

Original Article



Polyethylene Glycol-Assisted Purmorphamine Delivery on β -Tricalcium Phosphate Granules for Bone Tissue Engineering: An in Vitro and in Vivo Study

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Abstract

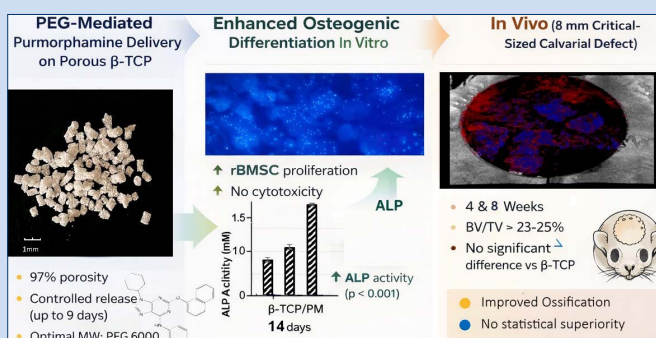
Introduction: We aimed to design and evaluate a polyethyleneglycol (PEG)-assisted purmorphamine (PM) delivery system in combination with β -tricalcium phosphate (β -TCP) granules.

Methods: Various PEG molecular weights in conjunction with β -TCP granules were examined to control PM delivery,

which showed that PEG with a molecular weight of 6000 g/mol was the most effective for PM release. Cell adhesion and morphology, cell proliferation, cytotoxicity, and differentiation were compared between PM-releasing β -TCP and control β -TCP. In vivo osteogenic capacity of PM- β -TCP granules in 8-mm critical-sized rat calvarial defects was evaluated after 4 and 8 weeks.

Results: The in vitro findings demonstrated that sustained PM release enhanced rBMS cell proliferation and significantly increased osteogenic markers, including ALP activity. However, in vivo evaluation showed no statistically significant difference in new bone formation or angiogenesis between PM-treated and control β -TCP groups at either time point, although bone formation was observed in all groups.

Conclusion: Overall, the PEG-mediated PM delivery system promoted osteogenic activity in vitro but did not result in a significant improvement in bone regeneration in vivo under the conditions tested. These findings suggest that further optimization of dosage, release kinetics, and experimental conditions is required before clinical translation.



Introduction

Molecules weighing < 900 Daltons, classified as small molecules, are an effective approach to control the behavior of stem cells, as their small size allows them to be easily endocytosed by cells.¹ In addition, other advantages of

small molecules compared to growth factors include their low cost of fabrication, ease of storage, stability during shipping, and ease of incorporation into delivery systems.^{1,2} Growth factors, on the other hand, are expensive, have high functional sensitivity, and may lose their activity during

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encapsulation. Furthermore, the high doses needed to induce differentiation raise concerns about toxicity and side effects.²

Purmorphamine (PM) is a chemically synthesized small molecule and a pharmacological activator of a transmembrane protein called smoothened (SMO), and it can activate hedgehog signaling in a variety of cells.³ Hedgehog signaling is recognized as an important positive regulator in osteoblast differentiation and bone formation. Therefore, bone repair can be enhanced by precisely adjusting the degree of hedgehog pathway activation.^{4,5} Various studies have examined the effect of different PM dosages ranging from 2 to 5 μM on proliferation and osteogenic differentiation.⁶ However, the best improvement in osteogenic induction was reported at concentrations ranging from 2 to 3 μM of PM.^{5,6}

Although PM has been used in conjunction with scaffolds to improve bone tissue engineering efficacy,^{2,7,8} designing a proper delivery system is an important tool for efficient transfer of molecules.⁹ Existing delivery systems have several limitations, including poor control over release kinetics and compromised mechanical properties. To the best of our knowledge, PM has not been used in animal studies. Therefore, the delivery system that uses PM combined with β -tricalcium phosphate (β -TCP) is a novel delivery system that can improve poor control over release kinetics and provide a safer and more efficient delivery pathway.

Polyethylene glycol (PEG) is widely used as a carrier due to its biocompatibility, inertness, hydrophilicity, and desirable physicochemical characteristics.¹⁰ These properties ensure minimal immune response and optimal solubility in biological fluids, facilitating better drug absorption and distribution. Additionally, PEG's physicochemical properties, such as the ability to form hydrogels, aid in controlled drug release. PEG also exhibits low toxicity, low immunogenicity, and excellent stability, making it a preferred choice in biomedical applications, including bone tissue engineering. It can also be easily functionalized by selecting different molecular weights, allowing the desired release rate and time to be achieved. An increase in release rate has been reported with increasing PEG molecular weight.^{11,12}

β -TCP is a synthetic ceramic biomaterial that shows promising applications as a bone graft substitute. Due to its biocompatibility and biodegradability, it is not only osteoconductive but also osteoinductive.^{13,14} β -TCP exhibits a high affinity to cells and can extend the active time of growth factors in vivo.¹⁵ These properties make it a suitable scaffold and carrier for use in bone tissue engineering.

This study aims to develop a PEG-based delivery system for the controlled release of PM and evaluate its efficiency when combined with β -TCP granules in vitro. Furthermore, it aims to assess its effect on bone formation in a rat critical-sized calvarial defect model. This system is designed to provide enhanced control over release rates and offer a potentially more effective and safer approach for PM delivery.

We hypothesized that PEG-mediated PM delivery on β -TCP granules would enhance osteogenic differentiation in vitro and promote bone regeneration in vivo compared to β -TCP controls.

Methods

Details about materials and methods are provided in Supplementary file 1.

Fabrication of Highly Porous β -TCP Granule

β -TCP granules were manufactured by the foam-casting method according to the protocol used by Bastami et al.¹⁶ In brief, PU was immersed in the TCP slurry, then slightly squeezed, and dried at room temperature. The granules were subsequently sintered in a furnace, and heat treatment was done to achieve desirable ceramic sintering.

Preparation of PM Delivery System and in Vitro PM Release Behavior

PM was reconstituted in DMSO to make a 1-mM stock solution according to the manufacturer's protocol, and aliquots were stored at -20°C . PEG of different molecular weights (600, 1500, 3000, and 6000 g/mol) was sterilized with ultraviolet light for three cycles of 20 minutes each, then dissolved in sterile distilled water at a concentration of 2 wt%, and the solution was stirred until a homogeneous solution was achieved. Next, 1-mM PM was added to the solution and placed on a shaker at 125 rpm for 2 hours. β -TCP granules at a concentration of 0.125 g/mL were immersed in PM-PEG solution and incubated at 37°C under 5% CO_2 pressure for 24 hours. For the control group during in vitro and in vivo studies, all the above steps were performed except the addition of PM; that is, control β -TCP granules were loaded only with PEG.

