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The beneficial effects of thymoquinone on viability and differentiation of human adipose tissue-derived mesenchymal stem cells

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Abstract

Thymoquinone is the primary active substance extracted from Nigella sativa. It has been reported to have many beneficial and protective effects on several cell types. However, there are limited studies about its effects on mesenchymal stem cells (MSCs). The purpose of this study is to investigate the effect of thymoquinone on the cell viability, proliferation, and differentiation of MSCs obtained from human adipose tissue. MSCs were obtained from the biobank and treated with concentrations of 50 nM, 100 nM, 200 nM, and 300 nM thymoquinone. Cell viability, proliferation, and apoptosis analysis were performed. The effects of thymoquinone on the differentiation of human adipose tissue-derived mesenchymal stem cells (haMSCs) were investigated through adipogenic, osteogenic, and chondrogenic assays. As a result of our study, doses of 50 nM, 100 nM, 200 nM, and 300 nM thymoquinone have been demonstrated to enhance cell proliferation, prevent apoptosis, and contribute to adipogenic, chondrogenic, and osteogenic differentiation. Thymoquinone promotes the viability of haMSCs and induces differentiation into osteogenic, adipogenic, and chondrogenic lienages. Therefore, it is an auxiliary active ingredient that can be used safely in regenerative medicine.

Keywords: Thymoquinone, cell differentiation, human adipose tissue-derived mesenchymal stem cell

Introduction

Thymoquinone (TQ), isolated from Nigella sativa (black cumin) extract, is the primary bioactive component [1]. It has been utilized as a natural herbal medicine for a variety of illnesses for many years. Numerous studies (in vitro and in vivo) report that TQ has several beneficial effects across multiple systems and protects organs such as the liver and bone [2,3]. Additionally, TQ has demonstrated cytoprotective, anti-inflammatory, antidiabetic, and anticancerogenic effects [2,4-7].

Mesenchymal stem cells (MSCs) are special cells exhibiting self-renewal and differentiation capacity into various cell types, playing an important role in tissue repair and regenerative medicine [8]. Because of their antimicrobial, anti-inflammatory, and immunomodulatory properties, MSCs are widely used in regenerative medicine [9]. MSCs can be derived from various sources, with adipose tissue-derived MSCs being particularly favored for their cost-effectiveness, rapid procurement, and ease of obtaining [10]. Like other MSCs, adipose tissue-derived MSCs have the potential to differentiate into adipose, neural, cartilage, muscle, and bone tissues [11]. Despite the well-documented biological and pharmacological benefits of TQ on tissues, its impact on stem cells has not yet been fully clarified [12].

The current study aims to investigate the effects of TQ on cell viability and proliferation, as well as its influence on the adipogenic, osteogenic, and chondrogenic differentiation of human adipose tissue-derived MSCs (haMSCs).

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Material and Methods

This study was conducted after ethical approval obtained from the Ethics Committee of Erciyes University (Approval ID: 2019/44).

Cell culture and Used Reagents

HaMSCs were obtained from the tissue bank of Erciyes University Genom and Stem Cell Center (GenKok). HaMSCs from the -196°C nitrogen tank were quickly dissolved in a 37°C water bath (Memmert Water bath). It was prepared for centrifugation in a biosafety cabinet (Telstar Clean Air). It was centrifuged at 300 g for 5 min (Hettich, Rotina 380, Germany). After removing the supernatant, the pellet was resuspended. After pipetting, haMSCs were seeded into a 25 cm2 flask (TPP, Switzerland). The flask was removed to 37°C, 5% CO_2 incubator (Sanyo CO_2 Incubator, Model: MCO-19AIC (UV), Sanyo Electric, Co., Ltd., Japan).

Human adipose tissue-derived MSCs were cultured in low-glucose Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin, 0.025 µg/mL amphotericin B). Twice a week, the cell culture media was replaced. Upon reaching approximately 70% to 80% confluence, the cells were routinely passaged at a 1:2 ratio.

Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, D2650) was used to dissolve thymoquinone (Sigma-Aldrich 274666, ≥98 %) to create a stock solution. Then it is diluted in a medium that is FBS-free and added to haMSCs at certain concentrations for 24 hours.

