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*Original Article*

# Catechin hydrate prevents cadmium-induced collagen downregulation in mesenchymal stem cells *via* SMAD/RUNX2

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## *Abstract*

**Background and purpose:** Cadmium (Cd) inhibited osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs), while catechin hydrate (CH) improved the osteo-differentiation. Meanwhile, this investigation aimed to study the compensating effect of CH on Cd inhibition.
**Experimental approach:** BMSCs from Wistar rats were treated with Cd and CH, and then the viability and population doubling number were investigated. Based on them, 1.5 µM and 0.25 µM of Cd and CH were chosen, respectively. Following osteogenic induction, matrix production, expression of osteogenic-related genes, and expression of collagen-1 (COL1-A1) protein were analyzed. Moreover, the ability of CH to prevent Cd-induced oxidative stress, as well as the metabolic activity of the treated cells, was evaluated.
**Findings/Results:** The selected concentration of CH significantly ameliorated Cd-induced inhibition of BMSCs osteo-differentiation through preventing oxidative stress and ameliorating gene expression. Indeed, CH in the co-treatment group significantly improved the expression of *Smad1*, *Bmp2*, *Runx2*, *Oc*, *Alp*, *Col-1* genes as well as the production of COL1-A1 protein compared to the control group. Moreover, the co-treatment of CH and Cd significantly decreased MDA levels and boosted TAC levels and CAT, SOD activity compared to the control group. CH compensated for the Cd-induced metabolic changes as LDH, ALT, and AST activities significantly improved to the control level.
**Conclusion and implications:** CH prevented oxidative stress and counteracted the inhibitory effect of Cd on the osteogenic differentiation of BMSCs. Therefore, CH may be suggested to prevent Cd toxicity on bone.

**Keywords:** Cadmium; Catechin hydrate; Mesenchymal stem cell; Osteoblasts; Osteogenic differentiation.

## INTRODUCTION

Cadmium (Cd), with a half-life of 10-30 years, is an environmental pollutant (1,2), which, due to human activities, is released to the environment and contaminates soil and water (3). Due to the contamination of vegetables, fruits, and aquatic animals, the entire food chain would be polluted (4) and as a result, Cd enters animals as well as the human body. Cd contamination and toxicity cause malformation of bone microstructure. The exposure to high concentrations of Cd due to the consumption of polluted water was the causative factor of Itai-Itai disease in Japan (5), causing a reduction of bone mineral density (BMD), which brought about osteopenia and osteoporosis (6). Due to the activity of osteoblasts and osteoclasts, bone is in constant reabsorption and biosynthesis in physiologic and pathologic situations (7). Osteoclasts resorb bone and osteoblasts, which are formed by bone marrow mesenchymal stem cells (BMSCs), subsequently synthesize the organic and inorganic matrix to help bone reformation (8).

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Several mechanisms are proposed to explain bone intoxication with respect to Cd exposure, including 1. parathormone inactivation (9); 2. activation of osteoclasts (10); 3. inactivation of the kidney enzyme that synthesizes 1,25-dihydroxycholcalciferol (11); 4. inhibition of collagen production in bone matrix (11); and 5. inhibition of the proliferation and differentiation ability of BMSCs (12-14).

Endogenous or exogenous antioxidants neutralize reactive oxygen species (ROS), which, as a result, prevent biomolecule oxidation in the cell. Excessive utilization of antioxidants can endanger the cell, especially when oxidation is occurring (15). The cell's antioxidant system, comprising non-enzymatic agents such as vitamin E, vitamin C, and glutathione and enzymatic agents like catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase, prevents oxidative stress (16). The antioxidant system prevents the oxidation of membrane lipids, DNA, and proteins by inhibiting the formation or neutralizing free radicals. Cd toxicity induces an imbalance between free radicals and antioxidants (17), causing complications, such as osteoporosis, due to the reduction of proliferation and differentiation ability of BMSCs.

To improve antioxidant capacity, the plant antioxidants are the first choice to be consumed; many fruits, including apples, plums, grapes, berries, and vegetables such as carrots, red cabbage, and cauliflower, contain plant secondary metabolites or natural products (18). Flavonoids, as naturally occurring polyphenols, are a class of phytonutrients found in fruits, vegetables, grains, barks, roots, stems, and flowers of plants (19). One of the members of this family is catechin hydrate (CH), which compensates for the damage caused by the oxidative effects of hydroxyl ($HO^{\cdot}$), superoxide ($O2^{\cdot}$), peroxyl ($ROO^{\cdot}$) radicals, and hydrogen peroxide (H2O2) as non-radical ROS (20).

BMSCs are vital for the regeneration and repair of bone, since they are a cellular backup for osteoblasts, the matrix-producing cells in bone marrow (12,13). Therefore, damage caused by ROS impairs the BMSCs' differentiation ability, which, as a result, the synthesis of bone matrix would be impaired due to the activity of osteoblasts. Since Cd induces oxidative stress, it is crucial to protect BMSCs when Cd toxicity is a matter of concern. No related documents have been found with respect to the preventive effect of CH on Cd-induced impairment of osteogenic differentiation of BMSCs. Thus, this study aimed to investigate the protective effect of CH on the impairment of osteogenic differentiation of BMSCs caused by Cd toxicity. In the present study, we looked forward to investigating the effect of CH on metabolism, oxidative damage, and production of bone matrix, as well as expression of the genes and proteins (collagen 1 and alkaline phosphatase (ALP) activity) involved in osteogenic differentiation of BMSCs in the presence of Cd.

## MATERIALS AND METHODS

### *Extraction and culture of rat bone marrow cells*

Six to eight-week-old Wistar rats, purchased from Pasteur Institute (Tehran, Iran), were housed in the animal facilities of the University of Arak (Arak, Iran) with controlled conditions and free access to water and food. This study was conducted following the guidelines outlined in the Guide for Care and Use of Laboratory Animals of the Arak University and approved by the Committee on the Ethics of Animal Experiments at Arak Medical University (Ethical No. IR.ARAKMU.REC.1401.025). All surgical procedures were carried out under sodium pentobarbital anesthesia, with utmost efforts made to reduce suffering.