β -TCP granules were loaded with PEG-PM and PEG, placed in sterile distilled water, and incubated under cell culture conditions. At specific time points, supernatants were taken out, and the optical density of the samples was measured at 221 nm using a BioPhotometer (Eppendorf, Hamburg, Germany). The obtained values were converted to μM using a constructed PM standard curve.

In Vitro Cell Behavior Analyses

Isolation of rat bone marrow-derived mesenchymal stem cells (rBMSCs) was according to the Lennon et al.¹⁷ protocol. Cells in passages 3 and 4 were used for this study. Expression of mesenchymal stem cell surface markers (CD34, CD31, CD105, CD45, and CD44) was assessed by flow cytometry. The multilineage differentiation potential of harvested rBMSCs was also evaluated. For this purpose, rBMSCs were cultured in StemPro osteogenic medium, adipogenic medium, and standard medium for 20 days. Alizarin Red S (Bio-Idea, Iran) or Oil Red O (Bio-Idea, Iran) staining was used to detect calcified tissue and adipogenesis in cultures.

In vitro PM Delivery Assessment Cell Proliferation and Cytotoxicity

Sterilized β -TCP with and without PM were soaked in a growth medium, and rBMSCs were seeded at a concentration of 2×10^4 cells per scaffold. DAPI (4',6-diamidino-2-phenylindole) staining was performed on days 1, 3, 5, and 7 to visualize live cells. Cell proliferation and viability of rBMSCs on granules were assessed using the MTT(3-(4,5-Dimethylthiazol-2-yl)-2 and 5-diphenyltetrazolium bromide) assay after 1, 3, and 5 days. Three samples per group were included.

Cell Adhesion and Morphology

Surface electron microscopy (SEM) was used to observe cell attachment and morphology on scaffolds at intervals of 1, 3, and 5 days. Subsequently, the specimens underwent gold sputter-coating before being subjected to scanning electron microscopy for analysis.

Alkaline Phosphatase (ALP) Activity Assay

The osteogenic capacity of the scaffolds was evaluated with an ALP kit (Abcam, USA). ALP activity was quantified as μM of p-nitrophenol produced per hour per milligram of cellular protein at three specific time intervals: 1, 7, and 14 days.

In Vivo Osteogenesis

Implementation of β -TCP Granules with PM Release

The use of animals in experiments complied with the ARRIVE guidelines and the Iran National Committee for Ethics in Biomedical Research guidelines. The experiments were also approved by the Ethics Committee of the School of Dentistry, Shahid Beheshti University of Medical Sciences (approval No: IR.SBMU.DRC.REC.1398.028). Thirty-two healthy mature rats were used as models for calvarial bone defects. The sample size was determined based on previous similar studies using rat critical-sized calvarial defect models, as well as practical and ethical considerations to minimize animal use while allowing for group comparisons.

The rats were randomly assigned to four study groups as outlined below: defects were filled with β -TCP granules with sustained release of PM (β -TCP + PEG/PM), defects were filled with β -TCP granules loaded with rBMSCs with sustained release of PM (β -TCP + PEG/PM + rBMSCs), defects were filled with β -TCP granules loaded only with PEG (β -TCP + PEG), and defects were filled with β -TCP granules loaded with rBMSCs and PEG (β -TCP + PEG + rBMSCs).

To assess in vivo bone regeneration, critical-sized bone defects (8 mm in diameter) were created in each rat's calvarium. The cut bone was removed, and the β -TCP granules were gently crushed to fit the defect before being inserted into the rat's defects. Throughout the healing process, the rats were subjected to clinical examination to detect any signs of tissue swelling, dehiscence, or infections around the surgical sites.

They were sacrificed with carbon dioxide asphyxiation 4 and 8 weeks after surgery.

Micro-CT Evaluation

Harvested calvaria were gently washed with tap water and immersed in 10% formalin solution in labeled containers. To ensure blinding, all the samples were coded by an independent researcher, and subsequent analyses were performed without knowledge of group allocation. The calvaria bone samples underwent micro-computed tomography (μ -CT) scanning using a desktop μ -CT scanner (LOTUS-NDT, Behin Negareh Co., Iran) for this study. The 3D data acquired were reconstructed using the LOTUS NDT-REC software with the standard Feldkamp, Davis, Kress (FDK) algorithm.¹⁸ Parameters such as bone volume (BV), total volume (TV), and scaffold volume (SV) were included in our report.

Histomorphometry

Tissue sections (5 μm in thickness) were stained with hematoxylin and eosin (H&E). The stained sections were digitally scanned to measure the percentage of newly formed bone and remaining scaffold within the defect. A pathologist, unaware of the study, conducted a semi-quantitative analysis by scoring sections for connective tissue organization, distribution of bone trabeculae, and angiogenesis.

Statistical Analysis

All in vitro experiments involving MTT and ALP assays were conducted in triplicate (technical replicates) for each group. All data were expressed as mean $\pm$ standard deviation (SD). Two-way analysis of variance (ANOVA) and post hoc Sidak tests were used for inter- and intra-group comparison. Statistical significance was set at $P < 0.05$. Statistical analyses were performed using Prism 8.3.0 (GraphPad Software Inc., San Diego, CA, USA).

Results

In vitro PM Release Behavior

Figure 1 shows the standard curve of PM, prepared at concentrations from 0.1 to 5 $\mu\text{M}/\text{mL}$ and measured at 221 nm, where PM exhibits maximum absorbance. Using the line equation in Figure 1 ($R^2 = 0.9984$), the absorption values were converted to the concentration of PM. It also shows the release profile of PM with different molecular weights of PEG in the first 12 days. Released values of scaffold loaded with PM in combination with PEG 6000, which were $> 2 \mu\text{M}/\text{mL}$ in the first 5 days, were measured in a phosphate-buffered saline solution. Due to the enhanced release profile of PM with PEG 6000, characterized by more consistent and prolonged release kinetics, higher release efficiency, and improved in vitro and in vivo efficacy, all further tests were conducted using PEG 6000. β -TCP granules were fabricated by the foam-casting method and are shown in Figure 1. β -TCP granules produced with this method had $97.7 \pm 0.4\%$ porosity with a mean pore size of $191.9 \pm 74.6 \mu\text{m}$ (minimum: 72.8 μm , maximum: 334.8 μm).

In Vitro Cell Behavior Analyses

Characterization of Isolated rBMSCs

The osteogenic capability of rBMSCs was assessed by Alizarin Red S staining, and calcification was detected.

