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ORIGINAL RESEARCH ARTICLE

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Study of mesenchymal stem cells cultured on a poly(lactic-co-glycolic acid) scaffold containing simvastatin for bone healing

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ABSTRACT

Background: Tissue engineering is a promising alternative for the development of bone substitutes; for this purpose, three things are necessary: stem cells, a scaffold to allow tissue growth and factors that induce tissue regeneration.

Methods: To congregate such efforts, we used the bioresorbable and biocompatible polymer poly(lactic-co-glycolic acid) (PLGA) as scaffold. For the osteoinductive factor, we used simvastatin (SIM), a drug with a pleiotropic effect on bone growth. Mesenchymal stem cells (MSCs) were cultured in PLGA containing SIM, and the bone substitute of PLGA/SIM/MSC was grafted into critical defects of rat calvaria.

Results: The in vitro results showed that SIM directly interfered with the proliferation of MSC promoting cell death, while in the pure PLGA scaffold the MSC grew continuously. Scaffolds were implanted in the calvaria of rats and separated into groups: control (empty defect), PLGA pure, PLGA/SIM, PLGA/MSC and PLGA/SIM/MSC. The increase in bone growth was higher in the PLGA/SIM group.

Conclusions: We observed no improvement in the growth of bone tissue after implantation of the PLGA/SIM/MSC scaffold. As compared with in vitro results, our main hypothesis is that the microarchitecture of PLGA associated with low SIM release would have created an in vivo microenvironment of concentrated SIM that might have induced MSC death. However, our findings indicate that once implanted, both PLGA/SIM and PLGA/MSC contributed to bone formation. We suggest that strategies to maintain the viability of MSCs after cultivation in PLGA/SIM will contribute to improvement of bone regeneration.

Keywords: Bone regeneration, Mesenchymal stem cell, PLGA, Simvastatin

Introduction

The aging population in the world makes bone fractures a substantial public health issue, thus increasing the need for strategies for bone regeneration (1). Autologous grafts (i.e., tissue or organs of the individual) remain the gold standard for stimulating bone regeneration (2), but these procedures are limited by lack of donor area, by morbidity and by pain. Therefore, development of artificial bone is indispensable. Various synthetic materials have been developed for bone regeneration; however, issues such as late or incomplete regeneration are still persistent. Among the synthetic materials developed are ceramics such as hydroxyapatite, bioactive glasses, tricalcium phosphate and polymers (3).

The most frequently used polymers for bone tissue regeneration are polylactic acid (PLA); polyglycolic acid (PGA); poly(lactic-co-glycolic acid) (PLGA), which is a copolymer of PLA and PGA; polycaprolactone (PCL); polyethylene (PE); polyethylene glycol (PEG) and poly(methyl methacrylate) (PMMA); among others (3, 4). PLGA is very interesting as a biomaterial since this polymer degrades in the presence of water and its by-products are completely eliminated by the body (4). Furthermore, it possesses good mechanical properties, low immunogenicity and toxicity, and can be structured to degrade in the time necessary for the growth of tissue (5, 6).

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However, these materials are not considered bioactive – i.e., they do not induce tissue growth (7). For that reason, various substances have been added to polymers to accelerate the healing process, such as growth factors, drugs and ceramics (8-9). Among the drugs used is simvastatin (SIM), a member of the statin family, used in the treatment of cholesterol problems (8). Studies have shown that SIM plays a role in osteoblast cell differentiation and mineralization, thus stimulating the regeneration of bone tissue (10-12).

Bone marrow-derived mesenchymal stem cells (MSCs) have also been associated with biomaterials in bone tissue engineering. These cells are easily expanded in vitro and have the capacity to differentiate into osteoblasts, chondrocytes and adipocytes (13). The use of MSCs in tissue engineering is based on evidence that these cells exert a paracrine effect on the implanted site. Studies have shown that the secretion of cytokines and growth by MSCs stimulate resident cells and promote matrix remodeling and the differentiation of native progenitor cells (14). Additionally, after implantation of any biomaterial, an inflammatory response is expected to occur, which can be reduced with the use of MSCs due to the immunomodulatory properties of these cells (14, 15).

Therefore, this study examined whether the presence of MSCs in a PLGA scaffold with SIM has a synergistic effect on the regeneration of bone tissue.

Material and methods

Sample preparation

Scaffolds were obtained by dissolution of PLGA in chloroform (Merck) (10%, m/v). Subsequently, SIM (Merck) was added to a concentration of 2% (w/w). Sucrose particles were used to obtain pores. The solution was reversed in a Petri dish to allow solvent evaporation.

SIM release study

To study the release of SIM, 90-mg scaffolds were immersed in 3 mL of phosphate-buffered saline (PBS) and incubated at 37°C. Every 5 days, 1 µL of supernatant was removed and measured using a UV spectrophotometer at a wavelength of 240 nm, and the solution was exchanged with new PBS after each reading point. The amount of SIM released was calculated from a standard curve containing known amounts of the drug.

Isolation, culture and characterization of MSCs

MSCs were isolated from the femur and tibia of 3 male Wistar rats about 3 months of age. All experiments of this study were approved by the Ethics Committee for Experimentation on Animals of the Pontifical Catholic University of São Paulo (2014/37). After sacrifice with halothane, the femur and tibia of animals were dissected. In a laminar flow cabin, bone epiphyses were cut. The bones were centrifuged at 1,500 rpm for 10 minutes. Then, bone marrow was suspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). The cells were maintained in an incubator at 37°C containing 5% CO_2. For characterization of MSCs, the cells were differentiated in an inducing medium for either osteoblasts (StemPro® Osteogenesis Differentiation Kit; Invitrogen) or adipocytes (StemPro® Adipogenesis Differentiation Kit; Invitrogen). After 14 days in differentiation medium, the MSCs were stained with alizarin red S and oil red O, respectively.

