

The Consequence of Vitamin E Exposure on *In Vitro* Cadmium Toxicity in Rat Bone Marrow Mesenchymal Stem Cells

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This investigation aimed to examine the protective function of vitamin E on rat bone marrow mesenchymal stem cells (MSCs) treated with cadmium chloride. Rat bone marrow MSCs were extracted using flashing-out and cultured in DMEM containing 10% FBS and 100 U/ml Pen/Strep. At the end of the third passage, cells were divided into 4 groups including control, cadmium chloride, cadmium chloride + vitamin E and vitamin E, for a period of 5, 10, 15 and 21 days in the osteogenic media. The cell viability, bone matrix mineralization and intracellular calcium were measured using MTT assay, alizarin red staining, von kossa staining and calcium kit, respectively. Alkaline phosphatase activity was also estimated. Morphology and DNA cleavage were studied with the help of fluorescent dye and comet assay. Data were analyzed using one way ANOVA. The viability and bone matrix mineralization of the cells treated with cadmium chloride was reduced significantly in comparison with the control group. Chromatin condensation, reduction of nuclei diameter and cytoplasm shrinkage were observed in cadmium group. The intracellular calcium and alkaline phosphatase activity of the cells decreased significantly with cadmium when compared to control group. A significant increase of these parameters was found in the group of cadmium chloride+ vitamin E compared to the control ones. Results show that vitamin E exhibits protective role against the toxic influence of cadmium on all studied parameters in rat bone marrow MSCs.

Keywords: Bone marrow, mesenchymal stem cells, Vitamin E, cadmium, toxicity, rat

Cadmium (Cd) is a heavy metal and found in concentration range of 0.1 to 1 ppm in the earth's crust. Cd has not been set up as a pure

element, but in the environment it is represented as compounds such as cadmium oxide, cadmium chloride, and cadmium sulfate or sulfide (1). Cd is

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known for its toxic effects on living organisms (2). The main source of toxic exposure is by the inhalation route of Cd particles or fumes during industrial operations. It is likewise present in cigarette smoke, presenting a substantial source of vulnerability. Cd has been shown to cause severe damage to a variety of organs, including the lung, liver, kidney, testis, bone, brain and even to the placenta (3). The mechanisms responsible for Cd toxicity are cell type dependent (4). Depending on the cell type and concentration, it may induce cell death via apoptosis, characterized by typical features such as caspase activation and DNA fragmentation, or necrosis typified by ATP depletion and plasma membrane permeabilization (5). Exposure to Cd was first associated with bone disease. In fact, Japan witnessed an outbreak of itai-itai disease, manifested by severe renal dysfunction with parallel osteomalacic and osteoporotic lesions. Although, numerous studies have since suggested that exposure to Cd may increase the risk of osteoporosis in humans and experimental animals (6). Vitamin E is a lipid-soluble antioxidant that is incorporated into cell membranes to prevent lipid peroxidation. Vitamin E exists in eight different forms (four tocopherols and four tocotrienols). All forms of vitamin E feature a chromanol ring with a hydroxyl group that can donate a hydrogen atom to reduce free radicals and a hydrophobic side chain allowing penetration into biological membranes. α -Tocopherol is traditionally recognized as the most biologic antioxidant in humans. Use of antioxidants has been suggested to be beneficial in oxidative stress-associated diseases. It has been reported that oxidative stress levels are negatively associated with bone mineral density and that antioxidant levels are lower in osteoporotic patients (7, 8). Because the protective effects of vitamin E with respect to Cd toxicity have not been well known and have not been investigated in MSCs, the present study was

undertaken to determine whether this antioxidant could reduce the toxicity of Cd in rat bone marrow mesenchymal stem cells (MSCs).

