

# Cadmium chloride treatment of rats significantly impairs membrane integrity of mesenchymal stem cells via electrolyte imbalance and lipid peroxidation, a possible explanation of Cd related osteoporosis

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## ABSTRACT

**Objective(s):** Bone marrow mesenchymal stem cells (MSCs) play an important role in bone health. Cadmium causes osteoporosis, but the exact mechanisms of its effect on MSCs are not known.

**Materials and Methods:** Rats were treated with cadmium chloride (40 mg/l) in drinking water for six weeks, and then the biochemical and morphological studies on MSCs were carried out as a cellular backup for osteoblasts. Viability and proliferation properties of the cells were evaluated using MTT assay, trypan blue, population doubling number, and colony forming assay. Morphology of the cells and biochemical parameters including activity of metabolic (ALP, AST, and ALT) and antioxidant enzymes (SOD, CAT, and POX) as well as the MDA level (as an indication of lipid peroxidation) were investigated. In addition, intracellular calcium, potassium, and sodium content were estimated. Data was analyzed statistically and  $P < 0.05$  was taken as the level of significance.

**Results:** The results showed a significant reduction in viability and proliferation ability of extracted cells when compared to the controls. In addition, it was revealed that the cadmium treatment of rats caused a significant reduction in nuclear diameter and cytoplasm area. Also, there was significant increase in (ALT) and (AST) activity and intracellular calcium and potassium content but no change was observed with sodium content and ALP activity. The results showed [a] significant reduction in the antioxidant enzyme activity and increases in the MDA level.

**Conclusion:** Based on the present study, reduction of viability and proliferation ability of MSCs might be a causative factor of osteoporosis in industrial areas.

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## Introduction

Cadmium (Cd) belongs to group IIB of the periodic table and is found in a concentration ranging from 0.1 to 0.5  $\mu\text{g/g}$  in the earth's crust (1). Cd as an environmental pollutant is a heavy metal with multiple industrial uses such as in Cd batteries, pigments, and plastic stabilizers (2). Agricultural and industrial activities cause soil and subsequently ground and drinking water to be contaminated with Cd (3). Due to its highly soluble nature, Cd is readily taken up by plants resulting in accumulation in the food chain (4) which finally makes it the primary source of Cd exposure in humans (5). Vegetables and cereals are the main sources of dietary Cd and to a lesser extent it is found in meat products and fish (except for crustaceans and mollusks, which accumulate large amounts from contaminated aquatic environments) (6). Other sources

of Cd exposure are smoking, occupational exposure, and household dust (7). Cd is a major component of tobacco, containing 1 to 2  $\mu\text{g/g}$  dry weight, which during smoking causes deposition of Cd in lung tissues or absorbed into the systemic blood circulation of smokers (8). In industrial areas, exposure to Cd takes place due to contamination via industrial factories such as zinc (Zn) smelters, battery manufacturing, metal recovering factories, Cd refining companies, and paint and pigment production units as well as via waste incineration and fossil fuel combustion (3).

In 2004, WHO released a report recommending an acceptable daily intake level of 3  $\mu\text{g/l}$  of drinking water for chronic exposure (4) where in some industrial areas the level of Cd in the water reached 18.4 mg/l (9). In most studies, the half-life of Cd in humans is estimated to range from 15 to 20 years

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(10). Therefore humans are exposed to a large quantity of Cd which may cause serious problem to societies.

Cd exerts toxic effects on the kidney, the skeleton, cardiovascular system, and the central nervous system and is also classified as a human carcinogen (3). Cd has been demonstrated to stimulate free radical production (3, 11), reduce cellular antioxidant deference (3), and has also undesired effects on cell cycle progression (12), cell proliferation (2), differentiation (13), DNA replication, and repair (14). Cadmium toxicity has been related to Itai-Itai disease where it causes bone density reduction and osteoporosis in Japan (15). Several mechanisms have been proposed to explain the bone malformation related property of this environmental pollutant. Cd causes osteoclasts activation (16), can be replaced with calcium in the hydroxyapatite (17) to reduce its strength, may interfere with collagen production in the bone (18), reduce PTH stimulation (19), and increase urinary calcium excretion (20).

