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Aberrant Expression of DNA Repair Genes in Lead-Exposed Human Renal Proximal Tubular Epithelial Cells

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Abstract

Lead (Pb) is a common environmental pollutant with potential genotoxic properties. However, the molecular mechanisms underlying Pb genotoxicity related to inhibition of DNA repairs are not fully elucidated. This study aimed to investigate the impact of Pb on DNA repair genes and proteins expression. The acute and chronic Pb-exposed human renal proximal tubular cell models (RPTEC/TERT1 cells) were established. The cell viability, cellular Pb level, changes in expression of DNA repair genes and proteins (XRCC1, hOGG1, and ERCC1) were measured. The cell viability assay showed a significant decrease in cell viability in a dose-dependent manner. The AAS analysis showed cellular Pb levels indicating bioaccumulation of Pb in treated cells. RT-PCR and Western blot results demonstrated that the mRNA and protein levels of XRCC1 significantly decreased after acute and chronic exposure. hOGG1 and ERCC1 showed a significant decrease in mRNA level after acute exposure, whereas no significant change in their protein levels after both acute and chronic exposure was observed. In conclusion, our study provides the first evidence to show that acute and chronic Pb exposure results in altered expression of DNA repair genes and proteins and support the possible genotoxic properties of Pb to inhibit the DNA repairs.

Keywords: Lead (Pb); DNA repair genes; Human renal proximal tubular epithelial cells

1. Introduction

Lead (Pb), a toxic heavy metal, is one of the most common environmental pollutants and a widely used heavy metal in several industries. Human is commonly exposed to Pb through ingestion and inhalation. Pb can be accumulated in various soft tissues such as kidneys, livers, lungs, brain, and heart, where it exerts the mechanisms of toxicity and induces a broad range of biochemical, physiological, and genetic dysfunctions in several organs (Singh *et al.,* 2018). The major mechanism of genotoxicity of heavy metals is primarily involved with oxidative DNA damage due to the ability of heavy metals to generate reactive oxygen species (ROSs) and interfere with antioxidant defenses resulting in oxidative stress (Ercal *et al.,* 2001). A common feature of DNA damage induced by heavy metals is the oxidized bases damage, while bulky DNA lesions can be a minority occurred due to generated ROSs (Dobrakowski *et al.,* 2017, Hengstler *et al.,* 2003, Santos-Escobar *et al.,* 2019, Wang 2008). Pb-induced genotoxicity via oxidative stress was well demonstrated, especially in the renal cells model. 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 (Siddarth *et al.,* 2018),

and Pb-exposed rats showed DNA fragmentation, higher ROS content, and decrease in antioxidant capacity in rat kidney tissue (Kelainy *et al.,* 2019). Therefore, the renal proximal tubular epithelial cells (RPTEC/TERT1) were chosen as an in vitro model in this study. In general, the DNA damage induced by heavy metal can be repaired by base excision repair (BER) and nucleotide excision repair (NER) known to correspond with ROS-induced DNA repair pathways (Torgovnick and Schumacher 2015, Wang 2008). Therefore, this study focused on the genes that involve in the BER and NER pathways. In the BER, *hOGG1* encodes for the repair enzyme 8-oxoguanine DNA glycosylase-1 responsible for the recognition and excision of the oxidized base pairs (Kim *et al.,* 2012). The protein encoded by *XRCC1* involves the repair of DNA single-strand break by acting as a scaffolding protein to enhance ligation in the BER (Mateuca *et al.,* 2005). Alternatively, *ERCC1* functions in the NER by interacting with *XPF* as *ERCC1-XPF* complex responsible for incision of a damaged strand within bulky DNA adducts (Sekelsky *et al.,* 2000). Thus, the specific DNA repair genes along the ROS-induced DNA repair pathways play a crucial role to cope with these damages.

