

# Insights into the Role of Magnesium-Containing Bioactive Glass in Osteogenic Differentiation of Human Olfactory Ecto-Mesenchymal Stem Cells



Mojtaba Kargar<sup>1</sup>, Ali Afshari<sup>1</sup>, Seyed Abdolhamid Angaji<sup>2\*\*</sup>, Mahmood Barati<sup>3,4</sup>, and Sara Simorgh<sup>5,6\*</sup>

<sup>1</sup>Department of Cell and Molecular Biology Sciences, Faculty of Biological Sciences, Kharazmi University, Tehran, Iran

<sup>2\*</sup>Department of Cell and Molecular Biology Sciences, Faculty of Biological Sciences, Kharazmi University, Tehran, Iran

<sup>3</sup>Department of Medical Biotechnology, Faculty of Allied Medicine, Iran University of Medical Sciences, Tehran, Iran

<sup>4</sup>Shahid Beheshti University of Medical Sciences, School of Pharmacy, Department of Pharmaceutical Biotechnology, Iran

<sup>5</sup>Cellular and Molecular Research Center, Iran University of Medical Sciences, Tehran, Iran

<sup>6</sup>Department of Tissue Engineering and Regenerative Medicine, Faculty of Advanced Technologies in Medicine, Iran University of Medical Sciences, Tehran, Iran

**\*\*.** co-corresponding authors: Seyed Abdolhamid Angaji, [angaji@khu.ac.ir](mailto:angaji@khu.ac.ir)

**\***. corresponding authors: Sara Simorgh: [srsimorgh@gmail.com](mailto:srsimorgh@gmail.com)

Received: 28 Jun. 2026 Revised: 0 Aug. 2026 , Accepted: 14 Aug. 2026

## Abstract

**Introduction:** In recent years, bone regenerative medicine has emphasized the modification of stem cells using bioactive glasses (BGs) that release various ions. Evaluating the effect of these ions in vitro is important for understanding their impact on cellular osteogenic differentiation. Human olfactory ecto-mesenchymal stem cells (hOE-MSCs) possess a high potential for differentiation into osteoblasts and, therefore, merit investigation as possible candidates for cell therapy in bone defect treatment.

**Methods & Materials:** In this study, we examined the effect of magnesium (Mg) ions an essential element in the body on the osteogenic differentiation of hOE-MSCs. Mg-containing BG (BG-Mg) and Mg-free BG (BG-C, control) were synthesized and characterized using XRD, FTIR, DLS, FE-SEM, EDX, ICP-AES, TEM, and pH analysis. Following the culture and expansion of hOE-MSCs, their characteristics were evaluated by flow cytometry and multipotency assays to confirm the cells' identity. The viability of the stem cells in response to all BGs was determined using MTT and Live/Dead assays at concentrations of 1, 1.5, 2, and 5 mg/mL. Gene expression related to osteogenic differentiation was quantified using real-time PCR, and Alizarin Red S staining was employed to assess mineralized extracellular matrix (ECM) deposition.

**Result:** Following the characterization of both BGs, and verification of the results, biocompatibility testing was performed. The assays showed that both BGs showed the highest biocompatibility at a concentration of 1.5 mg/ml, which supported optimal hOE-MSC survival. In real-time PCR analysis, an increase in ALP gene expression was determined in the BG-C group compared to the control group with a significant difference (\*\*\*), and in Alizarin Red S staining, an increase in ECM in the BG-Mg group was also shown with a significant difference (\*) compared to the control group.

## 1. Introduction

Bone defects represent a major global challenge that poses significant challenges to patients' daily lives (1). One type of fracture involves the maxillofacial bone, which is associated with psychological and psychiatric problems. Treatment techniques such as wire fixation and the use of jaw screws and plates has complications and lead to a long healing period (2).

Autologous bone grafting is gaining limelight as a treatment meant to develop regenerative potential for new bone tissue so that cell growth may be stimulated (3). Yet, the limited availability of bone tissue culture hinders clinical application, and an urge for emerging strategies to enhance bone regeneration remains (4). Using stem cell technology for tissue regeneration has a strong potential for addressing these issues (3). Biomaterials may have a considerable role in tissue engineer-

Numerous polymeric substances and synthetic bone replacements have been identified and engineered to repair bone abnormalities.

Bioactive glasses (BGs) are particularly noteworthy among various bioactive materials due to their high ion-releasing tendency and excellent biocompatibility (3). Their amorphous nature allows for the release of vital ions known to enhance essential biological processes, including osteogenic differentiation (4). To enhance the bioactivity, various ions with significant efficacy and positive roles were added to the BGs (5). Notably, magnesium (Mg) ions can be incorporated into BG formulations, thereby improving the biological performance in bone tissue regeneration applications. Research suggests that Mg ions play a significant role in stimulating stem cell differentiation and a function in cellular homeostasis, and regulation of gene expression. The regulatory mechanisms governing the expression of genes associated with osteogenesis are crucial for promoting bone formation (6, 7).

The field of bone regeneration technology has seen recent studies focusing on different types of stem cells, primarily stem cells obtained from both adult and fetal tissues (8, 9). The three major mechanisms of tissue regeneration include immune system influence, distribution of paracrine molecules, and angiogenic properties (10, 11). There are multiple types of mesenchymal stem cells (MSCs) recognized for bone regeneration. Researchers are focusing on using human olfactory ecto-mesenchymal stem cells (hOE-MSCs) as a source for therapeutic applications. hOE-MSCs are harvested from the lamina propria of the olfactory mucosa and are characterized as a novel type of MSCs (12). Easy accessibility and potential for autologous transplantation help address concerns related to immune responses and ethical considerations in clinical applications (8).

Furthermore, the expression of genes involved in osteogenesis is a complex process that facilitates the transformation of bone marrow-derived MSCs into osteoprogenitor cells (9).

Mg-containing BG (BG-Mg) facilitates bone regeneration owing to its elevated biological effects. BG-Mg shapes a hydroxyapatite-like layer that enriches bone grafting and osteoblast activity, which is crucial for novel bone formation, and reveals superior biocompatibility. Furthermore, it assures the adequate supply of pivotal ions and oxygen required for bone regeneration and grants the sustained re-

lease of therapeutic ions. Mg and phosphorus (P) ions markedly boost the bioactivity of BGs, improving their efficacy as biomaterials for promoting osteogenic differentiation and bone regeneration.

