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# Design and Fabrication of Electrospun Scaffold Based on Gelatin Containing Calcium Phosphate Nanoparticles: in Vitro and in Vivo Study

**ARTICLE INFO**

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***Authors***

Fatemeh Pourebrahim[1]

Nafiseh Baheiraei*[2]

Mansoureh Movahedin[1]

1- Department of Anatomical Sciences, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
2- Tissue Engineering and Applied Cell Sciences Division, Department of Anatomical Sciences, Faculty of Medical Sciences, Tarbiat Modares University, Tehran 1411713116, Iran, n.baheiraei@modares.ac.ir

***Corresponding author:**
Nafiseh Baheiraei
Tissue Engineering and Applied Cell Sciences Division, Department of Anatomical Sciences, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.
E-mail: n.baheiraei@modares.ac.ir.
Orcid: 0000-0002-8760-5971

**ABSTRACT**

**Introduction:** Bone defects resulting from trauma, infection, fractures, and other factors present significant challenges that adversely affect organ function and lead to both physiological and psychological damage. Tissue engineering offers a promising alternative to traditional and limited methods. In this study, we aimed to introduce a novel electrospun bone graft composed of beta-tricalcium phosphate (βTCP) combined with gelatin (Gel) and polycaprolactone (PCL) scaffolds, leveraging the favorable functional properties of bone-inducing biomaterials to enhance cell proliferation, biocompatibility, and signal transduction.

**Methods:** We fabricated the composite scaffolds using the electrospinning technique to mimic the matrix fiber structure. The prepared scaffolds were thoroughly tested for their physicochemical properties and cytocompatibility. We conducted subcutaneous implantation in mice to evaluate the scaffolds' ability to induce angiogenesis.

**Results:** Compared to Gel-PCL scaffolds, human bone mesenchymal stem cells (hBMSs) cultured on Gel-PCL/βTCP scaffolds exhibited improved cell viability and adhesion. Histological evaluations confirmed the enhanced vascularization and good integration with the surrounding tissue in the βTCP containing samples following subcutaneous implantation. Introducing βTCP powder into the electrospinning solutions improved the biological and histological properties of our composite scaffold.

**Conclusion:** Our findings suggest that βTCP-containing scaffolds could have beneficial effects on bone tissue engineering, and Gel-PCL/βTCP represents a promising scaffold for accelerating the angiogenesis of damaged bone tissue.

**Keywords:** Tissue engineering, Scaffold, Electrospinning, Gelatin, Polycaprolactone, Beta-tricalcium phosphate



## INTRODUCTION

Bone defects resulting from trauma, tumor excision, infection, and other factors have emerged as a significant concern, severely impacting organ function and leading to physiological harm. These conditions necessitate safe and effective treatment (1). Annually, approximately 4 million people undergo bone grafts or replacement surgeries. In the United States, age-related bone disorders are projected to increase from 2.1 million in 2005 to 3 million by 2025. In Europe, the incidence of fractures has risen by approximately 28% from 2010 to 2025 (2).

Bone tissue regeneration and repair often involve autografts, which are considered the gold standard for treating bone defects. Autografts transfer tissue from one part of a person's body to another, thereby promoting bone growth and regeneration. However, several limitations are

associated with bone grafting, including the limited availability of donor tissue, complications at the donor site, pain, high treatment costs, and the risk of adverse effects (3). Allografts, which are sourced from cadavers or living donors, are readily available in various shapes and sizes; however, they lack osteoinductive properties and typically enhance only osteoconductivity. This procedure is associated with several drawbacks, including prolonged anesthesia, increased blood loss, a heightened risk of wound infection, potential complications at the donor site, an unwanted immune response, and the risk of disease transmission. Consequently, traditional technologies for treating bone defects often fail to fully meet the needs of patients (3).

Clinical interest in bone tissue engineering (BTE) has increased due to the limitations of traditional treatments and the growing human population. Tissue engineering employs biological alternatives to regenerate damaged tissues. This technology integrates various bone cells, biocompatible scaffolds, and growth factors. Successful bone tissue regeneration requires a scaffold that can temporarily replace the injured area, is compatible with the host tissue, possesses optimal porosity to facilitate the transfer of an adequate number of cells, and degrades at a suitable rate within the host environment. The proposed biomaterial must interact effectively with the surrounding tissue while minimizing the patient's immune response (1).

Among the various methods of scaffold design, electrospinning has emerged as a versatile and straightforward technique for fabricating micro- or nano-dimensional fiber scaffolds from solutions of polymeric, ceramic, or composite materials, including polymer-nanoparticle blends and melted polymer (4). Electrospun nanofibers have a wide range of biomedical applications, including drug delivery systems, wound dressings, tissue engineering scaffolds, various diagnostic instruments, *in vitro* models, air and liquid filtration systems, cosmetics, electrical applications, and renewable energies (5).

