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Research Paper: The Inhibitory Role of Di-2-ethylhexyl Phthalate on Osteogenic Differentiation of Mesenchymal Stem Cells Via Down-regulation of *RUNX2* and Membrane Function Impairment

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ABSTRACT

Background: Blood contamination of di-2-ethyl hexyl phthalate (DEHP) has been reported due to its release following medical procedures such as blood transfusion and vital liquid injection. We investigated the effect of DEHP on osteogenic differentiation of mesenchymal stem cells and their viability.

Methods: The rat bone marrow mesenchymal cells (MSCs) were cultured three times, and the third passage kept in the differentiation medium with the presence of DEHP. The viability of differentiated cells, sodium and potassium level, calcium concentration, total protein concentration, and the activity of lactate dehydrogenase, alkaline phosphatase, alanine transaminase, and aspartate transaminase were determined. Also, the concentration of malondialdehyde, total antioxidant capacity, the activity of superoxide dismutase and catalase were estimated. Finally, the level of matrix deposition and expression of *alkaline phosphatase (ALP)* and *runt-related transcription factor 2* (*RUNX2)* genes were evaluated.

Results: We observed a concentration-dependent and significant reduction of matrix mineralization based on alizarin red and calcium analysis. Besides, the expression of *ALP* and *RUNX2* gene was down-regulated, and alkaline-phosphatase activity reduced significantly. Also, we observed cell viability reduction but the elevation of lactate dehydrogenase activity and malondialdehyde level. Sodium level was elevated too, whereas the activity of transaminases, oxidative stress enzymes, potassium level, and total antioxidants decreased.

Conclusion: DEHP contamination reduced matrix mineralization due to the down-regulation of the genes involved in osteogenic differentiation and viability reduction via electrolyte and metabolic imbalance as well as induction of oxidative stress.

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1. Introduction

Polyvinyl Chloride (PVC), is used in more than 25% of medical appliances [1]. Since PVC is rigid, phthalate esters are used to facilitate its processing and increase the flexibility of the final product through intra-molecular modification. In the market, there are many plasticizers, but esters of phthalates are more popular and share more than 60% of the market [2].

In medical equipment, out of 25 different types of phthalate esters, di-2-ethylhexyl phthalate (DEHP) is used more than others. Researches have shown the presence of DEHP in the tissues, where medical solutions passing through medical equipment [3-7] and get injected. Also, DEHP is used in food packaging, flooring, and wall coverings [8]. Human toxicity due to DEHP mainly occurs through the ingestion of contaminated food following its processing and packaging. As it was quoted before, humans also come in contact with DEHP when using medical equipment during treatments such as blood transfusion and hemodialysis [9].

The amount of DEHP is around 4.65 mg/L in blood products [10-12], 3.1 mg/L in drug-containing solutions, and 5 mg/L in electrolyte or sugar-based solutions [13-15]. When DEHP is absorbed, it can be accumulated in the adipose tissues, gastrointestinal tract, lungs, kidneys, and liver [16]. DEHP is also found to be accumulated in tissues such as the heart, reproductive tract, muscles, spleen, and brain [17]. Since DEHP crosses the placenta, concern has been raised regarding the infants' health [18]. The number of live births was significantly reduced in pregnant mice exposed to DEHP, and it caused embryonic abnormalities up to 40% [19]. In another study, the gestational exposure of phthalate esters caused bone abnormalities such as the absence of tail bones, incomplete skull bones, and incomplete leg bones in rat pups [20].

In another study, it was shown that the DEHP reduced bone formation in rats due to the downregulation of collagen and *ALP* gene in osteoblasts [20]. Alkaline phosphatase and its ectoenzymes such as tissue non-specific alkaline phosphatase play an essential role in bone mineralization where it provides free phosphate to form hydroxyapatite crystals and also hydrolyzes pyrophosphate that acts as an inhibitor of matrix formation [21]. Osteoblast cells play an essential role in bone remodeling, and it is well known that the cellular back up to form these cells are bone marrow mesenchymal stem cells [22].

