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ARTICLE

SYNTHESIS, CHARACTERIZATION, AND EVALUATION OF NANO-HYDROXYAPATITE BASED EXPERIMENTAL CALCIUM SILICATE CEMENT AS A ROOT REPAIR MATERIAL.

Síntesis, caracterización y evaluación de cemento experimental de silicato de calcio a base de nanohidroxiapatita como material de reparación radicular.

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ABSTRACT:

Introduction: This study aimed to prepare a new root repair material including Portland cement, bismuth oxide, and nano-hydroxyapatite and analyze its physicochemical properties and its effects on the proliferation and differentiation of human dental pulp stem cells (hDPSCs).

Material and Methods: Bismuth oxide as a radiopaque component and nano-hydroxyapatite particles were added to white Portland cement at 20% and 5% weight ratio, respectively. Characterization of the prepared cement was done using con-ventional methods. To examine the bioactivity of this new material, atomic absorption spectroscopy (AAS) was used for the investigation of the rate of calcium ions dissolution in simulated body fluid media. The viability of hDPSCs was assessed by an MTT assay after 1, 3 and 7 days. The odontogenic potential of this substance was evaluated by measuring alkaline phosphatase activity and alizarin red S staining.

Results: Based on the bioactivity results, the cement presented high bio-activity, corroborating sufficiently with the calcium release patterns. The cell viability was significantly increased in new root repair ma-terial containing hydroxyapatite nanoparticles after 3 and 7 days ($p<0.05$).

Conclusion: Moreover, alkaline phosphatase activity increased over 7 days in all experimental groups. The new cement containing nano-hydroxyapatite particles could be a good root repair material.

KEYWORDS:

Materials testing; Dental pulp; Stem cells; Root canal filling materials; Nanoparticles; Hydroxyapatite.

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RESUMEN:

Objetivo: Este estudio tuvo como objetivo preparar un nuevo material de reparación de raíces que incluye cemento Portland, óxido de bismuto y nano-hidroxiapatita y analizar sus propiedades fisicoquímicas y sus efectos sobre la proliferación y diferenciación de células madre de pulpa dental humana.

Material y Métodos: El óxido de bismuto como componente radiopaco y las partículas de nano-hidroxiapatita se agregaron al cemento Portland blanco en una proporción en peso del 20 % y el 5 %, respectivamente. La caracterización del cemento preparado se realizó utilizando métodos convencionales. Para examinar la bioactividad de este nuevo material, se utilizó la espectros-copia de absorción atómica para investigar la velocidad de disolución de los iones de calcio en medio fluido corporal simulado. La viabilidad de las células madre de pulpa dental humana se evaluó mediante un ensayo MTT después de 1, 3 y 7 días. El potencial odontogénico de esta sustancia se evaluó midiendo la actividad de la fosfatasa alcalina y la tinción con rojo de alizarina S.

Resultados: Con base en los resultados de bioactividad, el cemento presentó alta bioactividad, corroborando suficientemente con los patrones de liberación de calcio. La viabilidad celular aumentó significativamente en el nuevo material de reparación de raíces que contenía nanopartículas de hidroxiapatita después de 3 y 7 días (p<0,05).

Conclusión: Además, la actividad de la fosfatasa alcalina aumentó durante 7 días en todos los grupos experimentales. El nuevo cemento que contiene partículas de nanohidroxiapatita podría ser un buen material de reparación radicular.

PALABRAS CLAVE:

Ensayo de materiales; Pulpa dental; Células madre; Materiales de obturación del conducto radicular; Nanopartículas; Hidroxiapatitas.

INTRODUCTION.

Nowadays, nanotechnology plays a vital role in different fields of regenerative medicine. Regeneration of hard tissues like bone and tooth is one of the most notable criteria in tissue engineering. Nano-hydroxyapatite particles have a comparable construction to the apatite crystals in bone and tooth structure.

Furthermore, it has the ability to release calcium and phosphate ions as a bioactive material in the regeneration process of bone and tooth.[1] Nano-hydroxyapatite particles as nontoxic and non-immunogenic materials show no side effects *in vitro/in vivo* used in appropriate doses.[2] Other features that convert it into an appropriate biomimetic material include high surface area, good solubility, and ideal bioactivity.[3]

Nano-hydroxyapatite particles have been progressively applied for diverse dental applications. For example, this material may be applied to remineralize early caries lesions on enamel, protecting teeth against caries and dental erosion.[4] Some reports have shown that the size and shape of hydroxyapatite particles have a significant part in their applications.[5-7]

The structural integrity of the tooth can be affected adversely by caries, trauma, or other iatrogenic causes such as perforation. Mineral trioxide aggregate (MTA) was introduced in the early 1990s and is widely used in different fields of endodontics, as well as treat-ment of perforation, root-end filling, pulp capping, and apexification.