Materials and methods

Marrow cell culture

In the present study, Wistar rats (6-8 weeks old) were purchased from Pasteur institute (Tehran, Iran) and kept in the animal house of Arak University under standard condition of light and food and were treated according to the institutional guidelines for animals care. The animals were sacrificed by excessive chloroform inhalation and then their tibia as well as femur were removed and cleaned from the adherent soft tissue. Then the two ends of the bones were cut off and bone marrow was flushed out using 2 ml DMEM (Dulbecco's Modified Eagles Medium, Gibco, Germany) supplemented with 10% FBS (Fetal Bovine Serum, Gibco, Germany) and penicillin-streptomycin (Gibco, Germany). Bone marrow content was centrifuged at 1200 rpm for 5 min and resuspended in 5 ml DMEM containing 15% FBS and antibiotics and then was plated in 25 cm² flasks and incubated at 37 °C with atmosphere of 5% CO₂. Two days after culture initiation, the first medium replacement was performed and then medium was changed two times per week till the bottom of the flask was covered with the cells (till confluency). The cells were trypsinized (trypsin-EDTA, Gibco, Germany) and passed to another culture flask as the first passage and then the cultures were expanded through two additional subcultures for more purification of the MSCs, which were used for further investigation.

Osteogenic induction

Mineralization was induced on confluent monolayers of cells by addition of DMEM containing 15% (v/v) FBS, streptomycin-penicillin and osteogenic supplements (1mM sodium glycerophosphate, 50 µg/mM L-ascorbate and 8-10

M dexamethasone) All the chemicals were purchased from Sigma-Aldrich company. Culture flasks were incubated for 21 days at 37 °C with 5% CO₂ and their medium was changed every 3 days.

Exposure to cadmium chloride and vitamin E

During assays, cells were cultured in separate culture dishes in presence of DMEM supplemented with osteogenic media for periods of 5, 10, 15 and 21 days according to the design of the test, which represented control, cadmium chloride, vitamin E and vitamin E + cadmium chloride groups.

Cell viability assays

The viability test on control and treated cells was carried out in a 96 well-plate using MTT (4, 5dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), where after 4 h of incubation, the mitochondrial succinate dehydrogenase in the living cells reduces the yellow color tetrazolium into purple formazan. Then, 100 µL of DMSO was added to each well of the plate and formazan crystals were dissolved at room temperature. The absorbance of solutions was measured on an automated microplate reader (SCO diagnostic, Germany) at 505 nm.

Intra cellular Ca²⁺ assay

Cells in plates including control and treated ones were first washed twice with PBS and then their calcium content was extracted with 50 µl of 0.5 N HCl for 24 h. The amount of calcium was determined using the commercial kit (DarmanKave, Iran) and the developed color was measured at 575nm using a spectrophotometer (T80+ PG instrument Ltd, England).

Analysis of morphological changes

Following cadmium chloride and vitamin E treatment in an osteogenic media for 21 days, the nuclear morphology of the cells was studied using Hoechst 33342 at room temperature after 5 min of incubation in the dark. Hoechst is a fluorescent dye

penetrating the cells through the intact plasma membrane and stains the DNA. Consequently, changes in nuclear morphology such as chromatin condensation and fragmentation can be investigated. The morphology of the cell cytoplasm was investigated using another fluorescent dye called acridine orange staining the nuclei in green and the cytoplasm in orange. After staining, the cells after were washed twice with PBS, examined and immediately photographed under an inverted fluorescence microscope (Olympus, IX70) equipped with camera using 40x magnification.

Comet assay

The control group as well as vitamin E and Cd-treated cells were collected by trypsin and diluted in PBS. Then the cells were mixed with low melting point agarose (1% w/v) and dropped on the microscopic slide layer. The cells were spread on the slide with the help of a cover slip and were placed on ice in order to be solidified. The cover slips were then removed and the slides were immersed in a lysis buffer (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris-HCl, 1% (w/v) sodium sarcosinate, pH 10; 1% Triton X-100 and 10% DMSO added just before use) in the dark at 4 °C. Then the slides were placed in a horizontal electrophoresis unit containing freshly prepared alkaline electrophoresis buffer (200 mM Na₂EDTA, 10 mM NaOH). The DNA was allowed to unwind for 20 min and the electrophoresis was performed at 25 V, 300 mA for 30 min. After neutralization (0.4 M Tris-HCl, pH 7.5), the DNA was stained with 50 µl of 20 µg/ml ethidium bromide. The slides were placed in a humidified air-tight container to prevent drying of the gel, before analysis. Using a fluorescence microscope (Olympus, IX70), the slides were observed and photographed with the help of digital camera. The percentage of the cells with comet in comparison with the control group were determined and statistically analyzed.