Mesenchymal stem cells are programmed to differentiate into different cell linage and play an essential role in repairing damaged bone tissue via osteogenic differentiation [23, 24]. Since DEHP is used in the manufacturing of medical appliances and food packages, the bone marrow mesenchymal stem cells might get intoxicated via contaminated peripheral blood. If the differentiation property of mesenchymal stem cells is affected, then the population of osteoblasts, which are the responsible cells to generate bone matrix, would reduce, and osteoporosis might happen.

Osteoporosis is a worldwide growing clinical problem (affecting over 2 billion people all over the world), and the incidence of osteoporosis is increasing dramatically in the industrialized world [25]. To the best of our knowledge, there is no study about the effect of DEHP as the widely-used phthalate [26] on the differentiation of mesenchymal stem cells. Therefore, in the present research, we investigate its impact on the differentiation of rat bone Marrow Mesenchymal Cells (MSCs) to osteoblast.

2. Materials and Methods

Cell extraction and isolation

Some 6-8 weeks old male Wistar rats weighing 140±20 g were purchased from Pasteur Institute (Tehran, Iran) and kept in the polyethylene cage at 27±3ºC with free access to food and water. To separate the femur and tibia, based on the animal laboratory ethical committee role and regulation, the rats were anesthetized with chloroform (Merck, Germany). Then following the removal of surrounding connective tissues, the two end of the bones were cut and the bone marrow was flushed out with the injection of 2 mL of Dulbecco's modified eagle's medium (DMEM, Gibco, Germany) supplemented with 15% fetal bovine serum (FBS) (Gibco, Germany) and penicillin/streptomycin (Gibco, Germany).

The extracted bone marrow was centrifuged for 5 min at 2500 rpm, then re-suspended in fresh culture media and plated in the culture flask when kept in the incubator at 37ºC with 5% continuous injection of CO2. The supernatant containing mostly red blood cells and non-adherent cells is decanted after 24 h, and fresh culture media was added, and then returned to the incubator. The culture media was replaced every three days until 14 days when the bottom of the flasks was covered with a monolayer of the cells. At this stage, the cells were detached from the flasks using trypsin-EDTA (Gibco, Germany) and washed with Phosphate Buffer Saline (PBS). The cells were re-suspended in fresh culture media and plated in new flasks and incubated. To obtain 90%-95% purity (analyzed by

flow cytometer, Germany, PARTEC Co.), two more passages were run by repeating the trypsinization method.

Osteogenic induction

The osteogenic differentiation of the cells was induced by culturing them in DMEM, containing FBS (15%), streptomycin/penicillin (1%), 1 mM sodium glycerophosphate, 50 µg/mL L-ascorbic acid, and 10-8 M dexamethasone (all the chemicals purchased from Sigma-Aldrich Company). The culture flasks were kept in the incubator at 37ºC with 5% CO_2, where the culture medium was changed every three days for 21 days [27].

Exposure to DEHP

The cells were cultured in a proper culture dish for 24 hours to make sure that the cells were attached to the bottom. Then the monolayer of the cells was treated with 100 and 500 µM of DEHP (Merck, Germany) and incubated for 21 days along with the control group, which was treated only with culture media (without DEHP). The selected concentrations were chosen based on the dose-finding analysis (not shown) from a range of concentrations between 0.5 to 2500 µM. Each sample was analyzed three times, and the mean value was reported.

Cell viability assay

In a microplate, the viability test was performed using (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In this analysis, the mitochondrial succinate dehydrogenase converts yellow tetrazolium into a violet crystal of formazan. To perform the analysis, 10 µL of tetrazolium solution (1 mg/mL of PBS) was added to 100 µL of FBS free culture media and kept for 4 hours in an incubator. After removing the culture media, 100 µL of dimethyl sulfoxide (DMSO) was added to each well, and 30 min later, the blue color solution was transferred to another well. The absorption of the solution was estimated using a microplate reader (SCO, diagnostic, Germany) at 505 nm.

