EFFECTS OF LEAD ON THE PROLIFERATION, PROTEIN PRODUCTION, AND OSTEOCALCIN SECRETION OF HUMAN DENTAL PULP CELLS *IN VITRO*

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Abstract. Teeth have been recognized as providing a useful long-term record of lead (Pb^{2+}) uptake. However, information regarding the effects of lead on dental pulp tissue cells that foster dentinogenesis is scarce. This study investigated the effects of lead on dental pulp tissue using human dental pulp fibroblasts *in vitro*. Dental pulp cells from the teeth of young patients (aged 17-24 years) were cultured and subsequently treated with lead glutamate. It was shown that, in serum-free conditions, all the tested concentrations of lead (4.5×10^{-5} M, 4.5×10^{-6} M, and 4.5×10^{-7} M) significantly increased pulpal cell proliferation. In the presence of 2% fetal bovine serum, increasing cell proliferation was observed only after exposure to a lead concentration of 4.5×10^{-5} M. However, protein, procollagen type I, and osteocalcin productions were significantly decreased. The alteration of cell population and protein production of affected human dental pulp shown in this study are toxic effects of the lead.

INTRODUCTION

Lead (Pb^{2+}) is known to be one of the most hazardous metals: it is a cumulative toxin that causes a range of often severe effects (Ibel and Pollock, 1986). The importance of lead as an environmental pollutant has led to an accumulation of evidence, especially during the last decade, about the effects of lead on the human body. It is known that people who have been chronically exposed to 0.75 μg of lead may suffer from impaired growth (Mooty *et al*, 1975; Frisanch and Ryan, 1991). The basis of lead toxicity is that lead, a metalic cation, binds with specific ligands (*eg* sulfhydryl-, amino-, and carboxyl-groups) of biomolecular substances that are crucial to various physiological functions. These lead-containing complexes interfere with normal physiology by competing with ion transport (Mahaffey *et al*, 1992). There are indications that the interaction of lead with calcium may interfere with normal calcium regulatory processes, *eg* calcium binding, extracellular protein interactions associated with mineralized tissues, and subcellular functions at the level of mitochondria, synaptosomes, and membrane vesicles (Fullmer *et al*, 1985).

Teeth have been recognized as a useful, long-term record of lead uptake (Needleman and Shapiro, 1974) and it has been shown that lead-poisoned children have very high level of lead in their circumpulpal dentin (Shapiro *et al*, 1973). The lead content of teeth has been of interest to many epidemiologists during the past decades; they have wondered whether children living in highly contaminated areas are more susceptible to dental caries (Davies and Anderson, 1987). Recently, a link between lead exposure at the time of dental formation and the increased prevalence of caries has been documented (Moss *et al*, 1999).

A number of researchers have successfully

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cultured fibroblasts from dental pulp. Although fibroblastic, these cells are capable of differentiating into odontoblast-like cells that form dentin in response to injury (Yamamura, 1985). These fibroblasts have several characteristics that are similar to those of osteoblastic cells, including high alkaline phosphatase activity (Nakanishi, 1991; Nakanishi *et al*, 1994) and the formation of calcified nodules during long term culture (Nakanishi, 1991; Kasugai *et al*, 1993); moreover, some bone-related proteins in osteoblasts, *eg* osteocalcin, bone sialoprotein, matrix Gla protein and osteonectin, are also found in the dental pulp cells (Butler, 1992). Although the inhibitory effects of Pb^{2+} on bone cell proliferation (Sauk *et al*, 1992) and the production of osteocalcin have been reported (Long *et al*, 1990), information regarding the effect of lead on dental pulp cells is scarce. This study was designed to determine whether lead could alter the proliferation and protein production of human dental pulp cells *in vitro*; in addition, the effect of lead on procollagen type I and osteocalcin production was examined.

MATERIALS AND METHODS

Cell culture

Human dental pulp cells were obtained from teeth extracted from young patients (aged 17-24 years) in the course of orthodontic treatment. After the dental pulps had been extracted under sterilization, they were washed twice with phosphate buffer saline solution (PBS) pH 7.4. The pulpal tissues were minced and then placed in 35 mm tissue-culture dishes.

