

## Protective Effect of Encapsulated Nanocurcumin-PEGOA against Oxidative Damage on Human Mesenchymal Stem Cells Exposed to Hydroquinone as a Risk Factor for Leukemia

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### Abstract

**Introduction:** Benzene a well-known environmental pollutant is a human carcinogen which is involved in the manifestation of a number of malignancies. Activation of benzene and its reactive metabolites such as hydroquinone (HQ) leads to continuous production of reactive oxygen species (ROS), causing oxidative Stress.

Curcumin, the yellow pigment of *curcuma longa*, has been shown to possess antioxidant activity in vitro and in vivo. However, poor bioavailability is the major drawback about of this drug. By using dendrosome, a nontoxic nanoparticle, we tried to deal with this problem. In the present study, we have investigated the protective effects of encapsulated nanocurcumin-PEGOA (ENC) against the HQ-induced oxidative damage in human mesenchymal bone marrow stem cells (hMSCs).

**Methods:** hMSCs were pre-treated with ENC and then exposed to HQ. Cell viability, intracellular ROS and lipid peroxidation extent were assessed by MTT assay, DCFH-DA fluorescent dye and thiobarbituric acid reactive substances (TBARS), respectively. Moreover, mRNA levels of antioxidant enzymes, catalase and hemeoxygenase-1 were evaluated by qRT-PCR.

**Results:** The results showed that treatment of MSCs with ENC (10  $\mu$ M) for 12 hours prior to HQ exposure, significantly attenuated the cell viability loss, suppressed the elevation of ROS and reduced the extent of lipid peroxidation caused by HQ compared with the control. Moreover, a significant increase in mRNA expression of antioxidant genes of catalase and hemeoxygenase-1 was observed after treatment with ENC.

**Conclusions:** These results suggest that 10  $\mu$ M ENC could protect hMSCs against toxic and oxidative effects of HQ. Therefore, ENC may have cancer protective effect.

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## INTRODUCTION

Cancer is related to a number of risk factors including oxidants. Nowadays, there is a tendency to find natural antioxidant compounds with minimal adverse effects to attenuate intracellular reactive oxygen species (ROS) accumulation [1]. Curcumin is a yellow polyphenolic pigment of the *Curcuma longa* plant which is largely insoluble in water [2]. Curcumin has been reported to exhibit a broad spectrum of pharma-

cological effects, including antitumor, anti-inflammatory and antioxidant activities with low toxicity [3-6]. Among these properties, its potent anti-oxidative effect is renowned [7]. Despite these attractive attributes of curcumin, insolubility in aqueous solutions is one of the major drawbacks associated with the use of curcumin as a therapeutic agent [2]. Nano-encapsulation of curcumin could be a proper strategy to increase its aqueous solubility in therapeutic applications. Dendrosomes, a family of nontoxic nanoparti-

cles, are suitable choices to increase the bioavailability of curcumin [8]. Previous studies in our laboratory revealed that dendrosome increases the curcumin uptake in the cells with no toxic effects [9].

Hydroquinone (HQ) is one of the serious benzene-derived primary metabolites produced by primary benzene hepatic oxidation [10]. Several studies have suggested that oxidative damages, which their effect on incidence of cancers have been reported, may be involved in the toxic effects of benzene [11, 12]. Benzene biological activation contains two stages: the first step occurs in the liver and yields the primary benzene metabolites, including phenol, catechol and hydroquinone [13]; in the next step, the primary metabolites transported to the bone marrow lead to the formation of ROS and induction of oxidative stress, which may play a crucial role in starting benzene toxicity [14, 15]. Benzene by toxic effect on hMSCs may associate with development of leukemia.

Oxidative stress can arise from overproduction of ROS by metabolic reactions that use oxygen and shift the balance between oxidant/antioxidant status in favor of the oxidants [16]. In healthy organisms, ROS production is counterbalanced by the antioxidant defense system to maintain an appropriate redox balance [17]. In human, the anti-oxidative defense mechanism to escape the consequences of cell damage caused by ROS includes enzymes, such as catalase and superoxide dismutase, hemoxygenase-1 and low molecular compounds, e.g. glutathione [18, 19]. However, the production of ROS in the body plays important roles in cellular signaling processes [20]. The generation of large amounts of ROS and free radicals without any affective neutralization system leads to oxidative damage in proteins, lipids and DNA [21]. Oxidative stress is an important factor associated with many acute and chronic diseases including cancer, diabetes, cardiovascular, and neurodegenerative diseases [22].

