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Polymer: Bioceramic composites optimization by tetracycline addition

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Abstract

The aim of this study was to evaluate the biocompatibility of composites of poly-lactic acid polymer (PLA) and copolymer of lactic and glycolic acid (PLGA), dispersed in a bioceramic matrix, Osteosynt® (BC), to which tetracycline (TC) was added. The in vitro test used direct contact test (ASTM F-813) and elution test (USP-XXIII, ISO 10993-5), and in vivo evaluation was performed after subcutaneous implantation in outbread Swiss mice. The 0.01% (w/w) TC addition did not affect composite cytotoxicity in vitro. The macroscopic and histologic evaluation in vivo after 1, 7, 13, 21, 28 and 56 days showed an initial intense infiltrate of inflammatory cells for most of the groups. The tissue showed normal pattern after 21 days for all the groups. TC addition exhibited significantly larger reduction of inflammation signs (Mann–Whitney test, $p < 0.05$) in the critical period of the resolution of the inflammatory process. Angiogenesis, cellular adsorption and fibrous deposit were observed on SEM evaluation. In conclusion, TC addition optimized composites polymer/bioceramic biocompatibility, contributing to anti-inflammatory response during the early phases of the wound healing process.

Keywords: Tetracycline; Bioceramic; Polymer; Composite; Biocompatibility

1. Introduction

The regulation of the inflammatory reaction is a key factor to be controlled after graft implantation. Besides its very well-known bacteriostatic activity, tetracycline exhibits anti-inflammatory effects and an ability to promote the attachment of fibroblast and connective tissue to root surfaces, biological properties such as anti-collagenase activity and inhibition of bone resorption (Wikesjö et al., 1986; Goodson, 1989; Seymour and Heasman, 1995; Esposito et al., 1997; Vandekerckhove et al., 1997).

The tetracyclines display important inhibitory effects on inflammation processes, such as: (1) inhibition of matrix metalloproteinases (MMPs) (Brundula et al., 2002; Popovic et al., 2002); (2) inhibition of tumor necrosis factor (TNF) (Popovic et al., 2002); (3) inhibition of inducible nitric oxide syntheses (iNOS) production, besides accelerate its degradation (Amin et al., 1997; Popovic et al., 2002; Sapadin and Fleischmajer, 2006); (4) inhibition of phospholipase A2 activity (Pruzanski et al., 1992; Chtarto et al., 2003; Scarabelli et al., 2004); (5) inhibition of mitogen-induced human lymphocytic proliferation by blockage of blast transformation (Thong and Ferrante, 1979); (6) suppression of neutrophilic migration and chemotaxis (Martin et al., 1974; Esterly et al., 1978); (7) inhibition of T-lymphocyte activation with resultant inhibition of T-cell proliferation (Kloppenburg et al., 1994); (8) up-regulation of anti-inflammatory cytokine IL-10 (Ritchlin et al., 2000).

Diverse attempts to obtain a material that fulfill biological requirements for its use have been described in the literature (Hench, 1998; Shikinami and Okuno, 1999; Burg et al., 2000; Nasr et al., 2000; Pataro et al., 2003). Various materials provide a good approach for guided bone healing, but there are always remaining concerns about mechanical stability, long-term biocompatibility, and local or systemic inflammatory reaction during the in vivo degradation. Additionally, metals

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alone or coated with bioinert or bioactive ceramics have been also related, but metal corrosion, ceramics–metal interface wear, dense fibrous tissue formation introduced problems in their use (Burg et al., 2000; Rodrigues et al., 2003).

Biphasic micro-macro porous bioceramics of β-tricalcium phosphate (β-TCP) and hydroxyapatite (HA) have demonstrated neither antigenicity nor cytotoxicity (Burg et al., 2000). Biodegradable polymers such as poly (L-lactic acid) (PLA) or poly (D,L-lactic-co-glycolic acid) (PLGA) have been used in order to provide plasticity and different biodegradability profile. In addition, those materials also have a high potential as drug carriers, purity and the absence of biological contamination (Bajpai, 1992; Abrams and Bajpai, 1994; Athanasiou et al., 1996; Wong and Mooney, 1997; Wykrota et al., 1999).

