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Original article

Effect of gelatinous spongy scaffold containing nano-hydroxyapatite on the induction of odontogenic activity of dental pulp stem cells

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ABSTRACT

Tissue engineering methods may be applied for the repair or regeneration of damaged teeth by inducing cell proliferation and differentiation in endodontic regeneration. One of the main factors in tissue engineering is the scaffold used. An ideal scaffold should be able to facilitate predictable tissue development in endodontic regeneration. Thus, tissue engineering requires consideration for the composition and functionality of the scaffold suited for biological applications. The objective of this study was to assess the morphological, physico-chemical, and biological properties of a new gelatin (Gel)-nano-hydroxyapatite (nHA)-based scaffold to be applied in endodontic regeneration. The scaffold containing gelatin and nHA was prepared by a freeze-drying method. Conventional techniques, including scanning electron microscopy (SEM), Fourier-transform infrared spectroscopy (FT-IR), X-ray diffraction analysis (XRD), and dynamic light scattering (DLS) analysis were used to evaluate the physico-chemical properties of the fabricated Gel-nHA sponge scaffold. Biological examination for cell survival and differentiation of dental pulp stem cells (DPSCs) was evaluated by the MTT assay and ALP activity techniques, respectively. The produced Gel-nHA scaffold had spongy properties that all functional groups of gelatin and hydroxyapatite were present in the sponge. In biological studies, the viability of the cells grown on Gel-nHA scaffold had no different change from the control group on days 2nd, 4th, and 6th. Besides, after 14 days of cells cultured on Gel-nHA, alkaline phosphatase (ALP) activity showed a significant increment. In summary, the Gel-nHA scaffold revealed favorable effects on odontogenic activity, implying a potential future for application in endodontic regeneration.

1. Introduction

Regenerative endodontics is one of the latest endodontic treatment modalities that has recently been presented and has found a position in the area of current dental treatments. However, contradictory results have called into doubt its validity and dependability in endodontic treatments (Bansal et al., 2015). This procedure uses cells with the ability to differentiate, such as stem cells, to try to rebuild lost and necrotic tissues. Despite being innovative and using modern technology, this therapeutic strategy has considerable drawbacks. One of the method's main shortcomings is its inability to regenerate tissues completely (Galler and Widbiller

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2020). One of the main aims of the tissue engineering technique is to induce regeneration to heal injured tissues by utilizing bioactive chemicals, cell resources, and scaffolds to restore bone function through the regeneration and adaptation to the host tissue process (Hill et al., 2006, Sharifi et al., 2020a; Sharifi et al., 2020b; Khezri et al., 2021).

Stem cells are defined as cells with self-renewal potential and multi-lineage differentiation (Lavik and Langer 2004, Vahedi et al., 2019, Vahedi et al., 2021). Dental pulp stem cells (DPSCs) have a high capability for differentiation and for the regeneration of the dentin–pulp complex (Gronthos et al., 2000). Scaffolding functions as a synthetic matrix, enabling cells to proliferate while still performing their specialized function. Natural materials such as chitosan, gelatin (Gel), collagen, hyaluronic acid and fibrin have all been utilized to fabricate scaffolds for tissue engineering (Ahmadian et al., 2020, Sharifi et al., 2021). Cellular scaffolds suited for cell implantation or injection should promote cell survival, induce bioactivity, and increase cell adhesion in the location of cell implantation or injection. In addition, scaffolds are composed of various kinds of polymers, which shield cells from the immune system and allow the exchange of nutrients and waste compounds (Azami et al., 2012, Henkel et al., 2013, Firouzi et al., 2020).

Gel is a natural biopolymer derived from animal skin, bone, and tendon collagen. Gel structures with varying physical and chemical characteristics emerge from differences in collagen supply and manufacturing procedures (Djagny et al., 2001, Alipour et al., 2021). Gel may also improve cell proliferation and differentiation (Zhu et al., 2004, Salamon et al., 2014), such as DPSC adhesion and proliferation, migration, and odontogenic differentiation, which increases mineralization, alkaline phosphatase (ALP) activity, and increases collagen expression (Qu and Liu 2013).

