Archives of Oral Biology 97 (2019) 42–51

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/archoralbio

Topical application of phenytoin or nifedipine-loaded PLGA microspheres promotes periodontal regeneration *in vivo*

Xiao-Heng Zhao[a], Franklin R. Tay[b], Yan-Jun Fang[a], Liu-Yan Meng[a,*,1], Zhuan Bian[a,*,1]

[a] State Key Laboratory Breeding Base of Basic Science of Stomatology (Hubei-MOST) & Key Laboratory for Oral Biomedicine of Ministry of Education (KLOBM), School and Hospital of Stomatology, Wuhan University, Wuhan, Hubei, PR China
[b] College of Graduate Studies, Augusta University, Augusta, GA, USA

ARTICLE INFO

Keywords:
Alveolar bone loss
Anti-epileptics
Bone remodeling
Drug delivery
Gingival overgrowth

ABSTRACT

Objectives: Gingival recession and alveolar bone loss are common manifestations of periodontitis. Periodontal regeneration is the ideal strategy for rehabilitating periodontal tissue defects and preventing tooth loss. The present study examined whether localized, topical application of gingival overgrowth-inducing drugs, phenytoin, nifedipine or cyclosporine, induces periodontal regeneration.

Methods: Polylactic-co-glycolic acid (PLGA) was used as the carrier for preparation of phenytoin, nifedipine or cyclosporine-loaded PLGA microspheres, using an oil-in-water emulsification technique. The drug-loaded microspheres were delivered to periodontal defects created on alveolar ridges mesial to the first maxillary molars of Sprague-Dawley rats. After eight weeks, the operation area in each rat, including the maxillary molars and periodontal tissues, was harvested and evaluated by micro-computed tomography, histochemical and immunohistochemical analyses.

Results: Physical parameters representative of periodontal regeneration, including the length of new alveolar bone ($p < 0.01$) and the area of new alveolar bone ($p < 0.01$) were significantly improved in the phenytoin group. Compared to other groups, the phenytoin group demonstrated increased expression of COL-1, VEGF-A, osteoblast and osteoclast markers (BMP-2, TGF-β1, OCN and TRAP staining), as well as decreased expression of MMP-8.

Conclusions: Results of the present study provided new evidence that localized, controlled release of phenytoin confers therapeutic benefits toward gingival recession and alveolar bone loss. Phenytoin appears to be a promising drug that promotes periodontal regeneration.

1. Introduction

Chronic Periodontal disease is in one of the major oral diseases that challenge the integrity of the contemporary human dentition. Gingival recession and alveolar bone loss are the main clinical signs of destruction of the tooth attachment apparatus. If untreated, the disease causes progressive deterioration of the attachment apparatus and subsequent tooth loss.

Gingival recession is a prevalent oral condition affecting middle- and older-aged adults (Weiser & Saltzman, 2014). According to a US national survey, 50% of adults (18 to 64) and 88% of seniors (65 and over) suffer from gingival recession in at least one oral site (Bloom, Simile, Adams, & Cohen, 2012). The frequency and extent of gingival recession increase with age. The pathogenesis of gingival recession is multifactorial, and may be caused by inflammatory destruction, inappropriate tooth brushing, incorrect occlusal relationships, improper flossing, plaque accumulation and traumatic injury (Chan, Chun, MacEachern, & Oates, 2015; Pradeep, Rajababu, Satyanarayana, & Sagar, 2012). Gingival recession may result in tooth hypersensitivity, root caries, gingival bleeding, plaque accumulation and poor esthetics. Thin gingival tissues, being more translucent and fragile, are more susceptible to recession. Histologically, thin gingival tissues are less fibrous and less keratinized than thick gingival tissues (Merijohn, 2016). Alveolar bone loss is often associated with gingival recession (Serino, Wennstrom, Lindhe, & Eneroth, 1994). Receded gingival tissues seldom regain their original heights without concomitant regeneration of the underlying alveolar bone.

Regeneration of alveolar bone loss remains a humongous challenge in periodontics and periodontology research. Periodontal regeneration mandates regeneration of gingival tissues and restoration of alveolar

* Corresponding authors.
E-mail addresses: mengliuyan@whu.edu.cn (L.-Y. Meng), bianzhuan@whu.edu.cn (Z. Bian).
[1] Both authors contributed equally to the work.

https://doi.org/10.1016/j.archoralbio.2018.10.002
Received 4 June 2018; Received in revised form 13 September 2018; Accepted 2 October 2018
0003-9969/ © 2018 Elsevier Ltd. All rights reserved.

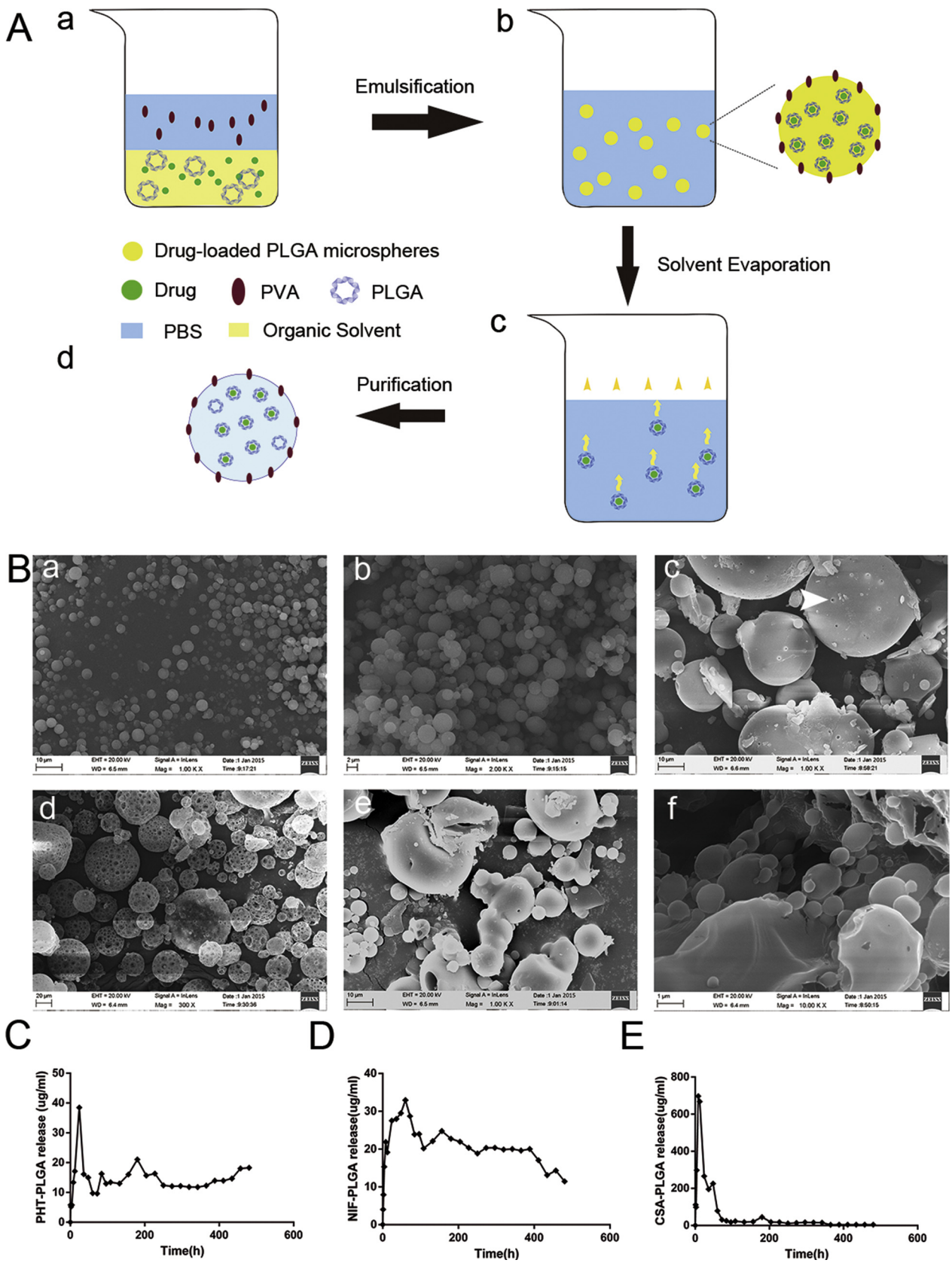

Fig. 1. Synthesis and characterization of drug-loaded PLGA microspheres. (**A**) Synthesis procedures of MPs preparation. Abbreviations: PBS: phosphate-buffered saline; PVA: polyvinyl alcohol; PLGA: polylactic-co-glycolic acid. (**B**) Scanning electron microscope images of PLGA microspheres. The diameter of the microspheres ranges from ~1–10 μm (a–b). Degradation of the microspheres occurred after exposing to a moist environment (c–f). White arrow: pores caused by solvent evaporation. (**C-E**) Concentration curve of phenytoin (PHT), nifedipine (NIF) and cyclosporine (CSA) released respectively from PHT-PLGA (**C**), NIF-PLGA (**D**), CSA-PLGA (**E**).

