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Research Article

Di-ethanolamine Causes the Mortality of Bone Marrow Mesenchymal Stem Cell Due to Metabolic Imbalance and Oxidative Stress

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Abstract

Background: Cell toxicity due to diethanolamine (DEA) is well known but no data are available regarding its mechanism. The present study investigated the cell viability and proliferation ability of rat bone marrow mesenchymal stem cells (BMSCs) treated with DEA.

Methods: At 3rd passages, BMSCs were treated for 12, 24, and 48 hours with 0.025 to 16 mM of bis(2-ethylhexyl) phthalate. The cell viability was estimated using the trypan blue and MTT, then 1, 4, and 16 mM, and 48 hours were selected as well. Next, other parameters were determined, including proliferation ability, cell morphology, sodium and potassium levels, as well as the concentration of calcium, total protein, and the activity of metabolic enzymes (i.e., alanine transaminase [ALT], aspartate transaminase [AST], and lactate dehydrogenase [LDH]). Finally, malondialdehyde (MDA), total antioxidant capacity (TAC), and the activity of antioxidant enzymes such as superoxide dismutase and catalase were measured based on the aim of the study.

Results: Based on the results, the viability reduced significantly from 0.6 mM at 12 hours and 0.2 for 24 and 48 hours ($P<0.05$). In addition, the proliferation ability showed a significant reduction ($P<0.05$) while the activity of LDH, ALT, and AST increased significantly ($P<0.05$). The level of electrolytes at 1 mM treatment demonstrated no change ($P>0.05$) whereas 4 mM concentration caused a decline in the calcium level while increased the sodium significantly ($P<0.05$). The results further revealed that the level of MDA increased although the activity of antioxidant enzymes and TAC represented a significant reduction ($P<0.05$). No change was detected in the morphology of nuclei while cytoplasm shrinkage and roundness were observable.

Conclusion: In general, the findings of this study showed that DEA reduced the viability and proliferation of BMSCs via metabolic change, electrolyte imbalance, and the induction of oxidative stress.

Keywords: Bone marrow mesenchymal stem cells, Di-ethanolamine, Cell viability, Cell proliferation, Oxidative stress

Introduction

Diethanolamine (DEA), as a secondary amine, is a dangerous environmental pollutant which contains two molecules of ethanol (1). This chemical compound, which is produced from ethylene oxide and ammonia, has been used in different industries since 1945 (2). The annual production of DEA is 1.5 million tones (3) and is utilized in the production of soaps, surfactants, washing liquids, detergents, cosmetics, shampoos, and pharmaceutical and metal industries (4).

Due to industrial use, this chemical compound enters the soil, air, water, and waste products (5-7), thus human beings are exposed to DEA via different roots. There is evidence of dermal abortion after 5-30 minutes after using body lotion containing 0.1% of DEA (8-11). In addition, it was reported that tissues such as the liver, spleen, kidneys, and brain can accumulate high concentrations of DEA (12,13). Different analyses revealed that the DEA oral treatment of rats via drinking water for 13 weeks caused microcytic anemia, the loss of kidney function, myelin sheath destructions in the brain and spinal cord, the disruption of seminiferous tubules, liver damage, and skin inflammation (14,15). Further, some studies reported that the DEA caused microcytic anemia, reduced erythrocyte, and reticulocyte number, as well as hemoglobin and hematocrit levels while increasing the hemolysis of red blood cells (16,17). Moreover, Niculescu et al studied the effect of 3 mM of DEA on mouse neural precursor cells and found that the DEA reduced the concentration of intracellular choline and caused proliferation reductions via apoptosis induction (18). Other studies have shown that DEA reduces the testicular steroidogenesis in male mice and inhibits phosphatidylcholine synthesis in Chinese hamster ovary cells (19,20). The cytotoxicity of DEA has

been mainly related to the alteration of choline hemostasis and membrane malfunctioning with the outcome of its carcinogenesis (13,21). According to our recent report, DEA at 1 and 4 mM concentrations reduced the osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) and induced cellular stress in the osteoblasts (22). BMSCs, with their proliferation and differentiation properties are considered as a cellular backup for bone repair and remodeling via osteogenic differentiation. Due to the interaction between the peripheral blood and bone marrow, these cells are found in the blood circulation, and researchers believe that BMSCs in addition to bones play an important role in the repair of tissues such as the heart and the liver (23,24). It should be mentioned that DEA with its hydrophilic property highly easily enters the blood and other tissues such as bone marrow. Therefore, it might cause the toxicity of bone marrow stem cells including BMSCs. Considering an increase in the use of DEA in different industries, humans and other animals are in danger as they are exposed to such pollutants. Different *in vivo* and *in vitro* studies have been conducted to investigate DEA toxicity (19,21,25-31) although, to the best of our knowledge, no report has been published on the effect of DEA on BMSCs. Given that BMSCs are extremely important in bone repair and renovation, this experimental study aimed to investigate the effect of DEA on the viability, proliferation, and the activity of enzymes involved in cell metabolism, as well as its ability for inducing oxidative stress.