Seeding of MSCs in scaffolds and cell proliferation assay

PLGA scaffolds, with or without SIM, were used in the cell proliferation assay. The scaffolds were cut into disks of 8-mm diameter and 1-mm thick, with the same dimensions for both in vitro and in vivo assays. Prior to each in vitro assay, the scaffold disks were sterilized for 1 hour under UVC light. Then cells were seeded in the scaffolds at a concentration of 2.6×10^4 cells/cm^2. The cell viability assay was performed by counting cell nuclei after 1, 7 and 14 days of cultivation. For this, cells were fixed with 4% paraformaldehyde and the nuclei stained with 4',6-diamidino-2-phenylindole (DAPI). The images were analyzed by laser scanning confocal microscopy (LSCM; Leica TCS SP8; Germany). To count all cells present in scaffolds, 3D image reconstruction was performed. The maximum depth where the cells were present was 400 µm. For implantation of the PLGA/SIM/MSC scaffold, cells were cultured for 7 days prior to implantation.

Implantation of scaffolds in murine model

PLGA/SIM/MSC scaffolds were implanted in the calvaria of Wistar rats to analyze their influence on the regeneration of bone tissue (Fig. 1). Altogether, 35 male Wistar rats about 3 months of age were used. The animals (n = 7) were divided into 5 groups: control (blank default), PLGA pure, PLGA/SIM, PLGA/MSC and PLGA/SIM/MSC. After anesthesia with 10% ketamine hydrochloride (40 mg/kg) and 2% xylazine hydrochloride (5 mg/kg), the calvaria were exposed and defects of 8-mm diameter were made with a trephine drill (Beltec®). The scaffolds were implanted, and the skin sutured. After 8 weeks, the animals were sacrificed, and the calvaria removed for histological analysis.

Histological analysis

The calvaria were fixed with 10% formaldehyde and decalcified under 10% ethylenediaminetetraacetic acid (EDTA) solution for 4 days. The samples were embedded in paraffin. The tissue was stained with hematoxylin and eosin (H&E). The processed calvaria tissue was serial sectioned into 6 slices of 4 µm, as presented in Figure 1F. The sections were examined with a light microscope (Nikon Eclipse E800).

Histomorphometric analysis

The quantification of new bone formation was performed by histomorphometric analysis: The calvaria tissue extracted from the bone defect was serially cut into 6 slices from different areas, as shown in Figure 1F. The histological sections were digitally acquired, the obtained images were demarcated and the new bone areas were quantified by NisElements software (Nikon Instruments Inc.).

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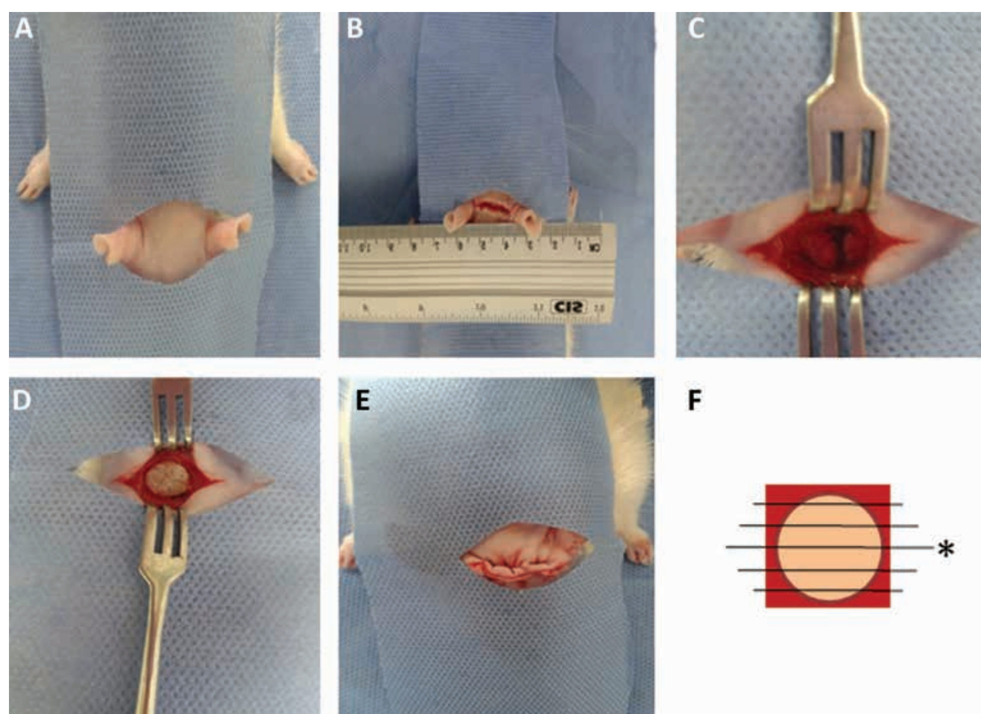

Fig. 1 - Surgical procedure: (**A**) Trichotomy, (**B**) incision, (**C**) defect with 8-mm diameter, (**D**) implant of scaffold, (**E**) suture. After 8 weeks, 1 cm^2 of the healing tissue in the implanted area was removed and processed for histology, and serial sections of each sample were performed (lines in (**F**)), as indicated in the scheme. The area indicated by the middle line (*) was separated by cleavage, and the middle sections were analyzed to generate Figure 5.

Statistical analysis

Statistical analysis was performed using 1-way analysis of variance (ANOVA). Results were expressed as means ± standard deviation (SD). The difference between groups was analyzed by Tukey's test, with p values less than 0.05 indicating significance (Prism version 6.03) (n = 5).

