Environmental lead exposure as a risk for childhood aplastic anemia

Maqusood Ahamed¹,*, Mohd Javed Akhtar¹, Sanjeev Verma², Archana Kumar², Mohammad K.J. Siddiqui³

¹ Indian Institute of Toxicology Research, Lucknow, India; ² Chhatrapati Shahi Maharaj Medical University, Lucknow, India; ³ Council of Science and Technology-UP, Lucknow, India.

Summary
Concern about environmental lead exposure as a significant public health threat has increased as evidence has accumulated regarding adverse health effects at successively lower levels. Aplastic anemia is a hematological disorder of unknown etiology with a high lethality rate. Lead is a known toxicant for the hematopoietic system. Oxidative stress appears to be the possible mode of lead toxicity. We evaluated the effects of blood lead level on oxidative stress parameters in children suffering from aplastic anemia disease. Seventeen children with aplastic anemia disease (15 male and 2 female, age 3-12 y) were recruited in the study group. Fifty one healthy children (45 male and 6 female, age 3-12 y) having normal blood profiles and not suffering from any chronic disease(s) were used as controls. Blood lead level and oxidative stress parameters were determined. Mean blood lead level was significantly higher while δ-aminolevulinic acid dehydratase (δ-ALAD) activity, a biomarker for lead exposure was significantly lower in the study group as compared to the control group (p < 0.05 for each). Thiobarbituric acid reactive species (TBARS), a marker of lipid peroxidation, was significantly higher while the antioxidant glutathione (GSH) level was significantly lower in the study group as compared to the control group (p < 0.05 for each). Activity of the antioxidant enzyme catalase (CAT) was significantly higher in the study group than in the control group (p < 0.05). There was a significant negative correlation of blood lead levels with δ-ALAD (r = −0.45; p < 0.05) and GSH (r = −0.32; p < 0.05), and a positive correlation with TBARS (r = 0.41; p < 0.05) and CAT (r = 0.37; p < 0.05). Although a causal pathway cannot be determined from this study, our results indicated that lead induces oxidative stress in children suffering from aplastic anemia. Lead-induced oxidative stress as an underlying mechanism for aplastic anemia warrants further research.

Keywords: Aplastic anemia, lead exposure, children, oxidative stress

1. Introduction
Aplastic anemia is a hematological disorder with a high lethality rate in which all cellular components of bone marrow origin are deficient (1). The annual incidence rate reported in the literature ranges from 1.5 to 24.6 cases per million with a range for more rigorous studies to be 1.5 to 5.0 (2). The determination of risk factors for aplastic anemia faces multiple difficulties. The evidence of myelotoxicity of several drugs, infectious agents, solvents, and other environmental toxins is circumstantial; there are no tests available that could confirm their cause-effect relationship. In addition, multiple exposures are the rule. For these reasons most cases are classified as idiopathic (3).

Exposure to lead can result in significant effects in multiple organs, with the hematopoietic system being an important target (4-6). Lead interferes with heme
biosynthesis, and it affects formation and function of erythrocytes. A shortening of erythrocyte life span was observed by Terayama (7) in experiments with rats. Grandjean et al. (8) reported a lead-dependent delay for the regeneration of human erythrocytes. Lead, furthermore, interferes with iron utilization for heme formation, and radio-iron studies showed that lead competes with iron for incorporation into erythrocytes (9). If lead was indeed toxic to the hematopoietic system, one would expect the risk of aplastic anemia might be associated with lead exposure.

Children are more susceptible to lead exposure than adults because of their hand to mouth activity, increased respiratory rates and higher gastrointestinal absorption per unit body weight (10). In the 1960s, a blood lead level of 60 μg/dL was considered safe. Due to increased understanding of lead toxicology, the acceptable blood lead level was reduced to 25 μg/dL in 1985 and 10 μg/dL in 1991 by the Center for Disease Control (CDC), USA. Despite these changes, subclinical effects of lead exposure have been reported at blood lead levels less than 10 μg/dL (10). This issue is further complicated by the fact that there is no demonstrated biological function of lead in humans. As such, it is arguable that a “safe” blood lead level cannot be defined.

Evidence suggests that cellular damage mediated by oxidants may be involved in some of the pathophysiology of human diseases including aplastic anemia (11-13). Free radical generation seems to be the possible mechanism of lead toxicity (14-18). In the present study we evaluated the effect of blood levels on oxidative stress parameters in children suffering from aplastic anemia disease.