This *in vitro* study evaluated the interaction between ion-releasing of BG-Mg and osteogenic differentiation of hOE-MSCs, hence making the first step towards promising possibilities to refine therapeutic methodologies to bone tissue regeneration. After characterization of both types of BGs involving BG-Mg and Mg-free control BG (BG-C) by X-ray diffraction (XRD), Fourier-transform infrared (FTIR), dynamic light scattering (DLS), field emission scanning electron microscopy (FE-SEM), energy dispersive X-ray spectroscopy (EDX), inductively coupled plasma-atomic emission spectroscopy (ICP-AES), transmission electron microscopy (TEM), and pH (millipore), the influence of the two types of BGs on osteoblast differentiation of hOE-MSCs was compared.

The primary aim of this research was to identify the optimal dose of BG-Mg to develop hOE-MSC survival, osteogenesis, and bone extracellular matrix (ECM) production for future application in vivo research studies to cure maxillofacial bone defects.

## 2. Materials & Methods

### 2.1. Materials

Tetraethyl orthosilicate (TEOS, purity >96%), triethyl phosphate (TEP, purity >98%), calcium nitrate tetrahydrate ( $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ , purity >97%), magnesium nitrate hexahydrate ( $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ , purity >99%), nitric acid ( $\text{HNO}_3$ , >99%), Alizarin red S (TMS-008-C), Live/Dead assay kit (CBA415), chloroform (purity 99.0–99.4%), isopropanol ( $\text{C}_3\text{H}_8\text{O}$ , 10723124), and acetic acid ( $\text{CH}_3\text{COOH}$ , 100063) were all purchased from Merck (Germany). Collagenase type 1 (BI-1603), penicillin-streptomycin (penstrep, BI-1203), Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12, BI-1011), trypsin/EDTA 0.25% (BI-1602), osteogenic differentiation medium (BI-1102), Oil Red O (BI-1802), adipocyte differentiation medium (BI-1101), and dimethyl sulfoxide (DMSO, BI-1407) were obtained from Bioidea Co (Tehran, Iran). Fetal bovine serum (FBS, AnaCell, AC-89), MTT assay kit (Cib Biotech, 3202-300), formalin (37%), whole RNA extraction kit (Fanavari Salamat Partogene (FSP), R2381601), cDNA synthesis kit (FSP, C2372902), ethanol (96%), sodium hydroxide (NaOH), phosphate-buffered saline (PBS) were sourced from the respective suppliers.

### 2.2. Ethical Approval

This project was granted approval by the ethics board of Iran University of Medical Sciences (code of ethics:

IR.IUMS.REC.1403.078) for the procurement of human cells, and was conducted in accordance with the university's guidelines. Written informed consent was obtained from all participants prior to the collection of human olfactory mucosa biopsies.

**Table 1:** Chemical compositions of the BGs (mol.%)

| BGs   | SiO <sub>2</sub> | CaO | P <sub>2</sub> O <sub>5</sub> | MgO |
|-------|------------------|-----|-------------------------------|-----|
| BG-C  | 64               | 26  | 10                            | -   |
| BG-Mg | 64               | 26  | 5                             | 5   |

### 2.3. Fabrication and Characterization of the BGs

The synthesis of two variants of BG 64S, specifically BG-Mg and BG-C, was performed using the sol-gel technique (10). The molar constituents of the BGs were verified to be 64%SiO<sub>2</sub>, 26% CaO, and 10%P<sub>2</sub>O<sub>5</sub> for BG-C, and 64%SiO<sub>2</sub>, 26%CaO, 5%P<sub>2</sub>O<sub>5</sub>, and 5%MgO for BG-Mg (**Table 1**).

For the synthesis of BG-C, a 2-molar HNO<sub>3</sub> solution was added to 50 ml of pure water to adjust the pH between 3 and 4. Then, 28.56 ml of TEOS was incorporated into the acidic solution and blended for 45 minutes to ensure thorough mixing. Subsequently, 3.392 mL of TEP and 12.28 g of Ca(NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O were sequentially added to the solution. Each component was then stirred for an additional 45 minutes using a magnetic stirrer to ensure thorough mixing. For the synthesis of BG-Mg, the amount of TEP was reduced to 50%. Following the incorporation of Ca(NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O, an additional 2.56 g of Mg(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O was introduced to the mixture. The solution was then subjected to a further stirring period of 45 minutes to ensure proper homogenization. Next, The combined solution was stirred for another 30 minutes to

facilitate the complete reaction. The resulting sol was placed at 25°C for 10 days to form a gel. The resulting gel was subsequently subjected to thermal treatment: first at 70°C for 72 h, followed by the next phase at 120 °C for 48 h. Finally, the resulting gel was calcined at 700°C for 7 h to ensure that the intended phase was stabilized for analysis.

#### 2.3.1. XRD, FTIR, and DLS Characterizations

The phase components of the two groups of BGs were thoroughly investigated using XRD (Philips PW1730). This analysis was conducted under specific conditions, utilizing a copper radiation source to obtain accurate and detailed diffraction patterns. The functional groups were characterized using FTIR (Perkin Elmer, Spectrum RX-I) across the wavenumber spec-

trum of 400–4000 cm<sup>-1</sup>. The size distribution of both types of BGs was determined by DLS (Malvern Zn series). To prepare the samples, 10 mg of each BG was weighed, immersed in 5 ml of absolute ethanol, and sonicated for 10 minutes.

#### 2.3.2. FE-SEM, EDX, and TEM Characterizations

The morphology and dispersion of the particles within both BGs by FE-SEM and EDX detectors (TESCAN, Mira 3 LMU). Additionally, the particle microstructure was also observed by TEM (LEO 906, Germany).

#### 2.3.3. Ion Release and pH Characterizations

3 mg of BG-Mg and BG-C respectively were immersed in 2 ml of DMEM/F12 medium (11). The ion release rates were analyzed at 24 and 48 h, with three independent replicates for each time point, to assess the effects of Mg ion on the ion release kinetics. The electrode was thoroughly washed and dried after calibrating the pH meter at pH 7 (12). Then, at time points of 3 h, 2 days, and 7 days, the sample was placed in a centrifuge tube filled with 10 ml of DMEM/F12 medium and BG for pH level measurement.

### 2.4. Extraction, Isolation, and Characterization of hOE-MSCs

A biopsy of a 2–3 mm of human nasal olfactory mucosa was performed during endoscopic surgery and subsequently transported to the laboratory using an appropriate transport medium (13). Physical digestion of the tissue was conducted using a Pasteur blade, followed by enzymatic digestion with collagenase type 1 in a sterile Petri dish. The detached cells were cultured and augmented in a fresh growth medium composed of DMEM/F12 supplemented with 10% FBS and 1% penicillin-streptomycin.

