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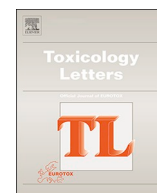
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Alterations of mineralized matrix by lead exposure in osteoblast (MC3T3-E1) culture

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ABSTRACT

The present study investigated the effect of lead (Pb) on bone ultrastructure and chemistry using an *in vitro* bone model. MC3T3-E1 preosteoblasts were differentiated and treated with lead acetate at 0.4, 2, 10, and 50 μ M. No abnormalities in either cell growth or bone nodule formation were observed with the treated dose of lead acetate. However, Pb treatments could significantly increase Pb accumulation in differentiated osteoblast cultures and upregulate expression of Divalent metal transporter 1 (*Dmt1*) in a dose dependent manner. Pb treatments also altered the expression of osteogenic genes, including secreted phosphoprotein 1, osteocalcin, type I collagen, and osteoprotegerin. Moreover, in mineralized osteoblast cultures, Pb was found to be mainly deposited as Pb salts and oxides, respectively. Ultrastructure analysis revealed Pb localizing with calcium and phosphorus in the mineralized matrix. In mineralizing osteoblast cells, Pb was found in the intracellular calcified vesicles which is one of the bone mineralization mechanisms. Pb was also present in mineral deposits with various shapes and sizes, such as small and large globular or needle-like mineral deposits representing early to mature stages of mineral deposits. Furthermore, Pb was found more in the globular deposits than the needle shaped mineral crystals. Taken together, our observations revealed how Pb incorporates into bone tissue, and showed a close association with bone apatite.

1. Introduction

Lead (Pb) is one of heavy metals causing a variety of human health issues. The use and disposal of Pb are subjected to regulation by laws to decrease risk of Pb toxicity in humans (Pirkle et al., 1998). Despite strict regulations, Pb poisoning is still a major concern in some occupational activities (Coon et al., 2006) and many waste management facilities, including electronic waste recycling plants (Zeng et al., 2016) and landfills (Kim and Williams, 2017). Pb not only has acute effects on health, but also results in long-term health complications dependent on the exposure doses and duration (Rosin, 2009). Exposure of lead can cause impaired physiological functions in many organs, including the kidney, (Zhang et al., 2013), nervous system (Oszlanczi et al., 2011) and bone (Carmouche et al., 2005; Oszlanczi et al., 2011; Zhang et al.,

2013). (Carmouche et al., 2005)

The biological fate of Pb in the bodies is mainly fixed in bone and other calcified tissues (Nascimento et al., 2016). Pb deposition in bone shows a wide range of the adverse effects including decreased ALP activities (Klein and Wiren, 1993). The biological fate of Pb in the bodies is mainly fixed in bone and other calcified tissue (Nascimento et al., 2016). Pb deposition in bone shows a wide range of the adverse effects including decrease ALP activities (Klein and Wiren, 1993) and inhibits osteonectin secretion in the bone (Sauc et al., 1992). Pb accumulation in the bone also decreases fracture healing process as it promotes chondrogenesis (Carmouche et al., 2005). It has been shown that, in Pb-exposed humans, bone resorption markers, including alkaline phosphatase, urinary hydroxyproline, and urinary deoxypyridinoline are upregulated (Akbal et al., 2014). Moreover, levels of Pb in the human

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blood are negatively associated with bone mineral density and strength (Bagchi and Preuss, 2005; Bjorå et al., 2001). It is interesting to note that Pb half-life in the bone is about 20 years. Therefore, the effect of Pb on the bone can be manifested many years after an acute or chronic exposure (Pemmer et al., 2013; Zoeger et al., 2006). A recent study in women has showed that previous exposures to Pb not only elevate circulating Pb levels, but also decrease bone mineral density during menopause, a period of high bone turnover in women (Potula et al., 2006). High levels of Pb in the human blood is also associated with osteoarthritis (Nelson et al., 2011) and osteoporosis (Akbal et al., 2014).

It has been demonstrated that Pb can be transported into enterocytes in the blood by Divalent metal transporter 1 (DMT1 or SLC11A2). DMT1 is known to be responsible for Fe^{2+} uptake (Liu et al., 2017). But it also transports other divalent cations with low-affinity, including copper (Cu^{2+}), zinc (Zn^{2+}), manganese (Mn^{2+}), cadmium (Cd^{2+}), and Pb^{2+} (Kayaalti et al., 2015). Multiple transcript variants encoding different isoforms, including DMT1A and DMT1B have been found for the human *DMT1* gene. The difference between the isoforms lies in the presence or the absence of an iron responsive element (DMT1 + IRE and DMT1-IRE) in the transcripts. The DMT1 isoforms present different expression patterns and metal binding affinity in tissues, suggesting specific functions in metal transport in different tissues (Lam-Yuk-Tseung and Gros, 2006). Whether DMT1 plays a role in Pb^{2+} uptake and trafficking in the bone cell remains to be elucidated. On the other hand, Pb in the bone structure can be detected using micro X-ray fluorescence (SR μ -XRF) and quantitative backscattered electron imaging (qBEI) analysis. It is worth noting that Pb deposition in the bone structure matrix is along with the cement line and around the border of Haversian canal (Pemmer et al., 2013). The cement line region faces interstitial fluid and is directly in contact with various metal ions. The deposition of Pb^{2+} in the bone matrix may be due to 1) Pb^{2+} may directly deposit into hydroxyapatite structure and/or 2) Pb^{2+} binds to bone associated proteins, such as Osteocalcin, and is then deposited in the region where Osteocalcin is localized (Dowd et al., 1994; Dowd et al., 2001; Pemmer et al., 2013). Both the mineral structure or matrix proteins are the building blocks of bone formation. Therefore, Pb deposition in these building blocks can alter bone chemistry and structure, resulting in interference of interactions among the building blocks. Although many studies have investigated the deleterious effects of Pb on bone functions, it remains largely unknown how Pb is incorporated and deposited into the bone structure.

The present study aimed to investigate the effects of Pb on bone ultrastructure and chemistry. We first determined sub-toxic levels of lead exposure in murine MC3T3-E1 preosteoblasts. We used MC3T3-E1 preosteoblasts in this study as these cells are osteogenic and form nodular structures mimicking the bone structure *in vivo* (Xu and Simon, 2005). We induced MC3T3-E1 cell differentiation to form bone nodules in the presence of sub-toxic levels of lead. Cell viability, morphology, and calcium deposits in the mineralized osteoblasts were determined. We also determined mRNA expression of DMT1 and osteogenic-associated genes. In addition, we used an X-ray absorption spectroscopy to analyze the chemical forms of Pb deposited in the mineralized cultures. Importantly, we performed ultrastructure analysis and electron dispersive x-ray spectroscopy, investigated cellular Pb trafficking, and analyzed accumulation of Pb in different stages of extracellular mineral deposits.

2. Materials and methods

2.1. Cell culture, osteoblast differentiation, and Pb treatment

MC3T3-E1 subclone 4 (ATCC[®] CRL-2593[™]) preosteoblasts were cultured and differentiated as described previously (Gentleman et al., 2009). Briefly, MC3T3-E1 preosteoblasts were maintained in modified α -Minimum Essential Medium Eagle (α -MEM) without ascorbic acid

(Gibco[®], ThermoFisher Scientific) supplemented with 10% fetal bovine serum (FBS, Gibco[®], ThermoFisher Scientific) and 1% penicillin/streptomycin (Gibco[®], ThermoFisher Scientific).

