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Reduced DNA Glycosylases Expression and Oxidative DNA Damage Induced by Lead

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Abstract

Exposure to lead (Pb) continues to be a significant worldwide problem. Pb is a highly toxic heavy metal affecting several organ systems in the body. There has been reported to have potential genotoxic properties to various cells. However, the underlying mechanisms of lead-induced toxicity are still unknown. The present study aimed to investigate the lead-induced cytotoxicity in human renal proximal tubular epithelial cells and its underlying DNA damage mechanisms. Lead exposure caused DNA damage as demonstrated by increased 8-OHdG/dG ratio in cells even at a relatively normal dose (10µg/dL). Lead also led to producing oxidative stress as characterized by increased intensity of the Reactive Oxygen Species (ROS) indicator. ROS overproduction should be the reason for lead-induced DNA damage. Therefore, the effects of Lead on ROS elimination should be the main reason for lead-induced oxidative stress in human renal proximal tubular epithelial cells. After lead acetate (PbAc) treatment, the cell viability significantly decreased in a dose-dependent manner, and the accumulation of cellular ROS was observed. 8-OHdG levels, a marker of oxidative DNA damage, were significantly increased by both acute and chronic Pb exposure. Interestingly, the mRNA expression of the 8-oxoguanine DNA glycosylase 1 (hOGG1) significantly decreased after acute and chronic exposure. In conclusion, our study provides the first evidence to demonstrate that acute and chronic Pb exposure results in the altered expression of DNA glycosylases genes indicating the impairment of DNA repair pathways and contributing to DNA damage. These findings should be useful for the more comprehensive assessment of the toxic effects of Pb.

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1. Introduction

Lead (Pb) a toxic heavy metal, is one of the most ubiquitous heavy metals and has been detected in virtually all biological systems and phases of the environment. It is also one of the most well known genotoxic toxicants and has been reported to have potential genotoxic properties, genotoxic endpoints have been extensively demonstrated in various experimental models. For instance, a significant increase in oxidative DNA damage is reported in the exposed renal cells[1,2]. Pb-induced oxidative DNA damage has been well illustrated in several studies[3-4]. On the other hand, the mechanisms underlying its genotoxicity related to DNA repair pathways are not fully elucidated. The major mechanism of genotoxicity of Pb has been reported to be associated with oxidative stress[5]. During oxidative stress formation, Reactive Oxygen Species (ROS) impair cells by oxidizing the membrane lipids and proteins as well as DNA. DNA damage is consistent with a facilitative role for Pb in carcinogenesis and even cell death[6]. A common feature of DNA damage induced by heavy metal toxicity is the oxidized base damage and single-strand break DNA (ssDNA) damage while bulky DNA lesions can be minority occurred[7]. Thus, the specific DNA repair proteins along the ROS-induced DNA pathway play a crucial role to cope with these damages induced by heavy metal toxicity.

In general, heavy metals are considered capable of interfering with the activities of several proteins and altering the expression pattern of numerous genes[8]. Interestingly, altered gene expression by Pb has been reported to affect proliferation, differentiation, detoxification, as well as DNA repair systems[9-11]. Thus, aberrant expression of DNA repair genes can be associated with DNA damage induced by Pb toxicity. Although the previous epidemiological and in vitro studies have demonstrated an association of Pb toxicity with DNA repair gene expression[12], the finding is still conflicting, and several DNA repair genes remain unexplored. Furthermore, the evidence in the molecular levels for the impacts of Pb toxicity on DNA repair genes expression in the experimental model is lacking.

Therefore, the main goal of this study was to identify the mechanisms by which lead triggered oxidative stress, induced DNA damage and cytotoxicity. To achieve this goal, we conducted a series of studies in cells to: 1) measure the effects of Pb on cell viability, ROS content and DNA damage; 2) measure Pb-induced DNA damage following ROS; and 3) determine Pb exposure on the DNA repair pathways by monitoring expression levels of the 8-oxoguanine DNA glycosylase 1 genes (hOGG1) known to correspond with ROS-induced DNA repair pathways in human renal proximal tubular cell models (RPTEC/TERT1) cells. The data in the present study might provide new insights into the mechanisms of Pb-induced cytotoxicity and should be useful for the more comprehensive assessment of the toxic effects of lead.