#### 2.4.1. Characterization of hOE-MSCs

The characterization of hOE-MSCs was conducted using flow cytometry analysis, and their multipotent differentiation capabilities were assessed through a multipotency assay.

The assessment of surface markers was carried out using five markers: two negative markers (CD34, and CD45), and three positive markers (CD73, CD90, and CD105) (14). Stem cells were fixed in 4% paraformaldehyde and permeabilized with cold methanol. Negative controls were treated with CD34 and CD45 antibodies, followed by incubation with CD73, CD90, and CD105 antibodies. The potential for differentiation into adipogenic and osteogenic cells was evaluated on day 21 (8). The cells were preserved in 4% formaldehyde solution. Osteoblasts were stained with Alizarin Red S and adipocytes were stained with Oil Red O.

## 2.5. Biocompatibility Analysis

Biocompatible dose assessment of the two types of BGs was performed by evaluating the viability of hOE-MSCs exposed to extracts of these biomaterials. (15) BG-Mg and BG-C extracts were obtained by immersing the materials in DMEM/F12 medium and incubating them for 48 h, followed by filtration.

### 2.5.1 MTT Assay

Extracts of 1, 1.5, 2, and 5 mg/mL were assessed in triplicate on

| Target gene | Primer sequences           | T <sub>m</sub> (°C) |
|-------------|----------------------------|---------------------|
| ALP         | F: AGACCAAGCGCAAGAGACAC    | 60.18               |
|             | R: GTGGAGACACCCATCCCATC    | 59.39               |
| RUNX2       | F: ACCAGATGGGACTGTGGTTACTG | 61.45               |
|             | R: AAGGTGAAACTCTTGCCTCGTC  | 60.38               |
| COL1A1      | F: GGCCAAGACGAAGACATCCC    | 60.32               |
|             | R: GTCATCGCACAAACACCTTGC   | 59.97               |
| GAPDH       | F: AACAGCCTCAAGATCATCAGCA  | 60.03               |
|             | R: ATGAGTCCTCCACGATACCA    | 58.25               |

days 1, 3, and 7 for precision (16). Initially, hOE MSCs were exposed to the BG-C and BG-Mg extracts. To evaluate cell viability on the specified days, MTT solution was added to the wells at 37°C for 4 hours. DMSO was then used to dissolve the formazan crystals, and absorbance measurements were recorded at a wavelength of 570 nm.

### 2.5.2. Live/Dead Staining

The cells were exposed to the doses with the highest viability determined using the MTT test. After 48 h, the wells were treated with fluorescence staining solution and incubated at 37°C for 10 minutes (17).

## 2.6. Osteogenic Differentiation

In total, 600,000 cells were seeded in each group, and differentiation media containing extracts were added.

### 2.6.1. Gene Expression Analysis

Total RNA was extracted using RNA-EZi (Fanavari Salamat Partogene, Iran) after adding optimal doses of BG-Mg and BG-C to the osteogenic differentiation medium on day 14 (13). A comprehensive quantitative and qualitative assessment of the isolated RNA was conducted via spectrophotometric analysis using a NanoDrop instrument (Thermo Scientific®). cDNA synthesis was performed using 2 µg of total RNA in a final reaction volume of 10 µL, following the FSP kit manufacturer's guidelines. Gene-specific primers were commercially synthesized and purchased from Qiagen. The PCR reactions were prepared in a total volume of 10 µL, which included 2 µL of cDNA and 5 µL of 2X

SYBR Green qPCR Master Mix (Fanavari Salamat Partogene, Iran), along with 10 pM of both reverse and forward specific primers. The amplicons generated by each primer pair were validated using sequencing. The amplification protocol began with an initial denaturation step at 95°C for 5 minutes, followed by 40 cycles. Each cycle included a denaturation phase at 95°C for 20 seconds, and an annealing/extension phase at 60°C for 1 minute. The analysis involved calculating the relative expression levels of the RUNX2, ALP, and COL1A1 genes in the experimental samples. All values were normalized to GAPDH levels, and the outcomes are presented as the average ± standard deviation (SD).

The primers required for the selected genes for real-time PCR were designed according to the sequences available in the NCBI Gene Bank using the NCBI website and Primer3 software (**Table 2**).

**Table 2:** Primer sequences for real-time PCR

### 2.6.2. ECM Mineralization

To assess the amount of calcium deposits, stem cells were treated for 21 days (18). Following washing with PBS, the stem cells were incubated in 4% formalin for 30 minutes for fixation. The stem cells were then washed a second time with PBS to remove excess residual contaminants, before addition of 150 µL of Alizarin red (pH 4.2) to each well, which was incubated at room temperature for 20 minutes. After that, we added 150 µL of acetic acid to each well and shaken the plates for 30 minutes to dissolve the calcium deposits. Then we transferred the total suspensions to a 85°C water bath for 15 minutes, before immediately placing the samples in an ice bath for 5 minutes. We centrifuged each sample at 20,000 g, and then this was nicely pipetted out to take 100 µL of the supernatant and mix with 50 µL of ammonium hydroxide to measure absorbance at 570 nm.

