Effect of Curcumin on Odontogenic/Osteogenic Differentiation of Dental Pulp Stem Cells

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Introduction: Stem cells have gained great attention in tissue engineering and curcumin is a natural phenolic product that had showed some positive effects on these cells. The aim of this study was to evaluate the effect of curcumin on the differentiation of human dental pulp stem cells (hDPSCs). **Materials and Methods:** In this experimental study, hDPSCs were isolated from human third molars and treated without and with different concentrations (5, 10 and 15 µM) of curcumin and dimethyl sulfoxide (DMSO) as solvent for curcumin. Proliferation of the cells was measured by methyl-thiazol-tetrazolium (MTT) assay. Osteo/odntogenesis were assessed by alkaline phosphatase (ALP) assay and alizarin red staining. The collected data were subjected to statistical analysis (two-way ANOVA and Bonferroni correction) at a significant level of 0.05 by using SPSS software version 21. **Results:** MTT assay showed that addition of curcumin at 5µM concentration to the medium had no significant effect on cell proliferation compared with control group. Higher concentrations significantly inhibited cell proliferation at days 2 and 14. ALP showed reduced cell activity at all concentrations compared with control group. However, curcumin at 5µm concentration increased the ALP activity compared with DMSO group. Alizarin red staining showed that curcumin had no effect on mineralization. **Conclusion:** Curcumin did not induce osteo/odontogenic differentiation of hDPSCs. However, low concentration of curcumin was not toxic and increased the ALP activity of the cells compared with the DMSO group.

Keywords: Curcumin; Dental Pulp Stem cells; Odontogenesis, Osteogenesis

Introduction

Nowadays, dentistry is based on non-cellular treatments. The use of autologous stem cells producing dentin, cement, bone and periodontal ligaments is likely to be an alternative to commonly used treatments in regenerative dentistry. Dentin tissue regeneration, which may be able to integrate with pre-existing dentin is a desirable goal in clinical dentistry that would overcome the drawbacks of the conventional treatments such as pulp capping and apexification (1-3). Dental pulp stem cells (DPSCs) were isolated from permanent human molar for the first time in 2000 (4). Since these cells have the ability to self-regenerate and differentiate into various cells such as fat cells, nerve cells and odontoblast, they are also called mesenchymal stem cells (5). In some studies, odontogenic activity, odontogenic differentiation, and the effect of different materials on modulation of these cells have been studied (1-3, 6-8). Most of these studies surveyed the use of various growth factors or manipulating expressed genes to affect the differentiation pathway (9-13). Moreover, several studies have tried to show that plant-derived compounds and metabolites can affect the osteo/odontogenic differentiation signaling pathways (2, 14, 15)

Curcumin is a natural phenolic product isolated from the rhizome of Curcuma Longa (Turmeric) that has been widely studied for its anti-inflammatory, anti-mutagenic, anti-metastatic, anti-oxidant, wound healing, anti-angiogenic, immunomodulatory, and antibacterial effects (16). At the cellular level curcumin modulates important molecular targets such as transcription factors, enzymes, cell cycle proteins, cytokines, receptors, and cell surface adhesion molecules (17-19). Since many of these factors participate in the regulation of

bone remodeling, curcumin may affect the skeletal system. Therefore, the theory of curcumin influences on osteoblasts and osteoclasts has been studied in several *in vitro* and *in vivo* studies (20-25).

Despite the different localization in situ of DPSCs and bone marrow mesenchymal stem cells (BMSCs), these cells share many features. Potent regulators of bone formation such as transforming growth factor-β, bone morphogenic protein 2, 4 have been implicated as promoters in development of odontoblasts. In the other hand, other growth factors involved in regulation, proliferation, and differentiation of odontoblasts (such as basic fibroblast growth factor, platelet-derived growth factor, epidermal growth factor, insulin-like growth factor, tumor necrosis factor-α, and IL-β1) also can affect osteoblastic cells. Furthermore, odontoblasts and osteoblasts express similar mineralized matrix proteins, such as dentin matrix protein 1, fibronectin, collagen type I, alkaline phosphatase, osteonectin, osteopontin, bone sialoprotein, and osteocalcin. Collectively, studies suggest that the biochemical pathways involved in differentiation DPSCs into odontoblasts are similar with that of BMSCs into osteoblasts (4).