Detection and quantification of mineralization

Alizarin red analysis

Using PBS, the cells were washed and fixed with formaldehyde solution (10% v/v) for 15 min, then 1 mL of Alizarin Red Solution (ARS) (40 mM, pH 4.1) was added to each well of the plate and kept for 20 min at room temperature. Then, 800 µL of acetic acid solution (10% v/v) was added and kept for 30 min with continuous gentle shaking. Next, the loosely attached cells were removed with the cell scraper and transferred to a 1.5-mL tube. The tube was vortexed for 30 seconds and overlaid with 500 µL of mineral oil (Sigma-Aldrich) then heated for 10 min at 85ºC. The content of each tube was kept on ice for 5 min and centrifuged for 15 min at 16000 rpm. Then 500 µL of supernatant was transferred to a new tube, and 200 µL of ammonium hydroxide (10% v/v) was added to neutralize the solution. In a microplate, the absorption of 100 µL of the solution was determined at 405 nm using a microplate reader (SCO diagnostic, Germany).

The absorption of unknown was calculated using linear equation Y=0.6899X+0.0921 with R2=0.9882 from the standard graph (19) plotted by different concentration of alizarin red (about 40 mM solution of ARS was diluted 20 times with a 5:2 solution of acetic acid (10%) and ammonium hydroxide (10%) to give a final concentration of 2 mM. Then a series of 5 different dilutions were made, and the absorption was estimated at 405 nm). In the above equation, Y stands for absorbance and X for concentration (mM) of the Alizarin Red Solution (ARS).

Determination of calcium concentration

After washing the treated and control group of cells once with PBS and then with double distilled water (dH_2O), the monolayer of cells was scraped and collected in a 1.5-mL micro-centrifuge tube followed by determining their weight. Based on the equal weight, the calcium content of the cells was extracted using 50 µL of HCl (0.5 N), and preserving them in the cold for 24 h. Using commercial kit (Pars Azmoon, Iran), the concentration of total calcium was measured colorimetrically with a spectrometer (Model T80+PG instrument manufacturing company, England) at 630 nm after 5 min. In this experiment, the calcium reacts with Arsenazo in neutral pH, and a blue color develops where its intensity is proportional to the calcium concentration. To calculate the concentration of calcium in the samples, a standard graph was plotted using various concentrations of $CaCl_2$ with the help of linear equation Y=0.0154X+0.0256 with R2=0.9958, where Y stands for absorption and X for concentration of calcium (mg/dL).

Cell content extraction

The control and treated cells were kept in osteogenic media for 21 days; then, the cells were washed quickly with Tris-HCl (20mM tris-HCl, pH=7.2) two times. The cells were scraped from the bottom of the flask, and the content was extracted using Tris-HCl. To break the cell membrane, the cell homogenate was kept at -20°C overnight. Eventually, the homogenate was thawed, and after making sure

of complete membrane break down, it was centrifuged at 12000g for 10 min. The total protein content of samples was determined using the Lowry method, based on a linear equation of Y=0.0009X+0.0391, with R2=0.999, drawn from a standard graph using bovine serum albumin as standard. Y stands for absorbance and X for the concentration of protein (µg). The biochemical analysis was run based on the equal amount of protein in each sample.

Determination of alanine transaminase, aspartate transaminase, and lactate dehydrogenase activity

The activity of Lactate Dehydrogenase (LDH), Aspartate Transaminase (AST) and Alanine Transaminase (ALT) was estimated using a commercial kit (Pars Azmoon, Iran), based on the equal amount of the protein, according to company instruction at 340 nm using a spectrophotometer (T80+, PG Instrument Ltd., England).

Determination of alkaline phosphatase activity

The activity of *Alkaline Phosphatase* (*ALP*) was estimated with p-nitrophenyl phosphate as a substrate according to company instruction (Pars Azmoon, Iran) and based on the equal amount of the protein, at 410 nm with a spectrophotometer (T80+, PG Instrument Ltd., England).

Determination of the Na+and K+concentration

Using a flame photometer (Model PFP7, England), the total concentration of sodium and potassium in the extracted samples from the treated and control groups of cells was determined. In flame-photometry, the emission light of Na+and K+when burning is measured using different filters. The concentrations of sodium (µg/mL) and potassium (µg/mL) were calculated by linear formula Y=0.0175X+0.0026 with R2=0.9988 and Y=0.4112X+0.00835 with R2=0.9985, respectively, when a standard graph was plotted using different concentrations of NaCl and KCl. In the above equations, Y represents the absorption of emitted light and X concentration of electrolyte.