The culture medium used was Dulbecco's Modified Eagle medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), L-glutamine (Gibco), and antibiotics comprising 10,000 units of penicillin G (Gibco) and 25 μg/ml of amphotericin B (Gibco) as fungisone. Cultures were grown at 37ºC in a humidified atmosphere of 5% CO_2. After growing from the explant and confluencing, the cells were harvested with 0.05% trypsin (Gibco) in PBS and subcultured weekly. Cells from the third to sixth passages were used. Lead glutamate was used in this *in vitro* assay since no visible precipitate was noted according to the report of Sauk *et al* (1992): a stock solution containing 4.5 x 10^{-2} M lead nitrate (Sigma) and 5.0 x 10^{-2} M glutamic acid in deionized water was prepared.

Cell proliferation study

The cultured dental pulp cells were seeded into 96-well culture plates (8 x 10^3 cells/well) with DMEM supplemented with 10% FBS, antibiotic and fungisone; the cells were incubated overnight at 37ºC. The following day, the medium was removed from each well and the experimental medium was added as follows: (a) DMEM + 2% FBS + 5.0 x 10^{-5} M glutamic acid; (b) DMEM + 0% FBS + 5.0 x 10^{-5} M glutamic acid; (c) DMEM + 2% FBS + lead glutamate; and (d) DMEM + 0% FBS + lead glutamate. The medium (a) and (b) are the controls for the with and without serum medium, respectively. The effects of lead glutamate on cell proliferation were evaluated at three different concentrations, 4.5 x 10^{-5} M, 4.5 x 10^{-6} M and 4.5 x 10^{-7} M. Cell proliferation on Day 1, Day 3, and Day 5 after treatment was determined by MTT colorimetric assay (Kasugai *et al*, 1990; 1991). The spectrophotometric absorbency was measured at 540 nm using an ELISA reader (Ceres UV 900 HDi, Biotek Instruments Inc).

Determination of protein production

The cultured cells, at a density of 8 x 10^3 cells/well, were seeded in 96-well plates in DMEM with 10% FBS. After the cells attached and spread, the medium was removed and the cells were incubated in serum-free DMEM with glutamic acid or three different lead concentrations as mentioned above. The protein content per cell and in the media samples was determined on Day 1, Day 3, and Day 5 after incubation by using the Bradford (1976) method. Bovine serum albumin standard solutions were prepared for the calibration curve.

Determination of procollagen type I peptide production

8 x 10^3 cells/well were seeded in 96-well culture plates as in the determination of protein

production. Collagen production was determined on Day 1, Day 3, and Day 5 after incubation using the sandwich assay of Procollagen Type I C-Peptide (PIP) EIA kit (Takara Shuzou, Osaka, Japan), which measured the amount of free propeptides, a reflection of the amount of collagen molecules synthesized. Briefly, sonicated cells, media samples, and standard solutions were added to wells that had been precoated with murine monoclonal antibody to PIP. After incubation and a washing step, horseradish peroxidase conjugated murine monoclonal antibody to PIP was added. The reaction between the peroxidase enzyme and the substrate resulted in color development. The concentration of PIP in samples could be determined by comparing their measurements (specific spectrophotometric absorbency at 450 nm) with those obtained from the standard curve.

Determination of osteocalcin production

8 x 10^3 cells/well were seeded in 96-well plates in DMEM with 10% FBS and cultured for 7 days. After the cells reached confluence, the medium was removed and the cells were incubated in serum-free DMEM with glutamic acid or three different concentrations of lead glutamate. Media were collected at 2, 4, 8, and 12 hours to determine the level of osteocalcin present by sandwich EIA assay (Takara Shuzou, Osaka, Japan). In brief, media samples and standard solutions were pre-incubated with murine monoclonal anti-Gla-osteocalcin antibody that was immobilized in the wells. After incubation and a washing step, anti-osteocalcin peroxidase was added and an assay was conducted using the method described for the determination of procollagen type I. The amount of osteocalcin in the samples was determined by comparing the measurement from spectrophotometric absorbency at 450 nm with those from the standard solutions.

Statistical analyses

Three cultures were used for each experiment, and 4 independent experiments were performed. The statistical significance of the differences in the effects of lead on cells was determined by analysis of variance and Duncan's multiple range test.

