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POTENTIAL OXIDATIVE STRESS DUE TO Pb EXPOSURE

A Senior Thesis

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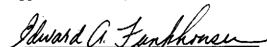

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Potential Oxidative Stress due to Pb Exposure

Abstract

The hazards of Pb exposure has been a topic of concern for many years. This research was developed to investigate the possibility of Pb induced oxidative stress. The research objectives were to observe Pb induced lipid peroxidation and Pb induced increases in oxidation of glutathione in K562 myelogenous leukemia cells at low Pb levels. The approach consisted of incubating K562 cells in solutions of 0, 300, and 700 ppb Pb dissolved in RPMI cell medium for a total of 96 hours. After this period of incubation, aliquots of cells were taken, placed in clean medium, and incubated for 96 hours. Samples were taken at 24 and 96 hours of Pb exposure, and at 96 hours after cessation of Pb exposure, then subsequently assayed for lipid peroxidation and glutathione levels. Lipid peroxidation was determined by the detection of malondialdehyde (MDA) spectrophotometrically at 535nm by the Thiobarbituric acid (TBA) assay, while oxidized and reduced glutathione levels were determined spectrophotometrically at 412 nm. Results showed no change in MDA levels in treated cells as compared to the control. The GSH:GSSG ratios were significantly greater for treated cells at 96 hours of exposure as compared to the controls, indicating a possible compensatory response.

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Introduction

The topic of lead (Pb) exposure has been a visible issue for many years. Even though lead-based paint and lead gasoline have been banned, the threat still exists at a high level. Lead enters our country in dishes, lead crystal, pewter and other sources, in very high concentrations. The paint in most homes contains lead which can easily be contacted through paint dust and chips by our families. Soil has been contaminated by this toxic element from lead paint on the exterior of homes and from home renovations. Pipes and solder hold extremely high concentrations, and result in contamination of water. Even though many medical manifestations of lead exposure are known, there is a great deal of knowledge to learn about the biochemical effects and mechanisms of Pb toxicity. This research is an attempt to explore possible mechanisms of Pb toxicity, specifically potential Pb-induced oxidative stress in mammalian cells.

Background

Sources of Pb

Sources of Pb exposure have been underestimated, there are many and they are quite varied. A government study shows that 3 out of 4 U.S. homes built before 1980 contain potentially toxic Pb-based paint. (Lehman, 1991) Pb-based paint was banned in 1978. The widespread presence of Pb in private housing, revealed by a Department of Housing and Urban Development survey, helped to prompt the government to increase its efforts to reduce Pb exposure. (Hines & Randal, 1989) In 1990 the EPA reduced the acceptable level of Pb in tap water from 50 ppb to 20 ppb. The growing understanding of the harmful effects of low levels of Pb in the bloodstream also has increased action to alleviate the problem of Pb exposure.

The major source of lead contamination is dust containing invisible lead-based paint, as identified by the EPA and Department of Health and Human Services (HHS). Exterior coats of Pb-based paint chalk off over time. The dust enters the soil and is tracked into the house. Interior paint dust is generated by wear, particularly in window wells. Greater quantities accumulate with scraping and sanding during a remodeling or renovation project. (Lehman, 1991)

Other sources of lead include dishes, lead crystal, pewter, bread bags, and some water supplies. Dishes manufactured in Mexico and the Orient contain extremely high Pb levels in the glaze and paint used for decoration.