Adipogenic induction was also confirmed by Oil Red O staining. The samples were imaged by inverted light microscopy (Figure 2). Flow cytometry analysis showed that the majority of the isolated rBMSCs significantly expressed CD44 and CD105, but they were constantly negative for CD31, CD34, and CD45 (Figure 3).

Cell Proliferation and Cytotoxicity

DAPI staining was performed on days 1, 3, 5, and 7 to visualize cell attachment and proliferation. As shown in Figure 3, rBMSC proliferation was higher on days 1, 3, and 5 on PM-loaded β -TCP granules (test) compared to non-PM-loaded β -TCP granules. Although no obvious difference was observed between the two groups on day 7, no cytotoxicity was observed in the test group. MTT assay performance after rBMSCs cultured for 1, 3, and 5 days also confirmed the DAPI staining results. Figure 3 shows that PM sustain release improved rBMSCs proliferation at all time points.

Cell Adhesion and Morphology

Scanning electron microscopy (SEM) imaging of granules was performed after 1, 3, and 5 days to assess cell adhesion and morphology (Figure 4). After 24 h, cell attachment was favorable on both PM-loaded and non-PM-loaded β -TCP granules. rBMSCs attached significantly to the granules, indicating that both PM and PEG 6000 support cell adhesion.

ALP Assay

The effect of PM sustain release on differentiation capability

of rBMSCs cultured on β -TCP granules into the osteoblast lineage was assessed using an ALP activity assay (Figure 5). The results showed that ALP expression was significantly higher in the PM-releasing group on days 1 ($P=0.02$), 7 ($P=0.0003$), and 14 ($P<0.000001$) after induction.

In Vivo Osteogenesis

Micro-CT Evaluation of Bone Regeneration in Vivo

All the animals survived during the experimental period, indicating good surgical tolerance. None of them showed signs of infection. Half of the rat calvarias were harvested 4 weeks after surgery, and the rest were removed 8 weeks after surgery.

Specimens were analyzed with micro-CT to evaluate the volume of newly formed bone (Figures 6 and 7). Bone volume fraction was used for comparisons, calculated as bone volume (BV) divided by total volume (TV). TV consisted of the scaffold, regenerated bone, pores, and soft tissue trapped in the analyzed 3D volume of interest selected from the structures. The values obtained for BV/TV mean $\pm$ SD are as follows. Four weeks after surgery (test group: 23.91 ± 0.37 , control group: 23.72 ± 0.89 , test + cell group: 23.25 ± 1.25 , control + cell group: 22.93 ± 1.82), 8 weeks after surgery (test group: 24.92 ± 1.07 , control group: 22.47 ± 1.96 , test + cell group: 23.68 ± 1.62 , control + cell group: 24.31 ± 1.17) (Figures 6 and 7). There were no significant differences between the groups in both time points in terms of BV/TV.

Histological Analysis of Bone Regeneration in Vivo

As indicated in Figures 6 and 7, there was no complete

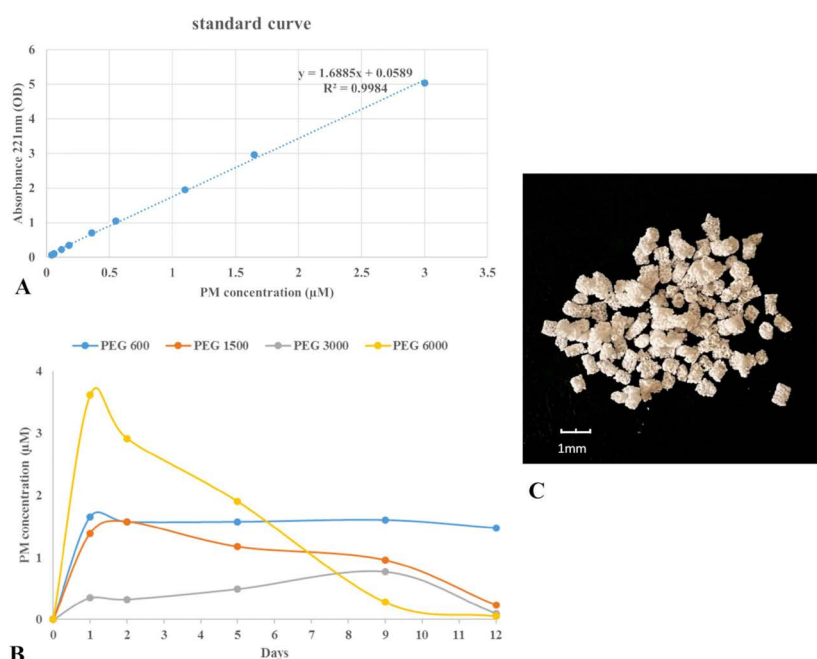


Figure 1. A. PM linear standard curve. Note the line equation at the top of the graph. The line equation and high correlation coefficient ($R^2=0.9984$) indicate a strong linear relationship, allowing for accurate conversion of absorption values to PM concentrations. B. PM release profile in the first 12 days. The groups are defined based on the molecular weight of the PEG used. PEG 6000 demonstrated superior performance, with released PM values exceeding 2 μ M/mL within the first 5 days. C. Highly porous β -TCP granule. The β -TCP granules, fabricated using the foam-casting method, are shown, with lengths between 1 and 3 mm, illustrating their highly porous structure ideal for tissue engineering applications