Gelatin (Gel) is an FDA-approved denatured collagen that offers numerous advantages, including low-cost availability, biocompatibility, excellent hydrophilicity, non-toxicity, biodegradability, and the ability to promote cellular adhesion, migration, proliferation, and differentiation. These qualities contribute to its widespread use in the preparation of tissue engineering scaffolds. The surface properties of Gel can be tailored to suit specific applications in tissue engineering (3). However, the mechanical properties and structural stability of electrospun Gel nanofibers are relatively weak, and their rapid solubility *in vivo* presents a significant drawback. Combining Gel with natural or synthetic biopolymers can enhance mechanical, physicochemical, and biological properties, as well as the rate of degradation (3, 6).

Polycaprolactone (PCL) is a tissue-compliant, semi-crystalline known approved by the FDA, recognized for its high bioabsorption capability and biocompatibility. It possesses suitable mechanical properties that demonstrate significant potential for various tissue engineering applications. PCL has also shown its effectiveness to serve as a suitable substrate for facilitating interactions with different types of cells during proliferation and adhesion. This polymer is a preferred choice for creating scaffolds in bone tissue engineering scaffolds due to its high activity, favorable mechanical properties, and absence of antigenic properties (7).Yao et al. reported that bone adhesion, proliferation, and differentiation of mesenchymal stem cells occurred on the mineralized scaffolds made of Gel and PCL, supporting their application in BTE (8). By producing a scaffold composed of Gel and PCL using the electrospinning method, scaffolds with enhanced bone differentiation, mechanical properties improved adhesion, and cell proliferation were achieved (9).

Calcium phosphate ceramics exhibit excellent biocompatibility, bioactivity, and bone induction capabilities due to their similarity to the mineral components of bone. During the bone regeneration process, β-tricalcium phosphate (β-TCP) is gradually released and is completely replaced as new bone tissue is stimulated, owing to its microstructure's resemblance to normal bone tissue and its absorbable characteristics

during regeneration. These bioceramics are utilized as fillers or scaffolds in dentistry and orthopedics. They can promote bone formation by enhancing adenosine signaling in phosphate metabolism, providing bone-inducing growth factors, facilitating osteoblast differentiation, and supporting extracellular matrix (ECM) mineralization (10, 11). Adding βTCP to PCL could improve the mechanical properties, biodegradability, and surface characteristics of the bone scaffold (12).

Considering the advantages of Gel in the application of BTE, which in addition to biocompatibility, provides suitable mechanical properties for the scaffold, as well as the studies conducted on the use of PCL as a polymer that has FDA approval, for bone and cartilage regeneration, this experiment will use the combination of these two polymers together. Adding βTCP to the mentioned composite can mimic the bone mineral phase, as well. In this study, Gel-PCL scaffold was prepared by electrospinning method and the effect of adding βTCP with different percentages (0.25, 0.5, 1, and 3%) on the properties of the scaffold was investigated.

## MATERIALS & METHODS

### Scaffold Synthesis

PCL (Sigma-Aldrich) with an average molecular weight of 80,000 g/mol was dissolved in a mixed solvent of acetic acid ($CH_3COOH$, Merck) and formic acid ($CH_2O_2$, Dr. Mojallali) in a 9:1 ratio. The solution was stirred at 700 rpm for 24 hours at room temperature to achieve a homogeneous 9 wt% solution. Gel powder (Sigma-Aldrich) was dissolved in a 75% acetic acid solution at a weight ratio of 9% (w/v), heated to 40°C, and stirred for 4 hours to obtain a clear, homogenous solution. Subsequently, the PCL solutions were mixed with the Gel solutions at room temperature for 8 hours. βTCP (Sigma-Aldrich) with different weight percentages (0.25%, 0.5%, 1%, and 3%) was added to the obtained result and stirred for 12 hours at room temperature at 700 rpm to finally obtain a transparent spinning solution. The prepared solution was then transferred to a standard 5-mL syringe fitted with an 18-gauge stainless steel needle having an inner diameter of 0.8 mm. The electrospinning was carried out using a 22kV high voltage applied to both the rotating collector and the needle, with a constant flow rate of 0.5 mL/h (Fanavaran Nano Meghyas Ltd., Co, Iran). The electrospun fibrous membranes were collected from the collector surface and left to dry off at room temperature overnight to remove the residual solvent. The electrospun nanofibers were cross-linked with saturated glutaraldehyde (Sigma-Aldrich) vapor for 24 hours at 25°C and then transferred to an oven at 45°C for 1 hour to remove the glutaraldehyde vapor.

### Scaffold Characterization

#### Physicochemical characterizations

The chemical composition and bonding of the scaffolds were evaluated using Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR; EQUINOX-55, Bruker, Germany). All spectra were recorded in the 400-4000 $cm^{-1}$ range at a scan speed of 64 scans per minute, with a resolution of 8 $cm^{-1}$ in a KBr-diluted medium. The structural phases of the fabricated samples were analyzed using X-ray Diffraction (XRD; D5000 diffractometer, Siemens Brucker, Germany) with a Cu anode at a fixed incident angle of 0.03° in a 2θ range of 5–105°. The mean average fiber diameter and the morphological characteristics of the scaffolds were also examined by using scanning electron microscopy (SEM; Tescan Vega II, Czech) after gold coating and the diameter of nanofibers was measured by Image J software.