RESULTS

For proliferation studies, cells were exposed to three concentrations of lead glutamate (4.5 x 10^{-5} M, 4.5 x 10^{-6} M, and 4.5 x 10^{-7} M) in DMEM/2% FBS or DMEM/0% FBS. A significant increase in cell proliferation was observed in cells exposed to lead at 4.5 x 10^{-5} M in DMEM/2% FBS on Days 1 and 3 (Fig 1A). It was noted that all the concentrations of Pb^{2+} did not significantly affect the dental pulp cell proliferation by Day 5 in the presence of 2% serum when compared to the control. However, in the absence of serum, three lead concentrations significantly increased the dental pulp cell proliferation on Day 1, Day 3, and Day 5 (Fig 1B); moreover, on Day 5, the stimulating effect of lead on the cell proliferation

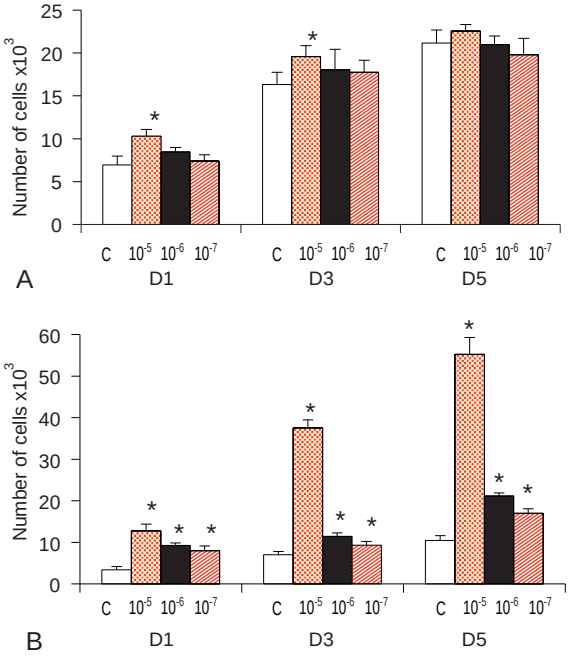

Fig 1–Effect of lead ($4.5x10^{-5}$-$4.5x10^{-7}$ M) in the presence (A) and absence (B) of 2% fetal bovine serum on the proliferation of human dental pulp cells. *Indicates significantly (p=0.05) different from control.

Table 1
Effect of lead on total protein production of human dental pulp cells.

Total protein ($\mu g/10^3$ cells)	Day 1				Day 3				Day 5			
	Control	Lead (M)			Control	Lead (M)			Control	Lead (M)		
		10^{-5}	10^{-6}	10^{-7}		10^{-5}	10^{-6}	10^{-7}		10^{-5}	10^{-6}	10^{-7}
Cell	4.59±0.83	1.63[a]±0.21	1.73[a]±0.12	1.98[a]±0.47	2.01±0.20	0.58[a]±0.05	1.26[a]±0.14	1.37[a]±0.21	1.68±0.05	0.30[a]±0.02	0.60[a]±0.08	0.61[a]±0.15
Media	9.76±1.37	3.92[a]±0.50	4.74[a]±0.60	5.31[a]±1.26	5.80±1.61	0.99[a]±0.04	2.09[a]±0.35	2.99[a]±0.17	2.73±0.26	0.16[a]±0.04	0.72[a]±0.03	0.98[a]±0.15

Cells were exposed to lead glutamate at concentration of 4.5 x 10^{-5}, 4.5 x 10^{-6} and 4.5 x 10^{-7} M. Values are mean ±SD for 12 cultures.
[a]$p < 0.05$ significantly different from control.

Table 2
Effect of lead on procollagen type I peptide production of human dental pulp cells.