Lead-crystal decanters holding alcoholic beverages initially tested at 89 ppb Pb, can leach 2,160-5,330 ppb Pb after faster months. (Raloff, 1991) Warm acidic liquids, including coffee and baby formula, can leach Pb levels approximately four times faster than cold to room temperature. (Elms, 1992; Raloff, 1991)

Bread bags also pose a threat of Pb poisoning. When bread bags are turned inside-out and are reused, the Pb contained in the ink can then come into contact with food stored in those bags. In some bags, if a minute fraction of the Pb were to rub off onto the food, a young child's intake could exceed the estimated maximum tolerable daily intake from all sources. Besides food contamination, the leaded printing ink will contaminate the environment. Lead emitted from incinerator smokestacks and leached from landfills contributes to general pollution problems. (Heim, 1991)

Toxicity of Pb

Lead has no known physiological or metabolic benefit. Many organs and metabolic systems are adversely affected by Pb exposure. The primary target organs for

lead toxicity are the brain and central nervous system. Effects of Pb on these organs are especially magnified during early childhood development. Low levels can result in delayed cognitive development, reduced IQ scores, impaired hearing, and inhibited growth. High levels of Pb can cause problems such as fatigue, irritation, sleep disturbance and constipation. Severe cases of Pb exposure have been reported to result in anemia, acute abdominal colic, acute and chronic encephalopathy (brain damage), and chronic nephropathy (kidney damage). (Allenby & Kizer, 1989)

Biochemical Manifestations of Pb Exposure

Lead interferes with cell metabolism by causing a deficiency of pyrimidine 5' nucleotidase (P5N), as well as other crucial enzymes (Considine, 1984). It has been suggested that the presence of Pb influences the membrane permeability to Pb (Simons, 1986; Elms, 1992). Studies of lead transport show that lead is rapidly taken up by red blood cells (Bambach, et al, 1942; Mortenson, et al, 1944; Clarkson, et al, 1958; Elms, 1992). Nearly all the lead in human blood is bound to the red blood cells (DeSilvia, 1981), and much of that is bound to hemoglobin (Barltrop & Smith, 1971). Lead binding to hemoglobin is unaffected by oxygenation (Simons, 1986). As a result of chemical similarities between Ca and Pb, bones serve as a repository for lead accumulated in the body. The lead released has a toxic effect when it is transferred to soft tissues. Work in this area has been inhibited by chemical problems associated with the insolubility of many Pb salts in water.

Objective

As a result of a growing concern about Pb exposure in our public and home environment, I have conducted independent original research on Pb toxicity. In previous research I conducted an analysis of potential toxicity in food containers to determine Pb exposure levels in a typical household. I found leaching levels ranging from 2.3 ppb Pb in a tin can to over 700 ppb Pb in a china plate. Therefore I determined the levels of Pb to be used in my experimentation would be 0, 100, 300, 500 and 700 ppb Pb. From this research using erythrocytes and cultured cells (K562 myelogenous leukemia cells) several important conclusions were drawn. Exposure to lead resulted in an uptake of lead into human erythrocytes at a concentration proportional to the exposure level; with a slight decrease in cell volume at exposure levels of 500 and 700 ppb Pb (as tested by hematocrit). The uptake of lead in the erythrocytes resulted in an alteration of the membrane permeability. Specifically iron (Fe) permeability was shown to be affected, resulting in a build up of iron in the cells. This build up increases as lead exposure increases. In liver cells, iron build-up has been found to produce free radicals which damage the cell DNA, resulting in mutation and sometimes carcinogenic behavior.

As a result of observing the build-up of iron in erythrocytes as a result of lead exposure, the question arose as to the possibility of this occurring in nucleated cells. If indeed a build-up of iron occurs in nucleated cells as a result of lead exposure, resulting in free radical production, free radicals may be a mechanism by which lead exposure, directly or indirectly causes adverse effects on organs and metabolic systems. By virtue of its chemical nature, Pb cannot produce free radicals directly. Therefore any free radical

production or build-up as a result of Pb exposure would have to occur indirectly. For example, a build-up of a substance such as Fe or by depleting and/or disrupting the cell's antioxidant defense systems. If the production of reactive oxygen species such as hydroxyl radical, hydrogen peroxide, and superoxide ions occurs as a by-product of Pb exposure, the cell's antioxidant defense systems could be depleted. This would leave the cell vulnerable to subsequent free radical production and oxidative damage (Elms, 1992; Ercal, et al., 1996). Therefore, the objective of this study was to investigate potential oxidative stress due to Pb exposure in mammalian cells.