Materials and Methods

Bone Marrow Mesenchymal Stem Cell Isolation

Male Wistar rats weighing 140 ± 20 g at the age of 6-8 weeks were used in the present investigation. The animal was purchased from Pasteur Institute, Iran and housed in the polyethylene box under the standard condition of 27 ± 3 ℃ with enough food and water. The rat was then anesthetized using chloroform (Merck, Germany) and sacrificed according to the Ethics Committee role of Arak University of Medical Sciences. The bones (femora and tibia) were surgically isolated, and its connective tissue was removed and then the bone marrow was flushed out by injecting 2 mL of Dulbecco's modified eagle medium (Gibco, Germany) containing 15% fetal bovine serum (Gibco, Germany) and penicillin/streptomycin (Gibco, Germany) when both ends of the bones were removed with a pair of surgical scizer. The extracted content was centrifuged for 5 minutes at 2500 rpm, then the cell pellet was homogenized in 5 mL culture media using a repeater pipette. Next, the homogenate was poured in culture flasks and kept in an incubator with 5% CO_2 at 37°C. The culture medium was replaced with the fresh one every three days for 14 days until the flask was covered with a monolayer of cells. Then, the cells were detached and centrifuged at 250 rpm using trypsin/ethylenediaminetetraacetic acid (EDTA, Gibco, Germany). To subculture the cells, the pellet was homogenized in a fresh culture medium and divided into two T25 flasks. Two more passages were carried out to enrich the BMSCs up to 95% purity (based on flow cytometry analysis). The subculture in the 3rd passage was used for further analysis.

Exposure to Diethanolamine

The cells at third passage were individually treated with culture media containing different concentrations (i.e., 0.025, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1, 2, 3, 4, 8, and 16 mM) of DEA (Merck, Germany). The treatment time for viability tests was 12, 24, and 48 hours, after which, the exposure time of 48 hours and concentrations of 1, 4, and 16 mM with 30, 50, and 80% of cell mortality were selected for further analysis based on the results of these tests. The exposure time for colony-forming assay and population doubling time due to their procedure was considered for 7 and 14 days as well as 2, 4, and 8 days, respectively. 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 concentrations of DEA (as it was mentioned previously) at different time intervals. First, the monolayer of the cell was washed with phosphate buffer saline (PBS) and then the cells were detached and collected in a 15 mL tube using trypsin/EDTA, followed by centrifugation at 2500 rpm for 5 minutes. After centrifugation, the cell pellet was re-suspended in culture medium and 50 µL of the homogenate was incubated at 37°C with the same volume of trypan blue (Sigma-Aldrich, America) for 2 minutes. The number of viable and dead cells (stained blue) was determined using the hemocytometer chamber and the percentage of viability was calculated as well.

MTT Assay

To confirm the trypan blue analysis, MTT assay (3-(4, 5-dimethy thiazo-2-yl)-2, 5 diphenyl tetrazolium) was used since two analyses are based on the different principles. The cells at a density of 1×10^4 were cultured in a 96-well plate and the treatment was carried out as it was explained previously. The monolayer of the cell was washed with PBS and 10 µL of tetrazolium (5 mg/1 mL of PBS) was mixed with 100 µL of fetal bovine serum free culture media in each well of the plate. The plate was incubated for 4 hours at 37°C, and then the medium was removed and washed with PBS. The crystals of formazan were extracted with 100 µL of dimethyl sulfoxide (Sigma-Aldrich, USA) and the absorbance was taken at 505 nm using the enzyme-linked immunosorbent assay reader (SCO diagnostic, Germany). A standard graph was plotted using a similar procedure

based on known number of viable cell. Then, the number of viable cells in each sample was calculated using the linear formula Y=0.0175X+0.0018 with R^2=0.999, where Y and X stand for absorbance and the number of viable cells.

Morphology

Cells with the density of 1×10^4 were cultured in a 24-well plate and incubated at 5% CO_2 for 24 hours, and then were treated with different concentrations of DEA for 48 hours. To study the morphology of cell nuclei, each well of the plate was washed twice with PBS and stained for 15 minutes with 100 µL of PBS containing 10 µL of the Hoechst solution (50 µg/mL, Sigma-Aldrich, USA). The morphology of the cytoplasm was investigated with acridine orange (5 µg/mL) for 2 minutes (the working solution was prepared by adding 10 µL of the acridine orange 100 µL of PBS. The morphology was analyzed using the inverted fluorescent microscope (Olympus, IX70) equipped with a camera (DP72), and a photograph was taken at 200 magnifications. In addition, measurements were conducted using Motic Image software (Micro-Optical Group Company, version 1.2) the diameter of the nuclei and the area of the cytoplasm were reported in µm and μm^2, respectively.

