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Early osteogenic differentiation of human dental stem cells by gelatin/calcium phosphate- *Punica granatum* nanocomposite scaffold

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Abstract

Background Tissue engineering for bone regeneration aims to heal severe bone injuries. This study aimed to prepare and assess the early osteogenic differentiation effects of a gelatin/calcium phosphate- *Punica granatum* nanocomposite scaffold on stem cells from human exfoliated deciduous (SHED) and human dental pulp stem cells (HDPSCs).

Methods The electrospinning method was used to prepare a gelatin/calcium phosphate nanocomposite scaffold containing pomegranate (*Punica granatum*) extract. The physicochemical properties of the scaffold were evaluated. The effect of the scaffold on the selected cells was done by the cell viability evaluation. A special alkaline phosphatase (ALP) kit was utilized to investigate the early osteogenic differentiation effects of the prepared scaffold on HDPSCs and SHED.

Results The results showed that the scaffold had uniformly accumulated in the networked form. Besides, the prepared scaffold did not have beads (structural defects). No new interactions were observed in the spectroscopic spectra of the scaffold and these peaks showed the successful formation of the fibrous nanocomposite as well. Furthermore, cell viability percentage was significantly higher for the scaffold compared with the control group (cells without any material) for both HDPSCs and SHED. Early osteogenic differentiation results specified that the ALP activity was significantly higher for the scaffold compared with the control group (cells without any material) for both HDPSCs and SHED.

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Conclusion The appropriate physicochemical assay and cellular results (cell viability and early osteogenic differentiation) for the prepared fibrous nanocomposite showed that the use of this nanocomposite can be considered in the construction of various scaffolds in bone and dental tissue engineering.

Keywords Scaffold, Nanofiber, Gelatin, Calcium phosphate, *Punica granatum*, Tissue engineering

Background

Bone tissue engineering through various scaffolds is a new therapeutic strategy in bone regeneration. Scaffolds serve as the basic framework in bone tissue engineering [1, 2]. The basis of regenerative medicine is tissue engineering using stem cells, scaffolds, and growth factors to regenerate bone tissue damage [3, 4].

Dental stem cells can multiply and regenerate under special laboratory conditions. These cells have shown odontogenic, neurogenic, osteogenic, adipogenic, and chondrogenic differentiation [5, 6]. A suitable scaffold for tissue engineering should be biodegradable, biocompatible, and mechanically strong. In addition, it should imitate the morphology and chemical composition of the extracellular matrix (ECM) that provides oxygen, nutrients, and growth factors to the cells to create a favorable environment for new tissue formation [7].

To design a scaffold, the bone tissue composition such as its chemical parameters, should be considered to provide control over the structural and physical properties of new biomaterials to improve the adhesion, proliferation, and differentiation of stem cells [8]. Calcium phosphate ceramics are biocompatible and allow attachment to mesenchymal cells due to their high propensity to absorb proteins. In addition, calcium phosphate ceramics are biodegradable and lead to osteogenic differentiation of mesenchymal cells due to sustained release of calcium and phosphate [9]. However, the use of calcium phosphate ceramics alone is limited due to their fragility. In order to overcome these limitations, polymer materials have been introduced to form composite scaffolds to increase the efficiency of repairing bone defects and increase the clinical efficiency of calcium phosphate [10].

Currently, polymers are considered among the desirable materials in tissue engineering. In tissue regeneration systems, the polymers act as an extracellular matrix in the early stages, which is eventually replaced by the natural extracellular matrix following the development of the relevant organ. They are used not only because of their similarity to the extracellular matrix but also as carriers of bioactive molecules to accelerate the regeneration process [11]. Gelatin is a natural polymer obtained from the incomplete hydrolysis of collagen and has biological properties such as biocompatibility and biodegradability [12]. This substance is extracted from the bone, skin, and connective tissue of animals [12]. It is proper for the growth, junction, and maintenance of the physiological function of cells. The materials resulting from its decomposition can be absorbed and metabolized without any adverse cellular and tissue reactions. Gelatin can also induce the proliferation and differentiation of osteoblasts [13, 14].