However, despite these wide applications of this substance, this material still has drawbacks such as prolonged therapeutic approach and setting time, high cost, the potential of discoloration, and poor handling.[8-10] MTA is one of the main members of the calcium silicate-based cements (CSCs) group, which induces biomineralization.[11,12]

Portland cement is another member of the CSCs and contains hydrophilic particles of dicalcium, tricalcium silicate, and tricalcium aluminate, like MTA.[13]

Different analyses have shown that MTA powder is approximately a mixture of Portland cement and bismuth oxide.[14] Different studies introduced Portland cement as an applicable alternative to MTA due to its similar biological, antimicrobial, and physicochemical properties.[14]

The pure form of Portland cement alone could induce dentinogenesis, but due to the lack of radiopacity, it is not appropriate for clinical application.[15] To overcome this drawback, many suitable radiopacifying agents have been introduced.[11,16] Considering the similarities between the properties of MTA and Portland cement, we decided to modify the Portland cement by using bismuth oxide and nano-hydroxyapatite particles.

Moreover, we aimed to investigate the bio-compatibility and mineralization potential in human dental pulp stem cells (hDPSCs) exposed to this experimental calcium silicate cement and compare them with MTA.

MATERIALS AND METHODS.

Sample preparation for in vitro studies

The experiment groups included:

Group 1: MTA (Angelus, Londrina, Brazil);

Group 2: White Portland Cement (WPC);

Group 3: White Portland Cement + Oxide Bismuth (WPC+BO) (WPC: Bi_2O_3 = 80:20);

and Group 4: nano-hydroxyapatite-based experimental Calcium Silicate Cement (WPC+ BO+ nHA) (WPC: Bi_2O_3: nHA= 80:20:5).

Under aseptic conditions, the mentioned powders in each group were mixed with distilled water in a water-to-powder ratio of 1:3. Each sample was pre-Zpared in the form of a disk (5 mm in diameter and 2 mm in thickness) and was kept in the incubator for 24 hours at 37°C in 100% humidity.

Preparation and characterization of nanohydroxyapatite based calcium silicate cement For the preparation of nano-hydroxyapatite-based experimental calcium silicate cement, white Portland cement (Sufiyan Cement Co, Tabriz, Iran) was mixed and homogenized with oxide bismuth and nano-hydroxyapatite particles with a 20% and 5% weight ratio. To find the surface charge of the cement, zeta potential was measured using a zetasizer (Malvern, UK) at 25°C. For this, a fresh suspension of the samples was diluted with distilled water and injected into the capillary cell of the zeta-sizer.

The cement's molecular structure and inter/intramolecular bonding were obtained over a Fourier transform infrared spectroscopy (FTIR) (Thermo Nicolet-6700) in the range of 400-4000 cm^{-1}. Furthermore, X-ray diffraction (XRD) (Philips TW 1710) was utilized for assessing the crystallinity and phase determination information of the cement at room temperature over the 2° range of 20 to 70° (3°/min for the scanning rate). The characterization was also performed for the pure nano-hydroxyapatite particles and the pure white Portland cement to compare with the prepared nano-hydroxyapatite based calcium silicate cement.

Cell viability

The prepared disks were placed in 96- and 24-well plates and sterilized by gamma radiation before cells were added for culture. Human dental pulp stem cells (passage no. 4) were obtained from the Stem Cell Research Center of Tabriz University of Medical Sciences.

For MTT tests, 5000 cells were seeded on the prepared discs of each group. After 1 day, 3 day, and 7 day, 50 µl MTT solution (2 mg/ml) replaced each group's 50 µl culture medium, and plates were incubated for 4 hours at 37°C and 5% CO_2. Then, MTT/medium solution was taken out of each well and 100 µl DMSO was added, the absorbance of dissolving blue formazan crystals was determined at 570 nm by a plate reader, and the percent of live cells for each group was reported.

Alkaline phosphatase (ALP) activity

The ALP activity of seeded hDPSCs on MTA, white Portland cement, White Portland Cement + Oxide Bismuth, and nano-hydroxyapatite-based experimental Calcium Silicate Cement was measured after 7 days. An alkaline phosphatase kit (Pars Azmoon, Tehran, Iran) was used to measure the final concentration of *p*-nitrophenol at the absorbance of 405 nm. The base of the experiment is:

p-Nitrophenylphosphate + H_2O → Phosphate + *p*-Nitrophenol

Briefly, the seeded cells were lysed in an alkaline lysis buffer that included magnesium chloride, diethanolamine, and NaCl.

After that, cells were incubated in *p*-Nitrophenylphosphate at 37O°C. The measured amounts of *p*-Nitrophenol were nor-malized by the total cellular protein.

Alizarin red S staining

In order to evaluate the matrix mineralization of hDPSCs, 21 days after seeding, cells were fixed in 70% (v/v) cold ethanol for 1 hour and stained with 40 mM alizarin red S for 30 min at room temperature. After three washes in dH_2O to remove the unbound stain, samples were airdried and photographed.

For quantification, the bound stain was extracted with 10% cetyl pyridinium chloride, and the absorbance of the solution was measured at 450 nm.

Bioactivity test

The prepared samples were soaked in simulated body fluid (SBF) (pH of 7.4 and 37°C) over 21 days in order to assess the dissolution rate of calcium ions, as their bioactivity, using atomic absorption spectroscopy (AAS).

Statistical Analysis

All experiments were carried out in triplicates, and data were determined as the mean ± standard deviation and compared using one-way ANOVA and Tukey test analysis with Prism software (version 8.0, GraphPad, San Diego, CA, USA); p-value<0.05 was considered statistically significant.