2. Materials and Methods

2.1. Chemicals

δ-Aminolevulinic acid (δ-ALA), reduced glutathione (GSH), 5,5'-dithio-bis-(2-nitro benzoic acid) (DTNB), thiobarbituric acid (TBA) and lead standards were purchased from Sigma Chemical Co., St. Louis, MO, USA. Hydrogen peroxide (H₂O₂) (30%) was obtained from E. Merck, Mumbai, India. All the other chemicals used were of the highest purity available from commercial sources.

2.2. Selection of patients

The study was designed to enroll children with aplastic anemia in the age group ≤ 12 y. A total of 17 cases of childhood aplastic anemia (15 male and 2 female, age 3-12 y) were enrolled at the Department of Pediatrics, Chhatrapati Shahuji Maharaj Medical University (CSMMU), Lucknow, India. Eligible patients were required to meet at least two of the following three criteria: white blood cell count ≤ 3.5 × 10⁹/L, platelet count ≤ 50 × 10⁹/L, and hemoglobin concentration ≤ 10 g/dL or hematocrit ≤ 30%. If the latter criterion was one of the two fulfilled, a reticulocyte count ≤ 30 × 10⁹/L was also required. Definite diagnosis and final acceptance of the cases required a characteristic hypocellular bone marrow biopsy (marrow cellularity < 30%) without gross marrow fibrosis and absence of infiltration by leukemia, lymphomatous, or carcinoma cells. These defined criteria are identical to those used in the International Agranulocytosis and Aplastic Anemia Study (IAAAS) (19).

2.3. Selection of controls

Fifty one healthy children (45 male and 6 female, age 3-12 y) from a similar socio-economic environment were used as the controls. Inclusion criteria for the selection of controls were hemoglobin concentration (≥ 12 g/dL), hematocrit (40-47%), red blood cell count (4.0-5.5 × 10¹²/L), white blood cell count (4.0-11.0 × 10⁹/L), platelet count (200-500 × 10⁹/L) and not suffering from any chronic disease(s).

Parent’s consent was obtained prior to sample collection and questionnaire administration. The Ethical Committee of CSMMU, Lucknow (India) approved the study. Parents of the subjects were interviewed by trained physicians or nurses to obtain relevant medical history, history of exposure to radiation, and a detailed case history of drug use. Patients who had received chemotherapy, immunotherapy or radiotherapy were not eligible as cases or controls. None of the subjects in either cases or controls were found enzyme-linked immunosorbent assay (ELISA) positive for anti-human immunodeficiency virus (HIV)-I and HIV-II antibody in the serum.

2.4. Blood collection and processing

Approximately 4.0 mL of venous blood was withdrawn from each subject. One milliliter was transferred to a vial containing ethylene diamine tetraacetic acid for routine blood investigation. Another 1.0 mL was transferred in a plain vial (without anticoagulant) for anti HIV-I and HIV-II antibody detection in serum by the ELISA method. These investigations were performed at the Department of Pathology, CSMMU, Lucknow. The remaining 2.0 mL blood was collected in heparinized vials as coded samples, and transported under ice-cold conditions to the Analytical Toxicology Laboratory, Indian Institute of Toxicological Research (IITR), Lucknow for biochemical assays and analysis for lead.

A portion of the blood was used for the preparation of the lysate. Briefly, blood was centrifuged at 2,500 rpm for 15 min at 4°C and the supernatant aspirated. The erythrocyte rich precipitate was washed three times with physiological saline (3:1, v/v) and lysed.
using double distilled water. The particulate material was centrifuged at 15,000 rpm for 90 min at 4°C, and the supernatant (erythrocyte lysate) was collected for the determination of catalase (CAT) activity. Another portion of the blood was utilized for assays of δ-aminolevulinic acid dehydratase (δ-ALAD), thiobarbituric acid reactive species (TBARS), GSH, and analysis of lead. The person analyzing for lead and carrying out biochemical assays was totally blind as to the case history of subjects.