Free radicals are very difficult to detect directly as a result of their short lifespan and low concentrations, therefore indirect methods of detection must be employed (Greenwald, 1985; Haliwell & Gutterridge, 1989). Two such methods, or indeces for identifying oxidative stress are determination of lipid peroxidation and determination of oxidized (GSSG) and reduced glutathione (GSH) levels (Armstrong & Browne, 1994).

Free radicals can easily damage lipids, producing reactive intermediates which can be detected and measured(Armstrong & Browne, 1994; Yagi, 1994). Malondialdehyde is produced in proportion to lipid peroxidation and can be used as an indicator of the extent of lipid peroxidation. Lipid peroxidation in Chinese Hamster ovary cells has been shown to occur at high Pb levels (10-1000 ppm) (Ercal, et al., 1996). In addition, in human skin fibroblasts lipid peroxidation was found to occur as a result of Pb exposure with an age-dependent action (Dominguez, et al., 1995). Sandhir and Gill observed increased lipid peroxidation, and decreased activity of oxidative enzymes in the liver of rats exposed to Pb (Sandhir & Gill, 1995).

Glutathione (GSH) is a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) which is found in most mammalian cells and participates in many biological processes, including protein and amino acid synthesis, sulphur-containing amino acid metabolism, and cellular antioxidant defense system by reducing disulfide linkage of proteins and other molecules, or by scavenging free radicals and reactive oxygen intermediates (Meister & Anderson, 1983; Meister, 1991; Yoshida, et al., 1995). GSH is synthesized by γ-glutamylcysteine synthetase and GSH synthetase. GSH can be oxidized to form GSSG by several pathways, including selenium-containing GSH peroxidase, transhydrogenation, and by reaction with free radicals (Meister & Anderson, 1983). Conversion of GSH to GSSG and depletion of glutathione can be used as an index of oxidative stress. Legare, et al. reported that low-level Pb exposure (0.1 and 1 uM) in cultured astroglia resulted in a reduction of glutathione content after 7 hours, and further reduction after 24 hours. By 48 hours glutathione content returned to control levels, and exceeded control levels after 6 and 9 days of Pb treatment, indicating a compensatory response. (Legare, et al., 1993) Ercal, et al. reported a decrease in GSH:GSSG ratio following high levels of Pb (10-1000 ppm) exposure, indicating Pb-induced oxidative stress (Ercal, et al., 1996).

From the previous results I have obtained of Fe build-up due to Pb exposure, the Pb induced inhibition of cytochrome oxidase and many other enzymes by disulfide bonds, and previous evidence of lipid peroxidation and altered glutathione levels in other cells and systems, I expected to observe mild oxidative activity resulting from Pb exposure, as tested by glutathione levels and using malondialdehyde as an index of lipid peroxidation.

Methods and Materials

K562 myelogenous leukemia cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and maintained at 37°C in 6% CO_2/94% air. Cell counts were taken using trypan blue and an hemocytometer. Flasks were seeded at approximately $3x10^5$ cells/ml with a total volume of 50 ml of cells and medium. This was the cell count at the start point of incubation. Cells were exposed to three concentrations of Pb acetate (0, 300, & 700 ppb Pb) for approximately 96 hours, and then Pb exposure was stopped and the cells cultured in Pb-free medium for another 96 hour period. This was to simulate short-term exposure, and the possible compensation by the cells after cessation of Pb exposure. Samples were taken at 24 and 96 hours of Pb exposure, and 96 hours after cessation of Pb exposure. These samples were tested for lipid peroxidation by the thiobarbituric acid (TBA) assay, and for oxidized and reduced glutathione levels spectrophotometrically. Protein was determined spectrophotometrically using a protein determination kit (BCA-1) purchased from Sigma.

