

Supplementary File 1. Materials and Methods

2.1 Materials

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and trypsin-ethylenediaminetetraacetic acid were purchased from ThermoFisher Scientific, USA. Dulbecco's phosphate buffered saline (PBS), dimethyl sulfoxide (DMSO), tricalcium phosphate (TCP), colloidal silica, carboxy methyl cellulose (CMC), sodium tripolyphosphate pentabasic (TPP), PM, and PEG were purchased from Sigma-Aldrich, USA. IgG₁-FITC-Isotype control, IgG₁-PE-Isotype control, IgG₁-PerCP.Cy5.5-Isotype control, CD105-PE, CD31-PE were purchased from eBioscience, USA. CD45-FITC, and CD34-PE were purchased from BioLegend, USA. Sterile distilled water were purchased from Caspian Tamin Pharma, Iran. Polyurethane foam (PU) was sourced from Safaom, Iran.

The osteogenic medium used in this study consisted of low glucose DMEM 10%, FBS 50 µg/ml, ascorbate-2 phosphate (Sigma-Aldrich, USA), 10⁻⁸ M dexamethasone, 10 mM β-glycerophosphate and 1% penicillin/streptomycin (ThermoFisher Scientific, USA).

2.2 Fabrication of highly porous β-TCP granule

β-TCP granules were manufactured by foam-casting method according to the protocol used by Bastami et al. (1). Further details about the methodology of manufacturing the scaffold and distribution of the pores are provided in the mentioned study by Bastami et al. In brief, PU was cut into granular shape in 2 mm diameter. Then, immersed in the TCP slurry consisting of dissolved TCP powder (1 g/ml), colloidal silica (30 wt. %), CMC (0.2 wt. %), and TPP (0.5 wt. %) in distilled water. The PU pieces were slightly squeezed and dried at room temperature for 24 hours. Granules were placed in furnace and heated to 600 °C with a rate of 0.5 °C/min, held

at 600 °C for an hour; then, the temperature was raised to 1200 °C with a rate of 5 °C/min and kept at 1200 °C for 3 hours to achieve desirable ceramic sintering. All granules were sterilized by immersion in 70% ethanol (diluted from absolute ethanol (Merck, Germany) with sterile distilled water) for 2 hours, then washed five times with sterile distilled water.

2.3 Preparation of PM delivery system

PM was re-constituted in DMSO to make 1 mM stock solution according to the manufacturer's protocol and aliquots were stored at -20 °C. PEG in different molecular weights of 600, 1500, 3000, 6000 g/mol was sterilized with ultraviolet light for three cycles of 20 minutes each, then dissolved in sterile distilled water at the concentration of 2 wt. %, and the solution was stirred until reaching a homogenous solution. Next, 1mM PM was added to the solution and placed on shaker with 125 rpm for 2 hours. β -TCP granules at a concentration of 0.125 gr/ml were immersed in PM-PEG solution and were incubated at 37 °C, 5% CO₂ 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.

2.4 In vitro PM release behavior

The release profile of PM from β -TCP granule was studied as follow. β -TCP granules were loaded with PEG-PM and PEG and were placed sterile distilled water, and incubated under cell culturing conditions. At specific time points, supernatants were taken out and stored at -20°C. The PM quantification was done for the samples at 221 nm using a BioPhotometer (Eppendorf, Hamburg, Germany). The absorbance values of scaffold with PM was calibrated with scaffold containing only PEG. A PM gradient from 0.1 up to 5 μ L/cc was prepared and read at 221 nm to create PM standard curve. Then, obtained values were converted to μ M using constructed PM standard curve.

2.5 In-vitro Cell behaviors analyses

2.5.1 Isolation and culture of rat bone marrow-derived mesenchymal stem cell (rBMSCs)

Two male 2-week-old Sprague Dawley rats were anesthetized with subcutaneous injection of 80 mg/kg Ketamine (Bremer, Germany) and 8 mg/kg Xylazine (KELA, Belgium). Then, rat femora and tibia were aseptically removed and kept in DMEM at a low temperature according to the Lennon et al. protocol (2). Then, the bones were washed with solution of PBS and 2.5 cc Pen-Strep three times and the bone marrows were flushed out by injecting DMEM into the cut head of bone several times. The substrate was collected and centrifuged and cultured in 75 cm² flasks. The specimen was then incubated at 37 °C, 5% CO₂. Cells at passage 3 and 4 were used for this study.