Heavy metals are considered capable of interfering with the activities of several proteins and altering the expression pattern of numerous genes (Korashy and El-Kadi 2005, McNeill *et al.,* 2004, Murata *et al.,* 1999). Interestingly, altered gene expression by Pb was reported to affect proliferation, differentiation, detoxification, as well as DNA repair systems (Abdullah *et al.,* 2014, Al Bakheet *et al.,* 2013, Gadhia *et al.,* 2012, Garcia-Leston *et al.,* 2012). Although the previous epidemiological and in vitro studies demonstrated an association of Pb toxicity with DNA repair gene expression (Abdullah *et al.,* 2014, Liu *et al.,* 2018, Singh *et al.,* 2021), 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 and protein expression in the experimental model is lacking.

Pb-induced oxidative DNA damage has been well illustrated in several studies (Kelainy *et al.,* 2019, Kucukler *et al.,* 2021, Liu *et al.,* 2018, Pawlas *et al.,* 2017, Siddarth *et al.,* 2018). On the other hand, the mechanisms underlying its genotoxic properties related to the inhibition of DNA repairs are not fully understood. Therefore, this study aimed to investigate the effects of Pb on DNA repair genes and proteins expression. The hypothesis is that Pb exposure might alter the expression levels of DNA repair genes and proteins. To test this hypothesis, the cell viability and bioaccumulation of Pb after acute and chronic lead acetate (PbAc) exposure were determined in human proximal tubular cells (RPTEC/TERT1), a target site of Pb toxicity. For the first time, the expression levels of genes and proteins (hOGG1, XRCC1, ERCC1) known to correspond with ROS-induced DNA repair pathways were measured.

2. Materials and Methods

2.1 Chemicals and reagents

Lead acetate (PbAc) and culture supplements were purchased from Sigma-Aldrich. RPTEC/TERT1 cells were obtained from American Type Culture Collection (ATCC) Culture medium, penicillin, and streptomycin were purchased from Gibco. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Molecular. Trizol reagent was purchased from Invitrogen. For cDNA synthesis and RT-PCR, the iScript Reverse Transcription supermix and iTag Universal SYBR Green Supermix kit from Bio-Rad Laboratories were used. Primary antibodies against human XRCC1, ß-actin, and HRP-conjugated secondary antibody were purchased from Cell Signaling Technology and primary antibody against hOGG1 was purchased from Abcam.

2.2 Cell culture and treatment

RPTEC/TERT1 cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) medium supplemented with 10 ng/mL epithelial growth factor, 5 ng/mL sodium selenite, 36 ng/mL

hydrocortisone, 5 µg/mL transferrin, 5 µg/mL insulin, 100 U/mL penicillin and 100 µg/mL streptomycin in humidified incubator under 5 % CO_2 at 37 °C. Cells were treated with PbAc for 24 h and 72 h (repeating treatment). For repeating treatment, the old medium containing PbAc was replaced with a fresh medium containing PbAc every 24 h until 72 h. Untreated (control) cells were included with only a culture medium. Thereafter, cells were harvested for experimental analysis. This study was approved as exemption review by the Human Research Ethics Committee of Thammasat University (Science), (HREC-TUSc).

2.3 Cell viability assay

The determination of cell viability was assessed by the MTT assay, as described by Mosmann (Mosmann 1983). Briefly, cells were seeded at 5 x 10^4 cells/well in a 96-wells plate and allowed to adhere for 24 h. The confluence monolayers were then treated with different concentrations of PbAc (0 - 200 µg/mL) for 24 h and 72 h (repeating treatment). After treatment, all concentrations of PbAc were removed and 25 µl of MTT (5 mg/mL) in Phosphate Buffered Saline (PBS) was added to 225 µl of the fresh culture medium to each well and incubated for 4 h under 5 % CO_2 at 37 °C. After incubation, the supernatant was removed and 100 µL of dimethyl sulfoxide (DMSO) was added to dissolve the insoluble formazan crystals. The absorbances were measured at 570 nm using an 800 TS microplate reader (BioTek Instruments). The cell viability was calculated as the percent of control. The experiment was repeated in three independent experiments.

