

**Original Article****Cadmium Treatment of Rats Caused Impairment of Osteogenic Potential of Bone Marrow Mesenchymal Stem Cells: A Possible Mechanism of Cadmium Related Osteoporosis***Mohammad Hussein Abnosi\*, Somayeh Gholami**Received: 25.04.2016**Accepted: 28.05.2016***ABSTRACT**

**Background:** The mechanism of cadmium induced osteoporosis is not well understood, so in this study, we examined the toxicity of bone marrow mesenchymal stem cell (MSCs) following treatment of rats with CdCl<sub>2</sub> in drinking water, to revile the effect of this chemical on differentiation potential of MSCs.

**Methods:** At the end of third passage, MSCs were grown in the osteogenic medium for 21 days. To study the differentiation property the viability, morphology, intracellular calcium, and matrix mineralization via quantitative alizarin red were evaluated. Besides, biochemical parameters including activity of alkaline phosphatase (ALP), aspartate amino transaminase (AST), alanine amino transaminase (ALT) as well as antioxidant enzyme such as superoxide dismutase, catalase, and peroxidase were determined too. In addition, level of lipid peroxidation based on determination of malondialdehyde (MDA) content was studied.

**Results:** The results showed significant reduction in the viability of cells after differentiation compared to control ( $P<0.05$ ). The treatment of rats caused significant reduction in nuclear diameter. There was significant increase in (ALT) and (AST) activity whereas activity of ALP reduced significantly ( $P<0.05$ ). The results showed significant reduction in the antioxidant enzyme activity and increases in (MDA). The mean bone matrix mineralization and intracellular calcium content of the MSCs also reduced significantly ( $P<0.05$ ).

**Conclusions:** Oral consumption of cadmium affects osteogenic differentiation potential of MSCs via membrane damage, reduction of calcium deposition and metabolic changes. Thus, it might be considered as a probable factor involve in cadmium related osteoporosis.

**Keywords:** Antioxidant Enzymes, Cadmium, Lipid Peroxidation, Osteoblasts, Stem Cells.

**IJT 2017 (1): 1-9****INTRODUCTION**

Cadmium (Cd) which exists in the earth crust is also released by industrial activity. It is one of the most toxic heavy metals, which causes a wide range of biochemical and physiological dysfunctions such as lung fibrosis, kidney tubular dysfunction, hypertension and osteoporosis in humans and laboratory animals [1, 2]. The bone is one of the major target of Cd toxicity [3], which its accumulation in the skeleton can happen during lifetime. Cd affects the bone tissue directly by interfering with the hydroxyapatite formation and affecting the activity of bone cells, indirectly to causes mineral metabolism disruption [4]. Osteoblasts are the main cells, which take part in the mineralization process of the bone matrix; therefore, the activity of osteoblasts is necessary to prevent osteoporosis [2]. Mesenchymal stem

cells have the ability to differentiate into many cell lines including osteoblasts [5] thus any damage to cause dysfunction of these cells would bring about matrix deposition impairment that finally results in bone deformation and repair malfunctioning.

Since the mesenchymal stem cells are cellular backup for osteoblasts production, therefore, the differentiation properties of mesenchymal stem cells are foremost important character to be considered in the bone health. In an in vitro experiment, the different

CdCl<sub>2</sub> in a dose and time dependent manner have caused significant reduction of viability and proliferation of rat bone marrow mesenchymal stem cells (MSCs). In the same investigation, the cadmium chloride caused significant reduction of

cell nuclear diameter and cytoplasm shrinkage as well as calcium elevation and DNA breakage, which all together could be the reason for cell mortality [2]. The treatment of MSCs with nanomolar concentration of Cd for a period of 21 days significantly reduced the viability, proliferation and differentiation ability of these cells with the same mechanism [5]. All these investigations have been conducted in vitro, however MSCs in their natural environment (i.e. bone tissue) are protected by many biological barriers, which may ameliorate and compensate the adverse effect of Cd when given orally. When we considered the fact that the MSCs are directly reachable via blood circulation in vivo, therefore the health of these cells can be threaten due to intestinal absorption of Cd.

