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Osteogenic activity of cyclodextrin-encapsulated doxycycline in a calcium phosphate PCL and PLGA composite

V.C.C. Trajano [a], K.J.R. Costa [a], C.R.M. Lanza [b], R.D. Sinisterra [c], M.E. Cortés [a,*]

[a] Restorative Dentistry Department, Faculty of Dentistry, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, CEP: 31270-901 Belo Horizonte, Minas Gerais, Brazil
[b] Department of Oral Clinical, Surgery and Pathology, Faculty of Dentistry, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, CEP: 31270-901 Belo Horizonte, Minas Gerais, Brazil
[c] Chemistry Department, ICEX, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, CEP: 31270-901 Belo Horizonte, Minas Gerais, Brazil

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ABSTRACT

Composites of biodegradable polymers and calcium phosphate are bioactive and flexible, and have been proposed for use in tissue engineering and bone regeneration. When associated with the broad-spectrum antibiotic doxycycline (DOX), they could favor antimicrobial action and enhance the action of osteogenic composites. Composites of polycaprolactone (PCL), poly(lactic-*co*-glycolic acid) (PLGA), and a bioceramic of biphasic calcium phosphate Osteosynt® (BCP) were loaded with DOX encapsulated in β-cyclodextrin (βCD) and were evaluated for effects on osteoblastic cell cultures. The DOX/βCD composite was prepared with a double mixing method. Osteoblast viability was assessed with methyl tetrazolium (MTT) assays after 1 day, 7 day, and 14 days of composite exposure; alkaline phosphatase (AP) activity and collagen production were evaluated after 7 days and 14 days, and mineral nodule formation after 14 days. Composite structures were evaluated by scanning electron microscopy (SEM). Osteoblasts exposed to the composite containing 25 μg/mL DOX/βCD had increased cell proliferation ($p < 0.05$) compared to control osteoblast cultures at all experimental time points, reaching a maximum in the second week. AP activity and collagen secretion levels were also elevated in osteoblasts exposed to the DOX/βCD composite ($p < 0.05$ *vs.* controls) and reached a maximum after 14 days. These results were corroborated by Von Kossa test results, which showed strong formation of mineralization nodules during the same time period. SEM of the composite material revealed a surface topography with pore sizes suitable for growing osteoblasts. Together, these results suggest that osteoblasts are viable, proliferative, and osteogenic in the presence of a DOX/βCD-containing BCP ceramic composite.

1. Introduction

Ceramic biomaterials with biphasic calcium phosphate (BCP) are biocompatible and bioactive. Their porosity and interconnectivity promote cell adhesion, migration, proliferation, differentiation and dispersion of nutrients and metabolites that act directly in the physiological cellular components, improving the quality the newly formed bone and making them ideal scaffolds for clinical applications (*e.g.*, periodontal, implants), in particular for the repair and reconstruction of bone in tissue engineering [1–3].

The physical properties of bioceramics can be made more suitable for clinical applications by incorporating biodegradable polymers, such as polycaprolactone (PCL) and poly (lactic-*co*-glycolic acid) (PLGA), into polymer matrix–bioceramic composites [4,5]. This strategy combines the biological properties of bioactive ceramics with the flexibility of thermoplastic polymers, reducing the brittleness of the ceramic material and enabling its use in clinical practice [6]. Studies examining the performance of composites composed of BCP ceramics and biodegradable polymers have shown that these biomaterials, when used in bone defects, promote osteogenesis, or bone neoformation [7]. Thus, composite matrixes composed of BCP and biodegradable polymers have great potential for use in tissue engineering.

Because local infection can impair a favorable environment for tissue regeneration, antibiotic could be administered at the site to create a favorable environment for regeneration. Locally-applied sustained-release tetracycline preparations can produce higher localized antibiotic concentrations in periodontal pockets than is seen with systemic administration. Although the higher concentration may improve antibiotic efficacy, it might also put cells in the periodontal ligament and alveolar bone cells at risk of cytotoxic effects [8].

Doxycycline (DOX) is a broad-spectrum tetracycline antibiotic with documented clinical efficacy for the treatment of bone infections. Additionally, DOX has been reported to enhance bone tissue regeneration processes [9,10]. However, the optimal dose for topical DOX administration is not clear given that doses that are optimal for cellular differentiation, antimicrobial activity, and protein expression are likely to differ. The weighing of these different effects is particularly important in local delivery applications [11–13].