2.4 Determination of cellular Pb level

Cellular Pb levels were measured using a graphite furnace atomic absorption spectrophotometry (GFAAS; Shimadzu AA-6800 series, Tokyo, Japan) method (Ivanenko *et al.,* 2012). Cells were seeded at 1 X 10^6 cells/well in a 6-wells plate and maintained until confluence. According to cell viability determined from the MTT assay, cells were treated with 40 µg/mL of PbAc for 2 h, 5 h, and 24 h and also treated with 10 µg/mL of PbAc for 2 h, 5 h, and 72 h. At the end of treatment, cells were washed three times with PBS and cell pellets were harvested by trypsinization. Cell pellets were washed in PBS three times by centrifugation at 115 g for 10 minutes. Washed cell pellets were lysed in a lysis solution, containing 0.1% (v/v) Nitric acid and 1% (v/v) Triton X-100 for 30 minutes together with continuous shaking followed by centrifugation at 115 g, for 10 minutes. Supernatants were then collected and diluted with a modifier, containing 0.7 % (w/v) Ammonium dihydrogen phosphate and 0.2 % (v/v) Triton X-100, to measure Pb levels (Yee *et al.,* 1994). Pb standard (Merck) was used to prepare different standard levels. Limit of detection (LOD) and Limit of quantitation (LOQ) achieve allowed deter-mi-nation of cellular Pb were 0.3 pg/cell and 0.1 pg/cell, respectively. The experiment was repeated in three independent experiments.

2.5 RNA isolation and cDNA synthesis

PbAc-treated cells and controls were harvested in TRIzol reagent following the manufacturer's recommendation (Yongqiang *et al.,* 2021). 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) and reverse transcribed into cDNA using iScript RT supermix kit according to the manufacturer's instruction using T100 Thermal Cycler (Bio-Rad Laboratories). The cDNA was stored at -20 °C for later use.

2.6 Quantitative real-time polymerase chain reaction (RT-PCR) analysis

The mRNA expression was quantified using a sybergreen RT-PCR kit according to the instruction provided in the kit of iTaq Universal SYBR Green Supermix (Baig et al., 2016). The sequences of primers were chosen from previous studies (Pesta *et al.,* 2012, Singh *et al.,* 2021) as shown in Table 1. The PCR was run in CFX96 Real-Time System (Bio-Rad Laboratories). The thermocycling conditions were denaturation at 95 °C

for 3 min, annealing at 55 °C for 10 s, and extension at 60°C for 30 s, for 40 cycles. ß-actin was used as an internal control for normalization. The relative change of DNA repair gene expression was determined using the $2^{-\Delta\Delta CT}$ method. RT-PCR reactions were run in duplicate and repeated in three independent experiments.

2.7 Western blot analysis

The protein expressions of XRCC1, hOGG1 and ERCC1 were determined by Western blot (Lei *et al.,* 2015). Protein extracts were prepared in an ice-cold lysis buffer containing a protease inhibitor cocktail (Roche). 40 µg of each protein sample were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were then blocked for 1 h with 5 % nonfat milk in TBST and incubated with primary antibodies at dilution provided in the manufacturer's instruction at 4 °C overnight. After overnight incubation and three times 10 minutes wash with TBST, the membranes were incubated with HRP-conjugated anti-rabbit immunoglobulin G antibody at room temperature for 1 h according to kit's instruction (Krishn *et al.,* 2022). The protein-antibody complexes were detected by enhanced chemiluminescence (ECL) reagent using ChemiDoc XRS systems with Image Lab software (Bio-Rad Laboratories) for quantification of the band intensity. ß-actin was used as an internal control for normalization. The fold change of DNA repair proteins in the treated cells was calculated compared to the control. The experiment was repeated in four independent experiments.

2.8 Statistical analysis

All data point was 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 and Discussion

3.1 PbAc-induced cytotoxicity in RPTEC/TERT1 cells

RPTEC/TERT1 cells were treated with various concentrations of PbAc. The MTT results revealed a dose-dependent decrease in cell viability after 24 h and 72 h of exposure (Figure 1). At ≥ 40 µg/mL concentrations of PbAc, cell viability decreased significantly after 24 h of exposure, while cell viability decreased significantly at ≥10 µg/mL upon 72 h of exposure. Therefore, 40 µg/mL as acute exposure and 10 µg/mL as chronic exposure were chosen as the optimum dose for 24 h and 72 h exposure respectively of all subsequent experiments.