In a recent study we showed that, the oral treatment of rats with 300 mg/kg/d of p-nonylphenol for three months resulted in significant reduction of viability and proliferation ability of extracted bone marrow mesenchymal stem cells (MSCs) due to caspase dependent apoptosis. The differentiation ability of these cells reduced significantly following alkaline phosphatase activity reduction [6]. In industrial area, chemical toxicity might be the main culprit of increasing number of bone complications. Cd toxicity has been blamed to cause osteoporosis in Japan, but to our best knowledge, no research work has been published to emphasize the effect of Cd on differentiation property of MSCs after Cd toxicity in vivo.

Thus in this study we investigated the effect of cadmium chloride ( $\text{CdCl}_2$ ) on viability, morphology, activity of metabolic enzymes and the level of oxidative stress in the cells as well as bone Matrix Mineralization following differentiation of the MSCs to osteoblast after oral treatment of rats. The data of this study would help us to pin point the possible mechanism behind the Itai-Itai disease and increasing number of osteoporosis in the industrial area.

## MATERIALS AND METHODS

### *Animal Treatments and Mscs Isolation*

Wistar rats were divided in two groups, namely treated (N=5) and control (N=5) and were kept in the animal house of Arak University, central Iran under standard condition of food, water, and temperature. Treated group received 40 mg/L of cadmium chloride in drinking water for

six weeks, whereas the control group was treated only with same amount of drinking water. After the treatment period the rats were anesthetized using diethyl ether and euthanized according to the laboratory animal protocol approved by Arak University. Then under sterile condition their femora and tibiae were removed surgically and using flushed out technique the bone marrow content were extracted in 3 ml of Dulbecco modified Eagle medium (DMEM) (Gibco company) supplemented with 15% FBS (Gibco company), and penicillin/ streptomycin (Gibco company). The bone marrow content was centrifuged at 2500 rpm for 5 min at room temperature and pellet of the cells were homogenized with 1 ml fresh culture media and transferred in a culture flask. After 24 h, unattached cells were washed off the flask with phosphate-buffered saline ( $\text{PBS}^+$ ) containing  $\text{Mg}^{++}$  and  $\text{Ca}^{++}$  and adherent fibroblast-like cells were allowed to grow for 10-14 d, with every three days of culture media replacement. Cells were passaged at 90% confluence by trypsinization (Trypsin/EDTA solution; sigma) and reseeded at a density of  $10^5$  in another plastic flask. The time required for cell to reach the passage (in course of days) and the number of cells (using hemocytometer chamber) in each passage was noted down.

### *Osteogenic Induction and Estimation of Osteogenic Property*

At the end of the third passage, MSCs extracted from control and treated rats were cultured in T<sub>25</sub> plastic flasks supplemented with DMEM-high glucose, 15% FBS, penicillin-streptomycin, 1mM beta-glycerophosphate (Sigma-Aldrich Company; America), 50  $\mu\text{g/ml}$  L-ascorbate (Sigma-Aldrich Company; America) and  $10^{-8}$  M dexamethasone (Sigma-Aldrich Company; America). Culture flasks were incubated at 37 °C with 5% CO<sub>2</sub> and their medium was changed every 3 d for 21 d. The following analysis including Cell viability, (AST, ALT, ALP) activity, SOD, CAT, POX activity, content of MDA, intracellular potassium and sodium, calcium concentration of matrix and quantification of mineralization were carried out.

### *Cell Viability Assay*

Cell viability was quantitatively determined by 3-(4, 5-dimethyl thiazol-2-yl)-2, 5 diphenyl tetrazolium (MTT) assay. After 21 days of

treatment the attached cells were washed with PBS and 300  $\mu$ l of fresh culture medium (without FBS) containing 30  $\mu$ l MTT was added, then the plate was incubated for 4 h. The resulting formazan crystals were dissolved in 100  $\mu$ l of DMSO and the absorbance was measured using ELISA reader (SCO diagnostic, Germany). After plotting the standard graph, the number of the viable cells were calculated using the linear formula  $Y = 0.0134X + 0.007$  with  $R^2 = 0.996$ , where Y stands for absorbance and X stands for number of the viable cells.

### **Cell Morphology**

At the end of the treatment period in order to stain the nuclei, the cells were washed with PBS and incubated with 50  $\mu$ l of Hoechst (0/05 mg/ml) (Sigma Company, Germany) for 5 min. Furthermore, to study the morphology of the cell cytoplasm, the cells were incubated with 50  $\mu$ l of acridine orange (0.005 mg/ml) for 5 min. After Hoechst and acridine orange staining, the cells were observed under invert fluorescence microscopy (Olympus, I X 70) with 400 magnifications. In addition, motic Image software (Micro optical group company version 1.2) was also used to measure the diameter ( $\mu$ m) of the cell nuclei and cytoplasm area ( $\mu$ m<sup>2</sup>).