Induction of ROS within the cells due to exposure to benzene and its metabolites has been well established [11]. Moreover, the major target tissue affected by benzene metabolite toxicity is bone marrow which contains hematopoietic and mesenchymal stem cells; so, the impressive toxicity of benzene metabolites affects this tissue [23, 24]. Excessive exposure to benzene has been known to damage the bone marrow resulting in decreases in the numbers of circulating blood cells, and ultimately, aplastic anemia. Indeed, benzene metabolites induce mutagenesis which can eventually lead to the development of leukemia [25, 26].

The objective of the present study was to examine the protective potency of ENC against the oxidative effects of HQ on human mesenchymal stem cells (hMSCs) as a main target of HQ toxicity.

## METHODS

### Chemicals

Curcumin was purchased from Merck KGaA (Darmstadt, Germany) with a purity of 95%. Hydroquinone was the product of Sigma-Aldrich (St. Louis, USA). The dendrosome nanoparticle, specified Den O400, was a gift from the Institute of Biochemistry and Biophysics, University of Tehran, Iran.

Dullbecco's modified Eagle's medium (low-glucose DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS) and trypsin-EDTA were purchased from Gibco BioCult (Paisley, Scotland, UK).

### Preparation of Drugs

Dendrosome and curcumin powder were mixed in 25:1 ratio of dendrosome weight/curcumin weight based on optimized protocol and then diluted with PBS to have 10  $\mu\text{M}$  ENC [8]. Aliquots of this solution were sterilized by 0.22  $\mu\text{m}$  filters and stored in a dark place at 4°C. HQ powder was dissolved in low-glucose DMEM medium supplemented with 10% FBS (10 mM) just before use and was kept away from light. The HQ solution was diluted to the desired (15  $\mu\text{M}$ -150  $\mu\text{M}$ ) concentration for experiments.

### Cell Culture and Drug Treatment

hMSCs (passage 1) were obtained from Royan Stem Cell Bank (RSCB), Iran and were cultured in low-glucose DMEM (Invitrogen/Life Technologies, Carlsbad, CA, USA) supplemented with 10% FBS at 37°C in 95% air and 5%  $\text{CO}_2$ . Sterile stock solutions of chemicals were prepared in DMEM just before use. For treatment with these chemicals, cells (passage 3) were seeded in either multi-well plates or 25  $\text{cm}^2$  cell culture flasks, and incubated until reaching 70%-80% confluence. The cells were then exposed to a range of chemical concentrations.

### Cell Viability Assay

Cell viability was determined by the methylthiazol tetrazolium (MTT) reduction assay (Sigma-Aldrich, St. Louis, USA) according to the manufacturer's instruction. Briefly, hMSCs were seeded overnight in 96-well culture plates at a density of  $1 \times 10^4$  cells/well. To test the cytoprotective effect of nanocurcumin against HQ-mediated cell death, the cells were pretreated with 10  $\mu\text{M}$  of nanocurcumin for 12 hours. The cells were then washed with PBS and stressed with different doses of HQ. After each treatment, media containing the treatment agents were removed and 5 mg/mL MTT in PBS was added to each well. After incubating at 37°C for four hours, the medium was removed and 200  $\mu\text{L}$  dimethyl sulfoxide solution (DMSO) was added to each well. The absorbance was measured at 570 nm in each well using a plate reader (Bio-RAD 680, USA).

All the experiments were performed in triplicate and cell viability was calculated as the percentage of cell viability of treated cells against control cells.

