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Original Article

Downregulation of Osteocalcin Gene in Chickens Treated with Lead Acetate II

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Lead is one of the persistent agents found extensively in the environment and has destructive impacts on the tissues. Regarding the high spread of lead, particularly in Iran, we investigated the effects of lead acetate II on the expression level of osteocalcin gene and histological changes of bone. 40 chickens were randomly divided into four groups. As well as the libitum, the control group was fed with distilled water, while treatment 1, 2, and 3 groups were fed with distilled water and 50, 100, and 200 ppm of lead acetate II, respectively. Alterations in the histological profile of bone including decreased level of bone and pyknosis in the nuclei of osteocytes, and decreased expression of osteocalcin gene are the results of exposing to the lead. Regarding the adverse effects of lead on the bone, the spread of this toxic metal must be limited to decrease its adverse impacts on the birds, especially chickens.

Keywords: Lead acetate, osteocalcin, bone, chicken

There are concerns about the outbreak of heavy metals and particularly lead in the environment (1). Lead (Pb) is one of the heavy metals with wide distribution in the lithosphere and environment (2). The properties including softness, low melting point and persistence are the reasons for high utilization of lead. Also, lead has harmful effects on the body. Combustion, lead-based paints and pipes are common ways of exposure to this toxic material (3). Lead is used in the gas to decrease its flaming likelihood and also to elevate the octane number. The most important feature of the lead that is harmful to the body, is its high half-life, so that upon exposure to it, creatures will have it in their body throughout their life. The time of exposure, age and sex are factors that determine the aggregation level of this material in the body (4, 5).

One of the strong connective and high specialized tissues that is formed by organic and inorganic ingredients, is bone (6). Physical and physiological alterations are factors that induce the different functions of specialized cells including osteoblasts, osteoclasts and osteocytes in the bone. The formation, resorption, and remodeling of the bone are respectively performed by osteoblasts, osteoclasts and osteocytes (7). The process of bone remodeling can be controlled based on the distribution of enzymes and proteins involved in the formation and resorption of bone. Furthermore, the osteoporosis occurs due to the imbalance between the formation and resorption of bone (8). The reproductive, nervous and cardiovascular systems are affected by the aggregation of lead in the body (9), but one of the major targets of the lead is bone. After exposure to the lead, bone slowly distributes it into the blood, causing poisoning (10). The compact

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or cortical bone localizes almost 70 percent of the lead (11). The volume of the spongy bone compared to the compact bone is the determinant factor of lead's level in the bone (12). Osteocalcin is of the non-collagenous proteins in the bone and tooth. It is generated by osteoblasts. Also, the metabolism of the bone and body is partly controlled by osteocalcin that is also an indicator of the condition of the bone, and is used for the evaluation of bone diseases (13). In this research, we investigated the impact of lead acetate II on the expression of osteocalcin gene and the general structure and organization of bone.

Materials and methods

Animals and experimental procedures

Forty chickens were purchased and housed in the laboratory for three weeks under conditions similar to the environment. Then, they were divided into four groups including control (fed with mineral water and at libitum), treatment I, and III (fed at libitum and with 50, 100, and 200 ppm lead acetate II, respectively in the water).

The chickens were cared and kept according to the instructions of National Institute of health that are applied for the laboratory animals. Also, the ethical and human principles were considered throughout the experimentations (14).

Histological studies

For microscopic slides preparation, the chickens were anesthetized, and the femur was separated. All samples were fixed in Bouin solution. Dehydration phases by reducing the level of the ethanol, clearing with xylene, paraffin embedment, sectioning, and microscopic slides preparation were performed respectively. Then, the horizontal and vertical sections were stained by hematoxylin and eosin (H & E). The effects of the lead on hepatocytes were carefully investigated and the accumulation of the lipids were studied as described previously (14).