In spite of increasing use of tetracycline as antibiotic agent, scarcely in vivo studies have been carried out to determine the local anti-inflammatory effects of tetracycline. Thus, the aim of the present study was to evaluate the tetracycline influence of biological response of composites of bioceramic:polymers, altering or no altering the macro and micro-aspects of the composite by degradation process and porosity to which TC was added.

2. Materials and methods

2.1. Materials

The bioceramic Osteosynt® (BC) with granulometry varying from 60 to 80 Mesh, corresponding to a size ranging from 180 to 250 μm, 65% (w/w) of hydroxyapatite – $Ca_5(PO_4)_3OH$ – and 35% (w/w) of β-tricalcium phosphate (β-TCP) – $Ca_3(PO_4)_2$ – was gently supplied by Einco Biomaterial, Ltd., Belo Horizonte, MG, Brazil (Enduro Inc., 1991). Biodegradable polymers DL-polylactic acid (viscosity of 0.36 dL/g into CHCl3 at 30 °C; lot #D020015) (PLA) and DL-co-polymers of lactic and glycolic acid (50:50 lactide:glycolide molar ratio; viscosity of 0.39 dL/g into HFIP at 30 °C; lot #D01079) (PLGA), Birmingham Polymers, Inc., Durect Corporation, Pelham, USA, were used as minor composite phase. Tetracycline was obtained from Sigma Co., St. Louis, MO, USA.

2.2. Methods

2.2.1. Preparation of polymer/bioceramic composite

The composites were prepared through the dissolution of the polymer phase in 200 μL of dichloromethane for each gram of the composite, and mixture with the bioceramics phase until to obtain a plastic and homogeneous mixture at room temperature in the weight ratio 1:3 (polymer:bioceramic) (Sinisterra et al., 2006). This weight ratio was chosen as mimetic system of organic/inorganic components of natural bone (Hollinger and Battistone, 1986; Lin et al., 1998).

For in vitro tests, composites with 0.01% (w/w) of tetracycline were prepared. A total of 150 mg of each composite were pressed in any given case in a press of 250 kg/m^2 within 5 min, into disk-like tablets with a diameter of 7 mm × 1 mm (CARVER hydraulic model #3912, Inc.). For in vivo tests, composites with 0.1% (w/w) of tetracycline were prepared into tablets with 50 mg of disk-like tablets with a diameter of 7 mm × 1 mm. The materials were sterilized using ethylene oxide.

2.2.2. Cytotoxicity evaluation

Cytotoxicity of tablets was determined by direct contact test and minimum essential medium elution test based on USP (1995) and ISO 10993-5 (1999).

2.2.2.1. Direct contact test. These assays of triplicate tablets of tests and control materials – HD polyethylene as a negative control and latex as a positive control – sterilized by ethylene oxide were carefully placed on a near confluence monolayer of L929 mammalian fibroblast cells. The plates were incubated under standard condition at 37 °C in a humidified incubator containing 5% CO_2. After 24 h, the tablets were removed and the cultures examined under a microscopic using a histological stain such as 2% crystal violet in 20% ethanol to detect morphological alterations, reduction in the cell layer density and cell lyses induced by the tested material.

Zone index (ZI) corresponds to the area or the light zone which cells were not stained by crystal violet. Lyses index (LI) express the percentage of degenerated or affected cells into toxicity zone that were established for each plate after microscopic analysis. Response index (RI) is usually assessed by the expression: RI = ZI/LI.

2.2.2.2. Minimum essential medium elution test. Extracts were prepared from test and control materials which is then placed on Petri plates with L929 mammalian fibroblast cells monolayers and incubated under standard condition at 37 °C in a humidified incubator containing 5% CO_2. After 24 h, the cells were examined for morphologic changes and cytolysis to determine a toxicity score.

2.3. In vivo experiment

In vivo studies were performed after the analysis of the initial in vitro study and in accordance with Institutional Ethical Council for Animal Research, CETEA of Universidade Federal de Minas Gerais (014/2004).

