Assessing Lead Effects on Fisher-344 Rats Using ICP-MS and Histology
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Abstract
As a way of laying the foundation for research in analyzing global gene expression patterns in Fisher 344 rat liver, the effects of lead toxicity on Fisher 344 rats were assessed using histopathology and tissue residues were measured for metal ions using ICP-MS. Accumulation of lead in blood, liver, kidney and bone marrow increased significantly in lead exposed groups. With the exception of kidney, the 90-days treatment groups showed markedly high levels of lead in blood, liver and marrow than the 30-days exposed groups. Potential interactions of calcium, iron and zinc and lead examined showed positive and negative correlations. Hepatic histopathology produced no evidence of necrosis nor changes in architecture of hepatocytes in the 0 ppm and 50 ppm for the 30-day duration of exposure in the case of the liver. In contrast, necrosis and alterations in the structure and disposition of the liver and kidney tissues were observed for the 500 ppm treatment group.

INTRODUCTION TO STUDY
The most ancient and relevant environmental poison to be used by man is lead. According to Jernigan et al [1], hundreds of millions of people have been affected by the toxicity of lead during the last 4500 years either as mining slaves, or as consumers of adulterated wine and food or from breathing urban air. Written archeological evidence exists that lead was used widely in the ancient world. In recent times, lead has been used in gasoline which makes it widely distributed in the environment.

The environmental significance of lead as a chemical species exhibiting various form of toxicity in humans has been well documented. [2-5]. As a result of its past uses history, lead is widely distributed in water, soil and air. This is particularly of great importance considering lead use in gasoline and paint was curtailed more than two decades ago. As a result, the potential exposure to significant lead levels, especially infants in the population, is high [6].

Pb exposure targets organ systems such as the skeletal, hematopoietic, renal, endocrine and nervous systems [7], thereby partitioning between soft and hard tissues in the body with approximately 95 % and 70 % being found in the bones and teeth of adults and children, respectively. Bone then serves as a pool to replenish excreted lead from blood. Some adverse conditions associated with lead poisoning include DNA damage, neurological impairment, abnormal heart function, osteoporosis among others [8-9]. In addition, a weakened immune defense system, sterility in male and females, abnormal fetal development, and glycosuria are also associated with chronic lead poisoning.

Lead perturbs the functions of enzymes and proteins of varied classes. Studies have shown that, lead exerts its influence physiologically and biochemically as a mimetic agent substituting for essential elements participating in metabolism such as calcium, iron and zinc. For instance, it directly interferes with zinc and iron in the biosynthesis of heme, in the function sulfhydryl group rich protein enzymes and in protein synthesis in general either directly or indirectly [8-9]. Lead binds to different kinds of transport proteins including metallothionein, transferin, calmodulin and calcium-ATPase. By associating with these proteins, it is transported to specific tissues where it causes its damage. Lead transport and assimilation are optimum when there is the dietary deficiency of essential metals such as iron or calcium or zinc. This is because lead is able to displace these metals in transport proteins and during specific protein-mediated physiologic processes [10].
Liver and kidney damage have been linked to lead toxicity although the exact toxicity mechanism is not entirely understood. Other than the use of histopathology to assess the effects of lead poison in hepatocytes, the use of other methods has been inconclusive. The objective of this study is to assess the interaction of lead with calcium, iron, copper, zinc, cobalt and nickel in blood, liver, kidney and bone marrow using rat model. Histopathology of the liver and kidney will also be examined. Inductively-coupled plasma spectrometry (ICP-MS) is a useful analytical tool for quantifying multi-elements from such matrices as geological, environmental and biological samples at sub parts per billion. That is, it has a quantitation limit of 1-100 parts per trillion and a linear dynamic range of about eight orders of magnitude. The range of analytes that can be employed in ICP-MS has recently been extended to both metals and nonmetals including radionuclides, rare earths, and some halogens like Br and I. ICP-MS works by generating ions in the plasma which are directed into the ion focusing region using turbomoelcular pumps backed by rotary pumps. Then electrostatic ion and extraction lenses sort the negative and positive ions so that positive ions are directed towards the quadrupole. Ions then enter the separation hardware, the quadrupole mass spectrometer where the electric field forces them into wavelike motion. Ions with stable trajectories are filtered according to their mass to charge ration (m/z) in the quadrupole. Finally, individual ions are detected by ion counting electron multiplier [11].

