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Full Length Research Paper

Effects of chronic lead acetate exposure on bone marrow lipid peroxidation and antioxidant enzyme activities in rats

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Occupational and environmental exposure of lead (Pb) is a serious health problem in developing and industrialized countries around the world. Toxic effect of lead is closely related to its accumulation in important tissues after its absorption into the blood. Present study looked into the effect of lead toxicity on bone marrow oxidative biomarkers in Sprague-Dawley rats. Rats were divided into four groups and in three experimental groups, they were given lead acetate in drinking water for 21 days in three different doses (200, 400 and 600 ppm). Effect of lead acetate on bone marrow lipid peroxidation and antioxidant enzymes were examined. Lead exposure for 21 days resulted in a significant increase ($P<0.05$) in lipid hydroperoxides and protein carbonyl contents of bone marrow and there was significant decrease ($P<0.05$) in bone marrow total antioxidants and superoxide dismutase, glutathione peroxidase and catalase enzyme levels. More significant increase ($P<0.05$) in lipid peroxidation and a decrease in antioxidant enzymes level were recorded with 600 ppm dose of lead. There was also a significant level of perturbations ($P<0.05$) in bone marrow antioxidant enzyme levels with low level of lead exposure for 21 days. Thus, the study confirms that exposure to lead will result in significant amount of toxic effect in the bone marrow, resulting in increased lipid peroxidation and depletion of antioxidant enzymes.

Key words: Lead acetate, Lead toxicity, oxidative stress, bone marrow, lipid peroxidation.

INTRODUCTION

Lead (Pb) is a ubiquitous environmental and industrial pollutant toxic to many biological systems. As one of the common, non-essential heavy metals present in the environment, lead is toxic to the human body. Although lead levels in blood continue to decline in our population in recent times, various groups are at a disproportionately higher risk for lead exposure. This is especially the case of the lower age groups (infants and young children), and the working class sector that has direct contact with lead in industries (Campbell et al., 2004; Godwin, 2001; Hu, 1991). Many researchers have reported that lead toxicity is associated with impaired functioning of the brain, kidneys, liver and the hematopoietic system (Bellinger, 2008; El-Nekeety et al., 2009; Sivaprasad et al., 2004). Prolonged exposure to low doses of lead is a proven serious risk factor for neurological, cardiovascular and reproductive diseases. Lead toxicity is closely related to its accumulation in various tissues and its interference with the bioelements that hamper several physiological processes (Berrahal et al., 2007; Pande et al., 2001).

There is experimental evidence to indicate that cellular damage mediated by reactive oxygen species can be involved in the pathology associated with lead toxicity (Bolin et al., 2006; Ercal et al., 2001). Lead is known to cause oxidative damage in several tissues by bringing

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about an imbalance in the generation and removal of reactive oxygen species (Adonaylo and Oteiza, 1999; Sharma et al., 2010; Upasani et al., 2001). A pathological free radical mechanism that leads to lipid peroxidation and degradation of phospholipids, thereby causing a loss of membrane integrity, is currently proposed as an important factor in organ damage from lead exposure (Sandhir and Gill, 1995). Even though there are many studies explaining the possible mechanism of lead-induced physiological, biochemical and behavioral dysfunctions, the mechanisms of many lead poisoning symptoms have yet to be clearly explained (Mateo et al., 2003; Wei et al., 2002). Recent *in vivo* studies on lead-exposed animals and workers have shown the generation of reactive oxygen species, stimulation of lipid peroxidation and decreased antioxidant defence system (Bolin et al., 2006; Patrick, 2006).