A total of 81 male outbread Swiss mice, 8 weeks old, weighing 28 ± 2 g were used for tablet implantation. The animals were anaesthetized with intra-peritoneum injection of a mixture of ketamine hydrochloride 40 mg/kg and sedative, analgesic and muscular relaxant Calmiun® 7.5 mg/kg (both from Agener, São Paulo, SP, Brazil). The mice were shaved over dorsal anterior surface and the site cleaned with a povidone–iodine alcoholic solution. Under aseptic conditions, a 10 mm incision was made through the skin and the subcutaneous with a blunt tip scissors, through which tablets were implanted. The composites testing groups were established: N1 (PLGA:BC); N2 (PLGA:BC + TC); N3 (PLA:BC); N4 (PLA:BC + TC) and as control groups: C1 (PLGA), C2 (PLA) and BC. Also, only animals incised (A) and not incised (X) served as controls. Each animal received two different tablets implanted in a dorsal region under aseptic con-

ditions following a triplicate assay (right and left, 2 cm from the incision place). The incisions were closed with absorbable sutures (4-0 Vicryl, Ethicon Co., USA). The animals were kept in cages and maintained ad libitum. The mice were killed after 1, 7, 13, 21, 28 and 56 days by ketamine overdose (100 mg/kg).

2.3.1. Histological evaluation

Immediately after animal sacrifice, the tablets and surrounding tissues were cut off and fixed in 10% phosphate buffered formalin solution for 24 h. All samples were alcohol-dehydrated, soaked in xylol and embedded in paraffin. Sections of 5 μm were stained with hematoxylin and eosin according to standard procedures. Microscopic analysis was performed on the tablets implant area by the same single blinded examiner, using a light microscope. The fields were randomly selected and were submitted to cell counting in 8, 12 and 15 high power fields (1000×). As the difference in cell counting between these three methods was insignificant, the cells were counted in eight high power histological fields. The epithelium/connective tissue interface in each case was randomly selected and the percentage of positive cells for the total inflammatory infiltrate was obtained. Since the data did not conform to a normal distribution, a nonparametric analysis was used to compare both groups (Mann–Whitney test). The values were considered significantly different when the p-value was <0.05. The error for cell counts was determined by six double counts in random sections.

2.4. Scanning electron microscopy (SEM)

The implanted tablets were removed at each follow-up interval and were immersed for 6 h in a 2% buffered glutaraldehyde solution, pH 7.4. The tissue that was involving the tablet was partially removed to compare its interface with the composites. After this period, the material were dehydrated with a graded ethanol series, recovered with gold 99% for 240 s and analyzed by an electronic microscope (JEOL-JSM 840A Edwards, 20.0 kV).

3. Results

3.1. Cytotoxicity evaluation

The data of Table 1 show the cytotoxicity levels determined by direct contact and elution tests. The Response Index (RI) of PLGA:BC composites, obtained in direct contact test, was greater than the PLA:BC in the presence and absence of TC. The cytotoxicity levels of all composites were relatively low and compatible with International Standards. TC addition, in a low concentration (0.01%, w/w), did not affect composites cytotoxicity.

The minimum essential medium elution test data (reactivity degree = 1) confirmed the low cytotoxicity of composites. The in vitro evaluation demonstrated that these materials could be tested in animals.

Table 1
Cytotoxicity of PLA, PLGA, BC or their composites with TC obtained from direct contact test and elution test in vitro

Material	Direct contact test			Elution test
	ZI	LI	RI	Reactivity degree
HD polyethylene	0	0	0/0	0
Latex	2	5	2/5	3
PLA	1	3	1/3	1
PLGA	1	4	1/4	1
BC	1	3	1/3	3
PLA:BC	1	1	1/1	1
PLGA:BC	1	4	1/4	1
PLA:BC + TC	1	1	1/1	1
PLGA:BC + TC	1	4	1/4	1

The cytotoxicity samples were evaluated on L929 mammalian fibroblast cells by direct contact test (ASTM F-813) and elution test (USP-XXIII). High-density (HD) polyethylene was used as negative and latex as positive controls. ZI, zone index; LI, lysis index; RI, response index; $n = 3$; PLA, DL-polylactic acid, PLGA, DL-co-polymers of lactid and glycolic acid 50:50; BC, bioceramic; TC, tetracycline 0.01% (w/w).