Table 1. Primer sequences of target genes for RT-PCR analysis.

Genes	Primers	Sequences	Accession Number
XRCC1	Forward	5′-CTGGGACCGGGTCAAAAT-3′	NM_006297
	Reverse	5′-CAAGCCAAAGGGGGAGTC-3′	
hOGG1	Forward	5′-AATTCCAAGGTGTGCGACTG-3′	NM_002542
	Reverse	5′-CGATGTTGTTGTTGGAGGAAC-3′	
ERCC1	Forward	5'-GAAATTTGTGATACCCCTCGAC-3′	NM_001369419
	Reverse	5'GATCGGAATAAGGGCTTGG-3′	
ß-actin	Forward	5′CCTGGCACCCAGCACAAT-3′	NM_001101
	Reverse	5′-GCCGATCCACACGGAGTACT-3′	

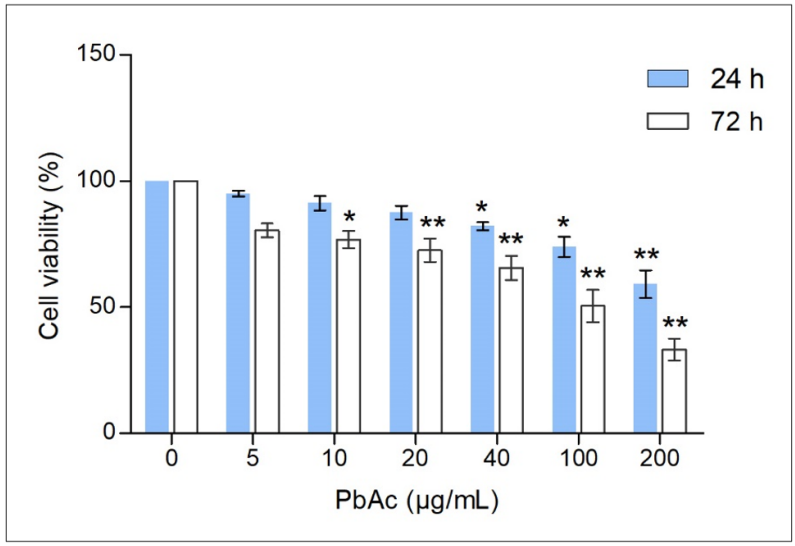

Figure 1. Cell viability (%) of RPTEC/TERT1 cells following PbAc exposure for 24 h and 72 h

3.2 Cellular Pb level in RPTEC/TERT1 cells after PbAc exposure

Cellular levels of Pb in RPTEC/TERT1 cells following PbAc exposure are shown in Figure 2. No cellular Pb content was observed in all controls. After exposure to 40 µg/mL of PbAc, a relative cellular Pb level at 5 h was higher than that of 2 h due to an increase in absorption rate that generally reaches a stationary state at about 10 h in cultured cells (Meshitsuka *et al.,* 1987) and a relative cellular Pb level at 24 h slightly decreased but still close to that of 5 h. In contrast, at the end of exposure to 10 µg/mL of PbAc, a relative cellular Pb level at 72 h was higher than that of 5 h as a result of repeating exposure every 24 h. Altogether, this study has previously indicated Pb levels accumulated in RPTEC/TERT1 cells after exposure to PbAc at different periods of incubation time. In agreement with our results, earlier studies have reported that renal cells are the target sites for Pb bioaccumulation initiating its mechanisms of toxicity (Albarakati *et al.,* 2020, Mabrouk 2018, Sabolic 2006). Moreover, repeating the administration of cadmium (Cd) in a longer period trended to produce more accumulation of Cd livers than in a shorter period (Habeebu *et al.,* 2000).