Results

SIM release study

SIM drug release from PLGA scaffolds is shown in Figure 2. This assay was performed for 1 month. Every 5 days, the supernatant was collected, and the SIM concentration measured by UV. This assay demonstrated that release from the SIM was slower over time.

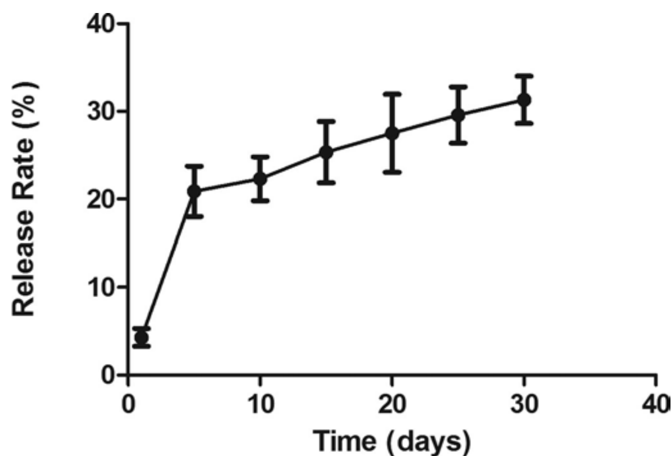

Fig. 2 - Release profile of simvastatin from poly(lactic-co-glycolic acid) (PLGA) scaffolds incubated in phosphate-buffered saline (PBS) at 37°C (n = 5).

Characterization of MSCs

Isolated MSCs were characterized as previously reported (16). These cells had to be adherent to a plastic polystyrene plate and capable of differentiating into bone and fat. The phenotype of the isolated cells after using the inductor medium for adipocytes (Fig. 3B) and osteoblasts (Fig. 3D) confirmed that these cells were MSCs.

Cell proliferation in the scaffolds

MSC quantification after seeding on scaffolds was performed by LSCM. After 1, 7 and 14 days the cells cultured over the materials were fixed, and the nuclei were stained with DAPI. Reconstruction of the 3D images was made up to 400-µm deep. After that, the nuclei were counted. In pure PLGA, cells were able to adhere and grow over time, while in PLGA/SIM, cells also had some adhesion but all died after 14 days (Fig. 4).

Implantation of scaffolds in murine model

After anesthetizing the animals, a skin incision was made exposing the skull. With the aid of a trephine drill, a defect of 8-mm diameter was made. Then, the scaffolds of PLGA pure, PLGA/SIM, PLGA/MSC and PLGA/SIM/MSC were implanted. The control remained empty (Fig. 1). The animals resumed their normal activities within 24 hours.

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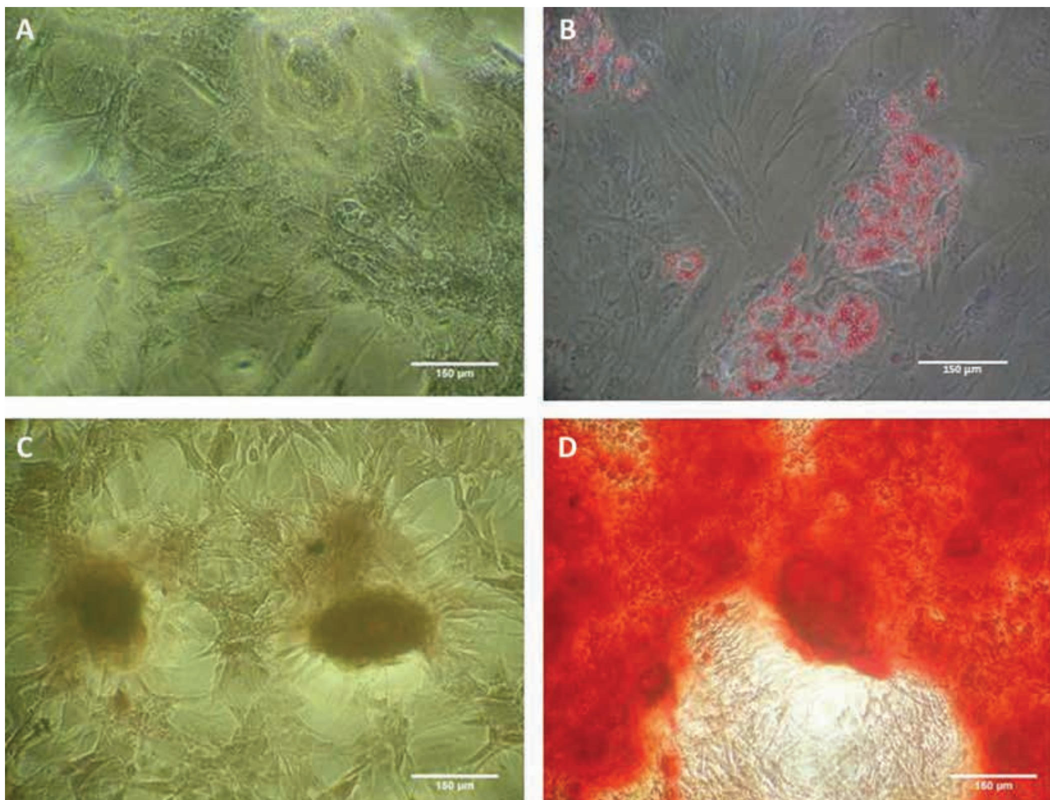

Fig. 3 - Characterization of mesenchymal stem cell (MSC) isolates. (**A, C**) Experimental controls, cells grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). (**A, B**) Depiction of ability to differentiate into adipocyte cells, after growth in inductor medium (**B**) (oil red O stain). (**C, D**) Illustration of differentiation into osteoblasts, after growth in inductor medium (**D**) (alizarin red S stain).