In an adult human, the majority of ingested lead (more than 95%) accumulates in the bone. With its half-life being in the order of decades, bone lead can remain elevated despite a decline in environmental exposure. Available data suggest that there is a close relationship between bone pathology and lead exposure (Hu, 1998; Jagetia and Aruna, 1998; Moussa and Bashandy, 2008; Oliveira et al., 2002). Thus, the skeleton is an important endogenous source of lead, and this source should be a subject of particular note when looking into the toxicity risks of lead. The present investigation was conducted to determine the effect of lead exposure on the antioxidant enzymes and lipid peroxidation in the bone marrow of rats. The hypothesis of the study was that lead (in the form of lead acetate) caused severe oxidative damage in the bone marrow arising from increased lipid peroxidation and homeostasis dysregulation of the antioxidant enzymes.

MATERIALS AND METHODS

Chemicals

Assay kits for lipid hydroperoxide, total protein, protein carbonyl content, super oxide dismutase, glutathione peroxidase, catalase and total antioxidant were purchased from Cayman Chemicals (Cayman Chemicals and Pierce Biotechnology, USA). Lead acetate was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Experimental design

Three-month old male Sprague Dawley rats weighing 150 to 200 g were purchased from the animal holding facility of University Kebangsaan Malaysia (UKM), Kuala Lumpur, Malaysia. The animals were used in the experiments after a review by the Institutional Research and Animal Ethics Committee. The rats were fed a standard diet and had free access to water before the start of the experiment. They were housed in stainless steel cages in a temperature-controlled room (22 ± 2°C) under a light cycle of 12 h light and 12 h darkness.

The animals were divided randomly into the following groups with eight animals in each group. Group 1: Control; Group 2: Rats exposed to lead acetate in drinking water (200 ppm) for 21 days; Group 3: Rats exposed to lead acetate in drinking water (400 ppm) for 21 days; Group 4: Rats exposed to lead acetate in drinking water (600 ppm) for 21 days. The lead doses were chosen to simulate low-dose lead exposures over a specific period of time (Ahamed and Siddiqui, 2007; Bokara et al., 2009). Control animals received distilled water over the same period. The animals were sacrificed at the end of the 21 day experimental period. The rats were anaesthetized, and blood was collected by cardiac puncture and transferred to 10 ml polypropylene tubes and stored at -20°C for blood lead estimation. The left and right femur bones of the rat were excised, the muscles and soft tissues on the bone were scrapped clean and the extreme ends of the femur were cut to reveal the marrow. A 3 ml syringe preloaded with phosphate buffer solution was used to flush out the bone marrow. The flushing and washing were repeated 3 to 4 times to ensure complete removal of the bone marrow contents (about 5 ml per rat) from the femur bone. The samples were maintained at -20°C (not longer than 7 days) before performing the various assays. From the bone marrow samples, lipid hydroperoxides (LPO), total antioxidants (TA), protein carbonyl content (PCC), superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) levels were assayed using ELISA kits (Cayman Chemicals and Pierce Biotechnology, MI, USA). Protein levels of the samples were estimated by a protein assay kit. Serum samples were assayed for blood lead levels by graphite furnace atomic absorption spectrophotometry.

Biochemical assays

Lipid hydroperoxide assay

A quantitative extraction method as provided in the kit method for lipid hydroperoxide assay was used to extract lipid hydroperoxides into chloroform and the extract was directly used. This procedure eliminates any interference caused by hydrogen peroxide or endogenous ferric ions in the sample and provides a sensitive and reliable assay for lipid peroxidation. The absorbance was read at 500 nm using a 96 well plate spectrophotometric reader and a dose response curve of the absorbance unit vs. concentration in nmol was generated (Cross et al., 1987; Halliwell, 1996).

Protein carbonyl content assay

In the protein carbonyl content assay kit, protein samples are derivatized by making use of the reaction between 2,4-dinitrophenylhydrazine (DNPH) and protein carbonyls. Formation of a Schiff base produces the corresponding hydrazone which was analyzed spectrophotometrically at 360 to 385 nm (Halliwell, 1996).