In recent years, gelatin along with dental stem cells have been used to regenerate bone tissue, dentin-pulp complex, dentin, and tooth root [15]. Specific bioactive compounds of plants can stimulate osteogenic differentiation of stem cells [16, 17]. Pomegranate (*Punica granatum*) has desirable therapeutic properties such as antimicrobial, anti-mutation, anti-inflammatory, anticancer, anti-diarrhea, and anti-diabetes properties. This substance with high phenolic content can improve bone healing as well. Also, its seed extract is effective in the proliferation and differentiation of osteoblasts prevents the activity of osteoclasts, and plays an important role in bone remodeling [18].

The introduction of new materials of natural and plant origin in the field of tissue engineering can establish a bridge between modern sciences and basic sciences. Many materials are introduced in this regard, but their effects are not determined clearly and scientifically. Therefore, in this study, we aimed to assess the effects of a gelatin/calcium phosphate- *Punica granatum* nanocomposite scaffold on the early osteogenic differentiation effect of human dental stem cells including dental pulp stem cells (HDPSCs) and stem cells from human exfoliated deciduous (SHED).

Materials and methods

Materials

Gelatin, calcium phosphate and 2,2,2 trifluoroethanol were purchased from Sigma- Aldrich Co. (Burlington, Massachusetts, United States). *Punica granatum* extract was prepared from Barij Essential Pharmaceutical Company, Iran, Kashan.

Methods

The electrospinning method was used to prepare a gelatin/calcium phosphate nanocomposite scaffold containing *Punica granatum* extract. A solution was prepared in 2,2,2 trifluoroethanol solvent with a ratio of 5-25-70% of gelatin, calcium phosphate, and pomegranate seed extract, respectively. This solution was transferred into a syringe. The syringe was located in the special place of the electrospinning machine and a nozzle with a micrometer tip was connected to its tip. The collector plate of the machine was sheltered with an aluminum sheet.

The key parameters were adjusted: a voltage of 20 kV, a working distance of 10 cm, and a flow rate of 1.5 ml/h. After pressing the start button, the prepared solution was thrown from the nozzle at a speed toward the rotating plate of the collector. During the process, the organic solvent was evaporated. The final product was prepared in the form of nanofibers on the collector and then the aluminum sheet was separated from the collector plate. Then the nanofibers were dried at room temperature and maintained at -18 °C for use in various evaluations. Figure 1 shows the produced scaffold on the aluminum foil surface.

Physicochemical assessments

The prepared scaffold was evaluated using Fourier Transform Infrared spectroscopy (FTIR, Vertex 70v, Bruker, USA). So, 2 µg of the prepared scaffold powder was placed in the special pan of the FTIR device and it was set at wavelengths of 400 to 4000 (cm^{-1}). Besides, X-ray diffractometer (XRD) patterns were determined at room temperature for gelatin, calcium phosphate, and *Punica granatum*. The nanofibers were affected by an X-ray diffractometer (Siemens, model D5000, Germany) and radiation with a wavelength of 1.5405 Å, a voltage of 40 kV, and a current of 30 mA, and their patterns were determined by the device.

The morphology of the prepared scaffold was evaluated using an electron microscope. The nanofibers were located on a scanning electron microscope (SEM, TESCAN, Warrendale) plate and a gold surface paper was covered on them. The ImageJ software was used to analyze the fiber diameters by measuring 100 fibers chosen randomly from a sample. Besides, the transmission electron microscope (TEM, Philips, TecnaiG220) was used to show the presence of nanoparticles on the fibrous nanocomposite.

The pore size was measured by bubble point pressure and the porosity was estimated by weight and volume of the sample [19].