RESULTS.

Physicochemical properties

Zeta potential measurements (Figure 1) showed a negative surface charge for the pure WPC, nanohydroxyapatite, and the cement (-20.70±5.39 mV, -9.54±7.80 mV, and -18.30±8.26, respectively).

The results for the XRD outline displayed the occurrence of sharp and high-intensity patterns with no additional peaks (Figure 2).

Figure 3 shows FTIR outcomes of the prepared samples. FT-IR peaks displayed all typical peaks of the materials with no additional peaks. The physicochemical assessments showed that there were no physicochemical changes (surface charge, crystallinity and functional groups) due to the addition of nano-particles to Portland cement

Biological properties

Cell viability

The viability of seeded hDPSCs after 1 day, 3 day, and 7 day were evaluated in all groups.

One day af-ter seeding of hDPSCs, the viability of hDPSCs in MTA, WPC, WPC+BO, and WPC+BO+nanoHA were 84.16%, 85.47%, 87.72%, and 102.81%, respecztively. The cell viability for evaluated groups after 3 days was 83.15%, 83.32%, 98.91%, and 120.42%.

However, these amounts reach 114.81% for the MTA group, 87.58% for the WPC group, 103.69% for the WPC+BO group, and 159.62% for the WPC+BO+nanoHA group. In the Figure 4, the nano-hydroxy-apatite based experimental calcium silicate cement enhances the viability of cells on all days of the experiment. However, this increase was significant after 3 day and 7 day.

Alkaline phosphatase activity

The ALP activity in studied groups were 249.87, 802.88, 444.76, 606.54, and 829.32 in Control, MTA, WPC, WPC+BO, and WPC+BO+nanoHA groups, respectively. Figure 5 ALP activity was significantly increased in MTA, WPC+BO, and nano-hydroxyapatite-based experimental calcium silicate cement groups compared with the control group (p<0.05).

Moreover, the ALP activity of hDPSCs seeded on MTA and nano-hydroxyapatite-based experimental calcium silicate cement did not show any difference, which means that the addition of nano-hydroxyapatite to Portland cement could enhance ALP activity as same as MTA.

Alizarin red S (ARS) staining

For the ARS assay, hDPSCs were exposed to MTA, WPC, WPC+BO, and WPC+BO+nHA groups. HDPSCs without any exposure were considered as the control group.

