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**Research Article**

### COLLAGEN TYPE II GENE EXPRESSION IN MESENCHYMAL STEM CELLS DELIVERED FROM RATS FEEDING WITH LEAD ACETATE II

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**Abstract:**

**Background and Objectives:** One of the most harmful heavy metals with deleterious effects on tissues is lead (Pb). In this project, the effects of lead acetate II oral doses on Collagen type II gene expression in mesenchymal stem cells (MSCs) in chondrogenic media were studied. Collagen type II is one of the most plentiful differentiated proteins of the extracellular matrix (ECM) in cartilage.

**Methods:** Twelve male Wistar rats were randomly divided into 3 groups. Except libitum feed, the first group were fed orally by 50 ppm lead acetate II, the second group were fed orally by 100 ppm lead acetate II while the control group was fed by distilled water for 2 months. After killing, the femoral bone marrow mesenchymal stem cells (BM-MSCs) were isolated then cultured in chondrogenic medium. Finally the gene expression of Collagen type II was evaluated by real time PCR.

**Results:** After 2 passages, Population Doubling Time of MSCs in the second group was more than the first while the control group was the least. Real time PCR demonstrated that after feeding rats bone marrow with lead acetate II, Collagen type II gene expression in MSCs in chondrogenic medium decreased significantly in the second group compared with the first, while Collagen type II gene expression in the control group was the most.

**Conclusion:** It seems that accumulation of lead acetate II with the doses 50 and 100 ppm in rat bone marrow in femur lead to reduce Collagen type II gene expression in MSCs in chondrogenic medium thus, public health is a priority.

**Kew words:** Bone Marrow, lead acetate, Mesenchymal Stem Cells, Real-Time PCR, Collagen type II

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## INTRODUCTION:

Although the standard amount of lead in water in World health organization (WHO) report is 10 ppm, in many regions level are higher. There are many factors that cause water pollution such as human activities particularly industrial contaminants and presence of lead components adjacency underground aquifer layer. At higher doses than WHO report, some tissues like brain, liver, kidney, reproductive system, cartilage and bone maybe damaged (1, 2). Lead is absorbed in different ways such as breathing, skin and mouth. In the oral way, the absorption of this heavy metal is higher, therefore it can easily enter into blood and consequently into soft tissues and bone. Also long-term toxicity is caused by lead accumulation in bone (3, 4).

One of the greatest interests in biology is adult stem cells. They are recognized by their self-renewal capacity and much plasticity. With these properties, they are able to procreate various mature progenitor cells retaining actively processes of homeostasis by replacing the damaged cells of tissues (5-8). Therefore, they can treat numerous degenerative disorders such as bone and cartilage diseases, various types of aggressive cancers, heart failures, muscular degenerative disorders, chronic liver, type 1 and 2 diabetes, Parkinson and Alzheimer (9, 10). Some tissues such as bone marrow have the source of stem cells with specific niches (8).

In adults, the chondrocytes are only cell types of articular cartilage occupying more than 5% of cartilage volume but 95% of this tissue volume is composed of extra cellular matrix (ECM). *Collagen type II* is the backbone of structure in this matrix. Fifty percent of cartilage protein and 80-90% of articular cartilage collagen are *Collagen type II* forming fibrils. The network of *Collagen type II* fibrils can entrap proteoglycan aggregate providing tensile strength for cartilage tissue (11, 12). Many joint treatments have been performed based on prevention of the *Collagen type II* degeneration or the reforming and the restoration of cartilage by mesenchymal stem cells (MSCs) (12, 13). *Collagen type II* plays an important role in these processes. In this research, we investigated on the level of *Collagen type II* gene expression in isolated BM-MSCs in a chondrogenic medium after feeding rats with lead acetate II for 2 months.

## MATERIALS AND METHODS:

### Animals and Housing:

For this original research, twelve male Wistar rats were purchased from Razi Vaccine and Serum Research Institute of Mashhad Maintained in standard condition comprising standard cages with 12:12 hour interval light/ dark cycle at 25° C. The

animals were divided randomly into 3 groups: the control group was fed by distilled water with libitum feed, the first group were fed with a dose of 50 ppm lead acetate II (Gibco Grand Island, NY) dissolved in distilled water along with libitum feed and the second group were fed with a dose of 100 ppm lead acetate II dissolved in distilled water with libitum feed. All groups were treated for 2 months. The research was in accordance with institutional animal care and use committee (IACUC). The studies on animals were done under ethical committee of Razi Vaccine and Serum Research Institute of Mashhad. We selected 50 and 100 ppm lead acetate II because some mines (like Forumad mine in Iran) have several time of WHO lead dose (1, 14) where some animals live there.