Tensile strength determination

The Hounsfield H5K-(UK) apparatus was utilized to assess the tensile strength of the nanofibers. The stretching rate of the device was set at 1 mL/min. The constructed samples were pulled to a cross-section of 10×2 mm with the help of two clamps of the device.

Cytotoxicity effect

Cells (HDPSCs and SHED) were purchased from Shahid Beheshti University (Tehran, Iran). Then, the effect of the scaffold on cells was assessed using a cell viability test. 5×10^3 cells/well were seeded on the prepared scaffold in 96-well microplates and incubated for 24 h at 37 °C in an incubator with 5% CO_2. After 72 h, the medium of the wells was replaced with fresh 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) solution, and microplates were maintained in an incubator at 37 °C for 4 h. Then, the MTT solution was replaced with DMSO. After the dissolving of the colored crystals, the solution was transferred to another plate, their absorbance was evaluated at 570 nm and the living cells percentage was assessed by comparing it to the control group (cells without scaffold).

Fig. 1 The produced scaffold on the aluminum foil surface

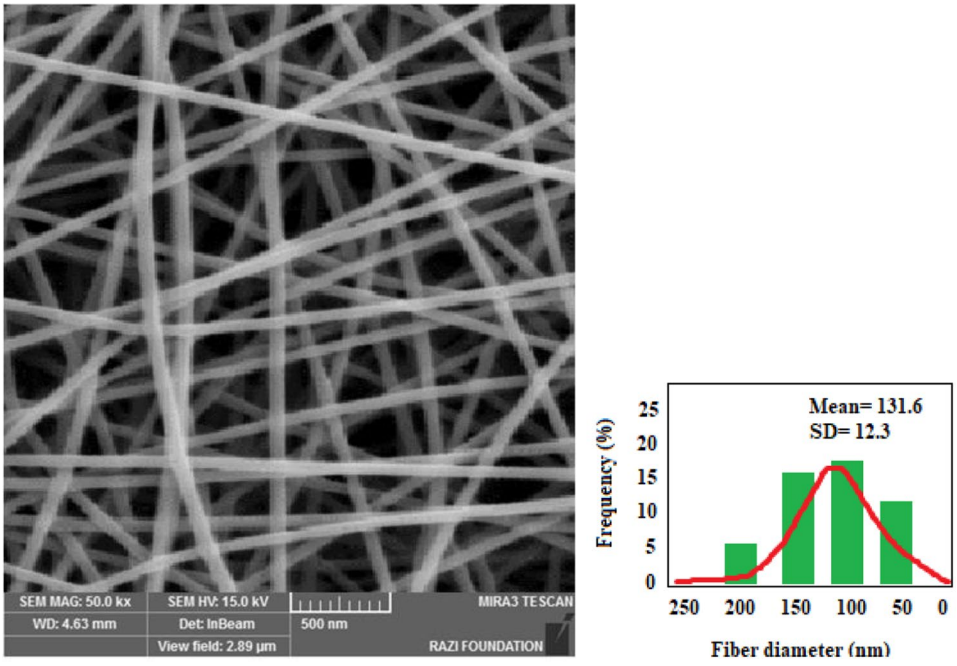

Fig. 2 The SEM image and diameter distribution of fibers

Biodegradation test

Total 100 mg of the prepared nanoparticles were weighed. The samples were reserved in an incubator at 37 °C and the humidity 50%. The degradation of the samples was measured every 5 days for the determination of weight loss. To identifying the sample weight loss, each sample was dried in a vacuum oven for 1 day [31]. The following equation was used to determine the weight loss:

$$\text{Weight loss } (\%) = \mathrm{w_a} - \mathrm{w_b}/\mathrm{w_b}$$

Where w_a is the dry weight of the sample before test, wb is the dry weight of the sample after the test.