* Corresponding author.
E-mail address: mecortes@ufmg.br (M.E. Cortés).

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Cyclodextrins (CDs) are toroidal molecules that can form inclusion complexes with guest compounds, such as drugs. The hydrophobic groups of the drug bind to the inner surface of the CD cavity, preventing physical and chemical degradation of the drug and intensifying its pharmacological effects [14]. CD inclusion complexes can also act as controlled drug release systems (CRSs), maintaining the drug concentration in its therapeutic range for an extended time with a single dose [15]. A CRS increases the solubility, stability and bioavailability of the encapsulated drug, while simultaneously reducing adverse effects and drug interactions [16]. The structural and thermodynamic parameters of the DOX/βCD complex were determined in order to understand the DOX upon inclusion in βCD interactions with *Staphylococcus aureus* cells. The DOX/βCD was found to be more active against *S. aureus* than pure DOX. Lower cytotoxicity and osteoblast cell proliferation of DOX/βCD was observed when compared to free DOX [17].

There are many carriers for the local and sustained delivery of antimicrobials, including bioresorbable polymers, collagenous, liquid crystalline, and bioglass- and nanotube-based carriers, as well as those composed of calcium phosphate, the mineral component of bone and teeth and composite [18,19]. Synthetic polymers such as polycaprolactone (PCL) and poly (lactic-*co*-glycolic acid) (PLGA) are widely employed and these types of materials have gained popularity due to the wide control over release kinetics, degradation rates, predictability/quality control, and mechanical properties [19].

In the present investigation, DOX/β-CD inclusion complex was embedded in a matrix composed of PCL/PLGA/BCP; the material was prepared by physical mixture for use as a pro-osteogenesis scaffold. We examined cellular responses to the DOX/β-CD-embedded material to gain insights into its pharmacological actions, particularly with respect to bone regeneration.

2. Materials and methods

2.1. Study design

A four-phase *in vitro* study was conducted. In phase one, we used methyl tetrazolium (MTT) assays to evaluate the cell viability of osteoblasts exposed to solutions of DOX or DOX/βCD (1–25 μg/mL) in culture for 24 h. Phase two was similar to phase one except that rather being exposed to a DOX or DOX-βCD solutions, the cultured osteoblasts were exposed to composites containing 1–25 μg/mL of DOX alone (BCP/PCL/PLGA/DOX) or DOX-βCD (BCP/PCL/PLGA/DOX/βCD) for 24 h before being subjected to MTT cell viability assays. Because 25 μg/mL DOX/βCD resulted in significantly higher cell viability than in untreated control cultures, this dose was used to assess osteogenic activity in the subsequent tests. In phase three, we evaluated the osteogenic activity of BCP/PCL/PLGA/DOX/βCD composite (25 μg/mL) after 1 day, 7 days, and 14 days in osteoblast cultures using assessments of cell viability (MTT assays), alkaline phosphatase (AP) activity, collagen production, and mineralization (Von Kossa). Finally, in phase four, composite morphology was evaluated by scanning electron microscopy (SEM).

2.2. Preparation of composites

The DOX/βCD inclusion complexes were prepared by adding an aqueous solution of DOX (molecular weight, 480.99 g/mol) to aqueous βCD (molecular weight, 1135 g/mol) while stirring, in a 1:1 equimolar ratio (DOX: βCD) [16]. The resulting solutions were distributed in 15 mL falcon tubes, flash frozen in liquid nitrogen, and lyophilized.

The PCL/PLGA/BCP-DOX/βCD composite was prepared as follows: 0.84 mg of lyophilized DOX/βCD was added to 10 mL of dichloromethane (25 μg/mL final [DOX]) in a closed beaker while stirring at room temperature. Next, 300 mg of 50:50 PCL:PLGA polymer mixture was added, followed by 1200 mg of BCP with granule size distribution from 60 to 80 mesh. Before the solvent was completely evaporated, the composites were placed into a 4 mm diameter cylindrical mold and sliced up after 48 h to yield 1-mm-thick disks weighing 150 mg. Then, the disks were sterilized using the ethylene oxide physic-chemical method.