The formation of mineralized nodules on MTA and

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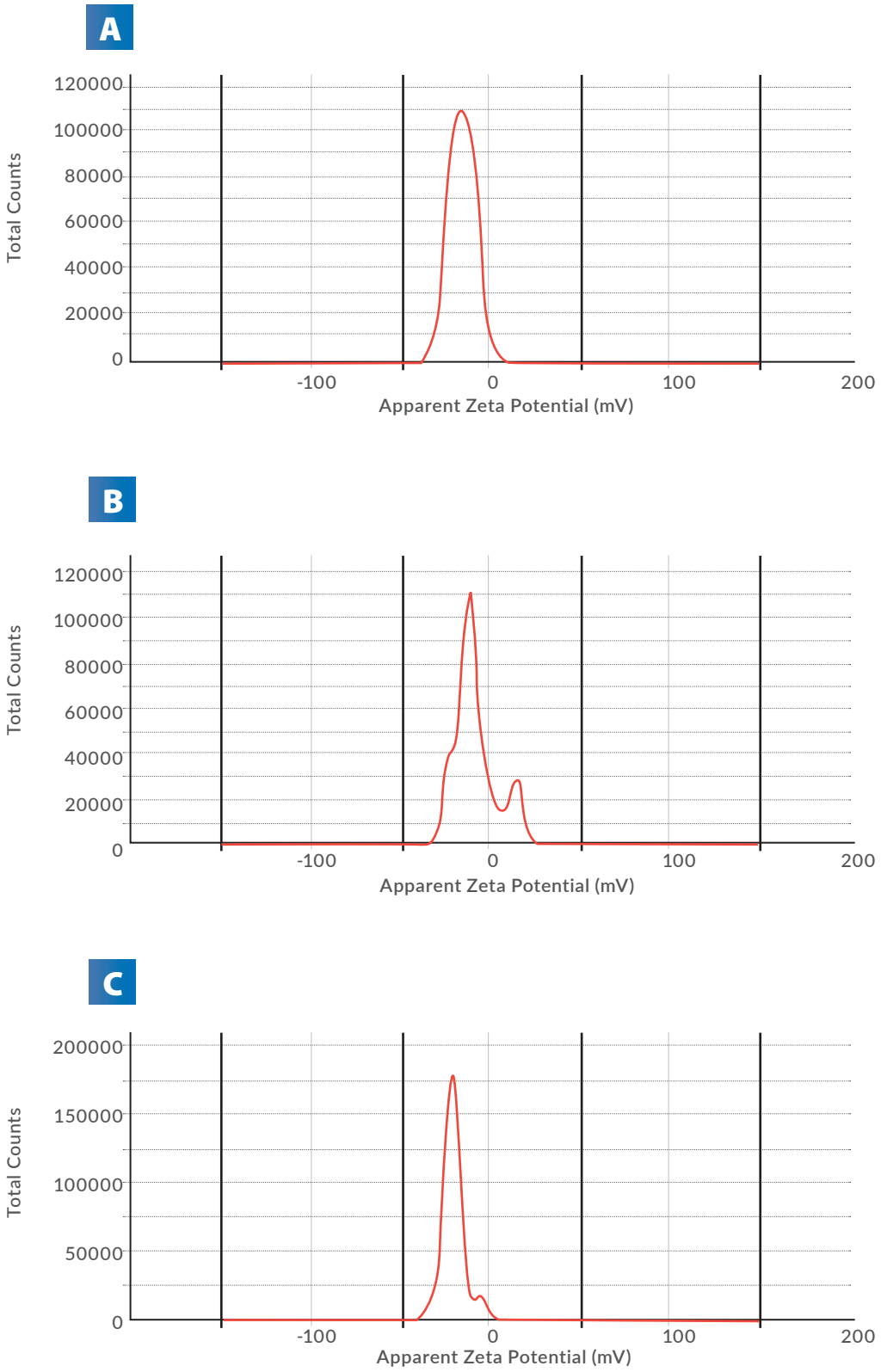
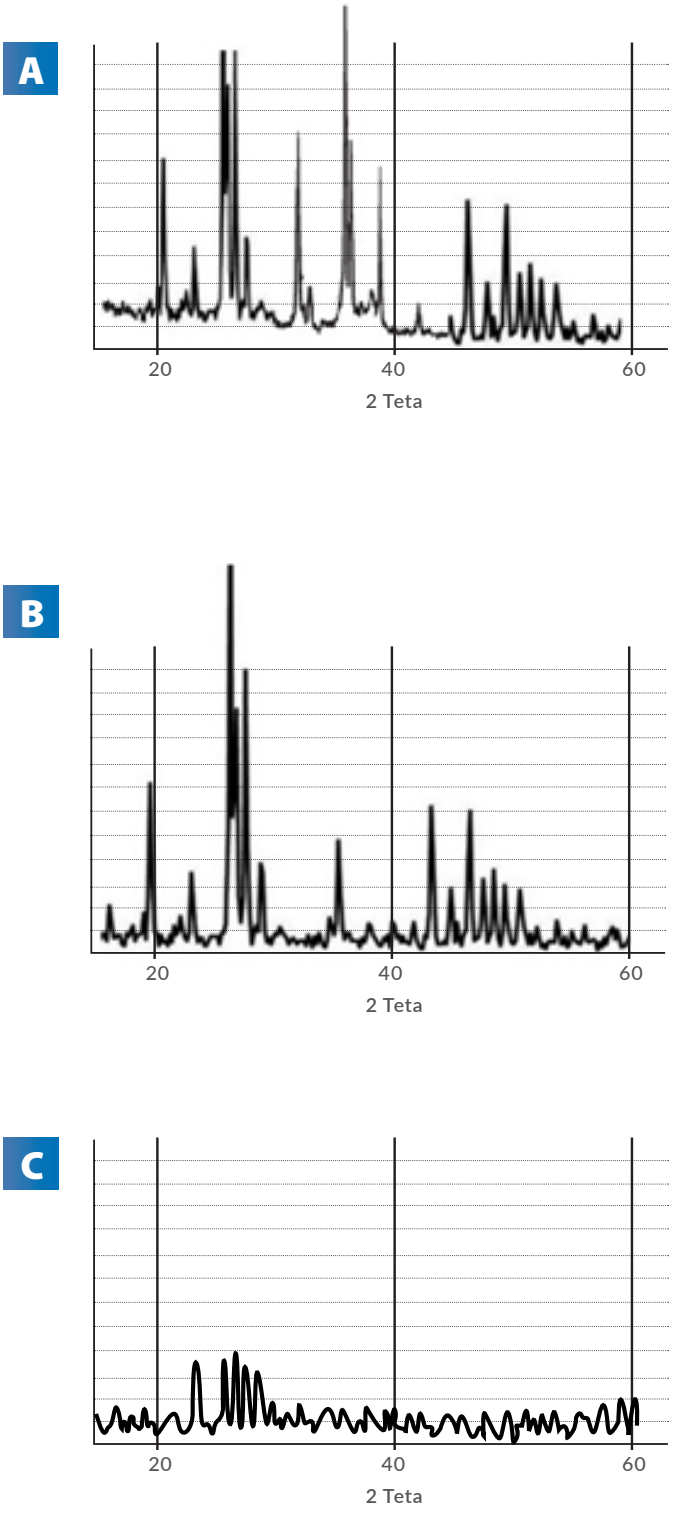
Figure 1. Zeta potential measurements for the prepared materials.



