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Original Article

Diethylhexyl phthalate induced oxidative stress and caused metabolic imbalance in bone marrow mesenchymal stem cells

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ABSTRACT

Introduction: Diethylhexyl phthalate (DEHP) is leaching form polyvinyl chloride to cause animal toxicity. In our previous study, DEHP caused osteoblasts mortality *in vitro*, since rat bone marrow mesenchymal stem cells (MSCs) is the cellular back up for osteoblasts; therefore its effect on MSCs was investigated.

Methods: MSCs were extracted from Westar rats and treated with 0.5 to 2500µM of DEHP for 12, 24 and 48h to study the viability. Then further investigations, including proliferation, cell morphology, sodium and potassium level, concentration of calcium, total protein, activity of metabolic enzymes (ALT, AST, ALP, LDH), malondialdehyde (MDA) level, total antioxidant capacity, activity of superoxide dismutase (SOD) and catalase (CAT) were measured using selected concentration (100, 500 and 1500µM).

Results: The 100µM of DEHP did not change the viability and biochemical factor after 48h but colony forming assay and population doubling number was significantly affected. Th 500µM only reduced the viability at 24 and 48h, while 1500µM caused the significant reduction at all the periods. These two concentrations, caused significant proliferation reduction as well as significant increase in calcium and sodium level, LDH activity and MDA level. In addition, we observed decrease in potassium, total protein, activity of metabolic enzymes and activity of CAT and SOD significantly.

Conclusion: DEHP has reduced viability and proliferation of MSCs through metabolic change, alteration in cellular ultrastructure, ionic imbalance and induction of oxidative stress.

Keywords:
Mesenchymal stem cell
Diethylhexyl phthalate
Oxidative stress
Cell survival
Enzymes assay

Introduction

Rat bone marrow mesenchymal stem cells (MSCs) are pluripotent cells that differentiate into cells, such as osteoblasts, adipocytes and chondrocytes, which can be used in autologous transplantation. These cells are found in bone marrow and other tissues such as adipose, synovial curvature as well as skeletal muscle and may easily be purified and expanded *in vitro* (Eslaminejad, 2007; Lin, 2012; De Sousa et al., 2014). Since MSCs are directly linked to peripheral blood and due to its important

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as cellular backup for osteoblasts, they are considered to be suitable for studying the effect of toxic substances on its differentiation and proliferation ability (De Sousa et al., 2014). On the other hand, since MSCs are playing important role in the bone homeostasis, therefore their well-being is the matter of concern with respect to bone tissue health and related complications.

More than 25% of all plastics used in medical applications are polyvinyl chloride (PVC) (Huber et al., 1996). PVC is used to produce medical devices such as flooring, wiring, medical purpose bags and tubes, syringes, pharmaceutical coatings, blood and plasma bags, dialysis equipment, catheters and gloves, infusion devices and urine bags. PVC is a relatively rigid and fragile polymer and phthalate esters are used as plasticizers in PVC production to facilitate processing and increase its flexibility in the final form by intra-molecular modification. While there are numerous plasticizers in the market, the main used group is the phthalate esters (Alto, 1996). Among 25 different phthalate esters, diethylhexyl phthalate (DEHP) is used more than any other phthalates in medical equipment. In the late 1970s, researches have shown that DEHP was found in the medical solutions via leaching through PVC made medical equipment (Jaeger and Rubin, 1970; Jaeger and Rubin, 1972; Autian, 1973; Hillman et al., 1975). DEHP is not chemically bounded to the PVC polymer and may leach when a medical device is heated or frozen and even when the PVC comes into contact with blood, drugs, or intravenous (IV) fluids. DEHP is extracted out from PVC, either by leaching or after an extracting material (blood, IV fluids) diffuses into the PVC matrix and dissolves the plasticizer (Rock et al., 1986; Nass and Heiberger, 1976; Lundberg and Nilsson, 1994). After absorption, DEHP is widely distributed in the body and since it is highly lipophilic then may accumulate in the organs (International Programme on Chemical Safety, 1992). Adipose tissue, lungs, digestive tract, liver and kidney are the main organs to accumulate DEHP, but this chemical compound has been also observed in the heart, spleen, genital and muscle as well as brain (Kluwe, 1982; European Union risk assessment report, 2008). Adult animal studies showed, high concentration exposure of DEHP resulted in concentration-dependent atrophy of the seminiferous tubules and testes (Singh Rowdhwal and Chen, 2018) as well as impairment of germ cell cyst breakdown, primordial follicle assembly (Liu et al., 2019) and induces apoptosis in granulosa cells (Tripathi et al., 2019). Investigations revealed that the DEHP induced oxidative stress was the main reason to cause renal lipidomic disruption (Gu et al., 2021), apoptosis of primordial follicle (Liu et al. 2019) and oocytes (Ambruosi et al., 2011) and reduced steroidogenesis in granulosa cell (Tripathi et al., 2019).

Investigation showed that, DEHP reduces the viability of rat calvarial osteoblasts (Bhat et al., 2013) and induces apoptosis in MC3T3 osteoblasts and mouse primary calvarial osteoblasts (Sabbieti et al., 2009). Recently, DEHP was found to interfere with osteoblastogenesis of MSCs in the mouse models (Chiu et al., 2018) and rat *in vitro* (Abnosi and Aliyari Babolghani, 2020). To the best of our knowledge there is no literature regarding the effect of DEHP on MSCs. Since in the bone marrow, these stem cells are the cellular backup for generation of osteoblasts therefore, in this study, the MSCs were treated with different concentrations of diethyl hexylphthalate to investigate the viability, proliferation, morphology, some biochemical properties of MSCs and its ability to induce oxidative stress.