Detection and quantification of mineralization

Cells in plates were washed with PBS and fixed in 10% (v/v) formaldehyde (Sigma-Aldrich) at room temperature for 15 min. The cells were then washed twice with excess of dH₂O, and 1 mL of 40 mM alizarin red solution (pH 4.1) was added per well. The plates were then incubated at room temperature for 20 min with gentle shaking. After pouring off the excessive dye, the plates were washed four times with dH₂O. Stained cells were investigated under light microscopy using an inverted microscope. To quantify the level of absorbed alizarin red, 800 μ L of 10% acetic acid (v/v) was added to each well, and the plate was incubated at room temperature for 30 min with gentle shaking. Then the loosely attached cells were scraped from the plate with a cell scraper and transferred into a 1.5 mL microcentrifuge tube. After vortexing for 30 s, the slurry was overlaid with 500 μ L mineral oil (Sigma-Aldrich), heated at 85 °C for 10 min, and then kept on ice for 5 min. The slurry was then centrifuged at 20,000 g for 15 min and 500 μ L of supernatant was transferred into a new microcentrifuge tube and 200 μ L of 10% ammonium hydroxide (v/v) was added to neutralize the acid. An aliquot of the supernatant (100 μ L) was read in triplicate at 405 nm by a microplate reader (SCO diagnostic, Germany) and quantified against standard graph. In order to prepare alizarin red standards (ARS) graph, working ARS (40 mM) was diluted 20 times with a mixture of 5:2 of 10% acetic acid and 10% ammonium to give a concentration of 2 mM. Then, using serial dilutions, standard solution of 2000 to 31.3 μ M was prepared and the absorption was taken at 405 nm using a microplate reader. The concentration of the unknown samples was calculated by using linear formula $Y=0.0004X+0.037$ with $R^2=0.997$ where Y is the

absorbance and X is the concentration (mM) of alizarin red.

Von Kossa staining

The presence of mineralized deposits in MSC cultures was demonstrated with von Kossa staining. On day 21, triplicate cultures of each experimental group were rinsed twice with Tyrode's salt solution, made with 10% (v/v) formaldehyde (Sigma-Aldrich) for 15 min, and washed three times with distilled water. 1 mL of 2% (w/v) silver nitrate (Sigma Chemical) was added per dish, and the cultures were located in a dark environment for 10 min.

Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity of control and treated cells in dishes was determined by p-nitrophenylphosphate (pNPP) hydrolysis method, using the ALP assay kit (DarmanKave, Iran). Cells were washed three times with PBS and homogenized in lysis buffer (0.25M Tris-HCl, Triton X-100, pH:7.5) and the samples were centrifuged at 12000 rpm for 10 min at 4°C. The supernatant was kept in -20 °C for further analysis of ALP activity and protein content. The total protein content of each sample was determined according to Bradford, using bovine serum albumin (BSA) as standard. ALP activity was determined in protein lysate based on equal amount of proteins using pNPP as substrate according to the kit instruction (DarmanKave, Iran). Absorbance at 410 nm was measured using spectrophotometer (T80+ PG instrument ltd, England) and then ALP activity was determined using a pNPP standard curve.

Statistical analysis

Statistical evaluation of the data was performed using one-way analysis of variance (ANOVA) Tukey's test, with the help of SPSS. Results were expressed as mean \pm S.D and $P < 0.05$ was accepted as the minimum level of significance.

Table 1. Cell viability assay

Groups	Number of cells in day 5	Number of cells in day 10	Number of cells in day 15	Number of cells in day 21
Control	12012 ^b ± 3.14	13541 ^b ± 1.60	15598 ^b ± 2.56	18152 ^b ± 1.06
Cadmium	11032 ^a ± 1.76	10251 ^a ± 1.91	9189 ^a ± 0.17	10084 ^a ± 0.53
VitaminE+Cadmium	12039 ^b ± 0.29	13941 ^{b,c} ± 7.61	15381 ^{b,c} ± 9.69	24891 ^c ± 1.76
Vitamin E	15559 ^c ± 12.03	17341 ^c ± 0.8	19939 ^c ± 3.65	37652 ^d ± 3.18

All values are expressed as the mean ±SD. a,b,c,d: means which are significantly different from each other (one-way ANOVA, Tukey's test, P< 0.05).