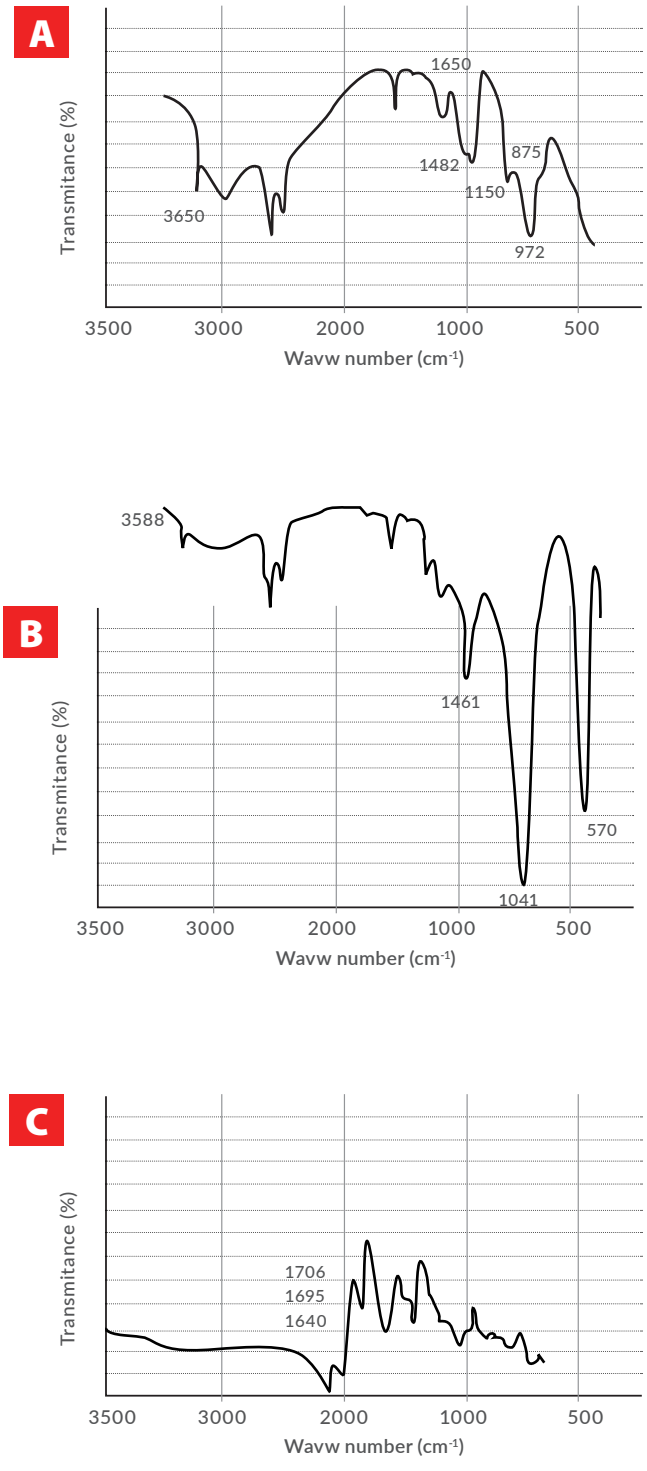
A: Pure WPC.
B: Nano-hydroxyapatite particles.
C:The cement

Figure 2. XRD outlines for the prepared samples.



A: Pure WPC. **B:** Nano-hydroxyapatite particles. **C:** The cement.

Figure 3. FTIR patterns for the prepared samples.



A: Pure WPC. **B:** Nano-hydroxyapatite particles. **C:** The cement.