Materials and methods

MSCs isolation

In the present experimental investigation male Wistar rats weighing 140±2 g at the age of 6 to 8 weeks were purchased from Pasteur Institute, Iran and housed in the polyethylene box under standard condition of 27±3ºC with enough food and water. The rat was anesthetized using chloroform (Merck, Germany) and sacrificed according to the ethical committee role of Arak University of Medical Sciences (IR.ARAKMU.REC.1395.365). The bones (femora and tibia) were surgically isolated and its connective tissue was removed. After cutting the both end of the bones with a pair of surgical scizer, the bone marrow was taken out by injecting 2ml of Dulbecco's Modified Eagles Medium (DMEM, Gibco, Germany) containing 15% fetal bovine serum (FBS, Gibco, Germany) and penicillin/streptomycin (Gibco, Germany). The extracted bone marrow was centrifuged at 2500rpm for 5min, then the palate was homogenized in 5ml culture media using repeater pipette. The homogenate was poured in the culture flasks and incubated in an atmosphere of 5% CO2 at 37°C. After 24h, the supernatant was discarded and fresh media was added. The culture media was replaced every three days for 14 days

till the bottom of the flask was covered with a monolayer of cell. Then using trypsin-EDTA (Gibco, Germany) the cells were detached from the bottom of the flask, washed with phosphate saline buffer (PBS), centrifuged, homogenized in fresh culture media and divided in two T25 flasks and kept in incubator till the flask was cover with cells. Two more passages were carried out to obtain approximately 95 % purity (based on flowcytometry analysis) then the cells at 3rd passage were used for further investigation (Abnosi and Aliyari Babolghani, 2020).

DEHP treatment

Following the cells being attached to the bottom of the culture dishes, treatment with different concentration of DEHP (0.5, 1, 10, 50, 100, 500, 1000, 1500 and 2500μM) in presence of control group (in absence of DEHP in culture media) was carried out. DEHP was purchased from Merck Company, Germany and each analysis was repeated three times in a bracket model.

Analysis of cell viability

Trypan blue staining

The cells were plated in a 24-well culture flask at a density of 5×10^4 and treated with different concentration of DEHP (as it was mentioned previously) for 12, 24 and 48h. Then the monolayer of the cell was washed with PBS and detached from the bottom of the flask with the help of trypsin/EDTA. The cells were collected in a 15ml tube and centrifuged at 2500rpm for 5min. Following centrifugation, the pallet was re-suspended in culture media and 50μl of the homogenized cell was incubated with the same volume of trypan blue (Sigma-aldrich, America) for 2min at 37°C. The number of viable and death cell (stained blue) was determined using hemocytometer chamber and the percentage of viable cell was reported. Trypan blue enters the cell when its membrane is damaged and under the light microscope the cell which is dead appears to be blue in color (Abnosi and Yari, 2018).

MTT assay

To confirm the trypan blue analysis we used MTT assay (3-(4, 5-dimethy thiazo-2-yl)-2, 5 diphenyl tetrazolium), as these two analysis are based on the different principles. In the present assay, the yellow tetrazolium is converted to blue crystal of formazan by dehydrogenases activity, specially succinate dehydrogenase activity, therefore, more crystal formation indicates the functionality of mitochondria and viability of the cell. The cells at a density of 1×10^4 were cultured in a 96-well plate and the treatment was carried in previously explained group for 12, 24 and 48h. Then, the monolayer of cell was washed with PBS and 10μl of yellow solution of tetrazolium (5mg/ml of PBS) was mixed with 100μl of FBS free culture media in each well of the plate. The plate was incubated for 4h at 37°C, then the media was removed and the plate was washed with PBS to remove the excess of dye. The formed formazan crystals were extracted with 100μl of dimethyl sulfoxide (Sigma Company, Germany) and measurement of the absorbance was taken at 505nm using ELISA reader (SCO diagnostic, Germany). A standard graph was plotted using similar procedure based on known number of viable cells, then the number of viable cells in each sample was calculated using the linear formula Y=0.0175X+0.0018 with R2=0.999. In the mentioned formula Y stands for absorbance and X stands for number of the viable cells (Abnosi and Yari, 2018).

Based on the viability tests, the concentration of 100, 500 and 1500μM of DEHP was selected as 0, 10 and 50 % mortality causing concentration to carry out further investigation at the required time period in each test.

Observation of cell morphology

Cells with the density of 1×10^4 was cultured in a 24-well plate and incubated in a CO_2 incubator for 24h (to make sure of their attachment to the bottom of the each well) the cells were treated with different concentration of DEHP for 48h. To study the morphology of the cell nuclei, each well of the plate was washed twice with PBS and stained with 10μl of Hoechst solution (50μg/ml, Sigma-Aldrich, USA) diluted in 100μl of PBS for 15min in dark at room temperature. Morphology of the cytoplasm was studied after 2min of incubation, using 10μl of acridine orange (5μg/ml) added to 100μl of PBS. After staining the cell, inert fluorescent microscope (Olympus, IX70) equipped with camera (DP72) was used and photograph was taken at 200 magnifications. With the help of Motic Image software (Micro Optical Group Company version 1.2) the diameter of the nuclei and the area of the cytoplasm were measured and reported at μm and μm^2 respectively (Abnosi and Yari, 2018).

Analysis of cell proliferation

Colony forming assay

Cells with the density of 5×10^4 were cultured in a 3 cm sterile plate for 24. After the cells were attached to the bottom of the plate, the culture media was replaced with DEHP contaminated media for 7 and 14 days with every 3 days of media replacement. Then to study the colony forming ability (CFA), the monolayer of the cells was stained with crystal violet solution (0.5 % in methanol) for 15min at room temperature. The plates were photographed using ordinary digital camera (macroscopic study) and light microscope equipped with camera (microscopic study). To estimate the colony diameter a graticule eyepiece was used and the diameter was reported in μm (Abnosi and Yari, 2018).