#### Cytotoxicity and Morphological Assessments

The cytotoxicity of the prepared scaffolds was evaluated using human bone mesenchymal stem cells (hBMSCs, National Cell Bank, Royan Institute, Iran). The cells were cultured in a medium consisting of Dulbecco's Modified Eagle Medium F12 (DMEM F12; Invitrogen), supplemented with 1% antibiotic penicillin/streptomycin (Sigma-Aldrich) and 10% (v/v) fetal bovine serum (FBS; Gibco). The culture medium was changed every other day, and the cells were passaged upon reaching 80%–90% confluency. Passage-3 cells were utilized for this experiment. Before cell seeding, the samples were sterilized with 70% ethanol (Zakaria Jahrom), and rinsed with deionized (DI)

water. Cell biocompatibility was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) colorimetric assay. For this evaluation, $3\times 10^3$ cells were seeded over scaffolds in a 96-well plate and incubated under standard culture conditions for 48 and 72 h. At each time point, the medium was discarded, and 100-µL of MTT solution (5 mg/mL in PBS) was added to each well. After four hours of incubation, the medium was removed, and the formazone precipitates were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich). The optical absorbance at 570 nm was recorded using a microplate ELISA reader (ELX808, BioTek, USA). Cells cultured on a tissue culture plate (TCP) were considered the control group. SEM was also used to analyze the morphology of cells cultured on the scaffolds. For this study, at 48 hours post-seeding, samples were rinsed with phosphate-buffered saline (PBS, pH=7) and cells were fixed with 2.5% glutaraldehyde (Sigma-Aldrich) for 1 hour at room temperature. Dehydration was subsequently carried out using a graded ethanol series before the samples were coated with gold for SEM observation.

### *In vivo* assessments and immunohistochemistry staining

The angiogenesis-inducing capability of the scaffolds was evaluated through subcutaneous implantation of the scaffolds after 2 and 4 weeks. Animal experiments were conducted according to the ethical committee guidelines for laboratory animals approved by the Ethics Committee of Tarbiat Modares University, Iran (IR.MODARES.AEC.1402.011). Four male NMRI mice (25–30 g, aged 7 weeks) were administered cyclosporine (Novartis Pharma AG, Switzerland, 15 mg/kg body weight) in their drinking water 3 days before scaffold transplantation. General anesthesia was induced via intraperitoneal injection of a combination of ketamine (Alfasan, The Netherlands; 0.04 mL/100 g body weight) and xylazine (Alfasan, The Netherlands; 0.02 mL/10 g body weight). A transverse incision, approximately 2 cm in length, was made on the back of the mice. The sterilized scaffolds with dimensions of 10×10 $mm^2$ (group 1: Gel-PCL; group 2: Gel-PCL/βTCP; n=3/group) were then implanted, followed by the closure of the incision with a surgical suture. After recovery from surgery, mice were allowed free access to food and water. The mice were randomly divided into two experimental groups and housed in individual cages. They were sacrificed after 2 and 4 weeks. Initially, local vascularization near and within the scaffolds was observed using a stereo microscope (SMZ 1000, Japan). For further histological investigation, the implanted scaffolds were explanted, and the surrounding tissue was fixed in 10% buffered formalin for 24 hours before being transferred to PBS at 4°C. The fixed tissues were embedded in paraffin, sectioned into 5 µm thick segments, and stained with Mayer's hematoxylin and eosin (H&E; Sigma) to assess cellularization.

To evaluate neovascularization, immunohistochemistry (IHC) was performed using the vascular endothelial growth factor (VEGF) as a marker. Anti-VEGF antibodies (1:100; Abcam, USA) were applied to paraffin-embedded tissue samples. In this experiment, the 5-µm paraffin sections were placed on poly-L-lysine-coated slides. Paraffin removal was conducted, followed by the inhibition of endogenous peroxidase incubating the sections with 10% hydrogen peroxide ($H_2O_2$, 30%, Sigma-Aldrich) in PBS for 10 minutes after deparaffinization. The tissue sections were then subjected to heat treatment at 95 °C in sodium citrate buffer (10M, pH=6) for 30 minutes to retrieve the antigens. After washing with PBS, the sections were incubated with primary antibodies at 4 °C overnight. The slides were subsequently washed with PBS and incubated with the secondary antibodies at room temperature (25 °C) overnight for 50 minutes. 3,3′-Diaminobenzidine (DAB) was used as a chromogen, and counterstaining was performed using Mayer's hematoxylin for 5 minutes.

### Statistical analysis

Data were expressed as the mean ± standard deviation (SD), and analyses were performed using one-way ANOVA. A post-hoc Tukey's test was conducted to determine intra-group differences over time. At least three samples were tested for each experiment. The statistical

analysis was carried out using GraphPad Prism 9 software. Differences with P-values of less than 0.05 were considered statistically significant.