Following a one-week adaptation, the animals were euthanized using sodium pentobarbital (90 mg/kg, intraperitoneally) followed by cervical dislocation, and then the bones, including tibias and femurs, were surgically removed. In sterile conditions, the bones were cleaned of tissues and then transferred to the laminar flow hood. Using a pair of surgical scissors, the bone ends were cut, and the content was taken out in a sterile tube using a 2 mL syringe containing culture media of Dulbecco's modified Eagle medium (DMEM, Gibco, Germany) consisting of 15% fetal bovine serum (Gibco, Germany) and 1%

penicillin/streptomycin (Gibco, Germany). After centrifuging the tube at 250 *g* for 5 min, the supernatant was removed. Then, the cells were suspended in culture media and placed in a T25 flask. The flasks were kept in an incubator at 37 ℃ under 5% $CO_2$ saturation and humid conditions. The media were replaced every 3 days with fresh media till the cells formed a monolayer and covered the bottom of the flask. Next, the cells were removed from the bottom of the flask using trypsin-EDTA, and then washed with phosphate-buffered saline (PBS, 20 mM, pH 7.2). Following centrifugation, the cells were suspended in culture media and placed in a T25 culture flask. The sub-culture was performed 2 times more, and at the 3rd passage the purity of the cells was confirmed based on differentiation to osteoblasts and adipocytes as well as the negative expression of CD 34, CD 45, and CD 14 and the positive expression of CD 73, CD 90, and CD 105 antigens (Fig. S1) using flow-cytometer (Partec PAS, Germany) and used for further analysis (14).

### *Cell viability*

A viability test was performed using trypan blue in non-osteogenic conditions to select the effective concentration for further analysis as follows. After the 3rd passage, BMSC cells ($5 \times 10^4$) were treated with Cd (Merck Company, Germany) at the concentrations of 0.5, 1, 1.5, 2, 4, and 5 µM and CH (Sigma-Aldrich, USA) at the concentrations of 0.06, 0.12, 0.25, 0.5, 1, 20, and 30 µM. After a 20-day period of incubation, the cells were detached from the bottom of the flasks using trypsin-EDTA. The cells were washed with PBS and homogenized in fresh culture media. Then, 50 µL of the hemogen was mixed with an equal volume of trypan blue (40 mg/mL in phosphate buffer, Sigma-Aldrich, USA), and after 2 min of incubation, the cell counting was carried out using a Neubauer hemocytometer. Trypan blue dye entered cells when the membrane was damaged, and cells appeared blue in color, whereas live cells were transparent (14). The percentage of live cells was calculated using the following equation:

$$Cell\ viability\ (\%) = \frac{Total\ number\ of\ cells - total\ number\ of\ death\ cells}{Total\ number\ of\ cells} \times 100$$

In osteogenic conditions, the cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, USA) assay. Briefly, BMSCs were seeded and treated with Cd at a concentration of 1.5 µM ($IC_{50}$) and Ca at a concentration of 0.25 µM (the highest proliferation ability) individually and together in a 12-well sterile plate. Following 20 days of treatment, 100 µL of MTT (5 mg in one mL of PBS) and 900 µL of DMEM (fetal bovine serum (FBS)-free) were added into each well and kept at 37 ℃ for 4 h. After removing the culture media, the formazan crystal was dissolved in dimethyl sulfoxide (at 25 ℃ for 30 min), and then each extracted solution (100 µL) was transferred to another 96-well plate, and the absorption was measured at 505 nm using a microplate reader (Medical SCO GmbH, Germany) (12). A standard graph was plotted in the same way using viable cells, and the formula $Y = 0.0071X + 0.0029$ with $R^2 = 9984$ was used to find out the total number of viable cells for each group. In the formula, Y is the absorption, and X stands for the sum total of viable cells.

### *Proliferation assay*

Using the population doubling number (PDN) formula, *i.e.*, $PDN = \log N/N0 + 3.32$, the proliferation ability of the BMSCs was calculated in the presence of Cd, CH, and the combination of Cd and CH in non-osteogenic conditions. In the formula, N0 is the number of cells cultured at the beginning, and N stands for the final number of harvested cells. The cell count was carried out using a hemocytometer chamber after 20 days of treatment (12).

### *Osteogenic differentiation and characterization*

Differentiation of BMSCs to osteoblasts was induced with DMEM-FBS media supplemented with sodium-glycerophosphate (1 mM, Sigma-Aldrich, USA), ascorbic acid (50 µg/mL, Sigma-Aldrich, USA), and dexamethasone (10 nM, Sigma-Aldrich, USA). The BMSCs were cultured in 6-well plates, and separately treated with osteogenic media having Cd (1.5 µM), CH (0.25 µM), and Cd (1.5 µM) + CH (0.25 µM). Then, the plates were kept in humid conditions at 37 ℃, which

contained 5% $CO_2$. The culture media was changed after 3 days, and the media replacement was carried out for 20 days (21).

Mineralization was checked and estimated using alizarin red as follows: the plates were washed using PBS and fixed with 10% formaldehyde for 15 min. Then, 1 mL of alizarin red reagent (ARR, 40 mM, pH 4.2) was added, and the incubation of the plates was carried out for 40 min. After washing the extra dye with PBS, the photograph was taken with a camera (DP-70, Japan) using a microscope (Olympus, Japan).

Alizarin red estimation was performed by adding 800 µL of 10% acetic acid into the wells, then the cells were removed by scraping the bottom of the plate after 30 min. The scraped cells were placed in a tube with a determined weight and vortexed vigorously, then boiled in a water bath at 85 °C for 10 min, while a layer of 500 µL of mineral oil (Sigma-Aldrich, USA) was placed on top of the solution. The tubes were placed on ice for 5 min, followed by centrifugation at 10000 *g* for 15 min. After that, 500 µL of solution (without mineral oil) was neutralized with 200 µL of 10% ammonium hydroxide. Then, absorption of the solution was measured with a microplate reader (SCO diagnostic, Germany) at 405 nm. To plot a standard graph, the determined concentrations of ARR were used. To dilute ARR, a mixture of acetic acid and ammonium hydroxide (5:2) was prepared as a stock solution with a concentration of 2 mM. Subsequently, 5 different concentrations were provided. After plotting the standard graph, the linear formula $Y = 0.3544X + 0.0246$ with $R^2 = 0.9988$ was used to determine the concentration of samples. In the above-mentioned equation, the Y axis is absorbance, and the X axis is concentration (µM) of the ARR (21).