Population doubling number

To study the population doubling number (PDN), 1×10^4 cells were plated in a 3 cm sterile plate for 24h. After the cells were attached to the bottom of the plate, the culture media was replaced with DEHP contaminated media for 2, 4 and 8 days. Then the plates were washed twice with PBS and using trypsin- EDTA the cells were detached from the bottom of the flasks. Number of the cells was determined using hemocytometer camber and the PDN was calculated using the formula PDN=logN/N0×3.31. In the mentioned formula N0 is the initial number of cells and N is the cell number after 2, 4 and 8 days (Abnosi and Yari, 2018).

Extraction of cell content

After 48h of DEHP treatment, the cells were removed from the flask using trypsine-EDTA and centrifuged at 2500rpm for 5min. The cells were washed with Tris-HCl (20mM tris-HCl, pH=7.2) and freeze overnight to break the cell membrane, then the homogenate was thawed and centrifuged for 10min at 12000rpm. The total protein content of the extract was estimated with the help of Lowry method, using bovine serum albumin as standard. The concentration of samples was calculated using the linear formula Y=0.0009X+0.0391, with R2=0.999. In the mentioned formula, Y stands for absorbance and X represents the concentration (μg) of the total protein in each sample (Abnosi and Yari, 2018).

The cell extract was used in the further analysis to estimate the metabolic enzymatic activity and electrolyte concentration as well as the degree of oxidative damage caused by DEHP based on the amount of malondialdehyde (MDA) and activity of antioxidant enzymes.

ALT, AST and LDH activity

The activity of lactate dehydrogenase (LDH), aspartate transaminase (AST) and alanine transaminase (ALT) enzymes in the cell extracted was determined using commercial kit (Pars Azmoon, Iran). The assay was carried out according to the recommended protocol by manufacturing company. Based on the equal amount of protein the analysis was carried out and measurement was taken at 340nm using spectrophotometer (T80+, PG Instrument Ltd., England).

Alkaline phosphatase activity

The activity of alkaline phosphatase was estimated based on the hydrolysis of p-nitrophenyl phosphate as substrate. The activity determination was carried out according to the commercial kit instructions (Pars azmoon, Iran), based on the equal amount of protein, at 410nm with the help of spectrophotometer (T80+, PGInstrument, Ltd., England).

Calcium concentration

Using commercial kit (Pars Azmoon, Iran), calcium concentration was measured colorimetrically based on the reaction between calcium and arsenazo in neutral pH. The intensity of blue color is proportional to the concentration of calcium which was measured after 5min using spectrophotometer (Model T80+ PG instrument, UK) at 630nm. To calculate the concentration of the samples, we used linear formula Y=0.0154X+0.0256 with R2=0.9958 (Y stands for absorption and X stands for concentration). The linear formula was derived from standard graph using different concentrations of $CaCl_2$ as standard. Finally, the calcium concentration was reported as mg/dl of extract.

Na^+ and K^+ concentration

Using a flame photometer (Model PFP7, England), total concentration of sodium and potassium in the extracted samples was determined. In flame-photometry, the emitted light from Na^+ and K^+ is measured using different filter. A standard graph using different concentration of NaCl and KCl was plotted and the concentration of sodium (μg/ml) and potassium (μg/ml) was calculated using linear formula Y=0.0175X+0.0026 with

R2=0.9988 and Y=0.4112X+0.00835 with R2=0.9985 respectively. In the equations Y stands for absorption and X represent concentration of the electrolytes.

Activity of antioxidant enzymes

Determination of superoxide dismutase (SOD) activity

A reaction mixture containing 6.1mg nito-blue tetrazolium (Sigma-Aldrich, N6876), 1.9mg methionine, 7.9mg riboflavin and 3.3mg EDTA at a final volume of 10ml was prepared in PBS. The 1ml of the reaction mixture was mixed with 50µl of cell extract and kept in a light box for 10min. A tube as blank and another as control free from extracted sample were also prepared in the same manner. The blank tube kept at dark for 10min and was used to adjust the spectrophotometer (Model T80+ PG instrument manufacturing company UK) at zero. Then the absorbance of each sample was taken at 560nm and the activity of SOD was reported as unit per minute for mg of protein required to cause 50 % inhibition (Abnosi and Yari, 2018).

Catalase (CAT) activity determination

Before starting the measurement, absorption of the reaction mixture containing 300µl of H_2O_2 and 200µl of 25mM potassium phosphate buffer with pH 7.0 was adjusted to 0.4. Measurement was taken for 2min based on the elimination of H_2O_2 absorption at 240nm after adding 50µl of sample using spectrophotometer (Model T80+ PG instrument manufacturing company UK). The activity of CAT was calculated for one minute using 39.4Mm $^{-1}$ cm $^{-1}$ min^{-1} as extinction coefficient (Abnosi and Yari, 2018).

Estimation of lipid peroxidation

To estimate the concentration of MDA as end product of lipid peroxidation, 100µl of sample and 1ml of the reaction solution (trichloroacetic acid 20%, thiobarbituric acid 0.5% in HCl) was kept in a boiling water bath for 30min. Immediately the mixture was kept on ice for 15 minutes and then it was centrifuged at 10000rpm for 10min. Using spectrophotometer (Model T80+ PG instrument, UK), first at 523nm then at 600nm, the absorption was measured. To calculate the concentration of MDA in µM/ml, the values were first subtracted from each other and then multiply by extinction coefficient (1.55×103 µmol^{-1} cm^{-1}) (Abnosi and Yari, 2018).