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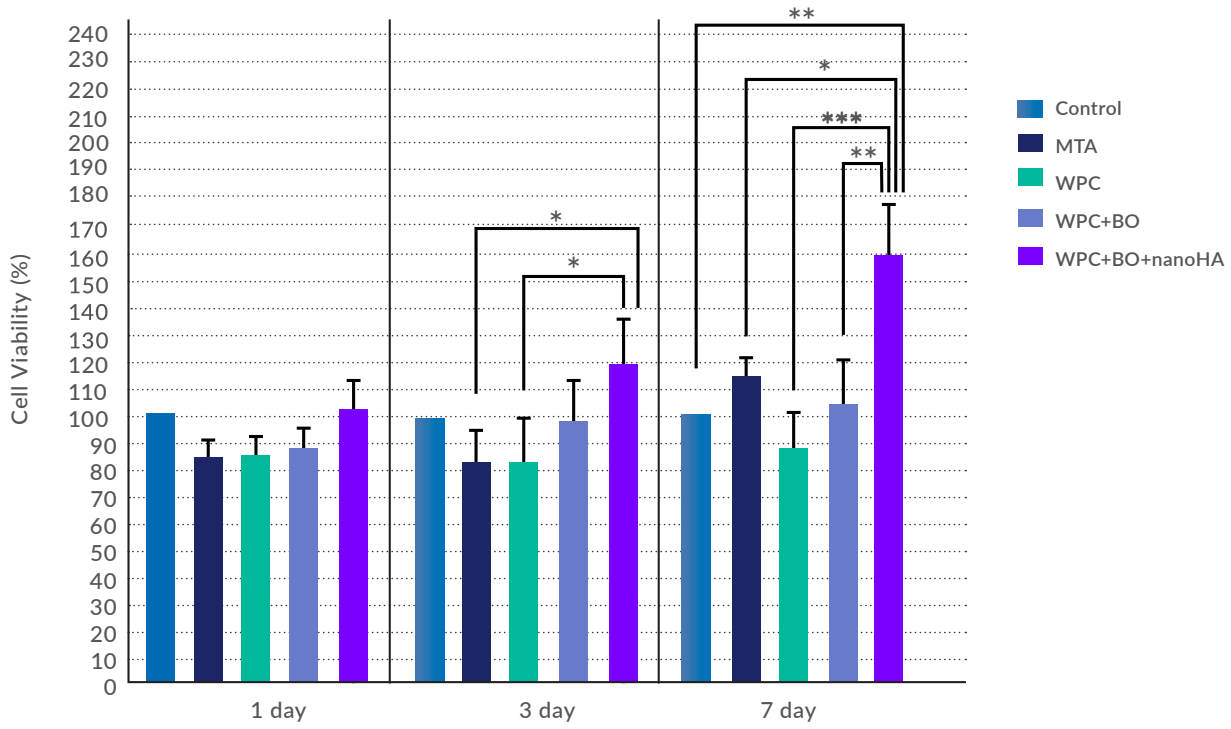
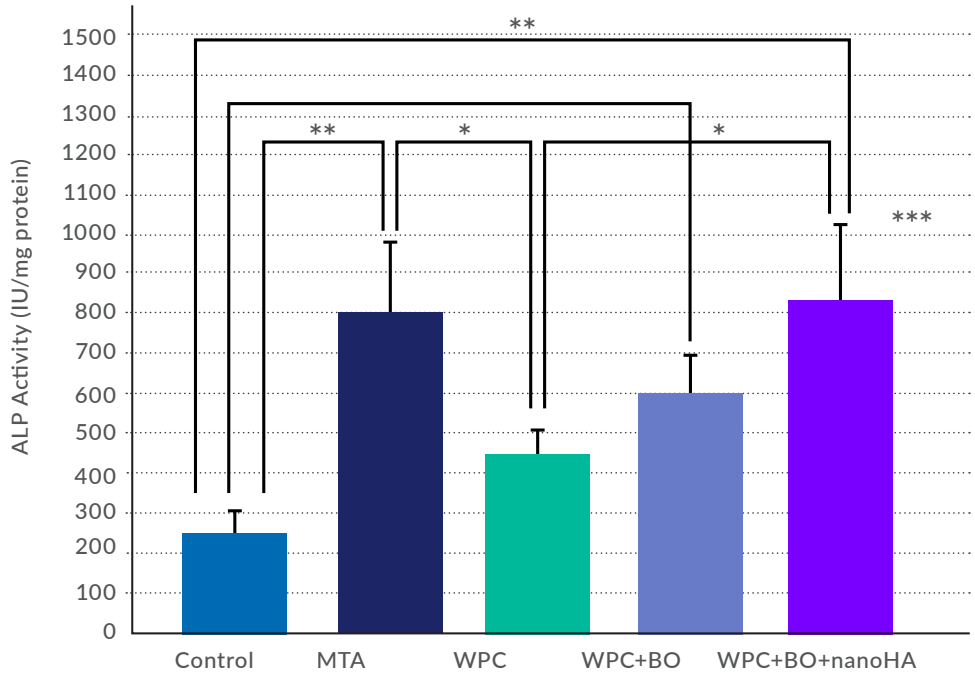
Figure 4. The viability of hDPSCs seeded on experimental groups after 1, 3, and 7days.



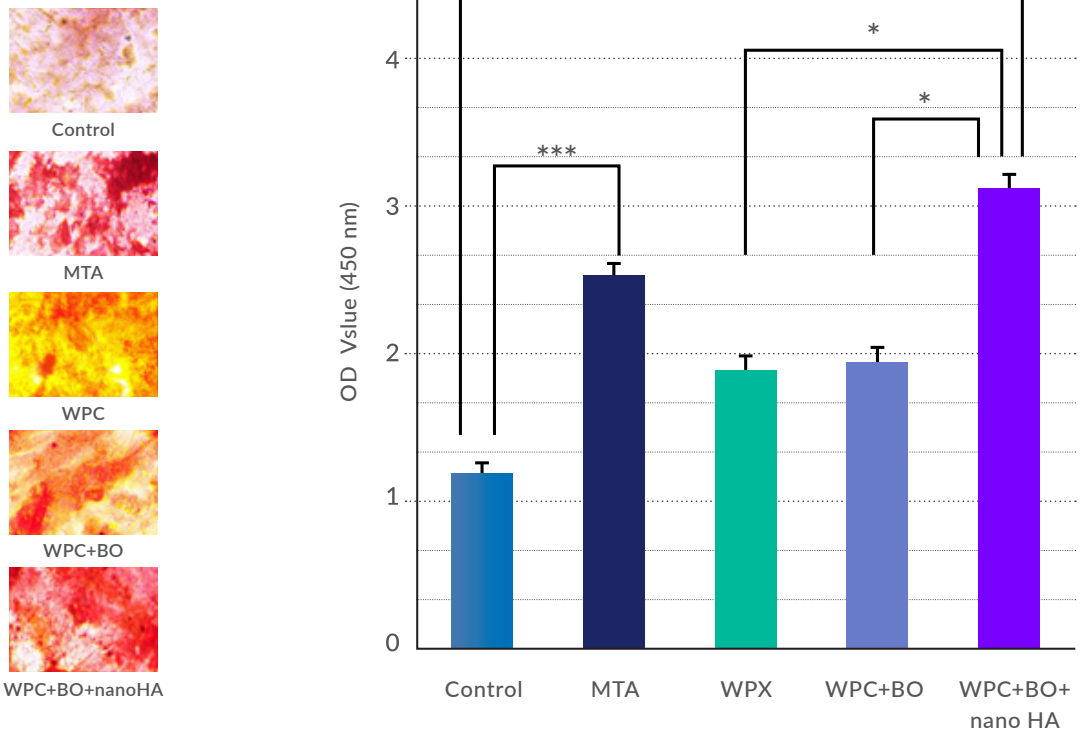
Asterisk (*): $p<0.05$. **(***):** $p<0.01$. **(***):** $p<0.00$.