### **Enzyme Activity Assays**

#### **Extraction of Intracellular Content**

MSCs washed 3 times with PBS and homogenized in lysis buffer (20 mM Tris-HCl, pH 7.2). Then the intracellular content was extracted using freeze-thaw method by incubating them in -20 °C overnight. Then the samples were centrifuged at 12000 rpm for 10 min at 4 °C and supernatant was kept in -20 °C for further analysis of enzyme activity as well as sodium, potassium and calcium content. The total protein content of each sample was determined according to Lowry, using bovine serum albumin (BSA) as the standard. All the analysis was carried out three times based on equal amount of protein.

The alkaline phosphatase (ALP) activity of the cells was determined based on p-nitrophenylphosphate (pNPP) hydrolysis method, using the ALP assay kit (Pars Azmon, Iran). Absorbance was measured at 410 nm using spectrophotometer (T80<sup>+</sup> PG instrument Ltd, England).

The alanine transaminase (ALT) and aspartate transaminase (AST) activity of the cells was determined using commercial kit (Pars Azmon, Iran). Absorbance was measured at 340 nm using spectrophotometer (T80+ PG instrument Ltd, England).

Superoxide dismutase (SOD) activity was measured by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium. One hundred  $\mu$ l of cell extract was added to 3 ml of reaction mixture (25 mM phosphate buffer (pH 8.6), 12 mM methionine, 75  $\mu$ M nitrobluetetrazolium, 1 mM riboflavin, 0.1 mM EDTA, 50 mM Na<sub>2</sub>CO<sub>3</sub>) and were illuminated for 15 min under fluorescent light, identical tube which was not illuminated served as blanks. One unit of superoxide dismutase activity was defined as the amount of enzyme required to cause 50% inhibition of nitro blue tetrazolium reduction, which was monitored at 560 nm. The SOD activity of the extract was expressed as unit activity min<sup>-1</sup> mg<sup>-1</sup> protein.

Peroxidase (POX) activity was assayed via oxidation of guaiacol in the presence of H<sub>2</sub>O<sub>2</sub>. The increase in absorbance was recorded at 436 nm. The reaction mixture contained 50  $\mu$ L of enzyme extract, 100  $\mu$ L H<sub>2</sub>O<sub>2</sub>, 100  $\mu$ L guaiacol (18 mM) and 100  $\mu$ L potassium phosphate buffer 100 mM (pH=7). POX activity of the extract was calculated using extension coefficient of 26.6 mM<sup>-1</sup>cm<sup>-1</sup> and expressed as unit activity min<sup>-1</sup> mg<sup>-1</sup> protein.

Catalase (CAT) activity was assayed at 240 nm by measuring the amount of hydrogen peroxide disappearance from the initial solution containing fix a enzyme extract was added to the reaction mixture contained crude enzyme extract, 10 mM H<sub>2</sub>O<sub>2</sub> and 25 mM sodium phosphate buffer (pH 7). The decrease in the absorbance at 240 nm was recorded for 1 min by spectrophotometer considering the extension coefficient as 39.4 mM<sup>-1</sup>cm<sup>-1</sup>. CAT activity of the extract was expressed as unit activity min<sup>-1</sup> mg<sup>-1</sup> protein.

#### **Estimation of Lipid Peroxidation Levels**

The level of lipid peroxidation in the cells was determined based on estimation of malondialdehyde (MDA) using 2-thiobarbituric acid. In 1 ml trichloroacetic acid (0.1%), 2.5  $\times$  10<sup>6</sup> cell was homogenized and then centrifuged at rpm 10000 for 10 min. The supernatant was treated

with 2.5 ml reagent mixture containing 0.5% (w.v) thiobarbituric acid, 20% TCA and 2.5N HCl and boiled at 95 °C for 20 min and cooled quickly. The absorbance was measured at 530 nm and the non-specific turbidity was corrected by subtracting A600 from A530 value. The concentration of MDA was calculated using formula  $A=\epsilon bc$  where (A) is absorption, (b) is path length 1 cm, (c) the MDA concentration and ( $\epsilon$ ) is the extinction coefficient ( $155 \text{ mM}^{-1} \text{ cm}^{-1}$ ).