Results

Effect of co treatment of vitamin E and cadmium on cell viability

Cell viability assay (Table 1) showed that the cells treated with Cd exhibited significant decrease of viability (P< 0.05) at the 5th, 10th, 15th and 21th days. Whereas the cells treated with vitamin E + Cd, showed no significant effect (P> 0.05) on the viability of the cells at the 5th, 10th, 15th and 21th days as compared to control group. The cells treated with vitamin E showed significant increase (P< 0.05) in cell viability from the 5th to the 21th day.

Mineralization based on alizarin red staining

Data showed that the mineralization of cells under osteogenic differentiation is minimum at the 10th day and reaches its maximum level at the 21th day in control group (Figure 1). The cells treated with Cd showed reduction in the mineralization

from the 10th to the 21th day based on quantitative (Table 2) as well as qualitative alizarin red estimation (Figure 1) as compared to control groups. Whereas the cells treated with vitamin E+ Cd, showed no significant effect (P> 0.05) on the mineralization of cells at the examined time periods based on quantitative (Table 2) as well as qualitative alizarin red estimation (Figure 1) as compared to control groups. Cells treated with vitamin E showed significant increase from the 10th to the 21th day based on quantitative (Table 2) as well as qualitative alizarin red estimation (Figure 1) as compared to control groups.

Intra cellular calcium concentration

Intra cellular calcium concentration (Table 3) showed that the cells treated with cadmium exhibited significant decrease of intercellular calcium concentration (P< 0.05) at the 5th, 10th, 15th and 21th days, whereas the cells treated with vitamin E+ Cd, showed no significant effect (P> 0.05) on

Table 2. Effect of cadmium and vitamin E on mineralization of MSCs cultured in osteogenic medium based on quantitative Alizarin red staining

Groups	Calcium(mg/dl) in day 5	Calcium(mg/dl) in day 10	Calcium(mg/dl) in day 15	Calcium(mg/dl) in day 21
Control	4.87 ^b ± 0.1	21.41 ^b ± 1.1	28.24 ^b ± 1.08	31.71 ^b ± 1.06
Cadmium	3.14 ^a ± 0.04	18.31 ^a ± 3.53	19.91 ^a ± 1.07	22.45 ^a ± 0.53
VitaminE+Cadmium	4.51 ^b ± 0.12	23.45 ^b ± 1.44	29.01 ^{b,c} ± 0.88	40.07 ^{b,c} ± 1.76
Vitamin E	6.19 ^c ± 3.31	27.12 ^c ± 0.7	30.41 ^c ± 1.32	45.25 ^c ± 3.18

All values are expressed as the mean ±SD. a,b,c: means which are significantly different from each other (one-way ANOVA, Tukey's test, P< 0.05).

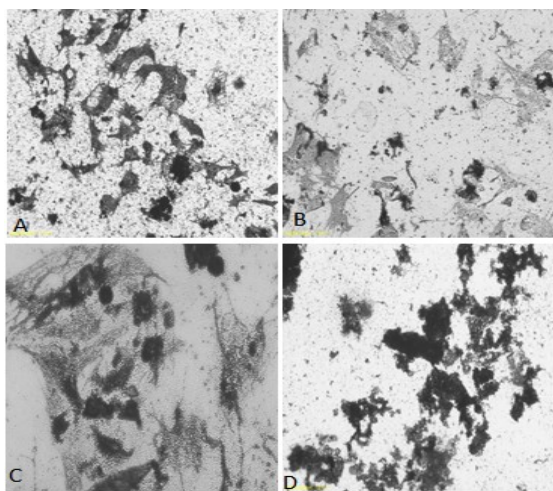


Figure 1. Von kossa staining for extra cellular calcium deposition. MSCs were analyzed after 21 days of osteogenic induction. A: control group; B: the group treated with cadmium; C: the group treated with vitamin E + cadmium; D: the group treated with vitamin E (with 40X magnification).

intercellular calcium concentration of the cells at the examined periods. In addition, the cells treated with vitamin E showed significant increase of intercellular calcium concentration ($p < 0.05$) from the 5th to the 21th day.