Histological analysis

The histological analysis was performed after 8 weeks of implantation. New bone formation is shown in Figures 5 and 6. Longitudinal sections were taken from the center of the defect and stained with H&E. It can be seen that in all treatments tested, the scaffold was still present. All materials were well accepted by the body. In the control, pure PLGA and PLGA/MSC groups, bone growth was always dependent on the edges (Fig. 5A, B and D, respectively), being more evident in the PLGA/MSC group. There was bone tissue growth independent of the edges in the PLGA/SIM scaffold with and without cells, indicative of osteoinduction (Fig. 6C and E). However, bone growth was less evident in PLGA/SIM/MSC than in the same material without cells.

Histological analysis of the defect borders is shown in Figure 6. It was noted that only animals given PLGA/SIM scaffolds had bone spurs surrounded by osteoprogenitor cells, suggesting maintenance of bone growth (Fig. 6B).

Histomorphometric analysis

The quantification of new bone formation was performed after 8 weeks of implantation (Fig. 7). The results demonstrated a major bone neoformation in the PLGA/SIM group as compared with the other groups. There was no statistically significant difference among the control (empty defect), PLGA, PLGA/MSC and PLGA/SIM/MSC groups with regard to new bone formation.

Discussion

Bioabsorbable polymers such as PLGA are widely used in tissue engineering (17, 18), but only PLGA is unable to induce the growth of tissue. Therefore, 2% of SIM was added to this scaffold to induce the growth of bone tissue. Some studies have shown that this SIM concentration was able to stimulate bone formation in vivo (19, 20). Furthermore, in vitro studies also showed that the addition of SIM increased the expression of osteogenic marker genes (11, 21-23).

Bone tissue engineering aims to use biomaterials as supports for cells. These cells can be isolated, cultured and expanded in vitro for use in biomaterials (24). MSCs have several advantages for this purpose. They are easily cultured in vitro and can differentiate into osteoblasts. Also, they have immunosuppressive properties, which may attenuate the inflammatory response induced by biomaterials (25).

After isolation of MSCs, cells must be characterized; one way to do this is to analyze the differentiation potential in mesodermal tissue cells, since it is known that they are able to differentiate into adipocytes and osteocytes when subjected to specific differentiation factors (26). The cytochemical analysis conducted in this study showed that the cells isolated were MSCs.

Before MSC culture in scaffolds, we needed to understand whether and how there would be a release of SIM by the PLGA scaffold; for this, we did an in vitro release assay. This assay showed that the release of SIM occurred slowly – about

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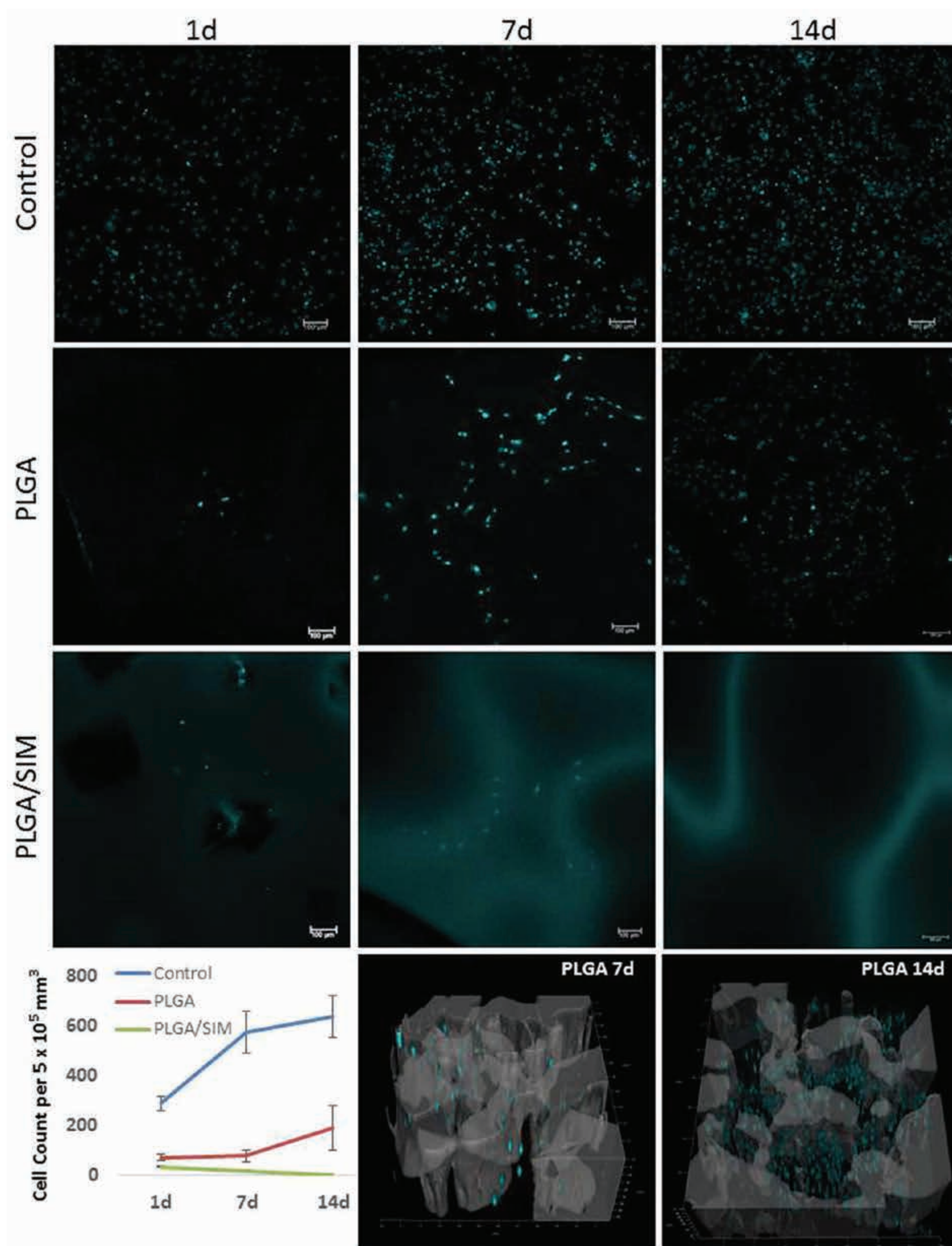

