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*Research Article*

# Influence of Curcumin Nanocrystals on the Early Osteogenic Differentiation and Proliferation of Dental Pulp Stem Cells

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Regenerative therapies are the developed method for regrowing, repairing, or exchanging damaged or diseased cells, tissues, or organs. Bone injury regeneration is a complicated procedure. For this reason, stimulation of the osteogenic differentiation impact on mesenchymal stem cells (MSCs) is a critical method. The goal of the current study was to test the biocompatibility and induction of early osteogenic differentiation by curcumin nanocrystals in human dental pulp stem cells (DPSCs) which are MSCs derived from dental pulp. In this study, curcumin nanocrystals were produced through the spray-drying technique and then were characterized using conventional methods. Then, cytotoxicity and proliferative properties of curcumin nanocrystals were evaluated in different concentrations at 48 hours, 72 hours, and 7 days which were measured by the MTT test, and a special kit was used to determine the activity of alkaline phosphatase (ALP). The results of this study displayed that the prepared nanocurcumin with the mean particle size of 128 nm, spherical morphology, and the negative surface charge did not show a toxic effect on dental pulp cells in most concentrations for 48 and 72 hours and was toxic only at a concentration of 25 $\mu$M. Also, comparing the toxicity of different doses at 7 days showed that at concentrations of 2.5, 5, 10, and 25 $\mu$M, cell growth was significantly inhibited. ALP activity was increased in two weeks compared to one week and also compared to that of the control group significantly ($p < 0.05$). Considering the positive possessions of nanocurcumin on the osteogenic differentiation of DPSCs and its low toxicity, this substance can be considered in the planning of regenerative protocols.

## 1. Introduction

The main cell types are mesenchymal stem cells (MSCs), which are used in regenerative therapies, and their main extraction sites are adipose tissue or bone marrow. The multipotent stem cells in contact with different factors such as bone morphogenic proteins (BMPs), vitamin D3, and bone growth proteins organize a broad set of tissues like the bone and cartilage and fat to maintain the body's balance of composition [1, 2].

Dental-derived mesenchymal stem cells (D-dMSCs) are now an attractive turning point for tissue regeneration because these cells have been stated to show a great capacity to differentiate into chondrogenic, adipogenic, and osteogenic lineages, with a special capability to increase mineralization of the bone [3].

Dental pulp stem cells (DPSCs) are isolated from dental pulp. These pluripotent cells can be differentiated into several cell types such as odontoblast, osteoblast, chondroblast, neuroblast, fibroblast, and myoblast adipocytes [4, 5].

DPSCs can be utilized in regeneration and tissue engineering procedures to treat various bone defects because of congenital malformations and trauma, tumors, and osteoporosis [6, 7].

DPSCs can differentiate into odontoblast-like cells for the formation of dentin in response to degradation, disruption, and mechanical erosion. Indeed, numerous investigations have revealed that mechanical stresses could impact the manners of DPSCs [8, 9]. The environmental signal transduction such as chemical and physical stimulants can be affected by the differentiation and formation of odontoblast-like cells derived from DPSCs [10].

Furthermore, DPSCs show a good perspective of the clinical future of these cells in tissue engineering due to their easy access, immunosuppressive properties, high proliferation potentiality, and differentiation capacity. However, the knowledge of growth factor development and signaling molecules is essential in the growth and reconstruction of various tissues [11, 12].

Bone injury regeneration is a complicated procedure. For this reason, induction of the osteogenic differentiation impact on MSCs is a critical method. Recent efforts have focused on the study of dental and bone tissue engineering as well as their structures. Therefore, the use and development of stem cells, biological materials, scaffolds, and growth factors have been considered [13]. Currently, oral-derived stem cells correlated with special biomaterials are applied in various tissue engineering methods [3, 14].

Plant-derived natural agents have several applications in medicine because of their beneficial therapeutic application [15, 16]. Curcumin is a natural compound of the turmeric plant. It is best known for its antiproliferative properties in the treatment of cancer. It also has antibacterial and antioxidant properties and stimulates mineralization [17, 18]. In a study, the cellular mechanism induced by curcumin on cell differentiation was investigated and it was found that this material induces ALP activity as well as mineralization and osteoblast differentiation [18]. The results of the Gu et al. study also indicate an increase in ALP activity and expression of osteoblast-specific mRNA and osteocalcin in bone culture medium and that curcumin reduces the differentiation of adipocytes [19].