Procollagen type I ($ng/10^3$ cells)	Day 1				Day 3				Day 5			
	Control	Lead (M)			Control	Lead (M)			Control	Lead (M)		
		10^{-5}	10^{-6}	10^{-7}		10^{-5}	10^{-6}	10^{-7}		10^{-5}	10^{-6}	10^{-7}
Cell	2.59±1.13	0.45[a]±0.11	0.54[a]±0.19	0.67[a]±0.31	1.89±0.28	0.25[a]±0.05	0.78[a]±0.21	0.91[a]±0.26	1.70±0.11	0.27[a]±0.03	0.50[a]±0.04	0.55[a]±0.04
Media	18.93±4.12	6.26[a]±0.77	7.17[a]±0.53	7.26[a]±0.58	6.84±1.13	0.99[a]±0.12	2.71[a]±0.46	2.97[a]±0.28	4.92±0.38	0.54[a]±0.05	1.03[a]±0.17	1.30[a]±0.12

Cells were exposed to lead glutamate at concentration of 4.5 x 10^{-5}, 4.5 x 10^{-6} and 4.5 x 10^{-7} M. Values are mean ±SD for 12 cultures.
[a]$p < 0.05$ significantly different from control.

Table 3
Effect of lead on osteocalcin production of human dental pulp cells.

Lead exposure time (h)	Osteocalcin (ng/10^4 cells)			
	Control	Lead (M)		
		10^{-5}	10^{-6}	10^{-7}
2	3.61 ± 0.10	3.42 ± 0.04	3.60 ± 0.46	3.68 ± 0.11
4	4.00 ± 0.32	3.47 ± 0.31	3.30 ± 0.64	3.43 ± 0.24
8	4.34 ± 0.25	2.01 ± 0.07[a]	2.48 ± 0.04[a]	2.85 ± 0.42[a]
12	5.67 ± 0.50	1.73 ± 0.07[a]	2.56 ± 0.09[a]	3.13 ± 0.21[a]

Cells were exposed to lead glutamate at concentration of 4.5 x 10^{-5}, 4.5 x 10^{-6} and 4.5 x 10^{-7} M. Values are mean ±SD for 12 cultures. [a]$p < 0.05$ significantly different from control.

was observed in a dose-dependent manner in the serum-free condition.

Based on proliferation results, serum-free medium was used for the assays designed to determine the effect of lead on protein content, procollagen type I, and osteocalcin productions. It was found that both the intracellular protein content and the protein production in media per cell were significantly decreased after exposure to lead for 1, 3, and 5 days (Table 1); a similar inhibitory effect of Pb^{2+} on procollagen type I production both in cells and media was also observed (Table 2).

To evaluate whether lead had an effect on cellular osteocalcin production, dental pulp cells were exposed to the three different lead concentrations for 2, 4, 8, and 12 hours after confluence. As shown in Table 3, lead significantly inhibited osteocalcin production after 8 hours, and a dose-dependent decrease was observed at 12 hours; however, no significant decrease in osteocalcin production was noted after 2 and 4 hours of Pb^{2+} exposure.

DISCUSSION

Various concentrations of lead (10^{-3} to 10^{-7} M) were evaluated in this study. Under light-microscopy, cellular damage or detachment from the culture well was observed when the lead concentrations were higher than 10^{-5} M. In addition, CDC-defined lead toxicity occurs at a blood level of ≥ 10^{-6} M, and therefore, in this *in vitro* assay, the 10^{-5} to 10^{-7} M of lead were used.

The present study demonstrated that lead, at a concentration of 10^{-5} to 10^{-7} M, increased the proliferation of human dental pulp cells *in vitro*. Although the measurement of mitochondrial dehydrogenase enzyme activity used in this study does not necessarily correlate to an increase of cell numbers, as usual in studies of the cytotoxicity of agents, the results of this MTT assay in our study corresponded to those of our neutral red colorimetric assay and to those of a dye exclusion test using tryphan blue staining in conjunction with hemocytometric counting (unpublished data). In addition, a linear relationship between the cell number and absorbency in the MTT assay has been reported elsewhere (Plumb *et al*, 1989; Kasugai *et al*, 1990; 1991).