Extra cellular calcium concentration

Extra cellular calcium concentration of the differentiated cells at the 21th day (Figure 1) was found decreased in the group treated with Cd compared to control group. In addition, the group treated with vitamin E+ Cd showed no extracellular calcium increase compared to control group, whereas the group treated with vitamin E showed an increase effect compared to control group.

Alkaline phosphatase activity

ALP activity (Table 4) showed that the cells

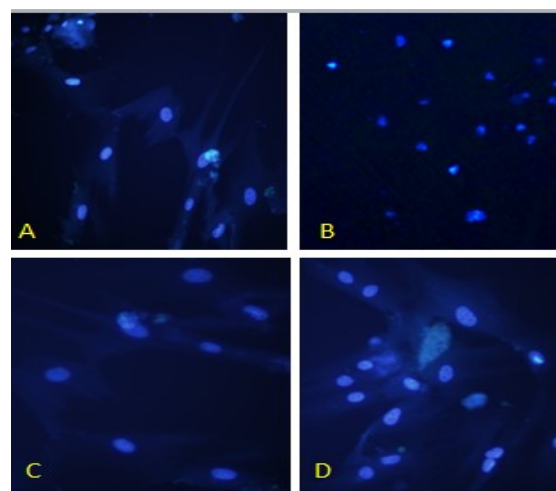


Figure 2. Cells in osteogenic medium stained with Hoechst. A: control group; B: the group treated with cadmium; C: the group treated with vitamin E + cadmium; D: the group treated with vitamin E (with 40X magnification).

treated with Cd exhibited significant decrease of alkaline phosphatase activity ($P < 0.05$) from the 5th to the 21th day, whereas the cells treated with vitamin E+ Cd, showed no significant effect ($P > 0.05$) on alkaline phosphatase activity at the examined time periods. In addition, the cells treated with vitamin E showed a significant increase of alkaline phosphatase activity ($P < 0.05$) from the 5th to the 21th day.

Morphological changes of differentiated MSCs

Morphological study of the nuclei of differentiated MSCs treated with cadmium after 21 days showed significant reduction ($P < 0.05$) in nuclei diameter and chromatin condensation as well as nuclear breakage (Figure 2). The groups treated

Table 3. Effect of cadmium and vitamin E on intra cellular Ca^{2+} concentration of MSCs cultured in osteogenic medium

Groups	Alizarin red in day 5	Alizarin red in day 10	Alizarin red in day 15	Alizarin red in day 21
Control	301 ^b ± 3.51	321 ^b ± 5.30	339 ^b ± 8.81	421 ^b ± 9.89
Cadmium	261 ^a ± 5.31	279 ^a ± 9.81	268 ^a ± 1.56	246 ^a ± 8.82
VitaminE+Cadmium	288 ^{ab} ± 3.41	304 ^{bc} ± 7.07	337 ^{bc} ± 5.3	429 ^{bc} ± 8.83
Vitamin E	352 ^c ± 7.07	398 ^c ± 3.41	421 ^c ± 3.51	506 ^c ± 7.07

All values are expressed as the mean ±SD. a,b,c: means which are significantly different from each other (one-way ANOVA, Tukey,s test, $P < 0.05$).

Table 4. Effect of cadmium and vitamin E on alkaline phosphatase level of MSCs cultured in osteogenic medium

Groups	Alkaline phosphatase activity(U/L) in day 5	Alkaline phosphatase activity(U/L) in day 10	Alkaline phosphatase activity(U/L) in day 15	Alkaline phosphatase activity(U/L) in day 21
Control	37.42 ^b ± 1.6	47.21 ^b ± 1.33	53.2 ^b ± 6.12	61.5 ^b ± 3.8
Cadmium	27.1 ^a ± 1.43	29.21 ^a ± 3.11	38.11 ^a ± 3.42	40.37 ^a ± 1.59
VitaminE+Cadmium	36.32 ^b ± 0.43	45.16 ^b ± 4.11	53.91 ^{bc} ± 3.53	65.50 ^{bc} ±3.18
Vitamin E	48.19 ^c ± 3.26	54.31 ^c ±4.21	69.11 ^c ± 2.54	87.75 ^c ± 1.41

All values are expressed as the mean ±SD. a,b,c: means which are significantly different from each other (one-way ANOVA, Tukey's test, P< 0.05).

with vitamin E+ Cd and vitamin E showed no significant morphological changes ($P > 0.05$)

compared to control. It can be also noticed that Cd causes remarkable changes in the morphology of cytoplasm (Figure 3) such as shrinkage and in some cells complete disappearance of cytoplasm compared to control cells, while the groups treated with vitamin E+ cadmium and vitamin E showed no significant effect as compared to control.