Early osteogenic differentiation

A special kit (Pars Azma, Tehran, Iran) was used to investigate the effect of the prepared scaffold on the level of alkaline phosphatase (ALP) activity of the cells (HDPSCs and SHED). The prepared scaffold was positioned in the bottom of the wells and then the suspension of cells was added. After two weeks, the activity of ALP was measured. Cells without any material considered as control groups for each types of cells and gelatin-hydroxyl apatite nanofiberous scaffold was used as well-known material for osteogenic differentiation.

Statistical analysis

The results were reported as descriptive statistics. The results of all tests were the average of three repetitions. A t-test was used to compare the means of two groups (cytotoxicity effect and early osteogenic differentiation). SPSS software version 24 was utilized for the analysis of data. The values of less than 0.05 were considered statistically significant.

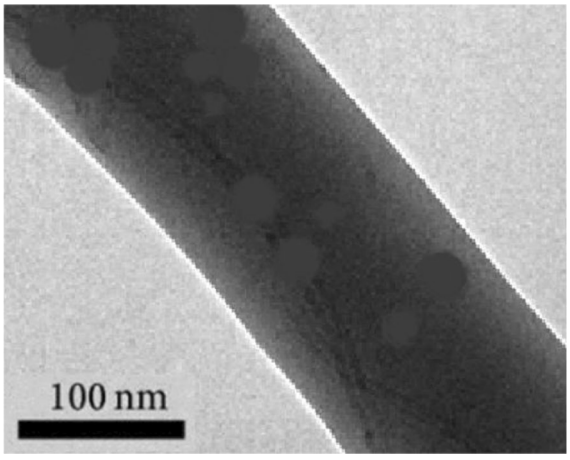

Fig. 3 TEM microscopic image for the prepared scaffold

Results

Physicochemical assessments

The mean fiber diameter was obtained from one samples which gave an average fiber diameter of 131.6 ± 12.3 nm. The SEM image and diameter distribution of fibers are shown in Fig. 2. The results displayed that the prepared nanofibers were accumulated in the form of porous and monodispersed network fibers. Besides, Fig. 3 shows the TEM image for these fibers. *Punica granatum* extract is

blended with gelatin in this structure and the presence of calcium phosphate nanoparticles on the fibers is evident in the TEM image.

Pore size and porosity (%) were determined 3–20 μm and 81%, respectively.

FTIR and XRD results of the prepared scaffold and the raw materials are shown in Fig. 4(A, B), respectively. All the main peaks of the raw materials were visible in the peaks of the final product, which indicates the successful preparation of this material. In the FTIR and XRD spectra, no new interactions were observed in the prepared nanofibers.