The main inorganic constituent of bone and enamel is hydroxyapatite (HA). HA particles have the ability to induce macrophages to release odontogenic and angiogenic growth factors (Honda et al., 2006, Kilian et al., 2008).

As angiogenesis and bone formation induction are crucial processes in bone regeneration, using nanohydroxyapatite (nHA) in the scaffold makes sense. Adding bioactive elements as fillers to scaffolds may enhance their physical and chemical characteristics; therefore, scaffolds incorporating inorganic and polymeric materials may increase mechanical strength, biocompatibility, and biodegradability (Sharifi et al., 2019). The nHA, which has a chemical and crystal structure similar to bone mineral is considered an excellent scaffolding material (Zakaria et al., 2013). Smaller nHA particles (<100 nm) seem more favorable to the host due to improved interfacial adhesion, cell proliferation, and cell adherence (Jiang et al., 2017).

In assessing the effectiveness of tissue engineering, the qualities of scaffolding are vital in cell survival or induction of differentiation, in addition to evaluate the characteristics of scaffolding. Tissue toxicity generally develops as a result of excessive absorption and the concentration of a chemical exceeding the tissue tolerance threshold and depletion of antioxidants reservoirs (Ma 2004, Ahmadian et al., 2017). According to several investigations, increasing cytotoxicity inhibits tissue regeneration and causes cell death. As a result, an evaluation of the impact of designed scaffolding on cell survival or toxicity is required. Changes in ALP activity must also be monitored to measure bone differentiation in scaffolding (Kanczler and Oreffo 2008, Polini et al., 2014). Several investigations have revealed that this enzyme plays an essential role in mineralizing organic tissue in bone and dental tissue samples (Liu and Chang 2002, Haque et al., 2005). Based on these examples, it is possible to infer that assessing the amount of this marker is critical in evaluating the success and efficacy of tissue engineering.

Thus, the aim of this study was to engineer the gelatin sponge scaffold containing nHA with an odontogenic specificity. Subsequently, this scaffold was studied to determine its effect on cell growth. Finally, the change in the level of ALP activity in DPSCs grown on the engineered scaffold was analyzed to assess longer-term effects.

2. Materials and methods

2.1. Ethics approval

The ethical code of this investigation is IR.TBZMED.VCR.REC.1400.391, which was accepted by the Ethic Committee of the Tabriz University of Medical Sciences.

2.2. Preparation of scaffold

Gelatin solution was prepared with a 1/10 w/v ratio of gelatin (Sigma Aldrich-USA) and distilled water at 65 °C. Then, the solution was stirred at 35 °C and 1 % w/v of glutaraldehyde (Sigma Aldrich-USA) was added and stirred. The resulting solution was poured into a mechanical stirrer. Then, 10 % w/v of nHA was added to the gelatin solution and stirred for 60 min. The resulting material was placed in a freezer at −20 °C for 24 h. The frozen material was then incubated in a vacuum oven for 24 h and dried.

2.3. Characterization of the scaffold

2.3.1. Scanning electron microscopy (SEM)

The morphological characteristics of the scaffold were examined using SEM (Razi Company of Tehran, Tehran, Iran). The samples were placed on a SEM plate and subsequently gold-coated in a high-vacuum environment and at an acceleration voltage of 10 kV. The magnification of the SEM was set to 100X.

2.3.2. Particle size

By an argon laser beam in a scattering angle of 90° and at 633 nm, dynamic light scattering (DLS, Malvern, Cambridge, Massachusetts, United Kingdom) was utilized to validate the nanoscale size accuracy of the nHA at 25 °C. The device's specific tube was filled with distilled water to generate a high-quality suspension of scaffolds.