Mesenchymal stem cells (MSCs) have the ability to differentiate into many cell lines including osteoblasts (2). Osteoblasts are the cells where they ensure the calcium deposition and bone matrix formation in the bone tissue (13). Any damage to these cells would bring about matrix deposition impairment and finally bone deformation and repair malfunctioning. Since MSCs are cellular backups for osteoblast production, therefore, the proliferation and differentiation properties of MSCs are the foremost important characteristic to be considered in bone health. In an *in vitro* situation, we have shown that the micromolar concentrations (5, 15, 25, 35, and 45  $\mu\text{M}$ ) of  $\text{CdCl}_2$  in a dose and time-dependent manner would cause significant reduction of viability and proliferation ability of rat bone marrow MSCs (2). 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). In another study, in our laboratory, it was shown that the treatment of MSCs with [a]nanomolar concentration of Cd for a period of 21 days would significantly reduce the viability, proliferation, and differentiation ability of these cells with the same mechanism (13). All the above investigations have been conducted *in vitro*, however, MSCs are protected by many biological barriers which may ameliorate and compensate the adverse effect of Cd when given through drinking water. But we may mention also the MSCs are directly reachable through blood circulation *in vivo*. Therefore a question remained to be answered: if the animal is intoxicated via oral consumption, would the toxic effect of Cd and the underlying mechanisms of toxicity be the same as in the *in vitro* situation? If so then one of the reasons of Itai-Itai disease and

osteoporosis in the industrial areas would be discovered. Thus in this study, the animals were treated orally with  $\text{CdCl}_2$  for a period of 6 weeks and then several biochemical and morphological investigations were conducted to answer this question.

## Materials and Methods

### Animal treatments and MSCs isolation

Wistar rats were divided into two groups, namely treated (N=5) and control (N=5), and kept in the animal house of Arak University under standard conditions of food, water, and temperature. Based on the previous study (21,22), the treated group received 40 mg/L of cadmium chloride in drinking water for six weeks, whereas the control group was treated only with the same amount of drinking water. After the treatment period, the rats were anesthetized using diethyl ether and euthanized according to the animal laboratory protocol approved by Arak University. Then under sterile conditions, their femora and tibia were removed surgically and the bone marrow content was extracted using the flushed-out technique in 3 ml of Dulbecco Modified Eagle's Medium (DMEM) supplemented with 15% FBS and penicillin/streptomycin. The bone marrow content was centrifuged at 2500 rpm for 5 min at room temperature and pellet of the cells was homogenized with 1 ml fresh culture media and transferred to a culture flask. After 24 hr, unattached cells were washed off the flask with phosphate-buffered saline (PBS<sup>+</sup>) containing  $\text{Mg}^{++}$  and  $\text{Ca}^{++}$  and adherent fibroblast-like cells were allowed to grow for 10-14 days, with culture media replacement every three days. 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 cells to reach the passage (in course of days) and the number of cells (using hemocytometer chamber) in each passage were noted down.

### Quantification of proliferation ability

To quantify the proliferation ability of the cells after the 3rd passage:

1) The colony forming assay (CFA) and the population doubling number were performed. To carry out colony forming ability,  $1 \times 10^4$  cells extracted from treated and control rats were separately seeded in 3  $\text{cm}^2$  sterile dishes. Cells were allowed to grow for 14 days, with culture media replacement every three days. After 14 days crystal violet staining (0.5 g crystal violet in 100 ml methanol solution) was performed and the number and diameter ( $\mu\text{m}$ ) of the colonies were estimated using light microscope equipped with a graticule eyepiece.

2) To estimate the population doubling number (PDN),  $1 \times 10^4$  cells extracted from treated and control rats were separately seeded in 3 cm<sup>2</sup> sterile dishes. Cells were allowed to grow for 5 days, with culture media replacement every three days, then the cells were washed twice with PBS, harvested with trypsin-EDTA, and the number of cells was counted using a hemocytometer chamber. Using the equation  $PDN = \log(N/N_0) \times 3.31$ , population doubling of the cells was determined. In the equation,  $N_0$  stands for the initial number of the cells seeded and  $N$  is the number of the cells harvested after 5 days (23).

