

# Gallic Acid Ameliorates Cadmium Effect on Osteogenesis by Activation of Alkaline Phosphatase and Collagen Synthesis

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## Abstract

**Objective:** We previously reported that cadmium (Cd) inhibits osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs). In addition, gallic acid (GA) improves BMSC differentiation. Here, we aim to study the ability of GA to prevent osteogenic inhibition induced by Cd.

**Materials and Methods:** In this experimental study, BMSCs were extracted and purified from Wistar rats and their viability was determined in the presence of Cd and GA. The results indicated that 1.5  $\mu\text{M}$  Cd and 0.25  $\mu\text{M}$  of GA were appropriate for further investigation. After 20 days in osteogenic medium, matrix production was analysed by alizarin red, calcium content, and alkaline phosphatase (ALP) activity. Osteogenic-related genes and collagen 1A1 (COL1A1) protein expressions were investigated. The preventive effect of GA on oxidative stress and metabolic change induced by Cd was estimated.

**Results:** GA counteracted the inhibitory effect of Cd on matrix production and significantly ( $P=0.0001$ ) improved the osteogenic differentiation ability of BMSCs. Also, GA prevented the toxic effect of Cd on osteogenic-related gene expressions and nullified the reducing effect of Cd on COL1A1 and ALP activity. A significant reduction ( $P=0.0001$ ) in malondialdehyde and lactic acid concentration showed that GA counteracted both oxidative stress and metabolic changes caused by Cd.

**Conclusion:** GA prevented the toxic effect of Cd, an environmental pollutant and a factor in osteoporosis.

**Keywords:** Cadmium, Gallic Acid, Mesenchymal Stem Cell, Osteoblasts

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## Introduction

Cadmium (Cd) is a heavy metal that has a half-life of more than 20 years in biological systems (1, 2). It is an environmental pollutant released mostly via human activities that contaminate soil and water. Cd enters the food chain (3) and causes contamination after consumption of vegetables and aquatic animals. Various adverse effects, including bone complications, have been reported following consumption of Cd contaminated food and water. Exposure to high concentrations of Cd decreases bone mineral density (BMD) and this may result in osteomalacia and osteoporosis (4). Bones undergo dynamic self-renovation attributed to activity governed by bone cells, osteoblasts, and osteoclasts (5). Osteoclasts resorb bone, whereas osteoblasts, which are differentiated from bone marrow mesenchymal stem cells (BMSCs), synthesise the bone matrix to assist with bone repair and renovation (6). Proposed mechanisms that explain bone-related Cd toxicity include: inactivation of the parathyroid hormone (7); significant increase in osteoclast activity (8); reduction in 1,25-dihydroxy vitamin D biosynthesis via inactivation of 1-hydroxylase (9); inhibition of collagen

production in the bone (9); and reduction in proliferation and differentiation ability of MSCs (10-12).

Several investigations have shown that Cd toxicity can generate reactive oxygen species (ROS) both *in vivo* and *in vitro* (4, 13-15). Cd induction of oxidative stress can deplete the cell's total antioxidant status and inactivate antioxidant enzymes, which lead to an accumulation of hydroxyl ( $\text{HO}\cdot$ ) radicals, superoxide ions ( $\text{O}_2\cdot^-$ ), and hydrogen peroxide (13, 16) that cause damage to membrane phospholipids, functional proteins, and DNA (17).

Antioxidants are endogenous or exogenous molecules that neutralise ROS and prevent its effects. Cells lack the ability to fight radicals when there is excessive use of endogenous antioxidants or a decrease in exogenous antioxidant intake, and this results in oxidative stress (18). The cell antioxidant system includes vitamin E, vitamin C and glutathione, and enzymes such as catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (19). This antioxidant system inhibits the formation or neutralisation of free radicals; therefore, it protects

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the cells from oxidative stress and ultimately prevents membrane lipid peroxidation and DNA/protein damage. Cd toxicity disrupts the balance between free radicals and antioxidants (20); this imbalance may cause osteoporosis by reducing the proliferation and differentiation properties of BMSCs.

Plant antioxidants are the first line of consideration to increase a cell's antioxidant capacity. Natural plant products are present in a variety of foods such as grapes, apples, plums, berries, and other vegetables. Phenolic acids are a family of phytonutrients found in tomatoes, apples, strawberries, peppers, bananas, and many other plants (21). A member of this family is trihydroxybenzoic acid, which is also called gallic acid (GA). GA efficiently prevents oxidative damage caused by hydroxyl (HO $\cdot$ ), superoxide (O $_2\cdot^-$ ), peroxy (ROO $\cdot$ ) and the non-radical hydrogen peroxide (H $_2$ O $_2$ ) free radicals (22).

BMSCs play an important role in bone repair and regeneration, and impairment of these cells has a tremendous impact on bone matrix production. Therefore, protection of BMSCs is very important after Cd toxicity. To the best of our knowledge, there is no published report that discusses the preventive effect of GA on Cd inhibition of osteogenic differentiation of BMSCs. The aim of the present study is to examine the toxic impact of Cd on osteogenic differentiation of BMSCs and assess the protective effect of GA on metabolic activity, oxidative stress, and matrix production as well as expressions of the genes involved in osteogenic differentiation after Cd exposure.

## Materials and Methods

### Extraction and rat bone marrow cell culture

This research was approved by the Ethics Committee of Arak Medical University, Arak, Iran (IR.ARAKMU.REC.1401.026). Wistar rats (6-8 weeks old) were obtained from Pasteur Institute (Tehran, Iran) and kept in an animal house at Arak University (Arak, Iran) under standard conditions for food and temperature. After a one-week acclimation period, the rats were euthanised using chloroform inhalation and their tibias and femurs were surgically removed. Under sterile conditions, the connective tissues were removed from the bones under sterile conditions and transferred to a clean room. The ends of the bones were removed and the bone marrow was extracted by a 2 ml syringe that contained Dulbecco's Modified Eagle Medium (DMEM) media [DMEM, 15% foetal bovine serum (FBS), and 1% penicillin/streptomycin (all from Gibco, Germany)]. The extracted bone marrow was centrifuged at 250 g for 5 minutes, then the cells were suspended in fresh medium and placed in T25 culture flasks. The flasks were incubated in an incubator at 37°C, 5% CO $_2$ , and 95% humidity. The medium was replaced every three days by fresh medium until the bottom of the flask was covered by a cell monolayer. At this time the cells were detached using trypsin-EDTA (Gibco, Germany),

centrifuged, and washed with phosphate-buffered saline (PBS, 20 mM, pH=7.2), then placed in new T25 culture flasks. This sub-culture was carried out two more times; at the third passage, flow cytometer [Germany, PARTEC (PAS)] analysis confirmed the purity of these cells. These passage-3 cells were kept for further analysis.