Identification of haMSCs by Flow cytometry

Flow cytometry was used on haMSCs to phenotypically characterize the cells. HaMSCs were cultured at a density of 1x 106 cells per well in a 6-well plate. They were then incubated with BD™ Accutase™ Cell Separation Solution cells for 10 minutes to eliminate non-specific binding. Subsequently, an antibody cocktail for MSC- negative markers (CD19-PE, CD45-PE, CD34-PE, CD11b-PE, HLA- DR- PE) and positive markers (CD73-APC, CD90-FITC, CD105-PerCP-Cy5.5) was added to the cells. After 20-minute incubation at 22°C, they were washed with BD Pharmingen™ Blot Buffer (PBS-Cell Wash) to remove excess antibodies and were resuspended at 300-500 µl in BD Pharmingen™ Blot Buffer (PBS-Cell Wash). Finally, the presence and characterization of MSCs were confirmed using a flow cytometry device (Beckman Coulter Novios, ABD).

Cell viability and proliferation assays

MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (Promega, Madison, WI) was used to observe cell viability and proliferation. After counting by a hemocytometer, the cells were seeded using sterile 96-well plates (5x 103 cells/well). Then, the cells were treated with increasing doses of TQ (50nM, 100nM, 200nM, 300nM). After treatment with TQ, MTS and phenazine methosulfate (20:1 v/v) containing solution was added to the cells. Following a 3-hour incubation at 37°C, viable cell number was calculated by measuring the absorbance at 450 nm, using Glomax Multi Elisa Reader (Promega Glomax Multi Detection System), depending on production of formazan by the cells.

Analysis of Apoptosis

Apoptosis was evaluated by flow cytometry and annexin V staining. The cells were seeded in a -6-well sterile plate (3x 103 cells/well). After TQ treatments (50nM, 100nM, 200nM, 300nM) for 24 hours, apoptosis was measured by annexin V/ propidium iodide staining following the manufacturer's protocol (Muse Annexin V Cell Death Kit, Luminex, USA). Using a fluorescence-activated cell sorting analysis, positive cells were found and measured.

Adipogenic Differentiation

Cells that reached the third passage were planted into a 6-well sterile plate at a density of 1x104 cells per well. Initially, they were cultured with an adipogenic induction medium kit for 3 days (PT-3102B, Lonza MD, USA). Following this, the cells were cultured with an adipogenic maintenance medium kit for 3 days (PT-3102A, Lonza MD, USA). This cycle was repeated for 21 days, with the medium being replaced every once in 3 days. TQ (50nM, 100nM, 200nM, 300nM) was applied to the medium every 3 days during the differentiation period (excluding the control group). Adipogenic differentiation of haMSCs was then evaulated by detecting oil droplets, using the Adipo Red Assay Kit (PT-7009, Lonza, USA). The oil droplets, indicative of adipogenic differentiation, were observed as orange-red in color under an inverted microscope (Leica DMI 3000, Leica Microsystems, Germany).

Osteogenic Differentiation

Cells that reached the third passage were inserted into 6-well sterile plates at a density of 1x104 cells/cm^2 per well. When cells reached 90-95% confluence, an osteogenic differentiation medium was used in its stead. This process was repeated by changing the medium at least twice a week for 21 days. During this period, TQ was added to the medium with each medium change (excluding the control group). After 21 days, 1% Alizarin Red dye was performed. To stain cells, the differentiation medium was taken out, and they were washed with PBS. Then, 2-3 ml of 70% methanol was added to each well. After 5-minute incubation at room temperature, the cells were washed with PBS and 1% Alizarin Red was added to each well. The cells were incubated for 20 minutes at room temperature and evaluated under an inverted microscope.

Chondrogenic Differentiation

Cells at the third passage were taken into a 15 ml conical tube, with $3x10^5$ cells per tube, and centrifuged. A chondrogenic differentiation medium was added. The differentiation process was maintained for 21 days with the medium being replaced at

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least twice a week. TQ was included in the medium changes during the differentiation period, excluding the control group. Chondrogenic pellets were extracted from the conical tubes and embedded in the cryomatrix. Frozen sections of 7µm thickness were obtained from the cartilage tissues embedded in the cryomatrix, kept at room temperature for 10 minutes, and fixed in acetone for 10 minutes. PBS was used twice to wash sections for 5 minutes each, followed by incubation in ethanol acetic acid for 5 minutes. The slides were washed twice with distilled water for 5 minutes, and stained with Alcian blue for 10 minutes. After staining, sections were washed twice with distilled water for 5 minutes each and covered with a water-based sealer.