RNA extraction

RNA was extracted using an RNA extraction kit (Parstous biotechnology, Iran). Briefly, the cells were lysed and then, RNA sedimentation was performed using isopropanol and chloroform. Finally, RNA was washed with 70 percent ethanol, and dissolved in water.

cDNA synthesis and real time PCR

cDNA synthesis was performed using Revert Aid H Minus first standard cDNA synthesis kit (Fermentas, Lithuania). 10 µl of RNA was incubated at 65 ℃ for 5 to 10 min. Then, 3 µl of 3X reaction buffer, 0.2 µg of osteocalcin gene primers, 0.5 µl of reverse transcriptase enzyme, 10 µM of 10 mM dNTPs, 3 µl of 50 mM MgCl2 were added to the solution. Then, this solution was incubated at 25 ℃ for 10 min and at 42 ℃ for 60 min, respectively. Finally, in order to stop the reaction, the solution was exposed for 10 min at 70 ℃. Then, synthesized cDNA was kept at -80 ℃. Real time PCR was performed using QuantiTect SYBR Green PCR kit in a Bio-Rad instrument. The sequences of the appropriate primers for aforementioned genes are presented in (Table 1). Finally, the expression levels of osteocalcin gene were calculated, according to the Livak technique (with the $2^{-\Delta\Delta Ct}$ formula) (15). GAPDH was used as a housekeeping gene.

Table 1. The sequence of primers used for gene expression level evaluation

Primer	Sequence 5' ⟶ 3'
Osteocalcin F	CGGAATTCGCGCCGGACG GCTCG
Osteocalcin R	CCGCTCGAGTCAGACGGG GCCGTAGAAGC
GAPDH F	AGGACCAGGTTGTCTCCT GT
GAPDH R	CCATCAAGTCCACAACAC GG

Statistical analysis

Statistical analysis was performed by using SPSS software version 18 (Chicago, IL, USA) and ANOVA test and T-test with $P < 0.05$ as a meaningful level.

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Results

The expression level of osteocalcin gene

The results of real time PCR demonstrated that lead decreased the expression of the osteocalcin gene. Furthermore, there was a reverse and dose dependent relationship between the dose of lead and the expression level of osteocalcin gene (Figure 1).

Histological effects

The investigation of cellular toxic effects of lead on osteocytes revealed the presence of nuclear pyknosis in the treatment III group (Figure 2). Furthermore, the decreased level of bone was observed in all three treatment groups with highest impact observed in the treatment III group, showing predisposition for osteoporosis (Figure 3). After the absorption of lead acetate II, the level of bone diminished (Figure 3) and consequently, the rate of chondrogenesis increased to compensate the loss of bone. Figure 4 shows the increased rate of chondrogenesis.

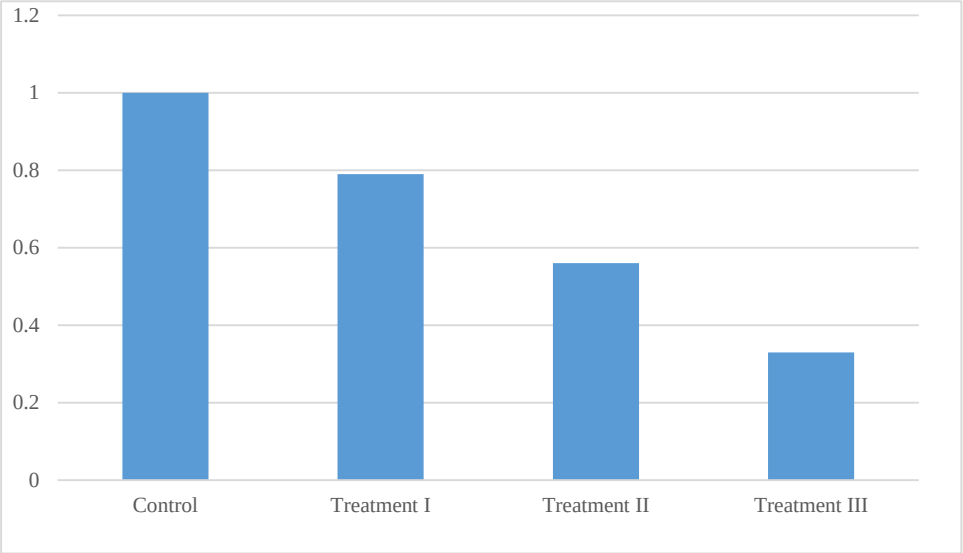


Figure 1. The expression level of osteocalcin in different groups. Treatment I, II, and III groups were fed at libitum and with 50, 100, and 200 ppm lead acetate II, respectively in the water. The expression of osteocalcin decreased significantly ($P < 0.05$) and dose dependently in comparison with the control group.