Lipid peroxidation - Thiobarbituric (TBA) Assay

The TBA assay detects MDA, one of the products of lipid peroxidation which is produced in a constant proportion to lipid peroxidation. One molecule of MDA reacts with 2 molecules of TBA, forming a chromogen which is detected spectrophotometrically.

Procedure

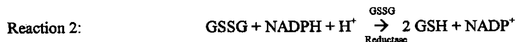
The assay was conducted by mixing a cell sample with 2 volumes of the TBA reagent consisting of 0.375% TBA(w/v) and 15% trichloroacetic acid(w/v) in 0.25 N HCl in a screw capped glass centrifuge tube. The mixture of cell sample and TBA reagent was placed in a boiling water bath for 15 minutes, cooled, centrifuged, and the absorbance of

the supernatant measured at 535 nm. Concentrations of the TBA-MDA adduct were determined by using a molar extinction coefficient of 156,000 M^{-1} cm^{-1}. (Greenwald, 1985 & Burdon, 1991)

GSH & GSSG Determination - DTNB-GSSG Reductase Recycling Assay

The DTNB-GSSG Reductase Recycling Assay was used to detect oxidized and reduced glutathione levels spectrophotometrically at 412 nm as described by Anderson. (Anderson, 1985) The detection of total GSH is accomplished by oxidizing GSH by 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to produce GSSG and 5-thio-2-nitrobenzoic acid (TNB) (Reaction 1). By the subsequent reaction with GSSG Reductase and NADPH, the GSSG is reduced to GSH (Reaction 2). TNB formation is followed at 412 nm. The rate of TNB formation is proportional to the sum of the GSH and GSSG present in the sample.

Reaction 1: $2\ GSH + DTNB \rightarrow GSSG + TNB$



Reaction 2: $GSSG + NADPH + H^{+} \xrightarrow[\text{Reductase}]{\text{GSSG}} 2\ GSH + NADP^{+}$

In order to assay for GSSG alone, samples undergo an additional preparation procedure. Once total GSH (GSH+GSSG) and GSSG levels are determined, the value obtained for GSSG is subtracted from the value obtained for total GSH to give the amount of GSH (reduced) in the sample.

Sample Collection

Cell samples were centrifuged at 0°C for 5 minutes (3000 g) immediately after collection, the incubation media removed, and the cell pellet washed with cold PBS. 1 ml of ice cold 5% 5-sulfosalicylic acid (5-SSA) was placed on the cell pellet for

deproteinization, and the sample sonified for 15-20 sec. The samples were centrifuged in a microfuge (10,000 g) at 0°C for 5 minutes and the supernatant removed and placed in a separate tube. The supernatant and pellets were stored at -20°C until analysis.

Reagents

The stock buffer used for oxidized and reduced glutathione determination consisted of 143 mM anhydrous sodium phosphate, 6.3 mM Na_4-EDTA hydrate at pH 7.5. Each time the stock buffer was used the pH was determined and corrected if necessary. All other reagents used were prepared in this stock buffer as follows:

Daily buffer: 0.248 mg/ml NADPH in stock buffer, prepared daily and stored at 4°C or on ice.

DTNB: 6 mM DTNB in stock buffer, stored frozen until use, then placed on ice.

GSSG Reductase: (yeast enzyme) diluted to 120 U/ml using stock buffer, prepared daily and stored at 4°C or on ice.

GSH Standards (for total GSH): prepared from a 500 uM GSH stock in stock buffer, and diluted to 0,1,2,3, and 4 nmol/10 ul using stock buffer and 5% 5-SSA.

GSSG Standards: prepared from a 500 uM GSSG stock in stock buffer, diluted to 0, 0.1, 0.25, 0.5, 0.75, 1 nmol/10 ul using stock buffer and 5%-SSA.