The aim of this study was to evaluate the effect of curcumin on osteo/odontogenic activity of DPSCs.

Materials and Methods

This study was approved by ethics committee of Research Institute of Dental sciences, Shahid Beheshti University of Medical Sciences and the reference number is IR.SBMU.RIDS.REC.1394.53.

Isolation and culture of hDPSCs

In this experimental study, normal human third molars were collected from adult (20-30 years old) at the Oral and Maxillofacial Surgery Department of Shahid Beheshti Dental School were immediately placed into a sterile 15 ml falcon containing physiological serum supplemented with 3% penicillin-streptomycin (Biosera, Lyle, France) and transferred to a cell culture lab with several ice packs. Under sterile condition, tooth surfaces were washed three times with sterile Phosphate-buffered saline (PBS) solution (0.01 mole/L, pH 7.4) (Inoclon, Tehran, Iran), and then was disinfected with 70% alcohol. The tooth was again washed with PBS solution. In order to expose the pulp chamber, the tooth was wrapped in a PBS-wetted sterile gauze and squeezed in a vise until cracked, and the dental pulp was carefully removed from the pulp chamber. Isolated pulp tissues were minced into 2-3 mm pieces. Afterward the tissue pieces were placed in a 6-well plate with media consist of alpha modification of Eagles medium (Inoclon, Tehran, Iran) supplemented with 20% FBS (Gibco BRL, Scotland, UK) and 1% penicillin-streptomycin and then incubated at 37°C in 5% CO2. After almost 5 days cell migration initiated and fibroblast-like cells observed. When the cell confluence reached to70- 80%, they were trypsinized and sub-cultured. DPSC from the third to fifth passages were used for this study.

Preparation of curcumin

Curcumin [1.7-bis (4-hydroxy-3-methoxyphenyl) -1, 6-heptadiene-3, 5-Dione] (Sigma-aldrich, MO, USA) was dissolved in dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany) and stored at -20°C. For experiments, curcumin was added to the culture media at indicated concentrations. DMSO was used in control experiments at 0.1%

Cell proliferation assay by MTT

Cell viability was assessed by using methylthiazole tetrazolium (MTT) method according to the manufacturer's (BIO-IDEA, Tehran, Iran) instructions.

The cells were seeded at a density of 5-10 × 〖10〗^3 cells per well and incubated to allow initial attachment for 24 hours. Then the culture medium was removed and replaced by different concentrations (5, 10 and 15 μM) of curcumin. These concentrations have been selected based on previous similar studies (21). The culture medium without curcumin was used as a control. Morphological changes of dental pulp stem cells were observed during experiment. At the time points (2, 14 days), 400 μL culture medium of RPMI1640 and 100 μL MTT was added to each well. After incubation for 4 hours, 500μL DMSO (Merck, Darmstadt, Germany) was put into wells. Finally, the optical density values were determined using a plate reader (ELx 800; Bio Tek, Winooski, VT) at the wavelength of 570 nm after removing the mixture in wells.

Osteogenic capability assessment by alkaline phosphatase activity (ALP)

In order to investigate the effect of curcumin on activity of ALP enzyme, different groups were compared at days 7 and 14. Briefly, DPSCs cultured in medium with the different concentrations of curcumin (5, 10 and 15 μM) were washed 2 times with PBS buffer to remove the cells from the surface of the plate, followed by the addition of lysis buffer. Then, cell protein

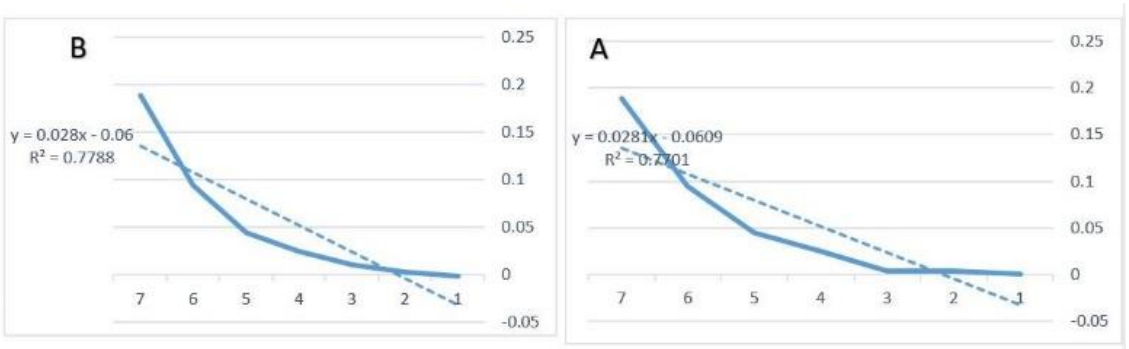

Figure 1: The alkaline phosphatase standard curve, day 7 (A), and day 14 (B)

