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Research Article

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Catechin Hydrate Mitigates DEHP Toxicity by Attenuating Oxidative Stress in Mesenchymal Stem Cells

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Abstract

Since di-2-ethylhexyl phthalate (DEHP) from medical bags and tubes induces oxidative stress in bone marrow mesenchymal stem cells (BMSCs). Therefore, the aim was to use Catechin hydrate (CH) as a natural antioxidant to prevent lipid peroxidation. In this experimental study, BMSCs were treated with 0.063, 0.125, 0.25, 1, and 10 µM of CH for 4 days. Based on its effect on cell viability, 0.25 µM of CH was selected for further analysis. The cell was treated with 0.25 µM of CH as well as 100 and 500 µM of DEHP individually and in combination for 4 and 8 days to study cell viability, population doubling number (PDN), total protein, malondialdehyde (MDA), total antioxidant capacity (TAC), catalase (CAT) and superoxide dismutase (SOD) activity, nucleus and cytoplasm morphology and expression of Nrf2 and NFkB genes. Viability and proliferation ability of BMSCs were significantly ($p<0.0001$) decreased by DEHP in a dose and time-dependent manner, while CH caused a significant increase ($P<0.0001$). DEHP decreased ($P < 0.0001$) total protein, TAC, and the activity of antioxidant enzymes, and increased ($P < 0.001$) the MDA level, as well as causing morphological changes. In addition, DEHP caused a significant decrease in NF-κB and an increase in Nrf2 expression. Although CH in simultaneous treatment was able to completely compensate for the toxic effect of DEHP at 100 µM, especially at 8 days, when compared to the control, it could not prevent the toxic effect at 500 µM. In addition, CH was able to compensate for the imbalance of the NFkB/Nrf2 caused by DEHP. The consumption of fruits, vegetables, and especially tea, which contains a low concentration of CH, may be an alternative medicine for patients undergoing hemodialysis and blood transfusion.

Keywords: Catechin hydrate; Di-2-ethylhexylphthalate; Bone marrow mesenchyme stem cells; Oxidative stress; NFkB/Nrf2.

1. Introduction

Catechin, a secondary metabolite of plants, is present in a variety of natural sources, including tea leaves, grape seeds, and the wood and bark of acacia [1]. In Asia and other parts of the world, plant leaves, such as those used in green and black tea, are widely consumed, serving as excellent sources of catechins. Following ingestion, the concentration of catechin in micromoles is measured in human blood serum [2], which reinforces the antioxidant properties of the plasma. No significant side effects have been reported following oral consumption of 800 mg/kg of catechin in the form of epigallocatechin gallate

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capsules under fasting conditions [3]. Therefore, catechin is considered safe and has shown a variety of applications in medicine, cosmetics, the food industry, and beyond. In the medical field, it has been utilized to prevent the effects of UV radiation on extracellular matrix degradation [4]. Additionally, it has protected against skin damage by activating collagen synthesis [5]; furthermore, catechin serves as an important antioxidant [6].

Catechin hydrate (CH), a potent plant antioxidant [7], inhibits free radical chain reactions by decreasing reactive oxygen species (ROS) such as hydroxyl radicals ($HO^{\bullet}$), hydroxide ions (OH^{-}), triplet oxygen ($^{3}O_2$), superoxide anions ($O_2^{\bullet-}$), peroxide ions (O_2^{-}), hydroperoxyl (ROOH), and nitric oxide ($NO^{\bullet}$). Since ROS is produced naturally in cells, it must be neutralized by the cell's antioxidant defense system, which includes enzymes such as catalase, superoxide dismutase, and glutathione peroxidase, as well as non-enzymatic compounds like vitamin C and glutathione, which combat the ROS [8]. ROS accumulation from external toxicants leads to biological hindrances and malfunctions. If the cell's antioxidant system is insufficient to neutralize these ROS, the application of exogenous plant antioxidants, such as CH, can bolster the system to prevent biological complications.

Di-2-ethylhexyl phthalate (DEHP) is the most widely used plasticizer among its various family members [9]. It is added to polyvinyl chloride (PVC) to enhance the flexibility of products such as plastic coverings, pastes, coloring agents, coating materials, adhesives, wall coverings, plastic tablecloths, plastic shoes, dolls, car upholstery, medical tubing, and blood storage bags [9, 10]. Since DEHP does not chemically bond with PVC, it easily diffuses into the environment, particularly with temperature fluctuations, leading to widespread contamination of the surrounding area [11]. Considering its omnipresence, the general population is exposed to this environmental pollutant through contaminated water, foods (especially a high-fat diet), plastics, or other products that contain DEHP. This environmental pollutant can enter the human body even during certain medical procedures. For example, blood products stored in plastic bags may contain 5-38 mg/L of DEHP [12, 13], as well as the plastic tubing used in dialysis procedures or respiratory aids for kidney failure or lung dysfunction. Medical equipment containing DEHP allows this chemical to enter patients' bodies through the bloodstream or respiratory airways [14].

In our previous investigation, it was revealed that DEHP reduces the viability of rat bone marrow mesenchymal stem cells (BMSCs) in a concentration-dependent manner when treated with concentrations ranging from 100 to 250 µM over 48 hours, attributed to membrane lipid peroxidation. While a concentration of 100 µM shows no significant changes in viability, nuclear diameter, or cell metabolism, its toxic effects increase with prolonged treatment time [15, 16]. We also found that the mechanism of DEHP toxicity involves the induction of caspase-dependent apoptosis and cell cycle arrest at the G1 stage, resulting from elevated P53 gene expression and the induction of oxidative stress [17].