### Cell viability assays

The viability tests on MSCs extract from treated and control rats were carried out after the 3rd passage using the (4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT colorimetric test and the trypan blue staining assay. To perform the MTT assay, the cells were cultured in an ELISA microplate in equal density and after 24, 48, and 72 hr the plates were washed with PBS followed by addition of FBS depleted fresh media to the plates. Then 10  $\mu$ l of MTT solution (5 mg/ml in PBS) was added to each well and the plate was incubated for 4 hr in an incubator. To extract crystals of formazan, 100  $\mu$ l of DMSO was added to each well and the plate was incubated for 30 min. The extracted solutions were transferred to another well and absorbance was taken at 505 nm using an automated microplate reader (SCO diagnostic, Germany). A standard graph was plotted and the linear formula  $Y=0.0134X+0.007$  with  $R^2=0.996$  was used to estimate the number of the live cells in the MSCs extracted from Cd-treated and control groups. In the formula  $Y$  stands for absorption and  $X$  is the cell number. In trypan blue staining assay the MSCs extract from Cd-treated and control rats were also harvested after a time interval of 24, 48, and 72 hr and washed with PBS, then the cells were stained with trypan blue solution (0.4 g/100 ml in PBS) for 2 min at 37°C. The total number of the cells and number of the live (transparent) and dead (blue in color) cells were estimated using a hemocytometer chamber and the percentage of the live cells was reported.

### Morphology

The MSCs after the 3rd passage were cultured in a 24-well plate and treated with Cd for 24 hr. Using a fluorescence microscope (Olympus, IX70) equipped with a camera (DP72), the nuclear morphology of the cells was studied with Hoechst 33342 (50  $\mu$ g/ml in PBS) following 5 min of incubation in the dark. In addition, the diameter ( $\mu$ m) of the cells was measured using the Motic Image software (Micro optical group company, version 1.2). Hoechst is a fluorescent dye which penetrates the cells through

the intact plasma membrane and stains the chromatin, where the changes in nuclear morphology such as chromatin condensation and fragmentation can be investigated. The morphology of the cell cytoplasm was investigated using the acridine orange solution (50  $\mu$ g/ml in PBS) which stains the basic proteins in the cell nucleus and cytoplasm. The stained cells were washed twice with PBS, examined, and immediately photographed by an inverted fluorescence microscope (Olympus, IX70) equipped with a camera (DP72) using 20X magnification.

### Extraction of intracellular content

MSCs extracted from Cd-treated and control rats after 3rd passage were washed 3 times with phosphate buffer (PB) and homogenized in lysis buffer (20 mM Tris-HCl, pH 7.2). The intracellular content of the cells was extracted using the freeze-thaw method (24) by incubating them at -20°C overnight. Then the samples were centrifuged at 12000 rpm for 10 min at 4°C and supernatant was kept at -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 analysis was carried out three times based on equal amount of protein.

The ALP activity of the cells was determined based on the p-nitrophenyl phosphate (pNPP) hydrolysis method, using the ALP assay kit (Parsazmon, Iran). Absorbance was measured at 410 nm using a spectrophotometer (T80+ PG instrument Ltd, England).

The ALT and AST activity of the cells was determined using a commercial kit (Parsazmon, Iran), where the production of NAD<sup>+</sup> following conversion of alanine and aspartate to pyruvate and oxaloacetate, respectively is measured. Absorbance was measured at 340 nm using a spectrophotometer (T80+ PG instrument Ltd, England).

SOD activity was measured by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium. 100  $\mu$ 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 nitroblue tetrazolium, 1 mM riboflavin, 0.1 mM EDTA, and 50 mM Na<sub>2</sub>CO<sub>3</sub>) and illuminated for 15 min under fluorescent light, an identical tube which was not illuminated served as blank. 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 (25).

POX activity was assayed by the 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  $\mu\text{L}$  of enzyme extract, 100  $\mu\text{L}$   $\text{H}_2\text{O}_2$ , 100  $\mu\text{L}$  guaiacol (18 mM), and 100  $\mu\text{L}$  potassium phosphate buffer 100 mM (pH=7). POX activity of the extract was calculated using extension coefficient of  $26.6 \text{ mM}^{-1}\text{cm}^{-1}$  and expressed as unit activity  $\text{min}^{-1} \text{mg}^{-1}$  protein (26).

Catalase activity was assayed at 240 nm by measuring the initial amount of hydrogen peroxide disappearance. 100  $\mu\text{L}$  of the enzyme extract was added to the reaction mixture containing crude enzyme extract, 10 mM  $\text{H}_2\text{O}_2$  and 25 mM sodium phosphate buffer (pH 7). The decrease in the absorbance at 240 nm was recorded for 1 min by a spectrophotometer with the extension coefficient of  $39.4 \text{ mM}^{-1}\text{cm}^{-1}$ . CAT activity of the extract was expressed as unit activity  $\text{min}^{-1} \text{mg}^{-1}$  protein (27).

