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Effects of Oral Dosage of Lead Acetate II on Osteocalcin Gene Expression in Rat Mesenchymal Stem Cells

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Abstract

Background: Lead (Pb) is a heavy metal that has devastating effects on many animal tissues. In this study we investigated the effects of orally-dosed lead acetate II on osteocalcin gene (*osteocalcin*) expression in mesenchymal stem cells grown in an osteogenic medium. Osteocalcin is an abundant bone matrix differentiation protein.

Methods: Twelve male Wistar rats were divided into three groups of four rats each. Two groups were fed orally with 50 or 100 ppm of lead acetate II with libitum feed and water for two months. The control group was fed with libitum feed and water only. Rats were euthanized and femoral bone marrow mesenchymal stem cells (BM-MSCs) were extracted. The cells were cultured in osteogenic medium and *osteocalcin* expression was determined by real-time PCR.

Results: Real-time PCR showed that *osteocalcin* expression was significantly less in the BM-MSCs of rats that received 100 ppm of lead acetate II than in the BM-MSCs of the other groups ($P<0.05$), and that *osteocalcin* expression was less in the BM-MSCs of the group that received 50 ppm of lead acetate II than in the control group.

Conclusions: Doses of 50 and 100 ppm of lead acetate II in rats caused a significant decrease in *osteocalcin* expression in BM-MSCs grown in osteogenic medium.

Keywords: Bone Marrow, Lead acetate, Osteocalcin, Real-Time Polymerase Chain Reaction, Stem Cells

Introduction

Lead is a heavy, toxic, and low-melting metal that is found naturally in soil. Its extensive use has caused environmental contamination and health problems worldwide (1, 2). Lead is ingested with food by both man and animals; its toxicity in animals depends on its chemical form, the route of administration, and the frequency and duration of administration (3), and is known to negatively affect a number of body systems; primarily the central nervous, hematopoietic, hepatic, and renal, producing serious disorders (4). Stem cells are found in several tissues including bone marrow, liver, kidney, skin, and adipose tissue, and are able to replenish themselves and differentiate into ectoderm, mesoderm, and endoderm. Bone marrow and adipose tissue are major sources of stem cells (5-8). Mesenchymal stem cells have been isolated and cultured from many species

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including mice, rats, cats, dogs, rabbits, pigs, and baboons (9). Isolated bone marrow stromal cells differentiate to a range of cell phenotypes including osteoblasts, chondroblasts, adipocytes, and fibroblasts. The mature osteoblast phenotype is determined by the ability of the cells to synthesize membrane-associated alkaline phosphatase, bone matrix molecules including collagen type I (COLL-I), and a variety of non-collagenous proteins, such as osteocalcin, bone sialoprotein (BSP), osteopontin, proteoglycans, and hormone and growth factor receptors, in particular the parathyroid hormone/parathyroid hormone-related peptide receptor (PTH/PTHrP-R) (10). Osteocalcin, or bone gamma-carboxyglutamic acid, is the most abundant non-collagenous protein in bone matrix and is produced only in osteoblasts. Osteocalcin contains 46-50 amino acids with three central glutamate residues. After translation, but before secretion, osteocalcin is post-translationally modified in that the three central glutamate residues are carboxylated, which is necessary for attachment to hydroxyapatite and deposit in bone extracellular matrix (11). *Osteocalcin* expression can be moderated by hormones and cytokines via signaling pathways, and transcription factors that bind and activate the *osteocalcin* promoter (1q25–q31) (12). In the present study, we examined *osteocalcin* expression in BM-MSCs from lead-exposed rats

Materials and Methods

Animals and experimental procedure

Twelve male Wistar rats were obtained from the Razi Vaccine and Serum Research Institute, Mashhad, Iran. The rats were housed in standard rat cages at 25° C and a 12:12 hour light/dark cycle during the two-month experimental period in the animal house of Islamic Azad University of Mashhad, Mashhad, Iran. The animals were divided into three groups of four rats each. The rats were fed as follows:

Group 1: libitum feed and distilled water containing 50 ppm lead acetate II (Gibco, Grand Island, NY),

Group 2: libitum feed and distilled water containing 100 ppm lead acetate II,

Group 3 (control): libitum feed and distilled water only (13).