bone in both morphology and function (Chen, Shelton, Jin, & Chapple, 2009). Different treatment options are currently available for regeneration of periodontal tissues, including barrier membranes, autografts, demineralized freeze-dried bone allografts, bovine-derived xenografts and combinations of membranes and fillers. Because of their unpredictable outcomes, these therapeutic strategies are seldom considered as effective regenerative procedures (Bosshardt & Sculean, 2009; Mitsiadis, Orsini, & Jimenez-Rojo, 2015).

Drug-induced gingival overgrowth is an adverse drug reaction that occurs in patients treated systematically with anti-epileptic drugs, calcium channel blockers or immunosuppressants such as phenytoin, nifedipine and cyclosporine (Brown & Arany, 2015; Dongari-Bagtzoglou, Research, Therapy Committee, & A. A. o. P., 2004). The potential of these drugs in causing gingival overgrowth is modified by the presence of bacterial plaque, gingival inflammation and genetic predisposition (Ramirez-Ramiz, Brunet-LLobet, Lahor-Soler, & Miranda-Rius, 2017). Despite differences in pharmaceutics and primary tissue targets, these drugs induce similar fibrous gingival overgrowth by enhancing collagen production and inhibiting collagen destruction (Mishra, Khan, & Mishra, 2011).

Compared with systemic administration, topical application of the aforementioned drugs has been shown to confer pharmaceutical benefits in the absence of systemic toxicity (Chan et al., 2007; Calabro et al., 2014; Kang et al., 2016). There is ample evidence showing that phenytoin induces bone turnover and increases the density of the periodontal ligament (Kanda et al., 2017; Karsten & Hellsing, 1997). Topical phenytoin has a better therapeutic outcome in wound healing (Chan et al., 2007; Pereira & Alchorne Ade, 2010) and promotes collagen deposition and neovascularization with increased expression of vascular endothelial growth factor and basic fibroblast growth factor in the wound tissue (Habibipour et al., 2003; Turan et al., 2004).

Nifedipine regulates periodontal ligament remodeling, inhibits bone resorption and increases bone density (Spolidorio et al., 2004; Zhang et al., 2008). Administration of cyclosporine has been associated with increased bone loss, increased bone remodeling (Nassar, Felipetti, Nassar, & Spolidorio, 2013), and induction of connective tissue growth factor (CTGF/CCN2) expression in gingival fibroblasts (Wu, Huang, Chen, Chang, & Deng, 2014). Results from the aforementioned studies suggest that topical application of phenytoin, nifedipine or cyclosporine may regulate periodontal tissue regeneration.

Polylactic-co-glycolic acid (PLGA) is a biodegradable polymer approved by the Food and Drug Administration and European Medicines Agency for human application because of its excellent biocompatibility (Hudson & Margaritis, 2014). Upon hydrolysis, the ester linkages in PLGA are severed, producing lactic acid and glycolic acid monomers that are metabolized by the Krebs cycle to produce carbon dioxide and water (Shenderova, Burke, & Schwendeman, 1999). The rate of PLGA degradation is affected by its molecular weight and the lactic acid-glycolic acid ratio (Kumari, Yadav, & Yadav, 2010). Polylactic-co-glycolic acid microspheres or nanoparticles, as well as their associated scaffolds, have been used as carriers for the controlled release of drugs in wound healing (Danhier et al., 2012). This biopolymer has been used in concert with active ingredients such as calcium phosphate (Carlo Reis et al., 2011), recombinant human growth/differentiation factor-5 (Park et al., 2012) and enamel matrix derivative (Plachokova, van den Dolder, & Jansen, 2008) for periodontal regeneration.

In the present study, gingival overgrowth-inducing drugs were incorporated into PLGA microspheres to evaluate the pharmacological effects on the gingival recession and alveolar bone loss in an *in vivo* murine periodontal defect model. The null hypothesis tested was that localized, topical application of gingival overgrowth-inducing drug-loaded PLGA microspheres has no effect on periodontal regeneration.

2. Materials and methods

2.1. Synthesis and characterization of PLGA microspheres

An oil-in-water emulsion solvent evaporation method was used to prepare PLGA microspheres (Naito et al., 2014). Briefly, polyvinyl alcohol (MilliporeSigma, St. Louis, MO, USA) was dissolved in 0.1 M phosphate-buffered saline (PBS) at 70–95 °C in a water bath to produce 0.5% polyvinyl alcohol (the water phase). Phenytoin (10 mg, MilliporeSigma), nifedipine (10 mg, MilliporeSigma) and cyclosporine (10 mg, Selleck Chemicals, Houston, TX, USA) was respectively dissolved in methylene chloride. Sixty milligrams of PLGA (lactide:glycolide ratio 50:50, Mw 30,000-60,000, MilliporeSigma) was added to produce a clear solution (the oil phase). The oil phase was decanted into the water phase at room temperature (Fig. 1A). The mixture was emulsified for 5 min with a homogenizer at 5000 rpm, forming a highly-fragmented, milky oil-in-water emulsion. The emulsion was stirred at 400 rpm at 35 °C for 4 h to evaporate the solvent. The microspheres were freeze-dried and sterilized with Cobalt-60 at 3 kGy. Morphology of the PLGA microspheres was examined using scanning electron microscopy (Quanta 200, FEI, USA) at 20 kV.

2.2. In vitro drug release

Biopolymer degradation and drug release were examined by incubating the PLGA microspheres in phosphate-buffered saline (PBS) containing 0.02% Tween 80 at 37 °C. Drug concentration was analyzed by reversed phase high-performance liquid chromatography (Model 1100, Agilent Technologies, Santa Clara, CA, USA) using a gradient elution method with mobile phases. A solution containing 60–80% methanol was used for phenytoin, 80–100% methanol for nifedipine, and a mixture of 21.5% acetonitrile, 2.5% methyl tert-butyl ether and 0.5% phosphoric acid for cyclosporine. Ultraviolet absorbance was measured at 220 nm for phenytoin, 235 nm for nifedipine and 210 nm for cyclosporine.

2.3. Murine periodontal defect model for in vivo delivery of drug-loaded PLGA microspheres

Forty male Sprague-Dawley rats (220–250 g) were obtained from the Animal Center of Wuhan University. Animal care was performed according to institutional guidelines. The experimental protocol was approved by the Research Ethics Committee of the School of Stomatology, Wuhan University and was performed in accordance with the policy on human care, use of laboratory animals and the “Animal Research: Reporting of *in vivo* Experiments” guidelines (Faggion, 2015). All rats received a standardized diet throughout the procedures. A randomized, single-blind controlled study was performed over an 8-week period. The rats were randomly assigned to 4 groups (N = 10) and designated as PHT-PLGA for phenytoin, NIF-PLGA for nifedipine, CSA-PLGA for cyclosporine and PBS-PLGA for the PBS-loaded control. The right maxillary molars and periodontal tissues of each rat were designated as the experimental side; the left maxillary molars and periodontal tissues were used as the control side. The rats were sacrificed at the end of 8 weeks after drug delivery. The experimental time was set at 8 weeks based on the time required for bone remodeling and degradation of the drug carrier.