Estimation of antioxidant enzymes activity

Determination of superoxide dismutase activity

Using nitroblue tetrazolium (NBT) (Sigma-Aldrich, N6876), the activity of superoxide dismutase (SOD) was estimated. One milliliter of the reaction mixture (6.1 mg NBT, 1.9 mg methionine, 7.9 mg riboflavin, and 3.3 mg EDTA dissolved in potassium phosphate buffer with the final volume of 10 mL) was mixed with 50 µL of cell extract and kept for 10 min in a lightbox. A blank and control were also prepared in the same manner without the addition of the extracted sample. The blank tube was kept at dark and was used to zero adjust the spectrophotometer (Model T80+PG instrument manufacturing company, England). The absorbance of each one of the samples was taken at 560 nm, and the enzyme activity was reported as unit per minute for mg of protein required to cause 50 % inhibition [28].

Determination of catalase activity

The reaction mixture containing 300 µL of H_2O_2 and 200 µL of potassium phosphate buffer (25 mM, pH 7.0) was prepared, and its absorption was adjusted to 0.4 before the measurement was started. The activity of Catalase (CAT) enzyme was estimated based on the elimination of H2O2 absorption for 2 min at 240 nm in the presence of 50 µL of a sample using the spectrophotometer (Model T80+PG instrument manufacturing company the UK). Taking 39.4 Mm−1cm−1min−1 as the extinction coefficient into the account, the activity of CAT was calculated for 1 min [28].

Determination of lipid peroxidation

Lipid peroxidation was determined based on the estimation of Malondialdehyde (MDA) as an indicator by mixing 1 mL of the reaction mixture, containing thiobarbituric acid (0.5%) and trichloroacetic acid (20%) in HCl with 100 µL of sample and keeping it on boiling water bath for 30 min. The temperature of the mixture was reduced by holding it on ice for 15 min then centrifuging it for 15 min at 12000 rpm. The absorption was measured first at 523 nm then at 600 nm using a spectrophotometer (T80+PG instrument manufacturing company, England). Then the values were subtracted from each other, and using the extinction coefficient (1.55×103 $\mu mol^{-1}cm^{-1}$), the concentration of MDA was calculated and reported as µM/mL [28].

Measurement of total antioxidant content

Total Antioxidant Content (TAC) of the samples were measured by mixing 1700 µL of the reaction solution (300 mM sodium acetate buffer (pH 6.3), 10 mM 2,4,6-Tri(2-pyridyl)-s-triazine (Sigma-Aldrich, USA)dissolved in 40 mM Hydrochloric Acid (HCl) and 20 mM Iron chloride), 850 µL distilled water and 150 µL of the sample. The mixture was kept at dark for 10 min, and the absorbance was measured using a spectrophotometer (Model T80+PG instrument Manufacturing Co. England) at 593 nm. Using different concentrations of iron sulfate ($FeSO_4.7H_2O$) (Merck, Germany), a standard graph was plotted, and TAC for each sample was calculated using

Table 1. Primers used for RT-PCR

Gene	Primers	Annealing Temperature (C°)	Product Length
ALP	F: CATGTTTCCTGGGAGATGGTA R: GTGTTGTACGTCTTGGAGAGA	58.4 59.4	144 bp
RUNX2	F: CCGCACGACAACCGCACCAT R: CGCTCCGGCCCACAAATCTC	64.6 64.6	289 bp
GAPDH	F: TCGTCTCATAGACAAGATGG R: GTAGTTGAGGTCAATGAAGGG	56.4 59.4	136 bp

F: forward primer; and R: reverse primer

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linear formulas Y=0.0078X+0.02, R2=0.9923, where Y stands for absorption and X for concentration [28].

Gene expression analysis

Reverse transcription PCR (RT-PCR) is widely used to investigate the effects of many chemical agents on gene expression. The total RNA from osteoblasts treated with 100 and 500 µM of DEHP for 21 days was extracted using a Super RNA extraction kit (YT9080). The concentration of extracted total RNA was determined by spectrophotometer (T80+PG instrument Manufacturing Co. England). The cDNA was made using the BioFACT (BR631-096) kit. Amplification of alkaline phosphatase (*ALP*), glyceraldehyde dehydrogenase (*GAPDH*), and runt-related transcription factor 2 (*RUNX2*) genes were performed using specific primers (Table 1) with Thermo Cycler (Eppendorf master cycler gradient, Eppendorf Co. Hamburg, Germany) programed as follows:

The initial temperature of 95° C for 5 min;

Denaturation temperature of 95° C for 1 min;

The annealing temperature of specific primer according to the Table 1 for 1 min;

Extension/Expansion temperature of 72° C for 1 min;

Final elongation temperature of 72° C for 7 min.