BMSCs, serving as a vital cellular backup for osteoblasts, are directly exposed to DEHP-contaminated peripheral blood. Given the widespread use of DEHP, BMSCs are exposed to this environmental pollutant; therefore, safeguarding BMSCs from oxidative stress induced by DEHP is crucial for the well-being of the general population and for patients undergoing medical procedures such as hemodialysis and blood transfusions. Our previous investigation revealed that DEHP induces oxidative stress, leading to lipid peroxidation of BMSC membranes. To date, no reports have been published indicating the use of catechin hydrate to mitigate the oxidative effects of this environmental pollutant to protect stem cells. Therefore, this study aims to utilize CH as a potent natural antioxidant to mitigate the toxic effects of DEHP on BMSCs.

2. Methods and materials:

2.1. Extraction and rat bone marrow cells culture

This experimental study received approval from the Ethical Committee of Arak University (approval number IR.ARAKMU.REC.1401.077). Wistar rats, sourced from the Pasteur Institute (Tehran, Iran), were housed in the animal facility at Arak University (Arak, Iran) and allowed to consume standard food under normal temperature and lighting conditions. After one week, the animals were sacrificed via cervical dislocation, and their tibias and femurs were surgically removed. Both ends of each bone were cut, and the marrow was

extracted by injecting 2 ml of culture media (DMEM containing 15% fetal bovine serum and 1% penicillin/streptomycin). The bone marrow content in the tube was centrifuged at 250 g for 5 minutes, and the cells were re-suspended in a T25 flask with fresh culture media and placed in a CO2 incubator. Every three days, the culture media was replaced until a monolayer of cells covered the bottom of the flask. Subsequently, the cells were harvested using trypsin-EDTA, washed with phosphate-buffered saline (PBS, 20 mM, pH 7.2), and subcultured in a new T25 flask with fresh media [17, 18]. This sub-culturing was performed two additional times, and the purity of the cells was assessed using a flow cytometer (Germany, PARTEC (PAS)) before further analysis. The cells were confirmed to be mesenchymal stem cells based on their ability to differentiate into osteoblasts and chondrocytes. They also exhibited surface antigens CD73, CD90, and CD103, while being negative for surface antigens CD34, CD45, and CD14 (see supplementary file 1). All culture materials mentioned above were procured from Gibco, Germany, and the entire procedure was conducted under sterile conditions.

2.2. Viability and proliferation of BMSCs

2.2.1. Selection of an effective concentration

Since trypan blue dye only penetrates damaged cell membranes, dead cells appear blue and can be counted in comparison to live cells. The trypan blue exclusion method was employed to assess cell viability in the presence of different concentrations of CH (0.063, 0.125, 0.25, 1, and 10 µM), with the results compared to those of a control group. Fifty thousand cells were cultured in each well of a 24-well plate, and treatment commenced once the cells had covered 70% of the plate's bottom. After 4 days, cells were detached using trypsin-EDTA and homogenized in the culture media. The homogenized cell sample (50 µL) was mixed with an equal volume of trypan blue (Sigma-Aldrich, USA) at a concentration of 40 mg/ml in phosphate buffer. After 2 minutes of incubation, cell counting was performed using a hemocytometer chamber, and the percentage of viability was recorded [18]. Additionally, the proliferation ability of the BMSCs in the presence of DEHP, CH, and a combination of DEHP + CH was calculated using the population doubling number (PDN) formula: PDN = log(N/N) + 3.32. In this formula, N represents the initial number of cultured cells, while N' (where N' denotes the final number of harvested cells) [19].

2.3. Experimental groups:

Based on the viability and proliferation tests conducted in this study, we selected a concentration of 0.25 µM CH for further analysis, both individually and in the presence of 100 and 500 µM of DEHP, as determined by previous studies [15-17]. Additionally, all analyses were conducted over 4 and 8 days to assess the effectiveness of CH at different time intervals, in the presence of a control group that received only culture media.

2.4. Morphological analysis

The nuclear and cytoplasmic morphology of cells treated with CH and DEHP, either individually or in combination, was examined using Hoechst (5 mg/ml in PBS) and acridine orange (1 mg/ml in PBS), respectively. The plates were washed with PBS, and then 10 µL of each fluorescent dye was added to separate wells containing 100 µL of PBS. The plates were incubated for 5 minutes and subsequently photographed using a digital camera (Olympus, DP72). Analysis of nuclear diameter (µm) and cytoplasmic area (µm²) was performed using Motic software (Micro Optical Group Company version 1.2) [18].

2.5. Extraction of Cell content

After 4 and 8 days of treatment, the cells were harvested using trypsin-EDTA, washed, and suspended in Tris-HCl buffer (TB) (20 mM, pH 7.2). The cell membranes were then lysed by freeze-thawing and centrifuged at 12,000 g for 10 minutes. Protein concentration was estimated using the Lowry method, and a standard graph was constructed using bovine serum albumin as the standard. The total protein concentration was then estimated using the linear equation $Y = 0.0056X + 0.0382$, $R^2 = 0.9854$, where Y represents the absorbance and X represents the protein concentration (µg). Given that each sample contained the same amount of protein, all further analyses of biochemical factors were performed [19].