#### 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.  $2.5 \times 10^6$  cells were homogenized in 1 ml 0.1% trichloroacetic acid (TCA) following centrifugation at rpm 4000 for 30 min. The supernatant was treated with 2.5 ml reagent mixture containing 0.5% (w.v) thiobarbituric acid, 20% TCA, and 2.5 N HCl and boiled at  $95^\circ\text{C}$  for 20 min and cooled quickly. The absorbance was measured at 530 nm wavelength and the non-specific turbidity was corrected by subtracting  $A_{600}$  from  $A_{530}$  value. The concentration of MDA was calculated using the formula  $A = \epsilon bc$  where (A) is absorption, (b) is path length 1 cm, (c) the MDA concentration, and ( $\epsilon$ ) is the extinction coefficient ( $155 \mu\text{M cm}^{-1}$ ) (28).

#### 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 (PFP7-GenWay company, England). Since  $\text{Na}^+$  and  $\text{K}^+$  emit lights 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 (29, 30).

#### Intracellular $\text{Ca}^{2+}$ assay

The intracellular calcium content was measured using a commercial kit (Darman Kave, Iran). In an alkaline reaction mixture, 2-cresolphthalein reacts with calcium and the color which has been formed is proportional to the concentration of calcium. The resulting color was measured at 570 nm using a spectrophotometer (T80+ PG instrument Ltd, England). A standard graph was plotted using a different solution

of  $\text{CaCl}_2$ , and the amount of calcium in the cell extract was estimated using the linear formula  $Y = 0.0658X + 0.0216$  with  $R^2 = 0.9967$ . Here Y is the absorbance and X is the concentration of calcium in the cell extract.

#### Analysis of data

Data was analyzed with SPSS software, using unpaired T-test, and the level of significance was taken at  $P < 0.05$ .

## Results

#### Time required for the cells to reach the next passage

The time (days) required for the cells extracted from the treated rats to reach the next passage was significantly longer ( $P < 0.05$ ) than the time for cells extracted from the control group of rats. In addition, the cells extracted from treated rats could not proliferate and grow enough to form a monolayer of the cells at the bottom of the culture flask beyond 6<sup>th</sup> passage. Thus no time was recorded for cells in the treated group compared to the cells extracted from control rats for the 6<sup>th</sup> passage (Table 1). Therefore the passage numbers which the cells extracted from Cd-treated rats could tolerate was reduced as compared to the cells extracted from control rats.

#### Effect of Cd on cell viability

Cell viability determination using MTT colorimetric assay showed, after 24, 48, and 72 hr, the viability of the cells extracted from treated rats was significantly reduced ( $P < 0.05$ ) compared to the control group of cells. In addition, the viability of the cells reduced significantly ( $P < 0.05$ ) from each other when the time passed (Table 2). The results were confirmed by cell viability determination using the trypan blue staining method also (Table 2).

#### Quantification of proliferation ability

The cells extracted from the treated rats showed a significant reduction ( $P < 0.05$ ) of number and diameter of the colonies (Table 3) compared to the cells extracted from the control rats. PDN of the cells extracted from the treated rats also showed a significant reduction ( $P < 0.05$ ) compared to the cells extracted from the control rats (Table 4).

**Table 1.** Effect of cadmium chloride on the time (days) required for the cells extracted from the control and treated rats to reach the next passage

	Control group	Treated group
passage 1	13 $\pm$ 0.57	16 $\pm$ 0.57*
passage 2	6 $\pm$ 0.57	9 $\pm$ 0.33*
passage 3	7 $\pm$ 0.57	10 $\pm$ 0.57*
passage 4	8 $\pm$ 0.57	11 $\pm$ 0.33*
passage 5	9 $\pm$ 0.57	13 $\pm$ 0.57*
passage 6	11 $\pm$ 0.33	Nil

Values are mean $\pm$ sd. means of the samples differ significantly from each control in the rows, (t-test, \* $P < 0.05$ ). nil: cell did not reach confluence after passage 5



**Table 2.** Effect of cadmium on viability of MSCs extracted from treated and control rats, based on MTT colorimetric and trypan blue staining assay