contents were obtained by centrifuging. According to the protocol of the kit (Greiner, Nlirtingen, Germany), the R1 (diethanoleamine) and R2 solutions (p-nitrophenylphosphate) were mixed and added to the protein content. The absorbance was measured by a plate reader at 405 nm to make a standard curve (Figure 1). Then the activity of the alkaline phosphatase enzyme was calculated in the unknown samples.

Mineralization assay by Alizarin Red S staining

Mineralization of DPSCs was observed by alizarin red S staining. Briefly After culturing DPSCs for 14 and 21 days in a culture medium with and without curcumin, the cells were washed twice with PBS, fixed with formaldehyde 10%, and stained with AR 2% (pH = 4.2-4.4) (Sigma-Aldrich, MO, USA) for 30 minutes at room temperature. After being washed with double distilled water, the stained image was acquired.

Statistical analysis

Each experiment was performed in triplicate. Results are expressed as the mean and standard error. Statistical analysis was performed by using SPSS 21.0 software. For cell proliferation, two-way ANOVA was used to analyze the effect of time and different concentrations. Multiple comparison among the groups was evaluated by Bonferroni test with applying Type I Error correction. A P value<.05 was used to identify significance of differences.

Results

Effects of curcumin on cell proliferation

We assessed the effect of curcumin on cell viability by using an MTT assay. As shown in Figure 2, after 48 hours, DPSCs exposed to the DMSO and 5μM curcumin did not show any differences compared to the control group (P = 1.00). However, the cells treated with 10 μM and 15 μM curcumin showed significantly lower optical absorption compared to 5μM curcumin (P=.014, P=.005; respectively).

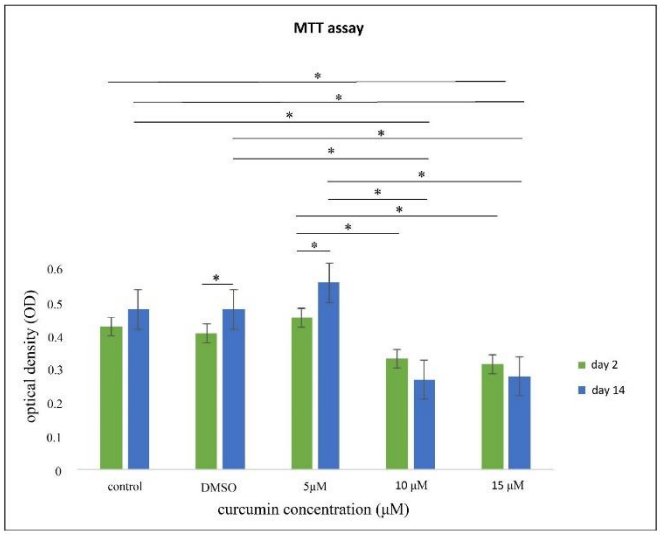

Figure 2: Effects of curcumin on cell proliferation measured using MTT assay. Statistical of significance of the differences between the results are indicated (* P<0.05)

After 14 days, there was no significant differences between the control group and the DMSO (P = 1.00). Optical absorption was increased at 5 μM concentration, which was not statistically significant compared to the control group (P = 0.26) but was significantly different from the second day (P=0.005).

The optical absorption of 10 μM and 15 μM curcumin was reduced compared to the control group, DMSO, and 5 μM concentration (all P≤0.001). However, there was no significant difference between the absorption of 10 μM and 15 μM with each other (P=1.00) and at different days (P=0.077, P=0.294). Morphological changes of cells during experiment are in consistent with these results (Figure 3).