In another investigation by Wang et al., the helpful effect of curcumin on enhancing neuronal stem cell proliferation and preventing stem cell apoptosis was investigated [20].

The biggest disadvantage associated with the curcumin application is its low bioavailability due to its poor solubility in water, low absorption, rapid metabolism, and inactive metabolites, which are the main obstacles in this field [21]. Nanotechnology has recently offered a variety of solutions to this problem [22]. Nowadays, nanoparticles of curcumin improve the beneficial properties of curcumin and reduce its disadvantages which lead to the maximum delivery of curcumin for multiple pharmaceutical purposes [23, 24].

To identify the induction of osteogenic differentiation, markers are used in osteoblasts and odontoblasts such as osteopontin, ALP, bone sialoprotein, dentin sialoprotein, and dentin protein matrix. An indicator of cell differentiation into osteoblasts is the increased ALP activity [25]. In most of the studies, this enzyme has been used as the main marker of osteogenic differentiation induction by biomaterials [26–28].

Therefore, stem cell osteogenic differentiation is of paramount importance. Therefore, this study is aimed at producing curcumin nanocrystals and assessing the early osteogenic differentiation induction in human DPSCs by measuring ALP activity.

## 2. Materials and Methods

*2.1. Preparation of Curcumin Nanocrystals.* In this research, curcumin nanocrystals were prepared according to our previous research. Briefly, ethanol (Merck, Germany) was used for dissolving curcumin (Sigma-Aldrich); then, hexane (Merck) was speedily added to it to attain a solution at 8 mg/ml. By volume, the ratio of hexane/ethanol was 30/1. Curcumin nanocrystals were achieved by solvent evaporation using a rotary evaporator. For spray drying of curcumin nanocrystals, a YC-015 experimental spray drier (Shanghai, China) was applied by the following operation: the outlet temperature of 80°C, inlet temperature of 150°C, and liquid feed rate of 1.5 mL/min. To keep the suspension homogenization, a magnetic stirrer was utilized. Dry curcumin nanocrystals were collected in the bottle and used for cellular examinations [29, 30].

*2.2. Particle Size Characterization.* The dynamic light scattering (DLS) method (Malvern, UK) was utilized for calculating the mean size of curcumin nanocrystals via an argon laser beam at 25°C. Freshly prepared dispersion of nanocrystals in distilled water was used for determination.

*2.3. Morphology Determination.* For evaluation of the morphology of the nanocrystal surface, it was used from SEM (SEM, TESCAN, Warrendale, PA, USA). The prepared nanocrystals were placed on stubs through the double-sided tape and covered with a gold layer under a voltage of 10 kV.

*2.4. Zeta Potential Measurement.* A zetasizer (Malvern, UK) was used for measuring the surface charge (zeta potential) of curcumin nanoparticles at 25°C. Freshly prepared dispersion of nanoparticles in distilled water was used for determination.

*2.5. DPSC Culture.* The certified and characterized DPSCs were purchased from Shahid Beheshti University of Medical Sciences in Iran. DPSCs (passage number three) were cultured in DMEM liquid culture medium comprising 10% fetal bovine serum (FBS) and 100 mg/ml streptomycin and 100 mg/ml penicillin and then incubated in 5% $CO_2$ at 37°C.

*2.6. Cell Proliferation and Viability Assay.* 3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) is used for evaluation of the viability of cells. It is a yellow dye that is easily absorbed by living cells and reduced via mitochondrial dehydrogenases. MTT assay was utilized for assessment of nanocurcumin effect on proliferation and

viability of DPSCs. DPSCs were seeded in 96-well plates (5000 cells/well) and exposed with nanocurcumin at concentrations of 01, 2.5, 5, 10, and 25 $\mu$M at times of 2, 3, and 7 days. After the treatment times, the medium was exchanged with 200 $\mu$l of MTT solution (0.5 mg/ml) and incubated at 37°C for 4 hours. Then, MTT solution was exchanged with 200 $\mu$l DMSO. Then after, the absorbance of all wells was read at 570 nm via plate reader and the percentage of living cells was calculated compared with the control (cells without nanocurcumin). All assessments were done in three replications.

*2.7. Induction of Osteogenic Differentiation in Human DPSCs.* DPSCs were cultivated in an osteogenic differentiation medium containing $\alpha$-MEM medium supplemented with 10% FBS (Gibco, Grand Island, NY), 100 $\mu$g/ml streptomycin, 100 IU/ml penicillin, 10 nM dexamethasone, 0.2 mM sodium L-ascorbyl-2-phosphate, and 10 mM $\beta$-glycerol phosphate. Every three days, the differentiation media was renewed. Nanocurcumin (0.5 $\mu$M) was added to cells in the differentiation media. The culture medium was changed every three days. The ALP activity was evaluated on weeks one and two.