2. Materials and Methods

2.1 Chemicals and Reagents

Lead acetate (PbAc), dichlorofluorescein (H_2DCF-DA), and culture supplements were purchased from Sigma-Aldrich (St. Louis, Mo, USA). Human renal proximal tubular (RPTEC/$TERT_1$) cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Culture medium, penicillin, and streptomycin were purchased from Gibco (Invitrogen, Carlsbad, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Molecular Probes (Invitrogen, Carlsbad, USA). Trizol reagent was purchased from Invitrogen (Carlsbad, CA, USA). For cDNA synthesis and RT-PCR, we used iScript Reverse Transcription supermix and iTag Universal SYBR Green Supermix kit from Bio-Rad Laboratories (Hercules, CA, USA). ß-actin, and HRP-conjugated secondary antibody were purchased from Cell Signaling Technology (Danvers, MA, USA) and primary antibody against $hOGG_1$ was purchased from Abcam (Cambridge, UK).

2.2 Cell Culture and Treatment

To gain insight into the molecular mechanism of DNA damage in the target organ of Pb-induced cytotoxicity in renal tubular cells, we assessed ROS formation, DNA damage, and DNA repair gene expression properties in human renal proximal tubular (RPTEC/TERT1) cells. RPTEC/TERT1 were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) medium supplemented with 10ng/mL epithelial growth factor, 5ng/mL sodium selenite, 36ng/mL hydrocortisone,

5µg/mL transferrin, 5µg/mL insulin, 100U/mL penicillin and 100µg/mL streptomycin in the humidified incubator under 5%CO_2 at 37°C. Cells were treated with PbAc for 24h and 72h (repeating treatment) in an incubator under 5%CO_2 at 37°C. For repeating treatment, the old medium containing PbAc was replaced with a fresh medium containing PbAc every 24h until 72h. Untreated (control) cells were included with only a culture medium. Thereafter, cells were harvested for experimental analysis.

2.3 Cell Viability Assay

The determination of cell viability was assessed by the MTT assay, as described by Mosmann *et al.*[13]. Briefly, cells were seeded 5×10^4 cells/well in a 96-wells plate and allowed to adhere for 24h. The confluence monolayers were then treated with different concentrations of Lead acetate (PbAc) (0-200µg/mL) for 24h and 72h. After treatment, all concentrations of PbAc were removed and 25µl of MTT (5mg/mL) in Phosphate buffered saline (PBS) was added to 225µl of the fresh culture medium to each well and incubated for 4h under 5%CO_2 at 37°C. After incubation, the supernatant was removed and 100µL of Di-Methyl Sulf-Oxide (DMSO) was added to dissolve the insoluble formazan crystals. The absorbances were measured at 570nm using an 800 TS microplate reader (BioTek Instruments, Winooski, VT, USA). The cell viability was calculated as the percent of control. The experiment was repeated in three independent experiments.

2.4 Determination of ROS Level

Production of cellular ROS was measured by a ROS-responsive fluorescent probe, H2DCF-DA, according to Kim *et al.*[14]. Briefly, seeded cells in a 96-well plate were loaded with 20µM H2DCF-DA in PBS for 45 minutes followed by washing with PBS. The cells were treated with various concentrations of PbAc (0-400µg/mL) for 5h. After Pb treatment, the cells were washed with PBS followed by measuring DCF fluorescent intensity at the wavelength pair of 488/530 nm using a Synergy LX multi-mode plate reader (BioTek Instruments, Winooski, VT, USA). The cellular ROS level was calculated and expressed as the percentage of fluorescence intensity compared to control. The experiment was repeated in three independent experiments.