Estimation of total antioxidant

A reaction mixture, composed of 300mM sodium acetate buffer (pH was adjusted to 6.3), 10mM TPTZ (Sigma-Aldrich, USA) dissolved in 40mM hydrochloric acid and 20mM iron chloride, was prepared. Then 1700µl of the reaction mixture was mixed with 850µl distilled water and 150µl of sample was added to the solution and kept in dark for 10min. The absorbance of the solution was measured at 593nm using spectrophotometer (Model T80+ PG instrument, UK). Using different concentration of iron sulfate ($FeSo_4.7H_2O$) (Company Merck, Germany), a standard graph was plotted and total antioxidant capacity of the samples were calculated using linear formulas Y=0.0078X+0.02, R2=0.9923. In the mentioned formula Y represent absorption and X stands for concentration (Abnosi and Yari, 2018).

Statistical analysis

SPSS software (version 16, Sun Microsystems Inc., USA) was used to analyzed data. The analysis was carried out with the help of one-way analysis of variance (ANOVA) and Tukey honestly significant difference test as post hoc test. Results were presented as mean±SD and $P<0.05$ was considered as the minimum level of significance.

Results

Effect of DEHP on cell viability

The treatment of cells at 12, 24 and 48h with DEHP significantly reduced ($P<0.05$) viability based on trypan blue And MTT assay. The results of trypan blue showed that the mean viability of the cells treated with 1500, 2000 and 2500µM was significantly low compared with the control group after 12h ($P<0.05$), but no significant ($P>0.05$) differences was observed difference between these concentrations. In 24h, the significant differences with control was begun ($P<0.05$) at 500µM and it reaches to maximum at 2500µM ($P<0.001$). Although at 48h, the toxicity also starts at 500µM, but it fallows a concentration dependent manner from 500 to 2500µM with respect to control group (Table 1). In addition, the results of MTT assay confirmed the results obtained from trypan blue staining (Table 1).

Based on the viability test, 100, 500 and 1500µM of DEHP and the 48h were selected for further investigation. The 100µM was the lowest concentration which

TABLE 1: Mean percentage (based on trypan blue staining) and number (based on MTT assay) of viable MSCs after treatment with various concentrations of DEHP in different time.

Conc. (µM)	TRYPAN BLUE			MTT (×10³)		
	hours 12	hours 24	hours 48	hours 12	hours 24	hours 48
Control	94.14^{a} ± 0.68	92.57^{a} ± 0.85	93.68^{a} ± 0.59	1.84^{a} ± 259.90	1.91^{a} ± 374.43	1.92^{a} ± 181.22
0.5	93.29^{ab} ± 1.01	92.41^{a} ± 0.39	93.23^{a} ± 0.71	1.84^{a} ± 46.07	1.93^{a} ± 349.41	1.93^{a} ± 342.77
1	93.70^{ab} ± 0.42	92.05^{a} ± 0.90	93.16^{a} ± 0.11	1.84^{a} ± 87.65	1.91^{a} ± 262.53	1.92^{a} ± 238.34
10	93.26^{ab} ± 1.26	91.87^{a} ± 0.16	93.45^{a} ± 0.64	1.84^{a} ± 103.42	1.90^{a} ± 348.89	1.94^{a} ± 347.99
50	92.91^{ab} ± 0.03	91.00^{a} ± 1.00	92.96^{a} ± 0.51	1.85^{a} ± 172.96	1.94^{a} ± 314.3	1.91^{a} ± 158.59
100	93.75^{ab} ± 0.22	90.63^{a} ± 1.08	92.72^{a} ± 0.66	1.84^{a} ± 351.52	1.88^{a} ± 259.04	1.90^{a} ± 174.34
500	93.15^{ab} ± 0.30	83.73^{b} ± 0.42	82.84^{b} ± 1.13	1.81^{a} ± 318.91	1.79^{b} ± 30.49	1.81^{b} ± 86.54
1000	92.36^{abc} ± 1.10	75.98^{c} ± 0.53	48.92^{c} ± 0.40	1.84^{a} ± 385.18	1.70^{b} ± 85.80	1.20^{c} ± 205.16
1500	91.69^{bcd} ± 0.45	73.89^{c} ± 1.18	46.71^{d} ± 0.83	1.79^{ab} ± 131.72	1.71^{b} ± 122.54	1.04^{d} ± 248.52
2000	90.69^{cd} ± 0.26	64.70^{d} ± 0.84	22.22^{e} ± 1.01	1.72^{bc} ± 248.74	1.53^{c} ± 348.30	5.24^{e} ± 31.54
2500	89.97^{d} ± 1.04	66.17^{d} ± 0.45	21.75^{f} ± 0.59	1.71^{c} ± 184.24	1.54^{c} ± 338.86	3.55^{f} ± 236.64

Values are mean±SD. In a column, same letter code indicates no difference in significant value (ANOVA, Tukey test, P<0.05).

TABLE 2: Mean cytoplasmic area (µm²) and nuclei diameter (µm), after 48h of treating MSCs with different concentration of DEHP.

Concentrations (µM)	Nuclear diameter (µm)	Cytoplasmic area (µm²)
Control (0)	16.6^{a} ± 0.48	1955.30^{a} ± 34.30
100	15.9^{a} ± 0.09	1950.72^{a} ± 6.52
500	14.8^{b} ± 0.29	1851.80^{b} ± 37.20
1500	12.8^{c} ± 0.37	1657.00^{c} ± 37.00

Values are mean±SD. In a column, same letter code indicates no difference in significant value (ANOVA, Tukey test, *P*<0.05).