The surgical site was exposed at the alveolar bone distal to the first upper molar in a sterile environment. Full thickness incision through the mesial gingiva was made with a number 11 blade. A pair of tweezers was used to separate the gingiva flap and expose the mesial alveolar bone. A 3-mm diameter, 1.5-mm deep bony defect was created with a low-speed handpiece on the mesial alveolar bone of each maxillary first molar (Shirakata et al., 2014; Zhang et al., 2012) (Fig. 2A). The bur was cooled with PBS to avoid osteonecrosis. One week after the surgical procedures, drug-loaded PLGA microspheres were placed

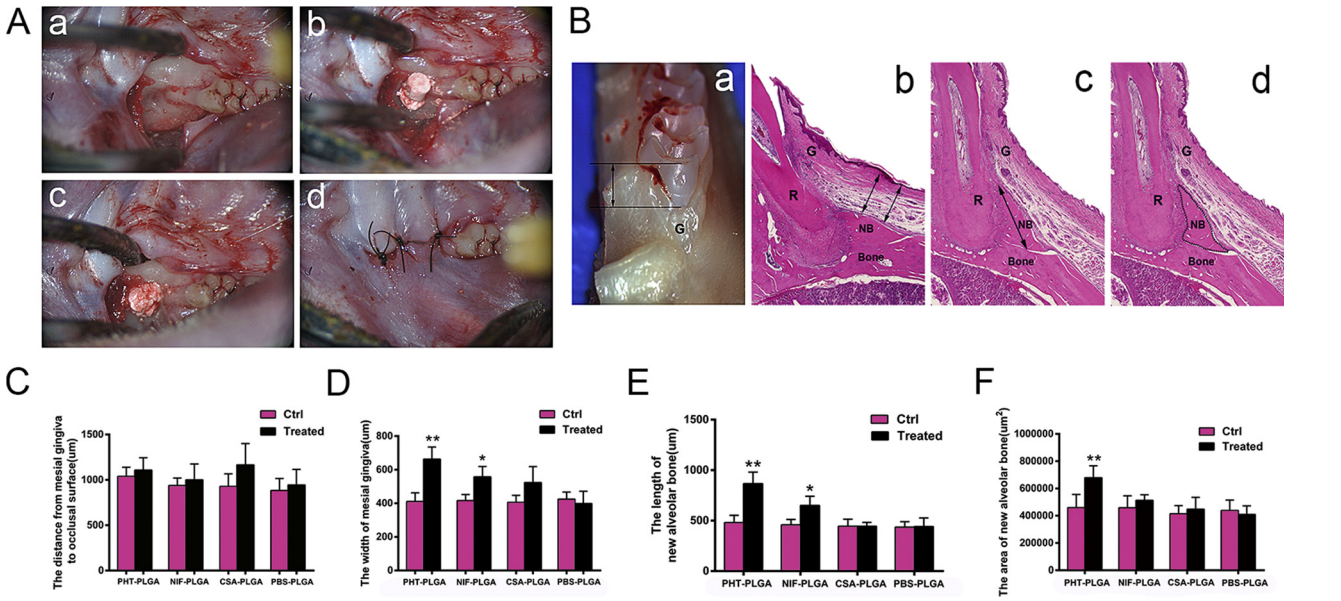

Fig. 2. Delivery of drug-loaded PLGA microspheres and local effects of phenytoin (PHT), nifedipine (NIF) and cyclosporine (CSA) on the gingiva and alveolar bone. (**A**) *In vivo* procedures. (a) preparation of alveolar bone defect; (b) delivery of drug-loaded microspheres to the alveolar bone defect; (c) compaction of microspheres into the defect; (d) wound closure. (**B**) Evaluation of periodontal tissue remodeling. Abbreviations: G, mesial gingiva; R, mesial root; NB, new alveolar bone. (a) measurement of the distance from the mesial gingival margin to the occlusal surface. (b) measurement of the distance from the alveolar bone periosteum to the basal membrane underlying the squamous epithelium. (c) measurement of the vertical distance from the bottom of the intrabony defect to the most coronal extension of new bone along the root surface. (d) measurement of the area of new alveolar bone. (**C-F**) Results of periodontal tissue remodeling after drug delivery. There was no significant difference in the height of the mesial gingiva among the four groups (**C**). The width of mesial gingiva and the length and area of new alveolar bone in the PHT-PLGA group were significantly larger than those in the control group (double asterisks; $p < 0.01$). The first two measurements were also significantly larger in the NIF-PLGA group than in the control group (asterisks; $p < 0.05$) (**D-F**).

inside the alveolar bone defects and compacted to ensure that the microspheres remained stationary. The wound was closed with 7-0 absorbable sutures (Covidien™, Medtronic, Dublin, Ireland). Defects were created on both sides of the maxilla and all surgical procedures were performed by a single operator.

During the experiments, two rats in the CSA-PLGA group died of abdominal distension because of overeating or intestinal obstruction. After the 8-week period, the remaining 38 rats were sacrificed with an overdose of pentobarbital sodium delivered *via* intraperitoneal injection. The maxillary periodontal tissues of each specimen, including the alveolar bone, molars and gingiva, were harvested. Adequate measures were taken to minimize the pain that the rats suffered during this process. After tissue fixation in 4% paraformaldehyde for 48 h, the specimens were observed under a stereomicroscope, and the distance from the mesial gingival margin to the occlusal surface of molars was measured.

2.4. Micro-computed tomography

Analysis of maxillary bones was performed using μ-CT 50 (Scanco Medical AG, Switzerland) at 80 kV, 80 mA, with an exposure time of 1600 msec/frame at 4 frames/view to obtain 900 views. The acquired images were reconstructed with 3D reconstruction software (Mimics Innovation Suite v17.1, Materialise, Leuven, Belgium) at 15-μm spatial resolution. For analysis of maxillary bones, the regions of interest were the mesial root and alveolar bone of maxillary first molars.

2.5. Hematoxylin-eosin and Masson trichrome staining

The 76 specimens (38 × 2) were demineralized and embedded in paraffin. Each paraffin block was sliced along the mesiodistal direction of the molar to generate 5 μm-thick serial sections. At least 30 sections of each block were obtained. Five sections were selected in the mid-buccolingual region of each molar, where the most optimal views of the mesial gingival tissues and alveolar bone could be obtained. The sections were stained with hematoxylin-eosin or Masson trichrome.

Histomorphometric analysis of the alveolar bone and gingiva were performed using hematoxylin-eosin stained sections. The cross-sectional area of the newly-formed bone above the site of the alveolar bone defect was defined as the area of new alveolar bone (Shirakata et al., 2014). The vertical distance from the bottom of the intrabony defect to the most coronal extension of new bone along the root surface was defined as the length of new bone (Shirakata et al., 2014). The vertical distance from the marginal gingiva to the occlusal surface of the maxillary molars was used as the indicator for gingival recession (Fig. 2B–a). The distance from the alveolar bone periosteum to the basal membrane underlying the squamous epithelium was defined as the width of gingiva (Bullon et al., 2007). Images were analyzed using Image-Pro Plus (Media Cybernetics, Rockville, MD, USA).

2.6. Immunohistochemical analyses

2.6.1. Osteoblastic-related and collagen-related markers

Immunohistochemistry was performed on paraffin-embedded sections using an avidin-biotinylated peroxidase enzyme complex-based kit (UltraSensitive SP IHC Kit, Maixin Biotech, Fuzhou, China) in conjunction with a DAB detection kit (Maixin Biotech) to produce a brown precipitate (Foster, 2012). Sections were counterstained with hematoxylin. Primary antibodies included transforming growth factor-β1 (TGF-β1, 1:30; Santa Cruz Biotechnology, Dallas, TX, USA), bone morphogenetic protein-2 (BMP-2, 1:200; Santa Cruz), type I collagen (COL-1, 1:400; Santa Cruz), matrix metalloproteinase-8 (MMP-8, 1:300; Abcam, Cambridge, MA, USA), osteocalcin (OCN, 1:150; Abcam) and vascular endothelial growth factor-a (VEGF-A, 1:200; Proteintech, Rosemont, IL, USA).