2.6. Determination of SOD activity

Superoxide dismutase (SOD) activity was assessed using nitroblue tetrazolium (NBT) (Sigma-Aldrich, N6876), as previously conducted [20]. Briefly, 50 µL of extracted samples, containing the same amount of protein, were mixed with 1 mL of a reagent containing NBT (6.1 mg), methionine (1.9 mg), riboflavin (7.9 mg), and EDTA (3.3 mg), all dissolved in potassium phosphate to obtain a final volume of 10 mL. After incubating the mixture in a light box for 10 minutes, the absorbance was measured at 560 nm. Separate blank and control samples were also prepared without the addition of the sample extract. The blank tube was kept away from light, while the control and other sample tubes were placed in the light box for 10 minutes. Using the blank tube, the T80+-spectrophotometer (PG company, England) was adjusted to zero, and measurements were carried out. To calculate enzyme activity, the absorbance of the control and sample was subtracted and divided by the absorbance of the control. Enzyme activity was reported as units per minute per milligram of protein required for 50% inhibition.

2.7. Catalase activity estimation

To assess catalase (CAT) activity, a reaction mixture was prepared using 300 µL H2O2 and 200 µL 25 mM potassium phosphate buffer (pH 7.0). The absorbance of the solution was adjusted to 0.4 before measurement. CAT activity was measured by adding 50 µL of the sample, containing an equal amount of protein, to the mixture and recording the decrease in absorbance at 240 nm after 2 minutes using a T80+ spectrophotometer (PG, UK). CAT activity was measured after 1 minute using 0.0436 $mM^{-1}cm^{-1}$ as the extinction coefficient [19].

2.8. Determination of lipid peroxidation

The level of malondialdehyde (MDA) was measured to assess the extent of lipid peroxidation. 100 µL of a sample containing equal amounts of protein was added to 1 mL of a reaction mixture (containing thiobarbituric acid (0.5%) and trichloroacetic acid (20%) in HCl), and the tubes were boiled for 30 minutes. The tube was then kept in an ice bath for 15 minutes and centrifuged for 15 minutes at 10,000 g. Using a T80+ spectrophotometer (PG instrument, England), the absorbance of the samples was measured at 523 nm and then at 600 nm. The concentration of MDA was determined after subtracting values multiplied by the extinction coefficient (155 mM^-1 cm^-1) and reported in µM/mL [18].

2.9. Estimation of total antioxidant content

Based on equal protein concentrations, total antioxidant content (TAC) was estimated by mixing 150 µL of the sample with 1700 µL of a reagent containing sodium acetate buffer (300 mM, pH 6.3), 2,4,6-Tri(2-pyridyl)-s-triazine (10 mM) (Sigma-Aldrich, USA) (dissolved in 40 mM hydrochloric acid), and ferric chloride (20 mM). Then, 850 µL of double-distilled water was added to the solution, and the mixture was incubated for 10 minutes (avoiding direct light) before measuring the absorbance at 593 nm using a T80+ spectrophotometer (PG Instrument Co., England). A standard graph was plotted using different concentrations of iron sulfate (FeSO4·7H2O) (Merck, Germany) to estimate the TAC level in the samples, as determined by the formula $Y = 0.0072X + 0.0011$, with $R^2 = 0.9965$, where Y represents absorption and X represents concentration [21].

2.10. Gene expression analysis

Total RNA extraction was carried out using a commercial kit (Super RNA extraction kit YT9080), and the cDNA was synthesized with the help of a BioFACT kit (BR631-096). Amplification of Nuclear factor erythroid 2-related factor 2 (Nrf2), Nuclear factor kappa B (NFkB), and glyceraldehyde dehydrogenase (Gapdh) genes was performed using a PCR instrument (Eppendorf master cycler gradient, Eppendorf Co., Hamburg, Germany) in triplicate with their specific primers (see supplementary file 2). The PCR program was carried out according to a previous study [22]. The PCR product was checked using agarose gel (1.5%), then the results were visualized and photographed using Gel documentation (Gel flash, Syngene bio imaging, England) and further analyzed by Gel Quant software (Gel Quant: 1.8.2).

2.11. Statistical analysis:

Data analysis was performed using SPSS (version 20), employing one-way analysis with Tukey's test as the post hoc test. The normality of the data was assessed

using the Shapiro-Wilk test. Graphs were created with GraphPad Prism, and results were expressed as mean ± SD, with $p<0.05$ considered the minimum level of significance. Significant differences were indicated as follows: *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$.

3. Results and Discussion:

3.1. DEHP effects on cell viability and proliferation

Viability analysis indicated that CH concentrations ranging from 0.063 to 0.25 µM did not significantly affect cell viability percentages, compared to the control group (**Table 1**). Conversely, proliferation analysis revealed that only the 0.063 µM concentration did not alter cell proliferation. However, concentrations of 0.125 and 0.25 µM of CH significantly enhanced cell proliferation, with the highest effect observed at 0.25 µM (**Table 1**). Higher concentrations of CH (1 and 10 µM) exhibited significant cytotoxicity ($p = 0.0001$), affecting both viability and proliferation (**Table 1**).

Table 1 provides an analysis to determine the optimal concentration of catechin hydrate (CH) by examining its effects on BMSC viability and population doubling number (PDN) over a 4-day treatment period.

Data are presented as mean±sd. Means in all the experimental groups are compared with the control in each column, and different letter codes represent significant differences with the control and with each other. Control is shown always with "a".

Table 1. Analysis to select the effective concentration of catechin hydrate (CH)

Concentration (µM)	4 hours of treatment	
	Cell viability	PDN
Control (0)	$96.66^{a}\pm0.58$	$2.86^{a}\pm0.03$
CH (0.063)	$96.33^{a}\pm0.58$	$2.93^{a}\pm0.04$
CH (0.125)	$96.66^{a}\pm0.57$	$3.06^{b}\pm0.05$
CH (O.25)	$97.66^{a}\pm0.55$	$3.27^{c}\pm0.02$
CH (1)	$89.00^{b}\pm1.00$	$2.11^{d}\pm0.03$
CH (10)	$73.66^{c}\pm0.54$	$1.53^{e}\pm0.11$

Data are presented as mean±sd. $p<0.05$ is taken as the minimum significant difference.