The Fisher 344 rat inbred strain was developed in 1920 to address the lack of reproducible animal model for cancer research. In 1970, the National Cancer Institute selected Fisher 344 rat as a replacement for the Osborne-Mendel rat model in cancer bioassay program because tumor latency due to chemical exposure is relatively short whilst maintaining good survivability. Fairly recent literature has indication that F344 rats are prone to exhibit inflammatory effects and mononuclear cell leukemia due to exposure of a range of chemicals and pharmaceuticals. Nevertheless, this animal model has been employed in as many cancer, toxicological, aging, neurological, organ transplant, heart disease etc studies in the literature [12-15].

This experiment is part of a larger project investigating the overall effects of Pb2+ on gene expression in the rat. Results from this investigation will enable us to determine what specific tissue levels of Pb in rat lead to alterations in gene regulation.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

Forty-eight six weeks post-weaning male Fisher 344 (F344) rats were exposed to 0 ppm, 50 ppm or 500 ppm of Pb2+, respectively, in the form of lead acetate through drinking water ad libitum for 30 and 90 days, respectively. Control drinking water was distilled water. Prior to commencing treatment, rat diet, control and treated water were analyzed by ICP-MS (model # 4202387, serial # A0126, manufactured by Perkin Elmer Instruments) for lead contamination and to verify accurate exposure levels. Rats were housed at the Western Michigan University Animal Facility. The animals were treated according to the principles outlined in the NIH Guide for the Care and Use of Laboratory Animals. There were eight rats assigned randomly to each treatment group. After each exposure period, rats were euthanized with CO2 and blood was collected by cardiac puncture for serum analysis using ICP-MS. Also, some of the liver, kidney and bone marrow were preserved for multi-elemental analysis. A portion of each of the livers and kidneys were also fixed in 10% neutral buffered formalin for subsequent histology analysis.

MULTI-ELEMENTAL ANALYSIS

Determinations of lead and other metal ion levels in blood, liver, kidney and marrow, an elemental analysis were carried out by ICP-MS. Approximately 1 g each of blood, liver, kidney and marrow was weighed into Teflon carousels containing 10 ml of 50 % nitric acid (ultra trace purity) and digested at high pressure in a microwave oven. After digestion, the samples were transferred to 50 mL conical tubes and diluted with 3 % nitric acid to the 50 mL mark. They were further diluted in a ratio of 1:10, 3 % nitric acid for final analysis. A 10 µL yttrium internal standard (10 µg/mL) was added to each sample just prior to inductively-coupled plasma mass spectrometry analysis. There were three replicates for each treatment and each sample was analyzed in triplicate.

HISTOPATHOLOGY

Fresh fixative was added to the samples and stored at 4 oC until ready for analysis. Briefly, the samples were passed through graded alcohol solutions for dehydration, xylene washed and then embedded in paraffin block cassettes. Then, tissues were sectioned in transverse and deparaffinated and stained with hematoxylin and eosin (H & E). Stained sections were examined under light microscopy to detect structural changes in the cells of the liver and kidney.
DATA ANALYSIS

Significant differences in lead, zinc, nickel, copper, cobalt as well as morphometric parameters such as body weight and organ weights were analyzed by student t-test and ANOVA. Regression analysis was also conducted to follow distributions of lead and the other metal ions in blood, liver and kidney as function of time and dose level. Data was presented as means ± standard deviation (SD) and differences were considered significant at P < 0.05 or P < 0.01. ANOVA and t-test were applied specifically to the data set shown in Tables 1, 2, 3 and 4. Figures 1, 2 and 3 were analyzed by ANOVA and Figures 4, 5 and 6 by regression analysis. H & E stained slides were observed under low- and high-power optical microscopes at the Biological Imaging Center, Western Michigan University.

RESULTS

LEAD EFFECTS ON SELECTED ANIMAL MORPHOMETRIC PARAMETERS

Selected measurements of animal health at time of sacrifice are reported in Table 1. Body weight gains as well as absolute liver and kidney weights were not significantly altered in both the 30-day and 90-day treatment groups relative to controls. The amount food consumed (gram) per gram of body weight gain was also not found to be significant. However, some significant differences were observed for the hepatosomatic and renal somatic indexes (organ wt./body wt.) in both the 30 and 90 days exposure period groups. In the 30-day treated rats, liver and kidney weights and the renal somatic index were decreased. In contrast, in the 90-day 500 ppm treated rats, liver weights, hepatosomatic and renal somatic indices respectively increased 8 %, 11 % and 5 % relative to controls.

METAL DISTRIBUTION IN BLOOD, LIVER, KIDNEY AND MARROW

In Table 2, lead accumulation in blood, liver, kidney and bone marrow were all increased significantly in lead exposed groups relative to the control groups. With the exception of kidney, the 90-day treatment groups also showed markedly higher levels of lead in blood, liver and marrow than the 30-day treatment groups. The amount of lead accumulated in blood was between 6-15-fold greater in the 90-day treated than the 30-day exposed group. This trend is similar to what was observed in the liver and bone marrow. In the kidney, it is a ratio of one-to-one.

