Original Article

Down Regulation of Osteocalcin Gene in Chickens Treated with Cadmium

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ABSTRACT

Background: Cadmium is one of the heavy metals with harmful effects on different body organs and systems. The aim of this study was to investigate the harmful effects of cadmium, as a heavy metal, on the histological structure of bone and the expression of osteocalcin gene.

Methods: Forty chickens were obtained, anesthetized and their femurs were surgically removed. The real time polymerase chain reaction (PCR) was used to study the osteocalcin gene expression.

Results: The osteocalcin gene expression rate were: 1.000± 0.1; 0.86± 0.01; 0.63± 0.09, and 0.41± 0.06 in the controls, experiment I, experiment II and experiment III groups, respectively ($P < 0.05$). Also, the nuclear pyknosis in osteocytes and decreased bone formation were observed in the histology slides of the chicken bones.

Conclusions: We conclude that cadmium adversely affected the chicken bones as evident by the decreased osteocalcin gene expression and the adverse effects on the bone histology. We recommend that plans be developed to prevent the outbreak of cadmium and other heavy metals in animal and human environment.

Keywords: Bone Formation, Cadmium, Gene Expression, Osteoblast and Osteocyte, Osteocalcin Gene.

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INTRODUCTION

Cadmium is one of the most harmful heavy metals, with high motility in the air, soil, and water with significant toxic effects even at low concentrations (1). In addition, it has been shown that cadmium results in harmful effects in human organs and tissues (2). Cadmium may be released to the environment as a result of mining of zinc, lead and copper (2). The International Agency for Cancer Research has considered cadmium as a class I carcinogen (3). Cadmium may spread in the environment and enter the human food chain (4). It has been shown that the average individuals in the U.S. may be exposed to 30µg cadmium daily; this level may be remarkably higher in China and Japan (5). It has been reported that the general population is at risk of exposure to cadmium, and poisoning with this heavy metal may readily occur among the wildlife (6). It has been shown that exposure to the cadmium in birds may lead to their limited growth, increased mortality, diminished egg production and poor eggshell quality (7).

Bone is one of the dense and strong connective tissues that is formed by organic and inorganic ingredients (8). Both physical and physiological alterations influence the functions of specialized cells, such as osteoblasts, osteoclasts and osteocytes in the bone. These cells impact the formation, resorption and remodeling of bones (9). The processes of bone formation, remodeling and resorption are controlled by circulating enzyme and proteins (9). Further, osteoporosis occurs due to an imbalance between the synthesis and resorption processes in the bones (10). Osteocalcin is produced by osteoblasts and is considered a non-collagenous protein in the bone and teeth. Osteocalcin plays a remarkable role in the regulation and metabolism of bones, and its level in the blood represents an index of bone health. Also, osteocalcin is a specific marker in the assessment of many bone diseases (11).

When cadmium is absorbed by human organs, its biological half-life would be longer than 10 years (12). Osteoporosis, renal dysfunction, diabetes, cancer, high blood pressure and reproductive disorders may result from exposure to cadmium (10). In workers exposed to cadmium, renal dysfunction has been reported even at the blood levels blow 10µg/l (11). For instance, excretion of low-molecular-mass proteins from the kidneys has been shown to occur following cadmium exposure (12). However, the impact of cadmium on the skeletal system, especially bones has not been studied genetically in details. The purpose of this study was to investigate the adverse effects of cadmium on the expression of osteocalcin gene and the resultant histological changes in chicken bones.

MATERIALS AND METHODS

Animals and Experimental Procedures

Forty chickens were obtained and housed in the laboratory for three weeks to get acclimated to the environment. The chickens were treated according to the guidelines of National Institutes of Health, applicable to the care of laboratory animals. They were divided into four groups of 10 each, i.e., controls, experimental groups I, II and III. The chickens were fed routinely as follows:

- Control: Libitum feed and mineral water

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- Experimental I: Libitum feed plus 1 ppm cadmium in water
- Experimental II: Libitum feed plus 5 ppm cadmium in water
- Experimental III: Libitum feed plus 10 ppm cadmium in water

Histological Studies

The histological studies were performed similar to those in our previous research (13, 14). The chickens were anesthetized and the femurs removed surgically. All bone samples were fixed in 10% formalin. After decalcification with ethylene diaminetetraacetic acid (EDTA), as the chelating agent, they were subjected to stages of dehydration, using 50%, 70% and 90% alcohol, respectively. Then, the samples were infiltrated with xylene and embedded in paraffin. Horizontal and perpendicular sections were stained with hematoxylin and eosin and prepared for histological examination.