Statistical Analysis

Every experiment was carried out in triplicate, and the results were presented as means with standard deviations. The normal distribution of data was evaluated using histogram, q-q plot, and Shapiro-Wilk's test. Statistical analysis was performed using the GraphPad Prism V8.02 program. For pairwise comparisons, Statistical significance was determined using the Student t-test; for multiple comparisons, ANOVA was used. p-values less than 0.05 were deemed statistically significant.

Results

Morphology and Characterization of MSCs were Achieved

HaMSCs were cultured in a low-glucose DMEM enriched with antibiotics and serum. The haMSCs displayed an adherent morphology and grew in colonies. Passage 3 haMSCs were shown to be positive for the surface markers CD44 (98.64%), CD73 (94.92%), CD90 (99.76%), and CD105 (94.47%) while they were shown to be negative for CD19, CD34, CD45, CD11b, HLA-DR (0.26%) (Figure 1).

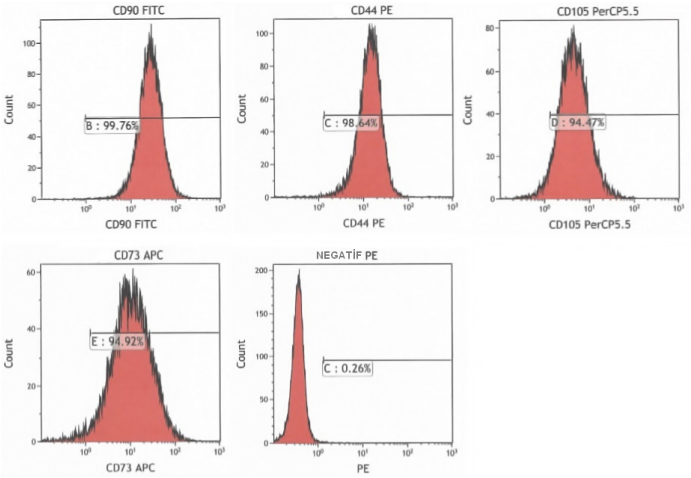

Figure 1. Adipose tissue-derived mesenchymal stem cell characterization showing negative and positive surface markers

Thymoquinone Increased Cell Viability at Lower Doses

To investigate the impacts of TQ on cell proliferation and viability of haMSCs, an MTS cell viability assay was conducted over 24 hours. The haMSCs were treated with TQ at doses of 40 nM, 50 nM, 100 nM, 200 nM, 300 nM, 400 nM, 500 nM, and 600 nM. The cell viability increased significantly at doses of 50 nM, 100 nM, 200 nM, and 300 nM of TQ. The results demonstrated a significant reduction in cell viability at doses of 500 nM and 600 nM of TQ (Figure 2) in comparison to non-treated (NT) and control (DMSO-treated) cells.

Thymoquinone enhanced cell proliferation at 50 nM, 100 nM, 200 nM, and 300 nM doses while inhibiting cell viability at higher doses. No significant difference was observed between the control and DMSO groups (p=0.1251). Comparison of the control group with the group treated with 40 nM TQ also revealed no significant difference (p=0.1598). However, significant differences were detected between the control group and the groups treated with 50 nM, 100 nM, 200 nM, and 300 nM TQ (p=0.0007, p=0.0155, p=0.0239, and p=0.0047 respectively). There was a decrease in cell viability in the group treated with 400 nM TQ, but it was not significant (p=0.0791). Hovewer, we observed a significant decrease in cell viability in the groups treated with 500 nM, and 600 nM TQ (p=0.0028; and p<0.0001, respectively). Notably, TQ significantly increased cell viability at 50nM concentration (Figure 2).

Given that TQ concentrations of 50 nM, 100 nM, 200 nM, and 300 nM enhance cell viability, these concentrations were selected for use in further assays.