EFFECT OF LEAD POISONING ON SOME ESSENTIAL TRACE METALS

As mentioned to in the introduction, lead exerts its toxic effects through mimicking the behavior of some other essential trace metals. We evaluated the responses of calcium, iron, cobalt, nickel, copper and zinc to varying levels of lead intoxication in some rat tissues. In Figure 1, the levels of zinc are shown in selected tissues as a function of lead exposure and time. The 30-day treatment group showed significant losses of zinc (P<0.05) in the liver at the
50 ppm Pb dose level, with kidney and marrow levels remaining statistically unaltered. For the 90-day exposure period group, zinc concentrations reduced significantly in liver, marrow (P<0.05) and kidney (P<0.01).

Not many significant alterations in calcium and iron levels in blood, liver, kidney or marrow were observed at either time point or in either treatment regime, except calcium was depressed in the blood of 90 day high dose animals (P<0.01) and iron (P<0.05) in marrow in the 90 day high exposure group (Tables 3 and 4).

The concentrations of nickel (ppb) in the selected tissues analyzed are presented in Figure 2. While long-term chronic (D) lead exposure resulted in significant nickel reduction in liver at 50 the ppm dose group only and marrow in the 500 ppm dose group only (P<0.05), short-term acute (C) exposure to lead did not yield any significant changes in nickel levels in liver, kidney or marrow tissues at any dose.

Similarly, cobalt levels (ppb) were not significant changed for the thirty days treatment group. Though, cobalt in the liver was significantly altered in the groups treated with lead for ninety days. For the 30-day treatment group, cobalt decreased 34 % in liver, and increased 12.5 % and 2.5-fold in kidney and marrow respectively. Blood, liver and marrow level cobalt decreased by 84 %, 85 % and 83 % respectively whereas that of the kidney increased by 7 % in the 90-day treatment group (data not shown).

Figure 5
Figure 1: Distributions of zinc (ppb) due to lead exposure for (A) thirty days and (B) ninety days. Significant decreases in zinc concentration due to lead intoxication were observed at both time points in the various tissues assayed. *P<0.05, **P<0.01

Figure 6
Figure 2: Levels of nickel in blood, liver, kidney and marrow (ppb) as a result of exposure to lead for 30 and 90 days. No significant differences were observed for the shorter (C) time period. On the contrary, significant differences were observed in the liver and marrow for the longer treatment period (D). *P<0.05

Figure 3 shows Cu (ppb) distribution in selected tissues at (e) 30 days and (f) 90 days. The 30-day exposure group showed a significant Cu reduction in the liver (P<0.05). Also, rats treated for 90 days showed marked changes in blood, liver and kidney, only in blood and kidney were copper reduced significantly.
The next several figures illustrate the relationship between various lead concentrations and essential trace metals in specific tissues. Though these relationships are too simplistic with respect to the direct influence of lead levels on the levels of these other metals or for explaining mechanisms involved in lead action, they nevertheless provide useful information as to the metal-targets of lead. Figures 4 and 5 show scatter plots of liver concentrations of lead on the horizontal axis against zinc, nickel and copper concentrations on the vertical axes for 30 day and 90 day exposures, respectively. Both figures were fitted to both linear and polynomial functions and the best function selected. The short-term (30d) exposure groups have R2 values ranging from 0.093 to 0.6109 (P<0.01). Similarly, the 90d experimental groups had R2 values in the range of 0.0245 to 0.2311 (P<0.01). The Pb-Ca and Pb-Fe relationships were curvilinear in nature.

**Figure 7**

Figure 3: Copper concentration in blood, liver, kidney and marrow (ppb) due to lead poison for 30 and 90 days. Copper levels in the short term treatment group were statistically changed only in the liver (E) whereas the other group was changed both in the blood and kidney. *P<0.05, **P<0.05

Although two of the long-term treatment groups reveal a weak negative correlation, the majority of the treatment groups showed a quadratic relationship indicative of dose-response, thus, reflecting the most accurate response of the cell to lead toxicity. These results confirm that the response of living organisms to the toxic effects of lead, like many other contaminants, is not likely to be a linear relationship. Whereas some of the treatment groups show positive associations, the others demonstrate negative interactions. Interactions of metals in blood and marrow were all negative.

