

Original Article**Expression of Collagen Type II and Osteocalcin Genes in Mesenchymal Stem Cells from Rats Treated with Lead acetate II***Hossein Rafiei¹, Milad Ashrafizadeh^{*2}**Received: 28.03.2018**Accepted: 13.05.2018***ABSTRACT**

Background: Lead is one of the sustainable metals with devastating effects on many tissues. This study, examined the adverse effect of lead poisoning on the gene expression of collagen type II and osteocalcin by mesenchymal stem cells (MSCs) cultured in chondrogenic and osteogenic media, respectively.

Methods: We used 18 male Wistar rats, divided in 3 groups. In addition to libitum feed as the control, treatment I and treatment II groups were fed by distilled water, distilled water with a dose of 50 ppm lead acetate II and distilled water with a dose of 100 ppm lead acetate II, respectively, over a 2-month period. The MSCs of rat femur were isolated in DMEM medium. After the second passage, the media were replaced separately with chondrogenic and osteogenic media over another 21 days. Then, Collagen Type II and Osteocalcin genes expression were investigated by real time PCR.

Results: Collagen Type II and Osteocalcin genes expression in treatments I and II groups showed meaningful decreases compared with that of the control group. Also, the concentration of collagen type II in treatment II group in chondrogenic medium was significantly reduced compared with Osteocalcin concentration in osteogenic medium.

Conclusion: We found that poisoning with lead and its accumulation at doses of 50 and 100 ppm in femoral bone marrow of rats decreased the expression of the collagen type II and osteocalcin genes in MSCs and in the chondrogenic and osteogenic media, respectively.

Keywords: Collagen Type II, Lead Acetate II, Mesenchymal Stem Cells, Osteocalcin.

IJT 2018 (5): 35-40**INTRODUCTION**

Mesenchymal stem cells (MSCs) are totipotent, with the ability to produce cell lines [1]. These cells are in an inactive and resting condition and they are activated as a result of in cellular injury. This feature has significant importance in tissue engineering and repair. These cells gain various potentials after each passage [2]. Studies have suggested that in spite of high proliferative potentials, these cells preserve their normal karyotype and telomerization activity, but they get old after frequent passages. One of the prominent features of mesenchymal stem cells is that they are able to differentiate into skeletal cell lines, i.e., bone, cartilage and fat cells. These cells have special niche that keeps them undifferentiated [3]. Stem cells are affected by factors and cells that control their proliferation in tissues and stabilize their growth condition [4]. Heavy metals influence the growth, differentiation and gene expression of stem cells and can produce different diseases [5, 6, 7].

Lead is one of the poisonous heavy metals, which is found increasingly in the environments of developing countries [8, 9]. This heavy metal is used in building materials, paint, some capacitors, bullets, soldering and liquefiable alloys, and in gasoline to prevent flaming up of the fuel and cause damage to vehicles engines. It is

also used to raise the octane grade in the form of lead tetra-ethyl compound. This element has a high half-life and remains in the body for a long time after ingestion, causing harmful changes in various tissues. Lead binds irreversibly with different cell organelles and replaces mineral elements, such as calcium. In addition, the accumulation of lead in the body depends on the duration of exposure, age and sex [5, 10]. For instance, lead readily accumulates in bones after ingestion.

Osteocalcin is a known non-collagenous protein in the bone and teeth. Osteocalcin is produced by osteoblasts and has a significant role in the metabolic regulations of the body and bones. The level of osteocalcin is regarded as an index of bone metabolism and is the specific index for the assessment bone diseases [11].

Collagen type II is one of the major connective tissue proteins, found richly in articular and hyaline cartilage. Collagen type II comprises 85-90% of collagens found in cartilage, and 50% of the proteins found in this tissue. This material makes a fibrous network, traps proteoglycans and produces a stretch-resistant structure in bones, cartilage and other connective tissues. Within cartilage, collagen is embedded in a jelly-like matrix. Proteoglycan molecules are also other components of this tissue, rich in carbohydrate chains and abundant negative charges that form a dense cloud of cations around of their molecules. This cationic cloud attracts a

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high amount of water into the cartilage matrix and provides a jelly-like environment, which serves as a shock and pressure absorbing mechanism within the connective tissues, especially bones and cartilage. Collagen fibers in this aqueous environment provide for the stretch-resistant property [12].

Since lead is present of in human and animal environments, and has harmful biological effects, this study was designed to examine the effects lead acetate (50 and 100 ppm dose) on the expression of collagen type II and osteocalcin genes, respectively, in chondrogenic and osteogenic cultures of rats' stem cells.

