}} = \text{units of ALAD enzyme activity},$$

Where 12500 is the blood dilution factor.

**Delta-aminolevulinic acid dehydratase (ALAD) genotyping**

Blood samples were analyzed for polymorphism as described elsewhere.\textsuperscript{22,23} Genomic DNA from each blood sample was extracted using a Qiagen genomic DNA purification kit (DNeasy, Catalogue no. 69506) following the manufacturer’s instruction. The resultant DNA products were purified before polymerase chain reaction (PCR) amplification. The PCR reaction mixture equivalent to $50 \mu$L contained $1 \times$ buffer (10 mM Tris-HCl, pH 8.8; 50 mM KCl), 2 mM MgCl$_2$, 0.2 mM dNTPs, 20 pmol each primer and 3U Taq DNA polymerase.

Forward primer, 5’-AGACAGACATTAGCTCAGTA-3’,

and reverse primer, 5’GGCAAAGACCACGTCCATTC-3’

The running conditions on a Gene Amp PCR system 9700 were; pre-denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, synthesis at 72°C for 1 min and final extension at 72°C for 5 min. The amplified products (916-bp region of genomic DNA) in volumes of 10 μL were digested overnight with MspI restriction enzyme (2.5 units) in a 20 μL reaction mixture containing 50 mM sodium chloride, 10 mM Tris-HCl, 10 mM magnesium chloride, 1mM dithiothreitol (pH 7.9) at 37°C. The fragments were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide and visualized under a UV illumination system. ALAD1-2 samples had both a 583- and a 512-bp fragment, whereas ALAD1-1 individuals had a single 583-bp fragment.

**Data analysis**

Results were expressed as means, correlations and the statistical significance was evaluated by one-way analysis of variance (ANOVA) using Minitab 19 statistical software, an equivalent open access alternative is Scilab-6.1.1 statistical software. In addition to maximizing data collection, missing data cases were completely omitted (listwise) from the data set prior to statistical analysis.

**Results**

Following genotyping of the samples for ALAD alleles, the outcome is shown in Table 1 with corresponding BLL, Hb levels, hematocrit, and ALAD enzyme activities. The results indicate that the ALAD1-1 isozyme was the most predominant with moderately high hemoglobin levels and seemingly normally functioning ALAD enzyme. The frequency of the ALAD2-2 isozyme is shown to be the least predominant as compared to the ALAD1-1 and ALAD1-2 isozymes. Comparing the hemoglobin levels across all the groups, it is apparent that members with ALAD2 allele have lower Hb levels compared to levels for ALAD1.

The results further indicate that members with isozyme ALAD1-1 had their ALAD enzyme activity functioning moderately normal as compared to the rest. Correlational analysis revealed that ALAD enzyme activity and hemoglobin levels strongly correlated with blood lead levels across all the genotypes (Table 2).

**Table 1. The gene distribution of ALAD (delta-aminolevulinic acid dehydratase) isozymes and the corresponding blood lead levels, ALAD enzyme activity, hemoglobin and hematocrit volume among the study participants.**

<table>
<thead>
<tr>
<th>Isozyme</th>
<th>Frequency of ALAD isozymes</th>
<th>Blood lead levels (mean) (μg/dL)</th>
<th>ALAD enzyme activity (mean) (Units/L)</th>
<th>Hemoglobin levels (mean) (g/dL)</th>
<th>Hematocrit volume (%)(mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALAD 1-1</td>
<td>0.889</td>
<td>8.8</td>
<td>39.6</td>
<td>8.9</td>
<td>27.6</td>
</tr>
<tr>
<td>ALAD 1-2</td>
<td>0.106</td>
<td>12.3</td>
<td>34.7</td>
<td>6.8</td>
<td>29.2</td>
</tr>
<tr>
<td>ALAD 2-2</td>
<td>0.005</td>
<td>14.1</td>
<td>33.8</td>
<td>6.1</td>
<td>32.9</td>
</tr>
</tbody>
</table>
Discussion

Various factors including duration (time) of exposure,24 levels of the environmental lead pollutant in the area, nutritional status, age, and genetics modulate one’s lead poisoning susceptibility.25,26 Even with similar environmental settings and confounding factors, variations in susceptibility to lead poisoning among individuals exist.27,28