3.3 Effects of Pb on the expression of hOGG1, XRCC1, and ERCC1

PbAc exposure at 40 µg/mL for 24 h represents acute exposure, while exposure at 10 µg/mL for 72 h represents chronic exposure. Interestingly, our results indicated that the relative mRNA and protein expressions of XRCC1 were significantly decreased by both acute and chronic exposure (Figures 3A, D, F). Moreover, the relative mRNA expressions of hOGG1 and ERCC1 were significantly decreased by acute exposure (Figures 3B, C), whereas their fold change of protein level showed no statistically significant change but still decreased (Figures 3E, F, H, I). Although the relative mRNA and protein expressions of hOGG1 and ERCC1 appeared to be decreased by chronic exposure, expression changes were not considered significant (Figures 3B, C). Overall, the relative mRNA and protein levels of all three DNA repair genes were decreased by both acute and chronic exposure to PbAc, even though not all of them were considered significant.

In this study, a significant decrease in mRNA and protein expression levels of XRCC1 in both acute and chronic PbAc-exposed cells was observed, while hOGG1 and ERCC1 mRNA and proteins showed no

significant change after chronic exposure. These differing effects might be due to the distinct sensitivities of DNA repair proteins under heavy metals toxicity (Candeias *et al.,* 2010). However, our results are consistent with the previous studies demonstrating the influence of heavy metal on the BER and NER pathways (Candeias *et al.,* 2010). For example, Gadhia et al. reported that Pb significantly reduced the expression of DNA repair gene Ogg-1 in mouse embryonic stem (mES) cells (Gadhia *et al.,* 2012). Xiangquan et al. demonstrated that the expression of DNA repair genes hOGG1 and XRCC1 were significantly inhibited in Pb-treated TK6 cells (Liu *et al.,* 2018). Zhou *et al.* reported the decreased expressions of DNA repair genes XRCC1 and hOGG1 during cadmium (Cd) induced malignant transformation of human bronchial epithelial cells (Zhou *et al.,* 2013). Moreover, XRCC1 and hOGG1 were significantly declined in the liver, kidney, heart, and lung tissues of Cd-exposed rats as compared with the control group (Lei *et al.,* 2015).

Epigenetics involves gene expression and regulation without DNA sequence changes (Hou *et al.,* 2011). Transcriptional regulation is administered through important epigenetic pathways, dictated primarily by DNA methylation, RNA regulation, and post-translational modification (PTM) of histones (Hou *et al.,* 2011, Jaenisch and Bird 2003). 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) (Gadhia *et al.,* 2012, Liu *et al.,* 2018, Rojas *et al.,* 2019, Zhou *et al.,* 2015, Zhou *et al.,* 2012). Therefore, the genotoxic properties of heavy metals, as well as Pb, may be involved with the epigenetic mechanisms affecting the expression of DNA repair genes. However, further studies are warranted. Moreover, some studies showed that heavy metal impaired DNA repair was mediated by aberrant expression through mutation in the exon of DNA repair genes (Zhou *et al.,* 2013). In this study, the decreased expression levels of DNA repair proteins were detected. It can be suggested that protein degradation might also play a role due to the ability of Pb to disturb calcium (Ca) homeostasis resulting in high expression of endoplasmic reticulum (ER) chaperones and ER stress (Meng *et al.,* 2018). Failure to resolve ER stress induces ER-associated degradation (ERAD) to eliminate unfold and misfold proteins (Meng *et al.,* 2018). However, further studies are warranted.