2.3.3. X-ray diffraction (XRD)

The raw materials and the prepared scaffold were evaluated at room temperature utilizing XRD patterns. The patterns of the samples were determined using an X-ray diffractometer (D5000, Siemens, Munich, Bavaria, Germany) set at 5405/1 Å wavelength, 40 kV voltage, and 30 mA. Their patterns were analyzed between zero and sixty 2θ values.

2.4. Fourier-transform infrared (FT-IR) spectroscopy

FT-IR spectroscopy (FTIR, Shimadzu 8400S-Japan, Kyoto, Japan) was used to identify the functional groups. The samples were combined with IR-grade potassium bromide and pressed using IR pellet production equipment. The wavelengths were then adjusted from 400 to 4000 (cm^{-1}).

2.5. MTT assay

In this study, to evaluate the toxic effect of scaffolding on DPSCs, an MTT assay was used. In order to perform these experiments, DPSCs were purchased from Shahid Beheshti University, which had a low passage number. The prepared scaffold was placed at the bottom of the 96-well plate and then the cells (5×10^3 cell/well) were added to each well in Dulbecco's Modified Eagle's Med-

ium (DMEM) medium containing 10 % fetal bovine serum (FBS) and 1 % antibiotics. The plates were incubated for 2, 4, and 6 days. Following this, the culture medium was removed and incubated with 200 µl of culture medium. Further, 50 µl of MTT solution (2 mg/ml PBS) was added and incubated for 4 h at 37 °C and kept away from light. After the incubation period, the above solution was removed and 200 µl of DMSO was added to each well. Their absorption was read at 540 nm and the percentage of living cells was measured by comparing the control (cells grown in the absence of the scaffold).

2.6. ALP activity

The sterilized scaffolds were put into the 24-well plate, and DPSCs were added to each well (5×10^4 cells/well). ALP activity was assessed after 14 days. Briefly, the wells were washed three times with PBS. A stock solution of cell digestion buffer, which has 1.5 M Tris-HCl, 1 mM $ZnCl_2$, and $MgCl_2x6H_2O$, was diluted in a solution of distilled water (1:10) and added to each well. Then, 1 % of Triton X-100 was added to the wells. Then, the plates were incubated at 37 °C for 30 min and were then stored at 4 °C overnight. Cell lysates were put into samples, vortexed for 2 min, and centrifuged for 5 min at 2000 rpm. Also, about 10 µl of cell lysate mixed with 190 µl of ALP solution (37.1 mg of pNPP in 20 mL of cell assay buffer) was added to each well of the 96-well plate. The sample was shaken at room temperature in the dark for 50 min. The Cytation 5 system was used to measure the absorbance of the ALP activity at 405 nm. The control group was cells grown in the absence of the scaffold.

2.7. Statistical analysis

To establish the significance of the experimental findings, one-way ANOVA and *t*-test statistical analysis were used for MTT tests and ALP activity tests, respectively. A statistically significant *p*-value of $p < 0.05$ was used. Tukey's post hoc test was used for the analysis between the groups for MTT test.

3. Results and discussion

3.1. Characterization of scaffold

Fig. 1a shows the produced sponge. SEM image of the sponge has presented in Fig. 1b. The microscopic structure of the prepared sponge under SEM showed a porous structure with the micrometric and nanometric pores. The micro/nano-structured porous sponges are recently in the core of attention to use as scaffolds. The micro/nano pores allow for *cell mobility* and metabolic processes (Sari et al., 2021). Nanometer-scale pores on the surface of the sponge can also leads to surface roughness (Xue et al., 2019).

3.2. Particle size

The size distribution for nHA particles is presented in Fig. 2. Based on the results, the mean particle size of 75 nm with a polydispersity index (PDI) value of 0.2 shows the relatively monodisperse particle size distributions (Jana et al., 2014). This index shows that values smaller than 0.05 are mostly highly monodisperse. PDI values bigger than 0.7 specify that the sample has a very wide-ranging particle size distribution and is possibly not appropriate to be investigated by the DLS method (Danaei et al., 2018).