Total antioxidant assay

Using the total antioxidant assay kit, aqueous and lipid soluble antioxidants were not separated and thus combined antioxidant activities of all its constituents were assessed. The assay relies on the ability of antioxidants in the sample to inhibit the oxidation of ABTS (2, 2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) to ABTS® + by metmyoglobin. The amount of ABTS produced was monitored by reading the absorbance at 405nm (Miller and Rice-Evans, 1997).

Superoxide dismutase assay

This assay kit utilizes a tetrazolium salt for the detection of superoxide radicals (O_2^-) generated by xanthine oxidase and

Table 1. Lead concentrations (mg/dL) in blood and bone marrow of control and lead-exposed rats.

Groups	Blood lead	Bone marrow lead
Group1	0.0038 ± 0.0006	0.0017 ± 0.0002 [a]
Group2	0.8941 ± 0.0042	0.5162 ± 0.0038 [a]
Group 3	1.5847 ± 0.012	0.9107 ± 0.0092 [a]
Group 4	2.2417 ± 0.027	1.1846 ± 0.0089 [a]

Values are Mean ± SD, Group 1: Control group,Group 2: Rats exposed to 200 ppm lead acetate in drinking water. Group 3: Rats exposed to 400 ppm lead acetate in drinking water , Group 4: Rats exposed to 600 ppm lead acetate in drinking water. Within each row, means superscript with different letters are significantly different ($P < 0.05$). a – Significantly different from blood lead levels – $P < 0.05$.

Table 2. Weekly total lead intakes of lead-exposed animals (Mean ± SD).

Groups	Week 1	Week 2	Week 3
Group 2	28.05 ± 3.16	33.46 ± 2.84	30.18 ± 1.45
Group 3	33.46 ± 2.84 [a]	43.71 ± 1.25 [a]	48.27 ± 2.52 [a]
Group 4	52.13 ± 2.42 [a, b]	56.31 ± 2.26 [a, b]	60.08 ± 2.10 [a, b]

Values are Mean ± SD. Group 1: Control group, Group 2: Rats exposed to 200 ppm lead acetate in drinking water Group 3: Rats exposed to 400 ppm lead acetate in drinking water, Group 4: Rats exposed to 600 ppm lead acetate in drinking water. Within each row, means superscript with different letters are significantly different ($p<0.05$), a – Significantly different from 200 ppm – $P <0.05$, b – Significantly different from 400 ppm – $P <0.05$.

hypoxanthine. One unit of SOD is defined as the amount of enzyme necessary to exhibit 50% dismutation of the superoxide radical. Oxidation rate of tetrazolium salt to Formazan dye by O_2^- is inversely proportional to the endogenous activity of SOD. The formazan dye stains the wells and its staining intensity was detected by absorbance spectrophotometry at 450 nm using a plate reader (Maier and Chan, 2002).

Glutathione peroxidase (GPx) assay

This assay kit measured GPx activity indirectly by a coupled reaction with glutathione reductase. Oxidized glutathione, produced upon reduction of an organic hydroperoxide by GPx, is recycled to its reduced state by glutathione reductase and NADPH. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. The rate of decrease in the absorbance at 340 was directly proportional to the GPx activity in the sample (Ceballos-Picot et al., 1992).

Catalase assay kit

The Cayman chemical catalase assay kit utilizes the peroxidatic function of catalase for determination of enzyme activity in the sample. This method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2. The formaldehyde produced is measured spectrophotometrically with 4-amino-3-hydrazino-5- mercapto-1,2,4-triazole (Purpald) as the chromogen (Wheeler et al., 1990).

Statistical analysis

All the results were expressed as means with standard deviation. Graph Pad prism 5.0 software used for statistical analyses. Comparison between all the groups were done using Kruskal Wallis one way analysis of variance test. Pair wise comparison between the different groups was done using Mann-Whitney –U test and a value of $P<0.05$ was considered as statistically significant.