This study therefore aimed at expounding on the relationship between genetic variations of proteins that code for ALAD enzyme and lead susceptibility among individuals (children aged 6–60 months) living in the same geographical area (Katanga Uganda). Based on the available literature about the stoichiometric inhibitory effect of blood lead ions on ALAD activity,13 we hypothesized that ALAD allele frequency distribution account for one’s blood lead levels. The extent of this ALAD enzyme inhibition is dependent on one’s ALAD protein genetics.13 From the fact that this enzyme (ALAD) is polymorphic with a G-to-C transversion at position 177 (db SNP ID: rs1800435) and two alleles (ALAD1 and ALAD2) and three isozymes; ALAD1-1, ALAD1-2, and ALAD2-2, dominating ALAD allele accounts for lead toxicity susceptibility. It is reported that delta-aminolevulinic dehydratase enzyme polymorphism differs by race, and geographical location. From the current study findings, we report significant correlations between ALAD genotype and Hb level, ALAD genotype and ALAD enzyme activity, and blood lead levels in magnitudes of ALAD1-1 (r = 0.42, p-value = 0.02) < ALAD1-2 (r = 0.62, p-value ≤ 0.001) < ALAD2-2 (r = 0.67, p-value ≤ 0.001) see (Table 2). Blood lead levels were significantly elevated in carriers of ALAD 2-2 isozyme than those of both ALAD1-2 and ALAD1-1 isozyme carriers (Table 1). The variations in lead burden among these three groups observed in Table 1 are attributed to the difference in the electronegativity of the amino-acids lysine and asparagine that code for these isozymes. From this study’s findings, we concur with reports of various previous studies from different regions that indicate a low prevalence of ALAD1-2 and ALA2-2 as compared to the ALAD1-1 genotype. Based on the study results in (Table 1), it is acceptable that having ALAD1-2 and ALAD2-2 isozymes as the less predominant phenotypes delay lead poisoning symptoms, like ALAD enzyme inhibition, than individuals who have ALAD1-1 as the dominating isozyme. We statistically analyzed data groups (ALAD1-1 vs ALAD1-2 and 2-2 individuals) using one-way analysis of variance (ANOVA). Stepwise regression and multiple analyses of variance were used to assess the contribution effect of different ALAD genotypes towards blood lead levels, ALAD enzyme activity, and hemoglobin levels of the study participants (Table 1). Compared to other isozymes, ALAD1-1 genotype, which is encoded by the less electronegative protein lysine, was the most dominant, and because of this, it binds fewer lead ions hence more susceptibility to lead poisoning. This is owed to the fact that lead ions bind ALAD with high electronegative charge tightly, hence reducing the number of bioavailable lead ions in circulation. Then, unbound lead ions circulating freely in the body systems end up affecting many vital organs. However, in times of oxidative and nutritional challenges, this tightly bound lead is released back into circulation resulting in manifestations like anemia, impaired intelligent quotient (IQ), etc.

These findings, therefore, reveal that ALAD polymorphism modifies lead kinetics by, for example, making ALAD2-2 isozyme predominant carriers lower the uptake of lead ions into cortical bones.

This study, therefore, concludes that ALAD polymorphism is of great importance in modulating the toxicokinetics of lead toxicity and therefore recommends a more detailed study on ALAD genotyping involving a bigger Uganda population.

Data availability

Underlying data

This study’s participants were minors, whose data public sharing is ethically restricted. However, the data that support these study findings are available from the corresponding author [J.K., joseph.kyambadde@gmail.com], upon presenting a clear written statement on the purpose of data requested for.

Table 2. Correlations between different ALAD (delta-aminolevulinic acid dehydratase) isozymes, blood lead levels, ALAD enzyme activity, hemoglobin levels and hematocrit volumes.

<table>
<thead>
<tr>
<th>Isozyme</th>
<th>Blood lead levels (μg/dL)</th>
<th>ALAD enzyme activity (Units/L)</th>
<th>Hemoglobin levels (g/dL)</th>
<th>Hematocrit volume (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALAD 1-1</td>
<td>r = 0.42, p-value 0.02</td>
<td>r = 0.66, p-value ≤ 0.001</td>
<td>r = 0.51, p-value ≤ 0.001</td>
<td>r = 0.11, p-value ≤ 0.07</td>
</tr>
<tr>
<td>ALAD 1-2</td>
<td>r = 0.62, p-value ≤ 0.001</td>
<td>r = 0.71, p-value ≤ 0.001</td>
<td>r = 0.69, p-value ≤ 0.001</td>
<td>r = 0.16, p-value 0.06</td>
</tr>
<tr>
<td>ALAD 2-2</td>
<td>r = 0.67, p-value ≤ 0.001</td>
<td>r = 0.71, p-value ≤ 0.001</td>
<td>r = 0.64, p-value ≤ 0.001</td>
<td>r = 0.12, p-value 0.11</td>
</tr>
</tbody>
</table>
Extended data
Dryad: A representative gel of ALAD following a restriction fragment length digestion, https://doi.org/10.5061/dryad.vt4b8gttz.5

Data are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).

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