## 2.7. Statistical Analysis

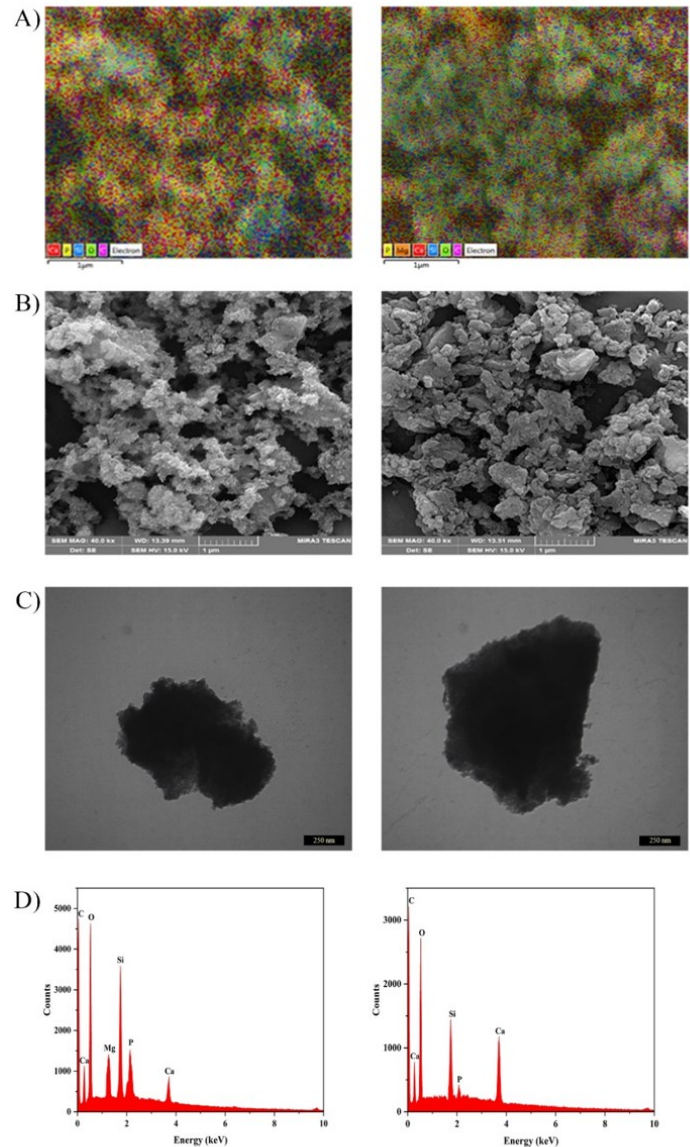
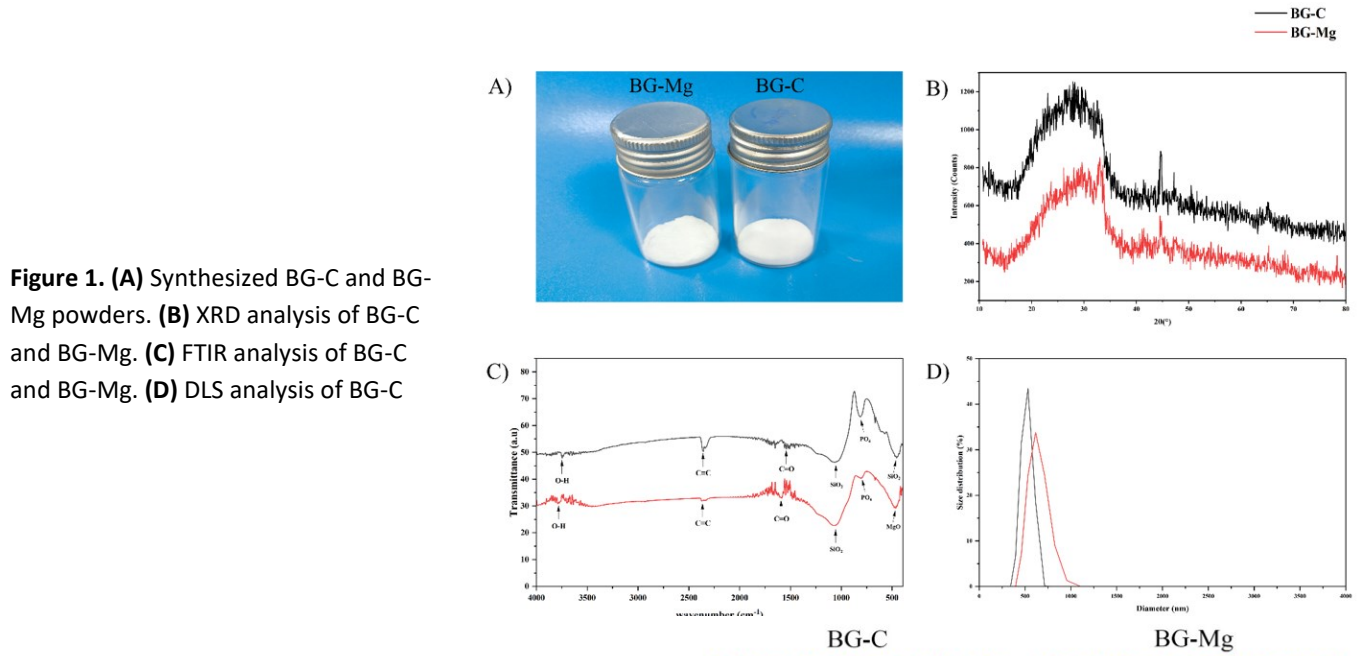
The statistical analysis for this study was conducted using Origin Pro 2024, Excel 2019, and GraphPad Prism version 8.4.3 software. All findings underwent one-way ANOVA and Kruskal-Wallis tests with Dunnett's post hoc test to evaluate differences among the groups. The results are presented as mean values accompanied by corresponding standard deviations. A significance threshold of  $p \leq 0.05$  was deemed statistically significant.

## 3. Results & Discussion

### 3.1. Characterization of the BGs

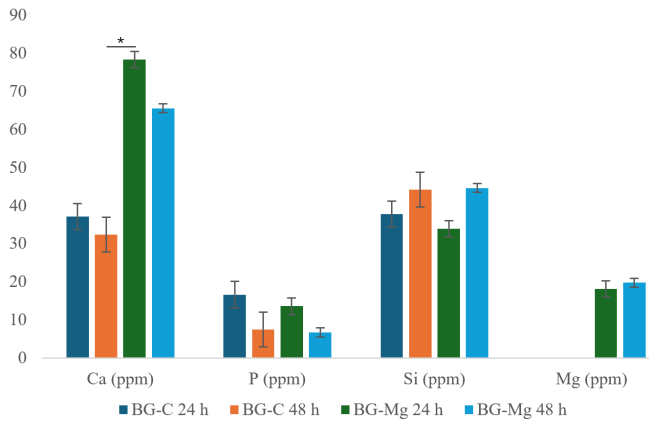
#### 3.1.1. XRD, FTIR, and DLS Analyses

Both BGs were evaluated after synthesis by XRD, FTIR and DLS tests and their results are shown (**Figure 1**). The XRD patterns



show the presence of a broad peak between 30° and 35° in the BG-Mg pattern, which, according to previous studies (10, 19), is due to the formation of a slightly different structure due to the formation of the wollastonite structure, which could be due to partial crystallization during calcination and also the existence of Mg ions in the BG-Mg structure. The peak in the hill-like structure of BG-C and BG-Mg in the 15°–35° region indicates the amorphous phase, which is the main phase of 64S BGs) **Figure 1B**).