### Cell viability

Cell viability was assessed by the trypan blue method under non-osteogenic conditions in order to select an effective concentration for further analysis. Passage-3 cells were treated with different concentrations (0.5, 1, 1.5, 2, 4, and 5  $\mu$ M) of Cd (Merck Company, Germany) and GA (0.06, 0.12, 0.25, 0.5, 1, 20, and 30  $\mu$ M; Sigma-Aldrich, USA) in the presence of a control group. After 20 days, the cells were removed from the flask by trypsin-EDTA and homogenized in fresh medium. Then, 50  $\mu$ l of the homogenized cell was mixed with 50  $\mu$ l (40 mg/ml in PBS) of trypan blue (Sigma-Aldrich, USA); after two minutes of incubation, the cells were counted using a haemocytometer chamber. Trypan blue passes through the cell membrane of nonviable cells (blue). The percentage of live cells was recorded.

We sought to study the effect of the selected concentrations, both individually and in combination, under osteogenic conditions. Cell viability was determined by the 3-(4, 5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich, USA) assay. Briefly, BMSCs were cultured in a 24-well sterile plate, then treated individually with 1.5  $\mu$ M (IC $_{50}$ ) of Cd or 0.25  $\mu$ M of GA, or their combination (1.5  $\mu$ M Cd+0.25  $\mu$ M GA). After 20 days the cells were washed with PBS and then incubated for 4 hours with 100  $\mu$ L of MTT (5 mg/ml in PBS) and 1 ml of culture media (without FBS). The culture media was removed and we added DMSO to dissolve the formazan crystals (30 minutes at 25°C). Then, 100  $\mu$ l of the extracted solution was transferred to an ELISA plate and absorption was read at 505 nm by an ELISA plate reader (Medical SCO GmbH, Germany). We used the linear formula  $Y=0.0032X+0.0073$  with  $R^2=9983$  to calculate the number of viable cells. In this formula, Y represents absorption and X is the number of viable cells.

### Proliferation assay

We used the population doubling number (PDN) formula to determine the proliferation ability of BMSCs in the presence of Cd, GA, and Cd+GA under non-osteogenic conditions.

$$PDN = \log N/N_0 + 3.32,$$

Where: N $_0$  is the initial number of cells cultured and N is the final number of cells harvested.

After 20 days of treatment, the total number of cells were counted using a haemocytometer chamber.

## Osteogenic induction and detection

Osteogenic differentiation was induced in complete DMEM medium that contained 1 mM sodium glycerophosphate, 50 µg/mL L-ascorbic acid, and 10 nM dexamethasone (all from Sigma-Aldrich Company). The six-well plates were incubated at 37° C and 5% CO<sub>2</sub> and the media was replaced every three days. After 20 days, mineralisation was detected and quantified by alizarin red analysis. The plates were washed with PBS and fixed in 10% formaldehyde for 15 minutes. Then, we added 1 mL alizarin red solution (ARS, 40 mM, pH=4.2) and the plates were incubated for 40 minutes. The excess dye was washed with PBS, and images were acquired with an inverted microscope (Olympus, Japan) equipped with a camera (DP-70, Japan). In order to perform quantitative alizarin red staining, 800 µL of 10% acetic acid was added to each well of the plate; after 30 minutes, the cells were removed with a cell scraper and collected in a 1.5 mL tube. The content of each tube was vortexed for 30 seconds, overlaid with 500 µL of mineral oil (Sigma-Aldrich), and then heated in a water bath (85°C) for 10 minutes. The micro-tubes were transferred to an ice bath for five minutes and centrifuged at 10 000 g for 15 minutes. We removed the supernatant (500 µL) and mixed it with 200 µL of 10% ammonium hydroxide to neutralise the solution. Absorption was read at 405 nm by a microplate reader (SCO Thec., Germany). Next, a stock solution of ARS was diluted with mixture of 10% acetic acid and 10% ammonium hydroxide (5:2) to give a final concentration of 2 mM, after which a series of five different dilutions were made. A standard graph of the known concentrations of ARS was plotted and we used the linear formula  $Y=0.0093X+0.3607$  with  $R^2=0.9985$ , where Y is absorbance and X is concentration (µM) of the ARS, to determine the absorption of the unknown.

## Determination of calcium concentration

The plates were washed with PBS and once with double-distilled water (ddH<sub>2</sub>O). The cells were scraped off the plates and collected in a preweighed 1.5 mL microcentrifuge tube to determine their weight by subtraction. An equal weight of the cells was used to extract the calcium with 50 µL of 0.5 N hydrochloric acid (HCl) for 24 hours at 4°C. Total calcium concentration was measured using a commercial kit (Pars Azmoon, Iran) and a spectrophotometer (T80 spectrophotometer, PG Instruments, Ltd., UK) at 630 nm. A standard graph was plotted using various concentrations of CaCl<sub>2</sub> and the concentration of unknown was determined using the linear formula  $Y=0.0145X+0.0256$  with  $R^2=0.9982$ , where Y is absorption and X is the concentration of calcium (mg/dL).

## Extraction of cell content for further analysis

After osteogenic differentiation, the T25 flasks were washed with tris-HCl buffer (TB; 20 mM tris-HCl, pH=7.2) and the cells were removed from the flask with a cell scraper. The cells were added to a microcentrifuge tube that contained 500 µl of TB. After freezing and

thawing, the cell membranes were broken and centrifuged at 12 000g for 10 minutes. The protein content was collected and estimated using the Lowry method. We used bovine serum albumin to plot a standard graph and the linear formula  $Y=0.00004X+0.0015$  with  $R^2=0.9973$  was used to calculate the protein concentration of each sample. In this formula, Y stands for absorbance and X is the concentration of protein (µg). Further biochemical analysis was performed based on the equal amount of protein in each sample.

## Determination of alanine transaminase, aspartate transaminase, and lactate dehydrogenase activities

We used a commercial kit (Pars Azmoon, Iran) to estimate the activities of aspartate transaminase (AST), lactate dehydrogenase (LDH), and alanine transaminase (ALT) according to the manufacturer's protocol. A standard graph was plotted and the 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 calculate the AST, LDH, and ALT activities, respectively, for each sample. In the formula, Y stands for absorbance and X for the enzyme activity (IU/L).