RESULTS

Blood and bone marrow lead levels

A significant increase ($P < 0.05$) in blood lead levels was observed in the lead acetate groups when compared with blood lead levels in the control animals. Animals fed with 600 ppm lead acetate had significantly higher ($P < 0.05$) blood lead levels than the other two lead treated groups. Bone marrow lead acetate also was significantly higher in all three lead treatment groups ($P<0.05$). Although the bone marrow lead concentrations were significantly lower than lead levels in the blood, there was significant elevation in bone marrow lead concentrations even with 200 ppm of lead acetate ingestion for 21 days ($P < 0.05$) (Table 1).

Lead intake

Table 2 shows the lead exposure, expressed as weekly lead-acetate intake per rat (calculated from the water consumption). The mean lead intake per week did not change significantly throughout the duration of experiment (Table 2). However, total lead acetate intake was significantly higher as the lead added to the drinking water was increased from 200 to 400 ppm, and

Table 3. Effects of lead acetate (Pb) on bone marrow oxidative parameters.

Group	Lipid hydroperoxides (nmol/mg protein)	Protein carbonyl content (nmol/mg protein)	Total antioxidants (µmol/mg protein)
Group 1	2.10 ± 0.26	12.124 ± 0.808	5.228 ± 0.247
Group 2	3.147 ± 0.181 [a]	18.177 ± 0.718 [a]	3.228 ± 0.484 [a]
Group 3	4.230 ± 0.125 [a, b]	22.863 ± 0.472 [a, b]	2.019 ± 0.713 [a]
Group 4	6.192 ± 0.814 [a, b, c]	25.195 ± 0.810 [a, b, c]	1.995 ± 0.617 [a, b]

Values are Mean ± SD, Group 1: Control group, Group 2: Rats exposed to 200 ppm lead acetate in drinking water, Group 3: Rats exposed to 400 ppm lead acetate in drinking water, Group 4: Rats exposed to 600 ppm lead acetate in drinking water. Within each row, means superscript with different letters are significantly different (p<0.05),a – Significantly different from control – P <0.05, b – Significantly different from lead 200 ppm – P <0.05, c – Significantly different from lead 400 ppm – P <0.05.

Table 4. Effects of lead acetate (Pb) on bone marrow antioxidant enzymes.

Group	Superoxide dismutase (U/mg protein)	Glutathione peroxidase (nmol/mg protein)	Catalase (nmol/min/mg of protein)
Group 1	3.937 ± 0.681	8.372 ± 0.189	4.816 ± 0.185
Group 2	2.008 ± 0.175 [a]	5.149 ± 0.219 [a]	3.174 ± 0.478 [a]
Group 3	1.941 ± 0.222 [a]	3.280 ± 0.318 [a, b]	2.547 ± 0.318 [a]
Group 4	1.017 ± 0.045 [a, b, c]	1.974 ± 0.048 [a, b, c]	2.002 ± 0.117 [a, b, c]

Values are Mean ± SD, Group 1: Control group, Group 2: Rats exposed to 200 ppm lead acetate in drinking water, Group 3: Rats exposed to 400 ppm lead acetate in drinking water, Group 4: Rats exposed to 600 ppm lead acetate in drinking water. Within each row, means superscript with different letters are significantly different (p<0.05),a – Significantly different from control – P <0.05, b – Significantly different from lead 200 ppm – P <0.05, c – Significantly different from lead 400 ppm – P <0.05.

subsequently to 600 ppm (P < 0.05).

Lipid hydroperoxides

When compared in pair -wise manner, there was a significant increase (P < 0.05) in bone marrow lipid hydroperoxide (LPO) levels in the Pb- fed rats than in the LPO levels in the control group (Table 3). The differences were more distinct, with 400 and 600 ppm doses than the 200 ppm dose of lead acetate (P < 0.05).

Protein carbonyl content

A significant increase in bone marrow protein carbonyl contents (PCC) was recorded after lead treatment (P < 0.05). The greatest change in protein carbonyl content was seen with 600 ppm dose of lead acetate (P < 0.05); exposure of even 200 ppm of lead for three weeks significantly increased bone marrow protein carbonyl levels by 50%, as compared with the levels in the control group (Table 3).