2.5. Biochemical assays

The European standardized method was used to determine blood δ-ALAD activity using δ-ALA as a substrate and expressed as μmol porphobilinogen (PBG) formed/min/L blood (20). The extent of lipid peroxidation in whole blood was determined by measuring the formation of TBARS by the method of Stocks and Dormandy (21) and expressed as nmol TBARS formed per mL blood using a molar extinction coefficient of 1.56 × 10^5 mol⁻¹ cm⁻¹. GSH was estimated in blood using Ellman’s method (22) and expressed as μmol/mL blood. CAT activity in hemolysate was assayed by the method of Sinha (23) using H₂O₂ as substrate and expressed as μmol H₂O₂ decomposed/min/g Hb.

2.6. Analysis of lead

Blood lead was determined using a graphite furnace atomic absorption spectrometer (Varian SpectrAA 250+, Varian Australia Pty Ltd., Victoria, Australia) (24). The instrument was calibrated using aqueous lead standards of 10, 20, 30, and 40 μg/L. The detection limit was 3 μg/L. Fifty microliters of blood were diluted 1:10 in the diluent in a 1.0-mL polystyrene auto-sampler tube. The diluent (Triton X-100, 0.1%, w/v; NH₄H₂PO₄, 0.2%, w/v; NH₃, 0.14 M) was prepared in deionized water. The calibration blank used was 0.2% nitric acid, 0.2% NH₄H₂PO₄ solution and the reagent blank was the diluent solution. Results for duplicate analysis of blood samples agreed within ±7%. Accuracy and precision of the method were checked by spiking the samples with known amounts of standard. Coefficients of variation were 6% and 4% at 10 and 40 μg/L, respectively.

The accuracy of the method for metal estimation was further controlled by participation in an inter-laboratory quality-assurance program (IIITR, Lucknow) wherein coded samples were analyzed regularly and results scrutinized by the quality manager. Further, a quality check sample was always run with each set of samples for lead analysis to maintain accuracy.

2.7. Statistical evaluation

All the data were normally distributed. The significance of differences of mean values of blood lead level and biochemical indices between the study and control groups were compared by Student's t-test. Linear regression analysis was performed to determine strength of relationship between the blood lead level and selected biochemical indices.

3. Results

All the subjects of this study were residents of Lucknow, India and its surroundings, where small-scale industries like smelting processes, recycling of batteries and their use in electronics might contribute substantial amounts of lead to the environment even after the phasing out of leaded-petrol (6,13). Table 1 represents the demographic covariates of the study and control groups. Age, sex, body mass index (BMI), and area of living in the two groups of children were not statistically different. Table 2 shows the comparison of blood lead level and biochemical indices between the study and control groups. Blood lead level was significantly higher in the study group when compared with the control group (p < 0.05). The δ-ALAD activity, a biomarker of lead exposure was significantly lower in the study group as compared to the control group (p < 0.05). Lipid peroxides in blood, as determined by TBARS level was significantly higher while the antioxidant molecule GSH was significantly lower in the study group than in the control group (p < 0.05 for each). Furthermore, antioxidant enzyme CAT activity was significantly higher in the children with aplastic anemia compared to the controls (p < 0.05).

Figure 1 indicates the relationship strength between blood lead level and biochemical indices. There were significant negative correlations of blood lead level with δ-ALAD (r = −0.45; p < 0.05) and GSH level (r = −0.32; p < 0.05) and a positive correlation with TBARS (r = 0.41; p < 0.05) and CAT (r = 0.37; p < 0.05) (See Figure 1).

4. Discussion

The hematopoietic system has been proposed as being one of the important targets of lead-toxicity (4,5). Erythrocytes have high affinity for lead and typically contain the majority of lead found in the blood stream (25). Exposure of children with pre-existing aplastic anemia to lead can have serious consequences, because lead can further impair their hematopoietic system. The existing experimental and epidemiological data on lead toxicity and the occurrence of significant levels of lead in the blood of Indian subjects, especially children, prompted us to study if there is an association between lead and childhood aplastic anemia (6,13,16,17). In the present study, we found that the blood lead level of aplastic anemia patients was significantly higher compared to...
Investigation of lead-induced oxidative stress as an underlying mechanism for aplastic anemia offers the potential to reveal more about the etiology of this complex disease and may provide opportunities for prevention of this disease of unknown etiology. Several lines of evidences suggest that cellular damage mediated by oxidants may be involved in some of the pathogenesis associated with lead intoxication (26,27). Oxidation phenomena and/or the formation of free radicals have been suggested to be causally related to various diseases (11-13). In the present study, activity of the δ-ALAD enzyme was significantly lower in aplastic anemia cases than in the controls. It has been