The above cycle was repeated 40 times.

The electrophoretic bands were analyzed using the software. The experiment was repeated three times, and the means were reported statistically.

Data analysis

Data were analyzed with 1-way ANOVA and Tukey test in SPSS v. 16 (Sun Microsystems Inc., USA). Results were presented as Mean±SD, and P<0.05 was considered as the minimum level of significance.

3. Results

Cell viability

The results of the MTT assay showed that the concentrations of 100 and 500 µM of DEHP after 21 days reduced the viability of differentiated MSCs significantly (P<0.05) compared with the control group (Table 2).

Mineralization analysis

Statistical analysis showed that concentrations of 100 and 500 mM of DEHP caused a highly significant (P<0.001) decrease in matrix mineralization compared with the control group based on alizarin red quantitative analysis (Table 3). Microscopic observations also confirm the results of quantitative analyses regarding the degree of matrix mineralization (Figure1).

Table 2. Effect of DEHP on cell viability differentiated MSCs after 21 days of treatment

Dose (µM)	Viability (Based on MTT)
Control	0.47±0.02[a]
100	0.27±0.04[b]
500	0.08±0.01[c]

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Data are presented as Means±SD, and in a column means with different letter codes differ significantly from each other (ANOVA, Tukey test, P<0.05).

Table 3. Effect of DEHP on the mineralization of BMCs based on quantitative alizarin red (mM) staining and matrix calcium deposition (mg/dL)

Dose (µM)	Mineralization (Based on Alizarin Red Assay)	Mineralization (Based on Calcium Concentration)
Control	0.24±0.006[a]	30.91±0.46[a]
100	0.14±0.002[b]	21.96±0.56[b]
500	0.08±00.01[c]	15.18±0.51[c]

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Data are presented as Means±SD, and in a column means with different letter codes differ significantly from each other (ANOVA, Tukey test, P<0.05).

Table 4. Effect of DEHP on total protein content (µg/mL) and activity (IU/L) of aspartate transaminase, alanine transaminase, lactate dehydrogenase, and alkaline phosphatase of MSCs after 21 days of treatment

Dose (µM)	Total Protein	AST	ALT	LDH	ALP
Control	390.32±11.13[a]	297.08±5.29[a]	26.01±0.3[a]	817.53±32.06[a]	450.31±42.11[a]
100	318.10±10.79[b]	270.04±12.57[b]	16.76±2.98[b]	1394.61±16.03[b]	249.96±11.14[b]
500	201.78±25.41[c]	178.64±9.09[c]	6.94±0.99[c]	1779.32±29.74[c]	73.18±10.20[c]

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Data are presented as Means±SD, and in a column means with different letter codes differ significantly from each other (ANOVA, Tukey test, P<0.05).

Abbreviations: AST, aspartate transaminase; ALT, alanine transaminase; LDH, lactate dehydrogenase; ALP, alkaline phosphatase.

However, it should be noted that the effect of DEHP was concentration-dependent, where 500 mM was more effective than 100 mM in reducing the mineralization. Analysis of calcium concentration of the cell-matrix also showed the same result where concentrations of 100 and 500 µM caused a significant (P<0.05) and highly significant (P<0.001) decrease in matrix calcium deposition of the differentiated MSCs respectively (Table 3).

Metabolic activity of the cells

The results showed that the total protein concentration in differentiated cells decreased concentration-dependent and highly significantly (P<0.001) in the treated groups compared to the control group (Table 4). It was also shown that the activity of aspartate transaminase in differentiated cells decreased significantly (P<0.05) due to treatment with 100 µM of DEHP whereas 500 µM of DEHP caused a highly significant (P<0.001) decrease of the activity of the same enzyme in comparison with the control group.