Total antioxidants

Compared to the control, there was a significant decrease in bone marrow total antioxidants after lead acetate exposure for 21 days (P< 0.05). A more significant decrease was seen with the 600 ppm dose of lead acetate than with either the 200 or 400 ppm dose (P<0.05) (Table 3).

Superoxide dismutase

Bone marrow superoxide dismutase (SOD) decreased significantly after all three lead treatments for 21 days (P < 0.05). Ingestion of 600 ppm of lead acetate had a more profound effect on bone marrow SOD levels, depressing the levels by more than 70% when compared to control rats (P < 0.05) (Table 4).

Glutathione peroxidase

Table 3 shows the bone marrow glutathione peroxidase (GPx) levels following lead acetate treatment. Lead acetate added to drinking water for 21 days decreased the glutathione peroxidase levels significantly (P < 0.05). The largest decrease was seen with 600 ppm of lead acetate exposure (P < 0.05) when bone marrow glutathione peroxidase levels decreased to 75% below the normal control levels, indicating the severe toxic effect

of lead in the bone marrow (Table 4).

Catalase

Bone marrow catalase (CAT) levels decreased significantly after lead acetate exposure for three weeks, with the 400 and 600 ppm dose ($P < 0.05$). For the 600 ppm treatment, the decline exceeded 50% as compared with the control. There was no significant difference in bone marrow catalase levels between the 200 and 400 ppm groups, but a significant decrease in catalase level was seen with 200 ppm lead acetate treatment and control ($P < 0.05$) (Table 4).

DISCUSSION

The results of the present study showed that there was a significant increase in blood and bone marrow lead levels following the consumption of lead acetate in drinking water for 21 days. Lead acetate absorbed from the gastrointestinal tract is carried *via* blood, mainly in the erythrocytes, to the bone where it accumulates (Freeman, 1970). Lead acetate present in drinking water resulted in severe oxidative stress in the bone marrow after 21 days with all three doses tested. There were increases in lipid hydroperoxides and protein carbonyl content in the bone marrow. At the same time, decreases were observed in total antioxidants and antioxidant enzymes like super oxide dismutase, glutathione peroxidase, and catalase levels.

Even though the exact molecular mechanism of lead toxicity on various tissues has still not been convincingly explained, evidence is accumulating to support the role of free radicals in the pathophysiology of lead toxicity. Oxidative stress appears to be a possible mode of the molecular mechanism for lead toxicity. This toxic metal induces disturbance in the physiological and biochemical state in different tissues, resulting in an alteration of the enzyme homeostasis and distortions in cell organelle functions. Lead is known to cause direct or indirect damage to mitochondria by depleting the endogenous thiol-containing antioxidant, glutathione. This will cause an increase in reactive oxygen species (ROS) that cause oxidative stress. The observed increases in lipid hydroperoxides and protein carbonyl contents in the present study are consistent with the lead-induced peroxidation of membrane lipids in the bone marrow (Ercal et al., 2001; Nyska and Kohen, 2002; Vinodhini and Narayanan, 2009). The generation of reactive oxygen species such as superoxide ion, hydrogen peroxide, and hydroxyl radicals (Hermes-Lima et al., 1991; Stohs and Bagchi, 1995), or the by-products of lipid peroxidation (lipid hydroperoxides and lipid aldehydes) (Adonaylo and Oteiza, 1999; Ercal et al., 1996) have been implicated in lead toxicity. Our study has confirmed that chronic lead exposure induces similar effects in the bone marrow that could result in severe oxidative stress.