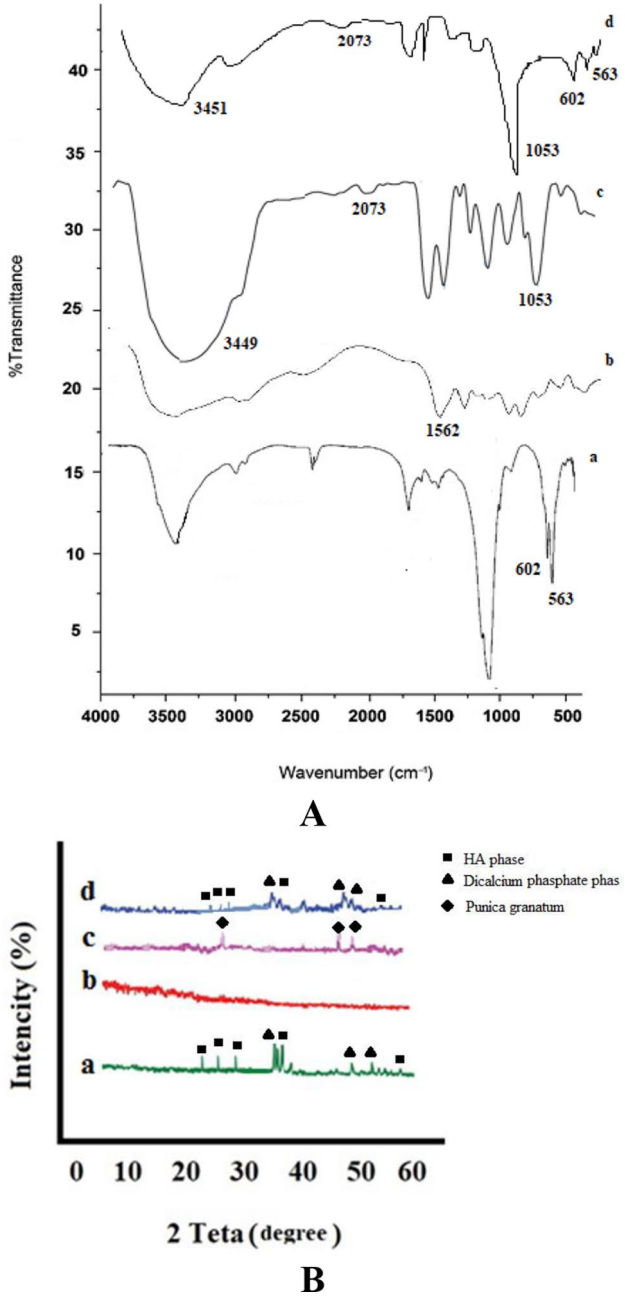

Fig. 4 FTIR peaks (**A**) and XRD (**B**) results for calcium phosphate (a), gelatin (b), *Punica granatum* oil (c), the scaffold (d)

Tensile strength

The produced scaffold has approximately high tensile strength in the waterless state, and its elongation at break was 40.60 ± 1.2 MPa. The tensile strength of the scaffold in the wet state decreased (35.24 ± 0.9 MPa). However, it remains within the optimal range for bone tissue engineering applications, ensuring sufficient mechanical stability in physiological environments [20, 21].

Cytotoxicity effect

The percentage of cell viability increased significantly when exposed to the scaffold for both types of cells (DPSCs and SHED) compared to the control group (cells without any material) ($p < 0.05$). Figure 5 shows this result.

Biodegradation

The sample weight loss was increased during the test. A 50% degradation of the scaffold was achieved after 30 days.

Early osteogenic differentiation

According to Fig. 6, a significant increase in ALP activity was observed for both types of cells (SHED (Fig. 6a) and DPSCs (Fig. 6b)) when exposed to the scaffold compared to the control group ($p < 0.05$) (cells without any material). There was no significant differences between the ALP activity of the scaffold and the gelatin- hydroxyl apatite scaffold as well-known material for osteogenic differentiation.

Discussion

Physicochemical characterization showed that the scaffold was prepared successfully. The diameter distribution of fibers was in the nanometric range (131.6 ± 12.3 nm). Besides, nanofibers have accumulated in the form of porous monodispersed network fibers without any beads. Besides, these results showed that *Punica granatum* extract is blended with gelatin in this structure and the presence of calcium phosphate nanoparticles on the fibers was observed in the TEM image. Recent studies have shown that osteoblasts have good attachments to nanofibers, and actively secrete an osteogenic matrix that prepares for bone growth [22, 23]. The study of Croisier et al. showed that the pore size is a main factor for the infiltration of osteoblasts and bone formation [24]. Abbasi et al. also reported that bone regeneration with porous scaffolds is highly dependent on the pore size of the scaffold, as bone growth was more prominent in scaffolds with a pore size of 100 μm in an in vivo study [25].