Fig. 4 - Mesenchymal stem cell (MSC) quantification after 1, 7 and 14 days of culture in the scaffolds. Cell nuclei were stained with DAPI (blue) and analyzed by laser scanning confocal microscopy (LSCM). The 3D reconstructions of poly(lactic-co-glycolic acid) (PLGA) pure scaffold merged with adhered cells are presented at the bottom of the image. 3D reconstructions of PLGA/simvastatin (SIM) with cells were not carried out due to SIM autofluorescence, blurring the visualization of the cells. Also, 3D reconstructions of PLGA after 1 day in culture were not performed because there were few cells to justify the reconstruction. The cell count graph represents the median and standard deviation of a triplicate assay.

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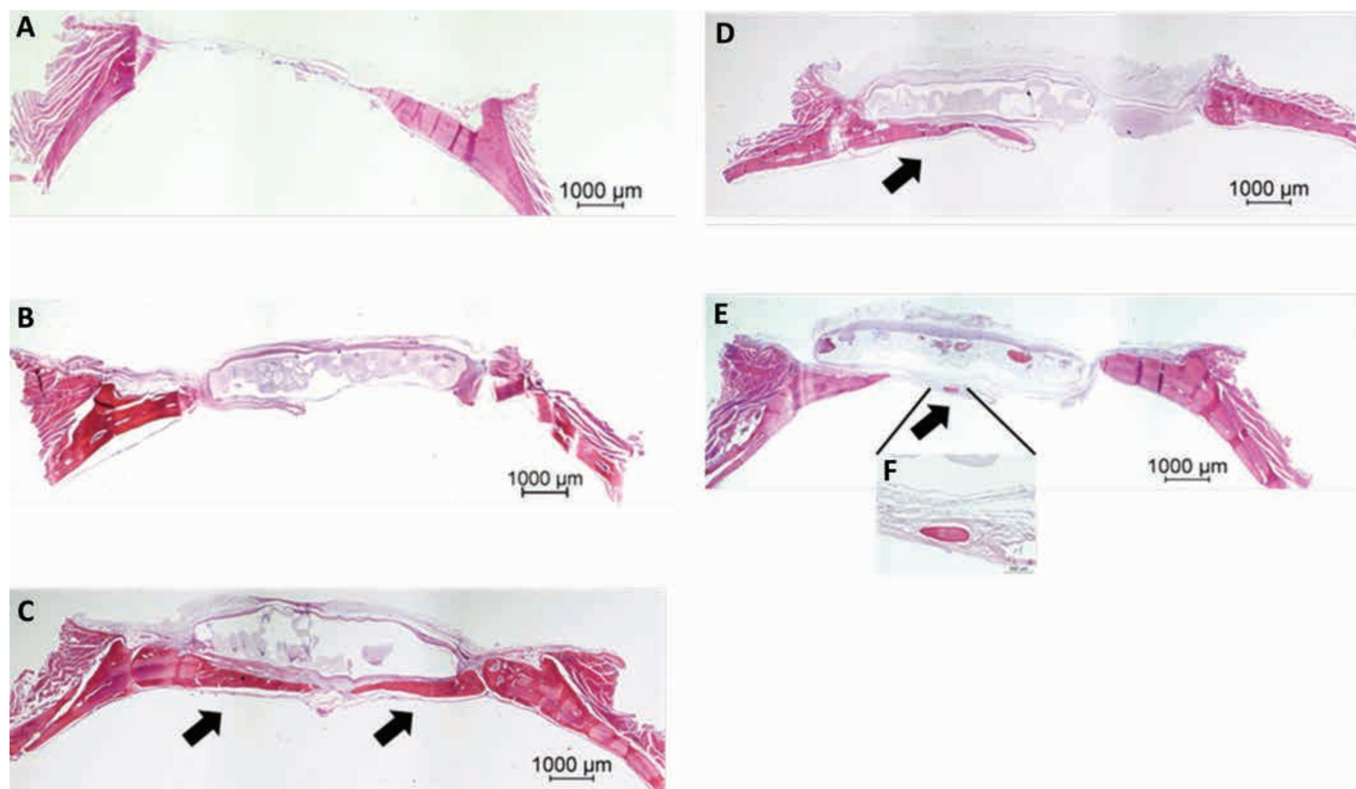

Fig. 5 - Histological analysis demonstrated the healing process of defects. (**A**) Control (empty defect), (**B**) poly(lactic-co-glycolic acid) (PLGA) pure, (**C**) PLGA/simvastatin (SIM), (**D**) PLGA/mesenchymal stem cells (MSCs), (**E**) PLGA/SIM/MSC, (**F**) detail of (**E**) showing a small fragment of newly formed bone. Arrows indicate newly formed bone. Sections stained with hematoxylin and eosin (H&E).