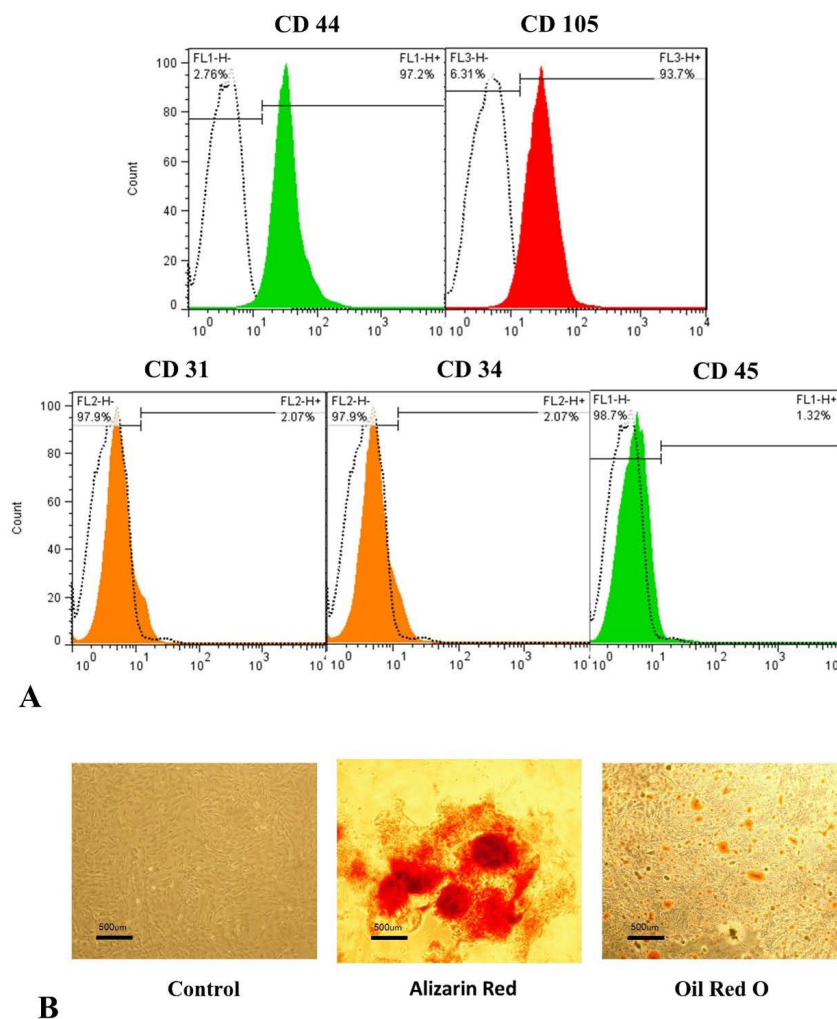


Figure 2. A. Flow cytometry histograms show significant expression of CD44 and CD105, and negligible expression of CD31, CD34, and CD45. This confirms the mesenchymal stem cell phenotype of the rat bone marrow-derived mesenchymal stem cells (rBMSCs). B. Alizarin Red S and Oil Red O staining of rBMSCs. The Alizarin Red S staining demonstrates mineralization, indicative of osteogenic differentiation, while the Oil Red O staining shows lipid accumulation, confirming adipogenic differentiation. The control represents the standard medium and shows the morphology of rBMSC

bone bridge formation within the defects in any specimen. However, a mixture of scaffold residues, fibrous connective tissue, and occasionally newly formed bone islands connected the edges of defects in all the treated groups. New bone formation area at 4 weeks as a percentage of total defect area was 7.79 ± 0.49 in defects treated with β -TCP/PM + rBMSC, 7.51 ± 1.086 in defects treated with β -TCP + PM, 7.92 ± 0.622 in β -TCP + rBMSC-treated defects, and 8.53 ± 1.149 in β -TCP-treated groups. New bone formation area at 8 weeks as a percentage of total defect area was 6.907 ± 0.640 in defects treated with β -TCP/PM + rBMSC, 9.64 ± 1.23 in defects treated with β -TCP + PM, 7.63 ± 1.85 in β -TCP + rBMSC-treated defects, and 10.90 ± 3.07 in β -TCP-treated groups.

Distribution of immature osseous foci of new bone trabeculae was distinctly seen in the center of all groups at both time points without any connection to the defect borders, except in β -TCP/PM 4 weeks after implantation, in which only new bone trabeculae were in contact with old bone of the defect edge. Fibrous connective tissue in all implanted defects represented well-organized collagen fibers and cellularity with generation of intramembranous bone foci. In β -TCP/PM and β -TCP/PM + BMSCs, the

dominance of the inflammatory response was more evident. New bone formation area as a percentage of total defect area in all groups did not show any significant difference between the groups. A summary of the results can be found in Figures 6 and 7.

Discussion

This study was designed to evaluate the effect of PM, a small bioactive molecule that activates the hedgehog pathway, delivered via a PEG-mediated system onto β -TCP granules to enhance osteogenesis in vitro and in vivo.

The designed delivery system successfully released PM from β -TCP granules. The results indicated that PM has no adverse effects on rBMSC proliferation, adhesion, and cell viability. In fact, it even led to a slight improvement in proliferation, although this was not statistically significant. Moreover, PM increased the differentiation of these cells, which can accelerate the healing process.

In the study by Woltje et al,¹⁹ PM suppressed rBMSC cell proliferation compared with rBMSC proliferation in the osteogenic medium with 2- μ M PM and rBMSC proliferation in the standard culture medium. However, in studies by Beloti et al,^{5,20} a 3- μ M concentration of PM had