The cells were seeded at 30,000 cells/cm² and differentiated in the osteogenic medium containing 50 $\mu\text{g}/\text{ml}$ ascorbic acid and 10 μM β -glycerophosphate in the complete medium. After 14 days, 1 μM dexamethasone (Sigma-Aldrich) was added into the osteogenic medium to complete the differentiation of MC3T3-E1 to osteoblasts. Pb treatments started at the same time of the addition of dexamethasone. Lead acetate (Sigma-Aldrich) were added to the osteogenic medium at different concentrations, including 0, 0.4, 2, 10, and 50 μM . Lead acetate was prepared as 0.1 M stock solution and filter-sterilized before use. The differentiation medium was changed every 2 days.

2.2. Pb toxicity assessment

Toxicity of Pb-treated MC3T3-E1 preosteoblasts was evaluated using xCELLigence[®] real-time cell analysis (ACEA Biosciences). The cells were seeded in E-Plates at 8,000 cells/well and cultured for 24 h prior to the Pb treatment. Lead acetate was added into the medium at 1, 10, 50, 100, 1,000, and 10,000 μM . Triton X was added at 1% final concentration in the control wells and used as positive controls. Real-time impedance traces were recorded every 30 min for 60 h. Cell death was indicated by a decrease in the impedance signal. Each treatment was performed in duplicate.

2.3. Cell viability and calcium staining

Differentiated osteoblasts were used to evaluate cell viability at days 7 and 14 after Pb was added into the differentiation medium. Cell viability was analyzed using a LIVE/DEAD Viability/Cytotoxicity kit for mammalian cells (ThermoFisher Scientific) according to the manufacturer's instructions. Calcium staining was performed using the osteoblast cells on days 3, 7, and 14 after the Pb treatment as described previously (Gregory et al., 2004). Briefly, the cells were fixed with 4% paraformaldehyde for 15 min at room temperature and washed twice with 1xPBS. Calcium deposits were stained for 15 min using Alizarin Red S (1% in double-distilled water, pH 4.1, Sigma Aldrich), washed with 1 xPBS, and dried overnight. Fluorescence or calcium staining was visualized using an inverted phase-contrast microscope (Eclipse Ts-2i, Nikon).

2.4. Gene expression analysis

Differentiated osteoblasts were collected on days 3, 7, and 14 after the Pb treatment. The cells were lysed in the TRIzol[®] reagent (ThermoFisher Scientific). Total RNA was isolated according to the manufacturer's protocol. cDNA was synthesized using an iScript cDNA synthesis kit (BioRad Laboratories) following the manufacturer's instructions. cDNA samples were diluted 5-fold with nuclease-free water prior to use in quantitative PCR analyses (qPCR). Gene expression was carried out using a SsoAdvanced SYBR[®] Green supermix (BioRad Laboratories). Primers were designed using Primer-Blast (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) or adopted from previous studies (Arsenijevic et al., 2012; Galy et al., 2013; Jadowiec et al., 2004; Mei et al., 2014; Tan et al., 2010; Yan et al., 2014). All primers were confirmed using melting curve analysis. qPCR was performed in triplicate on a CFX96 Real-time System (BioRad Laboratories). *Act- β* was used as an internal control. Fold differences were calculated using 2 ^{$\Delta\Delta\text{Ct}$} methods (Livak and Schmittgen, 2001).

2.5. Lead and calcium quantifications

Mineralized osteoblast cultures 14 days after the Pb treatment were collected and dehydrated using a freeze dryer (Gamma 2-16 LSC Plus, Christ). The dried cells were digested in 65% nitric acid at 200 °C under

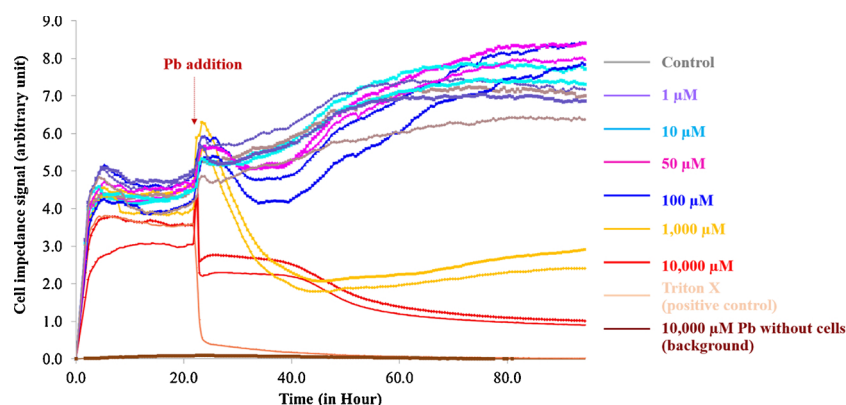


Fig. 1. Cell viability of MC3T3-E1 preosteoblasts with Pb treatment. Preosteoblasts was treated with lead acetate at 1, 10, 50, 100, 1,000, and 10,000 μM . Untreated and 1% Triton X-treated preosteoblasts were used as negative and positive controls, respectively. Each treatment was done in duplicate. Cell viability was determined by impedance signals using XCELLigence® real-time cell analysis. The impedance signals were measured every 30 min for 60 hours. Culture medium containing 10,000 μM lead acetate was used to measure the background signal.

pressure 800 psi for 15 min using a Microwave Digestion System (MARS 6, CEX Corp.). Pb and calcium contents were determined using an inductively coupled plasma mass spectrometry (ICP-MS; 7900 ICP-MS, Agilent) against reference standards. The calibration curves of Pb and calcium were linear with a regression coefficient of 0.9999 in the range of 1–500 ng/ml and 1–10 $\mu\text{g}/\text{ml}$, respectively. Pb and calcium contents were expressed in mg/g dry weight. The accuracy of measurement was evaluated using reference standard of Pb (Agilent Technologies) and calcium (Carlo Erba). 10 ppm Pb and 100 ppm calcium were spiked into digested cell solution to calculated % recovery, which were 104.60% and 107.65 % for Pb and calcium.

2.6. Characterization of lead compounds

Mineralized osteoblast cultures 14 days after the Pb treatment were dehydrated as described above. The forms of the Pb compounds in the rehydrated samples were characterized using an X-ray absorption spectroscopy (XAS). X-ray absorption near the edge structure (XANES) spectra of the Pb L_{3} edge was recorded at beamline 8 Synchrotron Light Research Institute, Thailand. The energy calibrations were carried out using metal Pb foil (13035 eV) for Pb L_{3} edge. PbO, PbO₂, and Pb₂O₄ were used to identify the unknown forms of lead oxide in the samples. PbSO₄ was selected to represent the lead salt compounds, such as halide, sulfate, chromate, and carbonate salts. The data reduction and peak fitting were performed using ATHENA data analysis (Ravel and Newville, 2005).