3.3. FT-IR analysis

FTIR results showed that all functional groups of gelatin and HA were present in the sponge (Fig. 3). The amide II peak in the sponge sample was related to gelatin (1634 cm^{-1}) (Tengroth et al., 2005). Also, it was revealed that the broad peak at 3200–3500 cm^{-1} was linked to the OH group's tension spectrum (El-Rahman and Al-Jameel 2014). The index peaks were associated with the HA phosphate group at 563 and 602 cm^{-1} (Hezma et al., 2019).

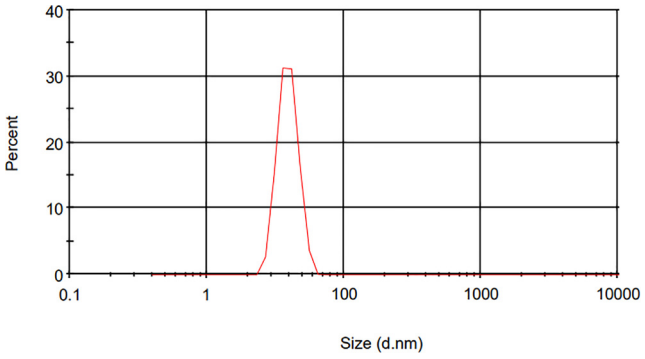

Fig. 2. The size distribution for nHA particles.

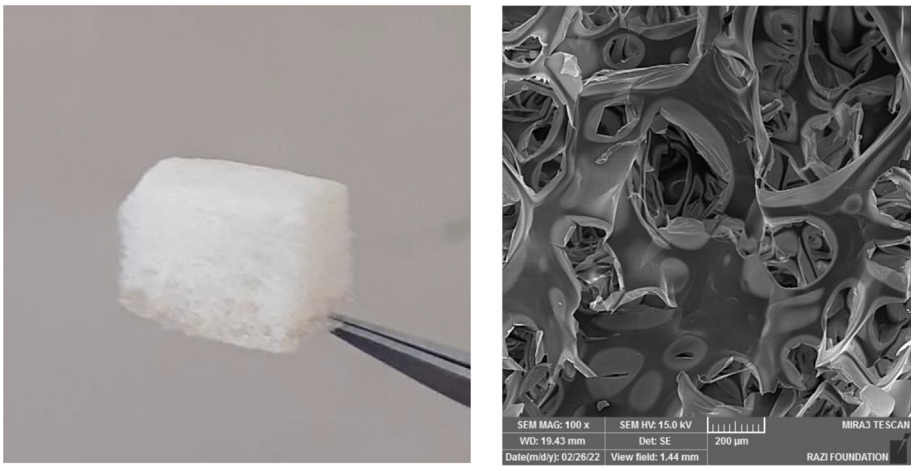

Fig. 1. The produced sponge (a). The SEM image of the sponge (b).

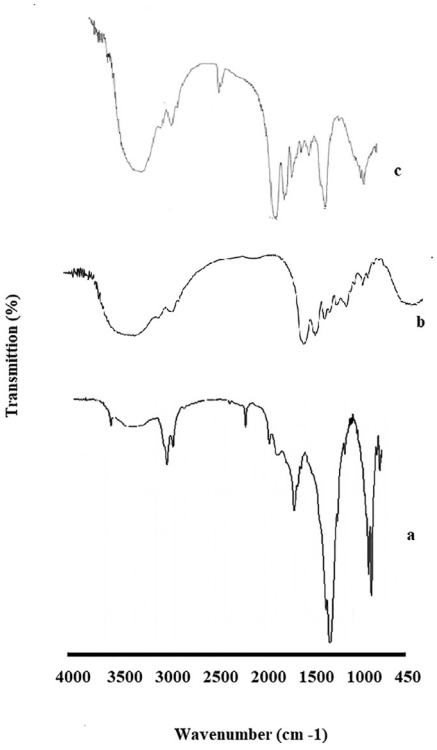

Fig. 3. FTIR results for nHA (a), Gelatin (b) and the prepared sponge (c).