Cell Proliferation Analysis

Cell proliferation was determined using two analyses as follow:

1. Colony Forming Assay

Cells with a density of 5×10^4 were cultured in a 3 cm sterile plate for 24 hours. Further, the attached cells were treated with DEA contaminated media for 7 and 14 days with every 3 days of media replacement. To study the colony-forming ability (CFA), the monolayer of the cells was stained with the crystal violet solution (0.5% in methanol) for 15 minutes at room temperature. Furthermore, photography was carried out using an ordinary digital camera (macroscopic study) and a light microscope equipped with a camera (microscopic study). To estimate the colony diameter, a graticule eyepiece was used and the diameter was reported in µm.

2. Population Doubling Number

The population doubling number (PDN), which refers to the total number of times the cells have doubled, was estimated when 1×10^4 cells were plated in a 3 cm sterile plate for 24 hours. The cells were treated with DEA contaminated media for 2, 4, and 8 days. Then, the plates were washed twice with PBS, and the cells were detached using trypsin/EDTA. The number of cells was determined using a hemocytometer camber and the PDN was calculated using the formula PDN = logN/N0×3.31 (32). In the above-mentioned formula, N0 is the initial number of cells and N denotes the cell number after 2, 4, and 8 days.

Extraction of Cell Content

After 48 hours of DEA treatment, cells were removed from the flask and centrifuged for 5 minutes at 2500 rpm. Then, they were washed with tris-hydrochloric acid (20 mM tris-HCl, pH=7.2) and frozen overnight to break the cell membrane. Then, the homogenate was thawed and centrifuged for 10 minutes at 12000 rpm. The total protein content of the extract was estimated by the Lowry method using bovine serum albumin as the standard. The concentration of samples was calculated using the linear formula Y=0.0009X+0.0391 with R^2=0.999. In the above-mentioned formula, Y and X represent absorbance and the concentration (µg) of the total protein in each sample, respectively.

Alanine Transaminase, Aspartate Transaminase, and Lactate Dehydrogenase Activity

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

Calcium Concentration

Using the commercial kit (Pars Azmoon, Iran), the calcium concentration was colorimetrically measured based on the reaction between calcium and arsenazo at a neutral pH value. The intensity of the blue color is proportional to the concentration of calcium, which was measured after 5 minutes using a spectrophotometer (T80+) at 630 nm. To calculate the concentration of samples, linear formula Y=0.0154X+0.0256 with R^2=0.9958 was used (Y and X stand for absorption and concentration, respectively), which was derived from the standard graph using different concentrations of $CaCl_2$ as the standard. Finally, the calcium concentration was reported as mg/dL of the extract.

Na+ and K+ Concentration

The total concentration of sodium and potassium in the extracted samples was determined using a flame photometer (Model PFP7, England). In flame-photometry, the emitted light from Na+ and K+ is measured using a different filter. A standard graph using different concentrations of NaCl and KCl was plotted, and then the concentration of sodium (µg/mL) and potassium (µg/mL) was calculated using linear formula Y=0.017X+0.0053 with R^2=0.996 and Y=0.316X+0.0001 with R^2=0.999, respectively. In the equations, Y and X indicate absorption and the concentration of electrolytes, respectively.

Activity of Antioxidant Enzymes

Superoxide Dismutase Activity

Using nitro-blue tetrazolium (NBT, Sigma-Aldrich, N6876), a reaction mixture containing 6.1 mg NBT, 1.9 mg methionine, 7.9 mg riboflavin, and 3.3 mg EDTA at a final volume of 10 mL was prepared in PBS. Next, 1 mL of the above-mentioned mixture was mixed with 50 µL of the cell extract and kept in a lightbox for 10 minutes. A tube as blank and another as the control (both tubes contained no extracted sample(were also prepared in the same manner. The blank tube was used to adjust the spectrophotometer (T80+) at zero. Then, the absorbance of each sample was taken at 560 nm and the activity of superoxide dismutase (SOD) was reported as unit per minute for mg of the required protein for 50% inhibition.

Catalase Activity

Before starting the measurement, the absorption of the reaction mixture (300 µL of H_2O_2 and 200 µL of 25 mM potassium phosphate buffer with a pH of 7.0) was adjusted to 0.4. After the addition of 50 µL of the sample, the measurement was taken for 2 minutes based on the elimination of H_2O_2 absorption at 240 nm using a spectrophotometer (T80+). Eventually, the activity of CAT was calculated for one minute using 39.4 Mm^{-1} cm^{-1} min^{-1} as the extinction coefficient.