2.3. Viability assay

Primary osteoblasts were isolated from the calvaria of 1–5-day-old neonatal Wistar male rats using the method described by Wong and Cohn [20]. The rats were obtained from the bioterium at the UFMG School of Pharmacy and their use was approved by the UFMG Animal Use Ethics Committee (protocol number 184/2012). The animals were anesthetized, and the calvaria were removed and placed in phosphate buffered saline (PBS) without calcium or magnesium. Osteoblasts were cultured in RPMI-1640 culture medium supplemented with 1% penicillin–streptomycin and 10% fetal bovine serum. In all tests, the cells were plated at 1.5×10^5 cells/well in 96-well plates and the Von Kossa test was performed in 24-well plates. To perform the MTT assay, a medium without serum supplementation was employed.

Osteoblast viability and proliferation were assessed by MTT assays, a standard colorimetric assay that measures color changes in response to the mitochondrial activity of viable and metabolically active cells. The MTT is a sensitive method for evaluating the cytotoxicity of materials detected as an absence or reduction in color change. The MTT was performed with the Vybrant® MTT Cell Proliferation Assay Kit (Invitrogen) by adding 10 μL of MTT final solution (5 mg/mL) to each well after 1 day, 7 days, or 14 days of incubation in the presence of a composite or an experimental solution, control and PCL/PLGA/BCP matrix without the DOX/βCD inclusion complex. After 4 h incubation, 100 μL of 10% sodium dodecyl sulfate/0.01 N HCL was added to each well to dissolve the formazan salts overnight. Optical density at 570 nm was measured by an ELX 800 Universal Microplate Reader (Bio-Tek instruments, Winooski, VT) [21].

2.4. Alkaline phosphatase activity assay

To assay whether free or βCD-encapsulated DOX mediates AP activity changes in osteoblasts, we prepared a solution of Nitro-Blue tetrazolium chloride and 5-bromo-4-chloro-3′-indolylphosphate *p*-toluidine salt (NBT-BCIP), which upon exposure to AP produces a purple insoluble precipitate. The supernatant from each well was removed and the cell layer was washed twice with PBS. Then 60 μL of NBT-BCIP solution was added to each well. After a 2-h incubation period, the cells were observed with an inverted light microscope and purple insoluble precipitates were solubilized with 60 μL of 10% sodium dodecyl sulfate/0.01 N HCL. After an 18-h incubation, optical density at 595 nm was measured on an ELX 800 Universal Microplate Reader [21].

2.5. Collagen production assay

The effect of free and βCD-encapsulated DOX on collagen production was determined as per the manufacturer's protocol with the Sircol Collagen Assay Kit Invitrogen®. Briefly, after 7 days and 14 days of exposure to the composite, 1 mL of cell culture supernatant from each plate-studied group was removed and placed in tubes preloaded of which 200 μL was stored cold (4 °C). Isolation and concentration reagent were added to each sample. The tubes were pre-loaded with 200 μL of cold medium during 24 h and centrifuged at 12,000 rpm for 10 min. The supernatant was removed from each tube. Then 1 mL Sircol Dye reagent was added to each sample and stirred for 30 min, followed by centrifugation at 12,000 rpm for 10 min. The supernatant was discarded and 750 μL of cold acid-salt wash reagent was added to each tube, again followed by centrifugation at 12,000 rpm for 10 min. The supernatant was discarded and 250 μL of the alkali reagent was added to each tube and stirred for 5 min. Finally, 200-μL aliquots of supernatant from each tube were transferred to 96-well plates. The optical density of the

aliquots was measured at 555 nm on an ELX 800 Universal Microplate Reader.

2.6. Mineralization of bone nodules assessed by Von Kossa assay

The Von Kossa assay was performed in triplicate to determine whether the osteoblast cells differentiated into mineralized nodules after 14 d of being cultured *in vitro*. The Von Kossa assay is a specific colorimetric assay for detection of mineralization nodules within an osteoclast culture. The culture medium was removed from 24-well plates and cells were washed three times with PBS and fixed with 100 μL of 10% formaldehyde for 5 min. The fixative was removed, the cells were washed with Milli-Q water, and 200 μL of 5% silver nitrate was added to each well. The 24-well plates were incubated for 1 h in a laminar flow cabinet with ultraviolet light irradiation. Cells were then washed three times with Milli-Q water, followed by a 3-min incubation with 200 μL of 5% sodium thiosulfate and then exposure to 200 μL of 1% safranin for 30 s. Visual analysis of the nodules was performed on an inverted light microscope (BEL Photonics®, BEL Equipment Ltda., Piracicaba, SP, Brazil) equipped with a 10× objective.