## Determination of alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was estimated according to a commercial kit (Pars Azmoon, Iran) by using the same amount of protein. The measurement was taken at 410 nm with a spectrophotometer (T80 spectrophotometer, PG Instruments, Ltd., UK). A standard graph was plotted and the linear formula,  $Y=0.0015X+0.0004$  with  $R^2=0.9993$ , was used to calculate the enzyme activity of each sample. In the formula Y stands for absorbance and X for the enzyme activity (IU/L).

## Determination of superoxide dismutase activity

Nitro blue tetrazolium (NBT; Sigma-Aldrich, N6876 was used to determine SOD activity. Briefly, 50 µL of sample was added to 1 ml of the reaction mixture (6.1 mg NBT, 1.9 mg methionine, 7.9 mg riboflavin, and 3.3 mg EDTA dissolved in 10 mL potassium phosphate); after 10 minutes of incubation in a light box, absorbance of each sample was read at 560 nm. The blank and control tubes were also prepared in the same manner without the sample. The blank was kept in the dark and after 10 minutes, the spectrophotometer (T80 spectrophotometer, PG Instruments, Ltd., UK) was adjusted to zero using the same tube. Activity of the enzyme was calculated as unit per minute for mg of protein required to cause 50% inhibition.

## Determination of catalase activity

We estimated CAT activity with a reaction mixture that consisted of 300 µL of H<sub>2</sub>O<sub>2</sub> and 200 µL of 25 mM potassium phosphate buffer (pH=7.0) with an absorption of the solution adjusted to 0.4 prior to the measurement. CAT activity was determined by adding the 50 µl of samples

to the mixture. Absorption was read after two minutes at 240 nm by a spectrophotometer (T80 spectrophotometer, PG Instruments, Ltd., UK). CAT activity was calculated for one minute using  $39.4 \text{ mM}^{-1}\text{cm}^{-1}$  as the extinction coefficient.

### Determination of lipid peroxidation

The level of malondialdehyde (MDA), as an indicator, was determined to estimate lipid peroxidation. 50  $\mu\text{L}$  of sample was added to 1 ml of reaction mixture (0.5% thiobarbituric acid and 20% trichloroacetic acid in HCl) and kept in a boiling water bath for 30 minutes. Then, the mixture was placed on ice for 15 minutes and centrifuged at 10 000 g for 15 minutes. Absorption of the samples was determined first at 523 nm then at 600 nm with a spectrophotometer (T80 spectrophotometer, PG Instruments, Ltd., UK). The absorption values were subtracted and we used the extinction coefficient ( $155 \text{ mM}^{-1} \text{ cm}^{-1}$ ) to determine the concentration of MDA ( $\mu\text{M}/\text{mL}$ ).

### Measurement of total antioxidant content

Total antioxidant content (TAC) was estimated based on an equal amount of protein. First, 150  $\mu\text{L}$  of the sample was mixed with 1700  $\mu\text{L}$  of the reaction solution [300 mM sodium acetate buffer (pH=6.3), 10 mM 2,4,6-Tris(2-pyridyl)-s-triazine; (Sigma-Aldrich, USA) dissolved in 40 mM HCl and 20 mM iron chloride] and 850  $\mu\text{L}$  distilled water. The mixture was incubated in the dark for 10 minutes, and absorbance at 593 nm was measured using a spectrophotometer (T80 spectrophotometer, PG Instruments, Ltd., UK). A standard graph was plotted using different concentrations of iron sulphate ( $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ; Merck, Germany). TAC of the sample was calculated using the linear formula,  $Y=0.0007X+0.0103$  with  $R^2=0.997$ , where Y stands for absorption and X for concentration.

### Protein extraction and Western blot analysis

Western blot was used to evaluate the level of collagen-1A1 (COL-1A1) in the protein extract. The cells were removed after trypsinization and lysed by freezing and thawing, after which the protein concentrations of the cell extracts were determined by the Bradford method. Protein cell extracts were isolated on 12% SDS-PAGE gels and electro-transferred onto a polyvinylidene difluoride membrane. The membrane was blocked with 5% non-fat powdered milk and probed with antibodies for COL-1A1 (Invitrogen, Cat# PA5-86862) and  $\beta$ -actin as the internal control (Santa Cruz Biotechnology, sc-53483) overnight at 4°C. The procedure was followed by incubation with horseradish peroxidase-conjugated secondary antibody for one hour. The blots were developed using an enhanced chemiluminescence reagent and detected by X-ray. The intensity of the colour on the membrane was quantified using ImageJ software and presented as arbitrary units.

### Gene expression analysis

Reverse transcription polymerase chain reaction (RT-

PCR) was conducted after total RNA extraction (Super RNA Extraction kit YT9080) and cDNA synthesis using a BioFACT (BR631-096) commercial kit. *ALP*, osteocalcin (*OC*), runt-related transcription factor 2 (*RUNX2*), *SMADI*, bone morphogenetic protein 2 (*BMP2*), *COL1A1* and glyceraldehyde dehydrogenase (*GAPDH*) were amplified three times with an Eppendorf Mastercycler Gradient (Eppendorf Co., Hamburg, Germany) and specific primers (Table S1, See Supplementary Online Information at [www.celljournal.org](http://www.celljournal.org)). The program was: 95°C for five minutes, 95°C for one minute, annealing temperature of the specific primer for one minute, 72°C for one minute and a final elongation temperature of 72°C for seven minutes. Amplification was repeated for 35 cycles and the product was run on a 1.5% agarose gel. The bands were photographed with a gel documentation system (Gene Flash, Syngene Bio Imaging, England) and analysed by GelQuant software (GelQuant: 1.8.2).