***Estimation of calcium from matrix extract***

Before the extraction, the plate was washed using PBS followed by deionized water, and the cells were removed by scraping them, then collected in a pre-weighed tube to calculate the exact weight of the cells. The same weight of the collected scrap was used to extract the calcium content of the matrix using 50 µL of 0.5 N HCl for 24 h at 4 °C. After 5-min incubation, the concentration of calcium content was estimated using a commercial kit (Pars Azmoon, Iran) with a spectrometer (Model T80+, PG instrument, England) at 630 nm. Making various concentrations of $CaCl_2$, a standard graph was plotted, and the concentration of the samples was determined using the linear formula $Y = 0.145X + 0.256$ with $R^2 = 0.9982$, where the values on the Y axis are absorption, and the X axis is concentration of calcium (mg/dL).

***Extraction of cell lysate***

Following differentiation of BMSCs to osteoblasts, the flask was washed with Tris-HCl buffer (TB, 20 mM, pH = 7.2), and then the cells were collected with a cell scraper. The removed cells were suspended in 500 µL of TB. After breaking the cellular membranes, the whole content was centrifuged at 12000 *g* for 10 min. The protein content of the supernatant was estimated with the Lowry method. Using bovine serum albumin, a standard graph was plotted, and the protein concentration was calculated using the linear formula $Y = 0.0016X + 0.0018$ with $R^2 = 0.9968$. In the formula, Y and X are absorption and protein concentration (µg), respectively. Analysis of biochemical factors was performed, considering each sample had an equal amount of protein (12).

***Determination of enzyme activity***

The activities of aspartate transaminase (AST), lactate dehydrogenase (LDH), and alanine transaminase (ALT) were measured, based on the same amount of the protein, using commercial kits (Pars Azmoon, Iran) according to the instructions of the company. Standard graphs were plotted, and linear formulas $Y = 0.0016X + 0.0003$ with $R^2 = 0.9996$, $Y = 0.0013X + 0.0119$ with $R^2 = 0.9854$, and $Y = 0.0015X + 0.0006$ with $R^2 = 0.9986$ were used to estimate the activities of AST, LDH, and ALT, respectively. In the formulas, Y and X are the absorption and the activity of the enzyme (IU/L), respectively.

***Estimation of ALP activity***

Activity of ALP was determined with a commercial kit (Pars Azmoon, Iran) based on

the equal concentration of protein. The absorption was measured at 410 nm using a T80+ spectrophotometer (PG Instrument Ltd., England). Based on the standard graph, the following formula, Y = 0.0015X + 0.0004, with $R^2$ = 0.9993, was applied to calculate ALP activity. In the formula, Y and X are the absorption and activity of the enzyme (IU/L), respectively.

***Estimation of SOD activity***

SOD activity was evaluated using nitro-blue tetrazolium (NBT, N6876, Sigma-Aldrich, USA). Briefly, 50 µL of extracted samples containing the same amount of protein was added to 1 mL of reaction mixture containing 6.1 mg of NBT, 1.9 mg of methionine, 7.9 mg of riboflavin, and 3.3 mg of EDTA dissolved in 10 mL of potassium phosphate. Following incubation for 10 min in the light box, absorption of the samples was measured at 560 nm. In the separate tubes, preparation of blank and control was carried out in the same manner, but no sample extract was added. The blank tube was kept in the dark for 10 min, then the solution was used to adjust the spectrometer (Model T80+, PG instrument, England) to zero. Enzyme activity was estimated as 50% of color development inhibition per minute and reported based on the mg of protein (22).

***Estimation of CAT activity***

To estimate the activity of CAT, a reaction mixture including 300 µL of H2O2 and 700 µL of 25 mM potassium phosphate buffer, pH 7.0, was made, and the absorption of the solution was adjusted to 0.4 before the measurement. Activity of CAT was determined by adding 50 µL of samples containing an equal amount of protein to the mixture, and reduction of absorption was measured at 240 nm after 2 min using the spectrometer (Model T80+, PG instrument, England). The CAT activity was determined after 1 min using 39.4 $mM^{-1}cm^{-1}$ as the extinction coefficient (22).

***Estimation of lipid peroxidation***

As an indicator of lipid peroxidation, the malondialdehyde (MDA) level was estimated. One hundred µL of samples containing an equal amount of protein was added to 1 mL of reaction mixture containing 0.5% thiobarbituric acid and 20% trichloroacetic acid in hydrochloric acid (HCl), and the tube was boiled in a boiling water bath for 30 min. Then, the tube was kept in an ice bath for 15 min and centrifuged at 10000 *g* for 15 min. Using a spectrometer (Model T80+, PG instrument, England), the absorption of the samples was measured at 523 nm, then at 600 nm. The values were first subtracted, and using 155 $mM^{-1}Cm^{-1}$ as the extinction coefficient, the level of MDA was determined and reported as µM/mL (13,23).

***Estimation of total antioxidant content***

Based on equal concentration of protein in the samples, the total antioxidant content (TAC) was measured by adding 150 µL of sample to 1700 µL of reaction mixture, including 300 mM sodium acetate buffer (pH 6.3) and 10 mM 2,4,6-Tri(2-pyridyl)-s-triazine (Sigma-Aldrich, USA), which were dissolved in 40 mM HCl and 20 mM iron chloride. After adding 850 µL of double-distilled water, the mixture was incubated in the dark for 10 min. Then, the absorbance was measured at 593 nm with a spectrometer (Model T80+, PG instrument, England). Plotting a standard graph with different concentrations of iron sulfate ($FeSO_4.7H_2O$, Merck, Germany), the TAC level in the samples was estimated by the formula Y = 0.0072X + 0.0011 with $R^2$ = 0.9965, where Y is absorption, and X is the concentration (21).