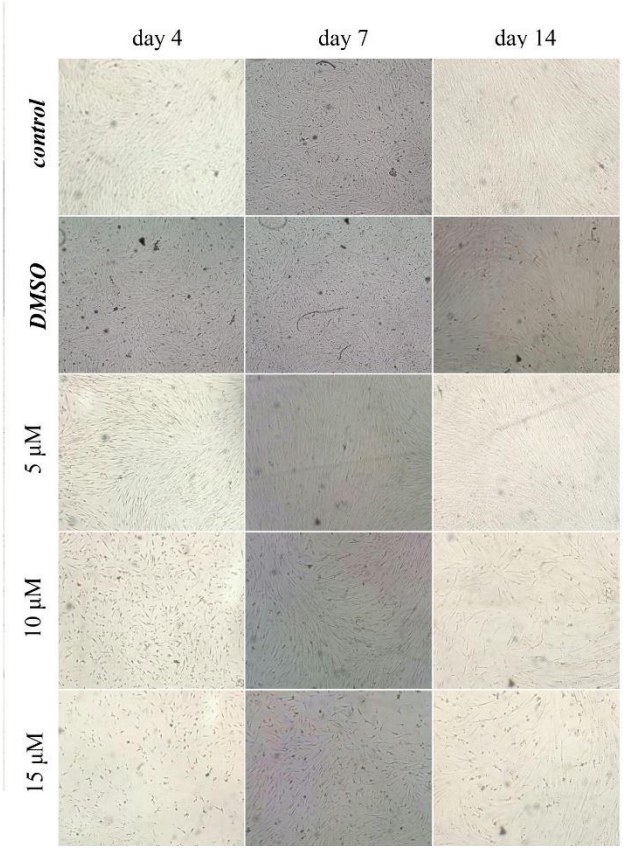

Figure 3: Morphological changes of cells during experiment

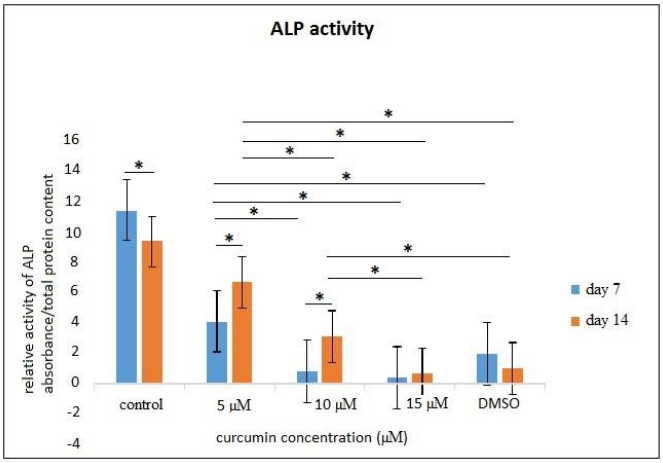

Figure 4: Effect of curcumin on alkaline phosphatase activity of human DPSCs. Statistical of significance of the differences between the results are indicated (* P<0.05). ALP activity of hDPSCs in control group was significantly higher than all of other groups a both days 7 and 14

Effect of curcumin on ALP activity of DPSCs

The activity of ALP in the samples was calculated. Using the Wilcoxon test, it was shown that the activity of the cells in the sham group (DMSO) was significantly reduced (P = 0.05). But curcumin at 5µM concentration significantly reduced the effect of DMSO and increased cellular activity. 10 and 15µM concentrations did not show these changes and even prevented increasing the cellular activity (Figure 4). These results and the results of MTT, represent the senescence of the cells, not associated with apoptosis.

Effect of curcumin on the mineralization of DPSCs

In undifferentiated stem cells, no extracellular deposition of calcium is seen, in contrast, during differentiation of DPSCs in the extracellular region, it occurs abundantly in the extracellular and intracellular conditions. Calcium depositioning is a positive sign of dentin formation and successful differentiation of pulpal stem cells into odontoblasts. Especially calcium can be stained with bright orange-red color using Alizarin. By using Alizarin staining, sediments on the surface of the extracellular matrix can be observed. In the presence of sediment, the color pattern of the treated samples varies with the control sample. The results showed that the color pattern of the treated cells with different concentrations of curcumin did not differ significantly from the control sample (Figure 5 and 6).