### Statistical analysis

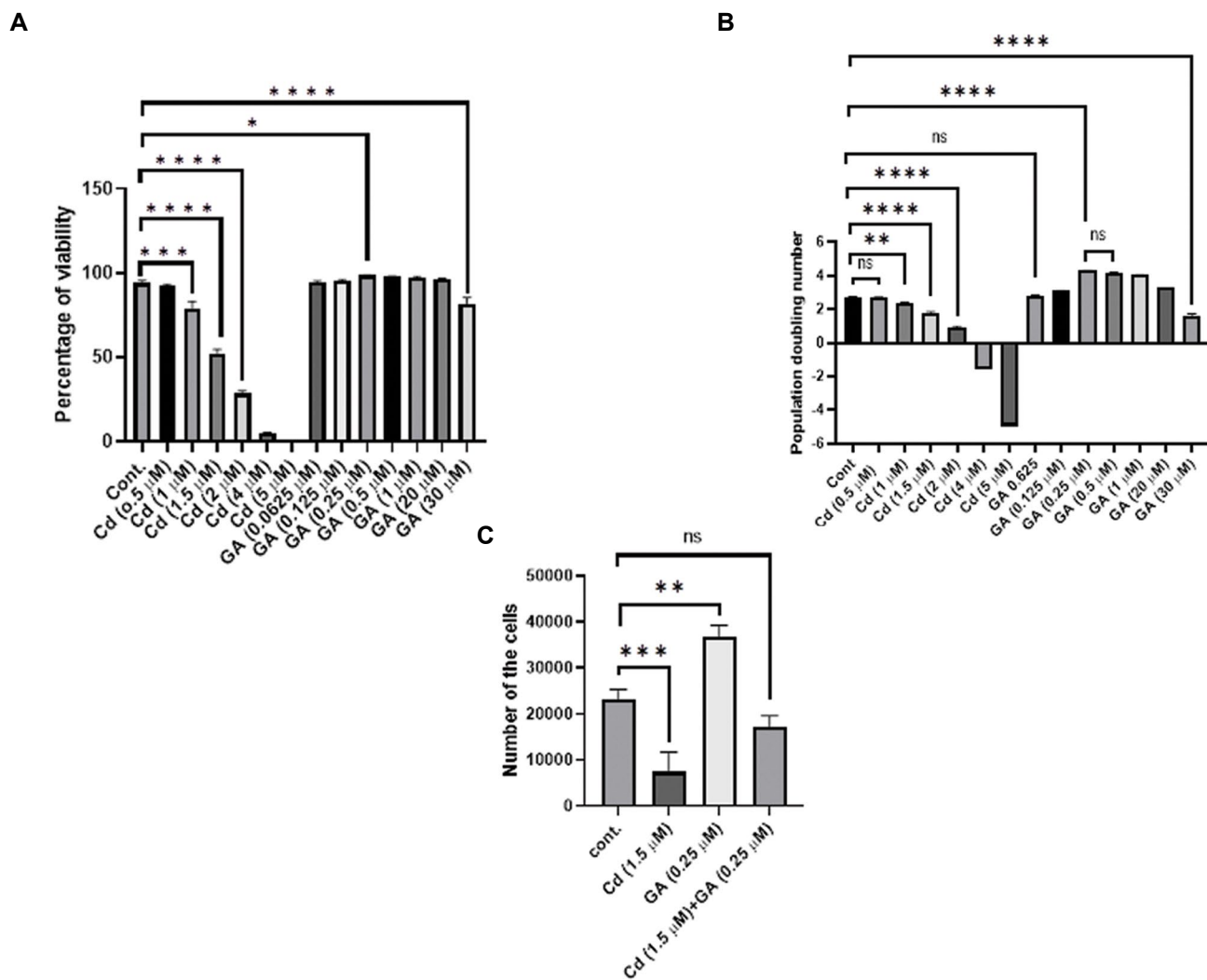
Data analysis was performed using one-way analysis of variance (ANOVA) and Tukey's tests with the help of SPSS software (version 20, IBM, USA). GraphPad Prism was used to plot the graphs (version 8.4.3 (686), GraphPad Software, Inc., USA). The results are shown as mean  $\pm$  standard deviation, and the minimum level of significance is  $P<0.05$ .

## Results

### Cell viability

Cd treatment caused a significant ( $P=0.001$ ), concentration dependent reduction of cell viability from 1  $\mu\text{M}$ . GA significantly increased cell viability from 0.25  $\mu\text{M}$  ( $P=0.04$ ). Although the highest increase in cell viability was observed with the 0.25  $\mu\text{M}$  GA treatment, the 30  $\mu\text{M}$  concentration significantly reduced cell viability ( $P=0.0001$ , Fig.1A). Cell proliferation analysis revealed that cells treated with Cd had a significant ( $P=0.001$ ) reduction in PDN from 1  $\mu\text{M}$ ; the highest reduction was observed at 5  $\mu\text{M}$ . Treatment with GA showed no significant changes at the 0.625  $\mu\text{M}$  concentration compared with the control; however, a significant increase in PDN was observed from the 0.125  $\mu\text{M}$  concentration ( $P=0.04$ ). The highest increase in PDN was observed with 0.25  $\mu\text{M}$  of GA. We observed a highly significant reduction ( $P=0.001$ ) at 30  $\mu\text{M}$  (Fig.1B). The 1.5  $\mu\text{M}$  concentration of Cd caused an almost 50% reduction in cell viability; therefore, we considered this concentration to be the  $\text{IC}_{50}$  and selected it for further analysis. PDN results indicated that 0.25  $\mu\text{M}$  of GA had maximum proliferation, and this concentration was also chosen for further analysis.

We used the MTT assay to study cell viability under osteogenic conditions. Co-treatment of the cells for 20 days with 1.5  $\mu\text{M}$  of Cd and 0.25  $\mu\text{M}$  of GA counteracted the toxic effect of Cd. Therefore, the change in cell viability in the co-treatment group was non-significant ( $P=0.06$ ) compared to the control group (Fig.1C).



**Fig.1:** Cell viability and proliferation. **A.** Effects of Cd and GA individually on cell viability of BMSCs after 20 days of treatment under non-osteogenic conditions (trypan blue assay). **B.** Effects of Cd and GA individually on BMSCs proliferation after 20 days of treatment. **C.** Effects of Cd, GA, and Cd+GA on cell viability of BMSCs after 20 days of treatment in osteogenic media using the 3-(4, 5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Data are shown as mean  $\pm$  SD for three replicates. Cd; Cadmium, GA; Gallic acid, BMSCs; Bone marrow mesenchymal stem cells, ns; Not significant, \*;  $P<0.05$ , \*\*;  $P<0.01$ , \*\*\*;  $P<0.001$ , and \*\*\*\*;  $P<0.0001$ .

## Osteogenic differentiation

Alizarin red staining of the cell matrix showed that Cd treatment reduced the osteogenic differentiation ability of the BMSCs compared with the control group (Fig.2IA, B). Treatment with GA increased the osteogenic ability (Fig.2IC) and co-treatment of the cells with Cd and GA counteracted the toxic effect of Cd (Fig.2ID). Statistical analysis confirmed this effect of GA in the co-treatment group. Alizarin red analysis results showed that GA treatment significantly improved matrix production ( $P=0.0001$ ), whereas treatment with Cd significantly reduced matrix production ( $P=0.001$ , Fig.2IIA).