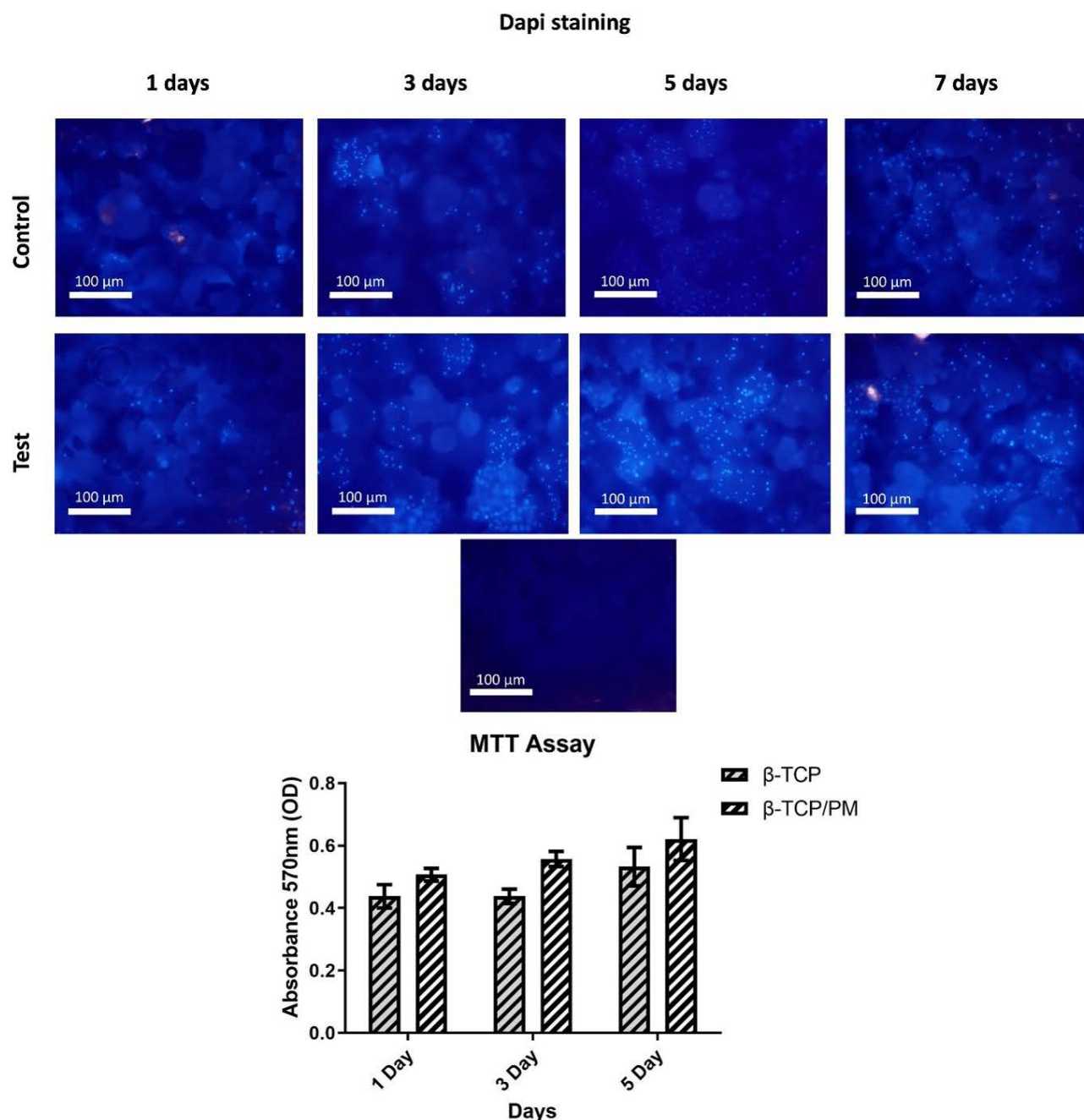


Figure 3. DAPI staining for test (PM-loaded) and control (non-PM-loaded) β -TCP scaffolds. The images show higher rBMSC proliferation on PM-loaded β -TCP granules compared to the control on days 1, 3, and 5. By day 7, no significant difference in proliferation was observed between the two groups, and no cytotoxicity was noted in the test group. MTT assay for evaluation of rBMSCs viability on β -TCP granules with/without sustain releasing of PM in stem cell growth medium (mean $\pm$ SD, $n=3$, analyzed using two-way ANOVA with multiple comparisons). The assay results confirm the findings from DAPI staining, indicating that PM sustained release improves rBMSCs proliferation at all evaluated time points

no effect on cell proliferation. In this study, an osteogenic medium was used, which favors cell differentiation more than cell proliferation. Rezai Rad et al.² showed that a 2- μ M concentration of PM increased the proliferation of human dental pulp stem cells (hDPSCs) in a standard medium. Similarly, in our study, PM supported the proliferation of rBMSCs in a standard medium. However, further investigations of different doses, especially lower doses, are necessary to improve cell proliferation efficiency.

The osteoinductive effects of PM were first discovered by Wu et al.²¹ They demonstrated PM's ability to induce osteogenesis in mouse progenitor cells and reported similar activity between PM and bone morphogenetic protein 4

(BMP-4).^{21,22} However, only one study, conducted by Plaisant et al.,²³ showed that PM reduced osteogenic markers in osteoblasts. This reduction may be attributed to the use of epidermal growth factor in the culture medium, which is known to inhibit bone differentiation.²⁴ Similarly, our study also demonstrated the significant effect of PM on the osteogenic capability of rBMSCs in vitro.

Most studies have used PM at concentrations ranging from 2 to 3 μ M.⁶ For instance, Wu et al.²¹ used a 2- μ M concentration of PM for 4 days, resulting in more than 80% of the cells expressing ALP, indicating the transformation of a large number of cells into the osteoblast lineage. Subsequent studies have demonstrated increased ALP,