MATERIALS AND METHODS

The Manner of Treatment of Rats by Lead Acetate II

Eighteen Wistar rats, supplied by Razi Vaccine and Serum Research Institute of Mashhad, were used in this study. After passing the standard lab conditions for environmental adaptation, rats were randomly divided into three groups: a) control, b) treatment I and c) treatment II, and were kept at the vivarium of the school, observing the guidelines set by the National Institutes of Health, Bethesda, MD, USA. Treatments I and II rats were fed distilled water plus standard lab foods. Based on the Iranian laboratory standard #1053, feeding animals a dose of lead at 10 ppm is permissible. Because of the increasing level of lead in some biological areas, the higher doses were chosen in order to study the effects of this element [13, 14].

Culturing Mesenchymal Stem Cells from Bone Marrow

After eliminating the non-adherent cells, the mesenchymal stem cells were isolated from the bone marrow aspirates under culture condition (These cells expressed CD73, CD90 and CD105, but they were negative for hematopoietic indexes such as CD14, CD19 and CD79). To observe animal rights, rats were killed by inhalation of diethyl ether. The femurs were removed from both sides by sterile scissors and the surrounding muscles were eliminated after the femurs were disinfected with alcohol 70%. The femurs were embedded in physiologic serum in Petri dishes under the laminar flowhood. Femurs were placed on a lab dish and using a needle containing 2 ml Dulbecco's Modified Eagle's Medium (DMEM) along with penicillin and streptomycin, the femurs were slowly punctured in the bone marrow cavity and the marrow aspirated. Then, 200-300 μ l of each marrow was mixed in a sterile test tube with 5 ml DMEM and was centrifuged at 1200 rpm for 5 minutes and the supernatant removed. A 5-ml DMEM, containing penicillin (1%) and streptomycin (1%) and 10% fetal bovine serum (FBS) were added to the test tubes and were placed in an incubator at the 37°C and 5% O₂ for 10 to 12 days. The medium was replaced every 3 days. The cells attached, were passaged 4 time.

Table 1. The number of cells after fourth passage.

	Control group	Treatment I	Treatment II
Sample 1	12.8 * 10 ⁵	11.4 * 10 ⁵	9.4 * 10 ⁵
Sample 2	13 * 10 ⁵	11.2 * 10 ⁵	9.48 * 10 ⁵
Sample 3	12.6 * 10 ⁵	11.4 * 10 ⁵	9.28 * 10 ⁵
Sample 4	12.6 * 10 ⁵	11.32 * 10 ⁵	9.36 * 10 ⁵
Sample 5	12.68 * 10 ⁵	11.28 * 10 ⁵	9.4 * 10 ⁵
Sample 6	12.8 * 10 ⁵	11.4 * 10 ⁵	9.4 * 10 ⁵
Average	12.74 * 10 ⁵	11.33 * 10 ⁵	9.38 * 10 ⁵

Doubling Time of Mesenchymal Stem Cells in Bone Marrow Culture

During the culture period (first and second passage), the population doubling time (PDT) of mesenchymal stem cells was calculated by following formula: $PDT = CT / (\log N / N_0) * 3.31$, where CT, N₀ and N are the duration of culture period, the number of cells at the beginning of culture and the number of cells at the end of culture, respectively [15].

Determination of Cell Density in Chondrogenic and Osteogenic Cultures

After the second passage, each group of mesenchymal stem cells with a density of 1000 cells at each cm² of chondrogenic and osteogenic cultures were incubated with six cells at 37°C and 5% CO₂.

Mesenchymal Stem Cells in Chondrogenic Medium

After the second passage, mesenchymal stem cells were incubated for 21 days in the chondrogenic medium, consisting of 5-ml DMEM plus penicillin (1%) and streptomycin (1%) and 10% FBS, 500 ng/ml BMP-6, 10 ng/ml TGF β 1, 1 mM dexamethasone, 0.05 mM ascorbic acid, 40 mg/ml proline, 50 mg/ml ITS, 1.24 mg/ml BSA and 5.35 mg/ml linoleic acid. The cultures were then incubated at 37°C and 5% CO₂ [16].

Mesenchymal Stem Cells in Osteogenic Medium

After the second passage, mesenchymal stem cells were incubated for 21 days in the osteogenic medium, consisting of 5 ml of DMEM PLUS penicillin (1%) and streptomycin (1%) and 10% FBS, 50 mg/ml ascorbic acid bisphosphate, 10 nmol. dexamethasone and 10 mmol beta glycerol phosphate. The culture medium was refreshed every second days [16].