3.2. Ameliorating effect of CH

DEHP treatment for 4 and 8 days significantly decreased cell viability, with a highly significant effect (**Figure 1A**). At the same time, Treatment with CH for the same durations improved cell viability only after 8 days of treatment. Conversely, in co-treatment groups, DEHP's effects were completely mitigated at 4 and 8 days and were similar to control levels. Although CH enhanced cell viability in groups treated with 0.025 µM CH and 500 µM DEHP compared to groups treated solely with 500 µM DEHP. However, the toxic effects of 500 µM DEHP were not fully mitigated compared to the control group. Regarding cell proliferation, a similar compensatory effect was observed (**Figure 1B**).

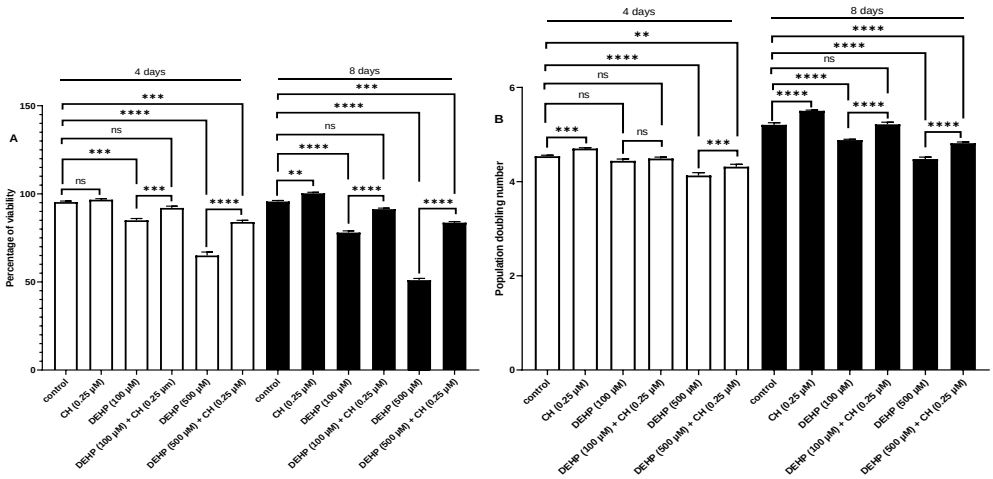

Figure 1. Ameliorating effect of 0.025 µM of CH on 100 and 500 µM of DEHP following 4 and 8 days of incubation: A) cell viability analysis using trypan blue assay, B) cell proliferation analysis using PDN calculation. All mean values were compared with the control, and the data are presented as mean ± SD, with the level of significance indicated as follows: (ns) $p> 0.05$, (***) $p <$, and (****) $p < 0.0001$.

3.3. Cell morphology analysis

Data analysis revealed a highly significant reduction in the nuclear diameter and cytoplasmic area of BMSCs ($p < 0.001$) after 4 and 8 days of treatment with 100 and 500 µM DEHP, compared to the control groups. In co-treatment groups, CH significantly mitigated the toxic effects of 100 µM DEHP on nuclear diameter and cytoplasmic area after 8 days ($p>0.05$) (**Table 2**). Although CH improved the toxic impact of 500 µM DEHP ($p<0.0001$) on nuclear diameter and cytoplasmic area, but this compensation was partial (**Table 2**) compared to the control groups. Microscopic observations indicated that the nuclei of cells treated with 100 and 500 µM DEHP for 4 and 8 days (**Figures 2C** and **E**) were smaller and more condensed compared to the control group (**Figure 2A**). Cytoplasmic shrinkage was also noted in DEHP-treated groups (**Figures 2C** and **E**). In groups treated simultaneously with CH and DEHP, most nuclei and cytoplasm exhibited normal morphology, resembling that of the control group (**Figures 2D** and **F**).

Table 2 provides a comparative analysis of the mean difference in BMSC nuclear diameter (µm) and cytoplasm area (µm) after 4 and 8 days of exposure to varying concentrations of DEHP and CH, alongside a control group.

Means in all the experimental groups are compared with the control in each column, and different letter codes represent significant differences with the control and with each other. Control is shown always with "a".

Table 2. mean difference of the BMSCs nuclear diameter (µm) and area of cytoplasm (µm) after 4 and 8 days of treatment with different concentrations of DEHP and CH in the presence of the control group.

Concentration (µM)	Nuclear diameter (µm)		Area of cytoplasm (µm)	
	4 hours of treatment	8 hours of treatment	4 hours of treatment	8 hours of treatment
Control (0)	$12.62^{a}\pm0.60$	$11.62^{a}\pm0.60$	$1010.23^{a}\pm57.57$	$947.80^{a}\pm11.65$
CH (0.25)	$13.01^{a}\pm0.62$	$12.31^{b}\pm0.52$	$1126.77^{b}\pm47.42$	$1197.57^{b}\pm9.07$
DEHP (100)	$8.58^{b}\pm0.54$	$6.78^{c}\pm0.39$	$829.53^{c}\pm14.23$	$732.47^{c}\pm72.52$
DEHP (100) + CH (O.25)	$10.77^{c}\pm0.81$	$10.78^{d}\pm0.27$	$936.73^{d}\pm9.66$	$933.60^{a}\pm7.35$
DEHP (500)	$6.72^{d}\pm0.48$	$4.89^{e}\pm0.54$	$635.33^{e}\pm52.70$	$467.23^{d}\pm32.82$
DEHP (500) + CH (0.25)	$9.58^{e}\pm0.64$	$8.69^{f}\pm0.35$	$825.70^{c}\pm18.16$	$846.57^{e}\pm9.26$

Data are presented as mean±sd. $p<0.05$ is taken as the minimum significant difference.