Time (hours)	Average number of living cells ( $\times 1000$ ) based on MTT Assay		Average percentage of living cells based on trypan blue staining assay	
	Control group	Treated group	Control group	Treated group
24	19.66 $\pm$ 0.37	13.70 $\pm$ 0.18**	98.50 $\pm$ 0.5	92.66 $\pm$ 2*
48	18.83 $\pm$ 0.33	12.66 $\pm$ 0.41**	97.00 $\pm$ 1	80.60 $\pm$ 1**
72	18.81 $\pm$ 0.62	10.00 $\pm$ 0.56**	96.72 $\pm$ 1	73.54 $\pm$ 2**

values are mean $\pm$ sd. means of the samples differ significantly from each control in the rows, (t-test, \* $P$ <0.05 and \*\* $P$ <0.001)

**Table 3.** Effect of cadmium on number and diameter of the colonies (mm) of the cells extracted from treated and control rats after 14 days

	Control group	Treated group
Number of the colonies after 14 days	50.0 $\pm$ 0.30	32.0 $\pm$ 0.36*
Diameter (mm) of the Colonies after 14 days	1.5 $\pm$ 0.1	1.0 $\pm$ 0.15*

Values are mean $\pm$ sd. means of the samples differ significantly from each control in the rows, (t-test, \* $P$ <0.05)

**Table 4.** Effect of cadmium on population doubling number of the cells extracted from treated and control rats

	Control group	Treated group
3 days	0.58 $\pm$ 0.026	0.21 $\pm$ 0.03**
6 days	1.05 $\pm$ 0.13	0.67 $\pm$ 0.05*
9 days	1.3 $\pm$ 0.15	0.99 $\pm$ 0.11*

Values are mean $\pm$ sd. means of the samples differ significantly from each control in the rows, (t-test, \* $P$ <0.05 and \*\* $P$ <0.001)

**Table 5.** Effect of cadmium on nuclei diameter and cytoplasm area of the cells extracted from treated and control rats

Time (hours)	Nuclei diameter( $\mu$ m)		Cytoplasm area ( $\mu$ m <sup>2</sup> )	
	Control group	Treated group	Control group	Treated group
24	11.86 $\pm$ 0.15	8.9 $\pm$ 0.36**	2476 $\pm$ 107	1155 $\pm$ 84**
48	11.46 $\pm$ 0.3	8.6 $\pm$ 0.37**	2171 $\pm$ 118	1138 $\pm$ 39**
72	11.53 $\pm$ 0.41	8.5 $\pm$ 0.62**	2059 $\pm$ 64	1119 $\pm$ 58**

Values are mean $\pm$ sd. means of the samples differ significantly from each control in the rows, (t-test, \*\* $P$ <0.001)

### Morphology

Morphological study of the nuclei of MSCs extracted from the treated rats showed chromatin condensation (Figure 1-B,D,F) as well as a significant reduction ( $P$ <0.001) of nuclei diameter (Table 5) when compared to the diameter of the nuclei of the cells extracted from the control rats.

It was also noticed that Cd treatment of the rats caused remarkable changes in the morphology of cytoplasm (Figure 2-f) such as cell roundness, cytoplasm destruction, and in some cells complete disappearance of cytoplasm content as well as reduction of the cytoplasmic area when compared to the control (Table 5).

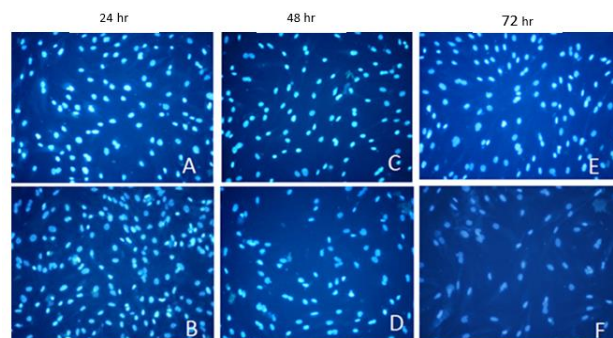
### Effect of cadmium on the activity of ALP, ALT, AST, SOD, CAT, POX and lipid peroxidation

A significant increase ( $P$ <0.05) of the activity of ALT and AST enzymes was observed in the cells extracted from the treated group of rats as compared to control ones (Table 6). Whereas the treatment of Cd showed no change in the alkaline phosphatase activity (Table 6).