## RESULTS AND DISCUSSION

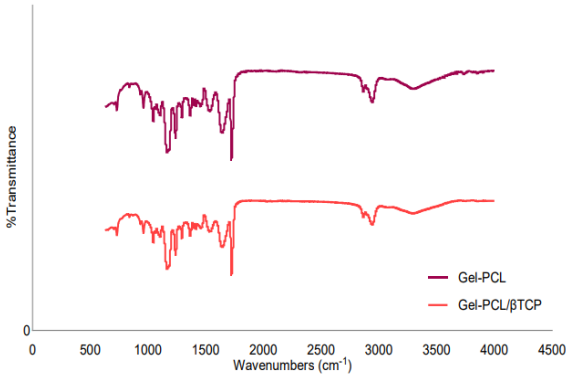
**Table 1:** Scaffold preparations and groups

| Abbreviations | Scaffolds |
|---|---|
| Gel-PCL | Electrospun Gel & PCL nanofiber |
| Gel-PCL/ %0.25βTCP | Gel & PCL nanofiber containing %0.25 βTCP |
| Gel-PCL/ %0.5βTCP | Gel & PCL nanofiber containing %0.5 βTCP |
| Gel-PCL/ %1βTCP | Gel & PCL nanofiber containing %1 βTCP |
| Gel-PCL/ %3βTCP | Gel & PCL nanofiber containing %3 βTCP |

### Scaffold characteristics

Table 1 shows the abbreviated names of the studied samples .The FTIR spectra of the Gel-PCL and Gel-PCL/βTCP nanofibers are presented in **Fig. 1**. Both composite nanofiber films exhibit characteristic peaks of Gel and PCL. The peaks observed in the range of 2800–2900 $cm^{-1}$ correspond to -$CH_2$- and -CH- bonds, while a peak around 1700 $cm^{-1}$ is attributed to the carbonyl stretching bond (C=O) in PCL. The strong band associated with the crystalline phase of PCL at 1240 $cm^{-1}$ was assigned to C−O and C−C stretching, whereas the peak at 1173 $cm^{-1}$ is linked to vibrations in COC, C–O, and C–C. The presence of characteristic peaks of Gel at 1660 $cm^{-1}$ for the C=O stretching bond of amide I, and at 1540 $cm^{-1}$ for the N-H bending and C-N stretching of amide II in the Gel-PCL composites, indicates the successful incorporation of gelatin and PCL. Peaks in the range of 3300–3500 $cm^{-1}$ range are associated with the O-H bond. Amide groups in gelatin can form hydrogen bonds with water molecules, suggesting that the hydrophilic functional groups in the gelatin molecule may enhance the hydrophilicity of the PCL composite nanofibers. These results demonstrate indicate that Gel and PCL exhibit strong molecular bonding. The absorption bands observed at wavenumbers 961.28, 1046.19, and 1107.28 $cm^{-1}$ can be attributed to the formation of βTCP, as these bands represent the phosphate ($PO_4^{-3}$) group in this material, consistent with previous studies. The absorption bands observed in the sample corresponded with the expected functional groups, aligning with previous studies. These similarities in the spectra confirm the presence of Gel, PCL, and βTCP in the analyzed samples.

**Fig. 1** FTIR patterns of the prepared scaffolds




Based on the XRD patterns of the Gel-PCL and Gel-PCL/βTCP scaffolds presented in **Fig. 2**, the Gel-PCL scaffolds exhibited a semi-crystalline state, characterized by two distinct diffraction peaks at 21.418° and 23.763, These peaks indicate the presence of the crystalline phase of PCL within the scaffold. Since Gel possesses an amorphous structure, it is not expected to display a diffraction peak. However, the inclusion of Gel, with its amorphous nature, diminished the sharpness of the peaks without altering the crystalline structure of PCL in the scaffold. The XRD pattern of the Gel-PCL/βTCP group revealed two peaks at 21.445° and 23.794°, accompanied by a slight increase in peak intensity. The characteristic reflection peak became sharper following the addition of βTCP, indicating the crystalline and single-phase structure of βTCP. These findings suggest that the scaffolds possess crystalline properties due to the crystallization of the PCL and βTCP structure, which aligns with previous studies (13).