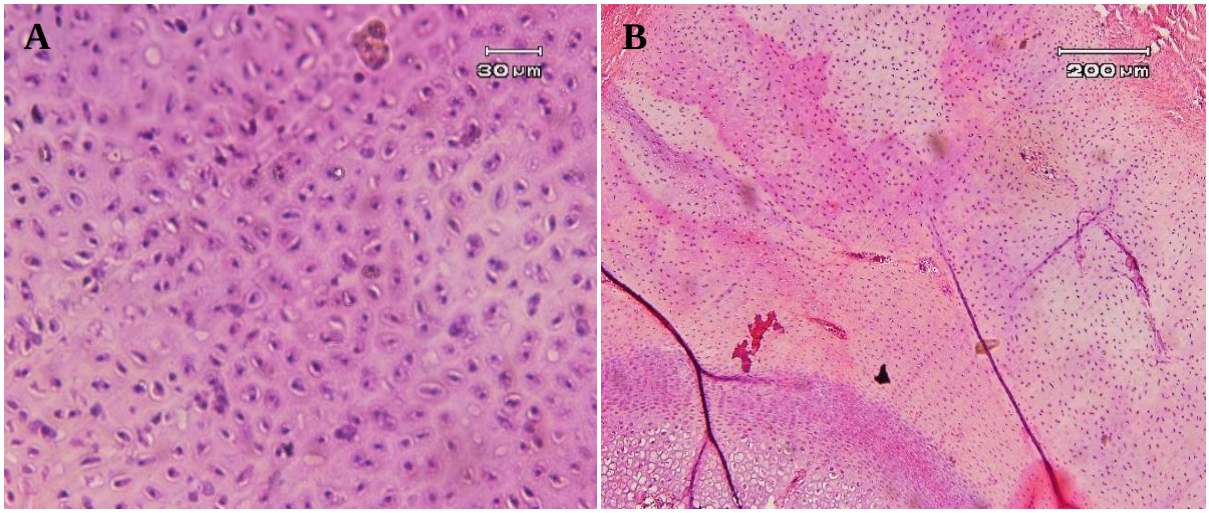


Figure 2. Cellular effect of lead on osteocytes. Pyknosis of nuclei was observed in the treatment III group (A). Normal non treated osteocytes are represented in the (B).

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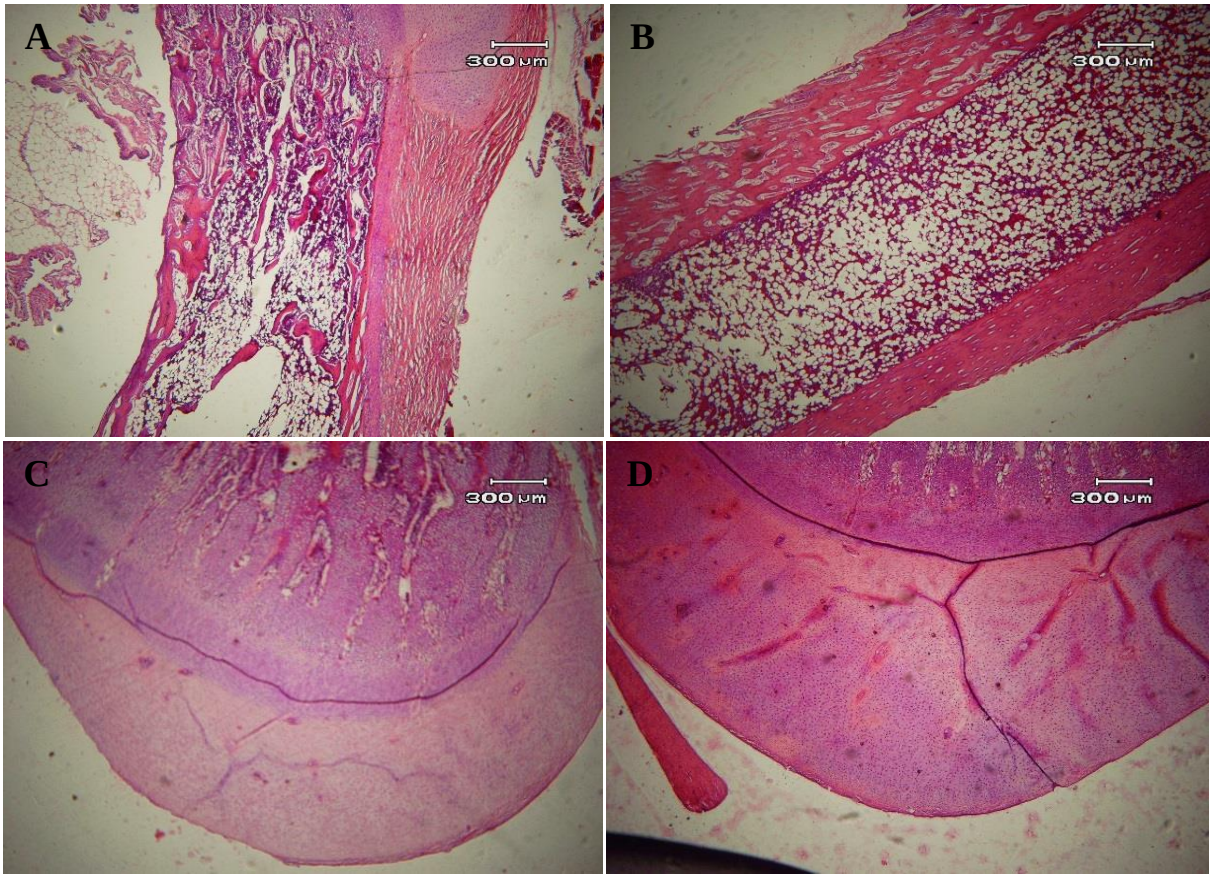