<table>
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<th>Covariates</th>
<th>Control group (n = 51)</th>
<th>Study group (n = 17)</th>
<th>p value</th>
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<tr>
<td>Age in year (range)</td>
<td>7.49 ± 1.91 (3-12)</td>
<td>7.65 ± 2.80 (3-12)</td>
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<td>BMI (kg/m²)</td>
<td>14.0 ± 2.8</td>
<td>14.4 ± 3.7</td>
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<tr>
<td>Sex</td>
<td>Male 45 (88)</td>
<td>Female 6 (12)</td>
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<tr>
<td>Area of living</td>
<td>Rural 0 (0)</td>
<td>Urban 51 (100)</td>
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</table>

Values represent mean ± S.D. (% of subjects). Student's t-test was applied for determining statistical significance between the cases and controls.

<table>
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<tr>
<th>Parameters</th>
<th>Control group (n = 51)</th>
<th>Study group (n = 17)</th>
<th>p value</th>
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<tr>
<td>Blood lead level (μg/dL)</td>
<td>4.23 ± 1.23</td>
<td>9.86 ± 2.04</td>
<td>&lt; 0.05</td>
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<td>δ-ALAD (μmol PBG formed/min/L blood)</td>
<td>4.10 ± 0.59</td>
<td>3.01 ± 0.31</td>
<td>&lt; 0.05</td>
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<td>TBARS (nmol/mL blood)</td>
<td>13.3 ± 3.9</td>
<td>23.3 ± 4.0</td>
<td>&lt; 0.05</td>
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<tr>
<td>GSH (μmol/mL blood)</td>
<td>24.9 ± 5.2</td>
<td>16.2 ± 3.9</td>
<td>&lt; 0.05</td>
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<tr>
<td>CAT (×10⁴ μmol H₂O₂ decomposed/min/g Hb)</td>
<td>82.3 ± 8.7</td>
<td>1.7 ± 5.3</td>
<td>&lt; 0.05</td>
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Values represent mean ± S.D. Student's t-test was applied for determining statistical significance between the study and control groups. Abbreviations: δ-ALAD, δ-aminolevulinic acid dehydratase; TBARS, thiobarbituric acid reactive species; GSH, glutathione; CAT, catalase.

Figure 1. Statistically significant correlations of blood lead level with selected biochemical indices. (A) Blood δ-ALAD activity plotted as a function of blood lead levels. (B) Blood TBARS levels plotted against blood lead levels. (C) Erythrocyte CAT activity plotted against blood lead levels. (D) Blood GSH levels plotted against blood lead levels. Blood lead level, μg/dL; δ-ALAD, μmol PBG formed/min/L blood; TBARS, nmol/mL blood; GSH, μmol/mL blood; CAT, ×10⁴ μmol H₂O₂ decomposed/min/g Hb.
suggested that inhibition of δ-ALAD enzyme by lead directs the accumulation of its substrate δ-ALA that can be rapidly oxidized to generate free radicals (6). On the other hand, lead per se has the capacity to stimulate ferrous ion initiated membrane lipid peroxidation (14). Lipid peroxides in the blood, as determined by TBARS level, were significantly correlated with blood lead levels in the children of the general population (16,28). In this study, TBARS level was significantly higher in aplastic anemia patients. Our finding is further supported by other experimental studies where blood TBARS level was higher due to lead exposure (14,15). Peroxidation of erythrocyte membrane lipids can be very damaging because it leads to alterations in the biological properties of the membrane, such as the degree of fluidity and can result in inactivation of membrane-bound receptors or enzymes, which in turn may impair normal cellular function, increase tissue permeability and shorten the life span of blood cells.

Lead can lower cellular concentrations of hematoproteins and GSH, thus reducing the reduct bufferng capacity of cells (29). In this study, GSH status significantly declined in the blood of aplastic anemia patients as compared to the controls. GSH is a tri-peptide containing cysteine that has a reactive thiol (-SH) group with reductive potency. GSH acts as a non-enzymatic antioxidant by direct interaction of the -SH group with free radicals, or it can be involved in the enzymatic detoxification reactions for free radicals as a cofactor (30). Evidence for involvement of free radicals in the pathophysiology of aplastic anemia also arises from the significant increase of erythrocyte CAT activity in children with aplastic anemia. CAT has been suggested to provide an important pathway for H2O2 activity in children with aplastic anemia. CAT has been

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References


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