2.7. Composite morphology

The morphology of the composites was assessed by SEM, which allowed for microstructural characterization of the surface, including qualitative assessment of interconnectivity, conformation, and morphology. The composites were coated with gold with a Sputter Coater (SPI Supplies) for 90 s at 13 mA. Images were acquired by a JEOL 6360LV® SEM machine at 15 kV and 750 mA at the Department of Physics, ICEX, UFMG.

All biochemical assays were performed with six replicates to enable statistical comparisons. Means and standard deviations (SDs) were calculated and analyzed in GraphPad Prism® 5.0 (GraphPad Software, San Diego, CA). Statistical significance was determined by analyses of variance (ANOVAs) and the Bonferroni *post hoc* tests. Differences were considered significant when $p \leq 0.05$.

3. Results and discussion

3.1. Phase 1: Cytotoxicity of DOX and DOX/βCD solutions on osteoblasts culture

MTT cytotoxicity assay results after a 24-h exposure to DOX suggest that cell proliferation was stimulated in osteoblasts cultured in a DOX solutions (1–25 μg/mL). This stimulation effect was statistically significant at 1 μg/mL DOX compared to untreated osteoblasts. Conversely, signs of cytotoxicity were observed with 25 μg/mL DOX (free, non-encapsulated) without significant stimulation of cell growth, corroborating the results of Honnorat-Benabbou et al. [22], Gomes & Fernandes (2006) [12], and Coelho et al. (2006) [8]. However, in this study, when a βCD inclusion compound of DOX was formed, 25 μg/mL DOX was non-cytotoxic and yielded greater cell proliferation ($p < 0.05$) than those that were noted with osteoblasts untreated (Fig. 1).

These results indicate that doses of DOX that are cytotoxic for free DOX can become non-cytotoxic when DOX is encapsulated in CDs. In addition to its antimicrobial activity, DOX has also been shown to possess anti-apoptotic, anti-inflammatory, and anti-collagenolytic effects that should favor the control of periodontal disease [12].

3.2. Phase 2: Composite cytotoxicity

The cellular viability of osteoblasts exposed to composites containing free DOX (BCP/PCL/PLGA/DOX) and DOX encapsulated in βCD (BCP/PCL/PLGA/DOX/βCD) at 1–25 μg/mL concentrations were determined after 24 h (Fig. 2). Composites containing free DOX in the BCP/PCL/PLGA matrix had no effect on osteoblast viability. However, when the composite matrix contained 25 μg/mL DOX encapsulated in βCD, osteoblast cell proliferation was induced significantly, ($p < 0.05$) compared to control cultures; this concentration was chosen to assess osteogenic activity in other *in vitro* tests. The induction of cell proliferation in osteoblasts by DOX/βCD was dose-dependent.

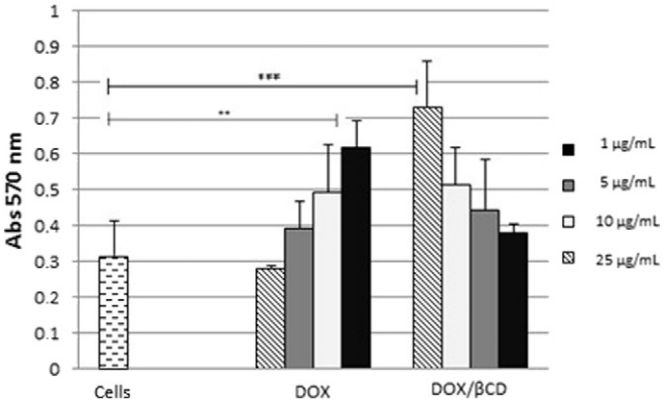

Fig. 1. Cell viability of the osteoblasts by MTT assay after 24 h of treatment with solutions pure DOX and DOX/βCD (1:1) at concentrations of 1; 5; 10; and 25 μg/mL.