***Western blotting analysis***

Using a Western blot, the expression of collagen-1 (COL-1A1) was determined in the cell lysate extract. Cells were scraped off using a cell scraper and lysed using freeze-thawing. Then, the concentration of protein was measured with the help of the Bradford method. Protein extracts were electrophoresed on 12% SDS-PAGE, and then the separated polypeptides were transferred onto the polyvinylidene difluoride membrane (Biocompare Company, USA). Blocking of the membrane was performed using 5% bovine serum albumin in Tris-HCl buffer (pH 7.2), and detection was carried out using COL1-A1 antibody (Catalog # PA5-86862, Invitrogen

Company, USA) and anti β-actin antibody (sc-53483, Santa Cruz Biotechnology, USA) at 4 °C overnight, where β-actin was used as a control. After washing, the incubation was continued with horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. Detection was carried out with the help of an enhanced chemiluminescence reagent (Mindray Company, China), followed by X-ray. Intensity of the color of the detected COL1-A1 was estimated with the help of ImageJ Software and presented as arbitrary units (21).

### *Detection of gene expression*

Following total RNA extraction using an extraction kit (Super RNA extraction kit, YT9080, Yeasen Biotechnology, China), the reverse transcription PCR (RT-PCR) was carried out to synthesize cDNA with the help of BioFACT kit (BR631-096, BioFACT Co., Ltd, South Korea). Amplification of ALP (*Alp*), osteocalcin (*Oc*), runt-related transcription factor 2 (*Runx2*), *Smad1,* bone morphogenetic protein 2 (*Bmp2*), collagen-1A1 (*Col1a1*), *and* glyceraldehyde dehydrogenase (*Gapdh)* were performed using a PCR instrument (Eppendorf Mastercycler gradient, Eppendorf Co., Hamburg, Germany) in triplicate by their specific primers (Table 1). The PCR was carried out at 95 °C for 5 min as the initial stage, followed by 35 cycles consisting of 95 °C for 1 min, annealing temperature of specific primer for 1 min, and 72 °C for 1 min. At the final stage, the elongation temperature was set as 72 °C for 7 min. PCR product was checked using a 1.5% agarose gel, then the results were visualized and photographed by gel documentation (Gel Flash, SYNGENE Bio Imaging, England) and further analyzed by Gel Quant software (version 1.8.2) (24).

### *Statistical analysis*

Data were expressed as mean ± SD, and analyzed by one-way ANOVA followed by Tukey post hoc test using SPSS (version 20). Graph Pad Prism was used to plot the graphs. *P*-values < 0.05 were considered significant levels.

### *Supplementary materials*

The supplementary materials for this article can be found online at: https://drive.google.com/file/d/1eIoITE8BMb-eDu1wN8YLyY8O5F_Mb7Q-/view?usp=drive_link

**Table 1.** Sequences of primer sets used for gene expression analysis.

| Gene name | Sequences | Size (bp) |
|---|---|---|
| ***Alp*** | Forward: CATGTTTCCTGGGAGATGGTA<br>Reverse: GTGTTGTACGTCTTGGAGAGA | 144 |
| ***Oc*** | Forward: AACGGTGGTGCCATAGATGC<br>Reverse: AGGAGGCTCTCTCTGCTCAC | 294 |
| ***Runx2*** | Forward: CCGCACGACAACCGCACCAT<br>Reverse: CGCTCCGGCCCACAAATCTC | 289 |
| ***Smad1*** | Forward: CCGCCTGCTTACCTGCCTCCTGAA<br>Reverse: GAACGCTTCGCCCACACGGTTGT | 246 |
| ***Bmp2*** | Forward: CGTCAAGCCAAACACAAACAGC<br>Reverse: GAGCCACAATCCAGTCATTCCAC | 106 |
| ***Col-1a1*** | Forward: ACGTCCTGGTGAAGTTGGTC<br>Reverse: CAGGGAAGCCTCTTTCTCCT | 362 |
| ***Gapdh*** | Forward: TCGTCTCATAGACAAGATGG<br>Reverse: GTAGTTGAGGTCAATGAAGGG | 136 |

## RESULTS

### *Cell viability*

Cd treatment caused cell viability reduction in a significant and concentration-dependent manner from 1µM, whereas CH increased the cell viability at 0.25 and 0.5 µM, significantly. The maximum increase in cell viability was observed at 0.25 µM of CH. It was also revealed that the viability of the cells reduced significantly at 30 µM CH (Fig. 1A). In addition to viability, PDN analysis revealed that the Cd treatment significantly reduced the proliferation ability of the cells from 1 µM, with the highest reduction at 5 µM. In treatment with CH, a significant increase in PDN was observed from 0.625 to 1 µM concentration compared with control, and the maximum in PDN was seen at 0.25 µM CH. The results showed no significant change in 20 µM of CH, whereas a highly significant reduction in PDN was observed at 30 µM of CH (Fig. 1B). Since 50% of cell viability was observed at a concentration of 1.5 µM of Cd, this concentration was considered $IC_{50}$ to carry out further analysis. In addition, based on the viability test and PDN analysis, the concentration of 0.25 µM of CH, which showed maximum proliferation, was also chosen for further analysis.

To study the viability of the cells in osteogenic conditions, the MTT assay was used. Data analysis showed that treatment of the cells with a combination of Cd and CH could compensate for the toxic effect of Cd. Therefore, no significant change in cell viability was observed in the Cd + CH group compared with the control group (Fig. 1C).