Maintenance and care of experimental animals complied with National Institutes of Health guidelines for the use of laboratory animals and was approved by the Ethics Committee of Razi Vaccine & Serum Research Institute.

Bone marrow cells isolation and osteogenesis

After two months on the experimental protocol, the rats were euthanized with diethyl ether. The femoral bones were isolated under sterile conditions and cut at both ends. The bone marrow from each group was collected by incubating the bones in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, USA) containing 1000 U/ml of penicillin G. The cell suspensions were centrifuged at 1200 × g for 5 min, the cells were resuspended in DMEM with 15% fetal bovine serum (FBS) (Sigma, USA) containing 100 U/ml penicillin and 100 µg/ml streptomycin, transferred to culture flasks, and incubated at 37° C in 5% CO2 (14).

For osteogenesis, the cultures were then incubated in α-MDM that was supplemented with 10% fetal calf serum (FCS), 10% human serum (HS), 100 U/ml penicillin, 100 µg/ml streptomycin, 12 mM glutamine, 20 mM glycerol phosphate (Sigma, St Louis, MO), 50 ng/ml thyroxine (Sigma), 1 nM dexamethasone (Sigma), and 0.5 M ascorbate 2-phosphate (Sigma). The media was changed 2 x per week for 21 days (15). The bone medium was replaced every two days with fresh medium. At days 7, 14, and 21 (16) the cells were harvested and RNA was extracted.

RNA isolation

The cells were pelleted and total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized for 1 h at 42 °C in a reaction mixture containing 5 µg of RNA, 100 U of ReverTra Ace (Toyobo, USA), 4 µl of 5X reverse transcriptase buffer, 2 µl of 10 mM dNTPs, and 1 µl of 5 pM oligo dT (Fermentas) adjusted with RNase-free water to a final volume of 20 µl (17).

The *osteocalcin* forward and reverse primer sequences were 5'-TGACAGACACACCATGAGAACCC-3' and

5'- AGCTCTAGACTGGGCCGTAGAAG- 3', respectively.

The glyceraldehyde phosphate dehydrogenase gene *(GAPDH)* was used as a control gene. Its forward and reverse primer sequences were 5´-CCTTCATTGACCTTCACTACATGGTCTA -3´ and 5´-TGGAAGATGGTGATGGCCTTTCCATTG -3´, respectively (18). The PCR was performed in a BioRad system (Hercules, CA, US) with the following conditions: initial denaturation at 95ºC for 5 min followed by 40 amplification cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 5 min. The conditions were identical for *GAPDH*.

Quantitative Real-time polymerase chain reaction

The numbers of cDNA molecules in the reverse-transcribed samples were determined by real-time PCR using a modified method with a QuantiTect SYBR Green PCR kit on an iCycler iQ Multicolor Real-time PCR Detection System (Bio-Rad). The reaction mixture contained 12.5 µl of Master SYBR Green I, 0.25 mM of each primer, and 2 µl of samples and/or ddH_2O to a final volume of 25 µl. A melting curve was obtained at the end of each run to discriminate specific from non-specific cDNA products. The cDNA content was normalized by subtracting the cycle numbers of *GAPDH* from the target gene (ΔCt = Ct of the target gene – Ct of *GAPDH*) and gene expression was measured using the Livak method and formula of $2^{-(\Delta\Delta Ct)}$ (19).

Statistical analyses

Statistics were analyzed by analysis of variance (ANOVA) and Dunnet t-test and the results were expressed as means ± standard errors of the means (SEM). Differences were considered significant for $P<0.05$.