RNA Extraction

The RNA was extracted, using a Kit from Parstous Biotechnology (Mashhad, Iran). According to the RNA extraction protocol, the cells were lysed and the RNA sedimented, using isopropanol and chloroform. Finally, RNA samples were washed with Ethanol 70% and diluted in water.

CDNA Synthesis and Real Time PCR

The cDNA synthesis was performed, using Revert Aid H minus standard (Fermentas, ThermoFisher Scientific, USA). For this purpose, 10µl of RNA was incubated at 65℃ for 5-10 minutes, and the following materials were added to the solution: 3µl of 3X reaction buffer, 0.2µg osteocalcin gene primers, 0.5µl reverse transcriptase enzyme, 10µM dNTP, and 3 ml $MgCl_2$, respectively. The solution was incubated for 10 minutes at 25℃ and then for 60 minutes at 42℃. To stop the reaction, the solution was loaded for ten minutes at 70℃. The synthesized cDNA was loaded on a PCR unit at 80℃ to analyze the real time process, using QuantiTect Syber Green PCR kit, equipped with a detector system (BioRad, USA). The appropriate primers for the above cDNA samples are presented in Table 1. Finally, the expression of osteocalcin gene was calculated based on Livak method.

Table 1. The osteocalcin gene primers and GAPDH*.

Gene	Sequence	Attachment temperature (℃)	Size of product (bp)	Number of cycles
Osteocalcin F	5/- CGGAATTCGCGCCGGACGGCTCG-3/	55-60	380	35
Osteocalcin R	3/- CCGCTCGAGTCAGACGGGGCCGTAGAAGC-5/			
GAPDH F	5/-AGGACCAGGTTGTCTCCTGT-3/			
GAPDH R	5/-CCATCAAGTCCACAACACGG-3/			

*Glyceraldehyde-3-phosphate dehydrogenase.

Statistical Analysis

We performed the statistical analyses, using ANOVA and T-test on SPSS software Version 18 (Chicago, IL, USA), with the significance level set at $p<0.05$.

RESULTS

In the course of our experiments, no chicken died prior to experiments in any group. The histological changes observed upon light microscopy of the stained bone samples were as follows:

Osteocalcin Gene Expression

The results of real time PCR demonstrated that the expression of the osteocalcin gene declined in the bone samples exposed to cadmium. Further, we observed that there was a reverse relationship between the cadmium dosage and the expression of osteocalcin gene, i.e., increasing the cadmium dosage caused a progressive decrease in osteocalcin gene expression.

Histological Alterations

We observed significant pyknosis in the osteocytes nuclei in the experimental group III (Figure 1). Decreased bone formation was observed in the three experimental groups, with the highest impact noted in group III (Figures 2 & 3).

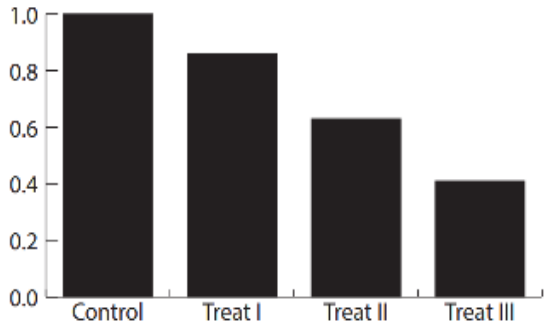

Figure 1. The expression of osteocalcin gene compared to the GAPDH samples of chicken bones ($P < 0.05$).