### **Determination of Sodium and Potassium**

A traditional and simple method for determining sodium and potassium in biological fluids involves the technique of emission flame photometry. Since  $\text{Na}^+$  and  $\text{K}^+$  emit light of different wavelengths, using appropriate filters the emission due to  $\text{Na}^+$  and  $\text{K}^+$  (and hence their concentrations) can be specifically measured in the same sample. The amount of sodium and potassium in the cell extract was estimated using the linear formula  $Y=0.005X+0.0592$  with  $R^2=0.992$  and  $Y=0.0201X+0.0039$  with  $R^2=0.996$  respectively. Here the Y is the absorbance and X is the concentration of each one of the electrolytes.

### **Intracellular Ca 2+ Assay**

Control and treated cells were plated in 24-well plates and washed twice with PBS. The cells were then exposed to 50  $\mu\text{l}$  of 0.5 N HCl for 24 h in order to extract their calcium content and using commercial kit (Darman Kave, Iran) the amount of intracellular calcium was determined. The resulting color was measured at 575 nm using a spectrophotometer (T80<sup>+</sup> PG instrument Ltd, England).

### **Bone Matrix Mineralization**

At the end of the treatment period, the cells were washed with PBS and fixed at room temperature in 10% (v/v) formaldehyde (Sigma-Aldrich) for 15 min. After washing the cells with  $\text{dH}_2\text{O}$  twice, 1mL of 40 mM alizarin red solution (ARS) (pH 4.1) was added per well followed by incubation at room temperature for 20 min with gentle shaking. The excess dye was then discarded and the plates were washed with  $\text{dH}_2\text{O}$  several times and the stained cells were observed using an inverted microscope and photographed by camera. To estimate the mineralization quantitatively, 800  $\mu\text{l}$  of 10% acetic acid (v/v) was added to each

well, and the plate was incubated at room temperature for 30 min with gentle shaking. Then the loosely attached cells were scraped into a 1.5 mL micro centrifuge tube and vortex for 30 sec. Then 500  $\mu\text{l}$  mineral oil (Sigma-Aldrich) was added and heated at 85 °C for 10 min followed by ice-cooling for 5 min. The slurry was then centrifuged at 20,000g for 15 min and 200  $\mu\text{L}$  of 10% ammonium hydroxide (v/v) was added to 500  $\mu\text{L}$  of the supernatant in new micro centrifuge tube to neutralize the acid. The absorbance of aliquots of the supernatant (100  $\mu\text{L}$ ) was read at 405 nm in a micro plate reader (SCO diagnostic, Germany) for three times and the amount of absorbed alizarin red was calculated using a standard graph.

To prepare the alizarin red standards graph, working ARS (40 mM) was diluted 20 times with a mixture of 5:2 of 10% acetic acid and 10% ammonium to give a concentration of 2000  $\mu\text{M}$ . Different standard solution ranging from 2000 to 31.3  $\mu\text{M}$  was prepared and the absorption taken at 450 nm using a microplate reader. The concentration of the unknown samples was calculated using the linear formula  $Y=0.099X+0.101$  with  $R^2=0.997$  where Y is the absorbance and X is the concentration (mM) of alizarin red.

### **Analysis of Data**

Data was analyzed with the help of SPSS software (version 16, Sun Microsystems Inc., America), using unpaired *t*-test. The results are shown as mean $\pm$ SD and  $P<0.05$  was determined as the minimum level of significance.

## **RESULTS**

### **Effect of Cd on Cell Viability**

Cell viability determination using MTT colorimetric assay showed, after 21 d the viability of the MSCs extracted from Cd treated rats differentiated to osteoblasts was significantly reduced ( $P<0.05$ ) compared to the control group of cells (Table 1).

**Table 1.** Effect of cadmium on viability of MSCs extracted from treated and control rats when differentiated to osteoblasts, based on MTT colorimetric assay.

	Average number of living cells ( $\times 1000$ )
Control group	11.69 $\pm$ 0.78
Treated group	9.25 $\pm$ 0.58*

Values are mean±SD. Means with asterisk (\*) differ significantly from control (ANOVA T-test, \* $P<0.05$ ).