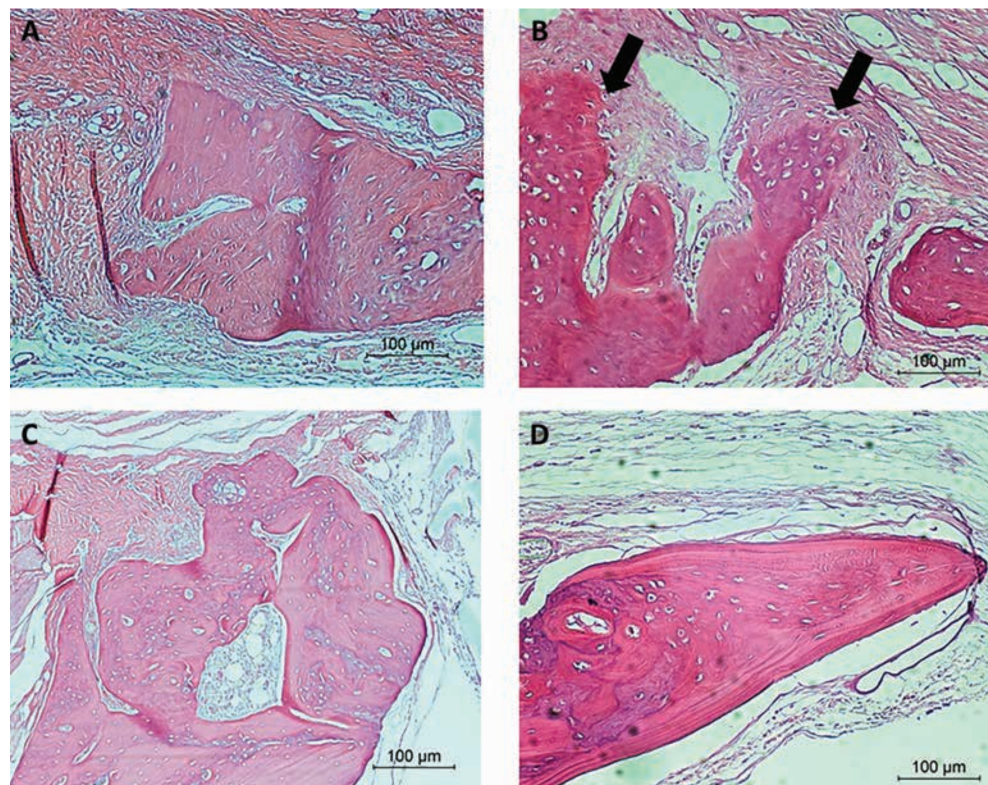

Fig. 6 - Histological analysis of the defect borders. (**A**) poly(lactic-co-glycolic acid) (PLGA) pure; (**B**) PLGA/simvastatin (SIM), showing bone beams surrounded by osteoprogenitor and osteoblast cells (indicated by arrows); (**C**) PLGA/mesenchymal stem cells (MSC), also showing bone beams; (**D**) PLGA/SIM/MSC. Sections stained with hematoxylin and eosin (H&E).

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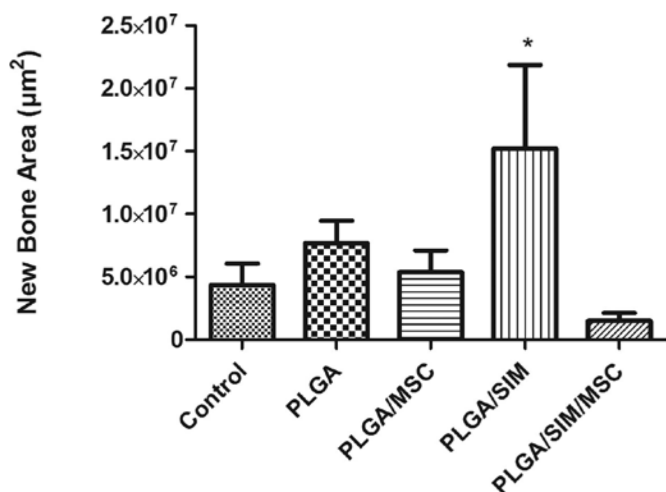

Fig. 7 - Bar graph of areas of newly formed bone after 8 weeks of implantation. Results are expressed as medians and standard deviation. All groups were compared to control (*p<0.01, vs control). MSC = mesenchymal stem cell; PLGA = poly(lactic-co-glycolic acid); SIM = simvastatin.

4% in 24 hours. The cumulative release was approximately 30% in 30 days. This slow release is ascribed to the hydrophobic characteristics of SIM (27). In fact, this release could also be correlated to the progressive degradation of PLGA, since it was shown that this polymer has a degradation rate of around 20% after 30 days (28), which is close to the release rate obtained by SIM after 30 days.

Our results demonstrated that when cells were cultured directly in the PLGA pure scaffold, the cell proliferation continuously increased over time. However, when cultured directly in the PLGA/SIM scaffold, there was 100% cell death after 14 days. We also performed an additional assay to evaluate the cell viability by MTT reduction, and in this experiment, the scaffolds were under indirect contact conditions. At each point in this colorimetric assay (1, 7 and 14 days), the PLGA/SIM and PLGA did not promote any cell death (data not shown). So, the cell death attributed to SIM, in these 2 analysis, was probably related to the direct release of SIM to the cells. Studies in vitro with osteoblasts and MSCs have demonstrated that SIM promotes cell viability and increases the expression of osteogenic marker genes such as ALP, OPC and RUNX-2 (21, 26, 29). However, in our study, the PLGA/SIM scaffold was toxic to cells in static conditions in vitro. Other research has shown that SIM reduces the viability of human MSCs in vitro and rat endothelial cells, through the depletion of mevalonate by inhibiting HMG-CoA reductase (30, 31). The mevalonate is related to cholesterol production and is required for cell cycle progression regulating the transition from G1 to S (32, 33).