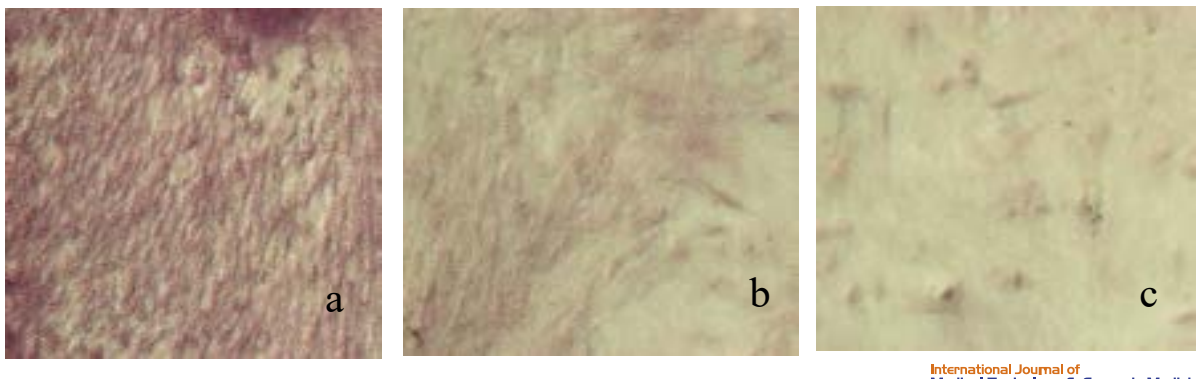

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Figure 1. Alizarin red staining of cell matrix after 21 days of culturing MSCs in osteogenic media

a. control; b. 100 µM of DEHP; c. 500 µM of DEHP

Table 5. Effect of DEHP on sodium (µg/dL) and potassium (µg/dL) levels of MSCs after 21 days of culture in osteogenic media

Dose (µM)	Sodium	Potassium
Control	7.41±0.52[a]	2.41±0.09[a]
100	10.75[b]±1.05[b]	1.67±0.09[b]
500	12.57[b]±1.05[b]	1.28±0.17[c]

Data are presented as Means±SD, and in a column means with different letter codes differ significantly from each other (ANOVA, Tukey test, P<0.05).

Table 6: Effect of DEHP on the TAC (µg/mL) and MDA (µM/mL) as well as activity of CAT (Unit min-1 mg-1 protein) and SOD (Unit min-1 mg-1 protein) of MSCs after 21 days of culture in osteogenic media

Dose (µM)	MDA	CAT	SOD	TAC
Control	0.29[a]±0.01[a]	1.63[a]±0.09[a]	4.32[a]±0.24[a]	21.14±0.33[a]
100	0.39±0.02	1.27[b]±0.09[b]	3.26[b]±0.19[b]	12.94[b]±0.25[b]
500	0.43[c]±0.01[c]	0.61[c]±0.03[c]	2. 11[c]±0.23[c]	9.31±0.32[c]

Data are presented as Means±SD, and in a column means with different letter codes differ significantly from each other (ANOVA, Tukey test, P<0.05).

MDA, malondialdehyde; CAT, catalase; SOD, superoxide dismutase.

However, the decrease in the activity of alanine transaminase and alkaline phosphatase was concentration-dependent and highly significant (P<0.001) following treatment of the cells with different concentrations of DEHP when compared with the control one. The activity of lactate dehydrogenase enzyme in differentiated osteogenic cells increased highly significantly (P<0.001) due to treatment with 100 and 500 µM of DEHP when compared with the control group (Table 3).

Sodium and potassium level

DEHP with concentrations of 100 and 500 µM caused a highly significant increase (P<0.001) in sodium concentration when compared with the control group. However, the amount of potassium of differentiated cells showed a significant decrease (P<0.05) when treatment with 100 and 500 µM of DEHP. It should be mentioned that the reduction of potassium in the treated group with 500 µM was highly significant (P<0.001) when compared with the control group (Table 5).

Evaluation of oxidative stress

Statistical analysis of the data showed that the treatment of the cells with DEHP caused a dose-dependent and significant (P<0.05) decrease of CAT and SOD activity compared with the control group. Activity reduction of CAT and SOD was highly significant (P<0.001) due to the treatment of the cells with a concentration of 500 µM (Table 6). Besides, the reduction of the Total Antioxidant C (TAC) apacity of the cell was concentration-dependent and highly significant (P<0.001) as compared with the control group. It was also observed that the amount of malondialdehyde in the differentiated cells treated with DEHP showed a significant concentration-dependent increase (P<0.05) compared with the control one. More increase in malondialdehyde was observed due to treatment with 500 µM (P<0.001) (Table 6).