Figure 5. ALP enzyme activity 7 days after hDPSCs were seeded into experimental groups. Cells cultured without scaffolding are considered as control. Data reported as mean ± SD performed in triplicate.



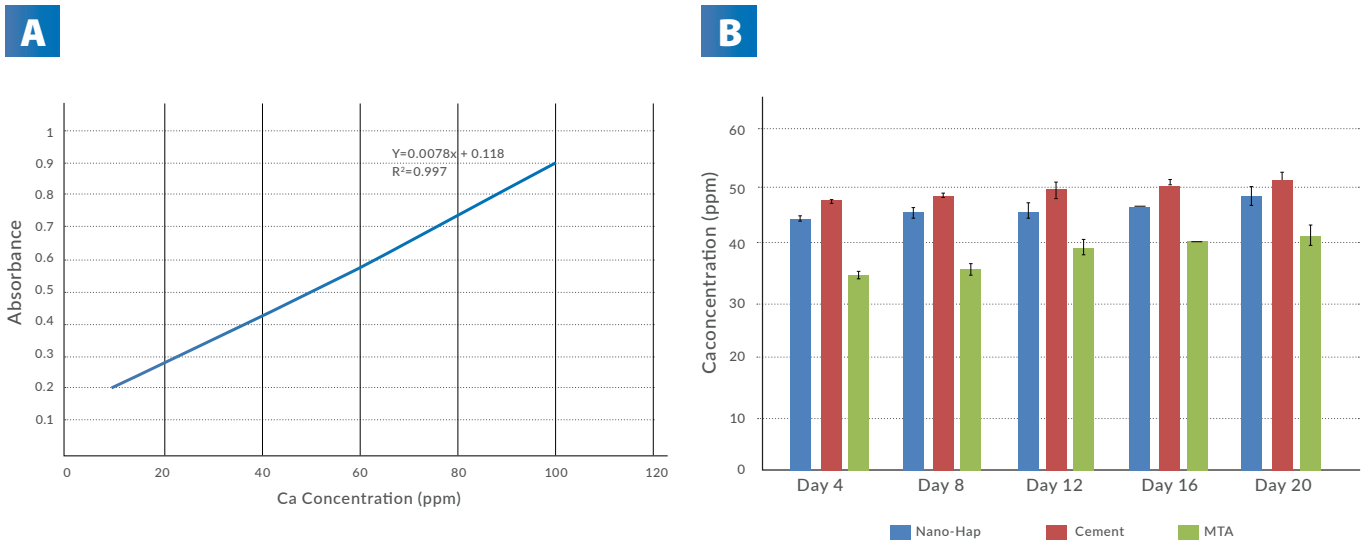
Asterisk (*): $p<0.05$. **(**):** $p<0.01$.

Figure 6. Alizarin red S staining of samples with the magnification of 200 µm. The OD values of seeded hDPSCs in Control, MTA, WPC, WPC+BO, and WPC+BO+nanoHA groups were 1.18, 2.53, 1.90, 1.96, and 3.12, respectively.



Asterisk (*): $p<0.05$. **(***):** $p<0.00$.

Figure 7. Release of calcium ions.



A: Calibration curve for calcium ions (absorbance). **B:** The ppm values for calcium ions compared to the groups (release of calcium ions from the prepared samples). **Asterisk (*):** Represents the statistically significant difference compared with MTA groups ($p<0.05$).

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nano-hydroxyapatite-based experimental calcium silicate cement was significantly higher than the control group, while there was no meaningful difference between them (Figure 6).

Moreover, the addition of nano-hydroxyapatite significantly incre-ased the mineralization of hDPSCs seeded on nano-hydroxyapatite-based experimental calcium silicate cement compared with WPC and WPC+BO cements ($p<0.05$).

Bioactivity test

Figure 7 shows the release of calcium ions from the prepared samples into SBF media.

More calcium ions were released from the nano-hydroxyapatite as compared with the prepared cement and MTA.

DISCUSSION.

Some reports have shown that the assessment of the physicochemical properties (surface charge, crystallinity and functional groups) is necessary to explain the adsorption, coagulation, stability, flotation and viscosity of a cement.[18] Zeta potential is an intrinsic possession of mineral materials in a liquid and is defined as the electrical potential at the hydrodynamic layer of a particle.[17] Our physicochemical assessments showed that there were no physicochemical changes (surface charge, crystallinity and functional groups) due to the addition of nanoparticles to Portland cement.