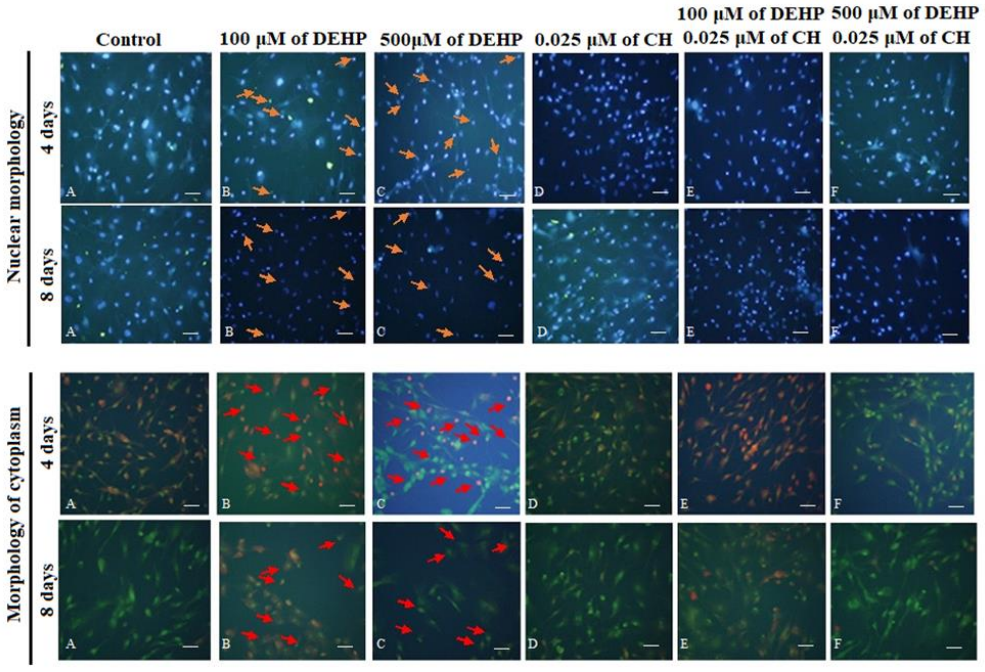

Figure 2. Morphological analysis: Photographs show the nuclear morphology and cytoplasmic morphology of the BMSCs after 4 and 8 days of treatment. A) control, B) treated with 100 µM of DEHP, C) treated with 500 µM of DEHP, D) treated with 0.25 µM of CH, E) treated with 0.25 µM of CH and 100 µM of DEHP, F) treated with 0.25 µM of CH and 500 µM of DEHP. Pink arrow shows the reduction in size of the nuclei and red arrow shows the shrinkage of the cytoplasm in groups treated with DEHP only (scale 50 µm).

3.4. Analysis of protein concentration

The concentration of extracted protein from BMSCs treated with DEHP significantly decreased ($p < 0.0001$) after 100 and 500 µM of DEHP, following 4 and 8 days of treatment. In co-treatments, cells treated with 0.025 µM CH and either 100 or 500 µM DEHP showed an increase in total protein over 4 and 8 days, indicating that CH significantly ($p < 0.0001$) mitigated the toxic effects of DEHP compared to the groups treated with DEHP alone. However, when comparing the compensatory effect of CH to the individual control group, the toxic effects of DEHP were not completely mitigated (**Table 3**).

Table 3 illustrates the mean differences in protein concentration (µg/ml) observed in BMSCs after 4 and 8 days of treatment with varying concentrations of DEHP and CH, compared to the control group.

Means in all the experimental groups are compared with the control, and different letter codes represent the significant differences with the control and among each other. Control is shown always with "a".

3.5. Estimation of total antioxidant capacity and malondialdehyde

In cells treated with DEHP, the concentration of MDA (**Figure 3A**) significantly increased ($p < 0.0001$) after 4 and 8 days of treatment with 100 and 500 µM of DEHP compared to the individual control groups. Conversely, DEHP treatment significantly ($p < 0.0001$) decreased the concentration of TAC (**Figure 3B**) after 4 and 8 days. Although co-treatment with CH mitigated the oxidative effects (as indicated by MDA levels) relative to cells treated with DEHP alone, it did not completely counteract the effects when compared to the control group (**Figure 3A**). However, treatment with CH led to a significant ($p < 0.0001$) restoration of TAC in the presence of DEHP (100 and 500 µM) after 4 days. After 8 days, TAC levels were observed to be fully compensated ($p > 0.05$) in the group treated with CH (0.25 µM) and DEHP (100 µM), reaching control levels (**Figure 3B**).

3.6. Antioxidant enzyme activity

Cells treated with 100 and 500 µM DEHP exhibited a highly significant decrease in the activity of catalase (CAT) and superoxide dismutase (SOD) after 4 and 8 days ($p<0.0001$) (**Figures 3C** and **3D**). Co-treatment with 0.25 µM CH in conjunction with 100 and 500 µM DEHP for both 4 and 8 days resulted in a highly significant increase in enzyme activity compared to the groups treated solely with DEHP ($p < 0.0001$). The addition of CH had a more pronounced effect on enzyme activity, with CAT activity at 0.49 ± 0.09 and SOD activity at 5.2 ± 0.18 following treatment with 100 µM DEHP. This effect was particularly notable after 8 days of treatment ($p > 0.05$), as the enzyme activities matched those of the control groups (**Figures 3C** and **3D**).