### BM-MSCs Isolation and Incubation:

After two months of treatment, the rats were anesthetized by diethyl ether and the mesenchymal stem cells of their femurs were isolated under sterile condition then cultured in Dulbecco's Modified Eagle Medium (DMEM) and incubated at 37°C and 5% CO<sub>2</sub> (15).

### Population Doubling Time of mesenchymal stem cells:

During the culture period (first and second passage), Population Doubling Time (PDT) were calculated base on following formula:

$$PDT = CT / (\log N/N_0) \times 3.31$$

CT, N<sub>0</sub> and N were respectively the period of cell culture, the number of the cells at the beginning of culture and the number of the cells at the end of culture (16).

### Chondrogenic Culture:

After 2 passages, the medium of MSCs was changed by chondrogenic medium. This medium consisted of DMEM, 1.25 mg/ml bovine serum albumin, 50 µg/ml ascorbic 2-phosphate, 100 nM dexamethasone, 500 ng/ml bone morphogenetic protein-6, 10 ng/ml transforming growth factor β3 and 50 µg/ml ITS. The cells were incubated at 37° C and %5 CO<sub>2</sub> for 21 days. The period of chondrogenesis for mesenchymal stem cells was reported 21 days. The bone medium was replaced every two days (17). We extracted RNA from 3×10<sup>6</sup> cells of control, first and second groups at the end of the 21st day (N= 8 for each group).

### RNA Isolation:

For each group, 3×10<sup>6</sup> cells were utilized for RNA extraction (N=8 for each group). Total RNA was obtained from the culture cells and the culture pellets by TRIzol reagent (Invitrogen) base on the manufacturer's protocol. Synthesis of cDNA was

done for 1 h at 42°C in a mixture of reaction including 5 µg total RNA, 100 U ReverTra Ace, 2 µl 10 mM dNTPs, 1 µl 5 pM oligo (dT) and 4 µl 5×RT buffer regulated with RNase-free water for 20 µl of total volume (18).

The forward and reverse primer sequences of *Collagen type II* were respectively 5'-GTGGAAGAGCGGTGACTAC-3' and 5'-TAGGTGATGTTCTGGGAGC-3'. Also, 5'-CCTTCATTGACCTTCACTACATGGTCTA -3' and 5'-TGGAAGATGGTGATGCCCTTCCATTG -3' were respectively as the forward and reverse primers of GAPDH (19, 20). For *Collagen type II*, PCR method was done by BioRad system (Hercules, CA, US). The condition of PCR was respectively, 95°C for 5 min for initial denaturation, followed by 40 amplification cycles. Each cycle comprised: denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and the step of an additional extension at 72°C for 5 min. The conditions for GAPDH were similar.

#### Quantitative Real-time Polymerase Chain Reaction:

The number of molecules of cDNA in the samples of transcription was detected by analysis of real-time PCR using a modified method along with QuantiTect SYBR Green PCR kit an Icyler iQ Multicolor Real-time PCR Detection System (Bio-Rad). The cDNA content was normalized using by subtracting

numbers of GAPDH cycle from target gene ( $\Delta Ct = \text{target gene } Ct - \text{GAPDH } Ct$ ). The level of gene expression was measured by  $2^{-(\Delta\Delta Ct)}$  formula (21). On days 7, 14 and 21, the gene expression of *Collagen type II* were compared between the first, the second and the control groups.