Estimation of Lipid Peroxidation

To estimate the concentration of malondialdehyde (MDA) as a lipid peroxidation indicator, 100 µL of the sample, and 1 mL of the reaction solution (trichloroacetic acid 20% and thiobarbituric acid 0.5% in HCl) were kept in a boiling water bath for 30 minutes. Then, the mixture was immediately kept on ice for 15 minutes and centrifuged for 10 minutes. Using a spectrophotometer (T80+), the absorption was measured at 523 and 600 nm, respectively. To calculate the concentration of MDA in µM/mL, the values were subtracted from each other and then multiplied by the extinction coefficient (1.55×103 μmol^{-1} cm^{-1}).

Estimation of Total Antioxidant

The total antioxidant content (TAC) of the cell was estimated using the FRAP method. A reaction mixture, composed of 300 mM sodium acetate buffer (the pH value was adjusted to 6.3), 10 mM tripyridyl-s-triazine (Sigma-Aldrich, USA) dissolved in 40 mM HCl, and 20 mM iron chloride, was prepared as the FRAP solution. Additionally, 1700 µL of this solution was diluted with 850 µL distilled water and then 150 µL of the sample was added to the diluted FRAP solution and kept in dark for 10 minutes. In addition, the absorbance of the solution was measured at 593 nm using a spectrophotometer (T80+). Using different concentrations of iron sulfate (FeSo4.7H2O, Merck Company, Germany), a standard graph was plotted, and the total antioxidant capacity (TAC) of samples was calculated using linear formulas Y=0.0078X+0.02, R^2=0.9923. In the mentioned formula, Y and X represent absorption and concentration, respectively.

Statistical Analysis

SPSS (version 16, Sun Microsystems Inc., USA) was used to analyze data by the one-way analysis of variance (ANOVA) and the Tukey honestly significant difference test as post hoc test. The results were presented as the mean ± standard deviation, and $P<0.05$ was considered as the minimum level of significance.

Results

Cell Viability

According to the trypan blue staining assay, a significant ($P<0.05$) dose-dependent reduction of cell viability was observed from 0.6 mM at 12 hours of treatment when compared with the control group. Further, a significant concentration reduction ($P<0.05$) of cell viability was found from 0.2 mM onward at 24 and 48 hours. Based on the results, the highest mortality was detected in the group of cells treated with 16 mM of DEA at all treatment time intervals (Table 1). The results of trypan blue analysis were also confirmed by MTT assay (Table 1).

Proliferation Ability Based on PDN and CFA

The PDN showed a significant ($P<0.05$) concentration-dependent reduction with 1 and 4 mM of DEA. It was observed that 4 mM caused a highly significant ($P<0.001$) reduction at 2, 4, and 8 days when compared with the control group. At the highest concentration of DEA (16 mM), the cells were detached from the bottom of the culture dish, therefore, no result was obtained and the PDN was reported as zero (Table 2).

The statistical analysis of data (Table 2) also demonstrated that 1 and 4 mM of DEA at days 7 and 14 caused a highly significant ($P<0.05$) decline in the number of the colony when compared with the control group while only the reduction of diameter was significant ($P<0.05$). In addition, macroscopic (Figure 1) and microscopic (Figure 2) observations of the BMSCs confirmed the statistical results. At 16 mM, no colonies were formed and no data were recorded accordingly.

With respect to viability and proliferation results which revealed the high mortality of 16 mM treatment, the data of this concentration was no more included in further analysis.

Morphology of the Cells

Following the Hoechst staining, the nuclei of the cells appeared in blue. Based on the obtained data, treated BMSCs with 1 and 4 mM of DEA caused no significant ($P>0.05$) change in the diameter of nuclei (Table 3). The microscopic observation confirmed the statistical results of the nuclear morphology (Figures 3B and 3C). In spite

Table 1. Mean Percentage of Cell Viability Based on Trypan Blue Exclusion Assay and the Mean Number of the Viable Cell Based on MTT Assay Following Treatment With Different Concentrations of DEA at Different Time Intervals