Results

Table 1 shows the ratio of *osteocalcin* expression in MSCs from rats in in groups I and II relative to controls after 7, 14, and 21 days in osteogenic medium.

Osteocalcin expression in MSCs from rats that were exposed to lead at both 50 and 100 ppm was significantly less than that in the control group (Fig.1). In addition, on each analysis day, *osteocalcin* expression in the rats that received lead acetate at 100 ppm was about half that of the rats that received lead acetate at 50 ppm. The lowest level of *osteocalcin* expression was seen in cells after 21 days of culture from the rats that received of 100 ppm lead acetate II.

Table 1. *Osteocalcin* expression in BM-MSCs in group I (lead = 50 ppm) and group II (lead = 100 ppm) compared to the control group (lead = 0 ppm) at culture days 7, 14 and 21 in osteogenic medium (N= 8).

day	day 7		day 14		day 21	
Fold difference / Sample	$2^{(-\Delta\Delta Ct)1}$	$2^{(-\Delta\Delta Ct)2}$	$2^{(-\Delta\Delta Ct)1}$	$2^{(-\Delta\Delta Ct)2}$	$2^{(-\Delta\Delta Ct)1}$	$2^{(-\Delta\Delta Ct)2}$
1	0.55± 0.1	0.28± 0.1	0.3± 0.1	0.17± 0.1	0.25± 0.1	0.12± 0.1
2	0.53± 0.1	0.26± 0.1	0.3± 0.1	0.17± 0.1	0.25± 0.1	0.11± 0.1
3	0.52± 0.1	0.29± 0.1	0.27± 0.1	0.17± 0.1	0.24± 0.1	0.11± 0.1
4	0.54± 0.1	0.28± 0.1	0.28± 0.1	0.16± 0.1	0.23± 0.1	0.12± 0.1
5	0.53± 0.1	0.26± 0.1	0.27± 0.1	0.15± 0.1	0.25± 0.1	0.12± 0.1
6	0.54± 0.1	0.27± 0.1	0.27± 0.1	0.17± 0.1	0.25± 0.1	0.12± 0.1
7	0.49± 0.1	0.25± 0.1	0.26± 0.1	0.16± 0.1	0.26± 0.1	0.12± 0.1
8	0.53± 0.1	0.27± 0.1	0.27± 0.1	0.18± 0.1	0.25± 0.1	0.13± 0.1

1. $2^{(-\Delta\Delta Ct)\ 1.}$ Ratio of *osteocalcin* expression in group I vs. control.
2. $2^{(-\Delta\Delta Ct)\ 2.}$ Ratio of *osteocalcin* expression in group II vs. control.