Table 3. mean difference of the protein concentration (µg/ml) of BMSCs after 4 and 8 days of treatment with different concentrations of DEHP and CH in the presene of the control group.

Concentration (µM)	**Protein concentration (µg/ml)**	
	4 hours of treatment	**8 hours of treatment**
Control (0)	71.05^{a}±0.08	73.56^{a}±0.67
CH (0.25)	73.91^{b}±0.29	76.88^{b}±0.06
DEHP (100)	55.22^{c}±0.41	54.10^{c}±0.91
DEHP (100) + CH (O.25)	63.98^{d}±0.56	70.01^{d}±0.81
DEHP (500)	29.95^{e}±0.17	32.62^{e}±1.06
DEHP (500) + CH (0.25)	44.26^{f}±0.34	46.45^{f}±1.04

Data are presented as mean±sd. $p<0.05$ is taken as the minimum significant difference.

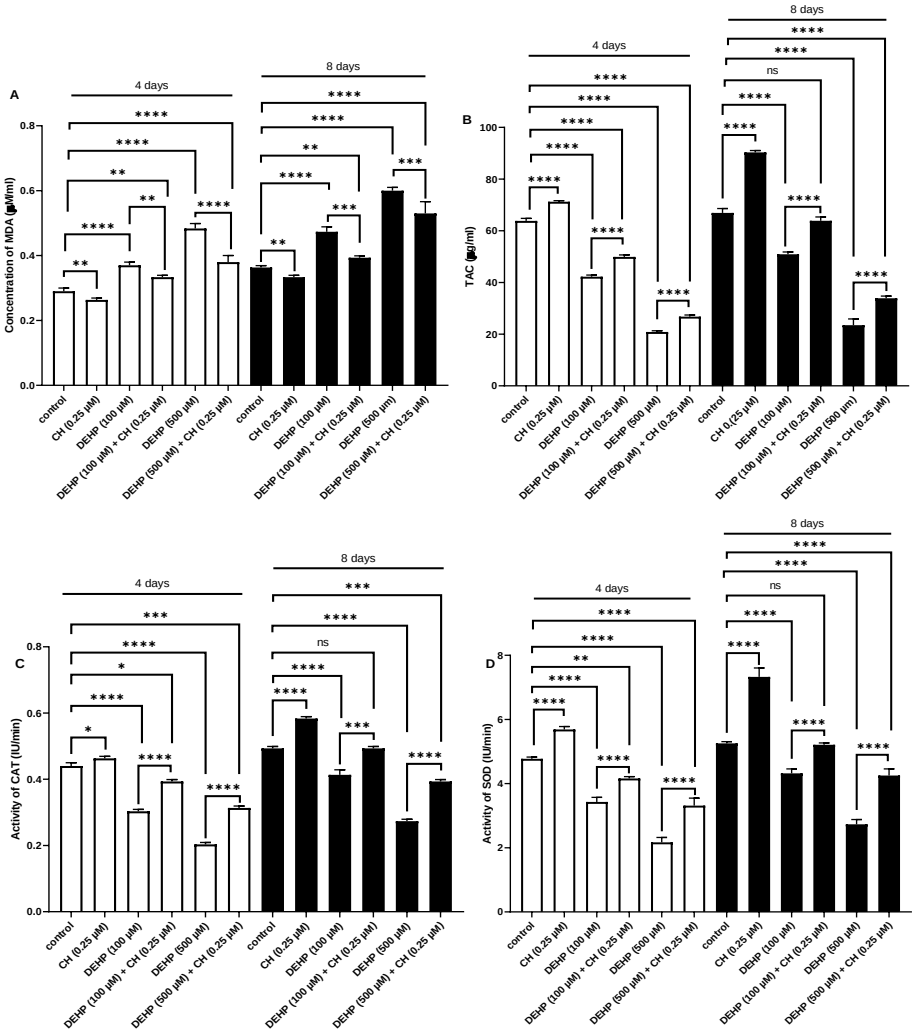

Figure 3. Mean A) concentration of MDA and B) concentration of total antioxidant capacity (TAC), C) activity of catalase (CAT), and D) activity of superoxide dismutase (SOD) in BMSCs treated with 0.025 µM of CH as well as 100 and 500 µM of DEHP following 4 and 8 days of incubation. All the mean values were compared with control and data is presented as mean ± SD with the level of significance as (ns) $p>0.05$, (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$ and (****) $p<0.0001$.

3.7. Gene expression analysis

The expression levels of Nrf2 significantly decreased following treatment with 100 µM ($p < 0.001$) and 500 µM ($p < 0.0001$) of DEHP after 4 and 8 days, compared to the control groups. Conversely, NFkB expression significantly increased with 100 ($p <0.001$) and 500 µM ($p<0.0001$) of DEHP after 4 days. This elevation remained significant ($p < 0.01$) after 8 days following treatment with 100 and 500 µM of DEHP, compared to the control group. In co-treated groups, CH increased Nrf2 expression and decreased NF-κB expression, achieving full recovery to control levels in the 100 µM treatment group ($p > 0.05$) after 4 and 8 days. Data analysis indicated that CH did not fully negate the toxicity of the 500 µM DEHP treatment (**Figures 4A** and **4B**). Gel electrophoresis images corroborated these findings (**Figures 4C** and **D**). The expression of GAPDH, used as a housekeeping gene, showed no variation (**Figures 4C** and **D**).