### Estimation of Lipid Peroxidation

To measure the extent of lipid peroxidation, a major marker of oxidative stress, the plasma content of malondialdehyde (MDA), an end product of membrane lipid peroxidation, was determined by the thiobarbituric acid-reactive substances (TBARS) assay in cell lysates. MDA forms a colored complex with thiobarbituric acid (TBA), which has the maximum absorbance at 532 nm. The absorbance is proportional to the extent of membrane lipid peroxidation. Briefly, the cells were plated onto six-well plates and were treated with the considered drugs. After each treatment, the cells were lysed in the lysis buffer and then 100  $\mu$ L of the sample was added to 200  $\mu$ L ice-cold 10% TBA for 15 minutes to precipitate the protein. The experiments were performed on ice. The precipitated samples were centrifuged for 15 minutes at 1400 rpm; 200  $\mu$ L of each Supernatant was mixed with an equal volume of 0.67% thiobarbituric acid and then boiled for 30 minutes and the absorbance was read in 532 nm. The remaining 50  $\mu$ L of supernatants were used for evaluating the protein content by Bradford assay. The lysis buffer used in these experiments contained EDTA (1 mM), Tris-HCl (150 mM, Merck), NaCl (150 mM), Triton X100 (1%, Sigma), DMSO (Gibco), and protease inhibitor PMSF (1 mM) in pH = 7.4.

### Estimation of Intracellular Reactive Oxygen Species

The levels of intracellular ROS were monitored using the DCFH-DA fluorescent dye (Sigma Aldrich, USA). DCFH is initially a non-fluorescent dye and it is converted to a fluorescent molecule called 2,7-dichlorofluorescein (DCF) by oxidation. DCF was then quantified by fluorophotometry. After the treatment, the cells were collected and washed twice with PBS and then DCFH-DA (10  $\mu$ M) was added into the medium for a further 30 minutes at 37°C and then it was analyzed by fluorescence spectrophotometer (PerkinElmer, Wellesley, MA, USA) with 485 nm excitation and 538 nm emission filters.

### Gene Expression

#### RNA Isolation, cDNA Synthesis and qRT-PCR

Total RNA was isolated from passage 3 hMSCs cultured

in 25 cm<sup>2</sup> flasks subsequent of each treatment. Total RNA was purified using 1 mL TRIzol reagent (Invitrogen). The quality and quantity of the extracted RNA were determined by 1% agarose gel and scientific NanoDrop 1000 spectrophotometer, respectively. Equal quantities of the total RNA from each sample were converted to cDNA using PrimeScript™ RT reagent (Takara) in the presence of oligo (dT) and random hexanucleotide primers. Primers were designed by Allel ID software. Beta-2 microglobulin ( $\beta$ 2M) was used as an internal control, which was stable during the experiment. All the primer sets had a calculated annealing temperature of 55°C. The list of primer sequences for specific and house-keeping genes is indicated in Table 1.

Real-time PCR was performed in MicroAmp 96-well plates (Applied Biosystems) using SYBR Green supermix (Applied Biosystems, USA) in triplicates with a 20  $\mu$ L final volume containing 10  $\mu$ L SYBR® Premix Ex Taq™ (2X), 0.4  $\mu$ L ROX, 1  $\mu$ L PCR primers, and finally 2  $\mu$ L diluted cDNA. The threshold cycle (Ct) (the fractional cycle number at which the amount of amplified target reached a fixed threshold) was determined and Ct value of the target genes were normalized by the Ct value of the housekeeping gene ( $\beta$ 2M). Relative gene expression was calculated as  $2^{-\Delta\Delta C_t}$ .

### Statistics

Statistical analyses were performed using GraphPad In-Stat software. All the experiments were conducted in triplicate and the quantitative data are expressed as mean  $\pm$  SD (standard deviation) for each group. The Student's t-test and paired t-test were performed to assess the differences between the means. P values less than 0.05 were considered statistically significant.