**Figure 1C** shows the FTIR spectra of BG-C and BG-Mg. Consistent with findings from previous research, the vibrations associated with SiO<sub>2</sub> were observed at 465 cm<sup>-1</sup> and 1061 cm<sup>-1</sup> for BG-C and at 1068 cm<sup>-1</sup> for BG-Mg (20, 21), while the P-O bending vibration was characterized in the range of 805–820 cm<sup>-1</sup>



(22, 23). The vibrations of 1594 cm<sup>-1</sup> and 1646 cm<sup>-1</sup> can be attributed to the asymmetric stretching of the carbonate functional units in BG-Mg and BG-C, respectively (10). A C≡C bond is observed between regions 2300 and 2400 cm<sup>-1</sup> (23). The bands at 3777 cm<sup>-1</sup> in BG-Mg and 3742 cm<sup>-1</sup> in BG-C correspond to the O-H resonances (24). The MgO band at 478 cm<sup>-1</sup> is also well characterized by Mg.

The particle size of both BGs was measured at 636 nm for the BG-C sample and 516 nm for the BG-Mg sample. The particles

| Variable         | Group | Mean | SD   |
|------------------|-------|------|------|
| pH after 3 hours | BG-C  | 7.51 | 0.05 |
|                  | BG-Mg | 7.55 | 0.07 |
|                  | C     | 7.4  | 0.01 |
| pH after 2 days  | BG-C  | 7.64 | 0.03 |
|                  | BG-Mg | 7.77 | 0.02 |
|                  | C     | 7.42 | 0.02 |
| pH after 7 days  | BG-C  | 7.94 | 0.04 |
|                  | BG-Mg | 8.06 | 0.06 |
|                  | C     | 7.45 | 0.02 |

synthesized via the sol-gel method exhibited sustained ion release capabilities and demonstrated superior efficacy in biological functions, such as bone repair, when compared to particles produced through alternative fabrication methods, as evidenced by previous studies (25, 26) **(Figure 1D)**.

### 3.1.2. FE-SEM, EDX, and TEM Analyses

The results in **Figure 2A** demonstrate that each component is represented by a unique color, and the spatial distribution of elements within the glass matrix of both types of BGs is consistently uniform. This uniformity indicates that both BGs have potential for biological applications. In **Figure 2B**, the FE-SEM micrographs of the BG-Mg and BG-C samples show that the particles of both BGs do not exhibit uniform morphology, which confirms the XRD results. The structure of BG-Mg is different from that of BG-C, which is attributed to the presence of Mg ions and the formation of distinct spectral bands compared to BG-C. (27)

The internal structure of the particles of both BGs is well demonstrated, confirming the amorphous nature of the structures of both samples and the absence of crystals in either structure. Moreover, the particle sizes were consistent with the DLS results for both samples **(Figure 2C)**.

The spectrum of each element is also displayed based on the radiant energy in electron volts (eV) and the reflection and energy reception by the device, and the corresponding graph is provided. In addition to the main structural ions of BG-C (silicon, calcium, and phosphorus), the distribution of carbon and oxygen elements was determined due to the presence of oxygen and carbon-containing functional groups based on previous studies **(Figure 2D)**.

### 3.1.3. Ion release and pH characterization

The ion release profiles of BG-C and BG-Mg at 24 h and 48 h are shown in **Figure 3**. Generally, BG-Mg demonstrated a raised rate of ion release compared to BG-C, while the release of P ions was lower in BG-Mg because of its reduced molar percentage in the sample. Remarkably, the discharge rate of calcium (Ca) ions in the BG-Mg group exceeded that of BG-C (\*  $p < 0.05$ ), whereas BG-C showed a higher release of silicon (Si) ions than BG-Mg.

**Figure 3.** Bar graph representing BG-C and BG-Mg ion release (ppm) at different time points. Data are presented as mean  $\pm$  SD (n=3). (\*  $p < 0.05$ ).

Moreover, the data illustrated in the graph supports the concept that the release of Mg ions positively affects cellular activity.

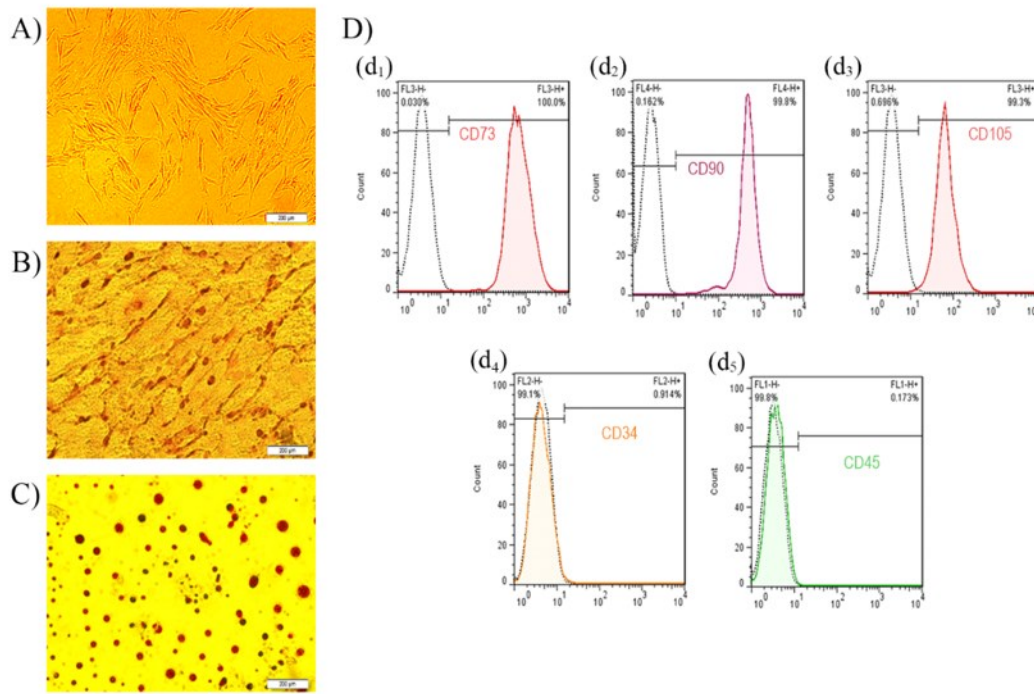


Figure 4. hOE-MSC confirmation. **(A)** Morphology of hOE-MSCs (scale bar: 200 μm); **(B)** hOE-MSCs differentiated into bone lineage (scale bar: 200 μm); **(C)** hOE-MSCs differentiated into adipocytes (scale bar: 200 μm); **(D)** Flow cytometric analysis to characterize hOE-MSC surface markers. The positive markers include CD73, CD90, and CD105, while the negative markers are CD34 and CD45.