3.4. Identification of crystallinity state

XRD patterns were determined at room temperature for the raw materials and the final product, respectively (Fig. 4). The produced sponge showed the peaks for both materials. In a previous study, XRD results showed that the structure of gelatin was essentially amorphous (Hezma et al., 2019). XRD pattern findings revealed that HA exhibited its index peaks at 26, 31, 39, and 41° according to pervious study (Hezma et al., 2019).

3.5. Cell viability

MTT assay results revealed that sponge was not cytotoxic, similar to the corresponding control groups (Cells were grown in the absence of scaffolds) ($p > 0.05$) (Fig. 5). Tukey's post hoc test also showed that there was no significant difference between the groups ($p > 0.05$). Sadeghinia *et al.* synthesized nanohydroxyapatite/chitosan/gelatin scaffolds which did not show any cytotoxicity on dental pulp stem cells and had proliferative effect (Sadeghinia et al., 2019). In our previous study, we prepared gelatin-hydroxyapatite nano-fibers which didn't show the cytotoxic effect on DPSCs and showed a significant proliferative effect (Sharifi et al., 2020a; Sharifi et al., 2020b). The non-toxicity of Gel-HA sponge on DPSCs was consistent with the results of other studies as well (Sharifi et al., 2020a; Sharifi et al., 2020b; Alipour et al., 2021).

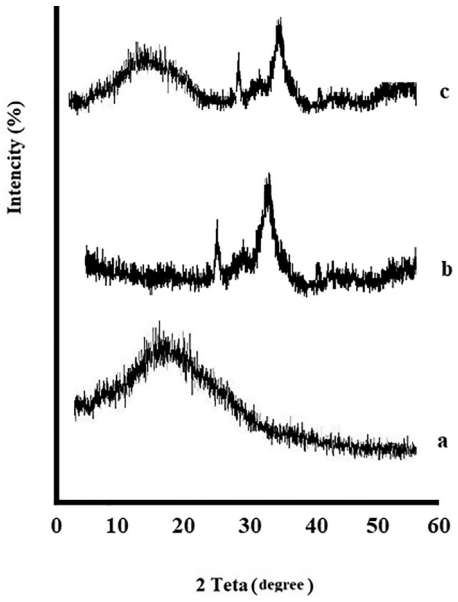

Fig. 4. XRD diffraction patterns, Gelatin (a), nHA (b) and the prepared sponge (c).

3.6. ALP activity

After 14 days of culture, the ALP activity of DPSCs cultured on Gel-HA rose considerably in contrast to the control groups ($p < 0.05$). This increase suggests that the DPSCs progressively differentiated into osteoblasts (Fig. 6). A previous study has shown

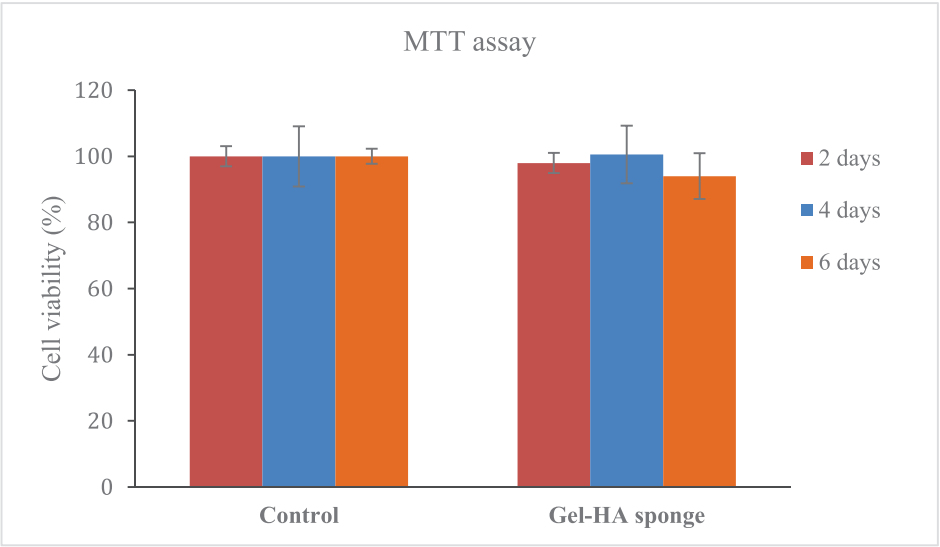

Fig. 5. The cell viability of seeded DPSCs on the Gel-HA sponge scaffolds.