Scanning Electron Microscope

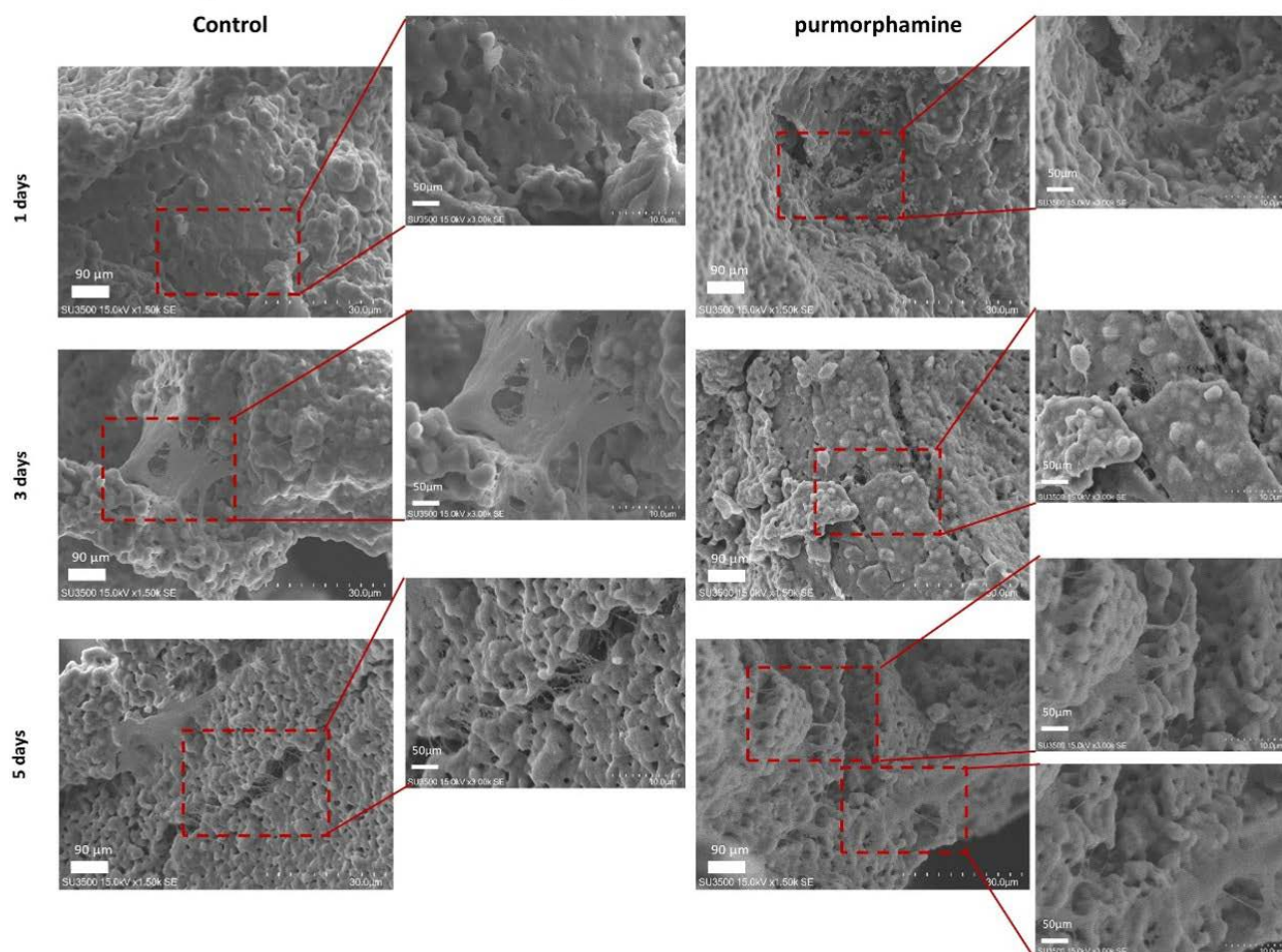


Figure 4. Scanning electron microscopy (SEM) imaging for cell adhesion and morphology assessment of β -TCP granules with/without sustained release of PM after 1, 3, and 5 days ($n=3$)

Cells exhibited favorable attachment and spreading in both groups, indicating that PEG 6000 and PM incorporation support cell adhesion and morphology

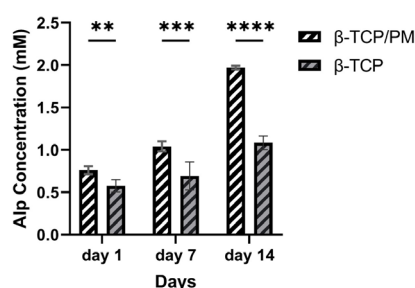


Figure 5. ALP assay for osteogenesis evaluation of β -TCP granules with/without sustained release of PM after 7 and 14 days (mean $\pm$ SD, $n=3$, analyzed using two-way ANOVA with multiple comparisons). The results demonstrated that alkaline phosphatase (ALP) expression was significantly higher in the PM-releasing group than in the control group at both 7 and 14 days of induction

runx-related transcription factor 2 (Runx2), osteocalcin (OCN), and osteopontin (OPN) proteins and genes when 2- μ M PM was added to the culture medium.^{5,20,22–24} Additionally, some studies have indicated that a 3- μ M concentration of PM is more effective in promoting osteogenesis compared to lower concentrations.^{7,8,25} PM exhibits dose-dependent osteoblastic differentiation, with the greatest effect observed between concentrations of 2 μ M and 3 μ M;²⁰ hence, a concentration range of 2–3 μ M

was used for this study.

The present study investigated PM release through a PEG-mediated system. PEG 6000 released 3- μ M PM for the first two days and 2- μ M PM for the following three days, with release at lower doses continuing until day 9. The results of this study showed that the higher the molecular weight of the PEG, the faster the release. This is in line with previous studies.^{11,12} Given that the most effective PM concentration reported in studies was 2–3 μ M, PEG 6000 was selected for this study. However, PEG 600 and PEG 1500 provided continuous release at lower doses, and their efficacy on proliferation and differentiation needs further investigation. According to a study by Beloti et al.,⁵ 1- μ M and 3- μ M doses of PM showed similar efficacy in the number and area of bone-like nodules. However, the 1- μ M dose induced less ALP activity.

To the best of our knowledge, only two studies have examined the effect of PM on in vivo osteogenesis. Gellynck et al.²⁶ placed the calcium phosphate beads in 200- μ M PM for 24 hours and subsequently placed them in a needle-sized lesion on the femur of a 14-day-old chick embryo. The femur was then inserted into the chicken chorioallantoic membrane, which remained vital for the next 7 days. The