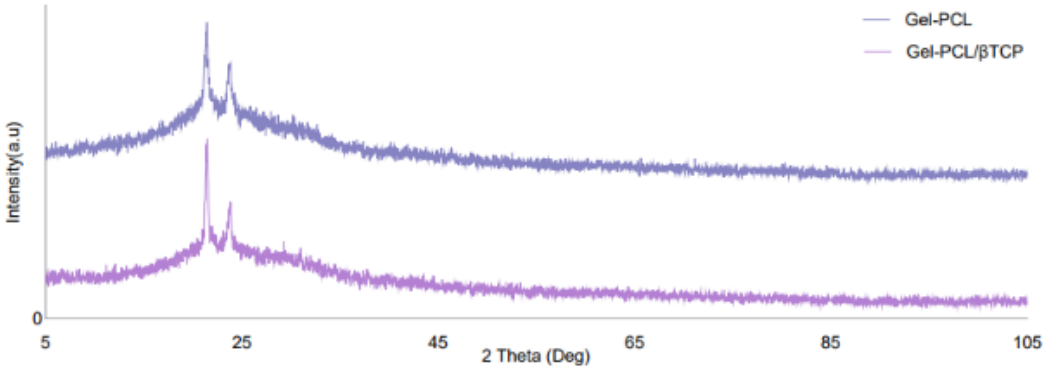



**Fig. 2** XRD patterns of the prepared scaffolds

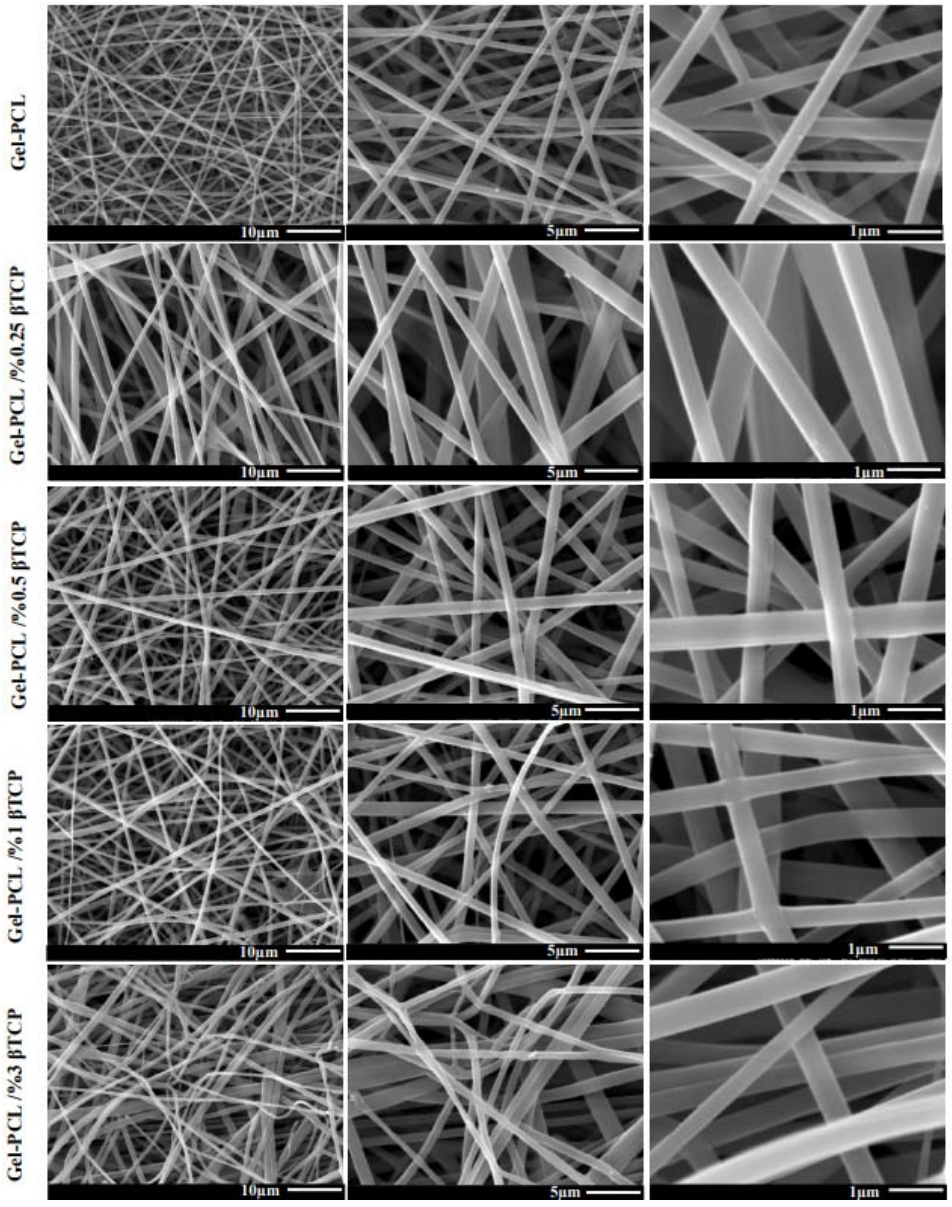



**Fig. 3** SEM images of the prepared scaffolds with different magnifications. Scale bar=10 µm, 5µm and 1µm

**Table 1** The mean fiber diameter of the scaffolds

| Sample | Mean Fiber Diameter(nm) |
|---|---|
| Gel-PCL | 417.5+0.1 |
| Gel-PCL/%0.25βTCP | 855+0.07 |
| Gel-PCL/%0.5βTCP | 777.5+0.17 |
| Gel-PCL/%1βTCP | 750+0.08 |
| Gel-PCL/%3βTCP | 775+0.17 |

The SEM images reveal that all nanofibrous scaffolds are free of beads, and exhibit a random orientation, as illustrated in **Fig. 3**. The mean diameter of nanofibers was shown in the supplementary information **Table** . The fibrous structure of the scaffolds with random orientation enhances the surface-to-volume ratio and mimic the fibrous structure of the ECM which promoting cell adhesion on the scaffold. Consequently, these scaffolds are well-suitable for applications in tissue engineering studies.

