Published in final edited form as:

Arch Environ Contam Toxicol. 2003 April ; 44(3): 417–420. doi:10.1007/s00244-002-2023-4.

Lead-Induced Cytotoxicity and Oxidative Stress in Human Leukemia (HL-60) Cells

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Abstract

Lead poisoning has been extensively studied over the years. Many adverse physiological and behavioral impacts on the human body have been reported due to the entry of this heavy metal. It especially causes the hematological effects to people of all ages. However, its molecular mechanisms of action remain largely unknown. Hence, the aim of the present study was to evaluate the cytotoxicity and oxidative stress induced by lead nitrate in a human leukemia cell line using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide], and lipid hydroperoxide assays, respectively. HL-60 cells were treated with different doses of lead nitrate for 24 h prior to cytotoxicity oxidative stress assessment. The results obtained from the MTT assay indicated that lead nitrate significantly decreases the viability of HL-60 cells in a dose-dependent manner. Similar result was obtained with the trypan blue exclusion test. Data generated from lipid hydroperoxide assay resulted in a significant increase ($p < 0.05$) in the production of hydroperoxides (degradation products of lipid peroxidation) with increasing doses of lead nitrate. Upon 24 h of exposure, the hydroperoxide concentrations in the sample [μM] (mean $\pm$ SE, $n = 3$) compared to untreated control were 6.7 ± 2 , 7.1 ± 1 , 14.7 ± 2 , 15.7 ± 1 , 16.2 ± 1 , and 15.2 ± 1 in 0, 10, 20, 30, 40, and 50. $\mu\text{g/mL}$ of lead nitrate, respectively. In summary, findings from this study demonstrated that lead nitrate is cytotoxic to HL-60 cells. This cytotoxicity is found to be associated with oxidative stress.

Introduction

Lead is a multi-targeted toxicant that affects many organ systems including; the gastrointestinal tract, hematopoietic system, cardiovascular system, central and peripheral nervous systems, immune system, and reproductive system. It is considered as the most clinically important heavy metals because it induces a broad range of physiological, biochemical, and behavioral dysfunctions. One of the major mechanisms by which lead exerts its toxic effect is through biochemical processes that include lead's ability to inhibit or mimic the actions of calcium and to interact with proteins (1). Many investigators have demonstrated that lead intoxication induced cellular damage mediated by the formation of reactive oxygen species (ROS) (2).

Although there are many studies showing evidence that lead is a multi-targeted toxicant, causing effects in the gastrointestinal tract, cardiovascular system, central and peripheral nervous systems, immune system, and reproductive system, little is known about the effect of lead on the hematopoietic system. Hence, the present study was designed to use human leukemia (HL-60) cells as models to determine whether exposure to lead is associated with the

prevalence of acute promyelocytic leukemia (APL), and to determine whether oxidative stress plays a key role in lead nitrate-induced cytotoxicity in this cell line.

Material and Methods

Chemicals and media

Reference solution (1000 ± 10 ppm) of lead nitrate (CAS No. 10099-74-8, Lot No. 981735-24) with a purity of 100% was purchased from Fisher Scientific in Fair Lawn, New Jersey. Growth medium RMPI 1640 containing 1 mmol/L L-glutamine was purchased from Gibco BRL products (Grand Island, NY). Fetal bovine serum (FBS), antibiotics (penicillin G and streptomycin), phosphate buffered saline (PBS), and MTT assay kit were obtained from Sigma Chemical Company (St. Louis, MO). Lipid peroxidation kit was purchased from Calbiochem-Novabiochem (San Diego, CA).