2.5.2 Characterization of isolated rBMSCs

Expression of mesenchymal stem cell (MSC) surface markers was assessed by flow cytometry. To do this, rBMSCs were treated with 0.05% trypsin-EDTA and re-suspended at concentration of 10⁵ per sample in PBS incubated for 30 min at 4 °C in the dark room with the following antibodies: IgG₁-FITC-Isotype control, IgG₁-PE-Isotype control, IgG₁-PerCP.Cy5.5-Isotype control, CD105-PE, CD45-FITC, CD34-PE, and CD31-PE. After incubation, cells were washed with PBS and flow cytometry was conducted with FACSCalibur (BD Biosciences, USA). Data were analyzed with FlowJo 7.6.1 software (BD, USA).

2.5.3 Multilineage differentiation potentials

rBMSCs were cultured in either StemPro osteogenic medium or adipogenic medium in order to evaluate their multi-lineage differentiation capability. After 20 days of incubation, the sample were rinsed with PBS twice and fixed with 4% paraformaldehyde (Sigma-Aldrich, USA) at room temperature for staining. After rinsing with distilled water, the cells were stained

with either Alizarin Red S (Bio-Idea, Iran) or Oil Red O (Bio-Idea, Iran) to detect the calcified tissue in cultures and adipogenesis. The cells cultured in standard growth medium were evaluated for morphology assessment.

2.5.4 Cell proliferation and cytotoxicity

Sterilized granules were soaked in DMEM supplemented with 15% FBS for 1 hour. Then medium was taken out and rBMSCs were seeded at a concentration of 2×10^4 cells per scaffold. The cells were cultured in cell culture medium at 37 °C in a humidified atmosphere with 5% CO₂, with the culture medium refreshed every other day. DAPI (4', 6-diamidino-2-phenylindole) staining was performed on 1, 3, 5, and 7 days to visualize live cells following manufacturer's instructions. The scaffolds were rinsed with PBS and fixed with paraformaldehyde (Sigma, USA), and rinsed with PBS again. Next, they were stained with DAPI stain solution for 2 minutes. They were washed with PBS. The cells were imaged with fluorescence microscope.

Both cell proliferation and viability of rBMSCs on granules were evaluated by 3-(4, 5-Dimethylthiazol-2-yl)-2 and 5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, USA) assay. After 1, 3 and 5 days, samples were rinsed with PBS. They were placed in DMEM solution with 10% MTT (5 mg/ml) for 4 hours, in dark room. Afterwards, sample medium was replaced with DMSO (100 μ L) solution and β -TCP granules were crushed. The sample optical density was recorded by ELISA reader (Biotech, USA) at 570 nm. Three samples per group were included.

2.5.5 Cell adhesion and morphology

Surface electron microscopy (SEM) was performed to observe cell attachment and morphology on scaffolds. After 1, 3 and 5 days of incubation, β -TCP granules were prepared for imaging as follows: Samples were rinsed with PBS and fixed with 4% paraformaldehyde

for 45 minutes at room temperature. Afterwards, the samples were dehydrated in the ethanol concentration sequence of 25%, 50%, 70%, 80%, 90% and 100% afterward air-dried overnight. Eventually, the samples were sputter-coated with gold and scanned using scanning electron microscopy (SEM, Hitachi su3500, Japan).

2.5.6 Alkaline phosphatase (ALP) activity assay

The osteogenic capacity of the scaffolds was assessed with ALP kit (Abcam, USA). The cell-scaffolds were incubated in standard medium for 2 days, then they were cultured in the osteogenic medium described earlier, for 1, 7, and 14 days. Three samples per group were included. The scaffolds were rinsed with PBS at each time point. To measure ALP activity, the cells were lysed using cOmplete™ Lysis-M buffer. P-nitrophenyl-phosphate (Fluka, Poole, UK) at pH 10.3 was utilized as the substrate for ALP. The absorbance of ALP was detected at 405 nm using an microplate reader (Stat Fax 2100, Awareness Technology Inc., USA).

2.6 In-vivo osteogenesis

2.6.1 Implementation of β -TCP granule with PM release

All animal experiments were in accordance with ARRIVE guidelines and Iran National Committee for Ethics in Biomedical Research guidelines and approved by the ethical committee of School of dentistry, Shahid Beheshti University of Medical Sciences (approval No: IR.SBMU.DRC.REC.1398.028). Thirty-two healthy mature male Sprague Dawley rats (11 to 12 weeks old), weighing 250-350 g were used as calvarial bone defect models. Animals were procured from Pasteur institute (Tehran, Iran). The rats were kept at a housing unit for 2 weeks previous to the surgery for acclimation period. Rats were randomly divided to four study groups as follows: defects filled with β -TCP granule with PM sustained release (β -TCP +PEG/PM), defects filled with β -TCP granule loaded with rBMSCs with PM sustained release

(β -TCP +PEG/PM + rBMSCs), defects filled with β -TCP granule loaded only with PEG (β -TCP +PEG), defects filled with β -TCP granule loaded with rBMSCs and PEG (β -TCP +PEG + rBMSCs). Animals were sacrificed at 4 and 8 weeks.