2.6.2. Tartrate-resistant acid phosphatase (TRAP) staining

Staining was performed with the TRAP assay kit (387A-1KT,

MilliporeSigma) according to the manufacturer's instructions. Deparaffinized and rehydrated tissue slides were incubated with light protection at 37 °C in a mixture of fast garnet GBC base solution, sodium nitrite, naphthol AS-BI phosphate, acetate and tartrate. The sections were counterstained with hematoxylin.

2.6.3. Sirius red staining

Deparaffinized and rehydrated sections were stained with a connective tissue staining kit (Picro Sirius Red, Abcam) and counterstained with hematoxylin to reveal the status of the newly-formed collagen tissues.

2.7. Statistical analyses

Quantitative data were expressed as mean and standard deviations. Data were analyzed for their normality and homoscedasticity assumptions prior to the use of parametric statistical methods; nonlinear transformation of the data sets was otherwise performed to conform to those assumptions. Data from the four groups were subsequently analyzed with one-factor analysis of variance and Holm-Šidák pairwise comparison procedures. Statistical significance was preset at $\alpha = 0.05$.

3. Results

3.1. Synthesis and characterization of drug-loaded PLGA microspheres

Drug-loaded PLGA microspheres appeared as white or yellow powders, consistent with the color of the drugs. The microspheres were predominantly 1–10 μm in diameter (Fig. 1B, a–b). Degradation, adhesion and fusion of the microspheres occurred after incubation in simulated *in vivo* condition for 24 h (Fig. 1B, c–f). The microspheres had a smooth spherical surface with pores that resulted from solvent volatilization. Drug release decreased with incubation time in both the PHT-PLGA (Fig. 1C) and NIF-PLGA MPs groups (Fig. 1D). Because of the high molecular weight or biologic degradation of CSA, drug release in the CSA-PLGA group remained at a very low level after the initial burst release (Fig. 1E).

3.2. Local effects of drug-loaded PLGA microspheres on the gingiva and alveolar bone

The vertical distance from the marginal gingiva to the occlusal surface of the maxillary molars was used as the indicator for gingival recession (Fig. 2B–a). No significant difference was detected among all groups (Fig. 2C). Mesial gingival width, length and sectional area of new alveolar bone (Fig. 2B, b–d) were measured in the paraffin sections according to previous methods (Bullon et al., 2007; Shirakata et al., 2014; Spoildorio, Spolidorio, Neves, Gonzaga, & Almeida, 2002). Compared with the PBS-PLGA group and control subgroups (further shown in the Masson trichrome-stained sections in Fig. 3A), the PHT-PLGA experimental group had significantly thicker mesial gingiva ($p < 0.0001$; Fig. 2D), longer length ($p = 0.0003$; Fig. 2E) and larger area of new alveolar bone ($p = 0.006$; Fig. 2F). The mesial gingiva ($p = 0.015$) and length of new alveolar bone ($p = 0.039$) were also significantly higher in the NIF-PLGA group, compared with its control subgroup, while there was no significant difference among the CSA-PLGA, PBS-PLGA and their corresponding control subgroups. Micro-computed tomography showed that there was more alveolar bone mass around the mesial root in the PHT-PLGA experimental group than in the corresponding control subgroup (Fig. 3B–C).

3.3. Effects of PLGA-loaded drugs on periodontal tissue regeneration

Immunohistochemistry of COL-1, MMP-8 and Sirius red staining were performed for the expression of extracellular matrix, fibrosis and collagen synthesis (Fig. 4A–C). The PHT-PLGA and NIF-PLGA experimental groups had increased COL-1 and reduced MMP-8 expressions in the gingival tissues (Fig. 4A–B). Under the polarized light microscope, the color intensity of type I collagen fibers (stained bright yellow or orange) in Sirius red-stained sections were also stronger in the PHT-PLGA experimental group when compared than the other groups (Fig. 4C), validating the increase in COL-1 expression. Overexpression of VEGF-A has various meanings, including angiogenesis, vasculogenesis and endothelial cell growth. Expression of VEGF-A was more pronounced in the PHT-PLGA experimental group (Fig. 4D), an indicator of the prominent activity associated with the vascular endothelial cells in this group.

Assays of BMP-2, TGF-β1, OCN and TRAP-staining were conducted to determine the expression of osteoblast and osteoclast markers in the new alveolar bone. The PHT-PLGA experimental group had stronger positive staining of the osteoblast-specific factors BMP-2, TGF-β1 and OCN, compared with other groups (Fig. 5A–C). Likewise, the PLGA experimental group also demonstrated more intensive TRAP-staining that was is associated with osteoclastic activities (Fig. 5D).

4. Discussion

In the present murine periodontal tissue defect model, a defect was made on the alveolar ridge mesial to the maxillary first molar on both sides of the maxilla, with critical damage of the mesial root of the first molar. In previous murine studies, a periodontal fenestration defect was usually created on the buccal surface of the mandibular alveolar bone to expose the distal and buccal roots of the first molar and buccal roots of the second molar (Pellegrini, Seol, Gruber, & Giannobile, 2009). Considering the craniofacial features of rats, it is more rational to establish the intraoral model in the maxillary teeth. Compared with the extraoral animal model, the anatomical and biological features of the present intraoral animal model better simulate human periodontal defects. There was also immediate contact of the tested drugs with periodontal ligament cells; the latter have been considered indispensable for periodontal regeneration (Sculean, Chapple, & Giannobile, 2015).

The use of localized controlled-release drug delivery systems such as PLGA enables the largest fraction of drug molecules to be delivered at or near the site of action and reduces systemic drug toxicity (Weiser & Saltzman, 2014). Depending on the monomer ratio and the polymer molecular weight, PLGA degradation may be tuned to occur in days or months (Makadia & Siegel, 2011). In the present work, the lactide:glycolide ratio was set to 50:50 and the molecular weight was approximately 30,000–60,000. Because of these attributes, the presently-employed PLGA takes approximately 2 months to be completely degraded. Particle size is also one of the main factors affecting the viability controlled-delivery system (Pagels & Prud'homme, 2015). The diameter of the PLGA microspheres is 1–10 μm, which is small enough to be delivered into alveolar bone defects of varying sizes and shapes.

It is critical that the gingival overgrowth-inducing drugs are released continuously at a slow, steady rate. Hence, PLGA was used for coupling these drugs through an emulsion and condensation method. This minimized potential deficiencies associated with localized topical application, such as too high concentration or too rapid degradation (Weiser & Saltzman, 2014). The drug-loaded PLGA-microspheres were delivered to the alveolar bone defect and compacted to ensure that they remained stationary and achieved immediate contact with periodontal ligament cells that repopulated the wound area. The PLGA microspheres also acted as a biological scaffold to guide the spreading of periodontal ligament cells along with the microspheres.