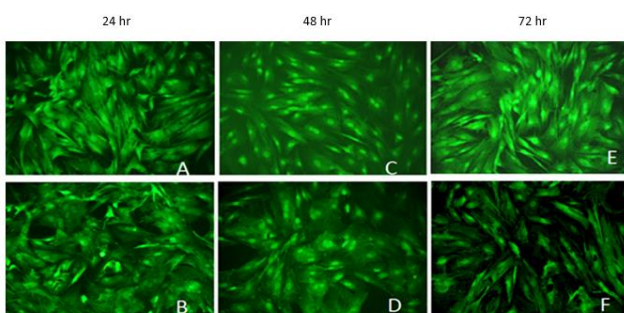
A significant decrease of SOD ( $P$ <0.05), CAT ( $P$ <0.05), POX ( $P$ <0.001) activity was observed in the cells extracted from the treated group of rats as compared with control ones. Also, it was shown that MDA level increased significantly ( $P$ <0.001) in the cells extracted from the treated group of rats as compared with control ones (Table 6).

### Effect of cadmium on intracellular sodium, potassium, and calcium

Cd treatment caused a significant increase in potassium ( $P$ <0.001) and calcium ( $P$ <0.05) content of the cells, whereas a significant decrease of intracellular sodium ( $P$ <0.001) was observed in the cells extracted from the treated group of rats as compared with control ones (Table 7).



**Figure 1.** Morphology of the cell nuclei A , C, E) cells extracted from control rats stained with Hoechst, nuclei appeared normal after 24, 48, and 72 hr B, D, F) MSCs extracted from Cd-treated rats stained with Hoechst, nuclei appear smaller as compared with control. (200X magnification)



**Figure 2.** Morphology of the cell cytoplasm A, C, E) cells extracted from control rats stained with acridine orange, cell cytoplasm are normal after 24, 48, and 72 hr. B, D, F) MSCs extracted from Cd-treated rats stained with acridine orange, cell cytoplasm shrinkage and roundness was observed after 24, 48, and 72 hr. (200X magnification)

**Table 6.** Effect of cadmium on oxidative stress enzymes (sod, cat, and pox), transaminases (ALT and AST), alkaline phosphatase (ALP), and lipid peroxidation (MDA)

	SOD (unit activity min-1 mg-1 protein)	CAT (unit activity min-1 mg-1 protein)	POX (unit activity min-1 mg-1 protein)	MDA μM	alp (IU/l)	alt (IU/l)	AST (IU/l)
Control	3.47±0.32	3.5±0.37	17.2±2.50	0.29±0.010	61.44±2.68	29.65±1.9	50.12±8.6
treated	2.41±0.19*	2.7±0.10*	7.4±0.85**	0.42±0.015**	67.6±2.69	45.11±5.1*	74.76±4.1*

Values are mean±sd. means of the samples differ significantly from each control in the column, (t-test, \*P<0.05 and \*\*P<0.001)

**Table 7.** Effect of cadmium on intracellular sodium, potassium, and calcium

	Ca mg/dl	Na μg/ml	K μg/ml
Control group	2.2±0.36	6.9±0.1	0.11±0.01
Treated group	3.5±0.32*	3.1±0.16**	0.27±0.03**

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

### Discussion

In the previous *in vitro* studies, it was shown that the treatment of the MSCs with high and low doses of Cd causes significant reduction of viability and proliferation ability in short and long periods of treatment (2, 13). The present study showed that *in vivo* treatment of rats with 40 mg/l of Cd for a period of 6 weeks also had the same impact on viability and proliferation property of the cells extracted from treated rats. There might be much reason to explain the cell viability reduction. 1) Either the reduction of viability was due to the loss of cell membrane integrity which was shown by trypan blue staining and imbalance of electrolytes (sodium and potassium). 2) It might be due to mitochondrial damage which was shown by MTT assay. 3) On the other hand nuclear and chromatin damage also might be another reason for cell viability reduction, as it was shown by the reduction of nuclear diameter. 4) In addition to nuclear diameter, cells extracted from Cd-treated rats showed significant reduction of cytoplasmic area, which along with nuclear condensation have been shown to be a sign of programmed cell death (apoptosis) (31-33). Also, elevation of intracellular calcium has been correlated with induction of apoptosis (34). The induction of apoptosis by Cd have been revealed in previous studies (35) therefore up- or down-regulation of the genes related to program cell death might be the main culprit of cell viability reduction beside above-mentioned reasons.