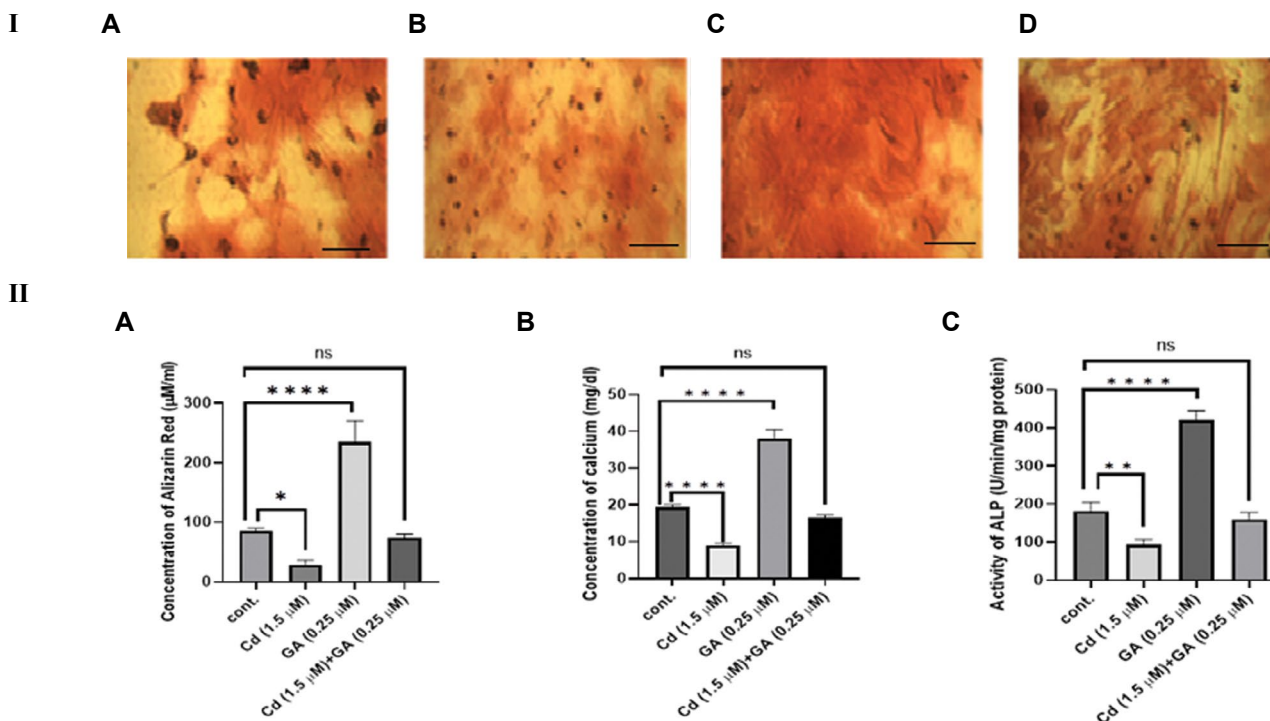
Calcium content (Fig.2IIB) and ALP activity (Fig.2IIC) analyses showed that co-treatment of the cells with 1.5  $\mu$ M of Cd and 0.25  $\mu$ M of GA improved the toxic effect of Cd. No changes could be observed in comparison to the control group. Treatment of the cells with only GA

significantly increased ALP and matrix extracted calcium content compared with the control group ( $P=0.0001$ ).

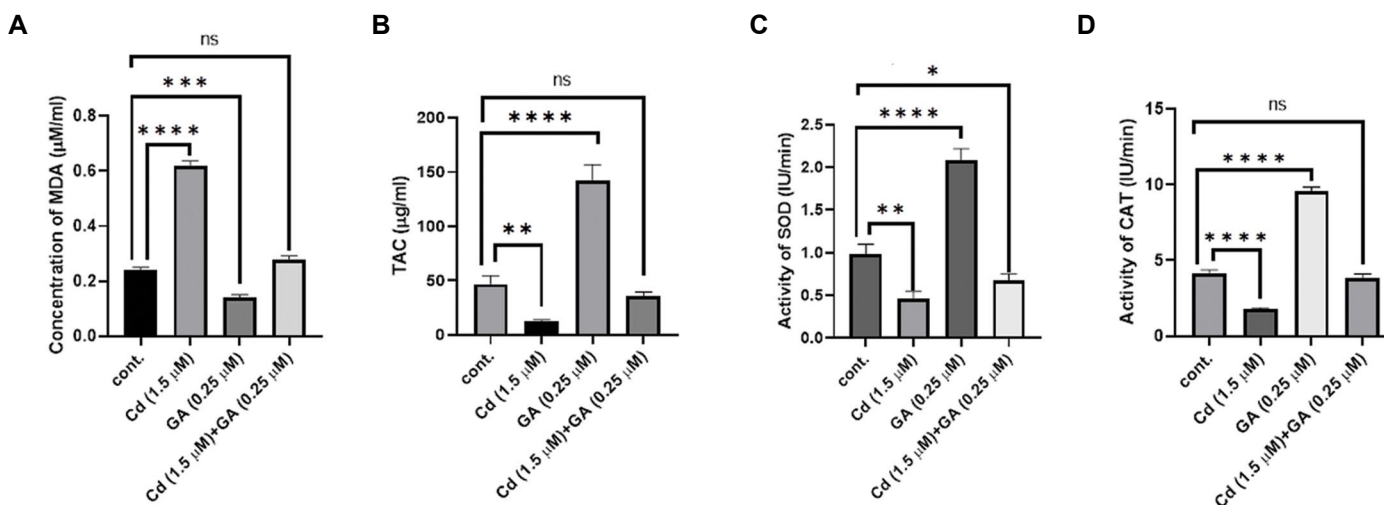
## Oxidative stress

Cells treated with Cd had a significant ( $P=0.00001$ ) increase in MDA (Fig.3A), whereas the level of TAC and activity of CAT and SOD were significantly reduced ( $P=0.01$ , Fig.3B-D). Treatment of the cells with GA significantly ( $P<0.001$ ) reduced MDA levels and significantly increased TAC levels and CAT and SOD activity ( $P=0.001$ ). In the co-treatment group, we observed that the level of MDA was non-significant ( $P=0.06$ ) in comparison with the control group. In addition, co-treatment of the cells counteracted the toxic effect of Cd with respect to TAC levels and antioxidant enzyme activities. SOD levels were significant compared to the control group.