3.3. Phase 3: Osteogenic activity of the BCP/PCL/PLGA/DOX/BCD composite on osteoblasts culture

3.3.1. Cell viability

Cell proliferation rate was determined by performing the viability assay at three time points. Cell proliferation rate was determined by performing the viability assay at three time points. The proliferative rate of osteoblasts cultured in the presence of the 25 μg/mL BCP/PCL/PLGA/DOX/βCD composite was increased significantly ($p < 0.05$) compared to that observed for control cells at all experimental time points (1 d, 7 d, and 14 d). The cellular proliferative rate of treated osteoblasts was time-dependent (Fig. 3) followed by an induction period of osteogenic differentiation, confirming prior results found by Gomes & Fernandes (2006) [12] and Pataro et al. (2003) [23].

Investigators have reported that the addition of βCD has multiple benefits, such as: reducing drug cytotoxicity, and besides reducing the adverse effects (morphological damage to dentin surfaces, tooth pigmentation, and unpleasant taste); and ensuring delivery of therapeutic doses to a specific location, such that it can be administered at a low concentration over a long period of time [16,23]. Other studies have shown a higher activity of encapsulated drugs relative to their free forms owing to the drugs being protected and stabilized in relation to physical and chemical decomposition by light, heat, and free radicals [24].

In CRSs that employ the biodegradable polymers PCL and PLGA, the drug is isolated from surrounding solution by a physical barrier

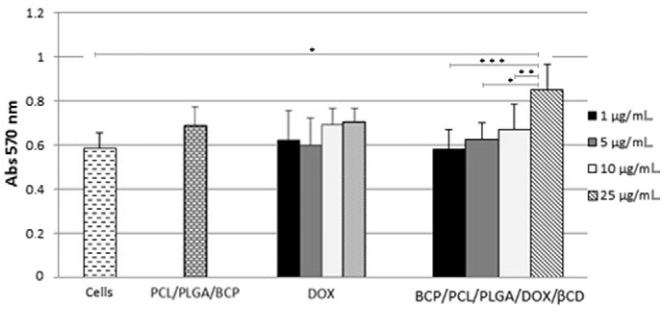

Fig. 2. Cellular viability of pure DOX (BCP/PCL/PLGA/DOX) and DOX encapsulated in βCD (BCP/PCL/PLGA/DOX/βCD) composites after 24 h.

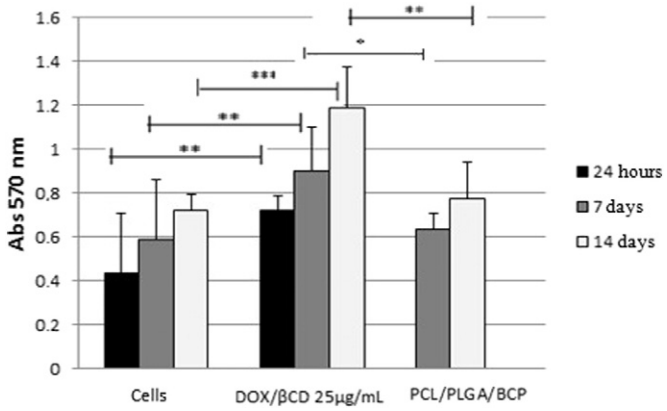

Fig. 3. Cell viability of the osteoblasts by MTT assay after 24 h, 7 and 14 days of treatment with BCP/PCL/PLGA/DOX/βCD composite at a concentration 25 μg/mL.

that prevents its rapid dissolution by biological fluids. The incorporation of BCP in polymeric matrices provides calcium (Ca^{2+}) and phosphate (PO_4^{-3}) ions for the formation of bone, favors the conversion of fibrous tissue into bone tissue with osteoconductive characteristics, and neutralizes by-products of degradation of the polymers. Several features can be optimized to reduce toxic side effects, limit the number of medicine administrations required for the desired therapeutic outcome, and encourage better adherence to treatment. These features include: (1) the ability to choose ceramic and polymeric materials that have similar characteristics to the tissue to be replaced; (2) the availability of composites with pore sizes compatible with the biological activity desired; (3) the use of materials that promote controlled and sustained release of therapeutic small molecules; and (4) polymer biodegradability. If these goals can be achieved, it may be possible to obtain a material that can stimulate osteoblast activity and bone healing [25,26].