2.7. Ultrastructure analysis

Mineralized osteoblast cultures 14 day after the Pb treatment were collected and prepared for ultrastructure analysis. The cells were fixed in 4% (v/v) glutaraldehyde in 0.1 M piperazinediethanesulfonic acid (PIPES) buffer at 4 °C for 2 h and incubated in 1% (w/v) osmium tetroxide made in 0.1 M PIPES buffer at room temperature for 1 h. The samples were dehydrated using ascending concentrations of ethanol series, infiltrated, and embedded in epoxy resin (Epon 812, Electron Microscopy Science). Ultra-thin sections were cut using ultramicrotome (EM UC7, Leica) with a 45° diamond knife (DiATOME). The sections were collected for transmission electron microscope (TEM) studies without post-staining. Mineral analysis was also carried out at ultrastructure levels. Briefly, the mineralized cultures were dehydrated in ethylene glycol (Boonrungsiman et al., 2012) for 3 h, immersed in absolute ethanol, and embedded in epoxy resin. The samples were sectioned and collected on copper grids. Bright-field TEM and electron dispersive x-ray spectroscopy (EDX) were performed on a JEOL 2100, operated at 120 kV. Pb, calcium, and phosphorus levels were quantified from the TEM sections ($n = 20$) using X-ray intensity of each element according to the Cliff Lorimer's equation.

2.8. Statistical analysis

Data were presented as the mean $\pm$ S.D. or S.E. Student's *t* test was used to determine differences in gene expression of the control and treatment groups. One-way analysis of variance (ANOVA) was used to compare the results among Pb treatments. Differences between groups in ANOVA were further analyzed using Tukey's honestly significantly difference (HSD) test. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Cell viability and bone nodule formation of Pb-treated MC3T3-E1 osteoblasts

To determine the sub-toxic level of Pb in MC3T3-E1 preosteoblasts, we first treated the cells with Pb at different concentrations (1, 10, 50, 100, 1,000 and 10,000 μM). Preosteoblasts treated with Triton X (1%) were used as a positive control for cell apoptosis. As shown in Fig. 1, cells treated with 1% Triton X for 20 h showed a dramatic decrease in cell impedance signals, indicating cell death (Fig. 1). Similar declinations, but less dramatic, of impedance signals were observed with cells treated with Pb at 10,000 μM for 20 h. At 1,000 μM Pb level, the declination of cell impedance signals were delayed compared to that of the cells treated at 10,000 μM Pb level. Treatment of MC3T3-E1 with 100 μM Pb caused a small reduction in cell impedance signals after about 30 h incubation with Pb. However, cells were recovered after 40 h and the signal was comparable to those in cells treated with 1, 10 or 50 μM Pb. In addition, the impedance signals obtained from cells treated with Pb at 1, 10, 50, and 100 μM were similar to that obtained from the untreated control. As the result, Pb concentrations lower than 100 μM were chosen as the doses for further treatments in the present study.

The minimum effect of Pb treatments at 0.4, 2, 10, and 50 μM on the cell viability in differentiated osteoblasts was further confirmed using a fluorescence-based LIVE/DEAD staining assay on days 7 and 14 after the Pb treatment. As shown in Fig. 2, no significant differences in the cell viability were observed in Pb-treated cells at all examined concentrations compared to the untreated control. We further investigated whether these sub-toxic Pb levels impaired the bone nodule formation and/or the extracellular matrix calcium deposit on days 7 and 14 after Pb treatments. The results from the bright-field and Alizarin Red staining images revealed the presence of bone nodules and extracellular matrix calcium deposits. The Pb treatment at all examined levels for 7 or 14 days showed no effect on the bone nodule formation and calcium deposits. Taken together, these results demonstrated that Pb concentrations at 0.4, 2, 10 and 50 μM had no detectable effects on the cell viability, nodule formation, and calcium deposits in mineralized osteoblast cultures.

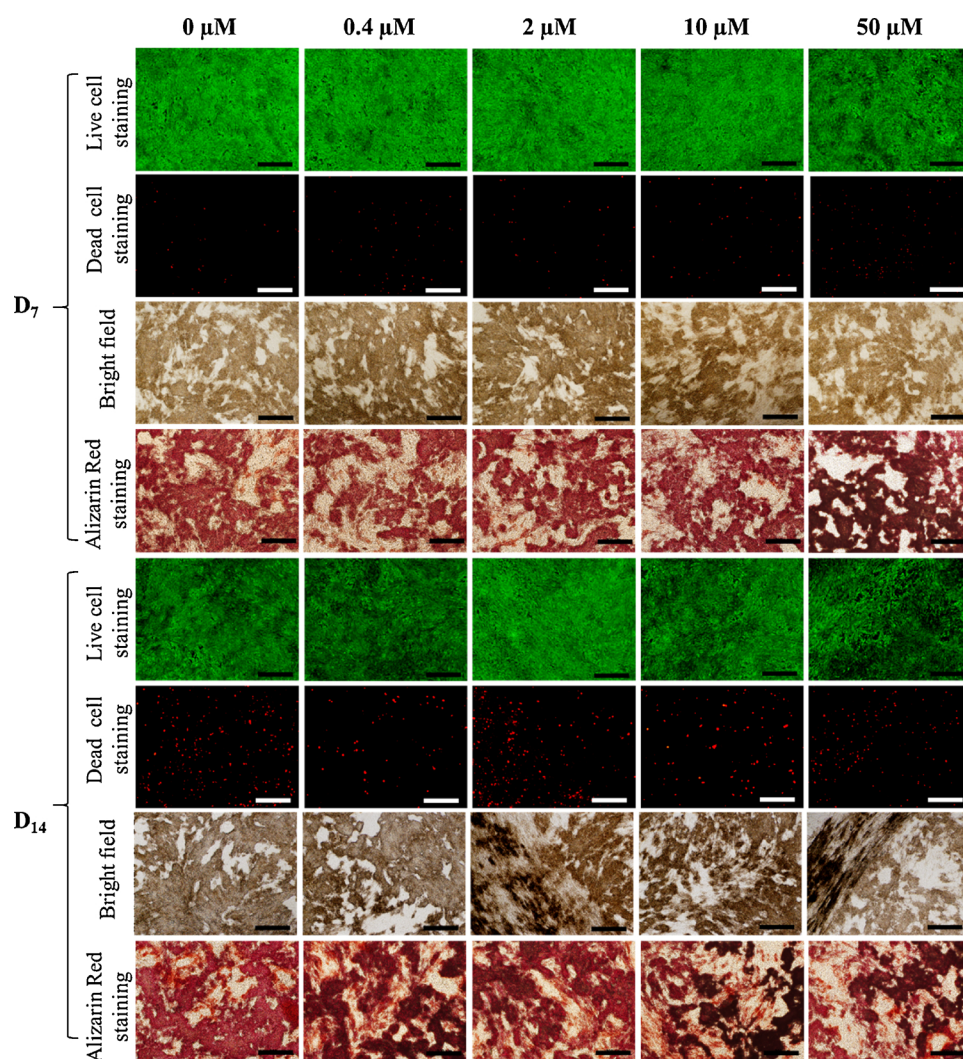


Fig. 2. The effects of Pb treatments on cell viability, morphology, and calcium accumulation in differentiated osteoblast cultures. The cultures were treated with 0.4, 2, 10 and 50 μ M lead acetate for 7 (D₇) or 14 (D₁₄) days. Untreated cultures were used for negative controls. Live/dead staining was performed and recorded using a fluorescence microscope. Live and dead cell populations were indicated by green and red punctures, respectively. The morphology of osteoblast cultures was examined using a bright field microscope. Calcium accumulation was determined by Alizarin Red staining and visualized as red crystal clumps. Representative photographs are shown. Scale bar = 200 μ m.