Tissue culture

In the laboratory, cells were stored in the liquid nitrogen until use. They were thawed by gentle agitation of their containers (vials) for 2 minutes in a water bath at 37°C. After thawing, the content of each vial of cells was transferred to a 25 cm² tissue culture flask, diluted with up to 10 mL of RMPI 1640 containing 1 mmol/L L-glutamine (GIBCO/BRL, Gaithersburg, MD) and supplemented with 10% (v/v) fetal bovine serum (FBS), and 1% (w/v) penicillin/streptomycin. The 25 cm² culture flasks containing 2×10^6 viable cells were observed under the inverted microscope, followed by incubation in a humidified 5% CO₂ incubator at 37 °C. Three times a week, they were diluted and maintained under same conditions at a density of 5×10^5 /mL and harvested in the exponential phase of growth. The cell viability was assessed by the trypan blue exclusion test (Life Technologies) and manually counted using a hemocytometer.

Cytotoxicity / MTT assay

Human leukemia HL-60 cells were maintained in RMPI 1640 containing 1 mmol/L L-glutamine, supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (w/v) penicillin/streptomycin, and incubated at 37°C in humidified 5% CO₂ incubator. To 180 µL aliquots in six replicates of the cell suspension (5×10^5 /mL) seeded to 96-well polystyrene tissue culture plates, 20 µL aliquots of stock solutions were added to each well using distilled water as solvent to make-up final lead nitrate doses of 0.8, 3.12, 12.50, and 50.00 µg/mL. Control cells received 20 µL of distilled water. Cells were placed in a humidified 5% CO₂ incubator for 24 h at 37°C. After incubation, 20 µL aliquots of MTT solution (5 mg/mL in PBS) were added to each well and re-incubated for 4 h at 37 °C following by low centrifugation at 800 rpm for 5 min. Then, the 200 µL of supernatant culture medium were carefully aspirated and 200 µL aliquots of dimethylsulfoxide (DMSO) were added to each well to dissolve the formazan crystals, followed by incubation for 10 min to dissolve air bubbles. The culture plate was placed on a Biotek micro-plate reader and the absorbance was measured at 550 nm.

Statistical analysis

Data were presented as means $\pm$ SDs. Regression analysis was performed to determine the lethal dose (LD₅₀)-chemical dose capable of killing 50% of the cell population. Statistical analysis was done using one way analysis of variance (ANOVA) for multiple samples and Student's t-test for comparing paired sample sets. *P*-values less than 0.05 were considered statistically significant.

Results

Effect of lead nitrate on cell viability

HL-60 cells were incubated with different concentrations of lead nitrate (0.8 to 50 µg/mL) for 24 h. After incubation, cell viability was determined by MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay. The viability of control cells was designated as 100%, and the others were expressed as percent compared to the control. As shown in (Fig 2), there was a slight increase in cell viability at 0.8 µg/mL compared to the control. When exposed to 3.12 µg/mL and higher concentration of lead nitrate, the viability of HL-60 cells decreased significantly and reached the statistical significance ($P < 0.05$).

Lipid hydroperoxide assay

The lipid hydroperoxide assay showed a significant ($p \leq 0.05$) increased in lipid hydroperoxide levels to HL-60 cells treated with lead nitrate compared to the control, indicating that lead increased cellular content of reactive oxygen species (ROS), as evidenced by the increase in lipid hydroperoxide (Fig 5, and 6). Overall, findings from these studies suggest that lead nitrate-induced cytotoxicity and oxidative stress in HL-60 cells through the generation of reactive oxygen species.

Discussion

Cytotoxicity of lead

In this study, MTT assay was used to determine the general cytotoxicity of lead nitrate in HL-60 cells. It showed that lead could gradually decrease the viability of HL-60 cells. According to the result of MTT assay, lead nitrate at concentration of 10, 20, 30, 40, and 50 µg/mL were used in the subsequent oxidative and DNA damage-related experiments. Data obtained from the present study clearly indicate that lead nitrate is highly cytotoxic to human leukemia (HL-60) cells. The LD₅₀ was computed to be 34.6 ± 5.2 µg/mL upon 24 hrs of exposure. These results support those of previous investigations reporting a marked reduction in the viability of HL-60 cells following exposure to higher levels of lead (3,4). We previously reported a similar trend with arsenic trioxide-treated HepG₂ cells in our laboratory (5). Data from a recent study also indicate that lead is highly toxic to immune cells by inhibiting cell adhesion property, and altering cell morphology in the splenic macrophages of mice (6). Although fatal lead poisoning occurs rarely in the United States, several epidemiological have pointed out that it represents a medical and public health emergency, especially in children consuming high amounts of lead-contaminated flake paints (7). Death in these lead-poisoned children has been associated with extreme lethargy with facial palsy and gasping respirations consistent with lead encephalopathy, and severe hematologic abnormalities (7).