Extracting the RNA

At the end of culture, the cells were separated in each medium by trypsinization procedure and the RNA was extracted. The process of RNA extraction was performed by using the extraction Kit (Pars Tous Biotechnology, Mashhad, Iran). Based on the protocol, first, the cells were lysed and RNA sedimentation was performed using isopropanol and chloroform procedures. Finally, the RNA sample was washed with 70% ethanol and mixed in water.

Synthesis of cDNA and Real Time PCR

Using cDNA synthesis kit (Revert Aid H Minus first standard cDNA synthesis kit, Fermentas) instructions, 10 μ l RNA was incubated at 65°C for 5-10 minutes. Next, the following materials were added to solution respectively: 3 μ l of 3X reaction buffer, 0.2 μ l of primers of osteocalcin or collagen type II genes, 0.5 μ l of reverse transcriptase enzyme, 10 mM of dNTP, 3MI of MgCl₂. The solution was then incubated for ten minutes at 25°C and for 60 min. at 42°C, respectively. To stop the reaction, the solution was left at 70°C for 10 min. The synthesized cDNA was loaded at 80°C. Real time PCR was performed, using the QuantiTect SYBER

Green PCR kit with a Bio-Rad detector system. Appropriate primers for the above mentioned genes are presented in Table 1. Also, the appropriate reference gene for normalizing during the real time PCR was GAPDH gene. The forward and reverse primers for this gene were 5'-CCTTCATTGACCTTCACTACATGGTCTA-3' and 5'-TGGAAGATGGTGATGGCCTTCCATTG-3'. Finally, the expression levels of collagen type II and osteocalcin genes were calculated and compared in treatments I and II groups. Further, the effect of lead acetate II was measured on the two genes under culture conditions.

Table 2. Primers for Osteocalcin and Collagen type II genes.

Gene	Direction	Sequence	Attachment Temp. (°C)	Product Size (bp)	Cycle No.
Osteocalcin	Forward	5'-GTCCCACACAGCAACTCG-3'	58	380	30
	Reverse	5'-CCAAAGCTGAAGCTGCCG-3'			
Collagen type II	Forward	5'-GGCTTAGGGCAGAGAGAGAAG-3'	63	315	35
	Reverse	5'-TGGACAGTAGACGGAGGA AAGTC-3'			

Statistical Analysis

Statistical analysis was performed, using SPSS software Version 18 (Chicago, IL, USA) and ANOVA test and *t*-test with $p < 0.05$ as a significant level. Also, related chart was shown in the Results section.

RESULTS

Stem cells Doubling Time

The average population doubling time (PDT) in the groups of control, treatment I and treatment II was respectively 45.92*10⁵+2, 48.14*10⁵+2 and 52.2*10⁵+2.

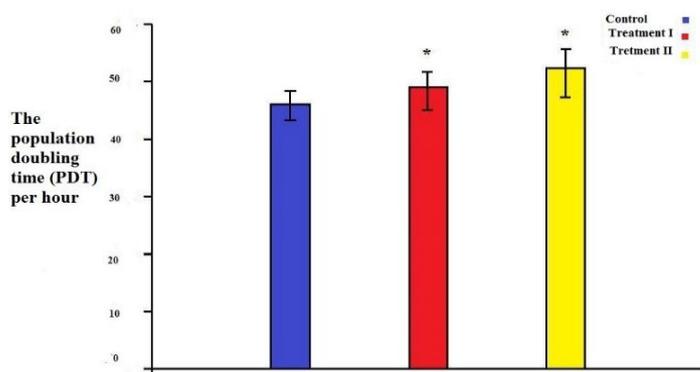


Figure 1. Comparison of the average population doubling time (PDT) per hour after the second passage for the control, and treatments I and II groups. The bars represent the significant difference in treatments I and II compared with the control ($P < 0.005$).

Comparison of Collagen Type II Gene Expression

The results of real time PCR indicated that feeding the rats with lead acetate II caused a downregulation of gene

expression for collagen type II in mesenchymal stem cells *in vitro* (relative expressions for treatments I and II were 0.31+0.09 and 0.08+0.12, respectively). Also, the 100 ppm dose had a higher inhibiting effect on collagen type II gene expression than the 50 ppm dose. See Table 2 and Figure 2.

Table 3. Relative expression of Collagen type II gene.