2.5 Determination of Oxidative DNA Damage

PbAc-treated cells and controls were lysed in DNase/RNase-free water containing 0.5% (v/v) Triton-X using a freeze-thaw lysis method involving freezing a cell suspension in liquid nitrogen and subsequent thawing at 37°C. The cell lysates were collected by centrifugation at 10,000 g at 4°C for 10 minutes. 8-OHdG, an oxidative DNA damage marker, was measured in lysates using Liquid Chromatography/Mass Spectrometry (LC/MS). The experiment was repeated in three independent experiments.

2.6 RNA Isolation and cDNA Synthesis

PbAc-treated cells and controls were washed with cold PBS and harvested in TRIzol reagent following the manufacturer's recommendation. Total RNA was extracted in chloroform and precipitated with isopropanol. The RNA pellets were washed with 75% ethanol and resuspended in DNase/RNase-free water. The total RNA was quantified in nanodrop one (Thermo Scientific, Wilmington, DE, USA) and reverse transcribed into cDNA using iScript RT supermix kit according to the manufacturer's instruction. Briefly, 1µg of total RNA was reverse transcribed into cDNA at 42°C for 30 minutes using T100 Thermal Cycler (Bio-Rad). The cDNA was stored at -20°C for later use.

2.7 Quantitative Real-Time Polymerase Chain Reaction (RT-PCR) Analysis

The mRNA expression was quantified using a sybergreen RT-PCR kit. cDNA templates were diluted 20 times and 4µL was mixed with RT-PCR mix to a total volume of 10µl according to the instruction provided in the kit of iTaq Universal SYBR Green Supermix. The sequences of primers were chosen from previous studies[14] as shown in Table 1. The PCR was run in CFX96 Real-Time System (Bio-Rad) under the defined condition of an initial denaturation (95°C for 3 min), followed by 40 cycles of denaturation (95°C for 10s) and extension (60°C for 30s). ß-actin was used as an internal control for normalization. The relative change of DNA repair gene expression was determined using the 2-ΔΔCT method. RT-PCR reactions were run in duplicate and the experiment was repeated in three independent experiments.

Table 1. Primers sequences of target genes for qRT-PCR

Gene	Sequences of primers	Accession number
hOGG1	Forward: 5'-AATTCCAAGGTGTGCGACTG-3' Reverse: 5'-CGATGTTGTTGTTGGAGGAAC-3'	NM_002542
ß-actin	Forward: 5'-CCTGGCACCCAGCACAA-3' Reverse: 5'-GCCGATCCACACGGAGTAC-3'	NM_001101

2.8 Statistical Analysis

All data point was generated from at least three independent experiments and presented as mean and the standard error of mean (mean ± SEM). One-way analysis of variance (one-way ANOVA) and student's t-test were used for comparison of differences among groups using GraphPad Prism analysis software (version 5.0). Comparisons were considered significant at *p <0.05 and highly significant at **p <0.01.

3. Results

3.1 PbAc-Induced Cytotoxicity in RPTEC/TERT1 Cells

This study aimed to determine the acute and chronic cytotoxic effects of Pb exposure on DNA repair genes. On the acute and chronic toxicity assay in RPTEC/TERT1 cells, LD50 (lethal dose 50%) was estimated at around 100µg/mL. LD50 level did not induce increase in the number of DNA damage, suggesting no genotoxicity. The MTT assay was used to select the optimum doses representing acute and chronic exposure of PbAc in the RPTEC/TERT1 cell model. Exposure times with PbAc were based on the cycle time of RPTEC/TERT1 cells. Cells show a kinetic half-life time (t1/2α) of 16 hrs. To be in line with the cells' exposure conditions, an incubation time of 24h and 72h were chosen for acute and chronic experiments, respectively. The MTT results revealed a dose-dependent decrease in cell viability after 24h and 72h of exposure (Figure 1). At the end of exposure, the concentrations at about 80% cell viability were chosen as acute and chronic doses. At ≥ 40µg/mL PbAc, cell viability decreased significantly after 24h of exposure, while cell viability decreased significantly at ≥ 10µg/mL upon 72h of exposure ($p < 0.05$ or $p < 0.01$) (Figure 1). Therefore, 40µg/mL (acute dose) and 10µg/mL (chronic dose) were chosen for 24h and 72h exposure respectively of all subsequent experiments.