## RESULTS

### MTT Assay

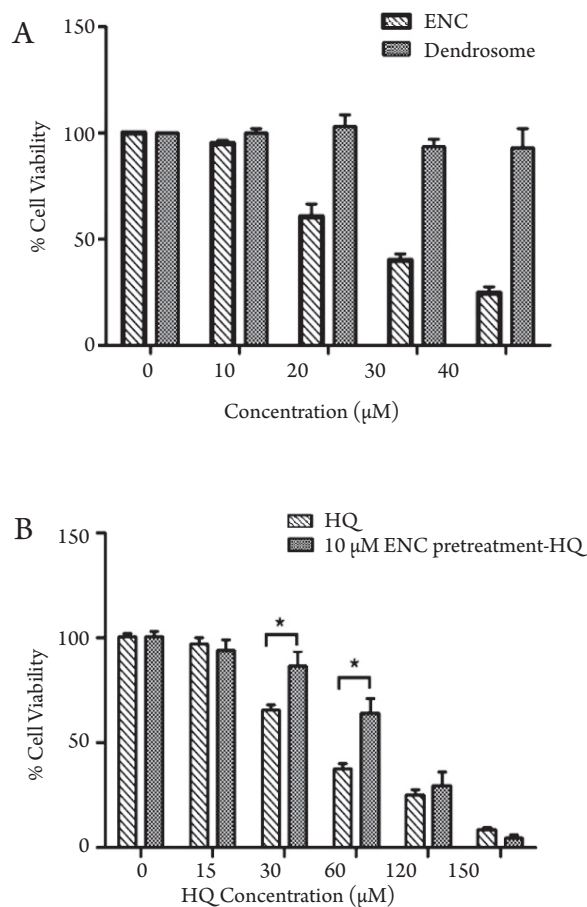
hMSCs were exposed to different concentrations of ENC (0-40  $\mu$ M) and dendrosome (0-40  $\mu$ M) separately in three replicates for 24 hours. In order to assimilate the effect of dendrosome carriers in all the treatments, cells in zero concentration of ENC were only treated with dendrosome; 20  $\mu$ M concentration of ENC significantly reduced the hMSCs viability (Figure 1A). Data from the MTT assay showed that 10  $\mu$ M ENC was the effective non-toxic concentration for subsequent experiments. Treatment of the cells with dendrosome did not show any toxic effect on hMSCs.

**Table 1:** Nucleotide Sequences of the Primers Used for Real-time PCR

Gene	Accession number	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size
$\beta$ 2M	NM_004048.2	AGATGTCTCGCTCCGTGG	TCTGCTGGATGACGTGAGTAA	109
CAT	NM_001752	ACACTGCCAATGATGATAACG	GTTCTTGACCGCTTTCITCTG	146
HO1	NM_002133.2	ATGACACCAAGGACCAGAG	GTAAGGACCCATCGGAGAAG	151



Moreover, in order to study the protective effect of ENC against the toxic effect of HQ, first the cells were pretreated with 10  $\mu$ M ENC for 12 hours; afterwards, the medium containing ENC was removed and the cells were exposed to different concentrations of hydroquinone (0-150  $\mu$ M) for an additional 24 hours. Results were compared with the cells exposed to the same HQ concentration without ENC pretreatment as control. As shown in Figure 1B, pretreatment of hMSCs with ENC protected the cells against the toxic effects of hydroquinone in 30  $\mu$ M and 60  $\mu$ M concentrations.



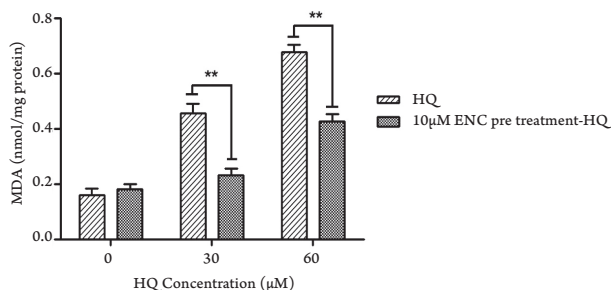
**Figure 1:** Evaluation of Cell Viability by MTT Assay.

(A) hMSCs were treated with different concentrations of ENC and dendrosome separately for 24 hours. (B) HQ: hMSCs were treated with different concentrations of hydroquinone for 24 hours, 10  $\mu$ M ENC pretreatment- HQ: hMSCs were treated with 10  $\mu$ M ENC for 12 hours and after removing the ENC, the cells were exposed to different concentrations of hydroquinone for 24 hours. Data is presented as mean  $\pm$  SD; n = 3, \*, P < 0.05.