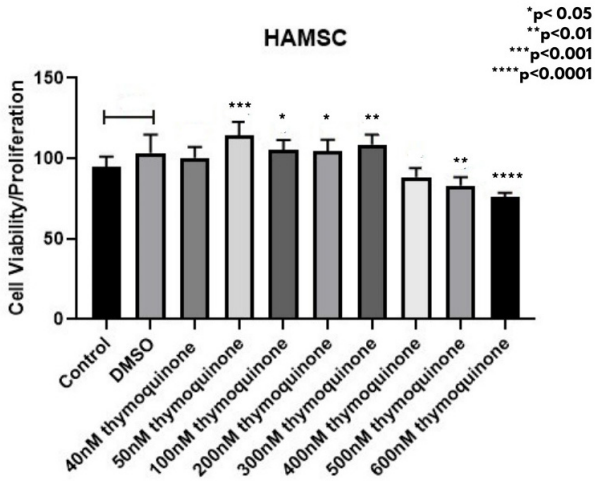

Figure 2. Cell viability/proliferation rates obtained as a result of the treatment of thymoquinone to adipose tissue-derived mesenchymal stem cells at different doses (*marks indicate the significant differences between treated groups compared to the control group)

Thymoquinone Inhibited Apoptosis and Induced Cell Survival at 50 nM Concentration

To determine whether TQ influences cell survival and affects apoptosis, we evaluated the apoptotic cell death in TQ-treated haMSC cells. We found that TQ at a concentration of 50nM

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inhibited apoptosis and promoted cell survival in haMSC cells ($p<0.001$). Conversely, TQ treatment at 100 nM induced apoptotic cell death ($p<0.001$) (Figure 3).

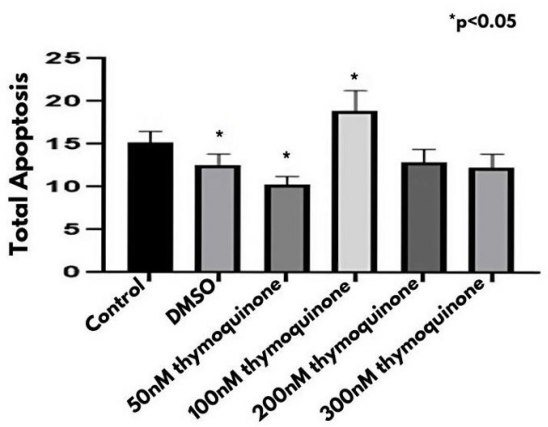

Figure 3. Effects of thymoquinone on apoptotic cell death. Human adipose tissue-derived mesenchymal stem cells were transfected with different concentrations of thymoquinone (50 nM, 100 nM, 200 nM, 300 nM) and analyzed by annexin V/propidium iodide staining and positively stained cells were assessed by FACS; the histogram shows the percentage of apoptotic cells in relation to the total number of cells (*marks indicate the significant differences between treated groups compared to the control group)

Thymoquinone Preserved Adipogenic Differentiation at Low Doses

To determine the effects of various TQ doses on adipogenic differentiation of haMSC cells, we conducted an adipogenic assay. We found that adipogenic differentiation was maintained at low TQ concentrations, whereas it was significantly impaired at higher concentrations (Figure 4).

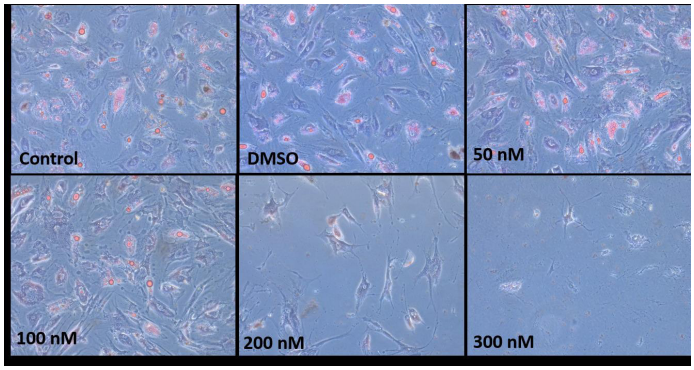

Figure 4. Oil droplets indicating adipogenic differentiation in human adipose tissue-derived mesenchymal stem cells after thymoquinone treatment (x20)

Thymoquinone Treatment Resulted in More Intense Calcium Deposits at Certain Concentrations

To evaluate the effects of various TQ doses on osteogenic differentiation of haMSC cells, we conducted an osteogenic assay. The results demonstrated that osteogenic differentiation was maintained in all TQ-treated groups. It was observed that the cells formed a more intense calcium deposits at TQ concentration of 50nM and 300nM compared to the control (Figure 5).