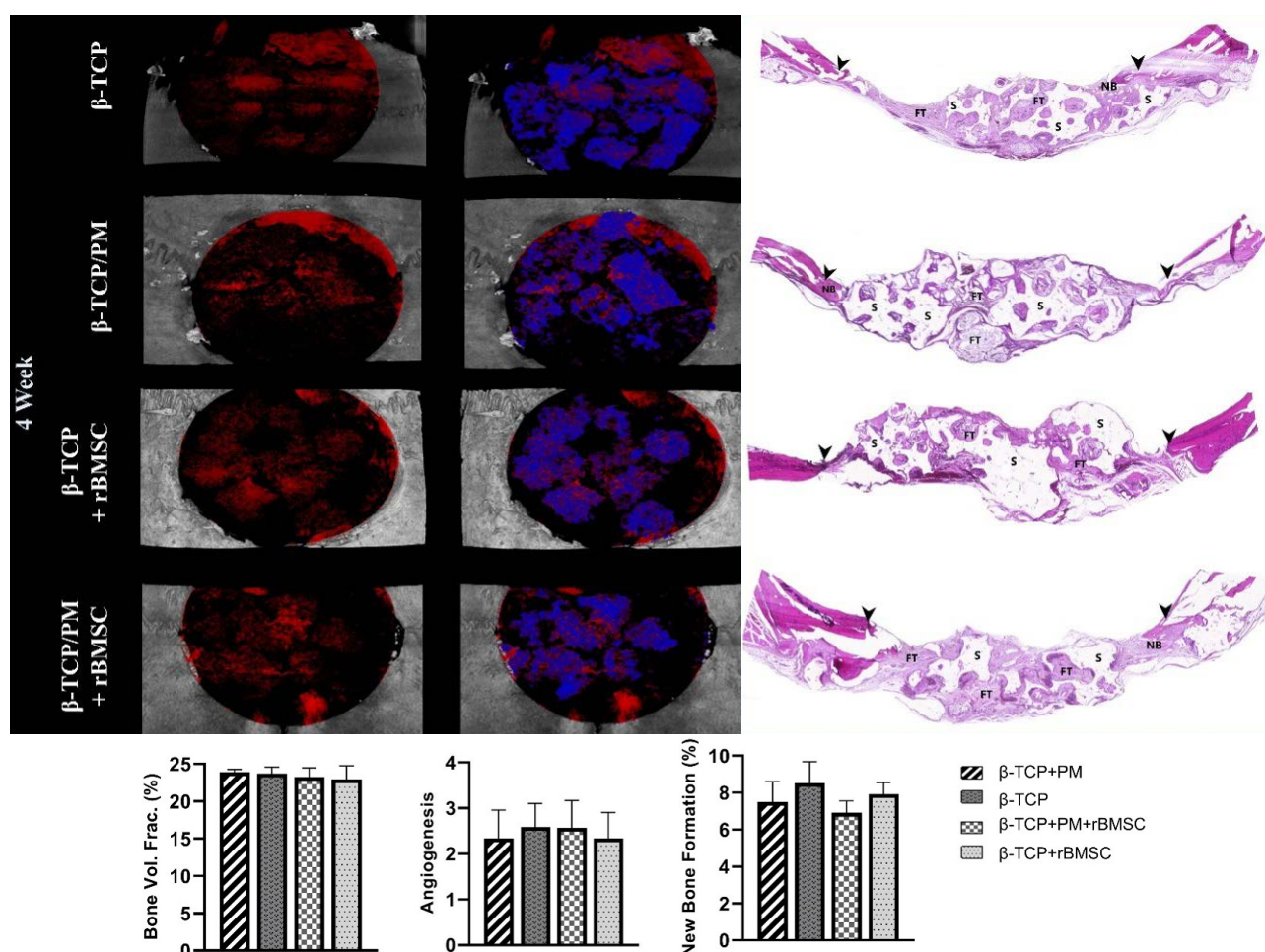


Figure 6. Reconstruction of calvarial defects 4 weeks after scaffold implantation. Micro-CT 3D images show newly formed bone (left), scaffold with new bone (middle), and corresponding histological sections stained with H&E (right). Bone volume fraction (BV/TV%) and histomorphometric analyses are presented (mean ± SD, n=4, analyzed using two-way ANOVA with multiple comparisons). New bone formation was observed in all groups, with comparable BV/TV values across groups

ratio of trabecular bone volume to bone marrow was higher in the PM group compared to controls. In another study, Faghihi et al.²⁵ investigated the effect of daily subcutaneous injection of 3- μ M PM into human mesenchymal stem cells (hMSCs) loaded on a β -TCP scaffold implanted in the rat neck. Real-time PCR results showed an increase in human osteoblast genes, including ALP, OCN, Runx-2, and collagen I, in the PM-injected group. However, this study was conducted with a small sample size of only 9 rats, which may not be sufficient to draw definitive conclusions.

The foam-casted β -TCP scaffold modulated the concentration of phosphate within a range suitable for cell proliferation by releasing calcium. Additionally, it exhibited favorable biocompatibility and porosity, facilitating cell adhesion and angiogenesis.¹⁶ Our study represents the first investigation into the effects of PM with β -TCP granules and rBMSCs in vitro and in a calvarial rat defect. Unlike other animal studies involving PM immersion or daily PM injection, our study uniquely considers parameters such as inflammation, fibrous tissue formation, and bone formation percentage.

Despite promising in vitro results, the PM-PEG delivery system developed, when evaluated alongside β -TCP granules and rBMSCs in a critical-sized rat calvarial defect,

did not significantly enhance osteogenesis in vivo compared to β -TCP granules alone. Although complete bone bridging was not observed in the specimens, new bone formation at the center of the defect in both groups suggested the osteoinductive properties of the studied scaffolds.

Some bioactive molecules, such as BMP-2, demonstrated their effect on bone formation in vivo in both short-term (4 and 8 weeks) and long-term (12–24 weeks) follow-ups, while others may have exhibited variable results in short-term assessments but showed good results in longer follow-up periods.²⁷

Kim et al.²⁸ investigated the effects of the KR-34893 bioactive molecule associated with an MgP ceramic scaffold in a 5-mm rat calvaria defect. This molecule significantly increased BMP-7 levels, with BMP-7 being identified as the primary molecular target for KR-34893. Results at week 4 did not exhibit a significant improvement compared to the control group, but at the 8-week follow-up, there was a dose-dependent increase in bone formation.

Heo et al.²⁹ examined the effect of alginate scaffolding and bone-forming peptide-1 (derived from BMP-7) on an 8-mm rabbit calvaria defect at 6 weeks. The percentage of newly formed bone in the hybrid scaffold (scaffold with bioactive molecule) was lower than that in the simple