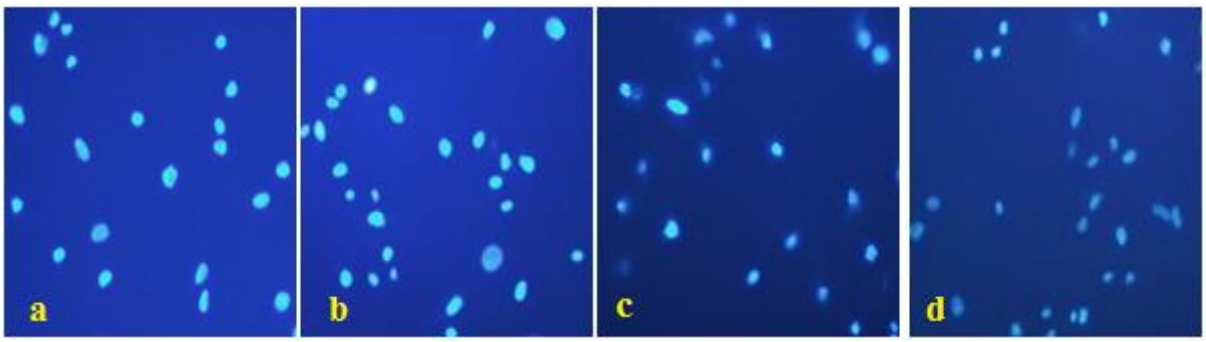

FIGURE 1. Fluorescent images. MSCs were stained with Hoechst, after 48h of treatment. a) control; b) 100µM; c) 500µM; d) 1500µM DEHP. Cell nuclear condensation and deformation was seen after treatment with 500µM and 1500µM when compared with control (X200 magnification).

TABLE 3: Mean diameter (mm) and colony number, after 7 and 14 days of treating MSCs with different concentration of DEHP.

Concentrations (µM)	7 days		14 days	
	Colony diameter (mm)	Colony number	Colony diameter (mm)	Colony number
Control (0)	$0.91^{a} \pm 0.02$	$199.33^{a} \pm 1.52$	$0.80^{a} \pm 0.30$	$255.66^{a} \pm 6.42$
100	$0.74^{b} \pm 0.03$	$150.33^{b} \pm 2.51$	$0.68^{b} \pm 0.01$	$94.00^{b} \pm 3.60$
500	$0.67^{c} \pm 0.02$	$83.33^{c} \pm 3.51$	$0.6^{c} \pm 0.04$	$61.66^{c} \pm 4.16$
1500	$0.00^{d} \pm 0.00$	$0.00^{d} \pm 0.0$	$0.00^{d} \pm 0.00$	$0.00^{d} \pm 0.0$

Values are mean±SD. In a column, same letter code indicates no difference in significant value (ANOVA, Tukey test, $P<0.05$).

TABLE 4: Mean population doubling number, after 2, 4 and 8 days of treating MSCs with different concentration of DEHP.

Concentrations (µM)	2 days	4 days	8days
Control (0)	$2.26^{a} \pm 0.08$	$3.12^{a} \pm 0.12$	$3.72^{a} \pm 0.05$
100	$2.23^{a} \pm 0.07$	$2.44^{b} \pm 0.13$	$2.79^{b} \pm 0.10$
500	$2.07^{a} \pm 0.08$	$1.86^{c} \pm 0.10$	$2.23^{c} \pm 0.07$
1500	$1.73^{b} \pm 0.05$	$1.56^{d} \pm 0.12$	$0.89^{d} \pm 0.09$

Values are mean±SD. In a column, same letter code indicates no difference in significant value (ANOVA, Tukey test, $P<0.05$).

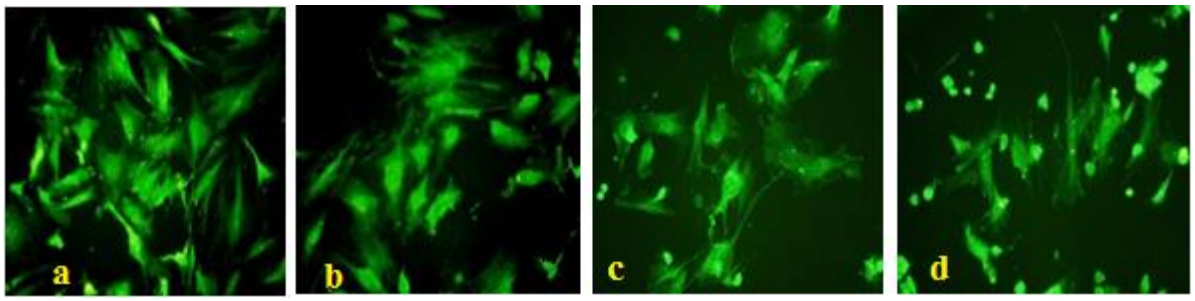

FIGURE 2. Fluorescent images. MSCs were stained with acridine orange after 48h of treatment. a) control; b) 100µM; c) 500µM; d) 1500µM DEHP. Cell cytoplasm morphological differences can be seen after treatment with 500 and 1500µM when compared with control (X200 magnification).

showed on effect on viability, the 500µM caused 12% mortality and the 1500µM reduced the viability almost 50%.

Morphology of the cells

Morphological changes of nucleus including, chromatin condensation and nuclear breakage were observed in the cells treated with 500 and 1500µM of DEHP (Figure 1). Treatment of the cells with 500 and 1500µM of DEHP also caused the cytoplasm shrinkage (Figure 2). In addition to microscopic results, statistical analysis of the data also showed a significant and concentration dependent decrease ($P<0.05$) in the diameter of the nucleus and the area of the cytoplasm of the treated cells (Table 2). The nuclear diameter and cytoplasm area reduction due to DEHP toxicity at higher concentration (1500µM) in was more obvious and highly significant ($P<0.001$). We observed no changes in the morphology of the cells treated with 100µM of DEHP as compared with control group.