Comet assay

Differentiated MSCs treated with Cd after 21 days showed significant increase ($P < 0.05$) in comet tail compared to control. In addition, the group treated with vitamin E+ Cd showed no significant effect ($P > 0.05$) in comet tail compared to control.

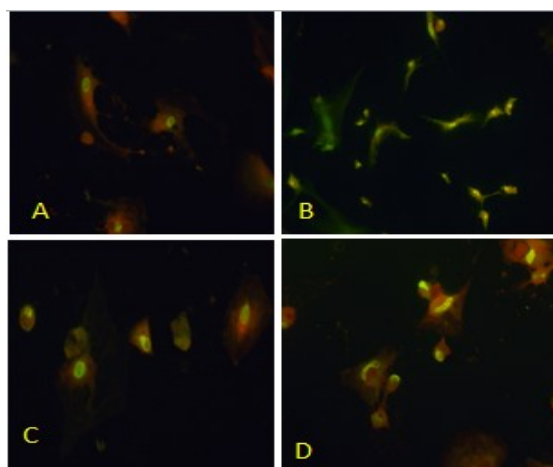


Figure 3. Cytoplasm morphology of cells using acridine orange staining. A: control group; B: the group treated with cadmium; C: the group treated with vitamin E + cadmium; D: the group treated with vitamin E (with 40X magnification).

The group treated with vitamin E showed significant reduction ($P < 0.05$) in comet tail compared to control (Figure 4 and 5).

Discussion

In this study, acute toxicity of Cd caused a significant reduction of viability based on MTT assay. This metal could cause intracellular reduction in the level of the main antioxidant compounds. Indeed, Cd inactivates enzymes and other antioxidants molecules by interacting with the thiol groups contained in these molecules (glutathione or proteins) or it can provoke homeostasis alteration of physiological metals such as copper, zinc and iron (4). It seems that inactivation of cellular antioxidant

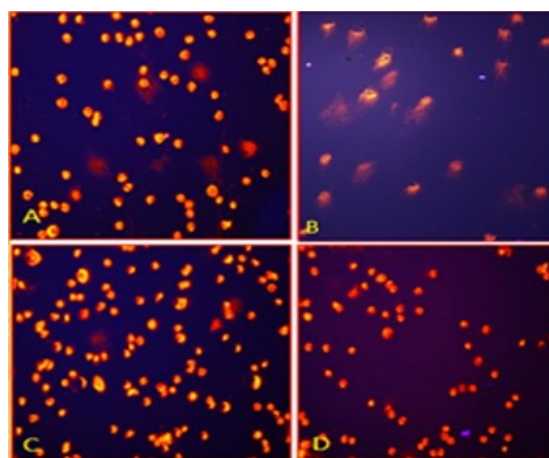


Figure 4. Comet assay. A: control group; B: the group treated with cadmium; C: the group treated with vitamin E + cadmium; D: the group treated with vitamin E (with 40X magnification).

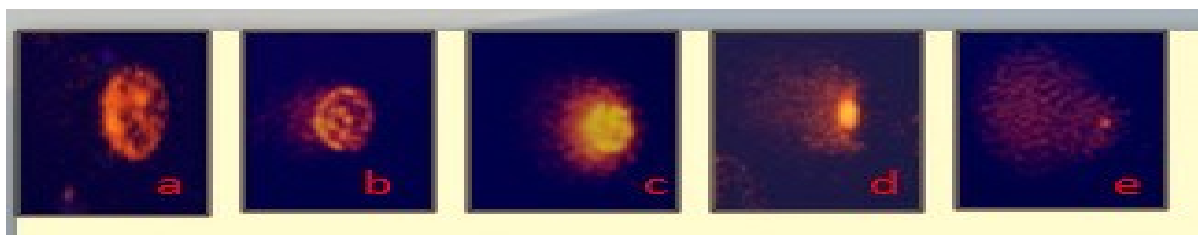


Figure 5. Degree of comet tail. a: degree 0; b: degree 1; c: degree 2; d: degree 3; e: degree 4.