### Measurement of Lipid Peroxidation Extent

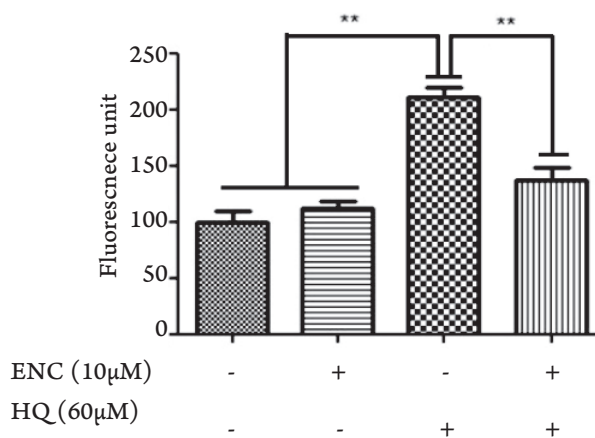
Measurement of MDA versus protein content in the cells is one of the oxidative stress sensing indicators which shows the extent of lipid peroxidation [27]. For this purpose, hMSCs were pretreated with 10  $\mu$ M ENC and then were exposed to 30  $\mu$ M and 60  $\mu$ M of hydro-

quinone. Lipid peroxidation in the cells was measured in terms of nmol/mg protein. The results were compared with the cells exposed to the same HQ concentration without ENC pretreatment. At zero concentration of HQ, cells were just treated with ENC. As shown in Figure 2, pretreatment of the cells with ENC reduced lipid peroxidation induced by HQ. The amount of protein in the cell lysate was measured by Bradford test [28] (Figure 3).



**Figure 2:** Malondialdehyde (MDA) Levels According to Protein Content in hMSCs After the Treatment.

HQ: hMSCs were exposed to hydroquinone for 24 hours, 10  $\mu$ M ENC pretreatment-HQ: hMSCs were treated with 10  $\mu$ M ENC for 12 hours and after removing the medium containing ENC, the cells were exposed to HQ (30 and 60  $\mu$ M) for 24 hours. Data is presented as mean  $\pm$  SD, n = 3; \*\*, P < 0.01.



**Figure 3:** Evaluation of ROS Production After Each Treatment Using DCFH-DA Assay.

In treatment with both ENC and HQ, hMSCs were exposed to 10  $\mu$ M ENC for 12 hours and after removing the medium, the cells were treated with 60  $\mu$ M HQ for 24 hours. Data are presented as mean  $\pm$  SD; n=3. \*\*, P < 0.01.

### Measurement of Intracellular ROS

Measurement of intracellular ROS using DCFH-DA probe showed that treatment of the cells with 60  $\mu$ M HQ significantly increased DCF fluorescent intensity, but treatment with 10  $\mu$ M ENC did not show any significant increase in the amount of intracellular ROS compared with the control group (cells with probe

only). Pretreatment of the cells with 10  $\mu\text{M}$  ENC and then treatment with 60  $\mu\text{M}$  HQ caused a significant decrease in the levels of intracellular ROS. Results were compared to the cells exposed to the same HQ concentration without ENC pretreatment.

### Changes in Gene Expression

Alterations in expression levels of antioxidant genes (*CAT* and *HO1*) were measured by real-time PCR technique. As presented in Figure 4, ENC treatment of hMSCs increased the catalase gene expression significantly 24 and 36 hours after the treatment compared to the control. This up-regulation in *HO1* gene expression occurred 12 hours after the treatment. Moreover, pretreatment of hMSCs with ENC and then exposure to HQ significantly elevated the mRNA level of *HO1* and *CAT* genes compared to HQ treatment alone. Therefore, this elevated expression can be attributed to the presence of ENC in the medium (Figure 5).