In Table 5 above, lead excretion through the feces increased 10 orders of magnitude from the 0 ppm to 50 ppm to 500 ppm treated groups. Clearly, this observation agrees with what we would expect that the more Pb exposure there is in a population to a particular chemical, the greater excretion amounts of that particular chemical.

The fecal amounts of zinc, copper, nickel and cobalt excreted were all greater in the control groups than in the treated groups. All three metals were minimally excreted in the 50 ppm exposure group relative to the controls.

**Figure 8**

Figure 4: Plots of Pb (ppb) versus (G) Zn, (H) Cu and (I) Ni (ppb) in liver for 30 days. Data fit to a polynomial function
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Figure 9
Figure 5: Plots of Pb (ppb) versus (J) Zn, (K) Cu and (L) Ni (ppb) in liver for 90 days. Data fit to both linear and polynomial function.

Figure 10
Figure 6: Plots of Pb (ppb) versus (M) Ca and (N) Fe (ppb) in the marrow for 90 days. Data fit to a polynomial function.

Figure 11
Table 5: Lead, copper, nickel and cobalt levels in feces. *P

<table>
<thead>
<tr>
<th>Dose</th>
<th>Lead (ppb)</th>
<th>Zinc (ppb)</th>
<th>Copper (ppb)</th>
<th>Nickel (ppb)</th>
<th>Cobalt (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 PPM</td>
<td>2113.8 ± 56.0</td>
<td>2047.8 ± 8.92</td>
<td>2067.6 ± 69.23</td>
<td>443.7 ± 25.71</td>
<td>52.4 ± 1.85</td>
</tr>
<tr>
<td>50 PPM</td>
<td>2119.5 ± 41.5</td>
<td>1509.8 ± 14.90</td>
<td>1728.0 ± 33.67</td>
<td>373.0 ± 2.93</td>
<td>37.9 ± 0.32</td>
</tr>
<tr>
<td>500 PPM</td>
<td>264.9 ± 131.0</td>
<td>1970.0 ± 42.47</td>
<td>2950.0 ± 11.15</td>
<td>264.6 ± 1.55</td>
<td>42.3 ± 0.34</td>
</tr>
</tbody>
</table>

Compared to the controls, zinc, copper, nickel and cobalt excretion were 27 %, 16 %, 38 % and 27 % less, respectively, in the low dose treatment group. Similarly, excretion of these metals was reduced by 23 %, 5 %, 34 % and 19 %, respectively, in the high dose group relative to controls.

HISTOPATHOLOGY

Images of H and E stained cells of the liver and kidney are shown in Figure 8. Necrotic tissue which is evidenced by nuclear shrinkage and fragmentation patterns were observed mostly in the long term treated group. The kidney tissues appeared to suffer more damage than the liver even at the same treatment. This is shown by H&E as hydropic degeneration and increase basophilia in renal epithelium.
DISCUSSION

Generally, the degree and duration of lead intoxication does not appear to be reflected in the body weight gain of the test species, although morphometric indices of tissue weights and their relative contribution to the total body weight gain might prove to be useful measures of frank toxicity to lead. In this study, no alterations were observed in total body weight gain, but rather, kidney and liver weights and their ratios of weights to total body weights at higher lead concentrations were affected. A change in body weight due to lead exposure is not clearly shown in the literature.

Figure 12

Figure 13
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Figure 14

![Image of tissue distribution](image)

Figure 15

![Image of tissue distribution](image)

For instance, whereas Miller et al [16], Corpas et al [8] and others did not find reduction in body weight as a result of increasing lead concentration, Adonaylo and Oteiza [17] observed lower body weights of rats intoxicated with lead. According to Corpas and coworkers, lack of evidence with respect to body weight gain does not necessarily mean lead has no effects. Instead the effects are rather intrinsic and continuously affecting the animal during its entire life at the tissue and cellular function level.

Tissue distributions of lead were consistent with applied doses and duration of exposure. In both time points, the 500 ppm exposed group accumulated greater lead levels than either the control or 50 ppm treatment groups in all tissues analyzed. Surprisingly, accumulated lead levels in kidney were almost the same irrespective of treatment time (30d vs. 90d). Lead accumulated in these tissues is a result of conjugation in the liver with metallothionein or other metal chelating proteins which are passed on to the kidney and other tissues, with the balance of the Pb being excreted either in feces or urine [5]. It is generally reported in the literature that a greater proportion of lead is excreted through the feces than urine [6]. The very high fecal content in our experimental animals is consistent with this observation.