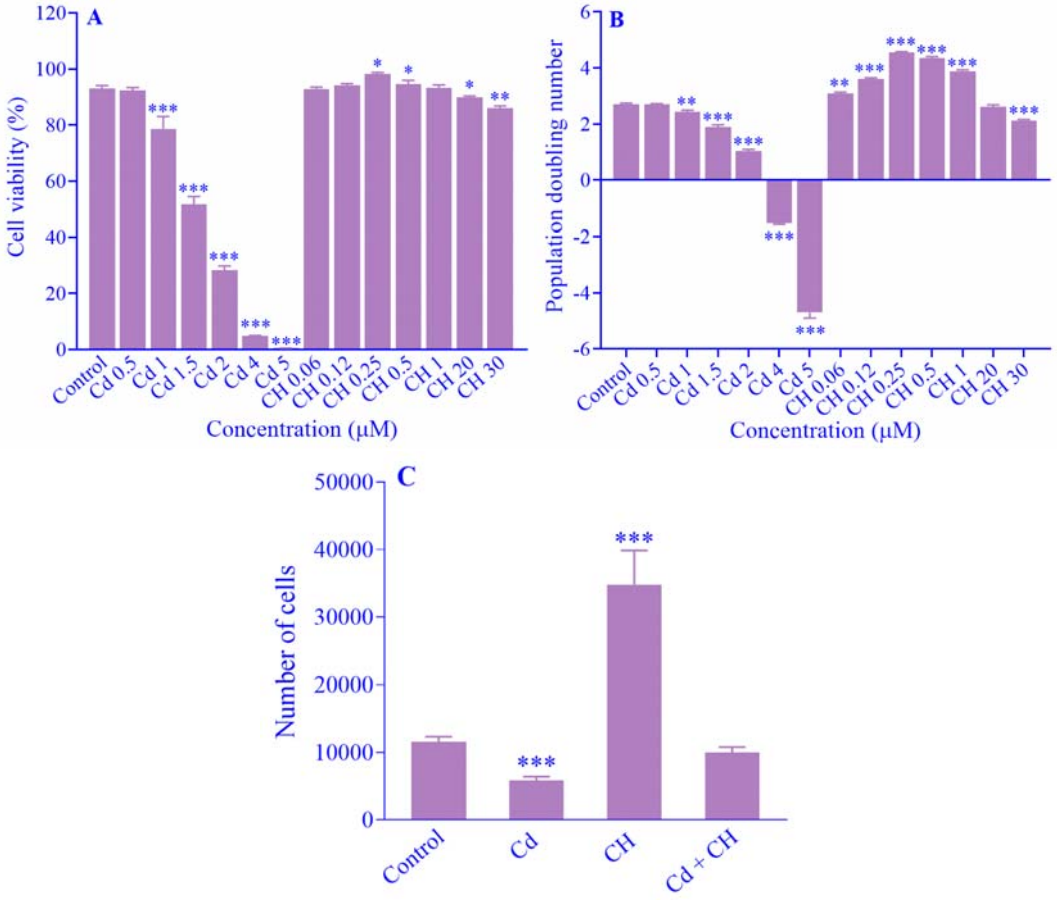


**Fig. 1.** Effect of various concentrations of Cd and CH individually on (A) cell viability and (B) population doubling number of BMSCs after 20 days of treatment in non-osteogenic condition using trypan blue assay; (C) effect of Cd, CH, and Cd + CH on cell viability of BMSCs after 20 days of treatment in osteogenic media using MTT assay. Data were shown as mean ± SD, n = 3. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ indicate significant differences compared to control. Cd, Cadmium; CH, catechin hydrate; BMSCs, bone marrow mesenchymal stem cells.

### *Differentiation of BMSCs to osteoblasts*

Based on alizarin red staining, it was observed that Cd reduced the osteogenic differentiation of BMSCs in comparison with the control (Fig. 2A and B). Whereas CH increased the differentiation ability of BMSCs to osteoblasts (Fig. 2C), and the treatment of cells with the combination of Cd and CH compensated for the inhibiting effect of Cd (Fig. 2D). In addition, data analysis revealed the CH compensating effect in the co-treated group. Therefore, alizarin red quantitative analysis showed that CH treatment significantly improved the production of matrix, while the Cd treatment significantly inhibited the production of matrix compared with the control (Fig. 3A).

Based on calcium content (Fig. 3B) and ALP activity (Fig. 3C) results, simultaneous treatment of the cells with Cd and CH had a drastic effect of Cd, where there was no variation between the Cd + CH and control cells. Treatment of the cells only with CH increased ALP activity as well as the level of calcium extracted from the matrix, significantly when compared with the control (Fig. 3B and C).

### *Oxidative stress*

BMSCs treatment with Cd caused a highly significant increase in MDA level (Fig. 4A), while a significant reduction of the concentration of TAC and the activity of antioxidant enzymes (CAT and SOD) was observed (Fig. 4B-D). Treatment of the cells with CH caused a significant reduction of MDA and elevated the TAC concentration as well as activities of CAT and SOD, significantly. The group treated simultaneously with Cd and CH revealed that the levels of MDA and TAC, as well as activities of antioxidant enzymes, were compensated and non-significant in comparison with the control group (Fig. 4).

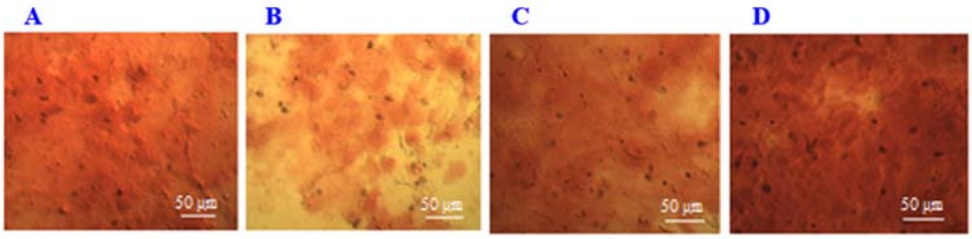


**Fig. 2**. Alizarin red staining of bone marrow mesenchymal stem cells after 20 days of treatment under osteogenic conditions. (A) Control, (B) treated with Cd, (C) treated with CH, (D) treated with Cd + CH. Cd, Cadmium; CH, catechin hydrate.

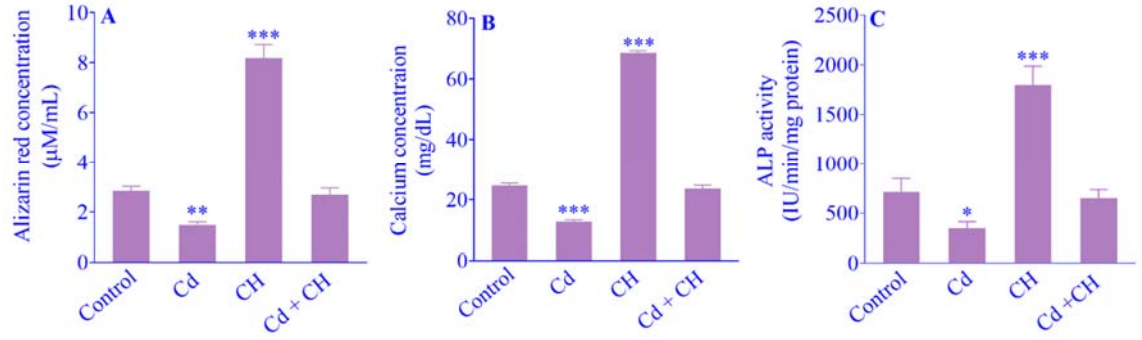


**Fig. 3.** Effect of Cd and CH on differentiation ability of BMSCs based on (A) alizarin red concentration, (B) calcium concentration, and (C) activity of ALP after 20 days of treatment in osteogenic conditions. Data were expressed as mean ± SD, n = 3. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ indicate significant differences compared to control. Cd, Cadmium; CH, catechin hydrate; ALP, alkaline phosphatase; BMSCs, bone marrow mesenchymal stem cells.