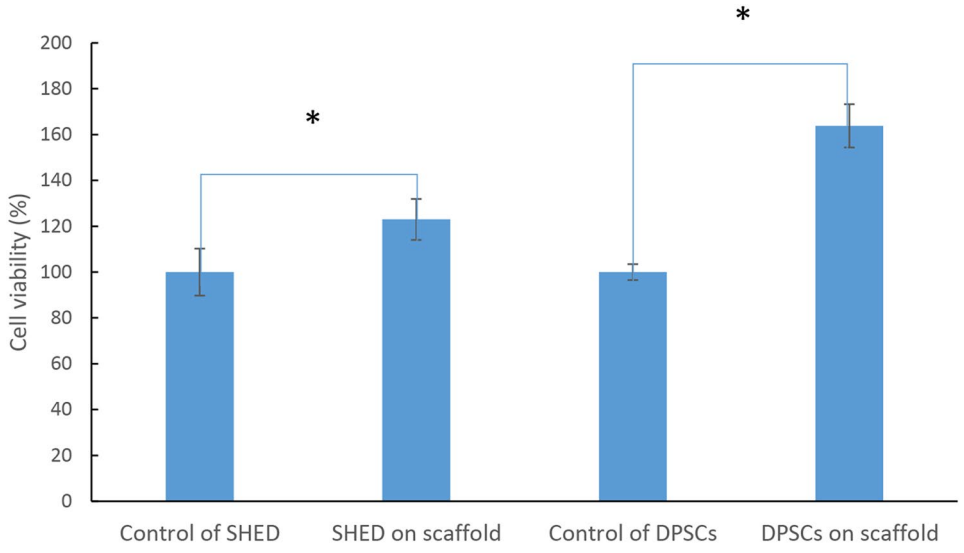

Fig. 5 Cell viability results for the prepared scaffold on DPSCs and SHED (*An asterisk indicates a significant difference)

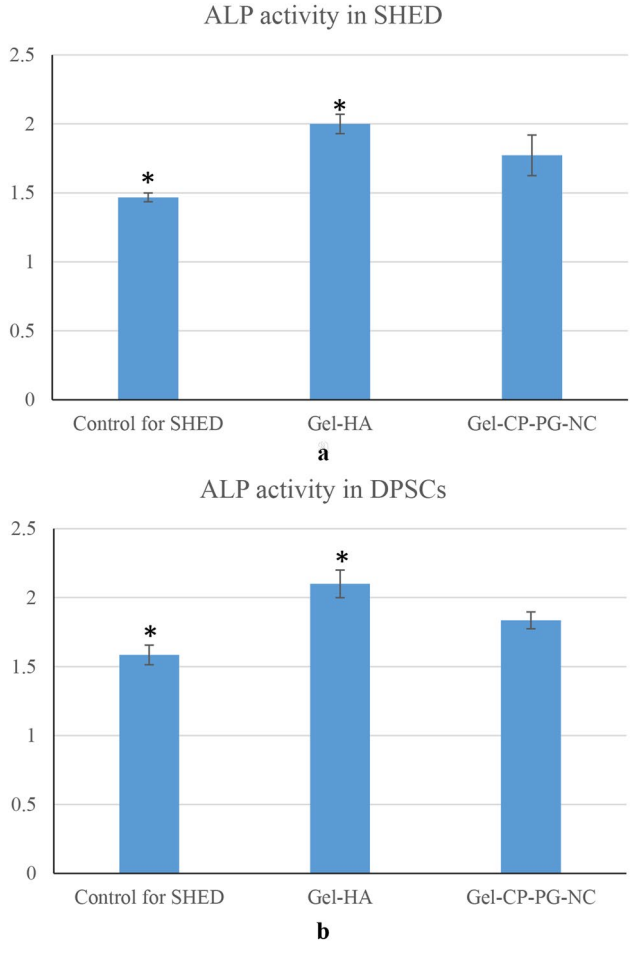

Fig. 6 Early osteogenic differentiation results for the prepared scaffold on DPSCs (**a**) and SHED (**b**), (*An asterisk indicates a significant difference with control group)

Calcium phosphates show a main role in biological calcification (formation of bones and teeth). Hydroxyapatite is the most stable phase of calcium phosphates. It has paying attention because of its similarity to the mineral phase of bones and teeth. Other crystalline calcium phosphate phases that often co-exist with HAP include dicalcium phosphate anhydrous, dicalcium phosphate dihydrate and octacalcium phosphate [26].