Through medical procedures, DEHP is leaching from PVC products, making contamination of the bloodstream unavoidable [12, 23]. In vitro studies on BMSCs have revealed a reduction in both viability and proliferation due to DEHP toxicity. Abnosi et al. (2022) demonstrated that DEHP at a concentration of 100 µM did not affect BMSC viability after 48 hours of treatment; however, longer exposure (4 days) at the same concentration significantly hindered cell proliferation. Furthermore, this study found that DEHP concentrations ranging from

500 to 2500 µM decreased both viability and proliferation even after a shorter exposure period (12 hours) [15]. Another study by Abnosi et al. (2020) confirmed DEHP's toxic effects on the viability of differentiated BMSCs, specifically in their differentiation into osteoblasts, after 21 days [16]. It appears that DEHP at high concentrations is hazardous even with brief exposure, whereas lower concentrations require extended exposure to produce similar effects. Both aforementioned studies align with the results of the present study, which showed that treating BMSCs with 100 µM of DEHP for 4 and 8 days significantly reduced the viability and proliferation of these cells. DEHP is a lipophilic compound [12] that can accumulate in tissues such as bone marrow, where lipids are predominant [24].

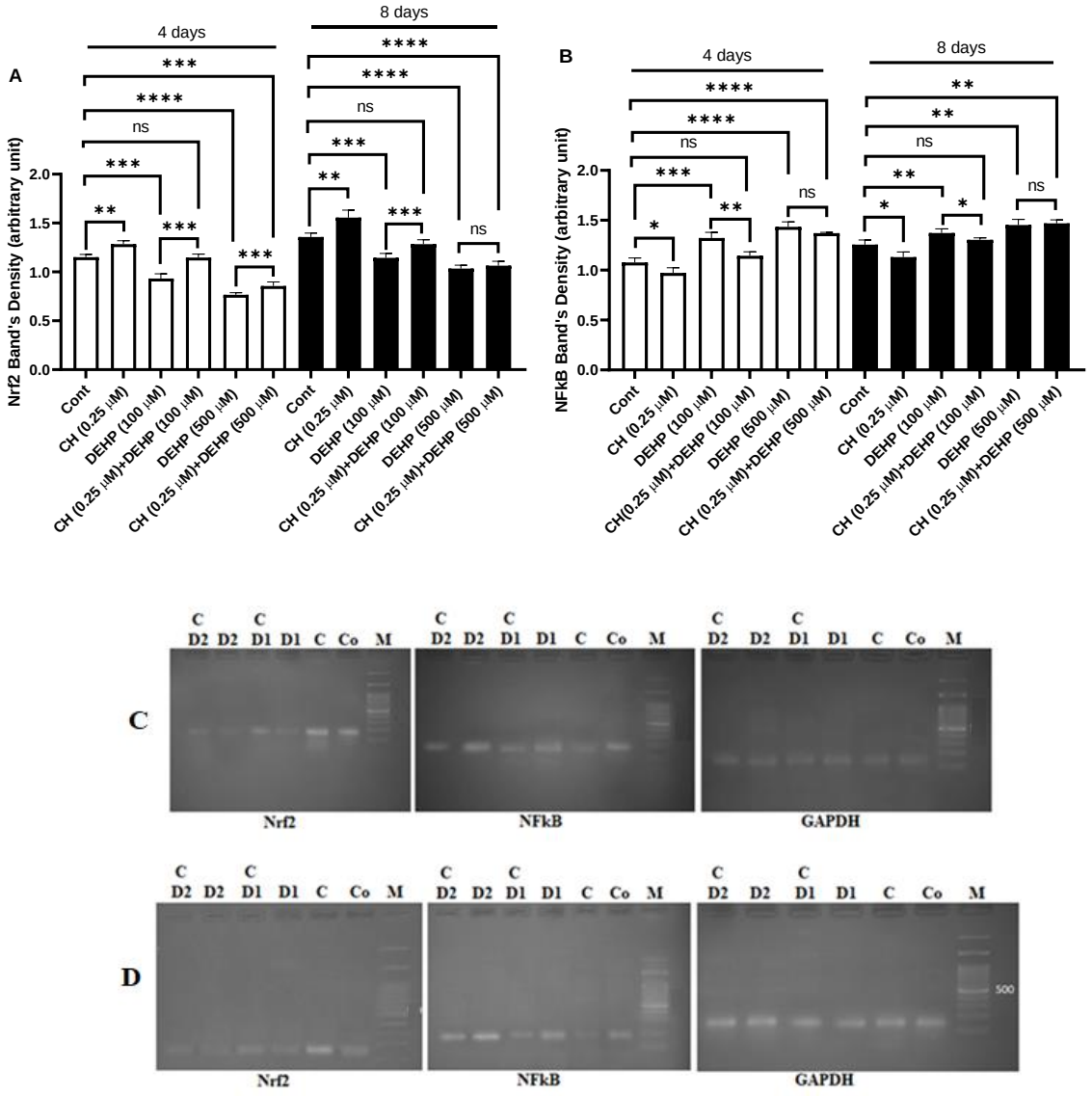

Figure 4. Graphs show the mean expression of A) Nrf2 and B) NFkB in BMSCs treated with 0.025 µM of CH as well as 100 and 500 µM of DEHP following 4 and 8 days of incubation. All mean values were compared with the control, and the data are presented as mean ± SD, with the level of significance indicated as follows: (ns) p> 0.05, (***) p <, and (****) p < 0.0001. As well as a photograph of agarose gel electrophoresis of the NFkB and Nrf2 gene in BMSCs treated for C) 4 days and D) 8 days of treatment. On the photograph (M) marker, (Co) control, (C) catechin hydrate, (D1) treated with 100 µM of DEHP, (CD1) treated with 0.25 µM of catechin hydrate and 100 µM of DEHP, (D2) treated with 500 µM of DEHP, and (CD2) treated with 0.25 µM of catechin hydrate and 500 µM of DEHP.