*2.8. ALP Activity Test.* For the detection of osteogenic differentiation, ALP is a reliable marker. After weeks one and two of treatment, the DPSCs grown in the osteogenic medium were detached and resuspended in an ultrasound breaker and were utilized for cell breaking. Then, cell supernatant was utilized for evaluation of the ALP activities utilizing a special detection kit (Nanjing) and a spectrophotometer (Bio-Rad) at a wavelength of 520 nm. Then, the relative activity of ALP was normalized to the total protein content.

*2.9. Data Statistical Analysis.* The results were reported as mean ± SD and frequency (percentage). Data normality was assessed using the Kolmogorov–Smirnov test. To compare the toxicity level and differentiation of nanocurcumin on cells, the $t$-test was utilized to evaluate the significance of MTT tests and one-way ANOVA was utilized to evaluate the activity of the ALP enzyme. Statistical analysis was done via GraphPad (version 17) software and a significance level of $p < 0.05$ was considered.

## 3. Results and Discussion

The prepared curcumin nanocrystals exhibited an average particle size of 128 nm (Figure 1(a)). Furthermore, the outcomes of SEM displayed that the curcumin nanocrystals have monodispersed and spherical small particle sizes (Figure 1(b)).

It was showed that some fine nanoparticles attached through spraying by van der Waals and electrostatic forces and caused larger particle sizes. Throughout spray drying, the nanoparticles were scattered in the arrangement of very small drops containing very fine particles. After the water vaporized, suddenly, these particles aggregated together and the aggregates were difficult to separate into single particles [30].

The physicochemical possessions (size, shape, and surface charge) of a nanomaterial have impact on its pharmacokinetics and distribution. These factors are also critical for the passive targeting and nonspecific cellular uptake of nanoparticles. According to Karaman et al., the shape of nanomaterials is a significant property regardless of both the different surface charges and doses tried [31]. They also validated that at lower doses, the shape of a nanoparticle is less principal; however, the charge-induced properties can influence the cellular uptake significantly [31]. Based on some reports, the nanoparticulated size and spherical shape lead to better uptake of nanomaterials in targeted cells that resulted in improved absorption and the reduced needed dosages [32–34].

The zeta potential of nanomaterials especially pharmaceutical nanoparticles has the main impact on the different possessions of these colloidal systems. The stability of nanoparticles, their release profile as well as their movement in the blood, and subsequently their absorption into body membranes are melodramatically related to the zeta potential [35, 36]. The prepared nanoparticles in this study showed a negative zeta potential of –18.3 mV (Figure 1(c)). The reports have shown that nanoparticles with negative zeta potential had higher cellular uptake than positive surface charge [35].

Advances in nanotechnology-based delivery systems are now widely used because of their promising potentiality and benefits. Due to its expansion, this technology has been considered in the field of dentistry, including the design of new materials for restorative dentistry and endodontic treatment. On the other hand, DPSCs have shown successful results in bone regeneration. However, some studies are still under investigation including the applications of DPSCs to improve bone regeneration *in vivo* and *in vitro* conditions and the vast potentiality of nanoparticles in enhancing the formation of high-volume bone by DPSC-based therapy [37]. Therefore, carrying out studies to obtain a method to reset the DPSC capacity alongside nanotechnology for more widespread clinical applications is significant. To the best of our knowledge, there are evidences, which state that curcumin may play a part in the mineralization of stem cells; therefore, the present study presents a new approach to improve the induction of osteogenic differentiation of DPSCs.

To test the cellular cytotoxicity of curcumin nanoparticles, the results of the MTT test indicated that nanocurcumin had no cytotoxic influence on DPSCs at concentrations of 0.5, 1, 2.5, 5, and 10 $\mu$M for 48 and 72 hours (Figure 2). Instead, some concentrations caused an increase in the growth of these cells. In other words, nanocurcumin had a proliferative effect on dental pulp cells at these concentrations. Also, nanocurcumin inhibited the growth of cells only at a concentration of 25 $\mu$M ($p < 0.05$). To investigate the cytotoxicity of nanocurcumin for a 7-day exposure (Figure 2), MTT test results indicated that nanocurcumin had no toxic effect on DPSCs at 0.5 and 1 $\mu$M concentrations ($p < 0.05$). In other words, the effect of nanocurcumin on these cells is time dependent and to some extent dose dependent.