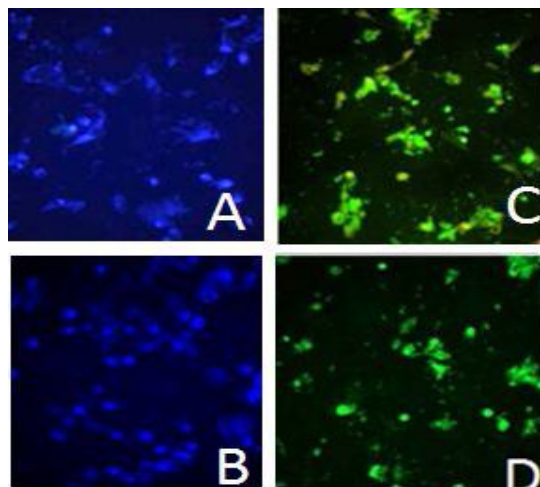
### Effect of Cd on Cell Morphology

Morphological study of the MSCs which have been extracted from treated rats and differentiated to osteoblasts showed chromatin condensation (Figure 1-B) but no change in the cell cytoplasm was observed (Figure 1-D) as compared to the cells extracted from the control rats (Figure 1-A and C). In addition measurement of the nuclei diameter ( $\mu\text{m}$ ) and cytoplasm area ( $\mu\text{m}^2$ ) also confirmed the microscopic observation where a significant decrease ( $P<0.05$ ) in nuclei diameter of the cells was shown (Table 2) as compared to control.

**Table 2.** Effect of cadmium on nuclei diameter ( $\mu\text{m}$ ) and the area of cytoplasm ( $\mu\text{m}^2$ ) of the cells extracted from treated and control rats following differentiation to osteoblasts.

Group	Nuclei diameter	Area of cytoplasm
Control group	8.6±0.65	320.6±27
Treated group	7.5±0.2*	299.6±16

Values are mean±SD. Means with asterisk (\*) differ significantly from control in each column (ANOVA T-test, \* $P<0.05$ )



**Figure 1.** Morphology of the cells A) cells extracted from control rats stained with Hoechst B) cells extracted from Cd treated rats stained with Hoechst, nuclei appear small as compared to control. Morphology of the cells cytoplasm C) cells extracted from control rats stained with acridine orange, D) cells extracted from Cd treated rats stained with acridine orange, no change in the cytoplasm was seen (20X magnification).

### Effect of Cadmium on the Activity of ALP, ALT, AST

The activity of ALT and AST enzymes was increase highly significant ( $P<0.001$ ) in the osteoblasts differentiated cells extracted from Cd treated rats as compared to control ones. In the same cells, we observed a significant decrease in the activity of ALP (Table 3).

**Table 3.** Effect of cadmium on activity of ALP (IU/L), ALT (IU/L), AST (IU/L) from osteoblasts differentiated cell extracted from Cd treated rats and control ones.

	ALP	ALT	AST
Control group	266.99±8.5	22.37±1.4	22.16±2.49
Treated group	217±8.5*	43.89±3.6**	59.55±3.97**

Values are mean±SD. Means with the asterisk (\*) differ highly significant control in each column (ANOVA *t*-test, \*  $P<0.05$  and\* \* $P<0.001$ )

### Effect of Cadmium on Oxidative Stress Enzymes and Lipid Peroxidation

Highly significant decrease ( $P<0.001$ ) of SOD, CAT and POX activity was observed in the MSCs which have been extracted from treated group of rats and differentiated to osteoblasts as compared to control ones. In addition, it was showed that MDA level increased significantly ( $P<0.001$ ) in the cells as compared to control cells (Table 4).

### Effect of Cadmium on Intracellular Sodium, Potassium, Calcium and Bone Matrix Mineralization

Cadmium treatment caused significant increase in potassium ( $P<0.05$ ) and decrease in calcium and sodium ( $P <0.05$ ) content of the osteoblasts differentiated cells extracted from treated group of rats as compared to control (Table 5).

In the osteoblasts differentiated cells extracted from treated rats, the quantitative analysis of Alizarin red showed highly significant ( $P<0.001$ ) decrease of matrix mineralization when compared to the control ones (Table 5), also the microscopic observation confirm this result (Figure 2).

**Table 4.** Effect of cadmium on scavenger enzymes and the level of lipid peroxidation in the MSCs extracted from treated rats after being differentiated to osteoblasts as compared to control.