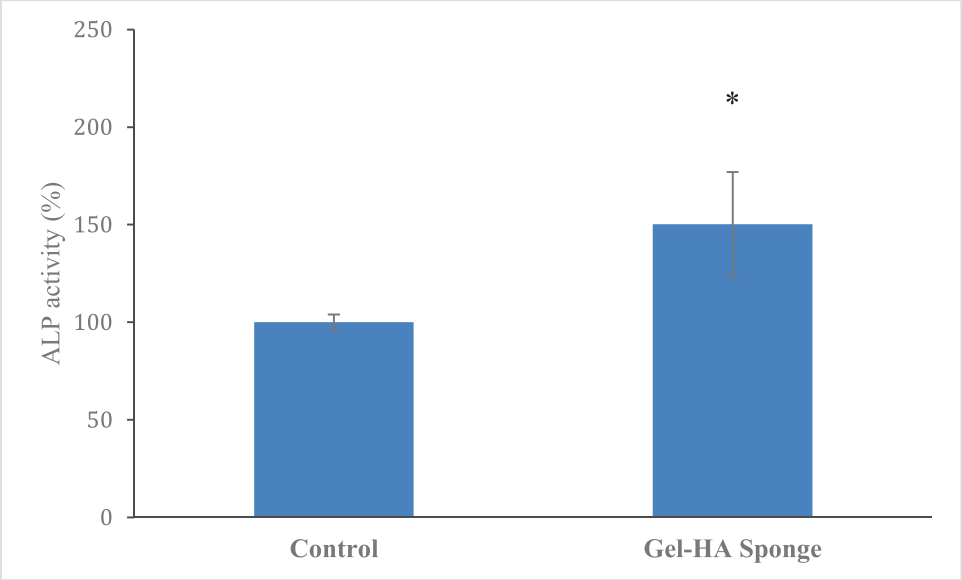

Fig. 6. The ALP activity in DPSCs seeded on the Gel-HA sponge scaffolds. * shows statistically significant ($p < 0.05$) difference with control.

that MSCs seeded on Gel-HA scaffold have a strong bio-mimetic potential as a hard tissue scaffold (Li et al., 2018). The combination of polymeric scaffolds by inorganic particles may induce a positive cellular response compared to neat scaffolds, for example in enhanced cell proliferation and early osteogenic differentiation of human mesenchymal stem cell (hMSC), as showed by the ALP expression (Raucci et al., 2019). Sadeghinia *et al.* showed nanohydroxyapatite/chitosan/gelatin scaffolds increased ALP activity of DPSCs after two weeks (Sadeghinia et al., 2019).

4. Conclusions

Our findings showed that the Gel/nHA sponge scaffold, in particular, has the best overall characteristics for endodontic regeneration applications. We believe that because this method of forming organic/inorganic hybrid scaffolds is reasonable and typically performed under mild conditions, while improving bioactive and mechanical properties, such a scaffold will have potential applications in tooth tissue regeneration, drug delivery, and other related medical fields. However, more studies should be conducted over a longer time period to develop the cell/gelatin scaffold build and explore the mechanical and physical properties that will be suitable for endodontic regeneration. In future, organic/inorganic hybrid materials may be accessible as a main part of the biomaterial scaffolds. Such advanced biomaterials can be used as scaffolding blocks with human stem cells in developing bioprinting application, antimicrobial and antioxidant therapy, including drug screening and disease modelling. However, more in vivo and clinical tests are needed to show these abilities.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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