3.2. Histology

The histological result is shown in Table 2. The tissue response varied highly among the groups at the first day, from a local severe inflammatory reaction to a slight response (Fig. 1). A decreasing tendency was observed at the subsequent days, moderate to slight mixed infiltrate cells. After 7 days a predominance of macrophages and mononuclear cells were observed, increasing the number of fibroblasts on the tissues surrounding the implantation site, principally at N1 and N3 (Fig. 2). The comparative analysis showed a significative difference between PLGA:BC (N1) and PLGA:BC with TC (N2) and PLA:BC (N3) and PLA:BC with TC (N4), with a lower presence of infiltrate cells in tetracycline groups ($p < 0.05$). This fact was observed during the resolution of the inflammatory process when tetracycline was added. After 13 days, a decrease in the mean cell number at all implanted groups was observed.

The tetracycline influence was clearly observed again at 21 day, when TC groups N2 and N4 presented lower presence of infiltrate than N1 and N3 ($p < 0.05$). Nevertheless, after 28 days,

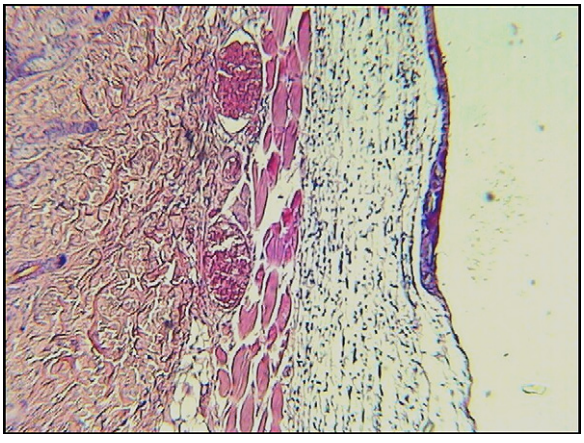

Fig. 1. Optic microscopy of subcutaneous mice tissue after 1 day of material implantation. PLA tablet. Intense infiltrate cells: 40×; H and E.

Table 2
Presence of inflammatory infiltrate cells by histological evaluation after implantation of tablets of PLA, PLGA, BC, or their composites with TC

	T1 (1 day)	T2 (7 days)	T3 (13 days)	T4 (21 days)	T5 (28 days)	T6 (56 days)
N1	513	166	65	92	66	30
N2	403	143	64	36	40	37
N3	372	177	70	58	43	29
N4	502	135	85	45	20	22
C1	434	95	90	90	37	N E
C2	23	133	68	44	29	19
BC	60	48	93	57	50	N E
A	16	14	4	NE	NE	N E
X	21	NE	NE	NE	NE	N E

Absent infiltrate, <30 cells; slight infiltrate, 31–140 cells; moderate infiltrate, 141–250 cells; intense infiltrate, >de 250 cells; NE, non-evaluated. N1, PLGA:BC; N2, PLGA:BC + TC; N3, PLA:BC; N4, PLA:BC + TC; C1, PLGA; C2, PLA; BC, bioceramic; A, animals only incised; X, animals not incised. PLA, DL-polylactic acid, PLGA, DL-co-polymers of lactid and glycolic acid 50:50; BC, bioceramic; TC, tetracycline 0.01% (w/w).