Compared with the PBS-loaded and other drug-loaded microspheres, the phenytoin-loaded PLGA microspheres were capable of inducing more collagen production in the gingival tissues and more extensive bone regeneration. Apart from the increased width of mesial gingiva associated with the use of the phenytoin-loaded PLGA microspheres, immunohistochemistry of tissue sections also identified increased expressions of COL-1 and VEGF-A, decreased expression of

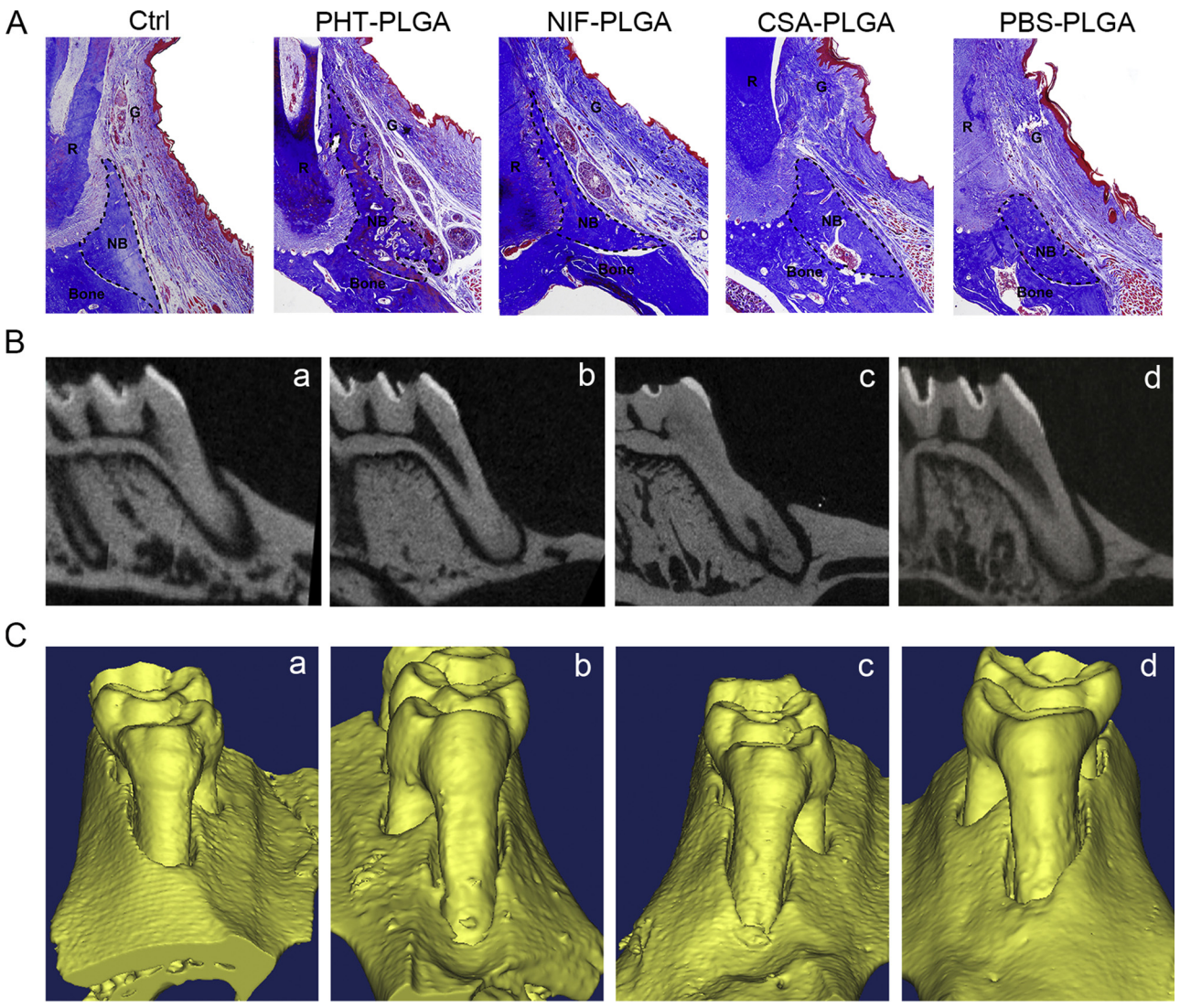

Fig. 3. Masson trichrome staining and micro-computed tomography of the control (Ctrl) and experimental groups. Abbreviations: PHT, phenytoin; NIF, nifedipine; CSA, cyclosporine; G, mesial gingiva; R, mesial root; NB, new alveolar bone. (**A**) Masson trichrome staining, showing new alveolar bone formation eight weeks after delivery of drug-loaded PLGA microspheres (inside the dashed lines). (**B-C**) Micro-computed tomography of coronal sections and 3-dimensional reconstruction of untreated alveolar bone (**B**-a, **C**-a), the alveolar bone defect (**B**-b, **C**-b), the control group (**B**-c, **C**-c), and the PHT-PLGA group (**B**-d, **C**-d).

MMP-8, increased expression of osteoblast markers, including TGF-β1, BMP-2, and OCN, and increased TRAP staining areas specific for osteoclasts. Accordingly, the null hypothesis that "localized, topical application of gingival overgrowth-inducing drug-loaded PLGA microspheres has no effect on periodontal regeneration" has to be rejected. Previous studies indicated that phenytoin-induced gingival overgrowths are the most fibrotic, whereas cyclosporine-induced lesions are highly-inflamed and the least fibrotic; fiber characteristics of nifedipine-induced lesions lie between those two ends of the spectrum (Trackman & Kantarci, 2015; Uzel et al., 2001). These outcomes are similar to the results obtained in the present study.

Although the risk of bone loss/fracture is considerably higher with the long-term use of phenytoin in the absence of vitamin D supplementation (Nakken & Taubøll, 2010), low doses of phenytoin induces proliferation and differentiation of human bone cells to stimulate bone formation (Nakade, Baylink, & Lau, 1995). At micromolar concentrations, phenytoin also inhibits osteoclast differentiation *via* suppression of Nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) signaling to prevent bone loss (Koide et al., 2009). When administered locally and released at a controlled low concentration, phenytoin-loaded PLGA microspheres promoted alveolar bone formation with increased expression of osteogenic markers. These findings indicate that phenytoin possesses dual effects on bone turnover that are dependent on the route of administration and dosage. The osteogenic stimulation effect of phenytoin is dose-dependent, being the most optimal at 5–50 μM in the *in vitro* setting (Nakade, Baylink, & Lau, 1996); osteogenic stimulation also requires long-term delivery of the medication at low concentrations. Although these effects were demonstrated in the present work, the optimal *in vivo* drug concentration that is necessary for stimulating the targeted tissues remains unknown. The optimal phenytoin concentrations required for bone proliferation, osteoblast differentiation, alkaline phosphatase activity, osteocalcin secretion and collagen synthesis had been reported to be different and varied from 1 to 50 μM (Nakade, Baylink, & Lau, 1995). Hence, determining the optimal phenytoin concentration for periodontal regeneration is an important topic for future studies.

The mechanism in which phenytoin increases bone formation and collagen synthesis is incompletely known. Transforming growth factor-β (TGF-β), connective tissue growth factor (CTGF/CCN2), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), angiotensin II (Ang II) and endothelin-1 (ET-1) are likely to be key driving forces (Nakken & Taubøll, 2010). The osteogenic effects of phenytoin are