molecules can result in an increase in reactive oxygen species (ROS) such as the hydroxyl radical, the superoxide anion or the hydrogen peroxide. The ROS will generate damage to lipids (lipid peroxidation), proteins and DNA. Direct interactions between Cd and DNA have been excluded (4). Yano et al. also observed that Cd significantly reduced the glutathione levels (10). A number of mechanisms of Cd toxicity have been suggested including increase of corticosterone, cell proliferation inhibition, apoptosis and necrosis (11). In mitochondria, Cd enters the structure of oxidative phosphorylation enzymes and alters energy production. Also, it can replace many metal nutrients such as Ca^{+2} entry into the cell. Cd induces lipid peroxidation by stimulating the production of superoxide anions, and causes chronic diseases followed by accumulation of free radicals and cell damage due to inhibition of antioxidants such as glutathione peroxidase and superoxide dismutase (12). Increased levels of malondialdehyde, protein oxidation and reduced activity of superoxide dismutase (SOD) in rat testes have been reported following a single subcutaneous injection of CdCl_2 indicating an increase in lipid peroxidation and a decrease in the removal of superoxide radicals. Vitamins C and E can protect the rat testis from Cd-induced oxidative damage. N-acetylcysteine, glycine, and glutathione-like compounds have also been shown to rescue various cells and tissues from this mechanism (12, 13). CdCl_2 caused significant time-dependent reduction of viability. In addition, morphological changes such as nuclear breakage,

chromatin condensation, and cytoplasm shrinkage were observed. DNA damages observed by the comet assay might be considered as a sign of apoptosis and a reason for significant reduction of viability.

Recent evidence indicated that Cd replaces zinc in P53 and impairs its DNA binding activity, which may result in cell cycle arrest after DNA damage. Therefore, the replacement of zinc by Cd in key regulatory factors may result in gene expression deregulation followed by cellular transformation (14, 15). Researches have shown that free radicals can inhibit calcium channel and disrupt calcium homeostasis which, itself might be a reason for significant reduction of calcium influx due to Cd toxicity (16). Cd can also cause bone damage, either via a direct effect on bone tissue or indirectly as a result of renal dysfunction (17). Vitamin E, is a natural antioxidant which protects cells from oxidative damage. It scavenges free radicals, terminating lipid peroxidation chain reaction (18-20). Mitochondria-targeted antioxidants modulate also the levels of ROS, thereby reducing mitochondria-driven cell death (21). Free radicals enhance also bone resorption by directly activating osteoclasts. An association between low activity of antioxidant systems and demineralization of bone, consequent to enhanced free radical levels was demonstrated (22). It was shown that exposure to an oxidizing agent such as ferric nitrilotriacetate, can reduce the level of bone calcium, which may be prevented by palm vitamin E supplementation. However, there are limited reports on the molecular

mechanism of the action of vitamin E on bone formation (23). ALP is a good index of bone formation and plays an important role in skeletal mineralization. Our data show that parameters of osteoblast differentiation were increased by α -tocopherol.

The present study suggests that α -tocopherol can enhance osteogenesis and osteoblast differentiation at the stem cell level. Therefore, vitamin E (α -tocopherol) has the potential benefit to be used in the prevention and treatment of postmenopausal osteoporosis and other forms of osteoporosis associated with increased cytokine levels as also suggested by Ahn and Hung (7). It was also shown that α -tocopherol may play a role in cell morphology maintenance by preventing actin depolymerization induced by oxidative stress. In fact, α -tocopherol maintains the cell plasma membrane and morphology by protecting proteins, especially by interacting with the thiol groups in actin (23, 24). Vitamin E deficiency was found to cause loss of bone calcium in growing female rats, and this could be due to increased free radical activity or decreased calcium availability for bone deposition (23, 25). The present study validate that Vitamin E plays a clearly protective effect against Cd-induced cytotoxicity in rat MSCs.

Conflict of interest

The authors declared no conflict of interest.

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