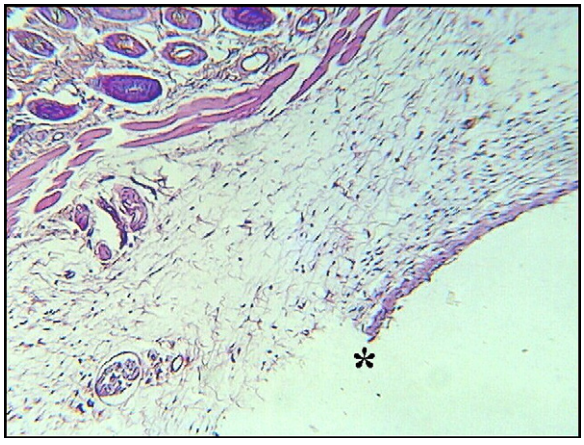

Fig. 2. Optic microscopy of subcutaneous mice tissue after 7 days material implantation. PLGA: BC + 0.1% (w/w) TC tablet. Moderate infiltrate cells. *Tablet zone: 40×; H and E.

as with 56 days, the tissue showed near normal pattern for all the groups and no signal of chronic inflammatory response was observed. In all the groups, a deposit of a fine layer of fibrous tissue surrounding the implantation area was detected, with a great concentration of fibroblasts, principally at N3, C2 and BC. BC and C2 showed the good biological response, with a slight infiltrate. Angiogenesis and cellular adsorption was noted at all test groups and BC. None of the groups showed necrotic tissue.

3.3. Tablets characteristics and imaging

3.3.1. Before implantation

Scanning electron micrographs of composites obtained through association among biodegradable polymers and bioceramic with tetracycline addition showed a semi-crystalline structure (Fig. 3(a and b)). The bioceramic:polymers presented a more crystalline aspect than polymer alone before implantation. The granular bioceramic has a brittle structure. Through macroscopic composite analysis, it was possible to affirm that composite preparation using low pressure led to consistent material.

3.3.2. After implantation

With elapsing time, composite bioerosion could be noted. All composites revealed micropores ranging from 5 to 10 μm and macroporosity bigger than 100 μm, as well as PLGA:BC with tetracycline tablet after 28 days implantation. Macroscopic and SEM analysis showed that PLGA:BC composites degraded

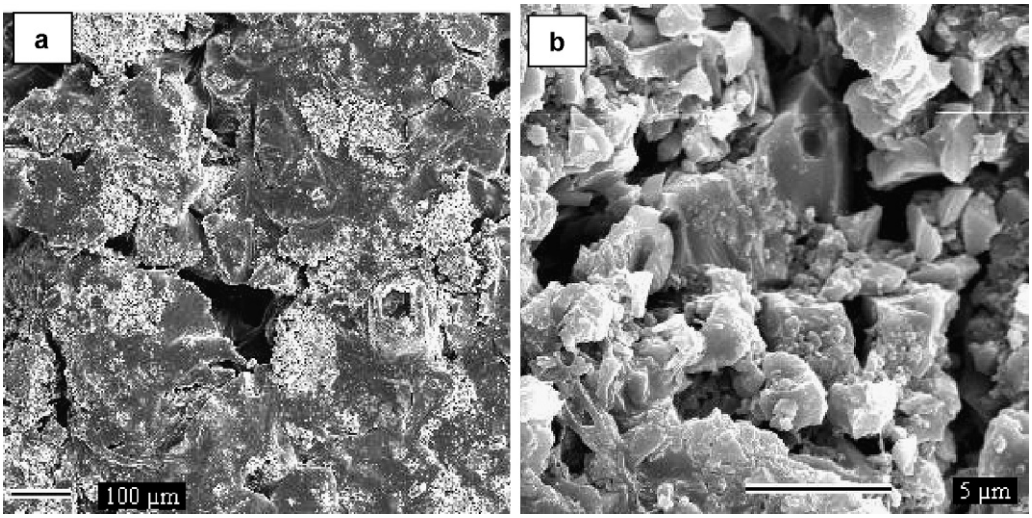

Fig. 3. Scanning electron micrographs of the tested surfaces before implantation of a PLA: BC + 0.1% (w/w) TC composite tablet showing a semi-crystalline structure.