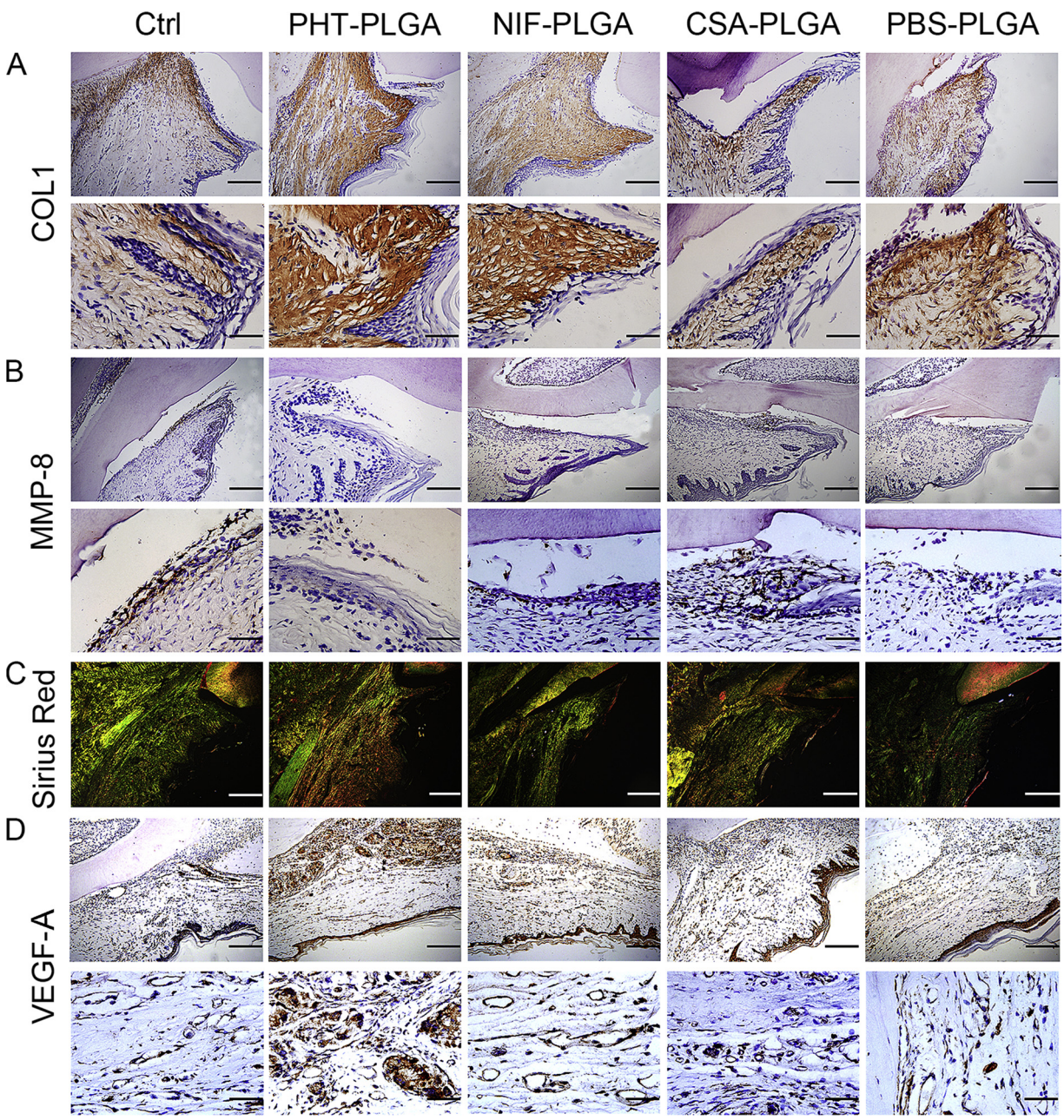

Fig. 4. Effects of PLGA-loaded drugs on extracellular matrix deposition and vascularization of gingival tissues. (**A**) Immunohistochemical assay of COL-1. (**B**) Immunohistochemical assay of MMP-8. (**C**) Sirius red staining. (**D**) Immunohistochemical assay of VEGF-A. Scale bars: 50 μm (upper row) and 100 μm (lower row) for each marker.

partially mediated through the release of TGF-β1 in bone cells (Nakken & Taubøll, 2010). CTGF/CCN2 is a critical modulator of matrix production during tissue remodeling (Norris et al., 2007). The levels of CTGF/CCN2 are highest in phenytoin-induced gingival overgrowths, intermediate in nifedipine-induced lesions and nearly absent from cyclosporine-induced lesions (Uzel et al., 2001). These findings are similar to the results of the present study, validating the inadequate anabolic efficiency of nifedipine and cyclosporine.

Phenytoin is a potential universal anabolic agent for most connective tissue-derived cells, including bone cells and gingival tissue. Nifedipine and cyclosporine do not represent impressive manifestations in the present preclinical study. Nifedipine results in a less fibrotic lesion than phenytoin, as well as lower staining levels of collagen density, with less alveolar bone mass and reduced osteogenic activity (Figs. 2F,). In addition, because nifedipine is susceptible to photolysis, it has limited clinical applications. Cyclosporine degraded rapidly in the HPLC analysis (Fig. 1E). As a cyclic polypeptide immunosuppressant agent consisting of eleven amino acids, cyclosporine is usually administered orally or with intravenous medication. The polypeptide probably degraded soon after intraoral topical delivery. Accordingly, phenytoin is the best choice among the three drugs examined for PLGA loading.

Although the presents findings provide new evidence suggesting that phenytoin possesses therapeutic effects on both gingival recession and alveolar bone loss, the work was conducted using an 8-week follow-up period. These findings should be validated using long-term *in vivo* follow-up protocols.

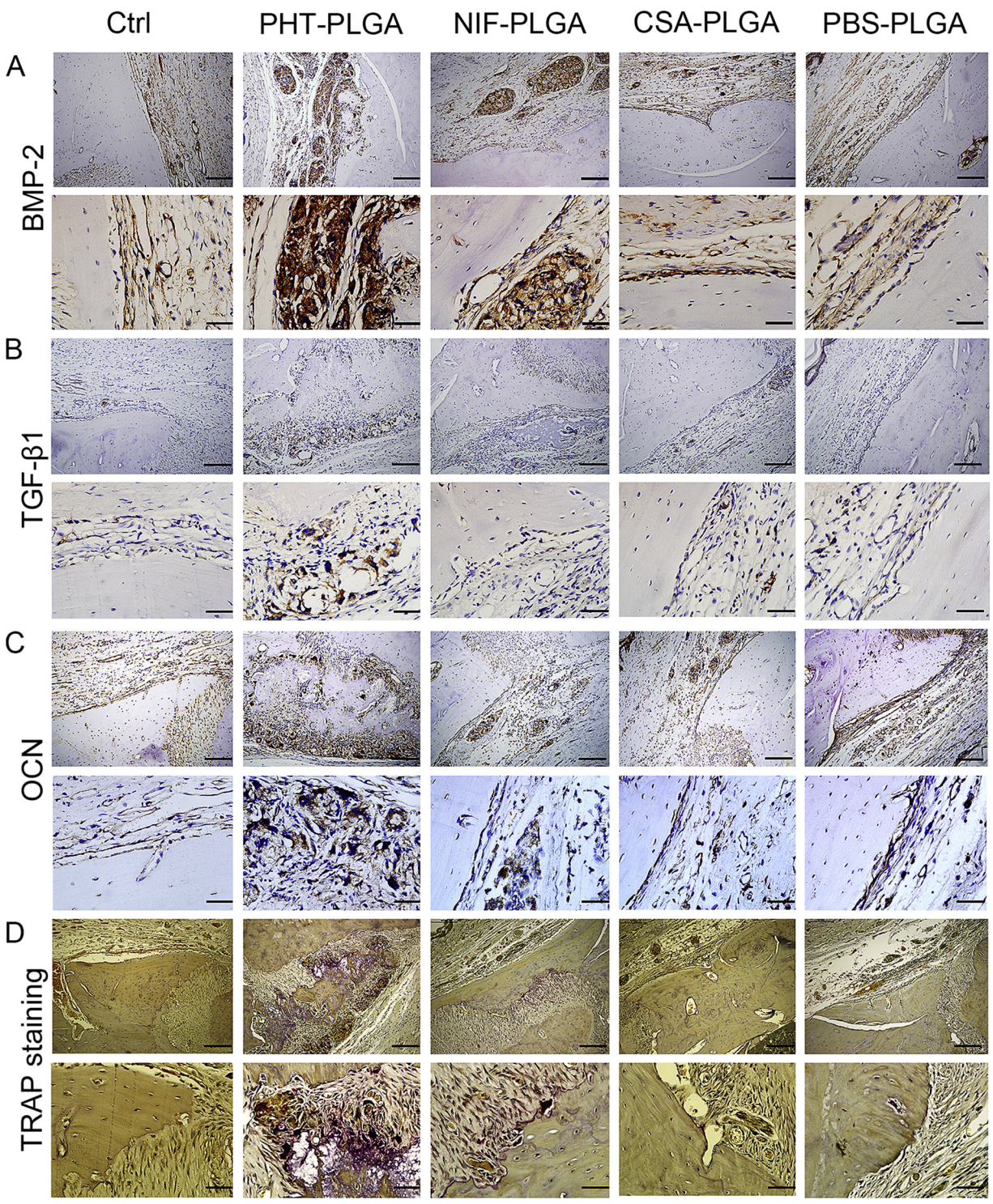

Fig. 5. Effects of drug-loaded PLGA microspheres on the expression of markers of alveolar bone remodeling. (**A-C**) Immunohistochemical assays of BMP-2, TGF-β1, and OCN. Brown precipitates were indicative of positive expression of osteoblast markers. (**D**) TRAP staining. Red precipitates were indicative of positive expression of osteoclast marker. Scale bars: 50 μm (upper row) and 100 μm (lower row) for each marker.