3.2. Pb and calcium accumulation in mineralized osteoblast cultures

Pb and calcium contents in the mineralized osteoblast cultures 14 days after the Pb treatment were determined using ICP. As shown in Table 1, significant differences in Pb contents were observed in mineralized cultures treated with various levels of Pb. The Pb levels in the mineralized cultures were shown to be dose dependent. Total Pb contents were detected in a range from 0.003 to 12.231 mg/g dry weight and total calcium levels ranged from 43.028 to 70.113 mg/g dry weight. Nevertheless, it seemed that the accumulation of Pb in osteoblast cultures had no notable effect on calcium deposits in the cultures treated with sub-toxic Pb concentrations. The Pb/calcium ratios were

0.00004, 0.0030, 0.0157, 0.0585, and 0.1888 in the osteoblast cultures treated with Pb at 0, 0.4, 2, 10 and 50 μ M, respectively. Accumulation of Pb in the mineralized osteoblast cultures was also supported by *Dmt1* gene expression analysis. We analyzed mRNA expression of *Dmt1* + IRE and *Dmt1*-IRE in the Pb-treated cells at 0.4, 2, and 50 μ M for 7 days. Upregulation of *Dmt1* + IRE and *Dmt1*-IRE were observed in the differentiated osteoblasts treated with Pb at 2 and 50 μ M (Fig. 3). Compared to the controls, the expression of *Dmt1* + IRE in the differentiated osteoblasts treated with Pb at 2 and 50 μ M increased by 1.38- and 2.18-fold, respectively. Additionally, the expression of *Dmt1*-IRE was upregulated by 1.33- and 2.17-fold in cells treated Pb at 2 and 50 μ M, respectively. Together, Pb treatments promoted Pb deposits in

Table 1

Lead (Pb) and calcium (Ca) contents in Pb treated mineralized osteoblast cultures.

Mineral contents	The levels of Pb treatment				
	0 μ M (Control)	0.4 μ M	2 μ M	10 μ M	50 μ M
Pb (mg/g dry weight)	0.003 $\pm$ 0.001	0.210 $\pm$ 0.003	0.860 $\pm$ 0.064*	2.517 $\pm$ 0.068*	12.231 $\pm$ 0.639*
% cv	33.33	1.42	7.44	2.70	5.22
Ca (mg/g dry weight)	65.513 $\pm$ 10.348	70.113 $\pm$ 7.438	54.984 $\pm$ 8.097	43.028 $\pm$ 5.452	64.779 $\pm$ 6.460
% cv	15.79	10.61	14.73	12.67	9.97
Pb/Ca	0.00004	0.0030	0.0157	0.0585	0.1888

% cv, coefficient of variation, * P < 0.05.

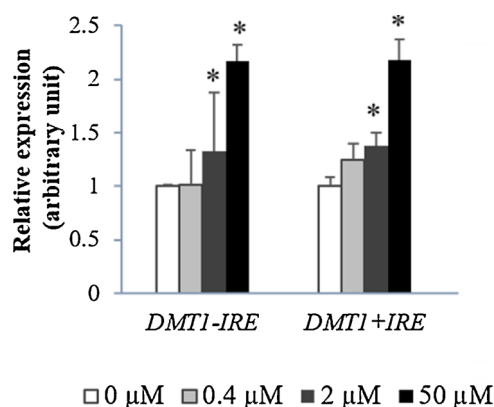
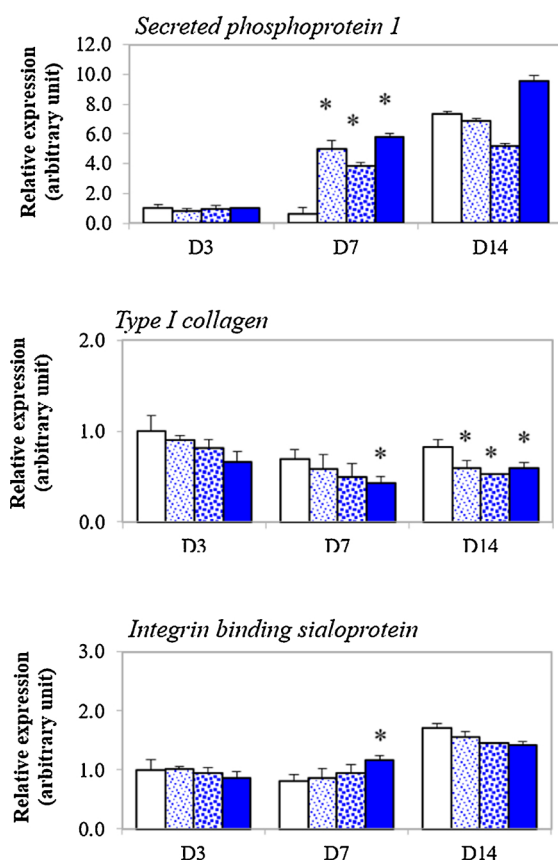


Fig. 3. The effects of Pb treatments on *Dmt1* + IRE, and *Dmt1*-IRE mRNA expression in differentiated osteoblasts. The cells were treated with lead acetate at 0, 0.4, 2 and 50 μ M for 7 days before use. Gene expression was measured by a SYBR-based quantitative real-time PCR. *Actb* was used as the internal reference. Three independent experiments, each with duplicate, were performed. Value are the mean $\pm$ S.E. *, $P \leq 0.05$.

mineralized osteoblast cultures and upregulated the expression of *Dmt1* + IRE and *Dmt1*-IRE in a dose dependent manner.

3.3. Effects of Pb treatments on the expression of osteogenic genes

Several studies have shown that acute or chronic Pb exposure



impairs bone metabolism and turnover (Pounds et al., 1991). Here, we investigated the effect of Pb exposure at 0.4, 2, and 50 μ M on the expression of osteogenic genes, including secreted phosphoprotein 1, osteocalcin, type I collagen, osteoprotegerin, integrin binding sialoprotein, and alkaline phosphatase. Quantitative real time RT-PCR (qPCR) was performed using differentiated osteoblasts collected in 3, 7, and 14 days after Pb treatments at 0, 0.4, 2, and 50 μ M. Pb at all examined concentrations significantly upregulated the expression of secreted phosphoprotein 1 by 4.97-, 3.87- and 5.8-fold, and the expression of osteocalcin by 3.45, 3.57 and 3.29-fold in 0.4, 2 and 50 μ M Pb treatment, respectively, after 7 day treatment (Fig. 4). However, no difference in the expression of both genes was observed at days 3 and 14 after Pb treatments. The qPCR results also demonstrated that Pb treatments significantly decreased the mRNA expression of type I collagen by 71.08, 63.85 and 71.08 % and osteoprotegerin by 57.21, 55.35 and 63.26% in 0.4, 2 and 50 μ M Pb treated cultures, respectively, at 14 days after Pb treatment. Pb treatment at 50 μ M also altered the gene expression of type I collagen and osteoprotegerin at day 7. In addition, we observed no effect of Pb treatments on the type I collagen and osteoprotegerin expression at day 3. The expression of secreted phosphoprotein1 and alkaline phosphatase was unaffected by the Pb exposure to osteoblasts. Together, these findings suggested that Pb treatments altered the expression of osteogenic genes, including secreted phosphoprotein 1, osteocalcin, type I collagen, and osteoprotegerin.