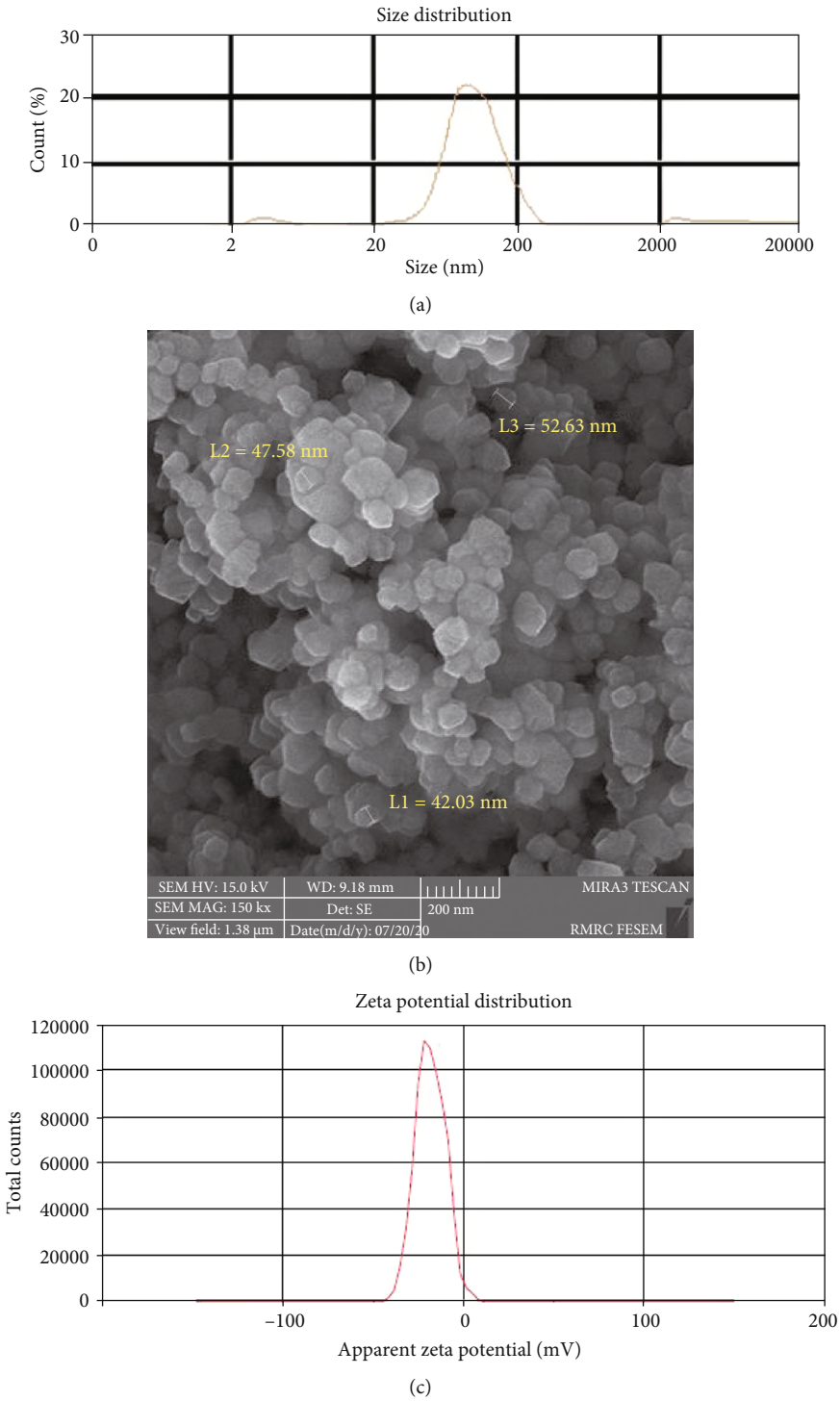


Figure 1: Physicochemical characterizations of the produced curcumin nanocrystals. (a) Distribution of particle size, (b) SEM (magnification of 150 kx), and (c) zeta potential.