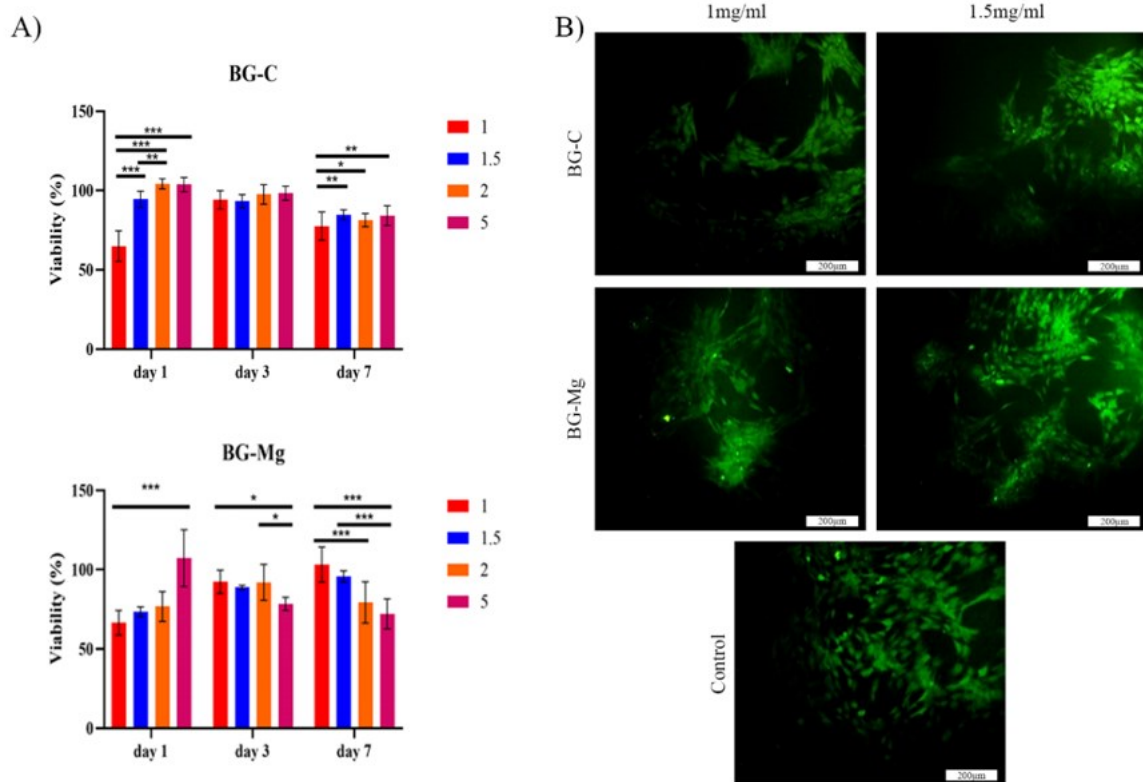


Figure 5. Biocompatibility of BGs. **(A)** MTT analysis of BG-C and BG-Mg assessed to 1, 3, and 7 days (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ). **(B)** Live/Dead assay of hOE-MSCs in 1 and 1.5 mg/ml BG-C, BG-Mg, and control group (scale bars: 200 μm).

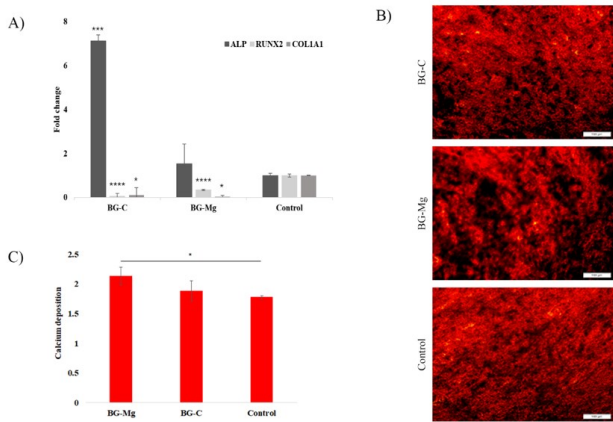
The results of the pH analysis of both groups at the desired time points are shown (Table 3).

Table 3: The pH values measured at 3 h, 2 days, and 7 days

The increase in pH in both BG groups indicates the alkaline properties of the desired ions in BG-C and BG-Mg. The greater increase in pH in the BG-Mg group compared to the BG-C group reflects the effect of Mg ions in alkalizing liquids and also affects the ion release rate of BG-Mg (28). A mildly alkaline microenvironment can affect cellular metabolic activity, proliferation, and osteogenic differentiation; however, excessive or prolonged alkalization may negatively affect cell viability and function. In the present study, the selected concentration of 1.5 mg/mL BG-Mg showed favorable cell viability based on the MTT and Live/Dead assays, suggesting that the observed pH increase did not result in substantial cytotoxicity under the experimental conditions.

### 3.2. hOE-MSCs confirmation

The morphology of the hOE-MSCs is shown in passage 3 (Figure 4A). Based on photographic analysis and corroborating previous studies, hOE-MSCs demonstrated the ability



to differentiate into osteocytes and adipocytes following a 21-day incubation period in a specific differentiation medium. This differentiation potential was validated through Alizarin red staining, which highlighted the presence of calcium deposits indicative of osteogenic differentiation, as well as Oil Red O staining, which effectively visualized adipocyte colonies (8, 13) (Figure 4B, C).

To characterize hOE-MSCs, the positive markers CD73, CD90, and CD105 were employed to confirm cell type identity, whereas the negative markers CD34 and CD45 were used to rule out contamination by other cell types. These markers were selected based on previous MSC lineage identification studies (Figure 4D). Analysis showed that the CD73, CD90,

and CD105 markers demonstrated approximately 99% expression, indicating the high purity of hOE-MSCs. In contrast, CD34 and CD45 markers demonstrated expression levels of less than 1%, confirming the absence of non-MSC populations, consistent with findings from previous studies (13, 29).