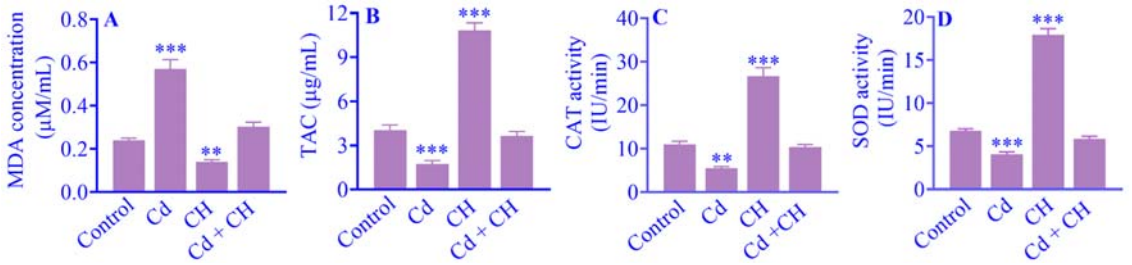


**Fig. 4**. Induction of oxidative stress induced by Cd and the oxidative prevention effect of CH on BMSCs based on (A) concentration of MDA, (B) TAC, (C) activity of CAT, and (D) activity of SOD after 20 days of treatment in osteogenic conditions. Data were shown as mean ± SD, n = 3. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate significant differences compared to control. Cd, Cadmium; CH, catechin hydrate; BMSCs, bone marrow mesenchymal stem cells; MDA, malondialdehyde; TAC, total antioxidant content; CAT, catalase; SOD, superoxide dismutase.

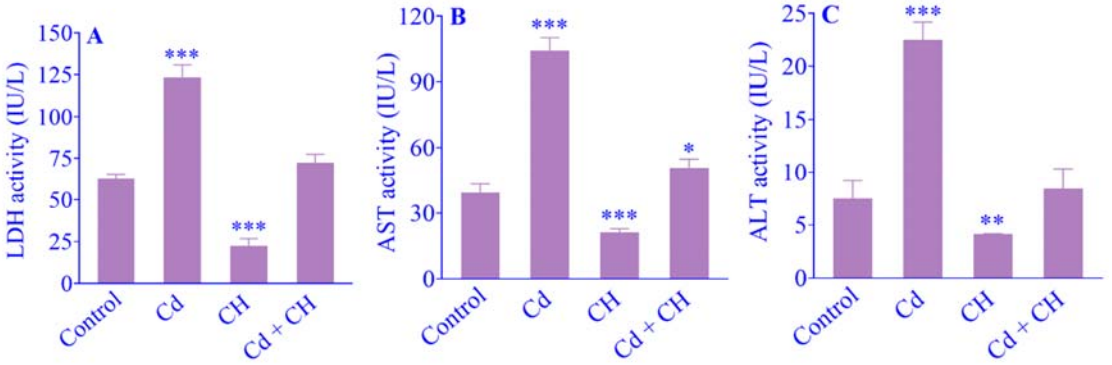


**Fig. 5**. Effect of Cd and CH on metabolic activity of BMSCs based on (A) activity of LDH, (B) activity of AST and (C) activity of ALT after 20 days of treatment in osteogenic conditions. Data were shown as mean ± SD, n = 3. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ indicate significant differences compared to control. Cd, Cadmium; CH, catechin hydrate; BMSCs, bone marrow mesenchymal stem cells; LDH, lactate dehydrogenase; AST, aspartate transaminase; ALT, alanine transaminase.

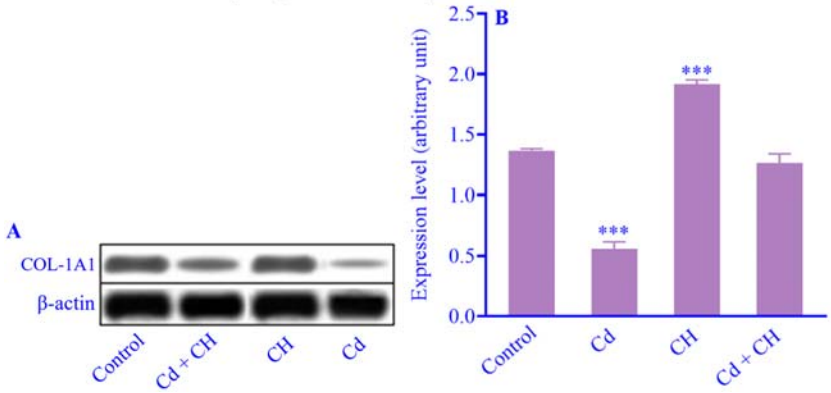


**Fig. 6.** Effect of Cd and CH on expression of COL1-A1 protein of BMSCs. (A) Western blot of COL1-A1 and β-actin (as internal control), (B) the expression level of COL1-A1 after 20 days of treatment in osteogenic conditions. Data were shown as mean ± SD, n = 3. $^{***}P < 0.001$ indicates significant differences compared to control. Cd, Cadmium; CH, catechin hydrate; BMSCs, bone marrow mesenchymal stem cells.

### *Metabolic activity*

Cd significantly increased the activities of metabolic enzymes, including LDH, AST, and ALT, compared to the control, but CH significantly reduced the activity of the enzymes. Although treatment of the cells with Cd and CH could compensate for the drastic effect of Cd, it could not restore the activity of AST when compared with the control group (Fig. 5).

### *Analysis of protein expression*

Western blotting was used to study the COL1-A1 expression in the cell extract, where it was shown that

Cd caused a highly significant reduction of this protein. Whereas CH increased the expression of COL1-A1 significantly in the differentiated BMSCs. Also, it was observed that the co-treatment with Cd and CH compensated for the expression of COL1-Al in cell extract in comparison with the control group (Fig. 6 and Fig. S2).