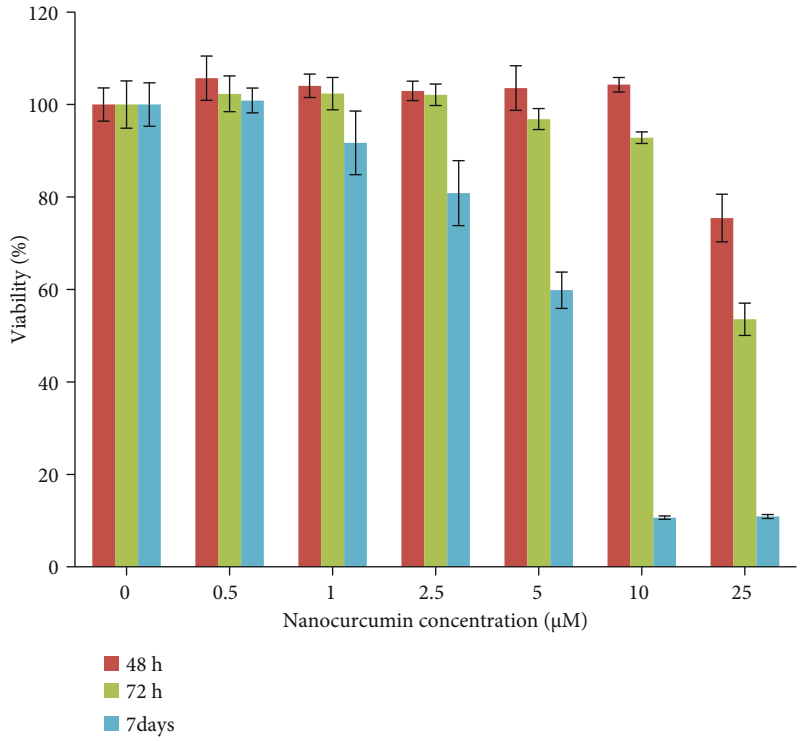


Figure 2: Comparative diagram of the MTT test for the evaluation of different concentrations of nanocurcumin toxicity on DPSCs at 48 hours, 72 hours, and 7 days.

It was found that curcumin nanocrystals did not have a significant cytotoxic influence on DPSCs in most concentrations and tested times.

The test result of measuring the activity of ALP on treated cells with 0.5 $\mu$M of nanocurcumin for one and two weeks (Figure 3) showed a significant increase in activity of ALP in two weeks compared with those for one week and the control group in each group ($p < 0.05$).

Recently, the nanoparticle application in the delivery of different medicinal agents has attracted the attention of investigators due to an increase in the drug bioavailability and the opportunity of targeting it. In the case of curcumin as a highly hydrophobic agent, the preparation of nanoparticles is of great importance for improving the aqueous dissolution of the drug. Recent studies on curcumin-containing nanoparticles have attracted attention, and research is expanding into the fabrication and application of nanoparticle-based systems for the delivery of curcumin [38–40].

In a report on human mesenchymal stem cells (hMSC) treated with hydroquinone, the protective influence of PEGOA nanocurcumin in contradiction of oxidative harm was investigated. The results exhibited that hMSC treatment with PEGOA nanocurcumin (10 $\mu$M) for 12 hours significantly reduced lipid peroxidation and increased the expression of antioxidant genes involving catalase and homoxygenase-1. These results indicate that PEGOA nanocurcumin can protect hMSC [41]. These results are intended for our study as a nontoxic effect of nanocurcumin and to protect and induce the growth of DPSCs.

Curcumin may exhibit anticytotoxic properties by modifying oxidative stress factors like reactive oxygen species (ROS) and the expression of antioxidant genes in a dose- and time-dependent manner. In another study, it was shown that the expression of antioxidant enzymes comprising catalase, glutathione peroxidase (GPx), and superoxide dismutase (SOD) was increased by increasing the curcumin concentration and incubation time in the RAW 264.7 macrophage cell line. Besides, the viability of cells was reduced meaningfully in high concentrations of curcumin (25 $\mu$M) but not in low concentrations (5 $\mu$M) [42]. The outcomes of the current study are in line with our study except that this study examined curcumin.

The results of a study on the toxicity of nanocurcumin against the baby hamster kidney normal cell line (BHK) showed that the rates of cell growth and proliferation gradually decrease with time increase so that this effect was more than 24 hours in 48 hours [38]. This study is in line with the present study that according to the obtained results, nanocurcumin in 7 days of exposure, even at low doses, is toxic to stem cells, while in high concentrations, at low times, it did not show toxicity.

Moghaddasi et al. by synthesizing the nanocurcumin showed that this material has high antioxidant activity. Examination of its cytotoxic effect in vitro condition revealed that it has very low toxicity in Neuro2A cells [43].