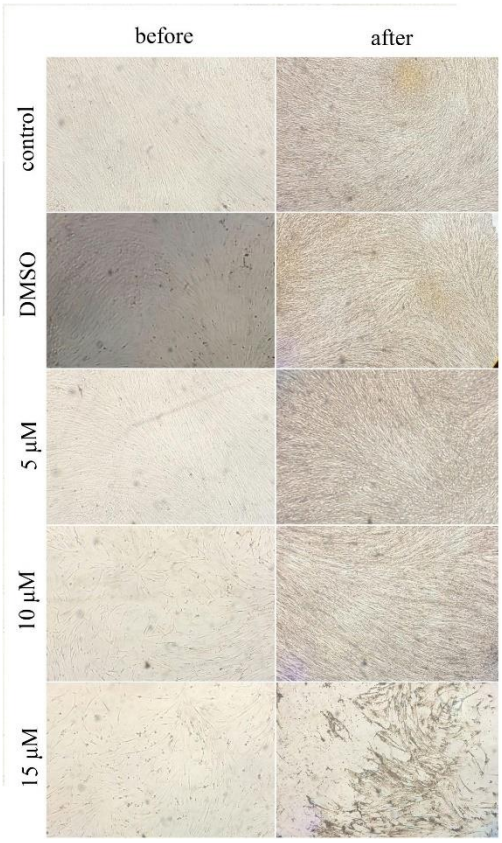

Figure 5: Alizarin red staining, day 14

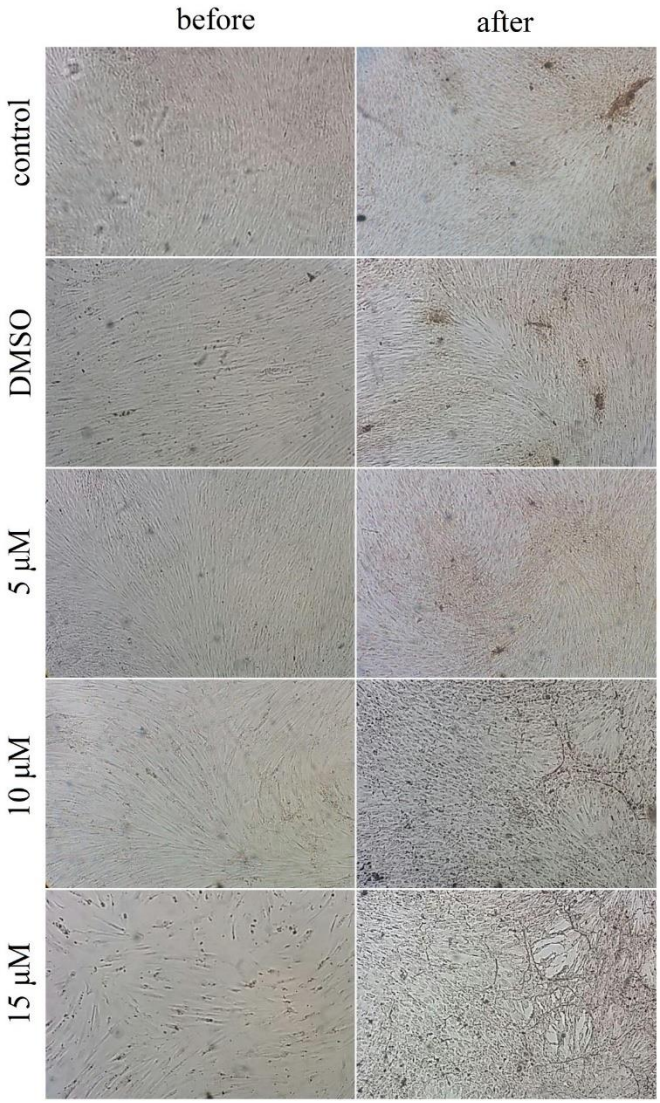

Figure 6: Alizarin red staining, day 21

Discussion

Tissue engineering approaches have gained great attention to restore or replace tissues with aid of progenitor cells with scaffold in response to appropriate signals. Recent advances in stem-cell biology have revealed the possibility of dentin regeneration using human adult DPCs as progenitor cells with the ability of forming a dentin/pulp-like complex (1).

Studies have shown that low concentrations of curcumin increase the osteoblastic differentiation of mesenchymal stem cells (21, 24, 26). Since the biochemical pathways involved in differentiation DPSCs into odontoblasts are similar to differentiation pathways of BMSCs into osteoblasts (4), we hypothesize that curcumin might have effect on the odontogenic activity of DPSCs.