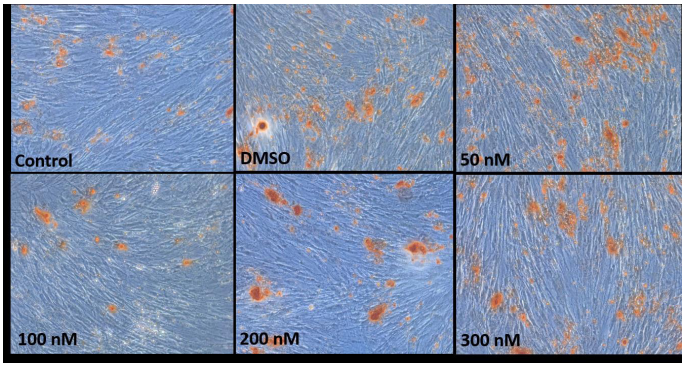

Figure 5. Alizarin Red staining of haMSCs to demonstrate osteogenic differentiation after thymoquinone treatment (x20)

Chondrogenic Differentiation was Induced at Higher Concentrations of Thymoquinone

To assess the effects of different TQ doses on chondrogenic differentiation of haMSC cells, we conducted a chondrogenic assay. We found that chondrogenic differentiation was more obvious in the TQ-treated groups than in the control. An increase in chondrogenic differentiation was observed with higher concentrations (Figure 6).

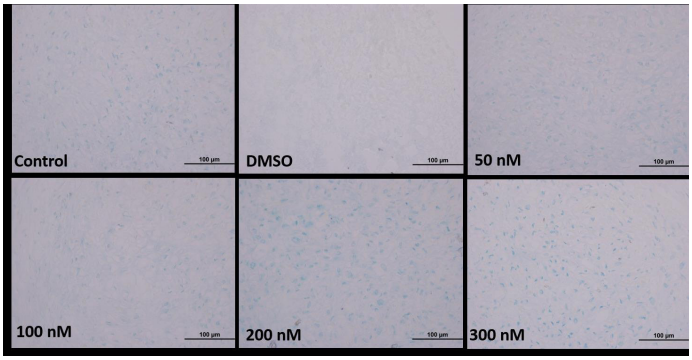

Figure 6. Alcian blue staining of calcium deposits indicating chondrogenic differentiation with different thymoquinone doses (x10)

Discussion

The current study revealed that TQ increased cell viability and induced cell survival in haMSCs at certain doses. In addition to that TQ was found to induce the adipogenic, osteogenic differentiation of haMSCs and chondrogenic differentiation.

MSCs are unique cells having an unlimited proliferation and differentiation capacity. The use of stem cell treatment has grown in popularity in recent years and has shown promising results in the treatment of many diseases [13]. Identifying the factors that influence the proliferation and differentiation capacities of MSCs is crucial for advancing stem cell therapy for several conditions.

Black cumin seeds (Nigella sativa) have been used for therapeutic purposes in traditional medicine because of their useful properties against certain diseases [2]. It has been reported that TQ treatment for 72 hours showed a protective effect against amyloid-β-induced neurotoxicity in primary neuron cultures

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[14]. Radad and colleagues found that TQ had a neuroprotective effect against 1-methyl-4-methylphenylpyridinium-induced dopaminergic cell death in primary mesencephalic cell cultures [15].

TQ has been shown to increase viability and reduce apoptosis in normal cells while decreasing cell viability and enhancing apoptosis in cancer cells. Studies involving breast cancer cell lines have demonstrated that TQ reduces proliferation, migration, and colony formation in triple-negative breast cancers [4]. TQ has also been shown to increase the chemosensitivity of cancer cells in colon cancers [16]. Therefore, it is suggested that TQ may be useful in treatment because of its cytotoxic effects on cancer cells and its potential to enhance drug efficacy [17]. All these studies indicate that TQ promotes proliferation in healthy cells and protects cells from potential toxic effects while decreasing cell viability and increasing apoptosis in diseased cells. However, there is limited data in the literature regarding whether TQ also has protective effects on stem cells.

A study on bone marrow stem cells stated that TQ reduces cell viability in a dose-dependent manner. Also, it has been determined that TQ treatment, particularly at high concentrations (100 nM–5 μM), induces cell death [5]. In our study, we examined the impacts of TQ on the viability and proliferation of haMSCs and their differentiation into specific lineages. We observed that TQ particularly at a concentration of 50nM, significantly increased cell viability. These results indicate that TQ supports cell regeneration by enhancing stem cell viability and preventing apoptosis. The obtained results were consistent with the literature.