Sample Preparation

Determination of total GSH requires no additional sample preparation - aliquots for the recycling assay are taken directly from the collected supernatant, but determination of GSSG requires an additional procedure prior to conducting the assay. GSSG levels are low compared to GSH, and therefore it is necessary to prevent artificial oxidation of GSH. This is accomplished by using 2-vinylpyridine to react with GSH in the sample,

leaving only GSSG to participate in the recycling assay. 100 ul of the sample (or standard) is treated with 2 ul of 2-vinylpyridine with vortexing, and 6 ul of triethanolamine (TEA) with vortexing. The final pH is determined with pH paper and must be between 6 and 7, otherwise a new sample must be prepared. More TEA is added if the pH is too low and less is added if the pH is too high. After incubating at room temperature for 1 hour the samples are assayed as described below.

Procedure

700 ul of daily buffer, 100 ul of DTNB solution, and 175 ul of water are pipetted into a cuvette, and the cuvette warmed at 37°C for approximately 10 minutes. 25 ul of the sample is added with mixing. GSSG reductase (5 ul for total GSH, 10 ul for GSSG) is added with mixing to begin the assay, and the cuvette immediately placed in the spectrophotometer. The formation of TNB is monitored over a 2 minute period. The amount of total GSH or GSSG is determined by comparing the rate of TNB formation, as indicated by the rate of change of absorbance, to a standard curve in which GSH or GSSG (nmol) is plotted against the rate of change of absorbance(dA/min) at 412 nm.

Results

Lipid Peroxidation - TBA Assay

The results from the TBA assay for detection of malondialdehyde are presented in Table 1. One-way Analysis of Variance (ANOVA) was performed comparing the 300 and 700 ppb Pb samples to the control for each respective time period with $\alpha = 0.05$. Results indicated that $P > 0.05$ for all tests performed. Therefore no difference was observed in

MDA levels for samples exposed to 300 and 700 ppb Pb as compared to the control for each time period.

GSH and GSSG Determination

The results of GSH and GSSG determination are segregated by exposure time; 24 hours (Table 2), 96 hours (Table 3), and 96 hours of recovery (Table 4). One-way Analysis of Variance (ANOVA) was performed comparing the 300 and 700 ppb Pb samples to the control for each respective time period for levels of GSH, GSSG, and the GSH:GSSG ratio ($\alpha = 0.05$). $P>0.05$ for GSH, GSSG, and GSH:GSSG ratio for samples taken at 24 hours of exposure and 96 hours of recovery (after cessation of Pb exposure), and for GSH and GSSG at 96 hours of exposure. $P<0.05$ for the GSH:GSSG ratios at 96 hours of exposure. The samples exposed to 300 ppb Pb had the highest ratio, 700 ppb Pb the next highest, and the control (0 ppb Pb) had the lowest ratio.

TABLE 1:

MDA levels in K562 cells exposed to 0, 300, & 700 ppb Pb

Pb exposure (ppb)	MDA levels(nmol/mg protein)		
	24 hour exposure	96 hour exposure	96 hour recovery
0	2.04±.03	1.17±.18	0.93±.04
300	2.03±.17	1.14±.02	0.96±.12
700	1.95±.05	1.26±.24	0.99±.01

NOTE:
P>0.05 for all time periods when comparing to the control for each respective time period.
(Using One-way Analysis of Variance)

TABLE 2:

GSH and GSSG levels in K562 cells exposed to 0, 300, & 700 ppb Pb After 24 hours of Exposure

Treatment (ppb Pb)	GSH (nmol/ mg protein)	GSSG (nmol/ mg protein)	GSH:GSSG ratio
0	79.58±4.2	1.019±0.16	78.76±8.24
300	77.95±2.97	1.109±0.14	71.05±11.78
700	99.09±21.92	0.938±0.03	106.1±27.1

NOTE:
P> 0.05 for all groups when compared to control
(Using One-way Analysis of Variance)