Our study indicated that prolonged exposure (8 days) to DEHP exacerbated its toxicity, confirming that the harmful effects of DEHP increase with continuous exposure. Since BMSCs are essential for producing osteoblasts in bone marrow, this environmental pollutant could impair bone tissue regeneration and remodeling [24]. Therefore, it is crucial to mitigate the severe effects of DEHP before it is too late, particularly during medical procedures. Catechin hydrate (CH), a natural plant product widely available through the consumption of fruits, vegetables, and some woody plant parts, may help to alleviate the toxic effects of DEHP when used continuously at low concentrations.

In the present study, we discovered that treatment with low concentrations of DEHP (100 µM) for 4 and 8 days affects the morphology of both the nucleus and cytoplasm of BMSCs, a finding corroborated by previous research [17]. Abnosi et al. (2022) demonstrated that oxidative stress induced by DEHP damages the cell membrane of BMSCs [15], which is considered the primary cause of reduced viability and morphological changes. Our study analyzed the same factor and confirmed the earlier findings. Oxidative stress occurs when there is an imbalance between reactive oxygen species (ROS) and the antioxidant system, which comprises both enzymatic and non-enzymatic components that protect cells [25]. While ROS are essential for cell signaling, excessive production can harm cellular macromolecules, including proteins, structural lipids, and nucleic acids [26]. The cell's antioxidant resources, such as vitamins and glutathione, reduce reactive species and neutralize them through self-oxidation, which depletes the cell's antioxidant reserves. In contrast, enzymes such as SOD and CAT convert oxygen radicals into water molecules [27], a process that occurs through gene activation [28].

We observed a significant decrease in the total protein content of the cells. While we recommend further investigation into the toxic effects of DEHP on microtubules and microfilaments, the reduction in protein content may be a critical biochemical factor responsible for the observed morphological changes, as cytoskeletal arrangements are vital for maintaining the normal shape of cells [29]. As previously mentioned, oxidative stress induced by DEHP may disrupt the cell's cytoskeleton, leading to protein disarrangement and alterations in the morphology of the BMSCs. Treatment with CH mitigated the cytotoxic effects of DEHP (100 µM) on cell morphology and protein levels, particularly after 8 days of treatment. Notably, the impact of DEHP at 500 µM improved, although it was not entirely compensated. CH is a potent antioxidant [30, 31], whereas DEHP has been shown to induce oxidative stress [15, 16]. In our study, CH's antioxidant properties were effective in reducing levels of MDA, a marker of lipid peroxidation, and enhancing the activity of antioxidant enzymes, especially after 8 days of treatment. Catechin's antioxidant activity is attributed to its ability to scavenge free radicals through its hydroxyl groups, occurring via two primary mechanisms: hydrogen atom transfer and single electron transfer [32]. Therefore, based on the mentioned mechanisms, catechin was able to mitigate the oxidative effect of DEHP and improve the viability and morphology of the BMSCs.

On the other hand, SOD and CAT work synergistically to neutralize reactive oxygen species (ROS) by first converting superoxide to hydrogen peroxide and then reducing H2O2 to water and molecular oxygen [33], thus collectively decreasing lipid peroxidation caused by oxidative stress. Nrf2 gene expression is highly dependent on ROS [34], and antioxidants also enhance its expression [35], although the mechanism remains unknown. Increased Nrf2 gene expression increases the level of Nrf2 protein in the cell, resulting in enhanced antioxidant activity. The Nrf2-ARE pathway regulates the expression of genes encoding antioxidant enzymes. Following the release of Nrf2 from Kelch-like ECH-associated protein-1, it translocates to the nucleus and binds to antioxidant response elements (AREs), initiating the expression of antioxidant-related genes [36]. There is significant cross-talk between Nrf2 and NF-κB in opposing directions; the elevation of NF-κB inhibits Nrf2 activity by forming a complex with the transcriptional co-activator CBP at the promoter region of the genes, leading to the inactivation of the Nrf2-ARE pathway [27]. Our results indicated that CH increased the expression of Nrf2 while downregulating NFkB gene expression to manage the oxidative stress induced by DEHP over a prolonged treatment period.

4. Conclusion:

The results of the present study indicate that CH, a potent antioxidant found in various plant products, mitigated the oxidative effects of DEHP by regulating antioxidant enzyme activity through the upregulation of the Nrf2-ARE pathway. The protective effect of CH reduced cell membrane lipid peroxidation and enhanced the viability, proliferation, and morphology of BMSCs, which serve as the cellular backup for osteoblasts in the bone marrow. Data analysis revealed that the effects of CH are more pronounced when used at low concentrations over extended periods of time. While further clinical research is necessary to understand the mechanisms by which CH mitigates oxidative stress caused by DEHP, prolonged consumption of low concentrations of CH may provide supplementary treatment for individuals exposed to DEHP, especially for patients receiving medical treatment where this environmental pollutant leaches out from medical facilities.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The present study was conducted based on the protocols recommended by the Guide for Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Committee on the Ethics of Animal Experiments of Arak University (Protocol Number IR.ARAKMU.REC.1401.077).

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Conflict of interest

The authors declare no conflicts of interest, financial or otherwise.

Authors Contributions

AMH; Conceptualization, Methodology, Writing-Original draft preparation, Software, and Supervision. AZ; Investigation, Validation. All authors read and approved the final manuscript.

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Using artificial intelligence chatbots

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