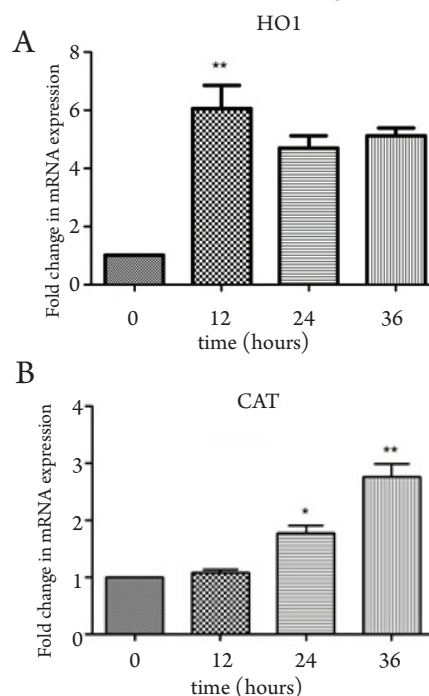
### DISCUSSION

Occupational or environmental exposures to benzene may cause several adverse health effects. Hydroquinone, a major benzene metabolite, is recognized to induce oxidative stress in the cells and tissues. Bone marrow, the niche of mesenchymal stem cells, is the main target tissue affected by benzene toxicity [14, 15]. ROS can promote many aspects of tumor development and progression. Indeed, cancer initiation has been related to oxidative stress by increasing DNA mutations, protein and polysaccharide damages and epigenetic changes [29, 30].

In recent years, there has been a trend toward the consumption of natural antioxidants, preventing the formation of radicals, scavenging them, or promoting their decomposition [31]. The supplementation of food with traditional antioxidants has been shown to be protective against cancer in a large number of studies [32-34]. The phytochemical curcumin, a major component of turmeric, has been known as a potent antioxidant used in herbal medicine [7]. Curcumin has been shown to exhibit several activities including antioxidant, anti-inflammatory and anti-carcinogenic [9, 35, 36]. A growing body of evidence has substantiated the neuroprotective, hepatoprotective and cardioprotective effects of curcumin in rat and mice [37-39]. Recently, dendrosomal ENC has been widely used to increase the bioavailability of this compound [8, 40].

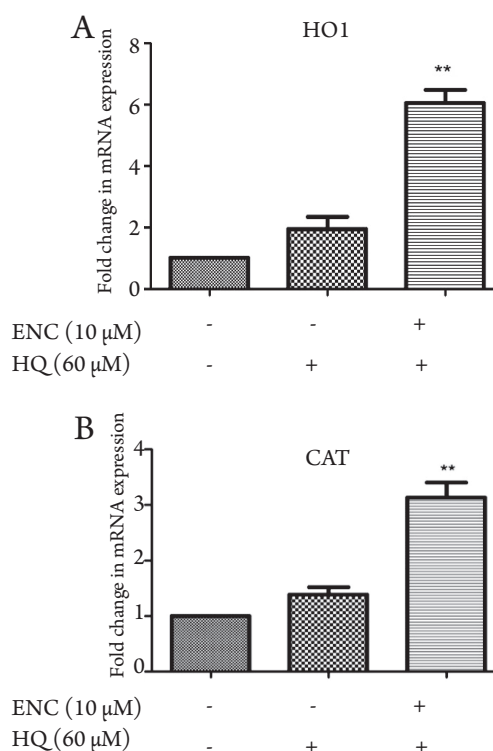
Here, we evaluated the protective effect of ENC on hMSCs exposed to HQ. In order to evaluate the toxic effect of ENC and HQ on hMSCs, cells were exposed to different concentration of chemicals and the percentage of cell viability was assessed by MTT assay. The results showed that HQ considerably decreased the cell viability, but pretreatment of the cells with ENC and subse-

quent treatment with HQ caused improvement in cell



**Figure 4:** The Effect of 10  $\mu\text{M}$  ENC on the Expression Level of Antioxidant Genes (*CAT* and *HO1*) in hMSCs at 12, 24 and 36 Hours After the Treatment.

Data are presented as mean  $\pm$  SD; n=3; \*,  $P < 0.05$ , \*\*,  $P < 0.01$ .