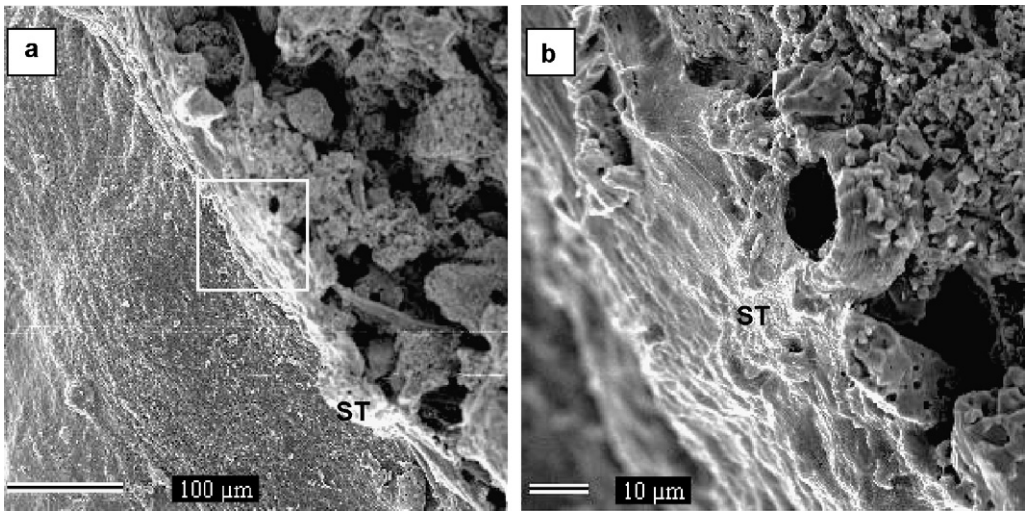

Fig. 4. Scanning electron micrographs of the tested surfaces of a PLGA: BC + 0.1% (w/w) TC composite tablet 56 days after implantation showing: (a) macroporosity and subcutaneous tissue (ST) adherence and (b) magnification of square zone and microporosity.

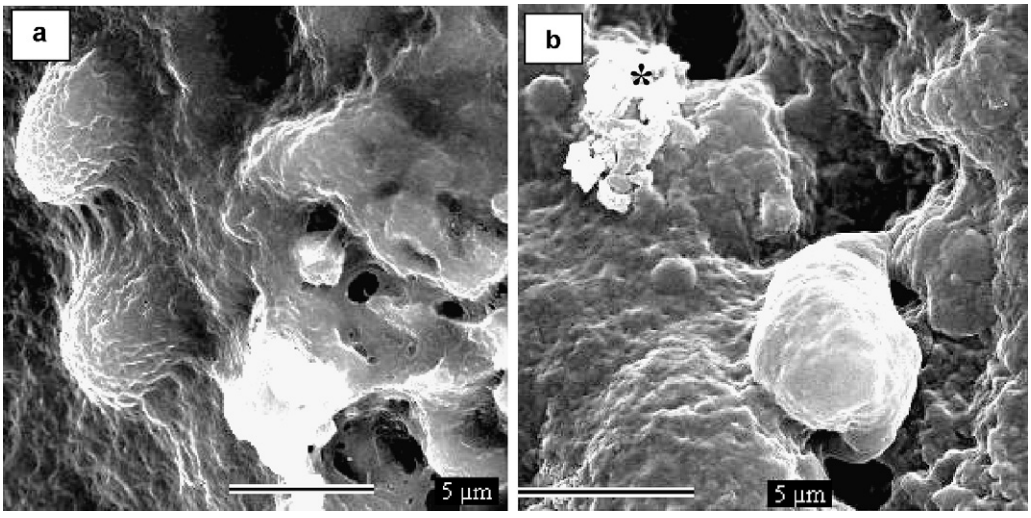

Fig. 5. Scanning electron micrographs of the tested surfaces showing cellular adsorption: (a) PLGA: BC tablet 13 days after implantation; (b) PLA: BC + 0.1% TC (w/w) 28 days after implantation. AT, animal tissue. *Possible TC crystals.

much faster than PLA:BC composites, independent of TC addition. At day 56, PLGA:BC composites were very eroded, with BC granules involved in a conjunctive tissue pocket (Fig. 4(a and b)). By contrast, PLA:BC tablets remained more consistent during all experiments, maintaining their original shape. Furthermore, a strong bond tissue surrounding the composites and BC tablets remained throughout the experiment. The addition of the polymer, PLA, to the composite showed the formation of a favorable surface to cellular adsorption (Fig. 5(a and b)).