Gene expression analysis

To investigate the difference in gene expression in response to DEHP toxicity, RT-PCR analysis was performed using total RNAs obtained from MSCs after 21 days of osteogenic induction (Figure 2 and Table 7). The results demonstrated the down-regulation of *ALP* and *RUNX2* following the treatment of the cells with DEHP (Figure 2). Also, the down-regulation of the gene expression was significant (P<0.05) due to treatment with 100 µM and highly significant (P<0.001) following treatment with 500 µM of DEHP (Table 7). The expression of *GAPDH* was not affected by the treatment of DEHP with either 100 or 500 µM concentration.

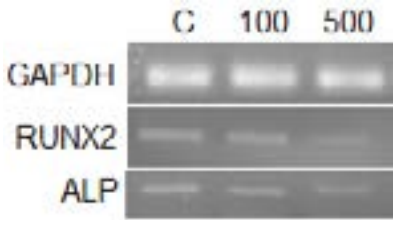

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Figure 2. Expression of genes involved in the osteogenic differentiation processes

BMCs, (C) control, (100) cells treated with 100 µM, and (500) cells treated with 500 µM of DEHP, respectively.

4. Discussion

Following DEHP treatment, the viability reduction based on MTT established that the function of mitochondria (the powerhouse of the cell) was affected. In the MTT assay, the dehydrogenase enzymes, especially mitochondrial dehydrogenase, are responsible for the reduction of yellow tetrazolium and the production of purple formazan crystals [29]. Depending on the effect of DEHP concentration in the study period, the decrease in viability can be attributed to inhibition of the dehydrogenase enzyme activity. Bhat et al. (2013) found out that DEHP with a concentration of 1000 µM reduced the viability of calvarial osteoblasts by 50% after 48 hours. They also reported that the treatment of the cells with DEHP reduced cyclin D1 and CDK2 proteins involved in the cell cycle [20].

In another study, Kanno et al. showed that the concentrations of 10-4 M (100 µM) reduced the viability of pre-osteoblasts (MC3T3-E1) by 50% after three days [30]. Our study on sodium and potassium electrolytes in differentiated osteogenic cells revealed that the concentration of electrolytes was significantly different from the control in 21 days, as sodium increased, and potassium decreased. Although multiple transporters transfer sodium to the outside of the cell [31, 32], the ATP-dependent potassium/sodium pump is the most vital transporter to balance these two electrolytes [33].

This transporter is extensively present in cells and consumes a large amount of intracellular ATP. Due to the activity of this transporter, three sodium ions are transferred out of the cell in exchange for two potassium ions transfer into the cell [33]. Due to the increase in the amount of sodium and the reduction of potassium in the differentiated mesenchymal cells caused by DEHP, this imbalance is probably due to impairment in the performance of potassium/sodium pump, which results in malfunctioning of the cellular, and organelle membrane and decreased cell viability.

Inadequate availability of ATP to balance the electrolyte, i.e., sodium and potassium level, might be a well-documented [33] reason of the membrane potential reverse, which reduces the cell viability [34]. At rest, the cell membrane is not impermeable to sodium ions, and during activity, a more massive amount of this ion enters the cell. This means that the maintenance of resting potential happens through an active extrusion of sodium by the pump at the ATP cost as the energy source. In the present study, the treatment of cells with DEHP increased the activity of the lactate dehydrogenase enzyme, which might be considered as a change in metabolism from aerobic to anaerobic.