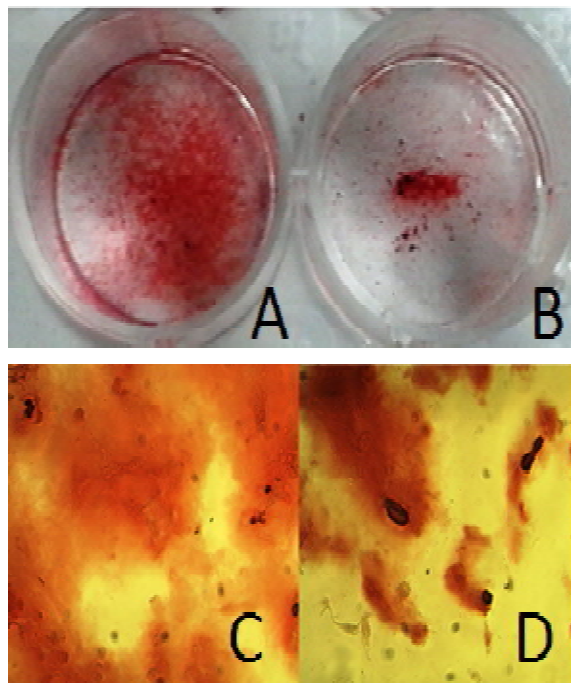
	SOD (unit min <sup>-1</sup> mg <sup>-1</sup> protein)	CAT (unit min <sup>-1</sup> mg <sup>-1</sup> protein)	POX (unit min <sup>-1</sup> mg <sup>-1</sup> protein)	MDA (μM)
Control group	33.83±1.7	173±17.5	36±0.75	0.1±0.01
Treated group	13.23±0.27**	55.7±6.3**	18.2±0.3**	0.24±0.1**

Values are mean±SD. Means with the asterisk (\*) differ significantly from control in each column, (ANOVA *t*-test, \*\**P*<0.001)

**Table 5.** Effect of cadmium on intracellular Na, K, Ca and matrix mineralization of MSCs extracted from treated and control when differentiated to osteoblasts.

	Ca mg/dL	Na μg/ml	K μg/ml	mean concentration of alizarin red (mM)
Control group	23.43±0.4	15.66±0.32	0.08±0.007	1.19±0.078
Treated group	15.53±0.5*	5.4±0.15**	0.12±0.02*	0.15±0.04**

Values are mean±SD. Means with asterisk (\*) differ significantly from control in each column, (ANOVA *T*-test, \* *P* <0.05 and \*\* *P* <0.001)

**Figure 2.** Photograph of matrix mineralization of MSCs extracted from (A) control and (B) Cd treated rats following differentiation to osteoblasts. Microscopic photograph of the matrix mineralization (C) control and (D) Cd treated.

## DISCUSSION

Cadmium exposure has been associated with bone diseases such as osteoporosis and osteomalacia [7, 8]. The mechanisms of Cd direct bone toxicity is not well clear, but researchers have shown that the Cd caused a) osteoblasts viability reduction and activation of osteoclasts [9], b) replacement of the calcium in

hydroxyapatite to reduce bone strength [10], c) interfere with collagen production in the bone [11], d) reduction of PTH stimulation [12], e) increase in urinary calcium excretion [13].

MSCs are stem cells found in the bone matrix and play important roles in the bone health via differentiate to osteoblasts, which are the main cells responsible for bone formation. Thus,

differentiation and proliferation impairment of MSCs could be considered as another cause of osteoporosis.

The treatment of MSCs with low and high concentration of cadmium chloride for the short (hours) and long (days) period caused the significant reduction of viability and proliferation in a dose and time dependent manner [2, 5]. In addition, low concentration in 21 days caused the osteogenic differentiation ability of MSCs to be impaired significantly [5].

In the present in vivo study, the treatment of rats with 40 mg/L of CdCl<sub>2</sub> for a period of 6 weeks in drinking water also caused significant reduction of viability and differentiation ability of the MSCs differentiated to osteoblasts, which confirm the previous in vitro studies [2, 5]. The effect which is left behind by cadmium toxicity would be quite persistence and can be seen in the vital properties of stem cell such as viability and differentiation even after several passages.