Group	Control	Treatment I	Treatment II
Calculations			
Δ Ct (reference-collagen type II)	0.133+0.02	1.813+0.02	3.79+0.01
$\Delta\Delta$ Ct	0	1.8+0.13	3.71+0.05
Mean Ration	1.000+0.11	0.31+0.09	0.08+0.12

The gene expression level compared to the GAPD H reference gene in the studied groups.

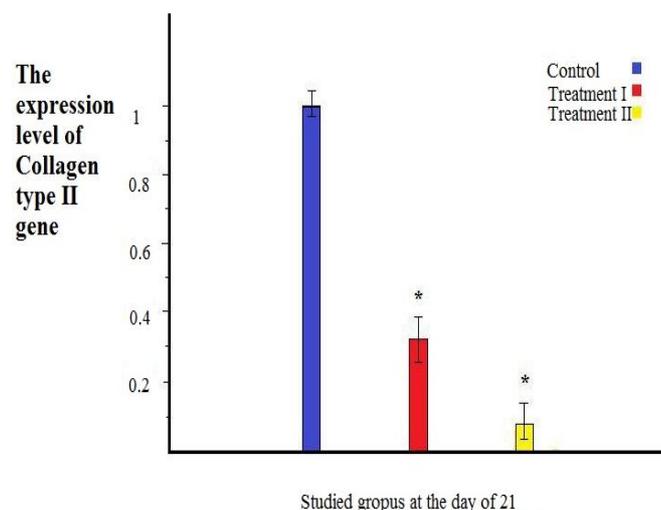


Figure 2. Comparison of average gene expression for collagen type II in the control, and treatments I and II groups after 21 days of incubation.

Osteocalcin Gene Expression

The results of real time PCR indicated that feeding the rats with lead acetate II decreased the expression of osteocalcin gene by mesenchymal stem cells *in vitro* (relative gene expression levels for treatments I and II groups were 0.31±0.15 and 0.19±0.07, respectively). Also, the 100 ppm dose of lead acetate II had a more inhibiting effect on Osteocalcin gene expression than that for of the 50 ppm dose (see Table 3 and Figure 3).

Table 4. Relative expression of osteocalcin gene.

Group Calculations	Control	Treatment I	Treatment II
ΔCt(Osteocalcin ref.)	0.01±0.123	0.015 ±-1.76	0.05 ±- 2.52
ΔΔCt	0	1.64±0.14	2.4 ±- 0.05
Mean Ratio	1.000±0.14	0.31±0.15	0.19 ±- 0.07

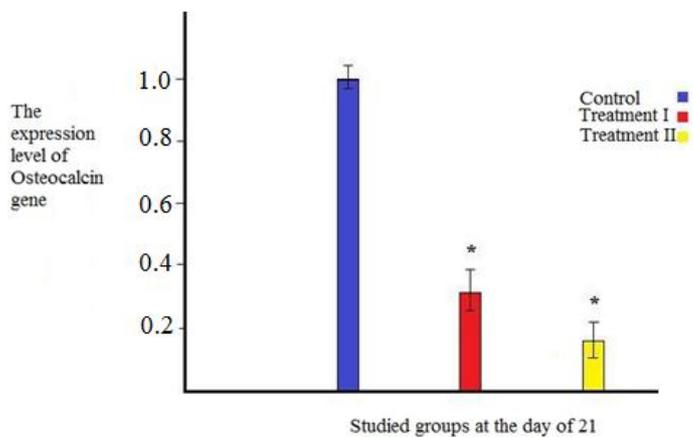


Figure 3. comparison of expression average for osteocalcin gene in the treatments I and II, and controls after 21 days of incubation. The bars show the significant differences in the treatment groups ($p < 0.005$).

DISCUSSION

We examined the effects of lead acetate II in the diet on the expression of Collagen type II and Osteocalcin genes by the mesenchymal stem cells of rats bone marrow *in vitro*.

Collagen type II is one of the most abundant proteins in articular cartilage and extracellular matrix, and is considered one of the main markers of cartilage differentiation [17]. Studies have shown that dexamethasone can partially decrease the expression of genes in cartilage matrix, particularly for Collagen type II. For chondrogenic differentiation of human mesenchymal stem cells, both TGFβ and dexamethasone must be present in culture environment. Chondrogenic differentiation of the mesenchymal stem cells proceeds in parallel for the two signaling pathways, which are called Wnt and TGFβ1. The collaboration occurs through the reaction of smad3 from TGFβ1 pathway and

betaketenin from Wnt pathway, the results of which is the differentiation to cartilage, increasing the proliferation of mesenchymal stem cells and the synchronous prevention of adipogenesis and osteogenesis [18].