3.3.2. AP activity

The AP activity levels of osteoblasts exposed to 25 μg/mL BCP/PCL/PLGA/DOX/βCD composite for 7 days and 14 days were greater than respective control levels ($p < 0.05$) and showed a time-dependence. The increased AP activity observed could serve as a source of high levels of phosphate ions, which are required for early mineralization processes. The AP activity of osteoblasts exposed to matrix without DOX did not differ from that of controls; however, AP activity levels with the matrix with differed between 7 days and 14 days (Fig. 4). Gomes et al. [12] also observed that AP levels increased over 14 days, and then decreased thereafter. Park [11] demonstrated that the higher levels of DOX caused a dose-dependent lower osteoblast differentiation and protein expression.

Studies have shown that the production of AP by osteoblasts in contact with different biomaterials can alter cellular growth, depending on the composition of the biomaterial. Qualitative and quantitative assessment of the secretion of this enzyme is an important parameter for analyzing effects on bone tissue, since it reflects the activity of osteoblasts in the presence of the biomaterial [27]. In this study, there was a statistically significant increase in the differentiation of osteoblasts exposed for 14 days to composite matrixes, suggesting that that this biomaterial promotes the deposition of bone matrix and osteogenesis. These results are consistent with those of Breyner et al. [28].

3.3.3. Collagen production

Osteoblasts exposed to 25 μg/mL BCP/PCL/PLGA/DOX/βCD composite exhibited greater collagen production than control osteoblasts by 14 d ($p < 0.05$). Collagen production levels were similar between cells exposed to matrix loaded with DOX alone and cells exposed to the control matrix, with both groups showing a difference in collagen production between days 7 and 14 (Fig. 5). We observed a significant, progressive increase in collagen secretion over time (7 days *vs.* 14 days), indicating that the cells underwent osteogenesis within 2 weeks in the presence of the composite.

Collagen production is an important event in the ossification process and its measurement has been considered an indicator of osteogenesis [15]. Zuk et al. (2002) [29] argued that increased collagen secretion after 2 weeks in culture can be correlated to osteogenic activity because various osteogenic cells, including osteoblasts, produce collagen. Collagen production precedes calcification of bone matrix *in vitro* and *in vivo*, indicating that an initial collagen matrix must first be formed for mineralization to occur. The high collagen production stimulated by the composites, in this study, indicates that the materials are suitable for bone growth scaffolding; a high proliferation rate is necessary to avoid fibrosis at the bone repair site, and collagen (type I) has been reported previously to promote osteoblast proliferation [30].

3.3.4. Mineralization of bone nodules

Von Kossa staining revealed mineralized nodules and positively stained cells in osteoblast cultures that had been exposed to 25 mg/mL BCP/PCL/PLGA/DOX/βCD composite for 14 d (Fig. 6). During these 2 weeks, the osteoblasts were incorporated progressively into the mineralized matrix but did not proliferate, as occurs with differentiation. Positive Von Kossa staining of mineralized structures is indicative of osteogenic cells in culture [31].

3.4. Phase 4: Composite morphology

SEM of the composites highly porous structures. Pore size is a key factor in promoting a uniform distribution of cells [32]. The micropores

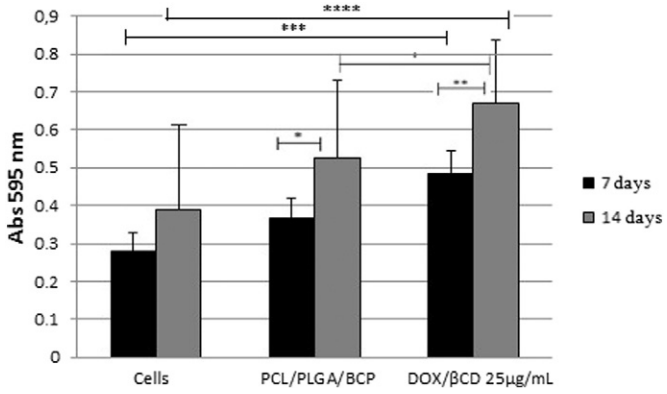

Fig. 4. Alkaline phosphatase production by osteoblasts after 7 and 14 days of treatment with BCP/PCL/PLGA/DOX/βCD composite at a concentration 25 μg/mL.