Concentration (mM)	% of Viability Based on Trypan Blue Staining			Number of the Viable Cell Based on the MTT Assay (×10³)		
	12 hours	24 hours	48 hours	12 hours	24 hours	48 hours
0	96.30^{a}±0.1	95.77^{a}±0.65	96.42^{a}±0.49	16.51^{a}±322.63	18.71^{a}±244.90	18.91^{a}±211.76
0.025	95.97^{a}±0.1	94.95^{a}±0.39	95.77^{a}±0.13	16.44^{a}±235.29	18.57^{a}±122.44	18.24^{a}±89.85
0.05	96.10^{a}±0.17	94.43^{a}±0.31	95.81^{a}±0.43	16.32^{a}±212.08	18.82^{a}±29.41	18.38^{a}±311.27
0.1	96.22^{a}±0.75	94.43^{a}±0.23	95.62^{a}±0.19	16.42^{a}±433.59	18.70^{a}±88.23	18.10^{a}±433.59
0.2	95.90^{a}±0.82	86.64^{b}±0.43	84.87^{b}±0.41	16.18^{a}±206.58	16.24^{b}±117.64	15.59^{b}±391.66
0.4	94.68^{a}±0.78	86.05^{bc}±0.38	84.67^{b}±0.41	16.08^{a}±411.76	16.12^{b}±323.98	15.32^{b}±269.56
0.6	87.83^{b}±0.57	84.80^{c}±0.75	84.39^{b}±0.37	15.18^{b}±301.86	15.95^{b}±391.66	14.83^{bc}±244.90
0.8	86.71^{b}±0.82	79.87^{d}±0.63	78.62^{c}±0.73	15.11^{b}±205.89	14.36^{c}±179.70	14.04^{c}±148.03
1	83.85^{c}±0.62	76.81^{e}±0.73	69.00^{d}±0.22	13.32^{c}±176.47	13.22^{d}±277.99	12.85^{d}±58.82
2	79.95^{d}±0.79	73.28^{f}±0.33	65.95^{e}±0.44	12.75^{c}±189.90	12.51^{e}±237.72	12.26^{d}±58.83
4	64.91^{e}±0.60	65.91^{g}±0.79	49.19^{f}±0.32	10.67^{d}±376.36	10.34^{f}±301.86	9.34^{e}±449.27
8	52.11^{f}±0.80	55.20^{h}±0.38	26.67g±0.43	8.77^{e}±222.70	8.67^{g}±212.08	6.99^{f}±148.03
16	46.45^{g}±0.69	32.73^{i}±0.44	20.18h±0.67	6.91^{f}±235.30	5.24^{h}±206.58	4.30^{g}±334.48

Note. DEA: Di-ethanolamine; MTT: Dye compound 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromidefor; Values are indicated as means ± standard deviations, and means with the same letter code do not differ significantly from each other within a column (ANOVA, Tukey's test, $P<0.05$).

Table 2. Effect of Different Concentraions of DEA on the Proliferation Ability of BMSCs Based on Mean Population Doubling Number and Mean Colony Fromng Assay (The number and diameter (μm) of the colonies) at Different Time Intervals

Concentration (mM)	Population Doubling Number			Colony Forming Assay			
				Number of the Colony		Diameter of the Colony	
	2 days	4 days	8 days	7 days	14 days	7 days	14 days
0	3.03^{a}±0.08	3.41^{a}±0.07	4.20^{a}±0.03	133.33^{a}±7.50	222.33^{a}±6.50	0.71^{a}±0.02	0.85^{a}±0.02
1	2.67^{b}±0.04	3.01^{b}±0.03	3.94^{b}±0.04	44.67^{b}±0.58	100.00^{b}±2.00	0.63^{b}±0.02	0.75^{b}±0.03
4	2.18^{c}±0.12	2.67^{c}±0.04	2.90^{c}±0.05	24.67^{c}±2.52	52.00^{c}±4.00	0.54^{c}±0.03	0.64^{c}±0.01
16	0.00^{d}±0.00	0.00^{d}±0.00	0.00^{d}±0.00	0.00^{d}±0.00	0.00^{d}±0.00	0.00^{d}±0.00	0.00^{d}±0.00

Note. DEA: Di-ethanolamine; BMSCs: Bone marrow mesenchymal stem cell; Values are represented as means ± standard deviations, and means with the same letter code do not vary significantly from each other within a column (ANOVA, Tukey's test, $P<0.05$).

Table 3. Mean Nuclear Diameter and Cytoplasm Area of BMSCs Treated With Different Concentrations of DEA

Concentration (mM)	Mean Area of the Cytoplasm	Mean Diameter of Nucleus
0	1721.03^{a}±10.50	15.06^{a}±0.37
1	1568.18^{b}±7.44	14.43^{a}±0.18
4	1648.02^{c}±3.54	14.50^{a}±0.23

Note. DEA: Di-ethanolamine; BMSCs: Bone marrow mesenchymal stem cell; Values are demonstrated as means ± standard deviations, and means with the same letter code do not differ significantly from each other within a column (ANOVA, Tukey's test, $P<0.05$).

of nuclear morphology, a highly significant ($P<0.001$) concentration-dependent reduction was observed in the cytoplasm area when the cells were treated with 1 and 4 mM of DEA (Table 3). Furthermore, the microscopic observation (Figures 3E and 3F) of the stained cell represented the morphological changes of cytoplasm, including shrinkage and roundness as compared with the control one.

Activity of Metabolic Enzymes

The activity of LDH, ALT, and AST in the groups of cells treated with 1 and 4 mM of DEA increased significantly ($P<0.05$) in a concentration-dependent manner when compared to the control one. It should be mentioned that the elevation in the activity of these enzymes was highly significant ($P<0.001$) at 4 mM of DEA (Table 4).