Conflict of interest

All the authors have approved the manuscript and declared no potential conflicts of interest with respect to the authorship and/or publication of this article.

Acknowledgments

The authors deny any perceived or actual conflicts of interest. The present work was funded by National Natural Science Foundation of China (81470727 and 81571438 to Zhuan Bian and Liu-Yan Meng), National key research and development program (2016YFC1000505) and Hubei Province's Outstanding Medical Academic Leader Program to Zhuan Bian, and Applied Basic Research Program of Wuhan, China

(2014062801011267) to Liu-Yan Meng. The authors thank Mr. Zhi-Yong Chen for animal technical support, Ms. Han-Ping Li for histological specimen preparation, Mr. Jian-She He for technical guidance in the HPLC assay and Ms. Qian Gao for her help in the animal experiment. The authors thank Dentsply Sirona Inc. (China) for providing a plugger to simplify the process of drug delivery.

References

Bloom, B., Simile, C. M., Adams, P. F., & Cohen, R. A. (2012). Oral Health status and access to oral Health care for U.S. adults aged 18-64: National Health Interview Survey, 2008. *Vital and Health Statistics Series, 10*(253), 1–22.. https://www.ncbi.nlm.nih.gov/pubmed/23077776.

Bosshardt, D. D., & Sculean, A. (2009). Does periodontal tissue regeneration really work? *Periodontology, 2000*(51), 208–219. https://doi.org/10.1111/j.1600-0757.2009.00317.x.

Brown, R. S., & Arany, P. R. (2015). Mechanism of drug-induced gingival overgrowth revisited: A unifying hypothesis. *Oral Disease, 21*(1), e51–61. https://doi.org/10.1111/odi.12264.

Bullon, P., Gallardo, I., Goteri, G., Rubini, C., Battino, M., Ribas, J., ... Newman, H. N. (2007). Nifedipine and cyclosporin affect fibroblast calcium and gingiva. *Journal of Dental Research, 86*(4), 357–362. https://doi.org/10.1177/154405910708600411.

Calabro, G., De Vita, V., Patalano, A., Mazzella, C., Lo Conte, V., & Antropoli, C. (2014). Confirmed efficacy of topical nifedipine in the treatment of facial wrinkles. *Journal of Dermatological Treatment, 25*(4), 319–325. https://doi.org/10.3109/09546634.2013.802759.

Chan, H. L., Chun, Y. H., MacEachern, M., & Oates, T. W. (2015). Does gingival recession require surgical treatment? *Dental Clinics of North America, 59*(4), 981–996. https://doi.org/10.1016/j.cden.2015.06.010.

Chan, F. C., Kennedy, C., Hanson, R. P., O'Sullivan, B., Kelly, J., & Bouchier-Hayes, D. (2007). Topical diphenylhydantoin sodium can improve healing in a diabetic incisional animal wound model. *Journal of Wound Care, 16*(8), 359–363. https://doi.org/10.12968/jowc.2007.16.8.27858.

Chen, F. M., Shelton, R. M., Jin, Y., & Chapple, I. L. (2009). Localized delivery of growth factors for periodontal tissue regeneration: Role, strategies, and perspectives. *Medicinal Research Review, 29*(3), 472–513. https://doi.org/10.1002/med.20144.

Danhier, F., Ansorena, E., Silva, J. M., Coco, R., Le Breton, A., & Préat, V. (2012). PLGA-based nanoparticles: An overview of biomedical applications. *Journal of Controlled Release, 161*(2), 505–522. https://doi.org/10.1016/j.jconrel.2012.01.043.

Dongari-Bagtzoglou, A., Research, S., & Therapy Committee, A. A. o. P (2004). Drug-associated gingival enlargement. *Journal of Periodontology, 75*(10), 1424–1431. https://doi.org/10.1902/jop.2004.75.10.1424.

Faggion, C. M., Jr. (2015). Animal research as a basis for clinical trials. *European Journal of Oral Sciences, 123*(2), 61–64. https://doi.org/10.1111/eos.12175.

Foster, B. L. (2012). Methods for studying tooth root cementum by light microscopy. *International Journal of Oral Science, 4*(3), 119–128. https://doi.org/10.1038/ijos.2012.57.

Habibipour, S., Oswald, T. M., Zhang, F., Joshi, P., Zhou, X. C., Dorsett-Martin, W., ... Lineaweaver, W. C. (2003). Effect of sodium diphenylhydantoin on skin wound healing in rats. *Plastic and Reconstructive Surgery, 112*(6), 1620–1627. https://doi.org/10.1097/01.PRS.0000086773.96319.DA.

Hudson, D., & Margaritis, A. (2014). Biopolymer nanoparticle production for controlled release of biopharmaceuticals. *Critical Review in Biotechnology, 34*(2), 161–179. https://doi.org/10.3109/07388551.2012.743503.

Kanda, J., Izumo, N., Kobayashi, Y., Onodera, K., Shimakura, T., Yamamoto, N., ... Wakabayashi, H. (2017). Effects of the antiepileptic drugs phenytoin, gabapentin, and levetiracetam on bone strength, bone mass, and bone turnover in rats. *Biological & Pharmaceutical Bulletin, 40*(11), 1934–1940. https://doi.org/10.1248/bpb.b17-00482.

Kang, H., Cha, K. H., Cho, W., Park, J., Park, H. J., Sun, B. K., ... Hwang, S. J. (2016). Cyclosporine amicellar delivery system for dry eyes. *International Journal of Nanomedicine, 11*, 2921–2933. https://doi.org/10.2147/IJN.S107569.

Karsten, J., & Hellsing, E. (1997). Effect of phenytoin on periodontal tissues exposed to orthodontic force–an experimental study in rats. *British Journal of Orthodontics, 24*(3), 209–215. https://doi.org/10.1093/ortho/24.3.209.

Koide, M., Kinugawa, S., Ninomiya, T., Mizoguchi, T., Yamashita, T., Maeda, K., ... Udagawa, N. (2009). Diphenylhydantoin inhibits osteoclast differentiation and function through suppression of NFATc1 signaling. *Journal of Bone and Mineral Research, 24*(8), 1469–1480. https://doi.org/10.1359/jbmr.090302.

Kumari, A., Yadav, S. K., & Yadav, S. C. (2010). Biodegradable polymeric nanoparticles based drug delivery systems. *Colloids and Surfaces B Biointerfaces, 75*(1), 1–18. https://doi.org/10.1016/j.colsurfb.2009.09.001.

Makadia, H. K., & Siegel, S. J. (2011). Poly lactic-co-glycolic acid (PLGA) as biodegradable controlled drug delivery carrier. *Polymers (Basel), 3*(3), 1377–1397. https://doi.org/10.3390/polym3031377.

Merijohn, G. K. (2016). Management and prevention of gingival recession. *Periodontology, 71*(1), 228–242. https://doi.org/10.1111/prd.12115 2000.

Mishra, M. B., Khan, Z. Y., & Mishra, S. (2011). Gingival overgrowth and drug association: A review. *Indian Journal of Medical Sciences, 65*(2), 73–82. https://doi.org/10.4103/0019-5359.103971.

Mitsiadis, T. A., Orsini, G., & Jimenez-Rojo, L. (2015). Stem cell-based approaches in dentistry. *European Cell & Materials, 30*, 248–257. https://doi.org/10.22203/eCM.v030a17.

Naito, Y., Terukina, T., Galli, S., Kozai, Y., Vandeweghe, S., Tagami, T., ... Jimbo, R. (2014). The effect of simvastatin-loaded polymeric microspheres in a critical size bone defect in the rabbit calvaria. *International Journal of Pharmaceutics, 461*(1-2), 157–162. https://doi.org/10.1016/j.ijpharm.2013.11.046.

Nakade, O., Baylink, D. J., & Lau, K. H. (1995). Phenytoin at micromolar concentrations is an osteogenic agent for human-mandible-derived bone cells in vitro. *Journal of Dental Research, 74*(1), 331–337. https://doi.org/10.1177/00220345950740010801.