**Fig.2:** Osteogenic differentiation. I. Alizarin red staining of BMSCs after 20 days of treatment under an osteogenic condition. A. Control. Treatment with B. Cd, C. GA, and D. Cd+GA (magnification: 20x, scale bar: 200 μm). II. Effects of Cd and GA on differentiation ability of BMSCs based on A. Alizarin red, B. Calcium concentration, and C. ALP activity after 20 days of treatment under an osteogenic condition. Data are shown as mean ± SD for three replicates. BMSCs; Bone marrow mesenchymal stem cells, Cd; Cadmium, GA; Gallic acid, ALP; Alkaline phosphatase, ns; Not significant, \*, P<0.05, \*\*, P<0.01, and \*\*\*\*, P<0.0001.



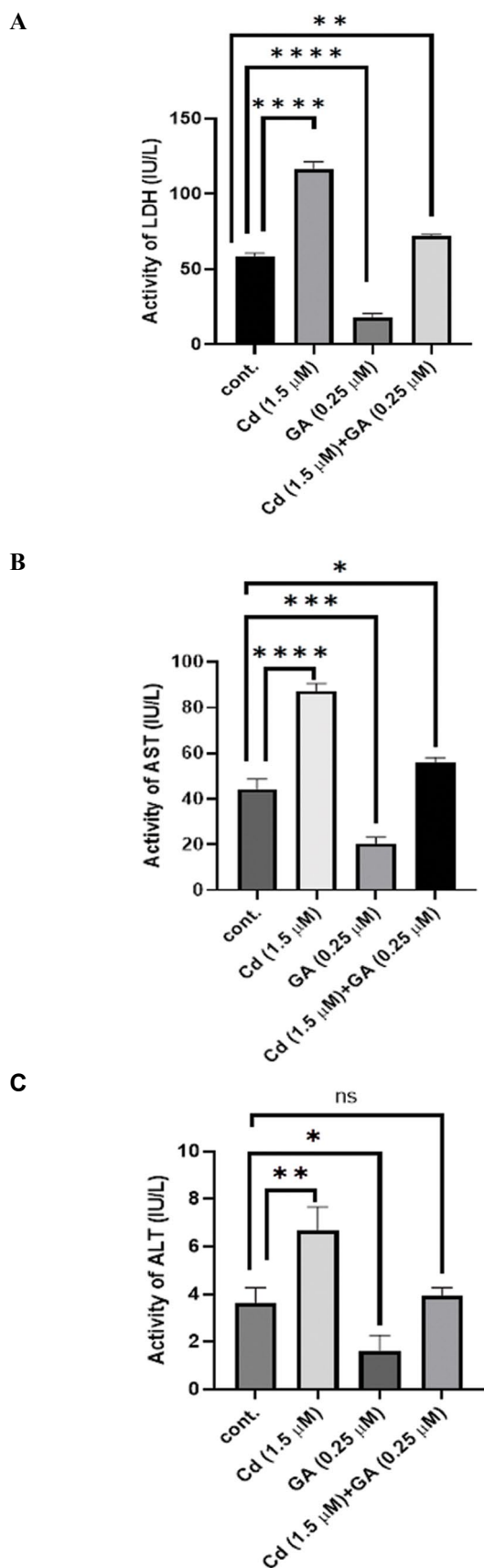
**Fig.3:** Effect of Cd and GA on BMSCs. A. MDA concentration, B. TAC, C. SOD activity, and D. CAT activity after 20 days of treatment under an osteogenic condition. Data are shown as mean ± SD for three replicates. Cd; Cadmium, GA; Gallic acid, BMSCs; Bone marrow mesenchymal stem cells, MDA; Malondialdehyde, TAC; Total antioxidant content, SOD; Superoxide dismutase, CAT; Catalase, ns; Not significant, \*, P<0.05, \*\*, P<0.01, \*\*\*, P<0.001, and \*\*\*\*, P<0.0001.

### Metabolic activity

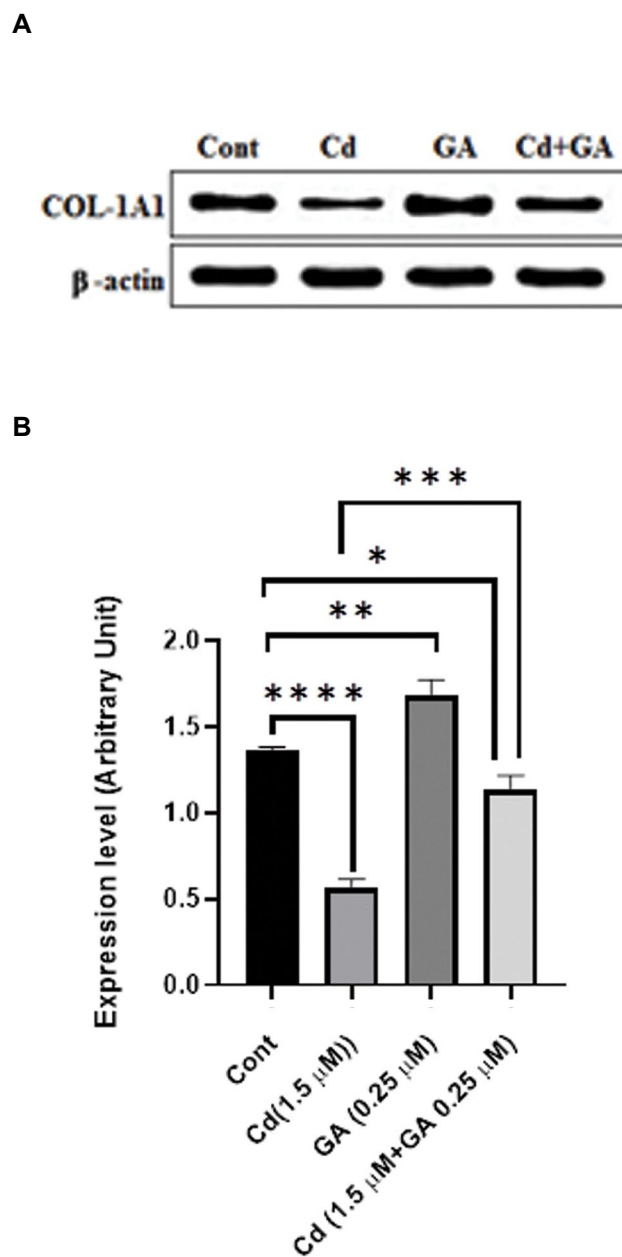
When compared with control group, Cd (1.5 μM) caused a significant (P=0.001) elevation in LDH, AST and ALT levels in the treated cells; however, 0.25 μM of GA significantly reduced the activities of these enzymes (P=0.001). Although co-treatment of the cells counteracted the toxic effect of Cd, it only restored ALT activity (Fig.4).