Electrolyte Level

The level of calcium and sodium showed no significant ($P>0.05$) difference when BMSCs were treated with 1 mM of DEA although 4 mM treatment caused a significant ($P<0.05$) reduction in calcium compared to the control and 1 mM groups (Table 5). With respect to potassium concentration, a significant increase ($P<0.05$) was found in both treated groups in comparison with the control group (Table 5).

Oxidative Stress

Based on data analysis, the treatment of BMSCs with 1 mM of DEA caused no significant ($P>0.05$) changes

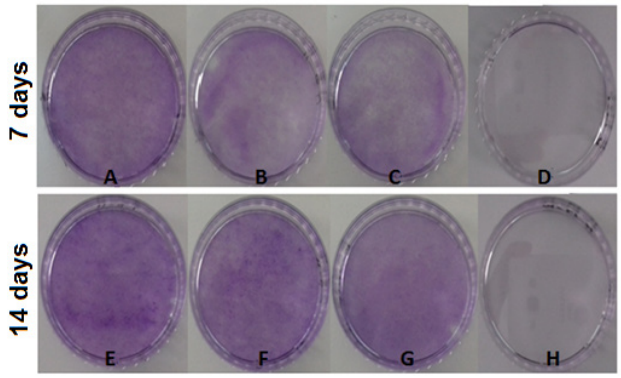

Figure 1. Macroscopic Observation of Colonies Formed by BMSCs Stained With Crystal Violet(A) Control (7 days of treatment), (B) 1 mM DEA treated group, (C) 4 mM DEA treated group, (D) 16 mM DEA treated group, (E) control (14 days of treatment), (F) 1 mM DEA treated group, (G) 4 mM DEA treated group, (H) 16 mM DEA treated group (ordinary camera).
Note. DEA: Di-ethanolamine; BMSCs: Bone marrow mesenchymal stem cells.

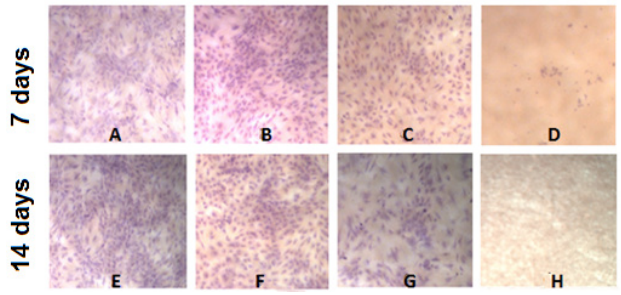

Figure 2. Microscopic Observation of Colonies Formed by BMSCs Stained With Crystal Violet (A) Control (7 days of treatment), (B) 1 mM DEA treated group, (C) 4 mM DEA treated group, (D) 16 mM DEA treated group, (E) control (14 days of treatment), (F) 1 mM DEA treated group, (G) 4 mM DEA treated group, and (H) 16 mM DEA treated group (magnification 40).
Note. DEA: Di-ethanolamine; BMSCs: Bone marrow mesenchymal stem cells.

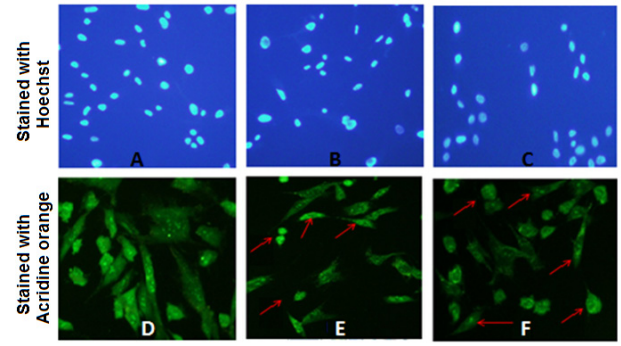

Figure 3. Florescent Staining of BMSCs Stained With Hoechst (A) control, (B) 1 mM DEA treated group, (C) 4 mM DEA treated group stained with acridine orange, (D) control, (E) 1 mM DEA treated group, and (F) 4 mM DEA treated group.
Note. DEA: Di-ethanolamine; BMSCs: Bone marrow mesenchymal stem cells. Arrows show shrinkage and roundness of the cytoplasm in treated groups (magnification 200).

in the level of TAC and MDA, as well as the activity of SOD while it significantly reduced the activity of CAT ($P<0.05$). Regarding the 4 mM concentration of DEA, a significant ($P<0.05$) reduction was observed in the TAC level and the activity of antioxidant enzymes (i.e., SOD and CAT). Conversely, the same concentration of DEA caused a significant ($P<0.05$) increase in the MDA concentration (Table 6).