### *Expression of osteogenic-related genes*

Expression of the investigated osteogenic-related genes including *Smad1*, *Bmp2*, *Runx2*, *Oc*, *Alp*, *Col-1* was grossly affected by Cd treatment, at which a highly significant reduction in the expression of the genes was observed (Fig. 7). In the co-treated group of the cells, CH could compensate for the reducing effect of Cd and restore the expression of *Oc*, *Runx2*, *Bmp2*, *Col-1*, and *Alp* genes when compared with control. Treatment of the cells with CH only significantly increased the expression of all the investigated genes (Fig. 7).

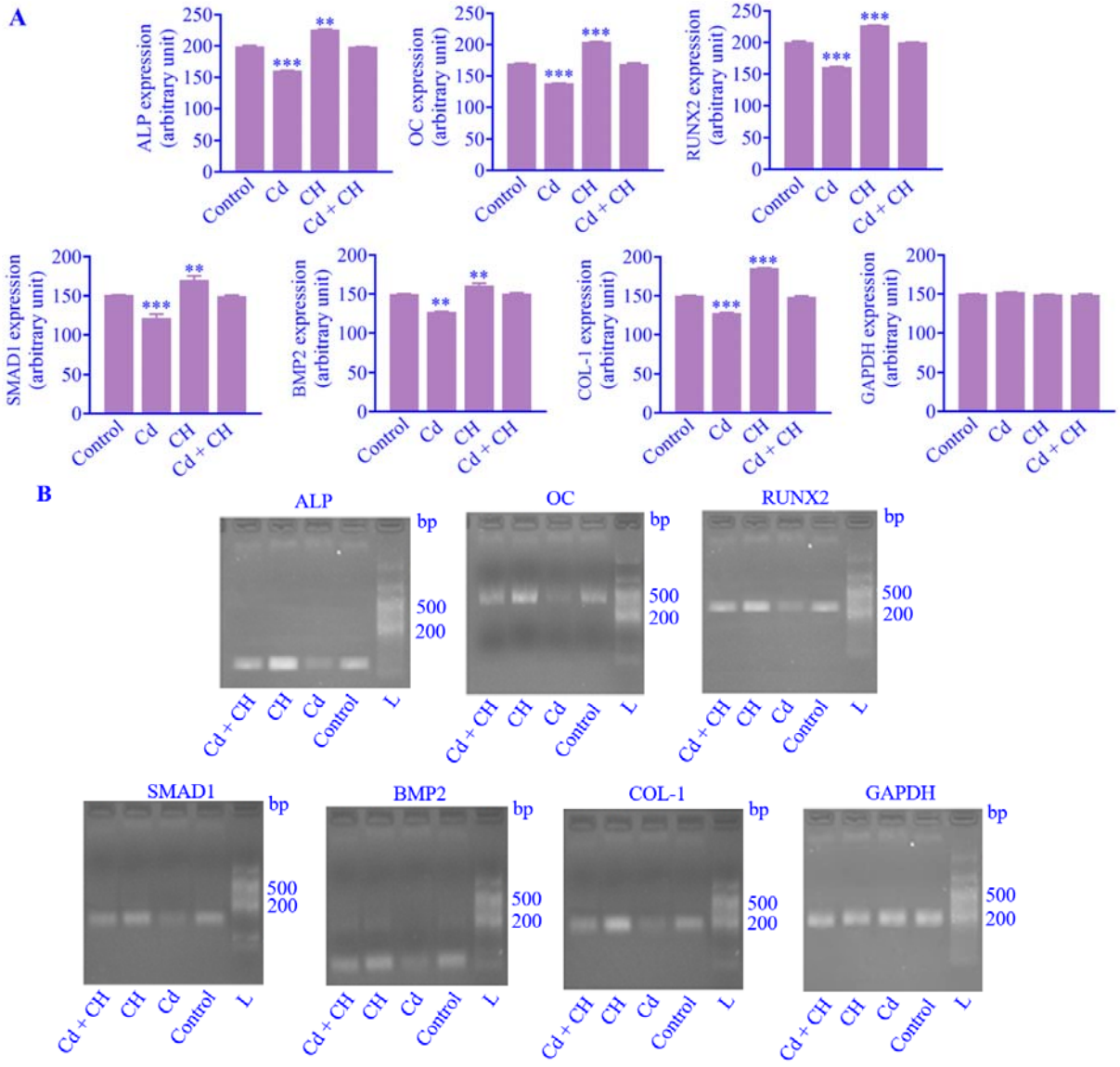


**Fig. 7**. Effect of Cd and CH on expression of the genes. (A) Expression of GAPDH, OC, RUNX2, SMAD1, BMP2, Col-1, and ALP in the BMSCs after 20 days of treatment in osteogenic conditions. Data were shown as mean ± SD, n = 3. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate significant differences compared to control. (B) The expression of osteogenic-related gene of BMSCs after 20 days of treatment under osteogenic conditions on the agarose gel. Cd, Cadmium; CH, catechin hydrate; BMSCs, bone marrow mesenchymal stem cells; L, ladder.

## DISCUSSION

This study revealed that Cd suppressed the differentiation of BMSCs to osteoblasts, while CH elevated this property of BMSCs. Following treatment of BMSCs with $CdCl_2$, the expression of the analyzed osteogenic-related genes significantly reduced. In this study, the activity of ALP, quantitative alizarin red analysis, measurement of calcium concentration of matrix, and detection of COL1-A1 expression confirmed the suppressing effect of Cd. Meanwhile, CH significantly increased the expression of genes related to differentiation of BMSCs to osteoblasts, the activity of ALP, and production of COL1-A1, which is an important enzyme and protein related to matrix production. In addition, the compensatory effect of catechin hydrate was revealed by estimating the matrix production ability based on quantitative alizarin red analysis and estimation of matrix calcium content, expression of osteogenic-related genes, activity of ALP, and detection of COL-1A1 expression.