The results of FTIR spectroscopy showed that the characteristic peaks at 563 and 602 cm^{-1} are related to the calcium phosphate groups [27]. It seems that crystalline calcium phosphate phases of our materials (both free calcium phosphate and scaffold) is dicalcium phosphate dihydrate [28]. The peaks at 3449, 2073, and 1053 cm^{-1} indicate the phenolic OH group, vibration of the alkyne group, and the vibration of aromatic amines in *Punica granatum*, respectively [29]. The broad peak at 3200–3500 cm^{-1} is also related to the stretching spectrum of the OH group [30]. Nisha et al. reported similar results about the position of the functional groups in the FTIR peaks of pomegranate seed oil [29]. Sivakumar et al. also obtained similar results [31]. The amide bands related to gelatin are seen at 1652 cm^{-1} [32]. Sharifi et al. reported similar results for the FTIR peaks of gelatin present in gelatin-hydroxyapatite nanofiber scaffolds [33]. Besides, according to Sadek et al., the presence of *Punica granatum* in a polycaprolactone scaffold showed promising effects on the antioxidant activity of the scaffold and led to a proper distribution of porosity and cross-linking and significant embedding of osteoblasts on the scaffold [34].

According to the X-ray diffraction pattern results, all the pure substances showed their characteristic peaks (calcium phosphate in pairs 26, 31, 39, 29, 43 and *Punica granatum* in pairs 29, 47, and 48 2Theta angel) [27, 35]. It seems that crystalline calcium phosphate phases of our

materials (both free calcium phosphate and scaffold) is dicalcium phosphate dihydrate co-exist with HAP [26] that is in a good match with FTIR results.

Gelatin did not show an index peak due to its amorphous nature [33]. Saadat et al. reported similar results for gelatin nanofibers containing pomegranate seed oil [35]. Also, the peak of all the materials can be seen in the peak related to the prepared nanocomposite. The reason for the reduction in the intensity of the peaks in nanocomposite is the conversion of the material to nanometer size [30] and also the amorphous nature of gelatin. Hazma et al. reported similar results for polylactic acid-hydroxyapatite-curcumin nanocomposite [27]. Also, the study of Sharifi et al. showed that the scaffold of gelatin-hydroxyapatite nanofibers showed all the peaks of the raw materials with a lower intensity [33].

Porous nanofibrous scaffolds for tissue engineering should have high porosity to allow accom-modation and attachment of large numbers of cells, as well as large interconnected pores to facilitate uniform distribution of cells and diffusion of oxygen and nutrients. The surface of nanofibrous film showed enhanced cell adhesion over that of the flat film [19]. Kikuchi et al. fabricated an artificial bonematerial with a bone-like nanostructure and chemical com-position; here a composite of nHA and collagen was synthe-sized under biomimetic conditions through a self-organizationmechanism between nHA and collagen [36]. Pore size and porosity (%) were determined 3–20 μm and 81%, respectively.

Scaffolds necessity to possess the biomedical demands detailed to their planned use and be suitably strong to endure the applicable stresses and strains. The produced scaffold has approximately high tensile strength in the waterless state, and its elongation at break was 40.60 ± 1.2 MPa. This may show a method of rapidly familiarizing the mechanical possessions of nanofibers [37].

The percentage of cell viability increased significantly when exposed to the scaffold for both types of cells (DPSCs and SHED) compared to the control group (cells without any material). Rostami et al. showed that pomegranate extract plays an important role in regulating the immunomodulating function of stem cells. It can improve the immunoregulatory property of adipose-derived mesenchymal stem cells through overexpression of miRNA-23 and miRNA-126 or downregulation of miRNA-21 and miRRNA-155, which plays a role in immune modulation pathways in MSCs [38]. The proliferative impact of *Punica granatum* extract is likely linked to its estrogenic components, such as quercetin, kaempferol, estrone, and estradiol, which are known to stimulate bone cell growth [39, 40]. Previous research has shown that *Punica granatum* extract can enhance the proliferation of MC3T3-E1 osteoblast cells by approximately twofold at a concentration of 250 μg/ml [41].