### Western blot analysis

Western blot analysis of the cell extract showed that the Cd caused a highly significant reduction of COL1A1 (P=0.0001), whereas GA significantly increased COL1A1 expression in the osteogenic differentiated BMSCs (P=0.01). Co-treatment of the cells could significantly (P=0.001) replenish COL1A1 expression in the cell extract compared with the Cd treated group (Fig.5).



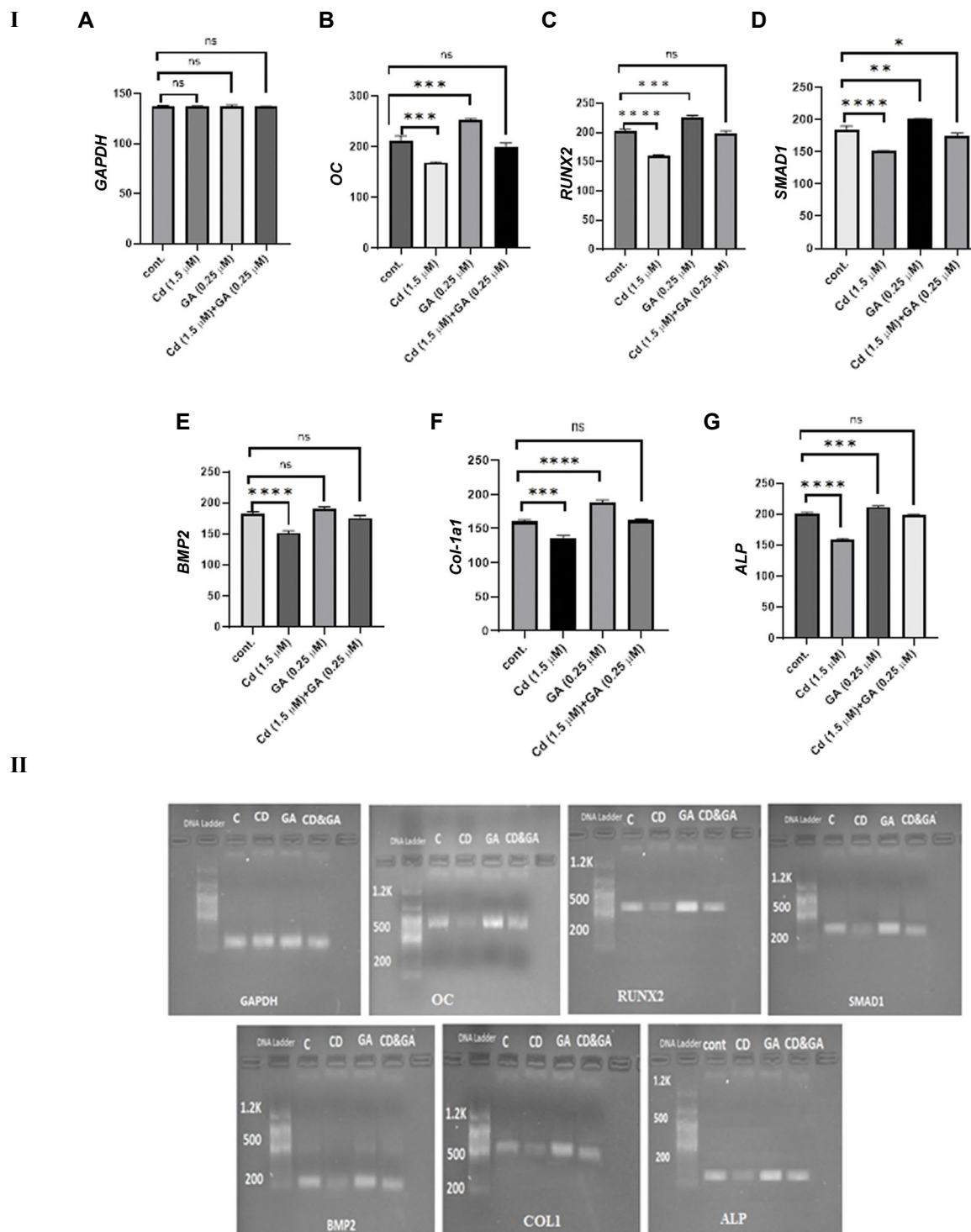
**Fig.4:** Effects of Cd and GA on metabolic activity of BMSCs. **A.** LDH, **B.** AST, and **C.** ALT activities after 20 days of treatment under an osteogenic condition. Data are shown as mean  $\pm$  SD for three replicates. Cd; Cadmium, GA; Gallic acid, BMSCs; Bone marrow mesenchymal stem cells, LDH; Lactate dehydrogenase, AST; Aspartate transaminase, ALT; Alanine transaminase, ns; Not significant, \*, P<0.05, \*\*, P<0.01, \*\*\*, P<0.001, and \*\*\*\*, P<0.0001.



**Fig.5:** Effects of Cd and GA on expression of COL1A1 protein in BMSCs. **A.** Western blot of COL1A1 and  $\beta$ -actin (internal control). **B.** Protein expression level (arbitrary unit) after 20 days of treatment under an osteogenic condition. Data are shown as mean  $\pm$  SD for three replicates. Cd; Cadmium, GA; Gallic acid, BMSCs; Bone marrow mesenchymal stem cells, ns; Not significant, \*, P<0.05, \*\*, P<0.01, \*\*\*, P<0.001, and \*\*\*\*, P<0.0001.

**Osteogenic-related gene expressions**

There was a highly significant reduction in expression of the osteogenic-related genes after treatment with Cd (P=0.0001, Fig.6). In the co-treatment group, GA counteracted the effect of Cd and restored *OC*, *RUNX2*, *BMP2*, *COL1A1* and *ALP* expressions compared with the control group. Treatment of the cells with only GA caused a significant increase (P=0.001) in *OC*, *RUNX2*, *SMAD1*, *COL1A1* and *ALP* expressions (Fig.6).