### 3.3. Determining the Dosage of BGs

To determine the optimal doses of BG-C and BG-Mg, the MTT test was first used to quantitatively measure cell viability, while the Live/Dead test was employed to examine the quality and appearance of the live stem cells.

MTT results were initially analyzed separately for each group on each day, and then all groups were compared on days 1, 3, and 7. Based on the one-way ANOVA accomplished on the groups, the significance of the differences among the various groups on days 1, 3, and 7 is indicated by an asterisk. A smaller  $p$ -value normally represents a more significant difference, implying that the results are unlikely to be owing to random variation and instead express a true impact. Data are presented as mean  $\pm$  SD. Statistical significance is indicated as follows: \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ . All groups were similarly compared with each other by one-way ANOVA analysis. The standard deviation of each group on different days is shown on the graphs, and the range of each significant difference is indicated by the lines with an asterisk. The groups without significant differences remained without lines and asterisks (Figure 5A).

By observing and reviewing the images taken from the Live/Dead staining, a dose of 1.5 mg/mL of BG-Mg and BG-C was considered the optimal dose for continuing the research (16, 30) (Figure 5B).

### 3.4. *In vitro* evaluations of the osteogenic activity of hOE-MSCs

The data obtained in this section were analyzed using the  $\Delta\Delta CT$  method (31). The data distribution was found to be normal for the RUNX2 gene and non-normal in the ALP and COL1A1 genes. To examine data with non-normal distribution, the Kruskal-Wallis test was used.

Regarding the ALPL gene that plays a role in the mineralization of bone precursor cells and their hardening, on day 14 of the osteogenesis process of hOE-MSCs, the effect of BG-C was greater than that of BG-Mg. After conducting the one-way ANOVA test, it was determined that this group had a significant difference (\*\*\*\*) compared to both other groups, which indicates a higher expression of the alkaline phosphatase enzyme in osteogenic differentiation. The RUNX2 gene is

one of the main genes involved in the osteogenesis process of hOE-MSCs. In this graph, during the 14-day period of osteogenic differentiation, BG-Mg had a higher expression than BG-C, but still lower than the control group. In addition, after running a one-way ANOVA, BG-Mg and BG-C were statistically significant (\*), and both groups were statistically significant from the control group (\*\*\*\*). In

Figure 6. Osteogenic differentiation of hOE-MSCs. **(A)** Osteogenic-related gene expression of RUNX2, ALP and COL1A1 on day 14. **(B)** ECM mineralization of BG-C, BG-Mg and C (scale bars: 100  $\mu$ m); **(C)** quantitative ECM mineralization (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*\*  $p < 0.0001$ ).

terms of the COL1A1 gene, BG-C had higher expression than BG-Mg, possibly due to BG-C having more P ions. Additionally, according to the one-way ANOVA analysis, both groups showed a significant difference (\*\*\*\*) compared to the control group and demonstrated a decrease. Mg alone does not enhance the expression of genes related to osteogenesis. Previous research (6, 32) indicated that the combination of Mg and strontium (Sr) promotes expression of crucial osteogenic genes, such as ALP, RUNX2, and COL1A1. This enhanced expression may be attributed to the presence of Sr ions, which play an essential role in osteogenesis (**Figure 6A**).

After 21 days of differentiation and Alizarin Red staining, the BG-Mg group displayed a higher calcium deposition rate than the other experimental groups. This finding highlights the role of Mg in promoting calcium deposition, which is a key component of the ECM at 21 days post-osteogenic differentiation (**Figure 6B**). Statistical analysis via one-way ANOVA indicated that the BG-Mg group exhibited higher calcium deposition compared to the other two groups, particularly in comparison to the control group (\*), a result that is confirmed by photographic documentation of the results of each group. Previous research has consistently supported the notion that Mg plays an active role in modulating calcium deposition formation and emphasizes its significance in bone ECM development (18) (**Figure 6C**).

#### 4. Conclusions

In this study, the effects of BG-Mg and BG-C on the viability, growth, and differentiation of hOE-MSCs were investigated. For this purpose, both BG powders were characterized using XRD, FTIR, FE-SEM, EDX, TEM, DLS, and ICP-AES analyses. Then, BG powders were applied to hOE-MSCs at concentrations of 1, 1.5, 2, and 5 mg/ml, ultimately, 1.5 mg/ml was determined as the optimal dose with the highest viability. In addition, BG-C with higher P content increased ALPL gene expression compared to the control gene and enhanced COL1A1 gene expression com-

pared to BG-Mg, indicating the effect of P ions on the mineralization process of bone differentiation. Furthermore, BG-Mg increased the expression of the RUNX2 gene compared to BG-C, indicating the influence of Mg on the RUNX2 factor as a regulatory factor in the osteogenic differentiation of hOE-MSCs. Finally, on day 21 of osteogenic differentiation, it was demonstrated that Mg was significantly different (\*) from the control group, indicating the effect of Mg on day 21 on the formation of calcium structures compared to other groups.

#### Acknowledgments

This work was conducted in collaboration with the Iran University of Medical Sciences and Kharazmi University. We are grateful for the support of the Cellular and Molecular Research Center of the Iran University of Medical Sciences through research Grant No. 28107.

#### Conflict of Interests

There are no declared conflicts of interest among the authors.

#### Author Contributions

**S. A. A.** and **S. S.** proposed the concept and basis of the study. **S. S.** and **M. K.** extracted and isolated hOE-MSCs. **S. A. A.** and **A. A.** designed the primers. **M. K.** and **M. B.** performed real-time PCR analysis. **S. S.** and **M. K.** wrote the first draft. All authors have read and approved the written manuscript.