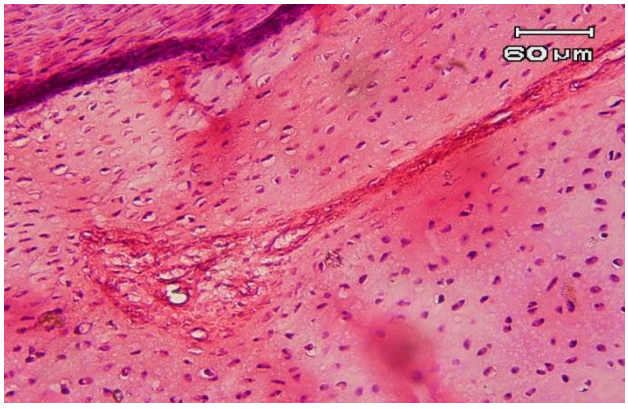

Figure 2. Pyknosis of osteocytes nuclei.

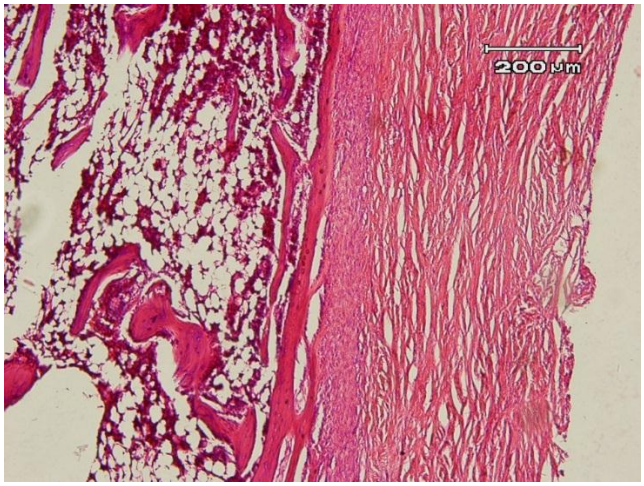

Figure 3. Decreased bone formation in chicken after exposure to cadmium.

DISCUSSION

During recent years, much attention has been performed towards the toxicity of compounds (15-17). Based on previous studies, exposure to cadmium may be related to poor density of bone minerals, osteoporosis, and the risk of fractures even at the low concentrations that may be prevalent in the environment (18). Further, the harmful effects of cadmium on osteoblasts and osteoclasts functions have already been reported (19-21). The presence of cadmium in animal products has been a concern in several countries and in some cases, its level in the manure and foods may exceed 130 mg/Kg (22, 23). The adverse effects of cadmium may be due to decreased glycogen synthesis in the liver and increased oxidative stress on the liver and kidneys (24, 25). Cadmium exerts harmful effect on the metabolism of bone cells and inhibits calcium incorporation and collagen synthesis. The importance and role of Ca++, phosphate and Mg++ have long been known on bone metabolism. Previous studies have shown that the concentration of cadmium precipitated in bones rises with increasing exposure to this element, i.e., 1, 5 and 50 mg/l. A previous epidemiological study has shown that low and chronic exposure to cadmium that occurs in industrialized countries is associated with osteoporosis and pathologic bone fractures. (26-28).

In this study, we found that the osteocytes and osteoblasts that normally participate in the maintenance and remodeling of bones were impaired after exposure to cadmium. This was evident by the detection of pyknosis in the nuclei of osteocytes and osteoblasts. Considering the decreased bone formation noted in the histological samples, we can safely conclude that the cadmium exposure impaired the bone forming ability of osteoblasts in the chicken bones. The results of this study demonstrated a disturbance in osteoblasts function by a decrease in genetic expression of osteocalcin after exposure to cadmium. Further, because of the importance of osteocalcin in the regulation of bone metabolism, the decreased expression of osteocalcin gene affected the metabolism of the bones, which can predispose to osteoporosis and osteopenia. Further, new evidence-based knowledge on this important topic awaits future research in animal and human bones.

CONCLUSIONS

Currently, there are concerns about the increasing use and outbreak of heavy metals, such as cadmium that is considered toxic and a class I carcinogen. We found that cadmium exerted harmful effects on the osteocytes and osteoblasts in chicken bones. This could be considered as a major predisposing factor in the development of osteoporosis. Our findings can serve as the basis for future studies to decrease or prevent the harmful effects of cadmium on bone in animals and humans. Also, our results can provide warning to the governmental policy makers to establish regulations and apply plans to prevent the outbreak of cadmium and other toxic heavy metals in the lives of people who are unaware of the risks.

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CONFLICT OF INTERESTS

The authors declare no conflict of interests in the course of conducting this research.

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