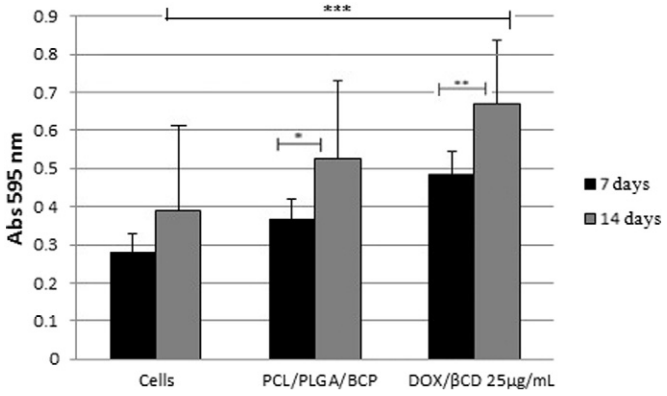

Fig. 5. Collagen production by osteoblasts after 7 and 14 days of treatment with BCP/PCL/PLGA/DOX/βCD composite at a concentration 25 μg/mL.

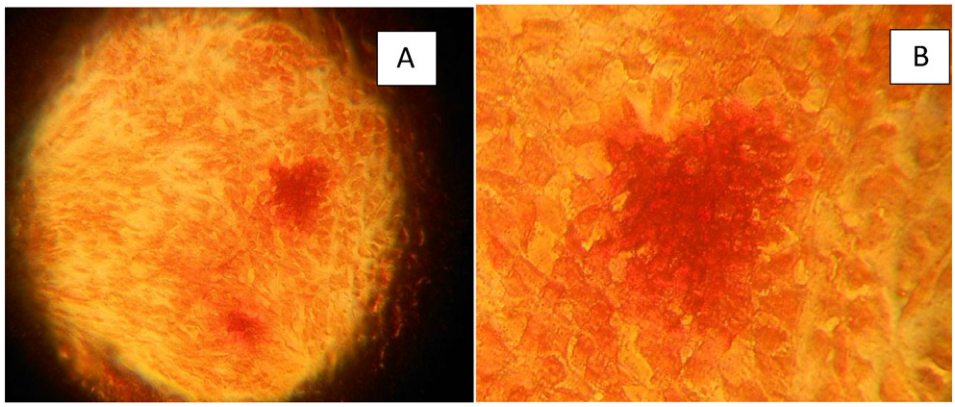

Fig. 6. View of mineralized structures by coloring of Von Kossa. (A) Image of two mineralized nodules after 14 days of inducing osteogenesis in the presence of BCP/PCL/PLGA/DOX/βCD composite, concentration 25 μg/mL. Magnification 20×. B) Magnification 400×.

in the BCP/PCL/PLGA/DOX/βCD composites were mostly of small diameter size (range, 1–10 μm) (Fig. 7C, D). Some intermediate-diameter pores (10–50 μm) were also visible (Fig. 7B). Interconnection micropores in the range of 1–10 μm were also noted.

Osteogenesis rate is affected by graft distribution as well as the size and number of interconnecting channels. The 1–10-μm pore size is desirable because: it maximizes the access area of cells; favors migration, cell adhesion, and growth; promotes extracellular matrix production and vascularity; increases tissue contacts, solubility, and molecular absorption; expands the capacity to exchange biological fluids and improves diffusion of secreted metabolites. To favor a large area per unit volume of cell anchorage pore size should accommodate the diameter of the cell suspension, typically 10 μm [33]. Meanwhile, the presence of some larger intermediate-sized pores (10–50 μm) may favor osteoblast adhesion, cell migration, growth, and extracellular matrix production [34].

3.5. The BCP/PCL/PLGA/DOX/βCD matrix

Although osteoblast cells have osteogenic capacity, alone they are not capable of forming functional tissue *per se*. A matrix is required to support colonization and osteogenic differentiation of stem cells and enable orderly cell growth [35]. Porosity is critical for cell migration, and bone matrix development can affect important mechanical properties of a polymer, potentially reducing its flexibility and increasing its fragility [36].