To assess the toxicity of curcumin, MTT test was used. The results showed that the low concentration of this substance, which in this study was 5µM, not only had no toxic effect on cells, but also it increased the proliferation of cells on day 14. However, higher concentrations had a toxic effect on cells and caused the degradation of cells. As shown in the studies, curcumin has different effects on different cells proliferation at different concentrations. In Son *et al.*, study, investigating the molecular mechanism of osteoblast differentiation by curcumin, the results of MTT test showed that Curcumin had no cytotoxic activity at concentrations up to 10 µM; however, curcumin at concentrations of 100 µM reduced cell viability. They suggest that curcumin induced osteoblast differentiation by increasing the expression of osteogenic genes (26). However, Notoya *et al.*, investigated the effect of curcumin on the metabolism of rat calvarial osteoblastic cells and showed that curcumin (5 to 10 µM) markedly inhibited the proliferation of cells by arresting them at the G1 phase of the cell cycle (22). In another study by Moran *et al.*, MTT showed curcumin (10, 20 and 30 µM) significantly decreased cell viability in a dose-dependent manner (20).

Similar to present study, Zhou *et al.*, demonstrated that resveratrol, a plant metabolite, has a stimulatory effect on cell proliferation at low concentrations (1-50 µM) but by increasing the concentration, at 10 µM inhibits the cell growth (14). Also, Mahmoudi *et al.*, showed that Ferula Gummosa Ethanolic Extract exhibited higher proliferation rates and maximum cell numbers with gradual decrease of cell proliferation by increasing concentration human BMSCs after treatment with low concentrations (1 µg/ml) (15).

ALP is a key regulator of the early stage differentiation of bone marrow stem cells. It is enhanced during osteogenic differentiation. It is also an isoenzyme involved in the inorganic pyrophosphate breakdown in order to provide the phosphate needed for mineralization (27). Present study showed that activity of alkaline phosphatase in the sham group (DMSO) was significantly reduced. 5µM curcumin significantly reduced the effect of DMSO and increased cell activity compared to the sham group (DMSO). This change did not appear in 10 µM and 15 µM. These results alongside MTT test represented that cell senescence was not associated with apoptosis. Gu *et al.*, investigated the effect of curcumin on osteoblast and adipocyte differentiation of rat mesenchymal stem cell and final results

showed that 10 µM and 15 µM concentrations of curcumin increase the rat MSCs alkaline phosphatase activity compared to the control group (21). Similar results were reported by other study, either (24, 26). In a study by Moran *et al.*, ALP activity increased constantly from day 7 with culture time. However, a significant decrease in the late stages of osteoblast differentiation (day 21) was observed (evidenced by the decelerating accumulation of calcium in osteoblasts) (20). Due to the increases of alkaline phosphatase (and pyrophosphate compounds) at the early stages, and its reduction in the final stages with simultaneous concentration of calcium and phosphate (hydroxyapatite), this test together the alizarin red staining can be used to determine the stage of cell differentiation (28).

The present study showed that treatment of DPSCs with curcumin had no effect on mineralization with no significant differences. The results of Gu *et al.*, were in consistent with this study (21). However, Yamaguchi *et al.*, followed differentiation of pre-osteoblastic cells into mineralizing osteoblasts in the presence or absence of curcumin analogues for 21 days. They found calcium deposition and potently enhanced mineralization by using alizarin red staining (29).The similar result was found by Ahmed *et al.*, (24) and Son *et al.*, (26). Considering that ALP activity has increased in the presence of low concentrations of curcumin, but no calcified nodule formation has been observed, it can be concluded that low concentrations of curcumin can play a role in the early stages of differentiation that is inconsistent with Gu *et al.*, study (21).

To assay odontogenic differentiation, it was necessary to evaluate expression of genes detecting human DPSCs odontogenic differentiation considering that we could not see differentiation of the cells by observing cells morphology and results of experiments, the expression of genes detecting hDPSCs odontogenic differentiation was not evaluated. Experiments in the odontogenic medium, with a greater number of repetitions at concentrations lower than 5µM curcumin should be done to achieve better results.

Conclusion

Low concentration of curcumin is not toxic and increases the ALP activity of the cells compared to the DMSO group, but it is cytotoxic at higher concentrations (10 to 15 µM) and does not cause cell differentiation.

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Conflict of Interest: 'None declared'.

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