After obtaining results of cell proliferation in vitro, we chose to implement scaffolds after 7 days of culture with MSCs since they had cells present in PLGA/SIM. Scaffolds remained deployed for 8 weeks. The control group (empty defect) showed extensive fibrosis at the site of the defect and a small amount of bone formation at the edges. In the PLGA pure group, a small amount of bone formation dependent on the defect edge was also observed. This was the expected result, since it is known that PLGA is not osteoinductive (34, 35).

The PLGA/SIM group showed significant bone growth at the edges, and the presence of osteoprogenitor cells surrounding bone spicules, suggesting maintenance of bone growth. This indicates that SIM was osteoinductive in vivo – i.e., it stimulated the differentiation of stem cells from the bone tissue cells (35). This result is consistent with several other studies that demonstrated the osteogenic potential of SIM (25, 36-38) and its ability to recruit autologous stem cells (39, 40). Based on this result and the in vitro proliferation assay, we suggest that the biological analysis of biomaterials in vitro should not be regarded as decisive.

In animals implanted with PLGA/MSC, no significant bone growth was observed. However, in the histological findings, increased osteoblastic cells were observed in newly formed bone as compared with control (empty defect), pure PLGA and PLGA/SIM/MSC, which did not present osteoblastic cells. This could be an indication that the MSCs could have a role in new bone formation, whether differentiating into osteoblastic cells or inducing other cells to differentiate into bone cells (14). In this study, it was expected that the combination of MSCs with SIM would promote a synergistic effect in bone tissue regeneration. However, this was not observed. Even with the presence of few cells in the PLGA/SIM/MSC scaffold, it was expected that these cells could grow in vivo. We believe that the interference in the regeneration process was due to the presence of dead cells in the scaffold. This result could probably be improved by maintenance of viable cells, which could be achieved by co-incubation with mevalonate or by pretreatment with inhibitor of nuclear factor kappa-B kinase subunit beta (Iκκ-β), as proposed by Li et al (30).

In conclusion, it was shown that the presence of SIM in PLGA scaffolds promoted the death of MSCs in vitro, and interfered with the in vivo results for PLGA/SIM/MSC. However, the use of MSCs in combination with a PLGA/SIM scaffold is still a promising possibility, since it was demonstrated that the presence of MSCs or SIM on a PLGA scaffold contributed to bone regeneration. This result can probably be improved by maintaining the viability of MSCs in a PLGA/SIM/MSC scaffold.