The results of our study also displayed that the activity of ALP in treated DPSCs with nanocurcumin for one week exhibited an improved amount compared with that in the control group significantly ($p < 0.05$).

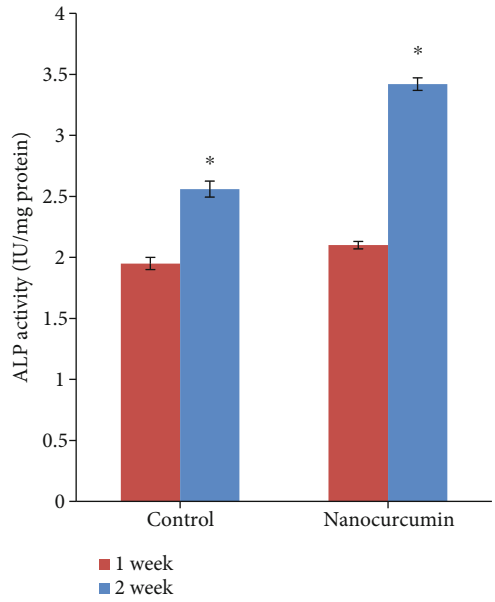


Figure 3: The result of measuring the activity of ALP on treated DPSCs with nanocurcumin in one and two weeks.

Our other recent study showed that curcumin upsurges the early osteogenic differentiation of DPSCs. These outcomes were demonstrated by analyzing the activity of ALP or osteogenic gene expression levels [44]. In another study, the cytotoxicity of curcumin and the osteogenic marker expression in C3H1-T1/2 cells were tested and it was shown that curcumin improved the expression of ALP and osteocalcin (OC) genes that then differentiated osteoblasts in cells [18]. This study is also in line with our study, which indicates the inductive effects of curcumin mineralization in previous studies. The study of Gu et al. revealed that curcumin induces the differentiation of rat mesenchymal osteoblast stem cells but prevents adipocyte differentiation [19]. Also, it was observed that curcumin and its analogues have an optimistic part in the rat's osteoblast differentiation by stimulation of Smad signaling and suppression of NF-$\kappa$B activity [17].

However, very limited investigations have been performed on the effect mechanism of nanocurcumin on mineralization induction of MSCs, and to the best of our knowledge, no clinical investigations have been performed. However, due to the inadequate clinical experiments to test the safety and effectiveness of curcumin nanoparticles in humans, significant research gaps have been identified in this area. Therefore, it is essential to perform several clinical experiments with a wide range of patients before introducing nanoformulations of curcumin to the medical markets. More attention may be paid to the improvement of tissue-specific nanomaterial delivery systems. Hybrid nanoparticles, consisting of two or more components that are composed of other materials, also need further study. It is also worth considering that whether nanocurcumin can be used as an inducing agent in regenerative therapies or in an appropriate formulation with other materials, which can enhance its potentiality for mineralization, has not yet been investigated. In this study, as the first step in this direction, the competence of nanocurcumin in using osteogenic differentiation induction processes in stem cells has been investigated. This was considered owing to its low toxicity and the property of osteogenic differentiation induction. The advance of these methods and organized experiments can be useful in identifying and introducing this effective material in treatment. The aggregation of nanoparticles during spray drying was the limitation of this study that should be eliminated in future studies.

## 4. Conclusions and the Future Perspectives

Due to the positive effects of nanocurcumin on osteogenic differentiation of DPSCs and its low toxicity effect, this material can be considered as an option in bone and tooth tissue engineering. Furthermore, curcumin nanoparticles can be used for the expansion of suitable scaffolds for stem cell-based bone, dental, and periodontal regeneration. These nanoparticles can improve the regeneration of dental tissues in advanced levels. For example, they may help maxillofacial surgeries to simplify dental surgical procedures in the future. Induction of the ALP activity showed by nanocrystals of curcumin indicates its potential to favor the osteogenic differentiation of DPSCs. Nevertheless, the evaluation of other odontogenic or osteogenic markers was not assessed so further studies are required to approve its effect on osteo/odontogenic differentiation.

## Data Availability

The data that support the findings of this study are available upon request from the corresponding author.

## Ethical Approval

The ethic consideration for this study was accepted by the Ethic Committee of the Tabriz University of Medical Sciences under the code of IR.TBZMED.VCR.REC.1398.391.

## Disclosure

The current study was done based on a thesis registered at Tabriz University of Medical Sciences (number 61366).

## Conflicts of Interest

There is no conflict of interest.

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