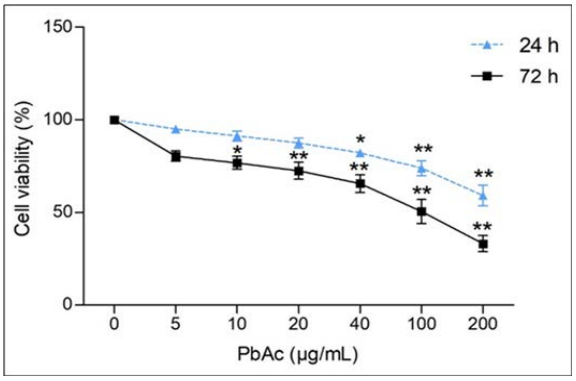


Figure 1. Cell viability (%) and cellular Pb levels accumulated in PbAc-exposed RPTEC/TERT1 cells. (A) Cells were treated with various concentrations of PbAc (0-200µg/mL) for 24h and repeatedly treated for 72h.

The results are presented as a percentage of the control. One-way ANOVA was used to determine significant differences among groups. All data are expressed as mean ± SEM of three independent experiments. The student's t-test was used for comparison of differences between groups and *$p < 0.05$ and **$p < 0.01$ are considered statistically significant.

3.2 Pb-Induced Oxidative DNA Damage in RPTEC/TERT1 Cells

To determine DNA damage status in PbAc-exposed RPTEC/TERT1 cells, the cellular ROS and 8-OHdG levels, a marker of oxidative DNA damage, were measured using H_2DCF oxidation assay and LC/MS respectively. After treatment with various concentrations of PbAc for 5h, the production of cellular ROS was significantly increased in a dose-dependent ($p< 0.05$) indicating Pb-induced oxidative stress as shown in Figure 2. To investigate oxidative DNA damage in cells after acute and chronic Pb exposure, PbAc exposure at 40µg/mL for 24h represents acute exposure, while exposure at 10µg/mL for 72h represents chronic exposure. As shown in Figure 2B, 8-OHdG levels were significantly increased by both acute and chronic PbAc exposure compared to the

control (p< 0.01). Altogether, we have demonstrated that acute and chronic Pb exposure induced DNA damage in RPTEC/TERT1 cells.

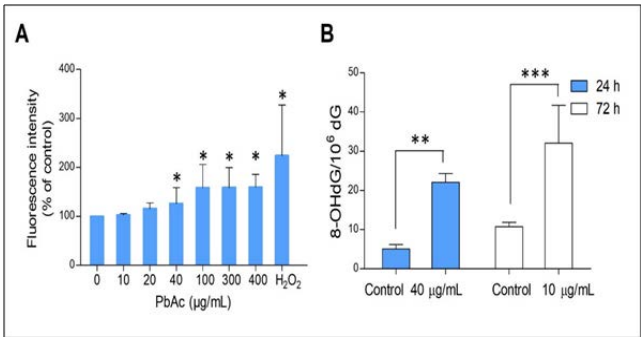


Figure 2. The cellular ROS and 8-OHdG levels in PbAc-exposed RPTEC/TERT1 cells. **(A)** The cellular ROS levels were determined by the measurement of fluorescence intensity. **(B)** The 8-OhdG levels were determined by LC/MS.

All data are expressed as mean ± SEM of three independent experiments. One-way ANOVA was used to determine significant differences among groups. The student's t-test was used for comparison of differences between groups and *p < 0.05 and **p < 0.01 are considered statistically significant.