3.4. Chemical analysis of Pb deposits in the mineralized osteoblast cultures

We performed chemical analysis of Pb deposits in the mineralized

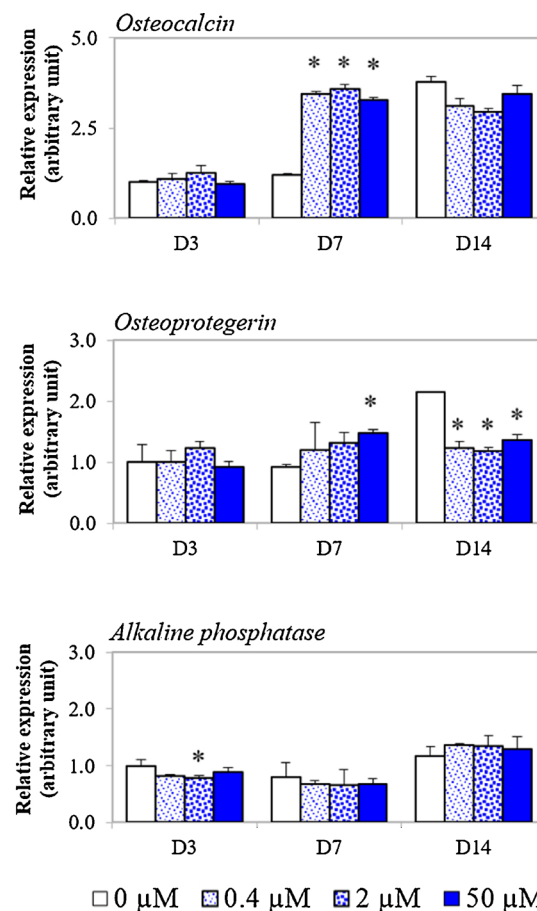


Fig. 4. The effects of Pb treatments on gene expression of secreted phosphoprotein 1, osteocalcin, type I collagen, osteoprotegerin, integrin binding sialoprotein and alkaline phosphatase in differentiated osteoblasts. The cells were treated with lead acetate at 0, 0.4, 2 and 50 μ M for 3, 7, and 14 days. The gene expression was measured by a SYBR-based quantitative real-time PCR. *Actb* was used as the internal reference. Three independent experiments, each with duplicate, were performed. Value are the mean $\pm$ S.E. *, $P \leq 0.05$.

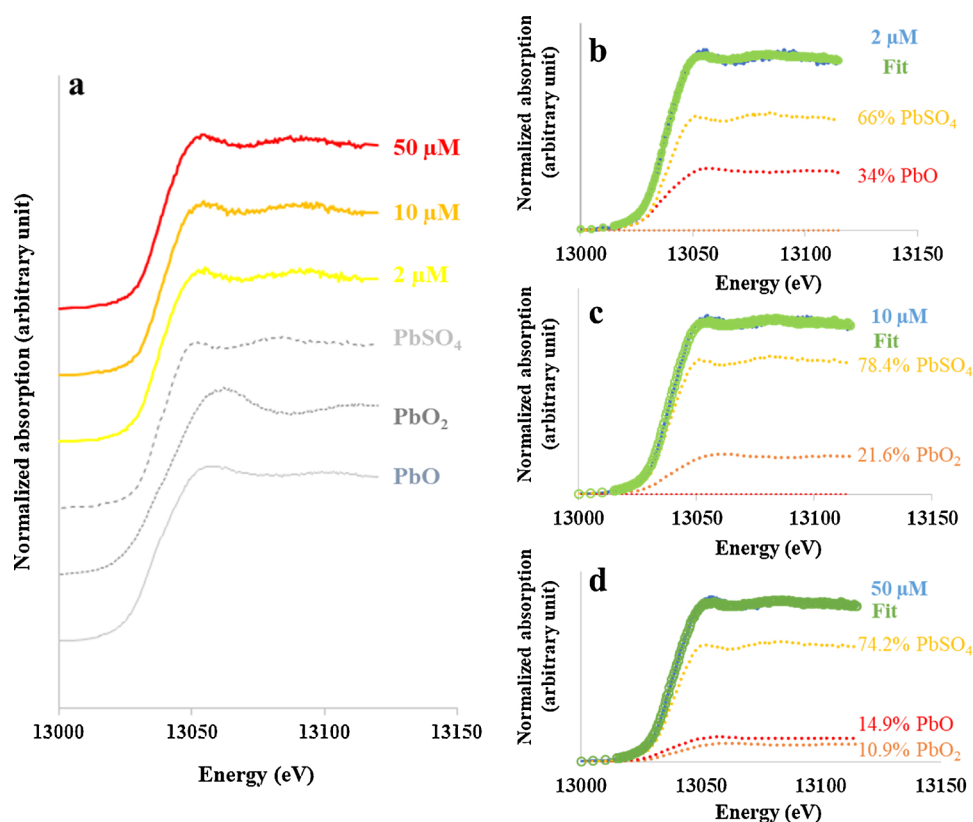


Fig. 5. Chemical analysis of Pb compounds in mineralized osteoblast cultures. Differentiated osteoblast cultures were treated with lead acetate at 2, 10 and 50 μM for 14 days. The dehydrated osteoblast cultures were subject to chemical analysis using X-ray absorption spectroscopy. a) Pb L₃ edge XANES spectra acquired from 2, 10 and 50 μM Pb treated osteoblast cultures are shown. The XANES spectra of PbO, PbO₂ and PbSO₄ are also displayed as the reference substances. The spectra acquired from the Pb-treated samples were fitted against the reference substances spectra to determine the levels of Pb compounds in Pb-treated cells at (b) 2 μM; (c) 10 μM; (d) 50 μM.

osteoblast cultures using XAS. XANES spectrum of Pb L₃ edge was acquired from the mineralized osteoblast cultures treated with Pb at 2, 10, or 50 μM. As shown in Fig. 5, differences in the spectrum profiles were not noticeable among the cultures treated various Pb levels (Fig. 5a). Linear combination fit (LCF) was applied to identify the chemical forms of Pb present in the mineralized osteoblast cultures at day 14 after the treatment. PbO, PbO₂, and PbSO₄ were used as the reference substances. The LCF data demonstrated that Pb was largely deposited as Pb salts, but some were deposited as Pb oxides (Fig. 5b-d). An increase in the Pb concentrations from 2 to 10 μM elevated the levels of Pb salts. However, the levels of Pb salt form remains unchanged although the Pb concentrations were increased from 10 to 50 μM. This suggests a possibility of Pb incorporation into bone mineral with a certain capacity. Taken together, Pb was largely accumulated as Pb salts and Pb oxides in the mineralized osteoblast cultures.

