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

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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].

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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