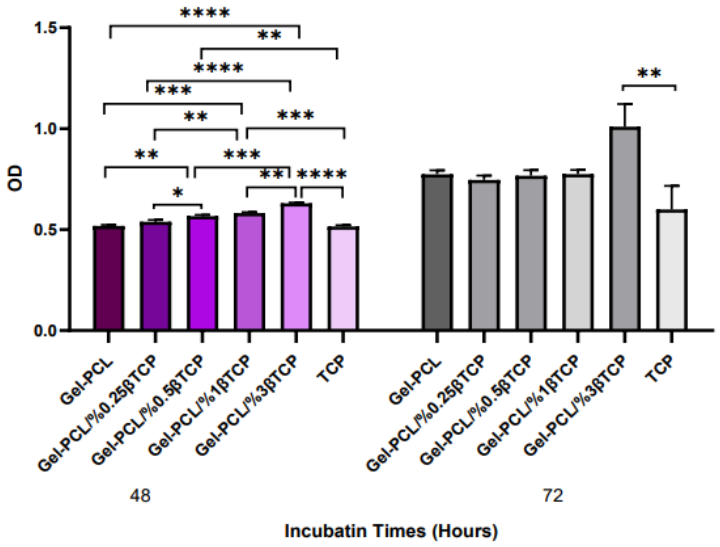



**Fig. 4** MTT assay results of prepared scaffolds on HBMSCs viability. *(p<0.05) ** (p<0.01) *** (p<0.001) and **** (p<0.0001) indicate significant difference compared with other groups. Data were presented as the average ± standard deviation (n=3)

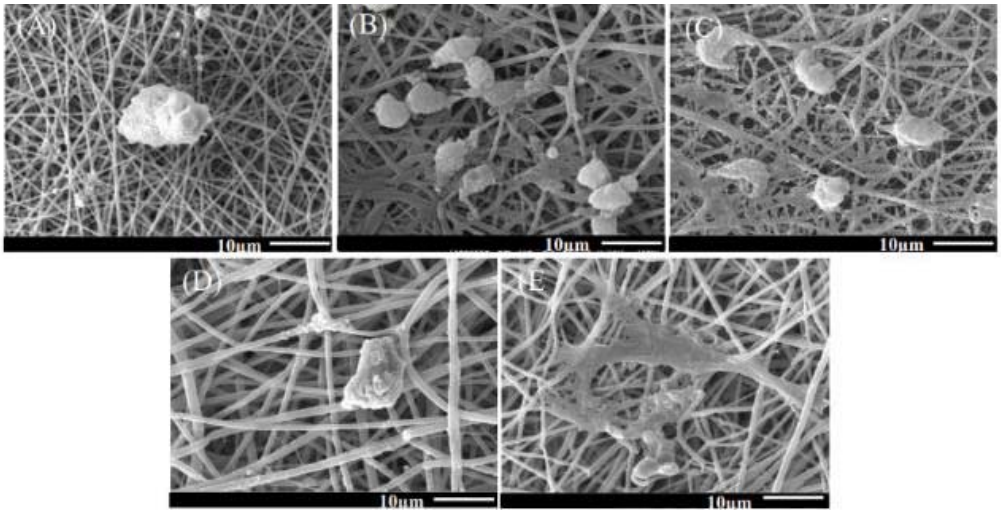



**Fig. 5** Scaffolds planted with hBMSCs, after 48 hours. Zoom 4000× A) Gel-PCL, B) Gel-PCL /%0.25 βTCP C) Gel-PCL /%0.5βTCP D) Gel-PCL /%1βTCP E) Gel-PCL /%3βTCP scaffold

## Cellular evaluations

The results of the cell viability test conducted on hBMSCs are presented in Fig. 4. Among the scaffolds, Gel-PCL/βTCP exhibited the highest cell viability, while Gel-PCL demonstrated the lowest viability compared to the other samples at both time points examined. The improvement in cell viability indicated that there was no significant toxicity associated with adding the βTCP, as this value remained above approximately 95% for all samples at both studied time intervals. The results of the MTT assay revealed increased viability of HBMSCs for all samples compared to TCP after 48hours and 72hours. The surface characteristics of βTCP containing scaffolds facilitate cellular adhesion and organization (12).

SEM micrographs of seeded HBMSCs were taken 48 hours post-seeding. As shown in

, all samples exhibited cell attachment on their surfaces, which was enhanced by increasing the concentration of βTCP. This finding suggests that the presence of βTCP facilitates and supports cell adhesion and growth by providing a suitable substrate. Cell adherence to the surface of the scaffolds was observed in all groups, indicating that the scaffolds created an appropriate environment for adhesion and cell growth. Notably, the Gel-PCL/βTCP group, which contained the highest percentage of βTCP among the samples, demonstrated superior adhesion and increased cell viability compared to the other groups (10).