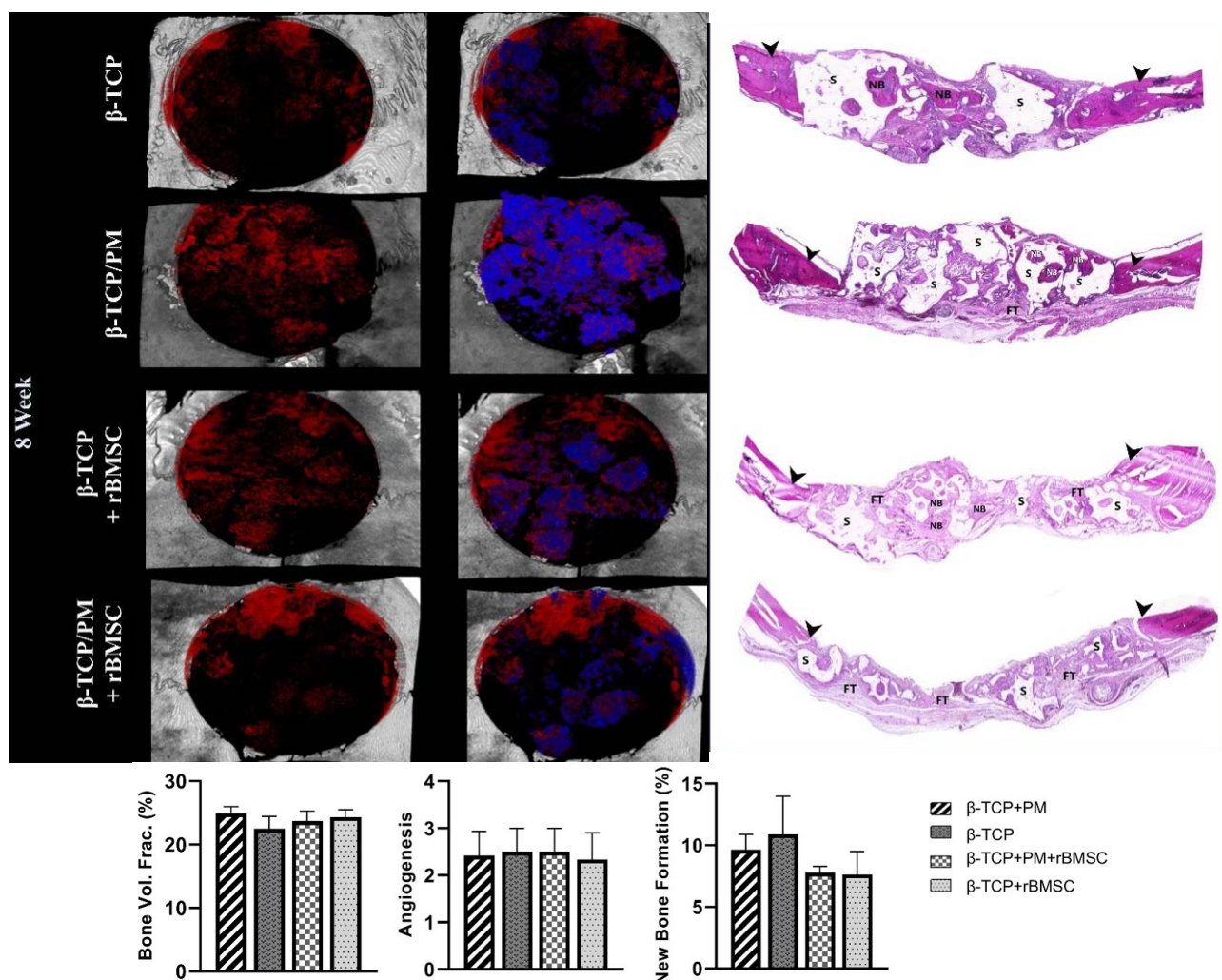


Figure 7. Reconstruction of calvarial defects 8 weeks after scaffold implantation. Micro-CT 3D images show newly formed bone (left), scaffold with new bone (middle), and corresponding histological sections stained with H&E (right). Bone volume fraction (BV/TV%) and histomorphometric analyses are presented (mean $\pm$ SD, n=4, analyzed using two-way ANOVA with multiple comparisons). Increased bone formation was observed over time, with no statistically significant differences between groups

scaffold. However, hybrid scaffold results were significantly better than pure scaffolds at a longer follow-up period (12 weeks).

Defect size was another influential factor in a systematic review and meta-analysis of critical-sized defects of rat calvaria. In a 5-mm central empty defect after 1 and 3 months, new bone formation was 18.29 ($\pm$ 2.85) and 21.44 ($\pm$ 8.27) percent, respectively. In an 8-mm empty defect, new bone formation was 9.81 ($\pm$ 9.4), 12.56 ($\pm$ 24.1), and 7.96 ($\pm$ 3.18) percent at 1, 2, and 3 months, respectively. A 5-mm calvarial defect can be considered a critical-sized defect. However, standardization is necessary for accurate comparison between studies because defect size, even in empty defects, affects the percentage of new bone formation.³⁰

Oxysterol, similar to PM, activates the hedgehog signaling pathway.³¹ Aghaloo et al.³² investigated the effects of poly(lactic-co-glycolic acid) alone or coated with low or high doses of oxysterol on 5-mm bilateral calvaria rat defects. After 6 weeks, the micro-CT evaluation showed new bone formation of 21% in the low-dose group and 22% in the high-dose group, similar to our findings.

The size of the defect, the animal model used, and

the biomolecule employed all influence the appropriate follow-up time to evaluate the maximum osteoinductive capacity of the bioactive molecule and scaffold complex. For PM, additional animal studies are needed to determine the most effective follow-up period. Furthermore, the use of an 8-mm critical-sized calvarial defect represents a highly challenging model with limited intrinsic healing capacity, which may have masked the potential effects of PM treatment.

One possible explanation for the lack of significant in vivo osteogenesis is that the effective local concentration of PM may not have been maintained within the defect site. Although the in vitro release profile indicated concentrations within the osteoinductive range (2–3 μ M), in vivo conditions such as fluid exchange and vascularization may have reduced the local PM concentration below the therapeutic threshold. The relatively short release duration of PM (up to 9 days) may also be insufficient to support the prolonged process of bone regeneration, which typically requires sustained signaling over several weeks.

One limitation of the study is that we did not use a sham, such as an autograft. Future studies can consider using a sham group to provide a more comprehensive evaluation

of the scaffold's efficacy compared to natural bone healing processes. Moreover, we did not use tests for demonstrating bone nodule formation (e.g., biochemical composition analysis: Raman spectrometry). They can be considered in further studies. Future studies can evaluate expression levels of key osteogenic genes and proteins such as Runx2, OCN, and OPN. Examining the release amount of the PM delivery system in a dynamic environment can provide conditions more similar to the clinical setting, allowing for the prediction of potential burst release. Furthermore, studying the effects of different concentrations of PM in vivo is necessary.

Potential inflammatory responses to PM should also be considered. If inflammation occurs, strategies such as controlled delivery systems or bioreactor-based preconditioning may help mitigate these effects. Additionally, lower concentrations of PM may exhibit fewer side effects while maintaining clinical efficacy. Furthermore, we acknowledge that a more accurate assessment of proliferation could have been achieved by quantifying DNA at different time points using a DNA quantification assay. While this specific test was not conducted in our study, it is recognized as a valuable approach to enhance the precision of proliferation measurements. It is ideal to normalize the MTT results to the amount of DNA. This normalization would provide a more accurate reflection of whether the overall increase in the fluorescence signal is due to more cells being present in the PM group or whether individual cells have increased metabolic activity as a result of the PM treatment. This point can be considered in future studies.

Another important consideration is the sample size and statistical power of the in vivo study. Although 32 animals were included, the study may still have been underpowered to detect subtle differences between groups, particularly given the biological variability inherent in animal models.

Collectively, these factors suggest that while PM demonstrates promising osteoinductive effects in vitro, optimization of dosage, release kinetics, and in vivo delivery strategies is essential before its full therapeutic potential can be reliably translated into in vivo applications.

Conclusions

The PEG-mediated PM delivery system developed in this study demonstrated controlled short-term release and supported osteogenic differentiation of rBMSCs in vitro, without adverse effects on cell viability. However, under the conditions tested, this system did not result in a statistically significant enhancement of bone formation or angiogenesis in a critical-sized rat calvarial defect model compared to β -TCP controls.

These findings suggest that while PM shows promising osteoinductive potential in vitro, its in vivo efficacy may be limited by factors such as release kinetics, local concentration, and the complexity of the biological environment. Therefore, studies should also include longer follow-up periods and evaluate PEGs with higher molecular weights to better assess translational potential.

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Competing Interests

The authors declare no conflicts of interest. The funders had no role in the design of the study, the collection, analysis, or interpretation of data, the writing of the manuscript, or the decision to publish the results.

Ethical Approval

It was approved by the Ethics Committee of the School of Dentistry, Shahid Beheshti University of Medical Sciences (approval No: IR.SBMU.DRC.REC.1398.028). Patient permission: N/A.

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Supplementary Files

Supplementary Files. Details of Materials and Methods

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