3.5. Pb localization in the mineralized osteoblast cultures

Our bright-field and Alizarin staining analyses had suggested that Pb treatments did not affect mineralization of the osteoblast cultures. These observations were also supported by TEM analysis. As shown in Fig. 6a and b, using the cross-section of the osteoblast cultures, we were able to demonstrate that the morphology of the osteoblasts and the mineralized extracellular matrix in the Pb-treated cultures were similar to that of the untreated controls. Moreover, we performed chemical analysis using EDX to determine whether Pb was in the osteoblast cells or in the mineralized extracellular matrix. As shown in Fig. 6, Pb was detected to co-localize with calcium and phosphorus in the mineralized extracellular matrix and intracellular vesicles but not in the other parts of the cells (6b, 6d and supplementary fig.1). On the other hand, no Pb deposit was detected intracellularly and extracellularly in the untreated controls (Fig. 6a and c). It is interesting to note that, in the differentiated osteoblasts, Pb was co-localized with calcium and phosphorus in the intracellular vesicles. These Pb-entrapped vesicles were likely the calcified vesicles due to the presence of calcium and phosphorus. The

intracellular calcified vesicles are known to play important roles in mineralization process (Boonrungsiman et al., 2012). Our results demonstrated, for the first time, that Pb was enriched in the intracellular calcified vesicles of inside differentiated osteoblasts.

Our EDX analysis suggested Pb deposits in extracellular mineralized matrix in osteoblast cultures. Here, we investigated the timeline of Pb depositing in the extracellular matrix during the mineralization process using TEM images and EDX analysis. It has been shown that a globular form represent the early stage of the mineral deposits while a needle-like form stands for more mature mineral deposits. As shown in Fig. 7, different morphologies of the mineral deposits, including small and large globular as well as needle shape minerals, were found in the Pb treated cultures. All these mineral deposits contained calcium and phosphorus as expected. Importantly, the EDX spectra showed Pb spreading in all mineral shapes, suggesting that Pb was co-localized with both calcium and phosphorus in early and mature mineral. We further examined the effect of Pb treatment on calcium and phosphorus deposits in the mineral matrix. As shown in Fig. 8a, we found a significant decrease in the ratio of calcium to phosphorus (Ca:P) within the globularly shaped minerals in the Pb treated cultures compared to the untreated controls. However, there was no differences in the Ca:P ratios among the cultures treated with different concentrations of Pb. Moreover, a downward trend in the Ca:P ratios in the needle shaped minerals was observed when the osteoblast cultures were exposed to Pb (Fig. 8b). Compared to the untreated controls, the decrease in the Ca:P ratio was most dramatic in the cultures treated with 50 μM Pb. Furthermore, we found that Pb was more abundant in the globularly shaped mineral than the needle-like shaped mineral in the Pb treated cultures at all examined concentrations (Fig. 8c). Taken together, we conclude that Pb was found to co-localize with calcium and phosphorus in in all stages of the mineral deposits. However, the Pb levels were higher in the early stage of the mineral than the matured form. Pb accumulation significantly decreased the Ca:P ratios in early stage of the mineral than the matured mineral.

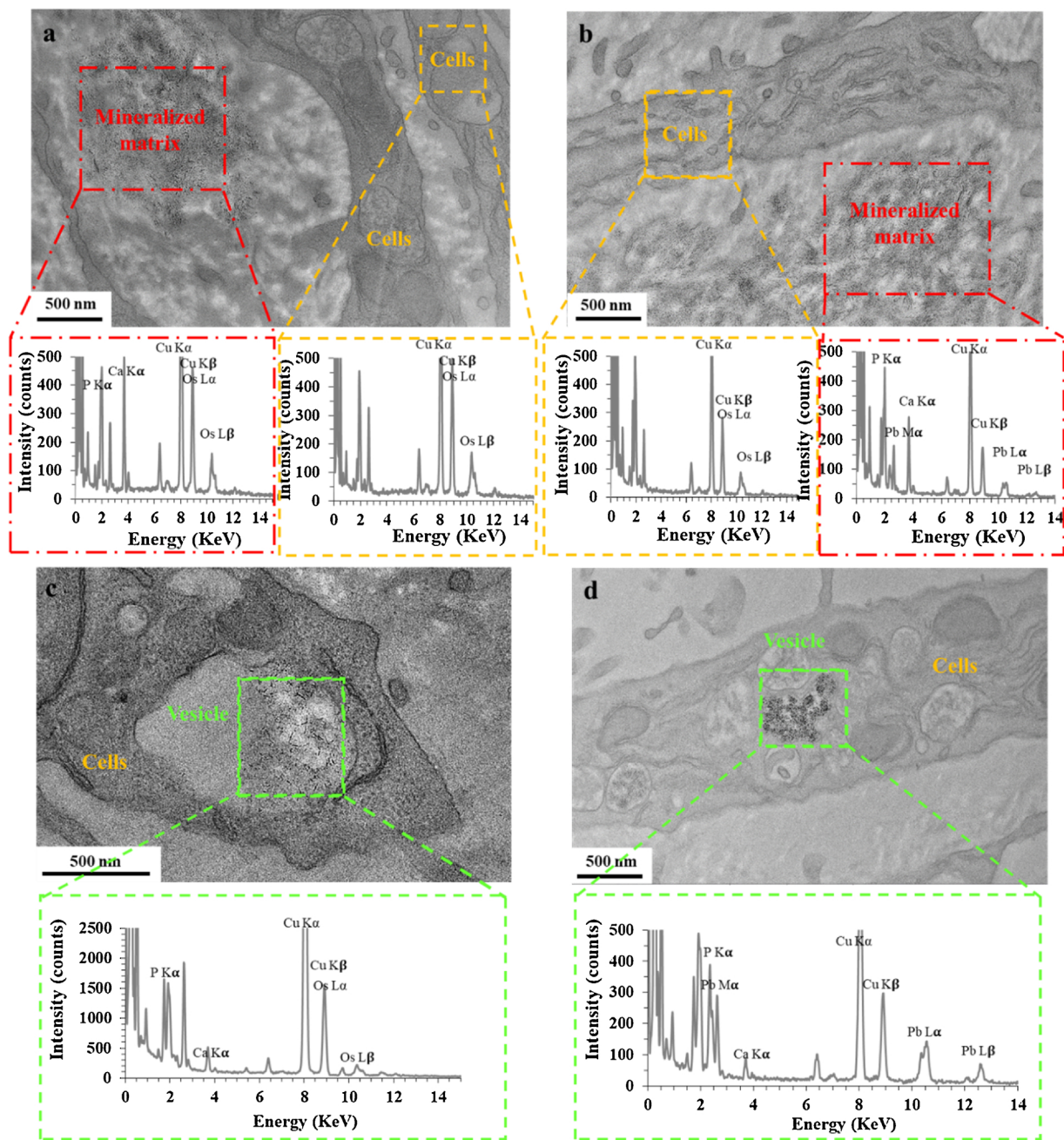


Fig. 6. Bright field TEM images of untreated and 10 μ M Pb-treated mineralized osteoblast cultures. a) Electron micrograph shows osteoblasts in adjacent to the mineralized matrix in the untreated mineralized osteoblast culture. The EDX spectra were taken from the region inside of the cell (yellow square) and mineralized matrix (red square), confirming the presence of calcium and phosphorus in the mineralized extracellular region, but not inside of the osteoblasts. b) The TEM image shows an osteoblast in adjacent to the mineralized matrix in 10 μ M Pb-treated osteoblasts. The EDX spectra were taken from the region inside of the cell (yellow square) and mineralized matrix (red square), confirming the presence of calcium, phosphorus and Pb in the mineralized region, but not inside of the cell. c) Calcified vesicles inside a lead-untreated osteoblast. The EDX spectrum was taken from intracellular calcified vesicles (green square), confirming the presence of calcium and phosphorus. d) Calcified vesicles inside an osteoblast treated with 10 μ M Pb. The EDX spectrum taken from intracellular calcified vesicles (green square), confirming the presence of calcium, phosphorus and Pb. Samples were stained with osmium tetroxide without post staining.