For assessment of in vivo bone regeneration, 8 mm in diameter critical-sized bone defects were created in each rat calvarium. Rats were weighed, anesthetized with intraperitoneal injection of 80 mg/kg Ketamine (Bremer, Germany) and 8 mg/kg Xylazine (KELA, Belgium). They also received 10 mg/kg Enrofloxacin (Rooyandarou, Iran) subcutaneously as prophylactic antibacterial therapy. After clipping the parietal region of rats with electrical clippers, the surgical field was disinfected with 10% povidone-iodine. Then a mid-sagittal skin incision was made using surgical blade (#15). Periosteum incised and elevated from bone with a periosteal elevator. We carefully used 8 mm-Trephine bur to create the defects and taking care of brain tissue and dura mater, under irrigation with sterile 0.9% saline to prevent thermal damage to the bone. The cut bone was removed and β -TCP granules were mildly crushed to fit the defect then they were inserted into rat defects. Finally, flap was sutured first in periosteal layer (4-0 PLGA, SUPA, and Iran) afterward in skin (4-0 Nylon, SUPA, Iran). Oral Enrofloxacin (Rooyandarou, Iran) was also given to rats for three consecutive days post-surgery for prophylaxis. The rats were monitored in a warm place with supplemental O₂ until regaining consciousness, then every three rats were placed in a cage (30 × 40 × 60 cm) with ad libitum feeding and transferred to their housing room. The housing room was kept at 22 centigrade and 12 hours light / 12 hours dark schedule. Rats were clinically examined for any tissue swelling, dehiscence or infections around the surgical sites both at the time of sacrifice and routinely throughout the healing procedure. At 4 and 8 weeks after surgery, they were sacrificed with carbon dioxide asphyxiation (flow rate 8lit/min until breathing movements cease for 1 minute) and their craniums were harvested.

2.6.2 Micro-CT evaluation

Harvested calvaria were gently washed with tap water and immersed in formalin 10% solution inside labelled containers. The solution was replaced 24h later and the samples were fixed for two weeks and then rinsed with PBS several times. Next, the calvaria bone samples were scanned using micro-Computed Tomography (μ -CT). A desktop μ -CT scanner (LOTUS-NDT, Behin Negareh Co., Iran) was used in this study. LOTUS-NDT has a cone beam micro-focus X-ray source and a flat panel detector. The X-ray tube voltage and its current were set to 80 KV and 85 μ A, respectively. No added filtration was used in this study. The total scan time was ~ 2 hours and the nominal resolution was 10 microns per pixel. All the protocol settings process was controlled by LOTUS NDT-ACQ software. The acquired 3D data was reconstructed using LOTUS NDT-REC by a standard Feldkamp, Davis, Kress (FDK) algorithm (3). Also, LOTUS NDT-3D was used for rendering of reconstructed images and by adding Bone Analysis Plugin (BAP) inside the software we reported bone volume (BV), total volume (TV) and scaffold volume (SV) parameters.

2.6.3 Histomorphometry

Harvested specimen were decalcified in 10% EDTA, dehydrated in an increasing grade of ethanol (70, 90 and 100%), cleared in xylene and embedded in paraffin. Tissue sections with a thickness of 5 μ m were prepared and stained with Hematoxylin and Eosin (H&E). The stained sections were scanned digitally to calculate the percentage of newly formed bone and remnant scaffold within the defect using an image analysis software Axiovision Rel. 4.8 (Carl Zeiss Microimaging, GmbH, Germany). Semi-quantitative analysis of tissue sections was performed by a pathologist blinded to the study groups. Each section was scored from 1 to 4 in order to evaluate: Connective tissue organization and maturation, distribution of newly formed bone trabeculae within the defect, and angiogenesis.

2.7 Statistical analysis

All experiments involving MTT and ALP assays were performed in triplicate. All data were expressed as mean $\pm$ standard deviation (SD). Two-way analysis of variance (ANOVA) and Sidak's post-hoc tests were used for inter- and intra-group comparison. When $p < 0.05$ the differences were considered statistically significant. Statistical analyses were performed using Prism 8.3.0 (GraphPad Software Inc., San Diego, CA, USA).

References

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3. Feldkamp LA, Davis LC, Kress JW. Practical cone-beam algorithm. *JOSA A*. 1984;1(6):612-9.