In the current study, the activity of ALP in scaffold samples was higher than in control groups, for both types of dental pulp stem cells (DPSCs and SHED). Indeed, ALP enzyme activity and calcium matrix concentration are considered two important factors in investigating the differentiation of mesenchymal stem cells into osteoblasts [42]. The presence of calcium ions is necessary for the activity of the ALP enzyme [42]. Then, the presence of calcium phosphate in the prepared scaffold strengthened the concentration of calcium ions. Siddiqui et al. showed that *Punica granatum* has a significant effect on proliferation of cells, content of collagen, activity of ALP, and mineralization of osteoblasts matrix in a dose-dependent manner in primary calvarial osteoblast cells achieved from neonatal rats. They suggested that *Punica granatum* has a stimulating influence on osteoblastic bone formation or potential activity against osteoporosis. They reported that *Punica granatum* increased DNA content in the S phase of the cell cycle and expression level of the *Runx2* gene in osteoblasts [43]. The results of the ALP assay were consistent with those of the MTT assay, reinforcing the link between cell proliferation and differentiation. These findings suggest that *Punica granatum* extract promotes osteoblast regeneration likely due to the presence of estrogenic compounds. Specifically, *Punica granatum*, which includes estrogen-like compounds such as daidzein and genistein, has been reported to enhance alkaline phosphatase activity in osteoblasts under in vitro conditions [39, 44].

Study limitations

This initial in vitro research was about the effect of osteogenesis scaffold on two types of stem cells and the scaffold was in contact with the cells for a shorttime, so the investigation of other factors (Runx2, OCN, BMP2) and Alizarin Red staining required a longer period of time. However, in future research, the effect of this scaffold on the expression of various genes related to osteogenesis will be investigated, as well as an in vivo study on laboratory animals. Besides, the long-term effects and biodegradability effects and compatibility with surrounding tissue, are also critical data that must be assessed in future studies.

The concentration of bioactive compounds (like polyphenols) in the scaffold, how stable are these compounds within the scaffold over time, and how do they impact osteogenic differentiation beyond cell viability should also be assessed in the future studies.

Conclusion

According to the results of the present study, gelatin-*Punica granatum*/ calcium phosphate nanocomposite scaffold show no cytotoxicity and had a proliferative effect, and also increased the activity of alkaline phosphatase enzyme in the studied stem cells (DPSCs and SHED). However, it seems necessary to conduct additional in vitro and in vivo studies before clinical trial studies in this regard.

Abbreviations

SHED	Stem cells from human exfoliated deciduous
HDPSCs	Human dental pulp stem cells
ALP	Alkaline phosphatase
FTIR	Fourier Transform Infrared spectroscopy
XRD	X-ray diffractometer
SEM	Scanning electron microscope
TEM	Transmission electron microscope
DLS	Dynamic light scattering
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide

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Author contributions

Atefeh Abedi and Solmaz Maleki Dizaj designed the study. Atefeh Abedi, Simin Sharifi, Mahsa Baghban Shaker, Maryam Jalili, Elaheh Dalir Abdolahinia, and Solmaz Maleki Dizaj performed experiments, analyzed data, and wrote the paper, with both authors contributing equally. All authors read and approved the final manuscript.

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Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethical approval

The study started after obtaining the code of ethics from the ethics committee of Tabriz University of Medical Sciences. The code of ethics of this project is IR.TBZMED.VCR.REC.1401.370.

Consent for publication

No humans/animals were used for studies that are the basis of this research.

Competing interests

The authors declare no competing interests.

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