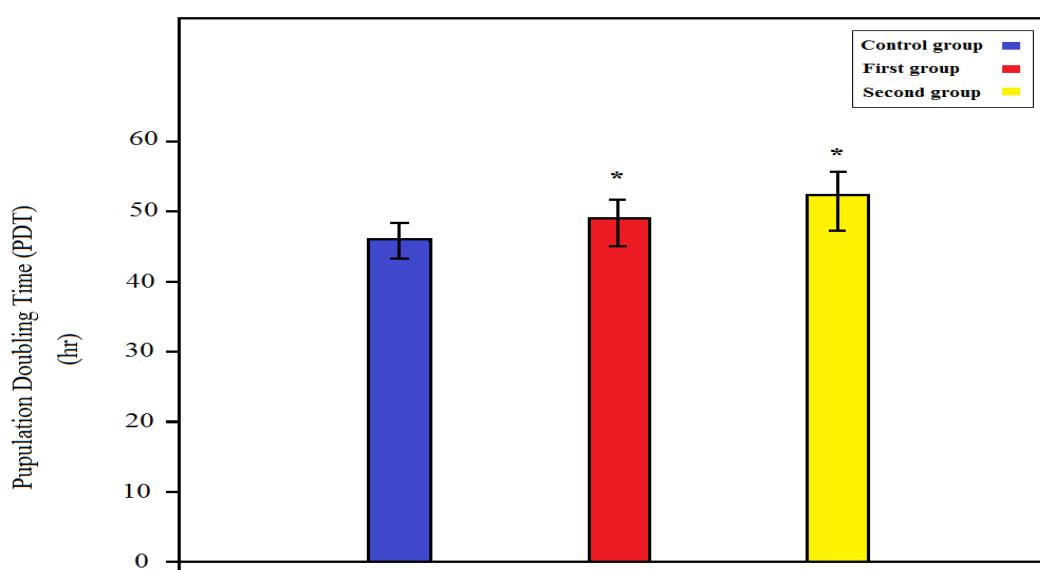
#### Statistical Analysis:

The significant differences between the first, the second and control groups were surveyed at  $P < 0.005$  by Anova and Dunnett t-test. Also comparison between days was done ( $P < 0.005$ ).

#### RESULTS:

PDT of the first, the second and the control groups were respectively  $52.2 \times 10^5 \pm 2$ ,  $48.14 \times 10^5 \pm 2$  and  $45.92 \times 10^5 \pm 2$  showing in fig 1.

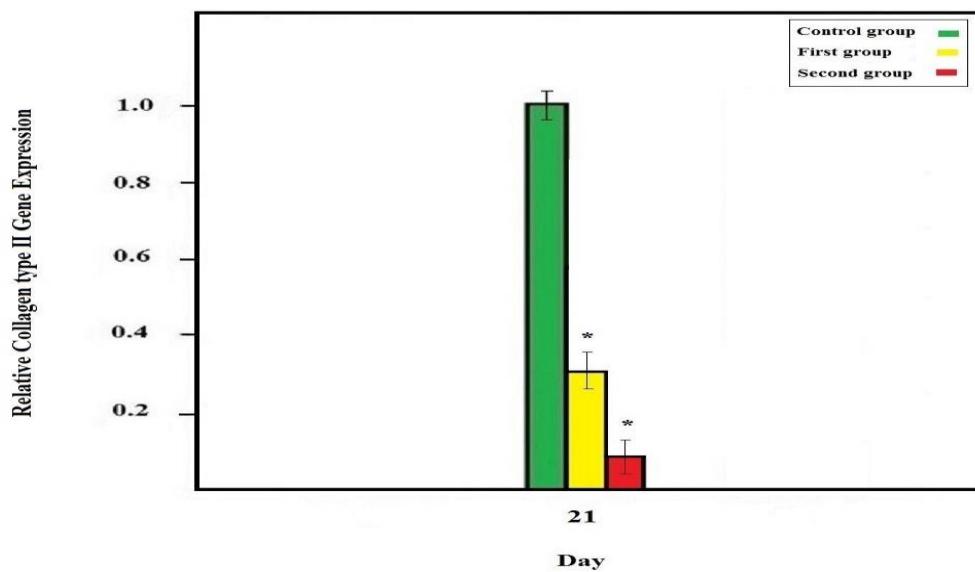
The first repeat of real time PCR showed that *Collagen type II* gene expression of the first group was almost one-third of the expression in the control, while the expression of the second group was less than one-tenth of the control ( $P < 0.005$ ). *Collagen type II* gene expression of the second group was one-fourth of the first ( $P < 0.005$ ). The second and third repeats of real time PCR were almost similar to the first repeat showing the table ( $N = 8$ ). Also, the median of *Collagen type II* gene expression in three groups is shown in fig 2.