3.3 Effect of Pb on the Expression of hOGG1

The current study investigated the effects of acute and chronic Pb exposure on hOGG1 expression in RPTEC/TERT1 cells. The mRNA expressions were determined by using RT-PCR. Interestingly, our results indicated that the mRNA of hOGG1 was significantly decreased by acute exposure compared to the control (p< 0.05 or p< 0.01) (Figure 3). However, hOGG1 expression was slightly decreased by acute and chronic Pb exposure. The mRNA and protein levels of DNA repair genes showed a tendency of decreased expression by both acute and chronic exposure to Pb. This is the first report to demonstrate that acute and chronic Pb exposure altered the expression of DNA repair genes in RPTEC/TERT1 cells.

4. Discussion

In this research work, we have assessed the ability of Pb to induce oxidative DNA damage in RPTEC/TERT1 cells using 8-OHdG levels, which is the sensitive and versatile method. Oxidative DNA modification was determined

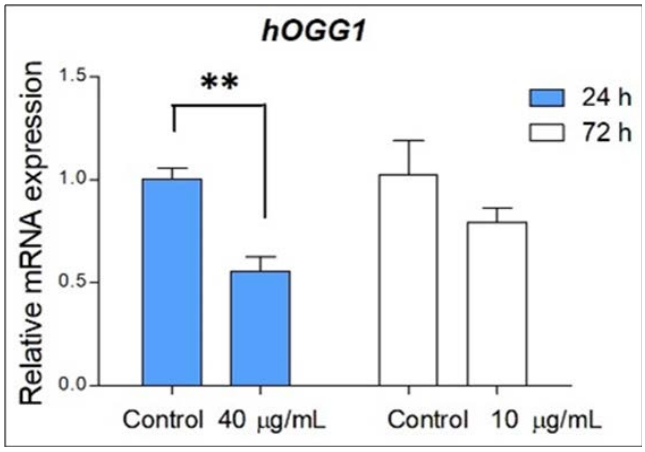


Figure 3. The effects of Pb on the expression of DNA repair genes and proteins in RPTEC/TERT1 cells under acute exposure (40μg/mL, 24h) and chronic exposure (10μg/mL, 72h). The relative mRNA expressions of *hOGG1* in control and PbAc-treated cells were determined using RT-PCR analysis. All data are expressed as mean ± SEM of at least three independent experiments. The student's t-test was used for comparison of differences between groups and *p < 0.05 and **p < 0.01 are considered statistically significant.

using DNA repair enzymes such as hOGG1. This is the first study to provide a potential molecular mechanism of genotoxicity of Pb related to DNA repair systems in human renal proximal tubular epithelial cells. We have observed that Pb oxidatively modified DNA bases in RPTEC/TERT1 cells alterations in the parameters examined. These findings confirmed previous results and other research works, which have shown prooxidative potential DNA damage and inhibition of DNA repairs of heavy metals[15-18]. These results were consistent with the previous studies, they have been extensively demonstrated that Pb-induced oxidative DNA damage plays a crucial role[1,3,4].

We have also reported that Pb induced oxidative DNA base modifications in RPTEC/TERT1 cells at 10μg/mL, which is considered to correspond to Pb concentration determined as normal blood lead of the general population[19]. ROS has been the major factor that can react with DNA bases, free nucleotides and deoxyribose. Literature data indicates that some by-products, which are created during oxidation of protein and lipids may damage to DNA including adduct formation[20,21]. Research works have suggested that Pb-induced DNA damage is associated with prooxidative potential of this substance. Pb-induced DNA damage via oxidative stress has been