**Fig.6:** I. Graph presents the effects of Cd and GA on gene expression (arbitrary unit). **A.** *GAPDH*, **B.** *OC*, **C.** *RUNX2*, **D.** *SMAD1*, **E.** *BMP2*, **F.** *BMP7*, and **G.** *ALP* of BMSCs after 20 days of treatment under an osteogenic condition. **II.** Image presents the agarose gel electrophoresis of the PCR product of the osteogenic-related genes of BMSCs after 20 days of treatment. Data are shown as mean  $\pm$  SD for three replicates. Cd; Cadmium, GA; Gallic acid, BMSCs; Bone marrow mesenchymal stem cells, ns; Not significant, \*,  $P < 0.05$ , \*\*,  $P < 0.01$ , \*\*\*,  $P < 0.001$ , \*\*\*\*,  $P < 0.0001$ , C; Control, Cd, GA, and Cd+GA; Combination of Cd and GA.

## Discussion

In the present study, we observed that Cd suppressed osteogenic differentiation while GA increased this property in BMSCs. Osteogenic-related gene expressions significantly reduced following treatment of BMSCs with  $CdCl_2$ . ALP activity, alizarin red staining, the calcium

concentration of matrix, and COL1A1 expression all confirmed the suppressing effect of Cd. On the other hand, treatment with GA significantly increased expressions of these osteogenic-related genes, and also increased the ALP enzyme and COL1A1 protein in osteoblasts that produce matrix. The increase in matrix formation



attributed to GA treatment was confirmed by alizarin red staining and measurements of calcium content, ALP activity, and COL1A1 expression.

Cd has been shown to reduce BMD in several different populations. In animal studies, Cd treatment resulted in changes to the bone microstructure, including an elevation in trabecular separation and reduction of bone vascularization (13, 14). Investigations in humans revealed that low Cd exposure had a significantly negative impact on cortical area and thickness as well as trabecular bone volume fraction, and an increase in trabecular separation (4). Cd causes direct and indirect effects on bone tissue; it reduces bone formation by inhibition of osteoblasts and activation of osteoclasts (11, 15, 16). The specific mechanism of bone damage by Cd is unclear, especially for osteogenic differentiation of BMSCs. We have previously reported that Cd causes membrane damage and metabolic impairment in BMSCs (10, 12). In the present study, Cd induction of peroxidation of membrane lipids increased the amount of MDA and reduced both antioxidant enzyme activities and TAC concentration in the BMSC-derived osteoblasts. Cd toxicity also altered the metabolic state of osteogenic differentiated BMSCs by activation of LDH to convert pyruvate and produce more lactic acid, which facilitates glycolysis (17).

Cd toxicity is related to a depletion in cell antioxidant capacity (18, 19) and generation of ROS ( $O_2$ ,  $H_2O_2$ , and HO.) radicals (20). Plant antioxidants may prevent lipid peroxidation due to ROS overproduction (21-23). GA is a potent plant antioxidant found in many fruits and vegetables; therefore, it is easily available to the public. GA has an oxidative preventive effect and an osteogenic improvement ability, whereas Cd inhibits BMSC osteogenesis (24). Here, we used GA to overcome the toxic effect of Cd. In this study, GA improved TAC of the osteogenic differentiated BMSCs and increased antioxidant enzyme activities. There was a significantly low level of MDA generated in the presence of GA compared to the control group. Therefore, GA in the co-treatment group could counteract the oxidative effect of Cd and prevent oxidative stress compared to the control group. In addition to lipid peroxidation, ROS can react with functional groups of proteins and inactivate enzymes (25). DNA and RNA damage has been reported in the presence of ROS generating agents (26). Thus, prevention of oxidation of macromolecule by ROS is a prime step to prevent the deleterious effect of free radicals.

Cells treated with GA improved BMSC osteogenesis after oxidative damage from Cd. We observed that Cd inhibition of osteogenic differentiation of BMSCs was reduced by GA treatment at the gene and protein levels. *RUNX2* is a key gene in the progression of osteogenic differentiation (27), which is regulated by *BMP2* and the *SMAD* family (28). Consequently, *RUNX2* activates *OC*, *COL1A1* and *ALP*, the genes that control matrix formation in newly produced osteoblasts (29). In the present study, following osteoinduction, expressions of

*BMP2*, *SMAD1*, *RUNX2*, *COL1A1*, *ALP* and *OC* up-regulated after GA treatment. Cd treatment resulted in down-regulation of the osteogenesis-related genes, whereas treatment with GA overcame the inhibitory effect of Cd on gene regulation and at the protein level of *COL1A1* and *ALP*.

In bone remodelling the balance between osteoblast-mediated bone formation and osteoclast-mediated bone resorption is important. Differentiation of BMSCs to osteoblasts ensures the availability of bone matrix to produce a strong and healthy bone. Therefore, any imbalance in osteogenic differentiation would result in decreased bone density (30), which is the main reason for osteoporosis. Osteogenic-related gene activation results in production of organic and inorganic components of bone matrix (31). Cd down-regulated gene expressions; as a result, it inhibited the organic (*COL1A1*) and inorganic (calcium) components required for matrix production. GA improved the gene expressions and restored *COL1A1* levels and *ALP* activity, which are main factors for production of bone matrix.

Previously, we reported the toxic effect of Cd on osteogenic differentiation of BMSCs (11, 32). The results of the current study support previous reports about the osteogenic induction property of GA (33, 34). Here, we confirmed the ability of GA to prevent the osteogenic inhibitory effect of Cd. In our previous study, we showed that the Cd changed the metabolic situation of BMSCs from an aerobic to an anaerobic state (10-12). We also showed that GA could restore the metabolic activity of differentiated BMSCs, as the cells in the osteogenic differentiated state need more energy for production via aerobic respiration.

## Conclusion

Cd induces oxidative stress that results in inhibition of osteogenic gene expressions. Cd also prevents *COL1A1* production and inhibits *ALP* activity, which prevents the production of organic and inorganic components of the bone matrix. GA is a strong antioxidant that prevents the oxidative effect of Cd and restores gene expressions. It also counteracts the inhibitory effect of Cd on matrix production by increasing *ALP* activity to deposit more calcium on the organic component (*COL1A1*).

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## Authors' Contributions

M.H.A.; Conceived the study, supervised the overall

direction and planning, designed the experiments, and wrote the manuscript. J.S.; Conducted statistical and gene analyses. F.N.; Performed the laboratory experiments and statistical analysis. All authors read and approved the final version of this manuscript.

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