Proliferation ability

The CFA showed that the treatment of the cells for 7 and 14 days caused a significant and concentration

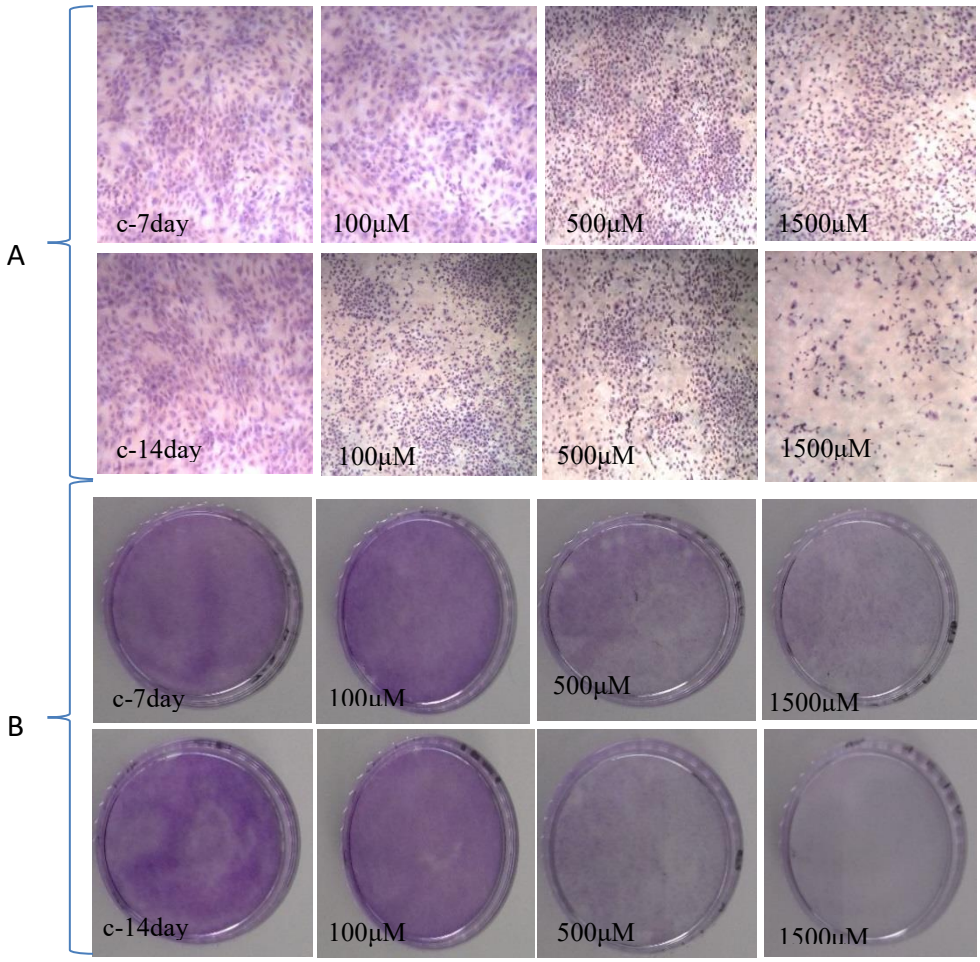

FIGURE 3. A-Colony micrograph images (100 magnifications) and B-camera image of MSCs stained with crystal violet after 7 and 14 days treated with different concentration of DEHP. C- stands for control.

dependent decrease (P<0.05) in number of the colony and its diameter when compared with the control group (Table 3). The effect of 1500µM was more devastating where no colony was observed due to its toxicity. In Microscopic image, the reduction in numbers and diameters of the colonies in the groups treated with DEHP also was confirmed (Figures 3A and B).

The results of PDN showed, only higher concentration (1500µM) could reduce the proliferation ability after 2 days of treatment when compared with the control. Whereas after 4 and 8 days, a concentration dependent significant reduction of the PDN was shown for all the concentrations (Table 4).

Metabolic activity of the cells

The results of biochemical studies showed that the total protein concentration in bone marrow mesenchymal stem cells significantly decreased (P<0.05) in 500 and 1500µM treatment groups compared to control group (Table 5).

Treatment of the cells with 100µM of DEHP showed no effect on the activity of the ALT, AST, ALP and LDH compared to the control group. Meanwhile, the activity of LDH enzyme was observed to increase significantly (P<0.05) in the groups of cells which been treated with 500 and 1500µM of DEHP. But, the activity of ALT, AST and ALP decreased significantly (P<0.05) in comparison to the control and 100µM treated groups when treated with 500 and 1500µM of DEHP. The results of alkaline phosphatase and alanine transaminase activity in the 1500µM treated group showed a highly significant decrease (P<0.00) compared to control and 100µM treated groups (Table 5).

Evaluation of oxidative stress

Statistical analysis of data showed that treatment of the cells with 500 and 1500µM of DEHP caused a significant decrease (P<0.05) in the activity of SOD and

TABLE 5: Mean total protein (µg), activity (IU/L) of enzymes (LDH, AST, ALP, ALT), Total antioxidant (TAC) (µg/ml), malondialdehyde (MDA) (µM/ml), activity of superoxide dismutase (SOD) (Unit min^{-1} mg^{-1} protein) and activity of catalase (CAT) (Unit min^{-1} mg^{-1} protein) after 48h of treatment with different concentration (µM) of DEHP.