TABLE 3:

GSH and GSSG levels in K562 cells exposed to 0, 300, & 700 ppb Pb After 96 hours of Exposure

Treatment (ppb Pb)	GSH (nmol/ mg protein)	GSSG (nmol/ mg protein)	GSH:GSSG ratio
0	54.93±12.34	0.805±0.14	67.91±3.43
300	59.43±2.05	0.618±0.03	96.15±0.711*
700	53.90±5.23	0.661±0.07	81.62±1.34*

NOTE:
*P< 0.05 compared to corresponding value of control
(Using One-way Analysis of Variance)

TABLE 4:

GSH and GSSG levels in K562 cells exposed to 0, 300, & 700 ppb Pb After 96 hours of Recovery

Treatment (ppb Pb)	GSH (nmol/ mg protein)	GSSG (nmol/ mg protein)	GSH:GSSG ratio
0	43.03±2.2	0.488±0.213	96.51±37.74
300	36.74±0.83	0.286±0.214	180.2±138.7
700	47.74±3.78	0.508±0.302	111.3±58.61

NOTE:
P> 0.05 for all groups when compared to control
(Using One-way Analysis of Variance)

Discussion

No apparent difference was observed in MDA levels in cells exposed to 300 and 700 ppb Pb as compared to the controls for each time level. This could be a result of the low levels used, since an increase in MDA levels has been observed at higher levels of Pb exposure. The TBA assay is used as a screening assay for lipid peroxidation and is not as sensitive as chromatographic methods. If extremely low levels of MDA formation occurs, this assay may not have the sensitivity to detect the formation. In addition, screening could be conducted more immediately after Pb exposure was initiated to see if initial MDA formation occurs with subsequent compensation by the cell.

The GSH:GSSG ratio was only found to be significantly different from the controls in the Pb treated samples taken at 96 hours of Pb exposure. The ratios for the Pb treated samples was higher than for the control (67.1 ± 3.43), with the 300 ppb Pb (96.15 ± 0.71) exposure level having a higher ratio than the 700 ppb Pb (81.62 ± 1.34) exposure level. Both sets of Pb treated cells exhibited increases in GSH levels above the control. This follows with the observations of Legare, et al. in astroglia - a compensatory response to the effects of Pb several days into exposure (Legare, et al., 1993). The lower level of exposure exhibited a greater compensatory response. This is not necessarily unusual, considering lower levels of some substances can cause an increase in production of a substance, while higher levels can cause an inhibitory response.

Considerations for future study of the effects of Pb on GSH and GSSG levels in K562 cells could include determination during the first 24 hours of exposure, and over long term exposure (several weeks), in order to better ascertain any trends that may occur.

In addition, many more samples need to be treated in order to attempt to decrease the high sample variance experienced in some parts of the experimentation.

Overall, lipid peroxidation as tested here in K562 cells treated with 300 and 700 ppb Pb was not observed as compared to controls. Alterations in GSH and GSSG levels and GSH:GSSG ratio in K562 cells exposed to 300 and 700 ppb Pb was only observed at 96 hours of exposure, with the Pb treated cells potentially exhibiting a compensatory response. The conclusions of this research leave much room for further investigation, especially involving GSH levels. More sensitive and more varied studies need to be conducted to fully determine whether or not potential oxidative stress occurs as a potential mechanism for low level Pb exposure. In addition, an essential component of this research should be to investigate the effect of low level Pb exposure on Fe levels in a variety of cell types.

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Co-Advisor: Dr. Evelyn Tiffany-Castiglioni - advisement, lab support, use of the spectrophotometer used for glutathione determination.
Dr. Martyn Gunn - advisement and use of cell culture facilities.
Dr. Lora Lindahl - advisement and assistance in learning the DTNB-GSSG Reductase. Recycling Assay and protein determination assay.
Dr. Michael Nazimec - use of the spectrophotometer used for the TBA assay.