**Figure 5:** Alterations in the Antioxidant Genes Expressions Occurred Due to Different Treatments.

In treatment with both ENC and HQ, hMSCs were exposed to 10  $\mu\text{M}$  ENC for 12 hours and after removing the medium, the cells were treated with 60  $\mu\text{M}$  HQ for 24 hours. Data are presented as mean  $\pm$  SD, \*\*,  $P < 0.01$ .

viability. However, at higher concentrations of hydroquinone ( $> 120 \mu\text{M}$ ), ENC was not able to inhibit cell death anymore. Similar results in mouse neuroblastoma cells showed that pretreatment of cells with  $25 \mu\text{g}/\text{mL}$  curcumin reduced the cell death induced by  $\text{H}_2\text{O}_2$  treatment dramatically [41]. Moreover, we assessed the protective effect of ENC on HQ pretreated cells. The results showed no significant differences between the two groups of HQ treated cells and ENC treatment on HQ pretreated cells (Data not shown).

Next, we sought to investigate the effect of ENC and HQ on levels of intracellular ROS. The results showed that treatment of hMSCs with HQ caused an increase in intracellular ROS compared to the non-treated cells. It seems that HQ could play a crucial role in the induction of oxidative stress in hMSCs. However, pretreatment of the cells with ENC and subsequent exposure to HQ reduced intracellular ROS, but ROS levels did not return to the levels of control cells. Therefore, it can be deduced that  $10 \mu\text{M}$  ENC may scavenge free radicals induced by HQ in hMSCs. Previously, other studies showed that curcumin was able to scavenge the additional ROS induced by  $\text{H}_2\text{O}_2$  or UVB radiation in human lens epithelial cells [7, 42]. ROS are highly reactive molecules and can induce lipid peroxidation and disrupt membrane lipid bilayer functions [43]. Lipid peroxidation products such as TBARS have been used as indirect biomarkers of oxidative stress [27]. Accordingly, the extent of lipid peroxidation was assessed by TBARS assay. Treatment of hMSCs with HQ showed a significant increase in the levels of lipid peroxidation, while pretreatment of the cells with ENC and then exposure to the same concentrations of HQ reduced the lipid peroxidation rates in the cells. Although, this extent did not decrease to the initial value (without treatment with HQ). These results suggest that ENC can provide a substantial protection against the oxidative damage induced by HQ on hMSCs.

According to the results mentioned above, we concluded that ENC may act as an antioxidant by enhancing the antioxidant enzyme gene expression. Analyzing the alteration in the expression of antioxidant genes, *CAT* and *HO1*, in different treatments showed that *CAT* and *HO1* were up-regulated after ENC treatment. Moreover, ENC pretreatment of the cells and subsequent exposure to HQ caused considerable up-regulation in *CAT* and *HO1* gene expression compared to the cells which were solely treated with HQ. Because of the fact that  $\text{H}_2\text{O}_2$  is one of the major species of ROS induced by benzene metabolites and since catalase has a main role in  $\text{H}_2\text{O}_2$  decomposition [11], it seems that catalase is one of the effective enzymes involved in the protective effect of ENC against the oxidative damage of HQ in hMSCs. There are some evidences that up-regulation of *HO1* by curcumin is mediated by elevated expression of NF-E2-related factor 2 (*Nrf2*) [44, 45]. *Nrf2* transcription factor plays a key role in the induction of

genes containing ARE sequences in their promoter and curcumin is also known as a potent activator of *Nrf2* [45].

Therefore, curcumin could exert antioxidative effects either directly as a chemical antioxidant due to its ability to scavenge reactive oxygen or by modulating cellular defenses, which themselves exert antioxidant effects such as up-regulation of antioxidative enzymes. Taken together, these data provides evidence that ENC is able to protect hMSCs against oxidative damaging effects induced by HQ. Therefore, ENC may have cancer protective effect.

However, previous studies showed that curcumin exhibited both antioxidant and pro-oxidant activity in a concentration dependent manner [46].

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## CONFLICT OF INTEREST

The authors declared that there was no conflict of interests.

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