Concentration (µM)	Control	100	500	1500
Total protein	360.69^{a}±9.44	343.29^{a}±17.25	299.21^{b}±18.33	204.77^{d}±11.77
AST	248.12^{a}±3.97	234.89^{a}±4.13	197.17^{b}±5.72	193.2^{b}±4.13
ALT	15.48^{a}±0.4	14.70^{a}±0.32	11.57^{b}±0.33	6.94^{c}±0.33
LDH	1691.16^{a}±8.01	1707.19^{a}±8.05	1806.04^{b}±9.25	2217.48^{c}±72.28
ALP	439.89^{a}±15.16	424.87^{a}±4.55	367.38^{b}±6.06	294.25^{c}±12.88
MDA	0.16^{a}±0.01	0.16^{a}±0.01	0.20^{b}±0.01	0.24^{c}±0.01
CAT	1.41^{a}±0.06	1.35^{a}±0.12	0.97^{b}±0.09	0.83^{b}±0.05
SOD	3.20^{a}±0.32	3.09^{a}±0.24	$2. 47^{b}$±0.08	1.86^{c}±0.09
TAC	14.01^{a}±0.32	13.28^{a}±0.26	11.57^{b}±0.19	10.42^{c}±0.41

Values are mean±SD. In a column, same letter code indicates no difference in significant value (ANOVA, Tukey test, $P<0.05$).

TABLE 6: Mean concentration of intracellular calcium (mg/dl), sodium (µg/dl), and potassium (µg/dl) after treating MSCs for 48h with DEHP.

Concentrations (µM)	Ca^{2+}	Na^{+}	K^{+}
Control (0)	1.37^{a}±0.07	2.49^{a}±0.22	0.66^{a}±0.09
100	1.34^{a}±0.04	2.42^{a}±0.25	0.69^{a}±0.08
500	1.66^{b}±0.06	3.78^{b}±0.52	0.22^{b}±0.04
1500	2.79^{c}±0.07	5.6^{c}±0.51	0.27^{b}±0.00

Values are mean±SD. In a column, same letter code indicates no difference in significant value (ANOVA, Tukey test, $P<0.05$).

CAT as well as the level of total antioxidant capacity compared to control and the group of the cell treated with 100µM after 48h. On the other hand, the level of MDA in treated samples with 500 and 1500µM showed a significant increase ($P<0.05$) compared to the control group. It is necessary to mention that the changes caused by 1500µM was highly significant ($P<0.001$) compared to the control and the group treated with 100µM of DEHP (Table 5).

Intracellular electrolyte level

The results of electrolyte analysis showed that calcium and sodium content in bone marrow mesenchymal cells treated with 500 and 1500µM of DEHP were significantly increased compared to control group ($P<0.05$). The effect of same concentrations of DEHP on potassium level was in opposite direction compared to sodium level. However, the 100µm of DEHP did not show any significant changes ($P>0.05$) with respect to sodium, potassium and calcium level (Table 6).

Discussion

Based on the trypan blue staining and MTT assay only high concentrations of DEHP (from 1500 to 2500 µM) at 12h reduced viability. Whereas, at 24 and 48h, in addition to high concentrations, low concentrations (from 500 onwards) also caused the same effect. Viability reduction after 12h of treatment with high concentration of DEHP revealed the potential of this pollutant to cause cell toxicity even at the short time. In addition to high concentration, the low concentrations also have its own profound effect to cause cell toxicity following long

exposure. In our research, treatment of the cells with 100μM of DEHP for 48h was safe and no harm was tracked except at proliferation analysis, which has been carried out for longer time (ie days). Other researchers have confirmed our results when working on cells other than MSCs. Kanno et al. (2004) showed that the 100μM of DEHP caused 50% viability reduction of MC3T3-E after 3 days. In addition, benzyl butyl phthalate, another family member of DEHP at concentration greater than 100μM reduced the proliferation of human osteosarcoma cells (Liu and Chen, 2010). In our previous study we also found that the 100μM treatment of the MSCs for 21 days caused 43% viability reduction of MSCs derived osteoblasts (Abnosi and Aliyari Babolghani, 2020).

In the present study, the results of PDN confirm the reduction of the cell population number after 4 and 8 days treatment with 100 and 500μM, while 1500μM reduced the number of cell population from 2 days. Also, the CFA at 7 and 14 days revealed that the treatment of cells with different concentrations of DEHP caused reduction of colony forming ability of MSCs. Based on these results, we might say, that in the long period (more than 2 days), in addition to viability also the proliferation ability of MSCs has been significantly affected by this environmental pollutant which proves the necessity of time requirement to complete the effectiveness of the DEHP toxicity. Sabbieti et al. (2009) showed that benzyl-butyl phthalate and D-butyl phthalate from the phthalate family induced caspase-dependent apoptosis in MC3T3-E1 cells. Also, it was quoted by another research, that the decrease in the number of the cells was associated with decrease in the levels of cyclic D1 and CDK2 proteins, which regulate the cell cycle (Wang et al., 2018). DEHP has been used as plasticizer to soften PVC which is used to make food containers and medical equipment and other commercial products (AuBuchon et al., 1988), thus continuous utilization of these industrial products was shown to be a certain rout of toxicity as DEHP gets released in to the biological solutions (Jaeger and Rubin, 1972; Hillman et al., 1975). DEHP contamination would activate the programed cell death to reduce MSCs viability and proliferation ability, therefore to fine the mechanism following analysis has been conducted.