Discussion

Although DEA at 12 hours of treatment caused a viability reduction in BMSCs with 0.6 mM of DEA, the viability reduction at 24 and 48 hours was started at 0.2 mM. It might be expressed in terms of IC50, namely, the concentration at which 50% of the cell growth was inhibited (33), which at 12 hours was equal to 16 mM. However, the IC50 for 24 and 48 hours is 10 and 4 mM, respectively. Therefore, it is noticeable that the toxicity of DEA depends on the treatment time indicating that the longer the treatment leads to the lower concentration of its toxicity. The trypan blue and MTT principles are based on different logics. More precisely, the trypan blue relies on the integrity of the cell membrane (34) while the MTT assay proves the well-function of the mitochondria (35). In the present study, both the analyses with different principles confirmed each other, therefore, the BMSCs treated with DEA lost their viability due to the disruption of the cell membrane and mitochondrial malfunctioning. Some studies reported that DEA causes choline deficiency and inhibits Phosphatidylcholine synthesis (20,27,29) which is the main phospholipid of cell and mitochondrial membrane (36). Therefore, the viability reduction based on trypan blue and MTT assay might be due to the

Table 4. Mean Activity (IU/L) of LDH, ALT, and AST After the Treatment of BMSCs With Different Concentrations of DEA

Concentration (mM)	LDH	ALT	AST
0	$1282.73^{a}\pm13.42$	$11.93^{a}\pm0.04$	$91.55^{a}\pm0.99$
1	$1369.86^{b}\pm11.12$	$14.51^{b}\pm0.60$	$112.69^{b}\pm1.69$
4	$1391.40^{c}\pm3.20$	$15.22^{b}\pm0.66$	$132.44^{c}\pm8.93$

Note. LDH: Lactate dehydrogenase; ALT: Alanine transaminase; AST: Aspartate transaminase; DEA: Di-ethanolamine; BMSCs: Bone marrow mesenchymal stem cell. Values are indicated as means ± standard deviations, and means with the same letter code do not differ significantly from each other within a column (ANOVA, Tukey's test, $P<0.05$).

Table 5. Mean Concentration of Calcium, Sodium, and Potassium After Treating BMSCs With Different Concentrations of DEA

Concentration (mM)	Calcium (mg/dL)	Sodium (µg/dL)	Potassium (µg/dL)
0	$1.53^{a}\pm0.13$	$1.67^{a}\pm0.52$	$0.329^{a}\pm0.067$
1	$1.48^{a}\pm0.10$	$1.67^{a}\pm0.52$	$0.446^{b}\pm0.067$
4	$1.11^{b}\pm0.15$	$1.97^{a}\pm0.52$	$0.446^{b}\pm0.067$

Note. DEA: Di-ethanolamine; BMSCs: Bone marrow mesenchymal stem cell. Values are shown as means ± standard deviations. Means with the same letter code do not vary significantly from each other within a column (ANOVA, Tukey's test, $P<0.05$).

Table 6. Mean Concentration of (TAC) and MDA, as well as the Activity of CAT and SOD After Treating BMSCs With Different Concentrations of DEA

Dose	TAC (μM mg^{-1} of Protein)	MDA (μM mg^{-1} of Protein)	CAT (Unit $min^{-1}mg^{-1}$ of Protein)	SOD (Unit $min^{-1}mg^{-1}$ of Protein)
0 mM	7.56^{a}±0.12	0.110^{a}±0.013	0.719^{a}±0.014	2.26^{a}±0.17
1 mM	7.30^{a}±0.26	0.125^{a}±0.042	0.609^{b}±0.026	2.10^{a}±0.17
4 mM	6.23^{b}±0.14	0.200^{b}±0.006	0.525^{c}±0.014	1.56^{b}±0.09

Note. TAC: total antioxidant capacity; MDA: malondialdehyde; CAT: catalase; SOD: superoxide dismutase; DEA: Diethanolamine; BMSCs: bone marrow mesenchymal stem cell. Values are indicated as means ± standard deviations. Means with the same letter code do not differ significantly from each other within a column (ANOVA, Tukey's test, $P<0.05$).

impairment of cell and mitochondrial membrane function.

In addition, it was the same with the proliferation ability of cells when both the PDN and CFA analyses reduced due to the DEA treatment. Although the elevation of cell proliferation in the liver tumor of $B6C3F_1$ mice was observed due to the dermal application of DEA (13), in the present study, DEA reduced the population growth in a concentration-dependent manner at different treatment times (i.e., 2, 4, and 8 days). The PDN in a certain time period is a factor which expresses the increase in the number of healthy individuals in a population. Accordingly, if the PDN reduces, the population growth also reduces and it might be the sign of senescence which causes the sudden reduction of the population over the longer period (37). On the other hand, the results of this study showed that DEA reduced the CFA of the cell which is an important factor for expressing the production of similar daughter cells from the same parent. In the CFA analysis, not only a decrease was observed in the colony number representing the reduction of the cell number but also in the diameter of colonies demonstrating the population density. More precisely, a reduction in the diameter of the colony means the reduction of the proliferation ability of a single cell which is inherited by the offspring for producing an abnormal population with the same ancestor (38). Therefore, CFA reductions in the course of time might produce a smaller population with reproductive malfunctioning. The reproductive ability of the cell from one side depends on the capability of internal organelles and biochemical balance, as well as the micro-environment where nourishes the cell. In our investigation, the micro-environment of culture media for control and DEA treated cells were similar. Thus, organelles' capability and biochemical balance are the main affected factors. Therefore, the present study particularly focused on the ability of DEA for oxidizing the membrane unsaturated fatty acids, the metabolic ability of the cell, and electrolyte balance to pinpoint DEA cytotoxicity.