## *In vivo* assessments and Immunohistochemical characteristics of VEGF

In vivo vascularization was assessed by retrieving the subcutaneously implanted scaffolds after 2 and 4 weeks. Although the amount of vascularity observed around the scaffolds in both groups appeared to be similar in the second week, a greater number of vessels were detected macroscopically around the Gel-PCL/βTCP scaffolds after 4 weeks. The scaffolds lost their distinct morphology after 4 weeks, however, degradation was pronounced in the Gel-PCL/βTCP scaffolds, indicating the scaffolds' rate of degradation over time (Fig. 6).

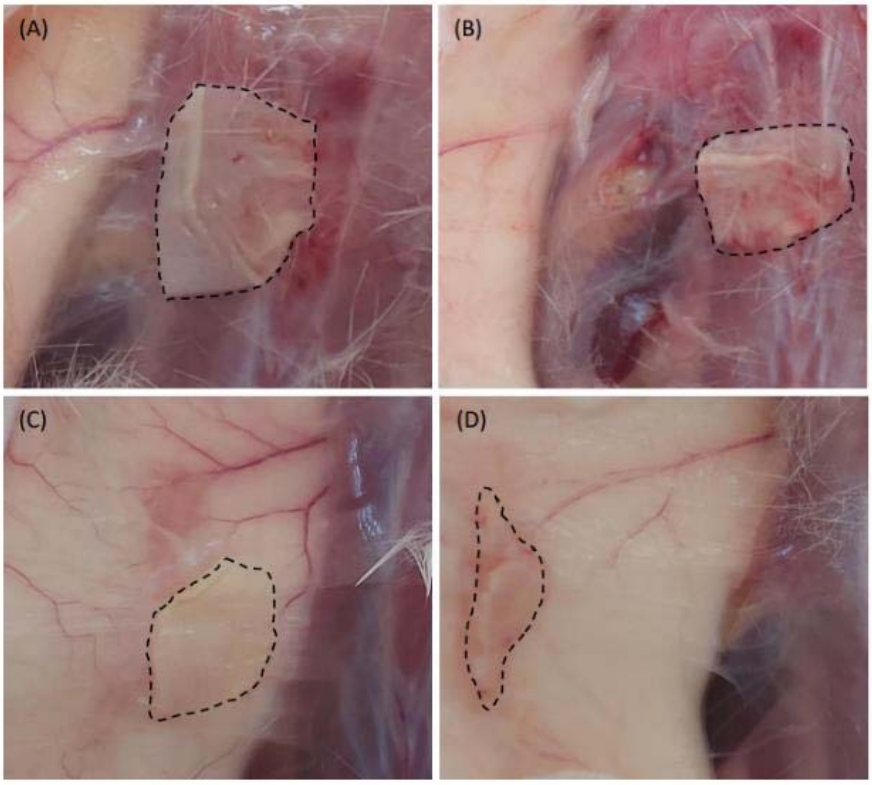



**Fig. 6** Gross morphology of the scaffolds after 2 (A, B) and 4 (C, D) weeks of subcutaneous implantation. Gel-PCL (A, C) Gel-PCL/βTCP (B, D). The dotted lines show the boundaries of the scaffolds

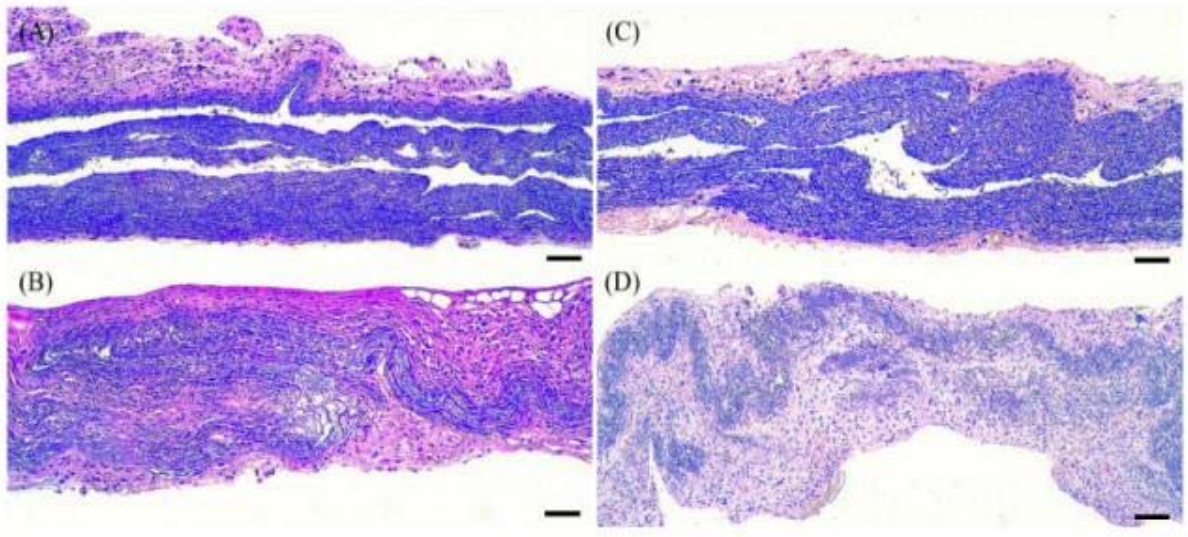



**Fig. 7** H&E staining in implanted scaffolds after 2 (A, B) and 4 (C, D) weeks; Gel-PCL (A, B), Gel-PCL/βTCP (C, D). (All panel 200 X)