Figure 3. Histological evaluation of the bone. Normal level of bone (A) was observed in control group, and decreased level of bone was observed in treated groups (B). **Histological analysis of chondrogenesis.** The purple color shows the points where chondrogenesis occurs. The intensity and area of chondrogenesis is higher in the treatment group (D) compared to the normal group (C).

Discussion

There is no evidence about the effects of lead on the histological feature of bone and expression of osteocalcin gene in chickens. The bones are major sites for lead storage in the body (16, 17). Two parts of bones are considered to store the lead. The exchangeable and non-exchangeable pools located at the surface and the cortical region, respectively. The exchangeable pool releases lead into the plasma, while the non-exchangeable pool does not. When the bone is re-absorbed, the lead moves to the surface in the non-exchangeable pool (18, 19). Bones participate to around 40-70% of lead releasing into the plasma in adults. In adults and children, 85-95% and 70%, respectively of the lead is stored in the bone, resulting in higher levels of lead in the soft tissues of children. It has been shown that lead reduces the generation of osteocalcin and prevents the activity of alkaline phosphatase in the osteoblasts (20, 21). Also, lead inhibits the expression of collagen type II and X in the chondrocytes, and changes the growth factors level. Besides, lead prevents the signaling responses during the maturation of chondrocytes (22, 23). Furthermore, lead stimulates bone resorption through osteoclasts (24). Lead might affect the substitution of calcium in the active sites of calcium signaling system and as a result, inhibits the physiological regulation. The effects of lead on the function of bone cells might be due to the disturbance of the calcium and cAMP signaling system in these cells (25). In *in vitro* studies, the harmful impacts of lead including the production of matrix (26), increasing the absorption of bone (26, 27), disturbance in the mineralization and organization of chondrocytes (28), and inhibiting the development of axial bone (27) were observed. Also, exposing to the lead delays the repairing of the

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broken bone by inhibiting the endochondral ossification (29). There is a relationship between high concentrations of lead and increased risk of unpredictable breaks. Also, it has been shown that lead participates to osteoporosis (30). In this research, we found that the osteocytes participating in remodeling of bone, were impaired in the presence of lead acetate II in the water as witnessed by osteocytes nuclei pyknosis. Also, the decreased level of bone suggests that the activity of osteoblasts was disturbed. Moreover, the decreased expression of osteocalcin that is normally produced by osteoblasts suggests that lead acetate may cause osteoblasts malfunction. Furthermore, because of the role of osteocalcin in the regulation of bone metabolism, the decreased level of osteocalcin gene diminishes the metabolism of bone and also predisposes bone to the diseases.

In conclusion, due to the increased use of lead and its spread in the environment, this research provides evidence on the destructive effects of lead on the birds. According to the histological features that show the harmful effects of the lead and because of the decreased expression of osteocalcin gene as a main regulator of bone metabolism, efforts should be done to prevent the outbreak of this compound in the environment.

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Conflict of interest

The authors declared no conflict of interest.

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