4. Discussion

The adverse effects of Pb exposure on the bone have been widely documented (Puzas et al., 2004). Pb not only alters bone-associated protein activities, but also impairs bone physiological functions. However, the process of Pb deposits into the bone structure remains to be elucidated. The present study demonstrated the effect of sub-toxic Pb treatments on Pb accumulation, osteogenic gene expression, Pb complex characterization, and Pb localization within the bone cells and extracellular matrix using an *in vitro* MC3T3-E1 osteoblast model.

In the present study, the sub-toxic levels of Pb treatments at 0.4, 2, 10, and 50 μ M showed no effects on cell viability, nodule formation, and mineral accumulation in differentiated MC3T3-E1 osteoblasts. Studies have shown that Pb displays a toxicity level lower than 50 μ M in cultured cells. It was also reported that rat primary osteoblasts treated with Pb at 20 μ M decreased cell viability and alkaline phosphatase activity (Ma et al., 2012). Changes in cell morphology, increased production of reactive oxygen species, and cell death become obvious when the rat primary osteoblasts are treated with Pb at higher Pb levels, such as 40 and 60 μ M (Abbas et al., 2013; Ma et al., 2012). However, it

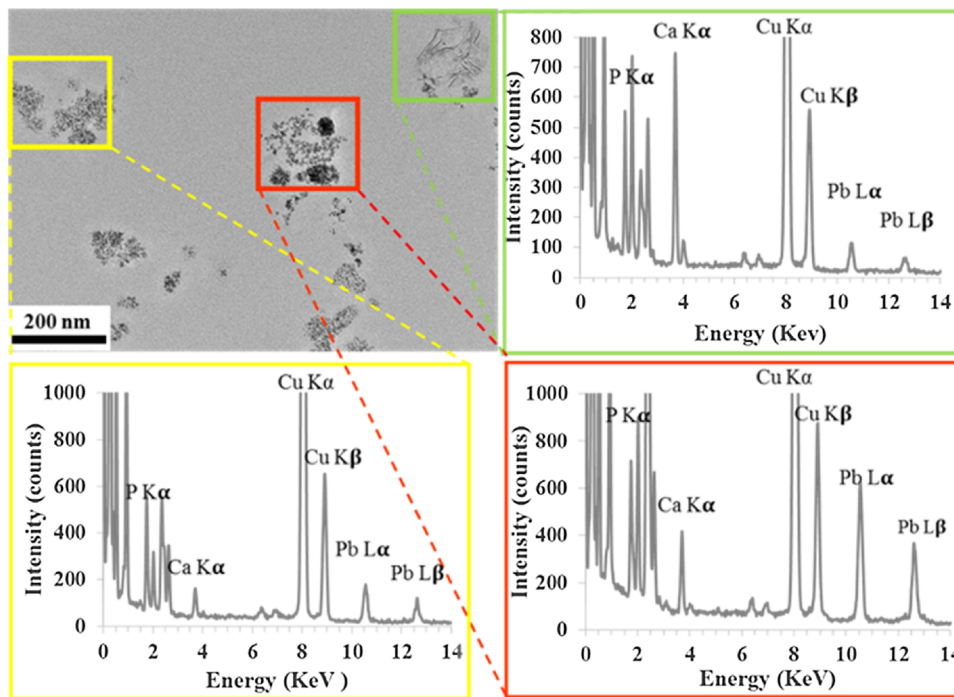


Fig. 7. A bright field TEM images of mineral deposits in 50 μM Pb treated mineralized osteoblast cultures. The mineral deposits are in different morphologies: small globular (yellow square), large globular (red square), and needle shaped (green square) in mineralized cultures treated with 10 μM lead acetate. The EDX spectra were acquired from the corresponding regions in the TEM photograph, confirmed the presence of Pb in these mineral deposits. Samples were processed without staining.

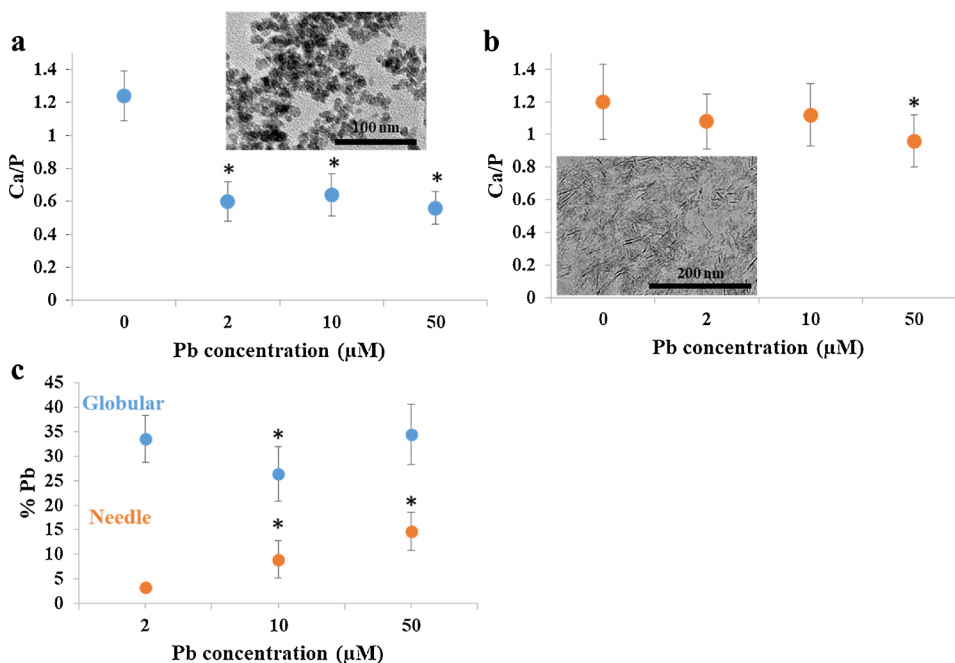


Fig. 8. Ratio of calcium to phosphorus (Ca:P) and Pb contents in globular and needle shaped mineral deposits in the mineralized osteoblast cultures treated with lead acetate at 2, 10 and 50 μM. Untreated mineralized osteoblasts were used as the negative control. a) Ca:P ratios in the globular mineral deposits in the mineralized osteoblast cultures. b) Ca:P ratios in the needle shaped mineral deposits in the mineralized osteoblast cultures. c) Pb contents in globular and needle shaped mineral deposits. The results are expressed as the percentage of Pb to sum of Pb, Ca and P in the globular (blue) and needle shaped (orange) mineral deposits. The untreated osteoblasts were used as a negative control. Value are the mean $\pm$ S.D., $n \geq 20$ per group, *, $P \leq 0.05$.

has been argued that the Pb toxicity level is dependent on cell origins, cell cultivation conditions, differentiation status, exposed durations, and Pb solubility (Aleo et al., 2006; Milgram et al., 2008b).