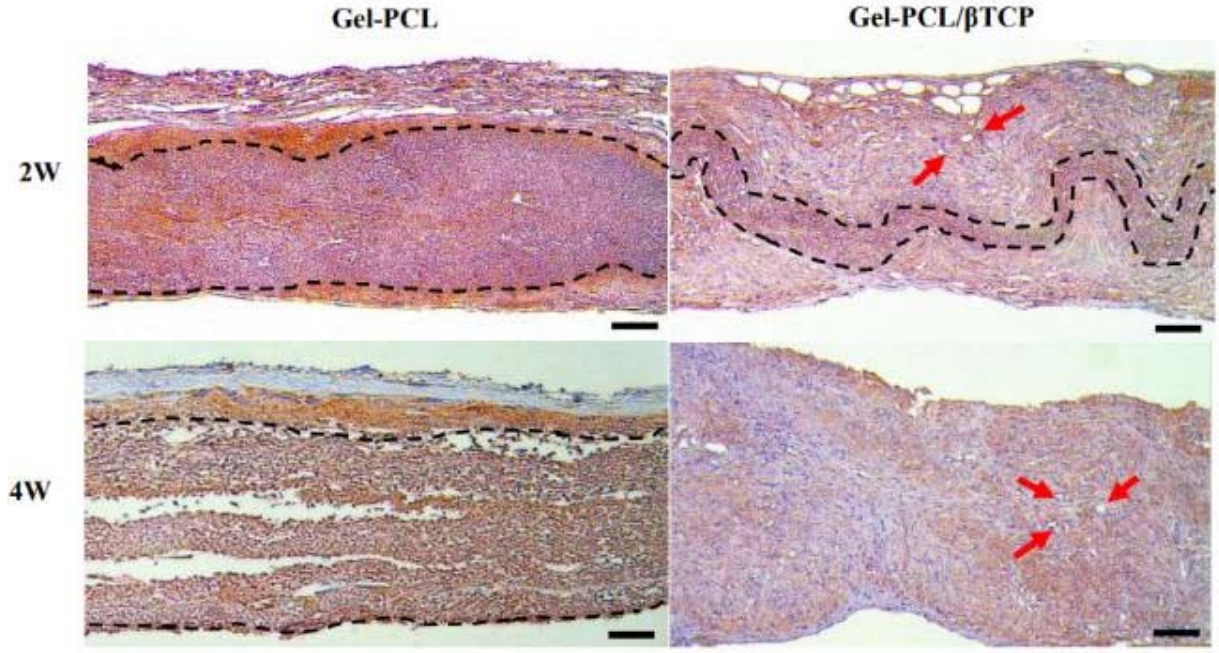



**Fig. 8** VEGF expression compared in subcutaneously implanted scaffolds after 2 and 4 weeks. The dotted line indicates the border between tissue and scaffold and arrows indicate newly forming lumens. In Gel-PCL/βTCP group, due to the cell's integration into the

The H&E images demonstrated improved perivascular localization in the βTCP-containing scaffolds compared to the Gel-PCL scaffolds. Additionally, both samples became cellularized after four weeks (see **Fig. 7**). Following the imaging of the samples, the results indicated the cells penetration into the both groups, However, the higher penetration of cells in the Gel-PCL/βTCP group after 4 weeks highlighting the potential of βTCP incorporation in promoting cell migration and tissue regeneration. These findings suggest that the addition of βTCP enhances the scaffolds' effectiveness in supporting cell proliferation over time (14).

IHC staining of tissue sections with VEGF was employed to investigate the formation of newly developed blood vessels. The results indicated that both forming and newly formed lumens were present in the cellularized areas of both groups. The Gel-PCL scaffolds exhibited lower VEGF expression compared to Gel-PCL/βTCP during both timepoints. The cell's integration into the scaffolds and the scaffolds degradation was promoted with the passage of time and subsequently the values for both groups increased after four weeks although the Gel-PCL/βTCP showing the highest expression, as confirmed in **Fig. 8**. and this finding aligns with cell proliferation and viability results observed *in vitro*. According to the results, adding βTCP into the scaffolds can promote the effective formation of functional blood vessels over time, which could be beneficial for tissue engineering.

## CONCLUSION

In this study, various concentrations of βTCP were incorporated into Gel-PCL scaffolds to develop a series of nanofiber scaffolds for BTE. The optimal concentration of βTCP for incorporation into Gel-PCL fibers and the preparation of suitable composite nanofibers was identified. *In vivo* evaluations further confirmed the enhanced vascularization properties of the samples following the subcutaneous implantation of Gel-PCL and Gel-PCL/βTCP scaffolds in mice. In conclusion, the findings suggest that βTCP has the potential to endow Gel-PCL scaffolds with the necessary angiogenic properties, which are crucial in the context of bone defects.

## ACKNOWLEDGEMENTS

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## DECLARATION

The authors have declared that there is no conflict of interest.

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