The BCP/PCL/PLGA/DOX/βCD matrix examined in the present study favored cell proliferation, delivered an osteogenic drug successfully, maintained a surface topography that allowed osteoblast metabolic activity and phenotypic expression of tissue characteristics, and exhibited osteoinductive and osteoconductive behavior. This work extends the prior findings of Coombes et al. (2004) [37], who also reported bone growth into a porous polymer device. Combining a microstructured

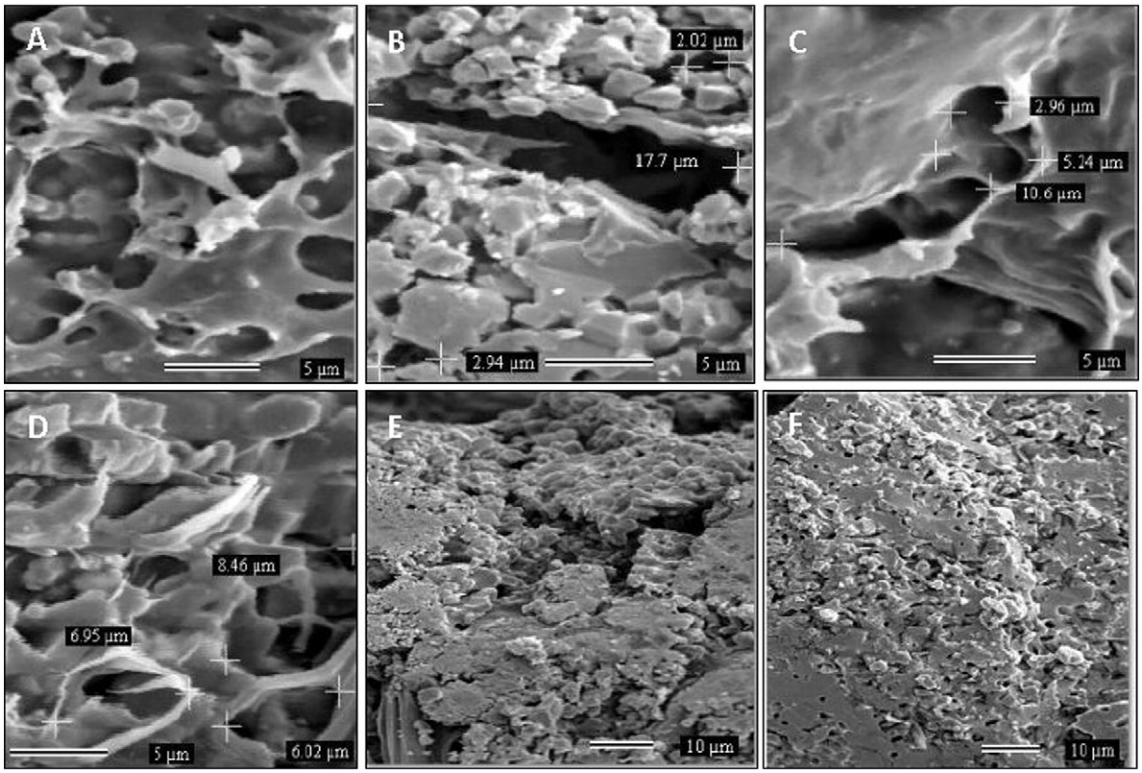

Fig. 7. SEM composite (BCP/PCL/PLGA/DOX/βcd). A, B, C, D with a 5× magnification; E and F with magnification 10×.

substrate with osteogenic molecules, the BCP/PCL/PLGA/DOX/βCD composite appears to have properties and provide conditions suitable for bone growth induction. The present findings suggest that BCP/PCL/PLGA/DOX/βCD composite material has the potential for application in orthopedic and periodontic bone regenerative therapies.

4. Conclusion

In conclusion, this study suggests that βCD-encapsulated DOX (25 μg/mL) incorporated in a BCP/PCL/PLGA/DOX/βCD composite material favored osteogenesis in all parameters assessed (*i.e.*, adhesion, cell growth, proliferation and mineralization). Furthermore, the material's microstructure had adequate porosity to support proliferation of osteoblasts, suggesting that the composite is a promising candidate for use in the tissue engineering of bone.

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Authors' roles

Study design: VCCT, CRML, RDS and MEC. Study conduct: VSB, KJRC. Data analysis: VCCT and MEC. Data interpretation: VCCT and MEC. Drafting manuscript: VCCT. Approving final version of manuscript: VCCT, CRML, RDS and MEC. VCCT takes responsibility for the integrity of the data analysis. Trial design: MEC and CRML.

Conflicts of interest

All authors state that they have no conflicts of interest.

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