Lead either bound to plasma proteins or the free salt form is introduced to the kidney through the apical membrane and in these forms it is cannot readily leave the blood stream through the basolateral membrane [18]. Another reason for the relatively high levels of lead accumulation in the kidney might be the indirect activities of metallothioneins and glutathione. These proteins have cysteine in their configuration which has an affinity for heavy metals [19]. Other workers have found that lead bioavailability in kidney and brain relates to binding to a low molecular weight protein that is rich in aspartic and glutamic dicarboxyl amino acids [20] other than metallothionein. According to Zalups [19], heavy metals such as lead can induce the synthesis of metallothionein and glutathione within the liver which then traps the metal ions within the cell by forming peptide conjugates. During the process of liver cell renewal, the heavy metal-metallothionein or metal-glutathione complexes are released into the systemic circulation and then delivered to the kidney [21]. This type of cycle is likely to result in higher levels of metal ions in the kidney than in most other organ systems.

The importance of trace metals to the normal function of the cell cannot be over emphasized. Essential trace metals exhibit a narrow range of concentrations within which they must function. Deficiencies result when their levels are below that level and when it is greater than that range of concentrations, the metals are toxic. As a result, trace metals are tightly controlled in the body to maintain homeostasis and normal cell metabolism. Levels of cobalt, copper, nickel...
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and zinc were altered in the various tissues analyzed as the result of lead exposure in the current experiments. It appears the metals most affected by lead intoxication are copper and zinc particularly during the long-term exposures. This is supported by the authors Goyer [22] and Peraza et al [10].

The interaction of lead with cobalt, cupper, nickel and zinc were all observed in these experiments. Cell homeostasis is maintained by adequate levels of cations such as Zn(II), Cu(II) and others. These metals are involved in various regulatory and physiological activities. Garza et al [23] notes that lead is able to substitute for other polyvalent cations that are involved in important molecular processes. According to them, lead has a higher binding affinity for chemical functional groups that would coordinate divalent cations in proteins. The ionic interaction of lead with these negatively charged acidic amino acid residues making it possible for lead to bind a wide variety of proteins results in a change in the structure and electric charge balance of proteins. The results presented here suggest that the interactions of lead with Zn, Cu, Ni and Co are time dependent. The entire 30 day treatment group showed that increasing lead concentration results in no increases of these essential divalent metals in rat tissues. The opposite was however true for the 90 day exposure group. In these animals the levels of Zn, Cu and Co were lowered. These trends are quite different from what was seen in other studies. For example, Goyer [22] reported that lead increases the excretion of zinc and reports a negative correlation between blood lead levels and the activity of zinc-containing heme enzymes. They suggest that lead replaces zinc on the enzyme. Again, he reported that lead exposed rats showed significant reductions in copper levels in the liver.

Examination of hepatic histopathology produced no evidence of necrosis or changes in cellular structure of hepatocytes in the 0 ppm and 50 ppm for the short-term exposure period. In contrast, both necrosis and alterations in cellular structure and cell distribution were observed in the liver and the kidney in both the 50 ppm and 500 ppm Pb2+ 90-day treatment groups. Analysis of liver and kidney of long-term exposed lead groups showed varying degrees of necrosis. The 90 day 500 ppm Pb2+ treated group showed signs of pyknosis and karyorrhexis of the liver. In the kidney, hydropic degeneration and basophilia of the renal tubule epithelia were observed. The 50 ppm treated in liver and kidney also revealed signs of pyknosis and karyorrhexis though these effects were not as pronounced as in the high dose groups. Literature reports suggest that the kidney is the most susceptible organ to lead toxicity. The work by Corpas et al [8], found no abnormalities in the liver structure or liver deposition of lead in young neonates intoxicated with lead. On the other hand, Jarrar and Mahmoud [5] found lead to have caused tubular and glomerular alterations in kidney. They observed anisokaryosis, nuclear pyknosis, and vacuolization among other histopathological effects in the kidney.

In summary, effects associated with lead exposure were observed to be both dose and time dependent in our study. Short-term exposures did not produce as serious damage, as did long-term, high dose intoxication levels. Post-hoc multiple comparison analysis would not significantly change these conclusions because the conclusions drawn from the study clearly delineates effects to be both time and dose dependent with short-term and low dose showing apparent no effects. Since post-hoc multiple comparison is typically undertaken to identify treatment parameters responsible for significance, this analysis is unnecessary and was therefore not employed in our study. Histopathology changes in tissue morphology were consistent with the lead concentration in liver and kidney. For instance, high lead concentration of lead in kidney cells results in pronounced cell necrosis in varied forms. There was a positive correlation between lead levels in tissues and the levels of other trace metals in the short-treatment period. However, a negative correlation between lead levels and other trace element levels in tissues was observed for chronic exposure levels in rat.

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