Ascorbic acid is one of the ingredients of osteogenesis culture media and is necessary more and less. The presence of this material causes the secretion of enriched matrix. While collagen is being synthesized, this material is necessary for adding hydroxyl groups to proline and lysine groups. Adding this supplement to the medium of mesenchymal stem cells stimulates the cell proliferation potential and expression of collagen type II [19]. In this study, we can relate the effects of lead acetate II in decreasing the expression of collagen type II by affecting one of the mechanisms mentioned earlier.

If growth factors of BMPs family with growth-factor TGFβ are added to the medium of mesenchymal stem cells, they stimulate the production of cartilage matrix. The differentiation of the cartilage in mesenchymal stem cells is derived from bone marrow, adipose tissue or synovium in the presence of growth-factor BMP6 [20]. Due to the decreased expression of collagen type II in mesenchymal stem cells due to exposure to lead acetate II, we can relate these effects to lead influencing the function of BMP.

As well as TGFα factor, the presence of proline and linoleic acid in the culture medium help the chondrogenesis and other factors which interrupt the pathways leading to cartilage synthesis or change in environment conditions, or alter the differentiation pathway of stem cells [21]. Perhaps, our findings suggest that the oral use of lead acetate at 50 and 100 ppm doses in rat for two months, had decreased the expression of collagen type II in mesenchymal stem cells by inhibiting or interrupting the pathways involved its synthesis.

In addition, glycerol phosphate plays an important role by providing the environment for some important genes of bone such as osteocalcin and the bone mineralization [22]. So, we can relate the decrease in the expression of this gene in Mesenchymal stem cells to the destructive effect or weakening these two materials or related signaling pathways.

Bones are the long-term deposit reservoirs for lead, which easily substitutes Ca^{2+} and interferes in the hormonal regulations of calcium, and affects the function of bone marrow and osteoblasts. This issue causes the long-term effects on the body organs and tissues of human and mammalian. Regardless of this, there is not much information available on the effects of calcium replacement by lead in bone tissue and subsequently in such processes as mineralization and changes in the structure and function of gene expression in bones [5].

By providing proper conditions for bone synthesis in mesenchymal stem cells medium, some signaling pathways may be activated, resulting in the activation of signaling proteins and the expression of osteoblast

transcription factors. One of the transcription factors which have been studied extensively is Runx2 and Cbfl. Following the expression of these transcription factors, the cell is committed to acquire the phenotype of bone. This transcription factor affects the bone genes, such as osteocalcin and causes the expression of its gene [23].

Lead is known as an inhibitor of bone synthesis *in vivo*, which adversely affects the bone repair and causes significant defect in the betaketenin signaling. This event suggests that Wnt/betaketenin plays an important role pathway in the inhibiting effects of lead on bone fracture repairs. Regarding the decrease of osteocalcin in mesenchymal stem cell in osteogenic medium as seen in this study, we can link this effect to the action of lead in these cells [24].

Lead acetate II decreases the percent of MAP-2 protein (a protein which is related to the microtubule-2) in the cell and inhibits the expression of its mRNA. This protein plays an important role in the structures of microtubule proteins and it is through this mechanism that MAP-2 can affect the stimulation and proliferation of the cells. It is likely that binding lead molecules to this protein and its mRNA in the cell may inhibit the cell proliferation [25]. As a result, we can explain the retarded expression of osteocalcin and collagen type II.

CONCLUSION

This study demonstrated that feeding rats with lead acetate II at doses of 50 and 100 ppm for two months inhibited the expression of osteocalcin and collagen type II genes in Mesenchymal stem cells in a dose dependent manner, during the differentiation process to becoming cartilage and bone cells *in vitro*. The expression of osteocalcin and collagen type II genes in mesenchymal stem cells declined dose dependently. We can also state that the rat model was an appropriate candidate to study the expression of collagen type II and osteocalcin genes during the differentiation of mesenchymal stem cells in the chondrogenic and osteogenic culture media. Based on our findings, we draw the attention of policy makers in Iran to the prevalence of environmental pollution with heavy metals, particularly lead, and the destructive effect of this heavy metal on the growth and development of skeletal tissues in humans and animals in the society.

ACKNOWLEDGMENT

We declare that there was no conflict of interest during the conduction of this study. The authors had no conflicting financial interests.

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