As it was proved that the low concentration of DEHP has no harm in the short time, thus due to its lipophilic property and its probable tissue accumulation at longer time, we discuss the effect of high concentration at short time to pin point the mechanism of its toxicity. In the present study, after treating the cells with 500 and 1500μM for 48h, it was observed that the morphology changed and decrease in nuclei diameter and cytoplasmic area were revealed. Since the morphology of a cell depends on the cytoskeleton arrangement, therefore the morphological changes following DEHP treatment probably are due to disruption of skeletal protein in the nuclei and cytoplasm. The cytoskeleton abnormalities might be due to changes in the amount of protein (Gross and Kinzy, 2007) or/and amount of calcium (Magee et al., 1987) within the cell. It should be noted that the calcium not only acts as a regulator of intracellular signaling waterfalls (Berridge et al., 2003) but also plays an important role in the cytoskeleton arrangement (Magee et al., 1987). In addition, the change in concentration of this electrolyte induces apoptosis in the cell (Hajnóczky et al., 2006) which causes the morphological changes. Study by Marchetti et al. (2002) showed that osteoblast from Py1a cells become more round and lose their appendix in comparison with control after being treated with phthalate ester. These changes are due to reduction of protein production and miss arrangement which causes configuration abnormality of the actin protein in the Py1a cell cytoplasm. It was also shown that the phthalate esters break down the microfilament which contribute to the collapse of the nuclear structure by affecting the actins (Sabbieti et al., 2009) since lamina and actin (nuclear micro-filament) are interconnected. In the present study, DEHP showed a significant decrease in protein level (data not shown) and potassium content while sodium concentration increased significantly. On the other hand, Dhanya et al. (2003) showed that DEHP inhibit sodium and potassium pumps, causing elevation of intracellular calcium. In addition, Palleschi et al. (2009) showed that DEHP can open transient receptor potential canonical (TRPC) channels (ie, TRPC3, TRPC4 and TRPC6), which increases the entry of calcium from the outside of the cell. As a result, increase in intracellular calcium can trigger the formation of mitochondrial pores and wastage of proton along mitochondrial membrane which cause the mitochondrial membrane rupture and induction of apoptosis via activation of caspase 9 (Hucho and Levine, 2007). Also, in a recent study it was shown that the DEHP caused mitochondrial dysfunction and swelling in the isolated kidney mitochondria (Ashari

et al., 2020) which might be the reason of its ability to induce apoptosis. The study on ALT and AST enzymes also showed that the activity of these enzymes was reduced especially at 1500µM. Reduction of the activity of aspartate transaminase might cause the decrease in oxaloacetate content which can reduce the production of ATP and cause an energy crisis in the cell. On the other hand, the increase in LDH activity after treating the cells with DEHP, showed an increased consumption of pyruvate through anaerobic metabolism where the amount of energy production is much lower than that of aerobic metabolism. Therefore, reduction of transaminases activity and impairment of acetyl-CoA catabolism by Krebs cycle caused the concentration of pyruvate to increase, which facilitates its conversion to lactic acid via lactate dehydrogenase. The study by Siddiqui and Srivastava (1992) showed the treatment with 1000 mg/kg of DEHP increased LDH activity. In the present study, the activity of alkaline phosphatase enzyme also decreased significantly with DEHP treatment which might be the result of metabolic acidosis. The reduction of enzyme activity due to DEHP toxicity has been mentioned by other researchers (Wang et al., 2018).

It was observed that DEHP increases the MDA level and reduces the total antioxidant capacity of the MSCs at concentration of 500 and 1500µM. This means, DEHP has been able to oxidize the unsaturated fatty acids in the cell membrane via production of free radical which in addition caused the elimination of total antioxidant capacity of the cell. Abnosi and Aliyari Babolghani (2020) found that the DEHP after 21 day caused elevation of MDA and reduction of total antioxidant capacity even at low concentration (100µM). They also found the antioxidant enzymes (CAT and SOD) activity was significantly reduced. Furthermore, we showed, DEHP caused an inhibition of the antioxidant enzymes CAT and SOD activity which was confirmed by Ashari et al. (2020) which has been reported recently. Studies showed that the DEHP induce oxidative stress in liver, brain, red blood cells (Dhanya et al., 2003), rat ovarian granulosa cells (Tripathi et al., 2019) as well as renal lipidomic disruption (Gu et al., 2021) which confirm the result of the present study. Antioxidant enzymes are the first line of defense against the reactive oxygen species, which along with other cellular antioxidant ameliorate the oxidative power of the free radicals. Therefore, DEHP induced oxidative stress which caused the lipid peroxidation of cell and organelle membrane might be the prime reason of cell death due to viability and proliferation reduction in MSCs. As the membrane phospholipids undergo oxidation at double bounds of unsaturated fatty acids, the membrane loses its integrity and functionality that bring about energy wastage, electrolyte imbalance, enzymes malfunctioning which finally cause the cell death.

Conclusion

The results of the present study revealed, that the effect of DEHP on the viability and proliferation of bone marrow mesenchymal cells is very complicated. The mechanisms of cell mortality due to DEHP can be defined as 1) electrolyte imbalance 2) metabolic shift 3) induction of oxidative stress and 4) cytoskeleton miss-arrangement. In today's medicine, MSCs are a promising hope for treatment of many diseases. Extraction and expansion of these cells for medical purpose takes place in the culture dishes made of PVC, since DEHP is presented in the medical and industrial equipment then the regenerative medicines conducted by MSCs are in danger. Also, presence of this environmental pollutant in many industrial products such as food container may increase the chance of osteoporosis since MSCs are cellular backup of the osteoblasts. Therefore, we strongly recommend a series of study to be conducted to fine the safe exposure limit of this environmental pollutant on MSCs *in vivo*. Finally, we suggest the usage of DEHP in various industries such as medical, food packaging and personal care products to be minimized.

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Conflict of interest

There are no conflicts of interest.

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