**Fig 1: Comparison between PDT median of MSCs in the first, the second and control groups after 2 passages in DMEM culture medium ( $N = 8$ ) ( $P < 0.005$ ).**

**Table 1 : Real time PCR comparison between collagen type II gene expression in MSCs after culture in chondrogenic medium by livak method (N= 8)**

day	First repeat		Second repeat		Third repeat	
Fold difference Sample \	$2^{(-\Delta\Delta Ct)1}$	$2^{(-\Delta\Delta Ct)2}$	$2^{(-\Delta\Delta Ct)1}$	$2^{(-\Delta\Delta Ct)2}$	$2^{(-\Delta\Delta Ct)1}$	$2^{(-\Delta\Delta Ct)2}$
Sample 1	0.31± 0.1	0.08± 0.1	0.32± 0.1	0.08± 0.1	0.31± 0.1	0.08± 0.1
Sample 2	0.33± 0.1	0.078± 0.1	0.3± 0.1	0.08± 0.1	0.31± 0.1	0.077± 0.1
Sample 3	0.32± 0.1	0.082± 0.1	0.3± 0.1	0.083± 0.1	0.28± 0.1	0.074± 0.1
Sample 4	0.31± 0.1	0.079± 0.1	0.31± 0.1	0.083± 0.1	0.29± 0.1	0.074± 0.1
Sample 5	0.28± 0.1	0.079± 0.1	0.28± 0.1	0.084± 0.1	0.29± 0.1	0.075± 0.1
Sample 6	0.29± 0.1	0.082± 0.1	0.31± 0.1	0.082± 0.1	0.33± 0.1	0.076± 0.1
Sample 7	0.31± 0.1	0.08± 0.1	0.28± 0.1	0.081± 0.1	0.3± 0.1	0.076± 0.1
Sample 8	0.31± 0.1	0.08± 0.1	0.32± 0.1	0.083± 0.1	0.3± 0.1	0.076± 0.1

**Fig 2: Comparison between Collagen type II gene expression median in MSCs in the first, the second and control groups after 21 days in chondrogenic culture medium (N= 8) (P <0.005).**<sup>1</sup>  $2^{(-\Delta\Delta Ct)1}$  : Collagen type II gene expression of the first group compare to control group<sup>2</sup>  $2^{(-\Delta\Delta Ct)2}$  : Collagen type II gene expression of the second group compare to control group

**DISCUSSION:**

There is a little information about lead toxicity in MSCs despite of wide investigation. More than 90% of lead accumulates in the bone. So, the main target of lead is bone marrow (22, 23). In this research, the effects of lead on *Collagen type II* gene expression in stem cells of bone marrow was investigated. First of all, the stem cells were isolated from rat exposed to lead acetate II with the dose of 50 and 100 ppm (23). After two passages and increasing stem cells, they were cultured in the chondrogenic medium. On day 21,  $3 \times 10^6$  cells of each group were utilized for RNA extraction ( $N= 8$  for each group). The cells were induced to chondrocytes using chondrogenic culture medium (24). The expression of *Collagen type II* was enhanced during these days. This gene was transcribed plentifully during chondroblast differentiation and cartilage formation and is substantially secreted to ECM where *Collagen type II* produces the fibrillar network to entrap the proteoglycan and provides tensile strength (25, 26). Many factors can have an affect on *Collagen type II* expression such as TGF-B, ascorbic acid and BMP6 (27, 28, 29). It has been found that much of the concentration of lead in water, air and environment has dangerous effects on various tissues such as bone marrow and stem cells (4, 23). *Collagen type II* expression levels plus might be an important key for lead toxicity of cartilage. Accumulation of this heavy metal in ECM provides a condition to reduce *Collagen type II* gene expression level other related genes in chondrocytes contributing to cartilage destruction. Another reason for *Collagen type II* repression is the high half-life of this element (31, 32). The fig shows that *Collagen type II* was downregulated in the first and second group compare with the control group. Also the expression of this gene in MSCs in the second group was reduced more than the first group. It's unclear how much lead causes a decrease in *Collagen type II* gene expression. It seems that probably lead directly targeted *Collagen type II* gene or impressed on transcriptional factors.

**CONCLUSION:**

With respect to the ability of lead metal to accumulate in bone, it might impress on BM-MSCs despite of isolation and culturing in chondrogenic medium that can decrease the *Collagen type II* gene expression level. Higher doses (100 ppm) of lead acetate II lead to more significant reduction in this gene than the low dose (50 ppm). It seems that lead can prevent *Collagen type II* gene expression. Also, lead caused to decrease PDT of MSCs. Hence, natural resources conservation and decreasing pollution are priorities for public health.

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We declare that there is no conflict of interests regarding the publication of this paper.

**Potential Conflict of Interest:**

The authors have no conflicting financial interests.

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