The cell membrane is the most important target of the free radical damage by xenobiotics (Halliwell and Gutteridge, 1989). Lead-acetate generation of free radicals may attack not only DNA in the cell, but also the polyunsaturated fatty acid residues of phospholipids in other organelles that are sensitive to oxidation (Sharma et al., 2010). Lead is known to cause oxidative damage in various peripheral organs by enhancing lipid peroxidation (Hamadouche et al., 2009; Landrigan et al., 2000). Lipid hydroperoxides are formed due of oxidation of lipid and cholesterol containing cellular molecules like cell membrane phospholipids, lipoproteins, glycolipid, cholesterol and other lipid-containing structures (Porter et al., 1995). The oxidation is usually caused by ROS like oxyl radicals, peroxyl radicals, and hydroxyl radicals. The balance between the production of oxidants and the scavenging of those oxidants by antioxidants determines the extent of lipid peroxidation (Hsu and Guo, 2002).

In addition to acute toxicity, lead is known to have an extremely long half-life in bone tissue (Pounds et al., 1991). However, its effects on free radical generation and oxidative damage in bone marrow have not been studied in detail. Bone marrow damage by lead acetate was thought to be caused by oxidative stress (Dowd *et al.,* 2001; El-Ashmawy et al., 2005). Lead being itself bivalently charged in its ionic form, is also known to displace bivalent ions such as Zn^{2+}, Cu^{2+} and Fe^{2+}. Since these transitional metal ions have variable oxidation states, this characteristic allows them to switch between oxidized and reduced states easily (Aleksandrov et al., 1996). Hence, they are important players in redox reactions involving, neutralizing oxidative stress in the marrow tissue.

Increased lipid hydroperoxides could be explained by lead-induced inhibition of free radical scavenging enzymes, leading to the accumulation of ROS to accumulate and cause increased oxidation in the bone marrow (Ribarov and Bochev, 1982). The reason for LPO increase could also be due to the combined inhibitory effects of the various antioxidant enzymes (GPx, SOD, and CAT), as observed in the present results. Protein carbonyls are formed when lysine, arginine, proline, histidine side chains of proteins are oxidized (Halliwell, 1996; Halliwell and Gutteridge, 1989), a development that we had observed in this study. Increased protein carbonyl contents in bone with lead ingestion could be due to increased ROS levels and reduced antioxidant enzymes in the bone marrow tissues.

In this study, the levels of bone marrow SOD, CAT and GPx antioxidants were reduced by lead acetate exposure for 21 days, thus rendering the tissues susceptible to the peroxidative damage. These antioxidant enzymes rely on essential trace elements and prosthetic groups for proper molecular organization and their enzymatic reaction because of which they are the potential targets for lead-induced toxicity (Ercal et al., 2001; Michiels et al., 1994).

GPx, CAT, and SOD are metalloproteins and complete their antioxidant functions by detoxifying the free radicals. In the present study, the activities of SOD, GPx, CAT and GST antioxidants were reduced by lead acetate, thus rendering the bone marrow susceptible to the peroxidative damage.

This study indicated that severe oxidative stress with increased lipid peroxidation and protein carbonyl content accumulation in bone marrow was seen with higher concentrations of lead acetate. Interference in the activities of bone marrow antioxidant enzymes was observed even with 200 ppm of lead in drinking water. A lead level of 0.5 mg/dL in the bone marrow was sufficient to produce a significant oxidant effect. Thus, the strong associations observed between bone marrow lead levels with oxidative biomarkers suggested that lead-induced oxidative stress should be considered as an important, if not the primary, cause of pathogenesis of lead–related bone marrow pathologies.

The present study confirmed that exposure to lead-acetate in drinking water for three weeks, produced significant alterations in bone marrow antioxidant enzymes and an increase in bone marrow lipid peroxidation in rats. The association of significant bone marrow oxidative stress with lead exposure suggests that an antioxidant may enhance the efficacy of therapeutic agents used in the treatment of lead toxicity. Hence, an ideal treatment for lead intoxication or lead-induced organ pathology should include both lead-chelating and antioxidant actions.

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