The ability of lead to increase cell proliferation in serum-free medium was found in the study of Sauk *et al* (1992), who investigated the action of lead on osteoblast-like ROS 17/2.8 cells. However, for high doses of lead (4.5 x 10^{-5} M and 4.5 x 10^{-6} M), these authors reported a decrease in the proliferation of rat osteosarcoma cell lines, whereas a stimulatory effect of lead was found in the present study. The differences in these findings may be explained by difference in the types of cells

used: although dental pulp cells have many biological and biochemical characteristics that are similar to those of bone-forming cells, they have the morphology and growth activity of the fibroblastic cell lineage and secrete some extracellular proteins that are different from those of bone cells (Butler, 1998). A recent report concerning the effect of lead glutamate on a rat kidney cell line (NRK-52E) showed an increase in cell proliferation over 10 days at 10^{-5}, 10^{-6}, and 10^{-7} M in a dose-dependent manner (Fowler, 2000). The mechanism underlying lead-induced increases in cell proliferation remains unknown; the effect may be modulated via transcription factors and kinases, since lead has been shown to activate nuclear transcription factor Kappa B and activator protein-1 as well as kinases such as mitogen-activated kinase, amino-terminal c-Jun kinase and protein kinase C in many cell types (Murakami *et al*, 1993; Ramesh *et al*, 1999). Moreover, epidemiological and experimental studies of human and animals have confirmed that inorganic lead components are associated with an increased risk of tumorogenesis (Silbergeld *et al*, 2000; Steenland and Boffeta, 2000); this risk can be present at doses that are not associated with organ toxicity. In addition, heavy metals can impair apoptosis; this suppression of apoptosis could facilitate aberrant cell accumulation, which may be important in the pathogenesis of malignancy or autoimmunity (Waalkes *et al*, 2000). It would be interesting to see whether Pb^{2+} could induce dental pulp tumor *in vitro*. In the presence of 2% FBS, the inhibitory effect of Pb^{2+} on dental pulp cell proliferation was not clearly demonstrated; in the serum-free condition, the effect was clear. Such a finding may be due to the effect of serum constituents on cell growth, as their presence may lower the concentration of unbound lead available to the cells.

In this study, lead was found to decrease protein content and procollagen type I peptide productions in both cells and medium. This may be the effect of lead's impairing the ability of cells to synthesize or secrete proteins; lead has been found to decrease cytoskeletal proteins in the primary culture of neurons and glia under serum-free conditions (Scortegagna *et al*, 1998). An alteration in rabbit kidney protein expression following lead exposure has been reported (Kanitz *et al*, 1999). However, Sauk *et al* (1992) showed a stimulatory effect of lead on rat osteosarcoma cells: they reported an increase in total protein production, but no significant alteration in collagen production. Since different kinds of cells were used, different tissues response to lead should be considered as a possible explanation for the variation between the studies' findings.

The present study demonstrated that lead directly affected the level of osteocalcin, a marker protein of bone formation, in the culture of human dental pulp cells. The ability of lead to impair osteocalcin production has been reported in osteosarcoma cells in both stimulated and basal conditions (Long *et al*, 1989; 1990; Angle *et al*, 1990). In this study, a dose of 4.5 x 10^{-6} M of lead glutamate reduced the level of osteocalcin by 55% (at 12 hours), whereas Long *et al* (1990) found a 70% reduction (at 24 hours) using an equivalent dose. This result supports that lead has an inhibitory effect on osteocalcin production. Nevertheless, the different levels of osteocalcin reduction found in this study and that in the study of Long *et al* (1990) could be due to different tissues responses to lead. This reduction of osteocalcin level could be possible by the effect of lead on two processes that occur in calcified tissues and other organ systems: (i) lead-intoxicated dental pulp cells may be less capable of synthesizing RNA and proteins, including osteocalcin; (ii) the secretion of osteocalcin requires prior post-translational vitamin K-dependent carboxylation; when this carboxylation or the secretory process to be impaired by lead, osteocalcin levels in medium would be reduced. Previous experiments in osteosarcoma cells have suggested that the site of the impaired production of osteocalcin is vulnerable to the toxic effect of lead (Markowitz *et al*, 1988).

In conclusion, the stimulation of cell proliferation, the decrease in protein content, and the reduction of collagen type I and osteocalcin production found in this study are due to the toxic effects of lead on human dental pulp cells.

ACKNOWLEDGEMENTS

The authors wish to express their especial gratitude to Assoc Prof Supa Rochanawuttanon and Dr Boonjira Anucul, Department of Oral Surgery, Mahidol University, for the collection of pulp specimens. The authors are deeply indebted to Prof Dr Mogens Kilian for his valuable criticisms and correcting the English. This study was supported by a grant from the Faculty of Dentistry, Mahidol University.

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