Cellular damage and viability reduction caused by ROS is due to inhibition of antioxidant defense systems such as SOD, CAT, and POX enzymes when the rate of reactive species generation exceeds the rate of their decomposition (36). It has been reported that Cd may induce oxidative damage in a variety of tissues by enhancing peroxidation of membrane lipids due to inhibition of the antioxidant

enzymes (3, 37). In the present research the MDA levels and SOD, CAT, and POX activities were used as markers of oxidative stress. The data showed that the cells extracted from the treated rats had significantly higher lipid peroxidation (LPO) and lower activities of SOD, CAT, and POX. 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 brings about the lipid peroxidation. Therefore LPO affects the integrity of the cell membrane and ultimately brings about viability reduction of the cells. These results are supported by others (37, 38), but what makes it more interesting is the effect that it still remains after several passages of the cell proliferation. Therefore it might be concluded that Cd affects the activity of scavenger enzymes via permanent down-regulation of their genes, although this has to be proven in future research works.

In the present study, viability reduction of the cells extracted from Cd-treated rats was not the only factor to be affected by Cd caused toxicity. The reduction of proliferation potential was observed when a significant decrease of PDN and colony forming ability had been estimated. This was also a mirror image of the increase in time requirement for the cells to reach confluency. In the present data, we showed that the time needed to reach the next passage increased significantly for the cells extracted from the treated rats compared to the cells extracted from the control ones. Impairment of the cell membrane, chromatin condensation, nuclear breakage, and cytoplasmic shrinkage due to inhibition of cytoskeletal rearrangement and assembly (31) as well as reduction of antioxidant enzymes activity are the culprits of viability reduction and ultimately proliferation impairment.

In general, researchers reported a significant increase in hepatic markers ALT and AST as well as bilirubin in rats treated with cadmium chloride. This elevation in marker enzymes indicates damage to the liver due to liver cell necrosis (39, 40). In the present study, the activity of the ALT and AST showed a significant elevation in the MSCs extracted from Cd-treated rats. Indeed, it is well known that the cells during proliferation require a high level of energy. It seems that the Cd toxicity caused the activity of metabolic enzymes to be elevated not in the direction to provide more energy but to divert the metabolic state to amino acid sources which might be futile to the cells.

ALP activity did not show any change while intracellular calcium content was elevated significantly, many investigators have shown that Cd is able to induce a perturbation in calcium homeostasis (41-43). Cd inhibits the pathways of cellular calcium influx and acts as a competitive ion to calcium at the voltage-dependent  $Ca^{2+}$  channels (2). The inhibition of transcellular calcium transport takes place at the basolaterally located  $Ca^{2+}$  pumps in the membrane proteins involved in the  $Na^{+}/Ca^{2+}$  exchanger (44), which is dependent on the correct operation of ( $Na^{+}$  and  $K^{+}$ )-ATPase, and the  $Ca^{2+}$ -ATPase. As it was mentioned before, in our study, the present data showed that the cells extracted from the treated rats have a significant increase in intracellular calcium and decrease in intracellular sodium in rats treated with cadmium chloride.

### Conclusion

We can say that there are no differences in mechanisms of Cd toxicity *in vivo* or *in vitro*. However in the *in vivo* situation, one would expect versatile and complex systems such as detoxification roles played by kidney, liver, spleen, and other organs to ameliorate or reduce the effect of such chemicals. As it was quoted before, several mechanisms have been proposed to explain the effect of Cd on bone such as; 1) Cd causes osteoclasts activation, 2) replaces calcium in the hydroxyapatite, 3) interferes with collagen production, 4) reduces parathormone stimulation of calcium absorption, and 5) increases urinary calcium excretion. The present study showed that the treatment of rats with Cd for a period of 6 weeks would bring about an accountable damage to proliferation and viability of the MSCs which might be considered as a prominent and significant mechanism for Cd related osteoporosis in industrial areas. Therefore we strongly recommend WHO and local health organizations to prevent industries from using Cd in formulation of industrial products, which cause the bone marrow MSCs viability and proliferation to decrease.

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### Conflict of interest

There is no conflict of interest.

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