Nakade, O., Baylink, D. J., & Lau, K. H. (1996). Osteogenic actions of phenytoin in human bone cells are mediated in part by TGF-beta1. *Journal of Bone and Mineral Research, 11*, 1880–1888. https://doi.org/10.1002/jbmr.5650111208.

Nakken, K. O., & Taubøll, E. (2010). Bone loss associated with use of antiepileptic drugs. *Expert Opinion on Drug Safety, 9*(4), 561–571. https://doi.org/10.1517/14740331003636475.

Nassar, P. O., Felipetti, F. A., Nassar, C. A., & Spolidorio, L. C. (2013). Evaluation of effect of cyclosporine a on the bone tissue with induced periodontal disease to ligature in rats. *Transplantation Proceedings, 45*(2), 778–782. https://doi.org/10.1016/j.transproceed.2012.02.029.

Norris, R. A., Damon, B., Mironov, V., Kasyanov, V., Ramamurthi, A., Moreno-Rodriguez, R., ... Markwald, R. R. (2007). Periostin regulates collagen fibrillogenesis and the biomechanical properties of connective tissues. *Journal of Cellular Biochemistry, 101*(3), 695–711. https://doi.org/10.1002/jcb.21224.

Pagels, R. F., & Prud'homme, R. K. (2015). Polymeric nanoparticles and microparticles for the delivery of peptides, biologics, and soluble therapeutics. *Journal of Controlled Release, 219*, 519–535. https://doi.org/10.1016/j.jconrel.2015.09.001.

Park, J. C., Wikesjö, U. M., Koo, K. T., Lee, J. S., Kim, Y. T., Pippig, S. D., ... Kim, C. K. (2012). Maturation of alveolar bone following implantation of an rhGDF-5/PLGA composite into 1-wall intra-bony defects in dogs: 24-week histometric observations. *Journal of Clinical Periodontology, 39*(6), 565–573. https://doi.org/10.1111/j.1600-051X.2012.01868.x.

Pellegrini, G., Seol, Y. J., Gruber, R., & Giannobile, W. V. (2009). Pre-clinical models for oral and periodontal reconstructive therapies. *Journal of Dental Research, 88*(12), 1065–1076. https://doi.org/10.1177/0022034509349748.

Pereira, C. A., & Alchorne Ade, O. (2010). Assessment of the effect of phenytoin on cutaneous healing from excision of melanocytic nevi on the face and on the back. *BMC Dermatology, 10*, 7. https://doi.org/10.1186/1471-5945-10-7.

Plachokova, A. S., van den Dolder, J., & Jansen, J. A. (2008). The bone-regenerative properties of Emdogain adsorbed onto poly(D,L-lactic-coglycolic acid)/calcium phosphate composites in an ectopic and an orthotopic rat model. *Journal of Periodontal Research, 43*(1), 55–63. https://doi.org/10.1111/j.1600-0765.2007.00994.x.

Pradeep, K., Rajababu, P., Satyanarayana, D., & Sagar, V. (2012). Gingival recession: Review and strategies in treatment of recession. *Case Report in Dentistry, 2012*. https://doi.org/10.1155/2012/563421 563421.

Ramirez-Ramiz, A., Brunet-LLobet, L., Lahor-Soler, E., & Miranda-Rius, J. (2017). On the cellular and molecular mechanisms of drug-induced gingival overgrowth. *The Open Dentistry Journal, 11*, 420–435. https://doi.org/10.2174/1874210601711010420.

Reis, C., Borges, E. C., Araújo, A. P., Mendes, M. V., Guan, V. C. L., & Davies, J. E. (2011). Periodontal regeneration using a bilayered PLGA/calcium phosphate construct. *Biomaterials, 32*(35), 9244–9253. https://doi.org/10.1016/j.biomaterials.2011.08.040.

Sculean, A., Chapple, I. L., & Giannobile, W. V. (2015). Wound models for periodontal and bone regeneration: The role of biological research. *Periodontology, 68*(1), 7–20. https://doi.org/10.1111/prd.12091 2000.

Serino, G., Wennstrom, J. L., Lindhe, J., & Eneroth, L. (1994). The prevalence and distribution of gingival recession in subjects with a high standard of oral hygiene. *Journal of Clinical Periodontology, 21*(1), 57–63. https://doi.org/10.1111/j.1600-051X.1994.tb00278.x.

Shenderova, A., Burke, T. G., & Schwendeman, S. P. (1999). The acidic microclimate in poly(lactide-co-glycolide) microspheres stabilizes camptothecins. *Pharmaceutical Research, 16*(2), 241–248. https://doi.org/10.1023/A:1018876308346.

Shirakata, Y., Eliezer, M., Nemcovsky, C. E., Weinreb, M., Dard, M., Sculean, A., & Moses, O. (2014). Periodontal healing after application of enamel matrix derivative in surgical supra/infrabony periodontal defects in rats with streptozotocin-induced diabetes. *Journal of Periodontal Research, 49*(1), 93–101. https://doi.org/10.1111/jre.12084.

Spoildorio, L. C., Spolidorio, D. M., Neves, K. A., Gonzaga, H. F., & Almeida, O. P. (2002). Morphological evaluation of combined effects of cyclosporin and nifedipine on gingival overgrowth in rats. *Journal of Periodontal Research, 37*(3), 192–195. https://doi.org/10.1034/j.1600-0765.2002.00028.x.

Spolidorio, L. C., Spolidorio, D. M., Nassar, P. O., Nassar, C. A., Holzhausen, M., & Almeida, O. P. (2004). Influence of age on combined effects of cyclosporin and nifedipine on rat alveolar bone. *Journal of Periodontology, 75*(2), 268–272. https://doi.org/10.1902/jop.2004.75.2.268.

Trackman, P. C., & Kantarci, A. (2015). Molecular and clinical aspects of drug-induced gingival overgrowth. *Journal of Dental Research, 94*(4), 540–546. https://doi.org/10.1177/0022034515571265.

Turan, M., Saraydýn, S. U., Bulut, H. E., Elagöz, S., Cetinkaya, O., Karadayi, K., ... Sen, M. (2004). Do vascular endothelial growth factor and basic fibroblast growth factor promote phenytoin's wound healing effect in rat? An immunohistochemical and histopathologic study. *Dermatologic Surgery, 30*(10), 1303–1309. https://doi.org/10.1111/j.1524-4725.2004.30404.x.

Uzel, M. I., Kantarci, A., Hong, H. H., Uygur, C., Sheff, M. C., Firatli, E., ... Trackman, P. C. (2001). Connective tissue growth factor in drug-induced gingival overgrowth. *Journal of Periodontology, 72*(7), 921–931. https://doi.org/10.1902/jop.2001.72.7.921.

Weiser, J. R., & Saltzman, W. M. (2014). Controlled release for local delivery of drugs: Barriers and models. *Journal of Control Release, 190*, 664–673. https://doi.org/10.

1016/j.jconrel.2014.04.048.
Wu, K. J., Huang, G. F., Chen, C. H., Chang, H. H., & Deng, Y. T. (2014). Cyclosporine a induces connective tissue growth factor expression in human gingival fibroblasts: Suppression by epigallocatechin-3-gallate. *Journal of the Formosan Medical Association, 113*(11), 828–832. https://doi.org/10.1016/j.jfma.2014.01.008.
Zhang, M. M., Feng, D. F., Shao, P., Sun, T. T., Wang, L., & Yang, B. F. (2008). Nifedipine regulated periodontal ligament remodeling: An experimental study in rats. *Acta Odontologica Scandinavica, 66*(6), 327–333. https://doi.org/10.1080/00016350802208398.
Zhang, L., Wang, P., Mei, S., Li, C., Cai, C., & Ding, Y. (2012). In vivo alveolar bone regeneration by bone marrow stem cells/fibrin glue composition. *Archives of Biology, 57*(3), 238–244. https://doi.org/10.1016/j.archoralbio.2011.08.025.