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References

1. Borgström F, Lekander I, Ivergård M, et al. The International Costs and Utilities Related to Osteoporotic Fractures Study (ICUROS): quality of life during the first 4 months after fracture. Osteoporos Int. 2013;24(3):811-823.
2. Hamajima S, Hayashi T, Sato Y, Sasaki K, Kawai T. Osteoanagenesis after transplantation of bone marrow-derived mesenchymal stem cells using polyvinylidene chloride film as a scaffold. Dent Mater J. 2011;30(5):707-716.
3. Shrivats AR, McDermott MC, Hollinger JO. Bone tissue engineering: state of the union. Drug Discov Today. 2014;19(6): 781-786.
4. Kranz H, Ubrich N, Maincent P, Bodmeier R. Physicomechanical properties of biodegradable poly(D,L-lactide) and poly(D,L-lactide-co-glycolide) films in the dry and wet states. J Pharm Sci. 2000;89(12):1558-1566.
5. Rani M, Agarwal A, Negi YS. Characterization and biodegradation studies for Interpenetrating Polymeric Network (IPN) of chitosan-amino acid beads. J Biomater Nanobiotechnol. 2011;2(1):71-84.
6. Hutmacher DW. Scaffolds in tissue engineering bone and cartilage. Biomaterials. 2000;21(24):2529-2543.
7. Fan J, Im CS, Cui Z-K, et al. Delivery of phenamil enhances BMP-2-induced osteogenic differentiation of adipose-derived stem cells and bone formation in calvarial defects. Tissue Eng Part A. 2015;21(13-14):2053-2065.
8. Garip S, Severcan F. Determination of simvastatin-induced changes in bone composition and structure by Fourier transform infrared spectroscopy in rat animal model. J Pharm Biomed Anal. 2010;52(4):580-588.
9. Simpson RL, Nazhat SN, Blaker JJ, et al. A comparative study of the effects of different bioactive fillers in PLGA matrix composites and their suitability as bone substitute materials: A thermo-mechanical and in vitro investigation. J Mech Behav Biomed Mater. 2015;50:277-289.
10. Walter MS, Frank MJ, Rubert M, Monjo M, Lyngstadaas SP, Haugen HJ. Simvastatin-activated implant surface promotes osteoblast differentiation in vitro. J Biomater Appl. 2014;28(6): 897-908.
11. Yan Q, Xiao LQ, Tan L, et al. Controlled release of simvastatin-loaded thermo-sensitive PLGA-PEG-PLGA hydrogel for bone tissue regeneration: in vitro and in vivo characteristics. J Biomed Mater Res A. 2015;103(11):3580-3589.
12. Tai IC, Wang YH, Chen CH, Chuang SC, Chang JK, Ho ML. Simvastatin enhances Rho/actin/cell rigidity pathway contributing to mesenchymal stem cells osteogenic differentiation. Int J Nanomedicine. 2015;10:5881-5894.
13. Fekete N, Rojewski MT, Fürst D, et al. GMP-compliant isolation and large-scale expansion of bone marrow-derived MSC. PLoS ONE. 2012;7(8):e43255.
14. Hanson S, DSouza RN, Hematti P. Biomaterial-mesenchymal stem cell constructs for immunomodulation in composite tissue engineering. Tissue Eng Part A. 2014;20(15-16):2162-2168.
15. Németh K, Leelahavanichkul A, Yuen PS, et al. Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent reprogramming of host macrophages to increase their interleukin-10 production. Nat Med. 2009;15(1):42-49.
16. Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006;8(4):315-317.
17. Davies JE, Matta R, Mendes VC, Perri de Carvalho PS. Development, characterization and clinical use of a biodegradable composite scaffold for bone engineering in oro-maxillo-facial surgery. Organogenesis. 2010;6(3):161-166.
18. Park SH, Park DS, Shin JW, et al. Scaffolds for bone tissue engineering fabricated from two different materials by the rapid prototyping technique: PCL versus PLGA. J Mater Sci Mater Med. 2012;23(11):2671-2678.
19. Ferreira LB, Bradaschia-Correa V, Moreira MM, Marques ND, Arana-Chavez VE. Evaluation of bone repair of critical size defects treated with simvastatin-loaded poly(lactic-co-glycolic acid) microspheres in rat calvaria. J Biomater Appl. 2015;29(7):965-976.
20. Maciel-Oliveira N, Bradaschia-Correa V, Arana-Chavez VE. Early alveolar bone regeneration in rats after topical administration of simvastatin. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2011;112(2):170-179.
21. Baek KH, Lee WY, Oh KW, et al. The effect of simvastatin on the proliferation and differentiation of human bone marrow stromal cells. J Korean Med Sci. 2005;20(3):438-444.
22. Wang Q, Zhang H, Li Q, et al. Biocompatibility and osteogenic properties of porous tantalum. Exp Ther Med. 2015;9(3): 780-786.
23. Zhang HX, Xiao GY, Wang X, et al. Biocompatibility and osteogenesis of calcium phosphate composite scaffolds containing simvastatin-loaded PLGA microspheres for bone tissue engineering. J Biomed Mater Res A. 2015;103(10): 3250-3258.
24. Crane GM, Ishaug SL, Mikos AG. Bone tissue engineering. Nat Med. 1995;1(12):1322-1324.
25. Zhu H, Yang F, Tang B, et al. Mesenchymal stem cells attenuated PLGA-induced inflammatory responses by inhibiting host DC maturation and function. Biomaterials. 2015;53:688-698.
26. Vater C, Kasten P, Stiehler M. Culture media for the differentiation of mesenchymal stromal cells. Acta Biomater. 2011; 7(2):463-477.
27. Nandagiri VK, Politecnico CM, Torino D, Daly J, Ciardelli G. Preparation and characterization of simvastatin loaded PLGA microparticles for tissue engineering applications. http://epubs.rcsi.ie/biolart/15/. Accessed November 1, 2016.
28. Ma X, Oyamada S, Wu T, et al. In vitro and in vivo degradation of poly(D, L-lactide-co-glycolide)/amorphous calcium phosphate copolymer coated on metal stents. J Biomed Mater Res A. 2011;96(4):632-638.
29. Chen PY, Sun JS, Tsuang YH, Chen MH, Weng PW, Lin FH. Simvastatin promotes osteoblast viability and differentiation via Ras/Smad/Erk/BMP-2 signaling pathway. Nutr Res. 2010;30(3): 191-199.
30. Li Y, Müller AL, Ngo MA, et al. Statins impair survival of primary human mesenchymal progenitor cells via mevalonate depletion, NF-κB signaling, and Bnip3. J Cardiovasc Transl Res. 2015;8(2):96-105.
31. Kaneta S, Satoh K, Kano S, Kanda M, Ichihara K. All hydrophobic HMG-CoA reductase inhibitors induce apoptotic death in rat pulmonary vein endothelial cells. Atherosclerosis. 2003;170(2): 237-243.
32. Langan TJ, Volpe JJ. Cell cycle-specific requirement for mevalonate, but not for cholesterol, for DNA synthesis in glial primary cultures. J Neurochem. 1987;49(2):513-521.
33. Quesney-Huneeus V, Galick HA, Siperstein MD, Erickson SK, Spencer TA, Nelson JA. The dual role of mevalonate in the cell cycle. J Biol Chem. 1983;258(1):378-385.
34. Quinlan E, López-Noriega A, Thompson E, Kelly HM, Cryan SA, OBrien FJ. Development of collagen-hydroxyapatite scaffolds incorporating PLGA and alginate microparticles for the controlled delivery of rhBMP-2 for bone tissue engineering. J Control Release. 2015;198:71-79.
35. Han F, Zhou F, Yang X, Zhao J, Zhao Y, Yuan X. A pilot study of conically graded chitosan-gelatin hydrogel/PLGA scaffold with dual-delivery of TGF-β1 and BMP-2 for regeneration of

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cartilage-bone interface. J Biomed Mater Res B Appl Biomater. 2015;103(7):1344-1353.

36. Miron RJ, Zhang YF. Osteoinduction: a review of old concepts with new standards. J Dent Res. 2012;91(8):736-744.
37. Fu YC, Wang YH, Chen CH, Wang CK, Wang GJ, Ho ML. Combination of calcium sulfate and simvastatin-controlled release microspheres enhances bone repair in critical-sized rat calvarial bone defects. Int J Nanomedicine. 2015;10:7231-7240.
38. Sukul M, Min YK, Lee SY, Lee BT. Osteogenic potential of simvastatin loaded gelatin-nanofibrillar cellulose-β tricalcium phosphate hydrogel scaffold in critical-sized rat calvarial defect. Eur Polym J. 2015;73:308-323.
39. Yueyi C, Xiaoguang H, Jingying W, et al. Calvarial defect healing by recruitment of autogenous osteogenic stem cells using locally applied simvastatin. Biomaterials. 2013;34(37):9373-9380.
40. Nyan M, Miyahara T, Noritake K, et al. Molecular and tissue responses in the healing of rat calvarial defects after local application of simvastatin combined with alpha tricalcium phosphate. J Biomed Mater Res B Appl Biomater. 2010;93(1):65-73.

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