reported. For example, a significant increase in DNA damage via ROS generation and reduction of antioxidant level and activity in Pb-exposed human renal proximal tubular epithelial (HK-2) cells was reported[2], and Pb-exposed rats showed DNA fragmentation, higher ROS level, and decrease in antioxidant capacity in rat kidney tissue[4]. In agreement with previous studies, this study demonstrated Pb-induced DNA damage in RPTEC/TERT1 cells. We observed a significant increase in 8-OHdG levels indicating oxidative DNA damage in cells following acute and chronic Pb exposure, and the production of cellular ROS was significantly increased in Pb-exposed cells. They also determined an increased hOGG1 expression, which is a specific enzyme catalyzing excision pre-mutagenic, oxidized lesion such as 8-oxoG. We have noticed that purine bases were more strongly oxidized than pyrimidines in cells treated with Pb. Similarly, Michałowicz and Majsterek *et al.*[22] observed that chlorophenols and their derivatives caused more severe oxidative damage to purines in comparison to pyrimidines in human cells. Formation of 8-hydroxylated purine lesions in DNA involves the well-known mechanism of ROS addition to the C8 of the purine base producing single lesions. Tandem lesions are created by addition of a peroxyl radical onto the C8 of an adjacent purine base followed by decomposition of the transiently generated endoperoxide. Electron transfer from mostly guanine to peroxyl radicals explains the high yield of formation of 7, 8-dihydro-8-oxo-2'-deoxyguanosine, which is observed in DNA[23].

In general, DNA damages generated by ROS can be repaired by Base Excision Repair (BER) and Nucleotide Excision Repair (NER) pathways[24,25]. Therefore, we focused on the DNA repair genes that involve in the BER and NER pathways known to correspond with ROS-induced DNA repair pathways. In the BER pathway, hOGG1 encodes for the repair enzyme 8-oxoguanine DNA glycosylase-1 responsible for the recognition and excision of the oxidized base pairs[26]. In this study, we observed the altered expression levels of DNA repair genes and proteins, and the change in gene and protein levels appeared to be parallel after both acute and chronic Pb exposure. A significant decrease in hOGG1expression at mRNA levels was observed after acute and chronic Pb exposure. Our results are consistent with a previous study demonstrating the influence of heavy metals on the DNA repair pathways, especially the BER pathway[27-29]. Although there are several reports on the decreased expression of DNA repair genes by heavy metals exposure, there are a few reports on DNA repair gene expression in Pb-exposed cells[3,9] and our results are consistent with the literature data demonstrating that Pb exposure inhibits its hOGG1 expression. A previous study reported that Pb significantly reduced expression of DNA repair gene Ogg-1 in mouse embryonic stem (mES) cells[9]. Moreover, a previous study demonstrated that the expression of DNA repair genes hOGG1 and XRCC1 were significantly inhibited in Pb-treated TK6 cells[3]. Epigenetics involves gene expression and regulation without DNA sequence changes[30]. Transcriptional regulation is administered through important epigenetic pathways, dictated primarily by DNA methylation, RNA regulation, and Post-Translational Modification (PTM) of histones[30,31]. Several previous studies demonstrated an interaction of heavy metals and aberrant expression of DNA repair genes via epigenetic mechanisms such as aberrant DNA methylation, modified histone modification, altered expression profiles of microRNAs (miRNAs), and long non-coding RNAs (lncRNAs)[3,9,18,32,33]. In contrast, some studies have shown that heavy metal-impaired DNA repair was mediated by aberrant expression through mutation in the exon of DNA repair genes. Moreover, protein degradation might also play a role due to the ability of Pb to disturb calcium (Ca) homeostasis resulting in high expression of ER chaperones and ER stress. Failure to Resolve ER stress induces ER-Associated Degradation (ERAD) to eliminate unfold and misfold proteins[24]. However, further studies are warranted.

5. Conclusion

In conclusion, the light of the research works showing the toxic and carcinogenic potential of Pb, the results of this study provide new evidence in Pb-induced genotoxicity, which may be involved in adverse health effects of these substances. This study indicates that acute and chronic Pb exposure reduces cell viability and induces oxidative DNA damage in renal proximal tubular epithelial cells. Moreover, our study provides the first evidence to demonstrate that acute and chronic Pb exposure results in the altered expression of DNA glycosylases genes indicating the impairment of DNA repair pathways and contributing to DNA damage. These findings should be useful for the more comprehensive assessment of the toxic effects of Pb.

6. Disclosure Statement

The authors declare no conflict of interest.

7. Funding

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