Yilmaz *et al.*,[19] reported the importance of zeta potential in the construction of surface morphology of cement interactions at the beginning of hydration. Despite the wide applications of MTT in endodontics, this material has disadvantages such as high cost, the potential of discoloration, and poor handling.[8,9] The similar biological and physicochemical properties of Portland cement with MTA have been demonstrated in different studies and then it can be applied as an alternative material for MT.[20]

However, for clinical application, this substance should be radiopaque. Therefore, bismuth oxide could be used as an opacifier agent, as it has been used in root repair materials for the past two decades.[21] It was shown that the addition of 20% (by weight) of this opacifier to Portland cement provided radiopacity higher than dentin, and it is the minimum amount recommended by ANSI/ADA.[22] On the other hand, the addition of nanoparticles has been accompanied by improved physicochemical properties of materials in various dental materials.[23,24]

Nano-hydroxyapatite is a biocompatible material, which is considered the main inorganic component of hard tissues. This nanoparticle forms 60%-70% and 90% (by weight) of bone and enamel, respectively.[25]

Nano-hydroxyapatite has the ability to release calcium and phosphate ions as a bioactive material in the regeneration process of bone and tooth.[26] Moreover, this nanomaterial has significant effects on osteo/odontogenic differentiation of different sources of mesenchymal stem cells.

Stimulation of odontogenic differentiation and biomineralization in the pulp tissue progenitor cells is a critical characteristic of endodontic restorative materials.[11] Therefore, the development of nanoparticle based biomaterials could be an attractive topic in endodontic fields.

Thus, in the current study, white Portland cement was combined with bismuth oxide and nano-hydroxyapatite particles, and the physicochemical and biological properties of this nano-hydroxyapatite-based experimental calcium silicate cement were evaluated and compared with MTA.

Evaluating the cytotoxic potential of reparative endodontic materials used in root canal treatment is the initial step due to the possibility of damages to adjacent periapical tissues and retarding wound healing.[27] Biocompatibility and non-cytotoxicity of Portland ce-ment were investigated in different studies.[28,29]

In the current study, the MTT results reported cytotoxic effects on hDPSCs in none of the experimental groups. However, nano-hydroxyapatite-based experimental calcium silicate cement demonstrated proliferative effects compared with other groups.

The study conducted by Kim *et al.*,[30] evaluated

the cytotoxicity of Portland cement with different percentages of bismuth oxide on human periodontal ligament stem cells. Their results showed that the cell viability of all experimental groups was the same as the MTA group after 72h.

The production of alkaline phosphatase enzyme as one of the functional enzymes increases through osteo/odontogenic differentiation. This enzyme is the primary marker of differentiation besides its essential role in the final mineralization in bone and tooth.[1] Moreover, alizarin red S staining confirmed the formation of mineralized nodules in the late differentiation stage.

The connection between the secreted calcium and ARS solution produces the orange nodules, specifying the mineralization sites.[31] The current study results showed that the alkaline phosphatase activity and calcium nodule formation ability of hDPSCs in MTA and nano-hydroxy-apatite-based experimental calcium silicate cement groups did not show any significant differences. In other words, this nano-hydroxyapatite-based cement could induce mineralization as well as MTA.

It was reported that calcium aluminosilicate and tricalcium silicate in Portland cements induced odontogenic differentiation of hDPCs.[9] In the study conducted by Lee *et al.*,[32] the addition of simvastatin and enamel matrix derivative to Portland cement significantly increased the ALP and ARS activates in human dental pulp stem cells. In the current study, the addition of nano-hydroxyapatite and bismuth oxide particles significantly increased the ALP and ARS activity.

Nano-hydroxyapatite particles are the main inorganic component of bone and tooth, which could mimic the natural extracellular matrix for induction of rege-neration in these tissues.[33] Based on the current study results, the addition of nano-hydroxyapatite particles to Portland cement enhanced the biological properties of this substance. As such, this new experimental composition is potentially able to induce mineralized barrier without cytotoxic side effects.

However, supplementary *in vivo* experiments are required to evaluate the histologic response of tissues to this material.

Limitations of the study

Despite attempts at homogeneous mixing of the initial materials, mixing the powders with nanoparticles might not have been done completely and could have had an impact on the physicochemical and biological results.

CONCLUSION.

Nano-hydroxyapatite-based experimental calcium silicate cement and MTA have similar biological properties *in vitro* situations. Both of these materials could induce differentiation and mineralization in human dental pulp stem cells.

The addition of nano-hydroxyapatite particles to Portland cement improved the biological properties of this material. This material is potentially able to induce mineralized barrier without cytotoxic side effects.

The new cement containing nano-hydroxyapatite particles could be a good root repair material. However, for clinical applications, further *in vivo* evaluations are essential.

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Conflict of interests:

The authors declare no conflict of interests.

Ethics approval:

All experimental protocols were approved by the Ethics committee of Tabriz University of Medical Sciences (TUMS), which complied with the Helsinki declaration, (Approval No. IR. TBZMED. VRC.REC. 1399.120).

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Authors' contributions:

Both authors contributed to the diagnosis, planning, execution and follow-up of the clinical case, as well as the writing of the article.

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