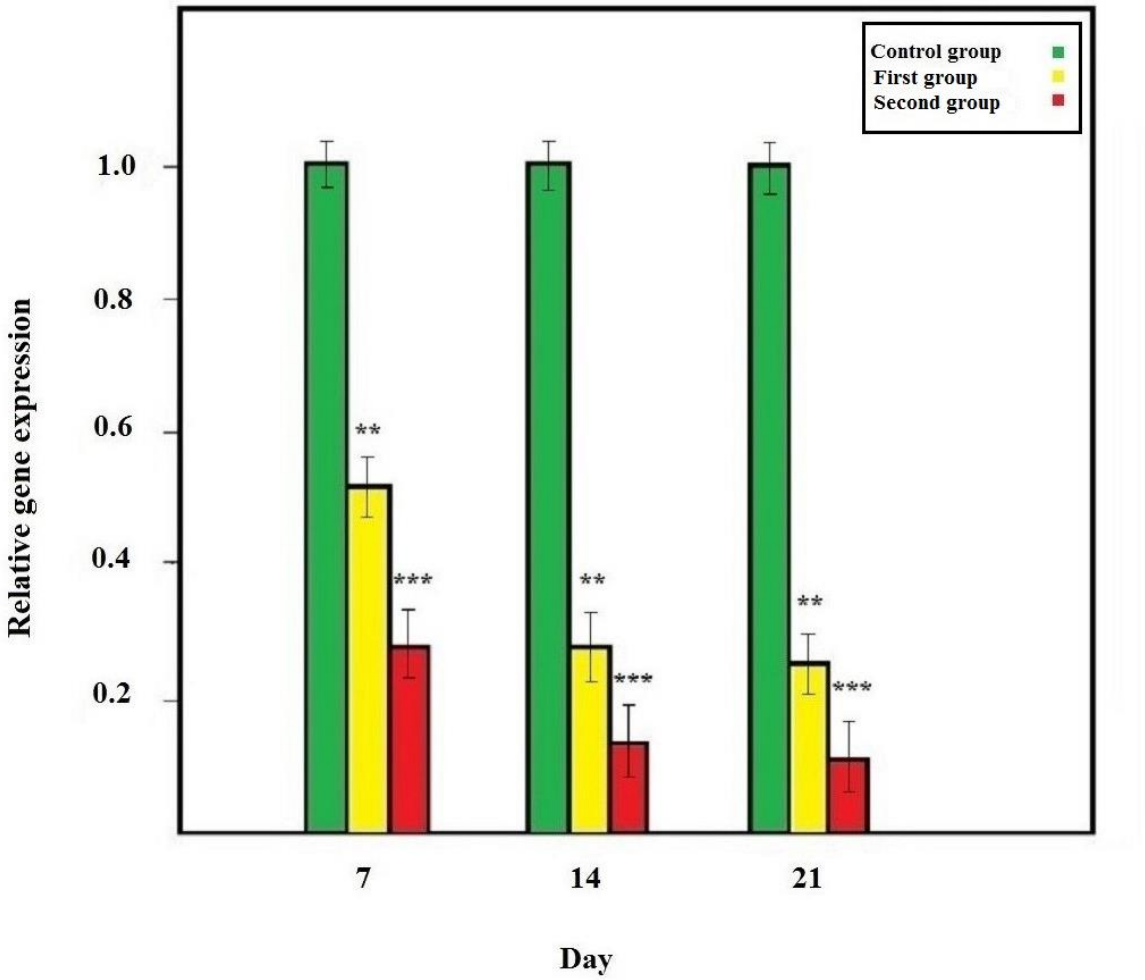

Fig. 1. Gene expression level, mean ± standard error mean (SEM) in bone marrow mesenchymal stem cells (BM-MSCs) in groups I and II (lead acetate = 50 and 100 ppm, respectively) relative to the control group (lead acetate = 0 ppm) after 7, 14, and 21 days of culture in osteogenic medium (N = 8).

Discussion

In this study, we investigated the effects of lead on *osteocalcin* expression in BM-MSCs extracted from rats that had been exposed to lead acetate II at concentrations of 50 and 100 ppm. After the second passage the stem cells began to differentiate and were then transferred to osteogenic medium. After 7, 14, and 21days (16), RNA was extracted from 3×10^6 cells from each group. In osteogenic medium BM-MSCs undergo osteogenesis (20-22) and *osteocalcin* expression increases (16). *Osteocalcin* is generally inactive during osteoblast proliferation, but is abundantly transcribed during osteoblast differentiation. Osteocalcin is released by osteoblasts during bone formation (23), mainly into osteoid where it binds hydroxyapatite. This binding is increased by $Ca2^+$ binding to osteocalcin (24). Low doses of lead are necessary for bone activity and osteogenic differentiation (24); however, increased lead in the environment has serious negative effects on tissues, especially bone and stem cells (1-5). In our study, osteocalcin expression was inversely proportional to lead intake. The mechanism by which this decrease occurs is not yet clear. Factors that affect *osteocalcin* expression include other genes, transcription factors, and heavy metals (24-27). Lead may act on these factors or directly target *osteocalcin*.

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