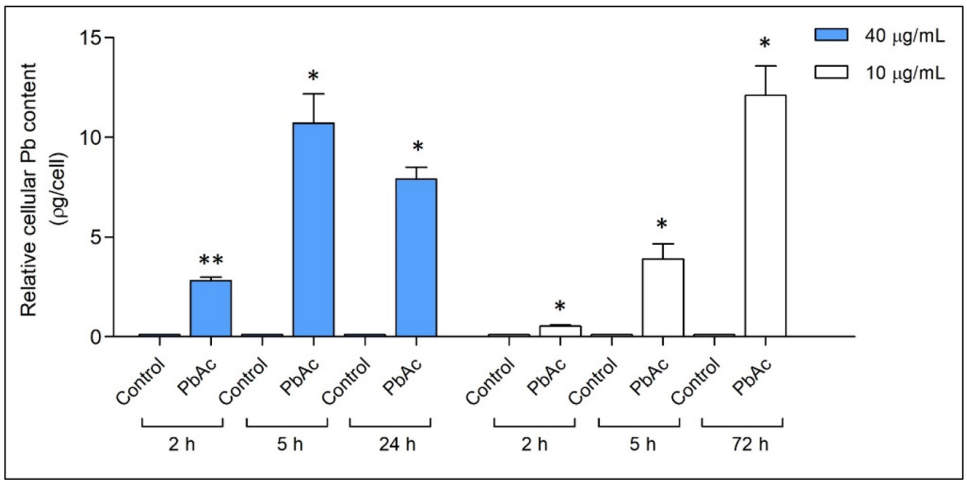

Figure 2. Cellular Pb levels accumulated in RPTEC/TERT1 cells after exposure to an acute exposure (40 µg/mL) and chronic exposure (10 µg/mL) dose of PbAc

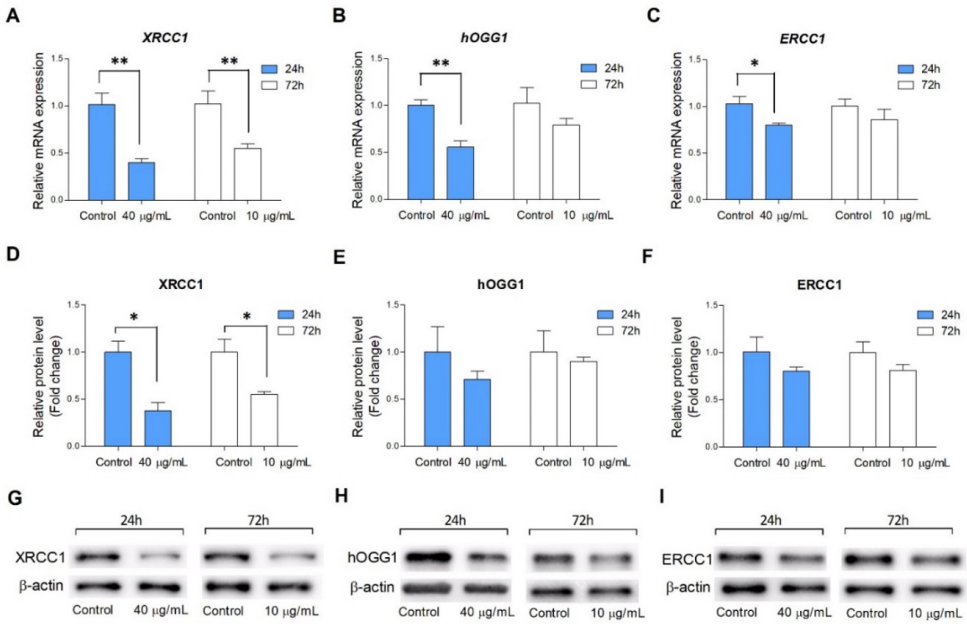

Figure 3. The effects of Pb on the expression of DNA repair genes and proteins in RPTEC/TERT1 cells following cute exposure (40 µg/mL, 24 h) and chronic exposure (10 µg/mL, 72 h). (A-C) The relative mRNA expressions of *XRCC1*, *hOGG1*, and *ERCC1* (D-F) Fold change of protein levels of XRCC1, hOGG1, and ERCC1. (G-I) Protein levels of XRCC1, hOGG1, and ERCC1

4. Conclusion

This study provides evidence in the molecular levels indicating the altered expression levels of DNA repair genes and proteins in the cells exposed to acute-high and chronic-low concentrations of Pb. The finding of this study concurs with the past relevant literature and also support the possible genotoxic properties of Pb to inhibit the DNA repairs. The limitations of this study include (a) the acute and chronic effects related to different concentrations of PbAc should be examined. (b) to examine the chronic effect, the exposure period should be prolonged. (c) the obtained results need to be verified in multiple experimental models in the future.

Disclosure statement

The authors declare no conflict of interest. This research was examined by the Ethical committee of Thammasat University, identified that this work-related cell culture is classified as Examption.

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