Our study indicated that Pb treatments significantly increased Pb deposits in a dose dependent manner in the mineralized osteoblast cultures cells both inside of the osteoblasts and the extracellular matrix. Pb can be transported into cells via anion exchange, passive transport, or cation channels (Kerper and Hinkle, 1997). It is worth noting that DMT1, a divalent metal transporter 1, is able to carry Pb into cells in addition to many other divalent ions (Bressler et al., 2004). In general, DMT1 plays a major role in iron uptake. DMT1 also can import other divalent metal ions into the cells. Overexpression of DMT1 remarkably elevates Pb contents in yeast and human fibroblasts (I. Bannon et al., 2002). In the current study, Pb treatments significantly upregulated

Dmt1 mRNA expression in mouse differentiated osteoblasts. Our observation was similar to a previous *in vitro* study showing that Pb exposure increased mRNA expression of *Dmt1* in the rat kidney and liver (Soto-Arredondo et al., 2018). Hence, we concluded that an increase in Pb contents in the mineralized osteoblast cultures can be partially explained by the upregulation of *Dmt1* expression.

Studies from others have demonstrated that Pb treatments decreased osteonectin secretion and type I collagen synthesis in osteoblasts (Sauk et al., 1992). Similarly, our results demonstrated alterations in osteogenic gene expression in Pb-treated osteoblasts in a time specific manner. mRNA levels of secreted phosphoprotein 1 and osteocalcin were significantly upregulated at day 7 after Pb treatments. It is worth noting that both phosphoprotein 1 and osteocalcin function in binding calcium and depositing it in the mineral matrix. Importantly, it

has been shown that osteocalcin has a higher affinity to Pb than calcium (Dowd et al., 1994; Dowd et al., 2001). We thus speculated that, during the process of mineral deposition at day 7 after Pb treatment, high availability of Pb may induce the mRNA expression of these osteogenic genes. On the other hand, we observed that type 1 collagen and osteoprotegerin were declined at day 14 after Pb treatments. Type 1 collagen plays a role as the main structural protein in the bone, contributing to bone toughness. The presence of osteoprotegerin inhibited osteoclast formation and prevented osteoporosis (Min et al., 2000; Udagawa et al., 2000). Hence, downregulation of type 1 collagen and osteoprotegerin in the fully differentiated osteoblasts may help shed light on why Pb is associated with osteoporosis and bone loss in humans.

Co-localization of Pb with phosphorus has been detected in lysosomes of rat osteoblasts (Milgram et al., 2008a). In the present study, we showed, for the first time, that Pb co-resided with calcium and phosphorus in the intracellular vesicles of the mineralized osteoblasts. During bone formation, osteoblasts produce mineral containing vesicles which include extracellular matrix vesicles and intracellular calcified vesicles to deposit minerals in the collagen matrix. The extracellular matrix vesicles are calcified extracellularly after minerals being exported from osteoblasts (Anderson, 2003). However, calcification of intracellular vesicles are accomplished within the osteoblasts and these calcified vesicles are enriched with both calcium and phosphorus (Boonrungsiman et al., 2012; Mahamid et al., 2011)(Boonrungsiman et al., 2012). Our study suggests that Pb is co-deposited with calcium and phosphorus in the intracellular calcified vesicles within osteoblasts. Although the mechanism underlying Pb transporting into the intracellular calcified vesicle is still unknown, the accumulation of Pb in the extracellular matrix is likely due to the release of intracellular calcium and phosphorus associated vesicles into the bone structure.

The formation of calcium phosphate in the bone structure is initially deposited as non-crystalline globular particles and then developed to crystalline bone apatite in needle shapes (Mhamid et al., 2010). We showed that Pb was in all shapes of mineral deposits, including globular and needle shaped minerals, as well as large mineral deposits. Pb treatments resulted in a reduction of the Ca:P ratio in the mineral deposits, particularly in the globular shaped mineral deposits. A decrease in the Ca:P ratio in both globular and needle shaped mineral deposits could be resulted from a replacement of Pb from calcium (Brès et al., 1986). In the hydroxyapatite structure, calcium ions occupy two non-equivalent positions, which are on the fourfold position at (1/3,2/3,0:001)(Ca(1)) and the sixfold position at (0:2462, 0:9938,1/4)(Ca(2)) (Kay et al., 1964). A X-ray powder diffraction pattern suggests that Pb ions mainly occupies Ca(2) position in the hydroxyapatite structure and the Pb substitution decreases the crystallinity (Andres-Verges et al., 1983). Studying Pb-associated hydroxyapatite using high resolution TEM also suggests that Pb can be added into both Ca(1) and Ca(2) positions (Brès et al., 1986). Taken together, the ability of Pb to replace calcium in the extracellular matrix can lead to a reduction in the ratio of Ca and P in the mineral deposits. Therefore, the mineral matrix in the bone structure could serve as a reservoir for Pb accumulation.

It is evident that Pb is largely accumulated in the tidemark of human articular cartilage, which is an active calcification front (Meier et al., 2011; Zoeger et al., 2006). Pb also deposits unequally among skeleton tissues. For example, it accumulates more in the bone than the cartilage (Lanocha et al., 2012). Replacement of Pb from calcium in the hydroxyapatite may explain why Pb can be accumulated in high concentrations in the bone. We speculate that Pb substitution could change chemical structures of the bone apatite. In the mineralized matrix, hydroxyapatite is closely associated with the collagenous matrix where it accommodates and regulates bone growth (Almora-Barrios and de Leeuw, 2010). Incorporation of Pb into the bone structures may impair the interaction between the collagen and hydroxyapatite as well as bone turnover and metabolism.

5. Conclusion

The present study reports the effects of sub-toxic levels of Pb acetate on bone structures and chemistry using an *in vitro* MC3T3-E1 bone model. Pb treatments at different concentrations ($\leq 50 \mu\text{M}$) promoted Pb accumulations in mineralized osteoblast cultures and upregulated the mRNA levels of *Dmt1* in a dose dependent manner. The mRNA expression of osteogenic genes, including phosphoprotein 1, osteocalcin, type 1 collagen, and osteoprotegerin was altered significantly. Pb deposited in the mineralized osteoblast cultures was identified as lead salts and oxides. Moreover, Pb was colocalized with calcium and phosphorus in the intracellular calcified vesicles, an important component of mechanisms of bone mineralization. Pb was also found in the extracellular matrix at all mineralization stages.

Conflict of interest

No conflict of interest is claimed.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.toxlet.2018.10.008>.

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