Lipid Hydroperoxide Assay

Lipid hydroperoxides derived from unsaturated phospholipids, glycolipids, and cholesterol are prominent intermediates of peroxidative reactions induced by activated species such as hydroxyl radical, lipid oxyl or peroxy radicals, singlet oxygen, and peroxynitrite (8). Once formed, Lipid hydroperoxides may undergo reductive degradation which either diminishes or enhances cytotoxic potential, depending on a variety of circumstances (8,9). In addition, lipid hydroperoxides or related peroxidation intermediates/products may trigger signal transduction pathways calling for either greater cytoprotection (exemplified by up-regulation of detoxifying enzymes) or deliberate termination (apoptotic death) (10). Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals, and is used as an indicator of oxidative stress in cells and tissues (11). Our results clearly showed that the treatment of HL-60 cells with lead nitrate resulted in a significant increase of lipid hydroperoxide levels, a

major degradation product of unsaturated phospholipids and glycolipids. Upon 24 hrs of exposure, the lipid hydroperoxide level value was computed to be $16.08 \pm 1.05 \mu\text{M}$ at highest dose tested. A series of recent studies showed that rats exposed to lead had an elevation of blood pressure accompanied by a marked increase of lipid peroxidation product (MDA) in the plasma and tissue; and a substantial reduction in urinary excretion of stable NO metabolites (NOx) (12). Metal-induced lipid peroxidation is mostly attributed to increased production of free radicals (13). Damage to cell organelles produced by lipid peroxidation has been demonstrated by many investigations. However, there is not a firm evidence about the mechanism of lipid hydroperoxide. Studies have reported that lead causes two types of unfavorable processes in biological systems. Firstly, it inactivates several enzymes by binding with their SH-groups. Secondly, lead ions, similar to other heavy metals can intensify the production of reactive oxygen species (ROS) leading to oxidative stress (14,15). These processes potentially affect and destroy cell structure through the metabolic pathway (16). In support of this proposition, our results indicate that excess lead nitrate increases the generation of hydroperoxides in HL-60 cells, indicative of oxidative stress, a biomarker of cellular injury.

Conclusions

Results from this study indicate at the cellular level that lead nitrate significantly reduced the viability of human leukemia (HL-60) cells in a dose-dependent manner. At the molecular level, lead treatment resulted in a significant increase in lipid hydroperoxide generation in HL-60 cells. Findings from this study indicate that lead nitrate is highly cytotoxic to HL-60 cells. This cytotoxicity is found to be mediated through oxidative stress, a biomarker of cellular injury.

Acknowledgments

This research was financially supported partly by a grant from J.S.U. Center for University Scholar, and by a grant from the U.S. Department of the Army (Cooperative Agreement No. W912H2-04-2-0002) through the CMC Program at Jackson State University. The authors thank Dr. Mary Coleman: Associate Dean of the College of Liberal Arts and Director, Center for University Scholar for her technical support.

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Lipid hydroperoxide assay

Figure 1.
Schematic Representation of the Steps in Lipid Hydroperoxide (LHP) Assay

Figure 2.
Cytotoxicity of lead nitrate to human leukemia (HL-60) cells

Figure 3.
LHP standard curve showing the net absorbance at 586 nm as a function of lipid hydroperoxide concentration.

Figure 4. Effects of different concentrations of lead nitrate on LHP generation in HL-60 cells.