The change in the activity of lactate dehydrogenase from one side and the aminotransferases (i.e., ALT and AST) from the other side showed the metabolic alteration from aerobic to anaerobic (39). Considering that the glucose in the culture media (Dulbecco's modified eagle medium) is the main available energy source to BMSCs, the main metabolic pathway is considered to be glycolysis which its end-product is converted to acetyl-CoA. Moreover, the acetyl-CoA enters the tricarboxylic acid cycle to produce electron carriers which, finally, produce ATP for cellular activity in the presence of oxygen through the electron transport chain (40). DEA caused mitochondrial malfunctioning (based on MTT results) as a result of which, the electron transferring process failed to happen properly. Therefore, the cells should convert pyruvate to lactate instead of acetyl-CoA to generate more NAD^{+} so that to ensure the progression of glycolysis via the elevation of LDH activity. In the present investigation, in addition to the elevation of LDH activity, an increase was observed in AST and ALT activity which is responsible for the production of alanine and aspartate via the amination of pyruvate and oxalate (41). Although metabolic changes from aerobic to anaerobic metabolism help the cell to generate ATP, it should be considered that the ATP production via anaerobic metabolism is far lesser than aerobic. Accordingly, the metabolic shift finally brings the cell to a poor energy state affecting the viability and proliferation ability of the cell.

DEA caused potassium elevation in addition to a metabolic shift. Based on the results, the concentration of sodium remained unchanged thus the change in potassium concentration could not be due to the effect of DEA on the ATP-dependent sodium/potassium pump. The sodium/potassium pump transports sodium and potassium simultaneously in a different direction (42), therefore, the change in the potassium concentration must be due to the effect of DEA on the other transporter independent from the sodium concentration. Given that there was a reduction in the calcium concentration while an elevation of potassium due to DEA, it seems that electrolyte imbalance is another reason for losing membrane integrity (based on trypan blue staining).

In addition to electrolyte imbalance, our investigation revealed DEA-induced oxidative stress in BMSCs. Although 1 mM concentration at 48 hours only caused a significant reduction in CAT activity, the 4 mM reduced the tricarboxylic acid, along with the activity of CAT and SOD. On the other hand, the MDA concentration as the lipid peroxidation indicator (43) increased significantly in addition to a reduction in antioxidative factors. Antioxidant enzymes, along with other non-enzymatic antioxidants are responsible for wiping out free radicals

from the cells. In the case of a decrease in antioxidant enzyme activity or the diminution of the non-enzymatic antioxidant concentration, free radicals attack biomolecules such as membrane lipids, proteins, and nucleic acids (44) impairing the cell function and causing apoptosis. Additionally, the membrane of the cell and its organelles are disrupted and the cell viability decreases in the case of lipid peroxidation. To the best of our knowledge, this is the first report which points out the ability of DEA to induce oxidative stress although Panchal and Verma indirectly pointed to the oxidative ability of DEA (19) and correlated that to choline deficiency.

Along with the impairment of membrane integrity, oxidative stress may cause more complex consequences such as protein malfunctioning and rearrangement (44) affecting cell morphology. In addition, cytoplasm morphology changed, and roundness, membrane breakage, and cytoplasm shrinkage were observed following treatment with DEA. It has also been reported that the reduction in calcium concentration due to DEA can be the reason for cell cytoskeleton miss arrangements (45). The dermal administration of DEA to pregnant rats and rabbits caused slight microcytic anemia with abnormal red blood cell morphology and hematological changes in rat and rabbit dams, respectively (10). In addition to the above-mentioned study, DEA caused morphological abnormality in the hepatoma fish cell line due to the retention of fat (steatosis) in these cells (21).

Conclusion

In conclusion, the DEA caused metabolic shifts from aerobic to anaerobic, electrolyte imbalance, and induced oxidative stress leading to cytoplasm morphological changes and proliferation, as well as viability reductions. Considering that BMSCs are the main cellular back-up for bone repair and remodeling, it is recommended that more investigation be conducted with respect to DEA cytotoxicity regarding BMSCs.

Authors' Contributions

MHA contributed to the conception, design, and interpretation of data and SH carried out the experimental work and ran the statistical analysis. Both authors read and approved the final manuscript.

Conflict of Interest Disclosures

There is no conflict of interests.

Ethical Issues

The Ethical Committee role of Arak University of Medical Sciences approved the study (No. IR.ARAKMU.REC.1395.367).

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