Thymoquinone protects healthy adipocytes by reducing the lipid content and cell size in mature adipocytes; It is reported to stimulate adipogenesis in immature cells [18-20]. Shahbodi et al. investigated the inhibitory effect of TQ on lipid differentiation in high doses in MSCs. The impacts of TQ on lipid accumulation and adipocytic differentiation of haMSCs were demonstrated in an in vitro model. Researchers reported that TQ may reduce the differentiation of adipocytic stem cells into mature fat cells through prevention of the expression of Peroxisome proliferator-activated receptor-γ (PPARγ) and Fatty Acid Synthetase proteins (FAS) at certain doses. They also reported that haMSCs are a promising tool for adipocyte differentiation research in obesity and similar diseases [21]. Similarly, we found more fat droplets in TQ-treated groups at low doses and fewer droplets at high doses.

A recently published study demonstrated the antioxidant and immunoregulatory effects of TQ in bone marrow-derived MSCs from mice. High doses of TQ have been shown to reduce cell proliferation [22]. Similarly, we also observed that high doses of TQ decrease cell viability in our study. We assume that high doses of TQ might reduce cell proliferation primarily through cell cycle arrest, while the concurrent decrease in cell viability is likely due to a combination of ROS-mediated oxidative stress and apoptosis induction. However, in our study, lower doses of TQ were also used, and the protective effect of TQ on the proliferation and survival of haMSCs was demonstrated at low doses. While low doses of TQ may trigger cytostatic effects, such as cell cycle arrest without substantial apoptosis, higher doses appear to tip the balance toward cytotoxicity, leading to apoptosis and necrosis. The biphasic effect of TQ could be due to its ability to differentially modulate cell signaling pathways at varying concentrations. There are limited studies in the literature regarding the effects of TQ on stem cells of human origin. In this context, our study highlights a significant research area and contributes to expanding the current knowledge in the field.

It has been reported that TQ accelerates osteogenic differentiation in osteoblasts. TQ increased the expression of early, middle, and late differentiation markers, indicating its involvement throughout the entire osteogenic differentiation process. TQ is deemed suitable for use at various stages of intervention. Furthermore, due to its non-toxic nature, TQ is considered a promising natural treatment agent with significant potential [23]. In studies involving MSCs derived from dental pulp, TQ was found to stimulate osteogenic differentiation and enhance differentiation [24]. Our results are consistent with these findings in the literature.

It has also been reported that TQ enhances the differentiation of stem cells into osteoblasts without altering bone tissue [25]. In our study, we used MSCs derived from adipose tissue and observed similar results. TQ accelerated osteogenic differentiation at low doses, consistent with previous findings. However, while the aforementioned study reported TQ's effect on osteogenic differentiation, its impact on adipogenic and chondrogenic differentiation was not studied. In contrast, our study investigated the effects of TQ on haMSCs and observed a pronounced impact in the TQ-treated groups. Specifically, it was observed an increase in calcium deposits and enhanced osteogenic differentiation compared to the control group.

An experimental study demonstrated that after TQ treatment the chondrogenic differentiation potential of haMSCs decreased, concurrent with a reduction in the activity of components within the NF- κB signaling pathway. TQ launched the suppression of NF-κB signaling, thereby impairing the chondrogenic potential of haMSC, contrary to previous findings in the literature [26]. The production of type II collagen, a marker of chondrogenic differentiation, was suppressed in a dose- and time-dependent manner following TQ treatment. Consequently, TQ was demonstrated to have the capacity to modulate chondrocyte differentiation [27].

In our study, we demonstrated the positive impact of TQ on the osteogenic and chondrogenic differentiation of haMSCs. Our findings revealed that TQ doesn't have any detrimental effects at low doses. These properties highlight the potential of TQ for application in regenerative medicine.

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Conclusion

In conclusion, we found that TQ stimulates the proliferation and viability of haMSCs and promotes their differentiation into osteogenic, chondrogenic, and adipogenic lineages, especially at lower doses. Given its several known benefits for healthy cells, TQ also has advantages for haMSCs. These beneficial features underscore the significant potential of TQ in the future application of haMSCs in regenerative medicine.

Conflict of Interests
The authors declare that there is no conflict of interest in the study.

Financial Disclosure
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Ethical Approval
The study was approved by the Ethics Committee at the University of Erciyes with approval ID: 19/127 on 23.01.2019.

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