Several investigations have shown that Cd reduces bone mineral density in several different populations (6, 13, 25-27). Some studies have reported that Cd treatment results in changes in bone microstructure, such as elevation of trabecular separation and reduction of bone vascularization (25,26). Investigation on humans also revealed that the low Cd exposure has drastically affected the cortical area and the thickness, as well as the trabecular bone volume fraction (6). Cd directly and indirectly affects the bone micro-structure, as it has been reported that the bone formation is reduced due to osteoblasts malfunctioning or osteoclasts overactivation by Cd toxicity (13,27,28). The molecular mechanism of bone damage due to Cd toxicity is not well understood, and it is also true regarding the effect of this heavy metal on the differentiation of BMSCs to osteoblasts. The previous studies showed that Cd disturbs the metabolism of BMSCs and damages the cellular membranes (12,14). In the current study, it was shown that Cd induced peroxidation of membrane lipids, where increased concentration of MDA, as well as reduced activity of antioxidant enzymes and concentration of TAC in BMSCs-derived osteoblasts were observed. Furthermore, Cd toxicity led to a metabolic imbalance in BMSCs during their differentiation into osteoblasts. This was evidenced by the excessive production of lactic acid, which resulted from the activation of LDH to enhance glucose consumption in the glycolytic pathway (29).

Cd toxicity often has been related to depletion of antioxidant capacity of the cells (15,30) and production of superoxide ion, hydrogen peroxide, and hydroxyl radicals (16) as ROS. It has been suggested to use plant antioxidants to prevent lipid peroxidation due to overproduction of ROS (18,20,31). CH is a potent plant antioxidant and present in cocoa, tea, broad bean, prune, strawberries, apricots, peaches, and barley (32), which are easily available to the public. Since Cd inhibited the osteogenic ability of BMSCs, the present study used CH to prevent the Cd-induced toxic effects due to the CH oxidative prevention effect as well as the osteogenic improvement effect (33). CH treatment caused improvement of the antioxidant capacity of BMSCs following differentiation to osteoblasts and also elevated the activity of their antioxidant enzymes.

In addition to peroxidation of lipids, ROS is able to attack the functional group of proteins, therefore inactivating the enzymes (34) and also damaging the nucleic acids (DNA and RNA) (35). Thus, using exogenous antioxidants to prevent the oxidation of macromolecules is a prime step. As it was observed significant low level of MDA generation was observed in the presence of CH, therefore, CH was used in the co-treated group, which compensated for the Cd-caused oxidative stress and was able to ameliorate its effect in comparison with the control.

Following oxidative damage caused by Cd, treatment of the cells with CH caused an improvement in the osteogenic ability of BMSCs. This study found that Cd inhibited osteogenic differentiation of BMSCs, where CH treatment compensated for this inhibitory effect of Cd on the gene and protein levels. *Runx2*, as an important gene involved in the differentiation of BMACs to osteoblasts (36), regulates the *Bmp2* and *Smad* family (37). In addition, *Runx2* causes the expression of *Oc* and *Alp* genes, which are involved in the formation of bone matrix by newly formed

osteoblasts (38). The present study showed that following osteo-induction, the expression of *Bmp2*, *Col-1*, *Smad1*, *Runx2*, *Alp*, and *Oc* genes was up-regulated following Ca treatment. It was also found that the Cd caused osteogenesis-related genes to be down-regulated, whereas treatment with CH could compensate for the inhibiting effect of Cd not only on gene regulation, but also in restoring the COL-1 protein level and ALP activity.

In bone remodeling, the behavior and mutual relationship between osteoblast and osteoclast, as bone-forming and bone-resorbing cells, respectively, is crucial. Following BMSCs differentiation to osteoblasts, the cells begin to produce bone matrix, which confirms the production of strong and healthy bone. Thus, if anything goes wrong during osteogenic differentiation of BMSCs, it might cause the bone to have low density (39), which might be a reason for osteoporosis. Osteogenic-related gene activation gives rise to the production of organic and inorganic components of bone matrix (40). Cd caused the down-regulation of gene expression, and as a result, the production of organic (COL1) and inorganic (calcium concentration) components required for matrix production was inhibited. CH, as a natural plant product, could improve the gene expression and restore the level of COL1 and the activity of ALP, two important factors involved in the biosynthesis of bone matrix.

The previous studies confirmed the toxic effect of Cd on osteogenic differentiation of BMSCs (12,41). In addition, some investigations also reported the osteogenic-inducing property of CH (33,42). The present study revealed that CH was able to compensate and prevent the toxic effect of Cd on the differentiation ability of BMSCs to osteoblasts. In addition, the previous studies confirmed that Cd causes the metabolism of BMSCs to be changed from aerobic to anaerobic (12-14). Meanwhile, this study proved that CH is able to ameliorate the metabolism of BMSCs following differentiation to osteoblasts; therefore, the energy state of these cells is restored, and since during the differentiation process, BMSCs need more energy, the presence of CH can be helpful when Cd toxicity is a matter of concern. It appears that CH, as a strong antioxidant, might have prevented enzymatic inhibition by deactivating oxidative stress and nullifying ROS. Enzymes play a vital role in cell growth, differentiation, and maturation within biological systems (43,44). The improvement observed in osteogenic differentiation of BMSCs in this study is likely attributed to the nullification of oxidative stress. Although no study has specifically investigated the impact of CH on the osteogenic inhibition effect of Cd, He's study demonstrated that geniposide, a plant antioxidant, could protect against osteoblastic injury induced by Cd. The study showed that geniposide prevented oxidative stress induced by Cd, thereby reducing apoptosis and increasing the expression of enzymes such as heme oxygenase 1 and NAD(P)H quinone dehydrogenase 1 (45).

## CONCLUSION

The current study determined that Cd led to oxidative stress, resulting in the suppression of osteogenic-related gene expression. Furthermore, the findings demonstrated that Cd hindered the production of COL1-A1 and suppressed ALP activity, both of which were crucial for generating the organic and inorganic components of the bone matrix. CH, a potent antioxidant, could counteract the oxidative stress induced by Cd, thus restoring gene expression and preventing Cd's toxic impact on the differentiation of BMSCs into osteoblasts by increasing ALP activity to produce more hydroxyapatite and organic components (COL1-A1) of the bone matrix.

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***Conflict of interest statement***

The authors declared no conflicts of interest in this study.

***Author's contributions***

M.H. Abnosi conceived the study and was in charge of overall direction and planning, designed the experiments and wrote the manuscript; J. Sargolzaei carried out the

statistical analysis and gene analysis; M. Eshragi carried out the laboratory experiments. All authors read and approved the final version of the manuscript.

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