To explain the drastic effect of Cd, morphological examination was carried out, which showed chromatin condensation might be related to epigenetic modifications and change in regulatory factors which finally cause the partial exclusion of nucleoplasmic proteins due to increased structural restrictions and dense environments [14]. It might prevent the expression of some genes necessary for cellular function [15]. In addition to morphological changes, we analyzed oxidative stress in the osteoblasts differentiated from MSCs (extracted from Cd treated rat) based on the MDA assay. When the unsaturated fatty acid in cell membrane is attacked by radical oxygen species (ROS), breakage of carbon skeleton would happen and the aldehyde compound of MDA is formed [16]. Presence of ROS in the cell is unavoidable, but the scavenger enzymes such as CAT, POX and SOD, which involve in the cellular defense mechanism would deactivate the ROS [17].

In the present study, the activity of these enzymes has reduced significantly, which might be the main reason of unsaturated fatty acid oxidation and production of MDA. Indeed, many investigators have revealed the oxidative stress property of the Cd in vitro and in vivo due to its free radical generation [18-21], here the lower activity of the scavenger enzymes which might have been caused by epigenetic modification could be the causative reason. Oral treatment of

Cd (5 or 50 mg Cd/L) for 6 months, decreased the bone antioxidative capacity and enhanced its oxidative status resulting in oxidative stress, especially at the higher exposure where the concentration of lipid peroxidation increased and that of total thiol groups decreased significantly [22]. Although the oxidative effect of Cd might be considered as a main reason, but we also observed that the activity of the metabolic enzymes such as ALT and AST increased significantly. These enzymes are involved in the metabolism of amino acids and also can provide Krebs metabolites such as oxaloacetate and  $\alpha$ -ketoglutarate [23], therefore elevation of their activity might have taken place to compensate the energy load for cellular maintenance. The Cd treatment caused the inhibition of hexokinase and phosphofructokinase activity; these are the main enzymes of glycolytic pathway which metabolise the carbohydrate to provide ATP to the cell [24], therefore when the cell is faced with shortage of energy, amino acid might have been used to compensate the ATP deficiency.

In addition to cell viability reduction, our results showed that CdCl<sub>2</sub> exposure caused impairment of bone mineralization based on quantitative alizarin red estimation and intracellular calcium reduction. The main finding regarding the reduction of mineralization was significant reduction of ALP activity in the differentiated osteoblast cells from MSCs of treated rats. Alkaline phosphatase is a marker enzyme of osteoblasts, and Cd treatment caused inhibition of this enzyme in osteoblast like cells (MC3T3-E1) [25]. Alkaline phosphatase is the enzyme responsible for deposition of Ca<sup>2+</sup> in the matrix of the osteoblasts [26, 27]; therefore, reduction of its activity might be considered as the main reason of matrix calcium reduction. Cadmium inhibits the pathways of cellular calcium influx, by acting as a competitive ion to calcium at the voltage-dependent Ca<sup>2+</sup> channels [2]. The inhibition of transcellular calcium transport takes place at the basolaterally located Ca<sup>2+</sup> pumps in the membrane proteins which involve the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger [28], that is dependent on the correct operation of the (Na<sup>+</sup> and K<sup>+</sup>)-ATPase, and the Ca<sup>2+</sup>-ATPase which in addition to ALP activity reduction could be another reason of lower calcium deposition. In our study, the cells extracted from the treated rats have significant decrease in intracellular calcium,

which, might be due to ALP activity reduction and imbalance of the Calcium pump. On the other hand, since the Cd treatment has taken place long before via drinking water, the effect on calcium, sodium and potassium pump might be due to membrane imbalance caused by oxidative stress. In addition to above mentioned reasons for inhibition of mineralization, impaired cellular metabolism and function also may lead to abnormal remodeling patterns and functional deficit of bone mineralization.

## CONCLUSION

There were five possible mechanisms raised to explain the cadmium-induced bone effects. But due to the results of this study and other in vitro investigation we may add one more mechanism of osteoporosis/osteomalacia induced by Cd. Irrespective of many biological barriers which might ameliorate and compensate the adverse effect of Cd, the MSCs are exposed to this heavy metal through blood circulation which Cd might have brought about the lipid peroxidation, metabolic changes and calcium imbalance to affect the viability and differentiation of these cells. Lipid peroxidation affected the integrity of the cell membrane and ultimately brought about viability reduction whereas metabolic changes and calcium imbalance caused the weakening of the matrix to reduce the differentiation.

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