On the other hand, the activity of ALT and AST (transaminase) enzymes also decreased significantly with DEHP treatment, which might indicate the unavailability of intermediates to ensure the entry of acetyl-CoA to Krebs cycle that ultimately leads to produce more lactic acid and less ATP. In anaerobic metabolism, the pyruvate, which produced in the glycolysis process, is converted to lactic acid by lactate dehydrogenase enzyme. Thus, glycolysis could be repetitively run to generate ATP in the cell [35] at the cost of carbohydrate expenditure. Siddiqui et al. showed that oral administration of DEHP to mice

Table 7. Effect of DEHP on the expression of genes involved in osteogenic differentiation of BMCs after 21 days of treatment

Gene / Doses	*ALP*	*RUNX2*	*GAPDH*
Control	0.05±0.47[a]	0.08±0.57[a]	0.05±0.93[a]
100 µM	0.04±0.35[b]	0.02±0.33[b]	0.03±0.93[a]
500 µM	0.01±0.08[b]	0.01±0.09[c]	0.05±0.93[a]

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Data are presented as Means±SD, and in a column means with different letter codes differ significantly from each other (ANOVA, Tukey test, P<0.05).

increased the activity of lactate dehydrogenase [36]. The lower ATP generation may result in malfunctioning of the Na+/K+ATPase pump, which results in the membrane potential imbalance and viability reduction.

Palleschi et al. study showed that the treatment of human granulocyte with DEHP increased the number of free radicals [37]. The result of our research also showed that the total antioxidant levels of treated cells decreased. On the other hand, the MDA level, which indicates the oxidation of unsaturated fatty acids in the cell membrane, increased [38]. The oxidative stress is an imbalance between oxidants (free radicals) and antioxidants, which lead to an increase in free radicals that suppress the antioxidant enzyme system [39].

The most basic and the most abundant antioxidant enzyme are catalase and superoxide dismutase, which can reduce free radicals and eliminates the effect of oxidative stress of these anions [39]. In the present study, the treatment of cells with DEHP decreased the activity of these enzymes. Another study also indicated a decrease in the activity of these enzymes [40]. It seems that in addition to electrolyte imbalance and inhibition of metabolic enzyme activity, the induction of oxidative stress could be another reason for the viability reduction of differentiated MSCs.

In the present study, the activity of *ALP* and calcium concentrations significantly reduced after treatment with DEHP (100 and 500 µM) compared with the control group after 21 days. The quantitative and qualitative results of alizarin red staining confirmed these results, which revealed the reduction of the calcium nodules formation in the cell matrix. The most important indices for osteoblast differentiation of mesenchymal cells are alkaline phosphatase activity [41] and calcium deposition [42].

ALP enzyme is a membrane enzyme that breaks down pyrophosphate in the cell, which also allows calcium entry. Since osteoblast plays a significant role in the formation of hydroxyapatite, i.e., Ca5(PO4)3(OH), the presence of phosphate in the cell is essential for the entry of calcium into the cell. Therefore, *ALP* activity is necessary for calcium presence in osteoblasts [43].

Bhat et al. reported that the treatment of rat calvarial osteoblasts with DEHP reduced the activity of alkaline phosphatase and collagen synthesis, decreased expression of *RUNX2*, and TAZ gene [20]. It has been well documented that *RUNX2* regulates the expression of genes involved in the differentiation of mesenchymal cells and plays an essential role in the commitment of mesenchymal cells into the osteoblast lineage [44]. Treatment of the cells with DEHP down-regulated *RUNX2* and *ALP* genes, which ultimately decreased cell differentiation. Therefore, considering the above findings and the confirmation of the other studies, DEHP would probably reduce the viability and kill the osteoblasts, and on the other hand, via down-regulation of *RUNX2* and *ALP* genes reduce the differentiation process of MSCs to osteoblast.

5. Conclusion

The osteogenic differentiation of MSCs to osteoblast was inhibited by DEHP via cellular metabolic imbalance and induction of oxidative stress which reduced their viability. In addition, the study of RUNX2 and alkaline phosphatase genes showed that the reason of matrix mineralization was the down regulation of the genes involved in osteogenic differentiation. According to the present study DEHP might be involved in prevalence of osteoporosis especially in the industrial areas. Therefore, it is strongly recommend that the use of DEAP in a variety of industries, including plastic medical devices, plastic food containers, and so on, should be prohibited even at low concentration.

Ethical Considerations

Compliance with ethical guidelines

The study was approved by Ethics Committee of Arak Medical University (Code: IR.ARAKMU.REC. 1395.365), Arak, Iran.

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Author's contributions

Study design, supervision, data collection, writing final draft of manuscript: Mohammad Hossein Abnosi; Investigation, data analysis, drafting the manuscript: Zahra Aliyari Babolghani.

Conflict of interest

The authors declared no conflict of interest.

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