#### References

- Vidal L, Kamleitner C, Brennan MÁ, Hoornaert A, Layrolle P. Reconstruction of large skeletal defects: current clinical therapeutic strategies and future directions using 3D printing. *Frontiers in bioengineering and biotechnology*. 2020;8:61.
- Priya J, Tripathi P, Hegde P. The Psychological Impact of Maxillofacial Trauma: Integrating Mental Health Support. *Post-Traumatic Stress Disorder-Latest Developments in Diagnosis, Causes, and Treatments: IntechOpen*; 2024.
- Pantulap U, Arango-Ospina M, Boccaccini AR. Bioactive glasses incorporating less-common ions to improve biological and physical properties. *Journal of Materials Science: Materials in Medicine*. 2022;33:1-41.
- Loty C, Sautier J, Tan M, Oboeuf M, Jallot E, Boulekbache H, et al. Bioactive glass stimulates in vitro osteoblast differentiation and creates a favorable template for bone tissue formation. *Journal of bone and mineral research*. 2001;16(2):231-9.
- Taye MB. Biomedical applications of ion-doped bioactive glass: A review. *Applied Nanoscience*. 2022;12(12):3797-812

6. Qi T, Weng J, Yu F, Zhang W, Li G, Qin H, et al. Insights into the role of magnesium ions in affecting osteogenic differentiation of mesenchymal stem cells. *Biological trace element research*. 2021;199:559-67.
7. Nandi SK, Mahato A, Kundu B, Mukherjee P. Doped bioactive glass materials in bone regeneration. *Advanced techniques in bone regeneration*. 2016;10:63266.
8. Veron AD, Bienboire-Frosini C, Feron F, Codecasa E, Devez A, Royer D, et al. Isolation and characterization of olfactory ecto-mesenchymal stem cells from eight mammalian genera. *BMC veterinary research*. 2018;14:1-11.
9. Ling M, Huang P, Islam S, Heruth DP, Li X, Zhang LQ, et al. Epigenetic regulation of Runx2 transcription and osteoblast differentiation by nicotinamide phosphoribosyltransferase. *Cell & Bioscience*. 2017;7:1-10.
10. Wu X, Meng G, Wang S, Wu F, Huang W, Gu Z. Zn and Sr incorporated 64S bioglasses: Material characterization, in-vitro bioactivity and mesenchymal stem cell responses. *Materials Science and Engineering: C*. 2015;52:242-50.
11. Davis HB, Gwinner F, Mitchell JC, Ferracane JL. Ion release from, and fluoride recharge of a composite with a fluoride-containing bioactive glass. *Dental Materials*. 2014;30(10):1187-94.
12. Khatri S, Mathew S, Nagaraja S, Hegde S, Ghosh S, Ravichandran K. Comparative evaluation of pH and Ca (+) ion release from MTA on interaction with platelet-rich fibrin and blood clot: an in vitro study. *F1000Res*. 2023;12:364.
13. Simorgh S, Milan PB, Saadatmand M, Bagher Z, Gholipourmalekabadi M, Alizadeh R, et al. Human olfactory mucosa stem cells delivery using a collagen hydrogel: As a potential candidate for bone tissue engineering. *Materials*. 2021;14(14):3909.
14. Ge Z, Qiu C, Zhou J, Yang Z, Jiang T, Yuan W, et al. Proteomic analysis of human Wharton's jelly mesenchymal stem/stromal cells and human amniotic epithelial stem cells: a comparison of therapeutic potential. *Scientific Reports*. 2024;14(1):28061.
15. Lutanova G, Palou MT, Kozankova J. Comparison of bioactivity in vitro of glass and glass ceramic materials during soaking in SBF and DMEM medium. *Ceramics-Silikáty*. 2011;55(3):199-207.
16. Moghanian A, Sedghi A, Ghorbanoghli A, Salari E. The effect of magnesium content on in vitro bioactivity, biological behavior and antibacterial activity of sol-gel derived 58S bioactive glass. *Ceramics international*. 2018;44(8):9422-32.
17. Breu A, Eckl S, Zink W, Kujat R, Angele P. Cytotoxicity of local anesthetics on human mesenchymal stem cells in vitro. *Arthroscopy: The Journal of Arthroscopic & Related Surgery*. 2013;29(10):1676-84.
18. Kargozar S, Milan PB, Amoupour M, Kermani F, Gorgani S, Nazarnezhad S, et al. Osteogenic potential of magnesium (Mg)-doped multicomponent bioactive glass: in vitro and in vivo animal studies. *Materials*. 2022;15(1):318.
19. León L, Stabile F. Processing of concentrated aqueous zirconia-bioglass slips by slip casting. *Ceramics - Silikaty*. 2017;61:1-10.
20. Oki A, Parveen B, Hossain S, Adeniji S, Donahue H. Preparation and in vitro bioactivity of zinc containing sol-gel-derived bioglass materials. *Journal of Biomedical Materials Research Part A: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials*. 2004;69(2):216-21.
21. Alasvand N, Simorgh S, Kebria MM, Bozorgi A, Moradi S, Sarmadi VH, et al. Copper/cobalt doped strontium-bioactive glasses for bone tissue engineering applications. *Open Ceramics*. 2023;14:100358.
22. Frost RL, Xi Y, Scholz R, López A, Lima RMF, Ferreira CM. Vibrational spectroscopic characterization of the phosphate mineral series eosphorite-childrenite-(Mn, Fe) Al (PO<sub>4</sub>)(OH) 2·(H<sub>2</sub>O). *Vibrational Spectroscopy*. 2013;67:14-21.
23. Youness RA, Taha MA, Ibrahim M, El-Kheshen A. FTIR spectral characterization, mechanical properties and antimicrobial properties of La-doped phosphate-based bioactive glasses. *Silicon*. 2018;10:1151-9.
24. Al-Ghamdi H, Alsaif NA, Afaneh F, Khattari Z, Abdelghany A, Rammah Y. Fabrication, structure, physical properties and FTIR spectroscopy of zirconate doped-borophosphate bioglasses. *Optical and Quantum Electronics*. 2023;55(11):1025.
25. Tamjid E, Bagheri R, Vossoughi M, Simchi A. Effect of particle size on the in vitro bioactivity, hydrophilicity and mechanical properties of bioactive glass-reinforced polycaprolactone composites. *Materials Science and Engineering: C*. 2011;31(7):1526-33.
26. Xia W, Chang J. Preparation and characterization of nano-bioactive-glasses (NBG) by a quick alkali-mediated sol-gel method. *Materials Letters*. 2007;61(14):3251-3.
27. Ghorbanzade Zaferani SP, Nabian N, Delavar M, Rabiee SM. Direct impregnation of MgO nanoparticles in 58S bioactive glass: bioactivity evaluation and antibacterial activity. *Iranian Journal of Science and Technology, Transactions A: Science*. 2021;45(3):885-98.
28. Luz GM, Mano JF. Preparation and characterization of bioactive glass nanoparticles prepared by sol-gel for biomedical applications. *Nanotechnology*. 2011;22(49):494014.
29. Tian J, Zhu Q, Zhang Y, Bian Q, Hong Y, Shen Z, et al. Olfactory ecto-mesenchymal stem cell-derived exosomes ameliorate experimental colitis via modulating Th1/Th17 and Treg cell responses. *Frontiers in immunology*. 2020;11:598322.