4. Discussion

A large amount of research concerning materials for bone replacement has been published (Hench, 1998; Shikinami and Okuno, 1999; Burg et al., 2000; Nasr et al., 2000; Rodrigues et al., 2003). The present composites prepared herein were 1:3 weight ratio polymer/bioceramic, inspired by natural bone organic/inorganic composition (Hollinger and Battistone, 1986; Lin et al., 1998; Sinisterra et al., 2006), rendered an advantageous biomaterial with a semi-crystalline structure and plastic behavior, besides being easily handled. Those are important characteristics of a biomaterial to be used for bone tissue engineering.

The composites porosity became greater with elapsed time. This process could be basically explained by the larger polymer covering the bioceramic particles and by the polymer degradation process that acts synergistically exposing the bioceramic matrix porosity, serving as a scaffold for cellular adsorption and angiogenesis. By analogy, mesenchymal cells penetrating the porous spaces of the sintered hydroxyapatite phase have the potential to express at least two distinct morphogenetic programs: the formation of fibrous tissue or the differentiation of bone. This choice is determined by environmental signal controlled by the geometry of the substratum onto which they attach, proliferate, and eventually differentiate. In this way, initiation of bone formation may be linked to concavities rather than planar

or convex surfaces of the material (Ripamonti, 1999; Ripamonti et al., 1999).

Polymer degradation rate depends on factors such as polymers chain formation, crystalline and hydrophobic characteristic (Wong and Mooney, 1997). The presence of an extra methyl group on PLA makes it more hydrophobic and consequently shows lower degradation rate than PGA. As much lower degradation occurs, fewer degradation products will be in contact with the cells and tissue, resulting in less cytotoxicity and inflammatory response. Thus, the smaller in vitro cytotoxicity level of PLA:BC composites when compared to PLGA:BC ones could be explained by these facts.

Diverse studies related that calcium phosphate bioceramics are highly biocompatible with cells and tissues (Wykrota, 1991; Burg et al., 2000; Lobo, 2002; Rodrigues et al., 2003). In this study, BC biocompatibility was demonstrated by the results obtained by direct contact test and in vivo evaluation. However, we founded a high cytotoxic level of BC using the eluation test. This fact could be associated to lack of cohesion of the BC granules on tablet conformation. Thus, it may release considerable amounts of Ca^{2+} ions, thereby increasing its concentration on the eluate and producing a cytotoxic effect.

The in vivo results shown an initial inflammatory response observed in all groups. This process could be associated to the surgery trauma for tablet implantation, probably followed by biodegradable acid product release from the polymer.

The sustained cellularity observed at all implanted group sites (N1, N2, N3, N4, C1, C2 and BC) at day 13 suggest protein modifications at the implantation site, with lymphocytes recruitment. In this way, this period correspond to the necessary time to develop an adapted immunological response, which could be seen at day 21. Notwithstanding, there was not an increase of the inflammatory process, but an enlargement of tissue cellularity with the purpose to resolve the inflammatory process. On the other hand, these composites were implanted in a heterotrophic site, the subcutaneous tissue, with a hyperactive inflammatory behavior. Although, it was verified a slight inflammatory response, that could classify these composites as biocompatible materials. Minor inflammatory responses are expected by the implantation of these materials into an orthotropic site, such as osseous sites.

The present study showed that superficial bioerosion initially occurs on the composites as soon as it contacts the body, by erosion to the center of the device. The tablet shape exhibits a major superficial area/volume proportion, increasing polymer hydrolysis, which could reduce the pH. During this stage, an initial high release of tetracycline from the superficial area of the tablets is expected, which also could help to lower the pH. As the polymeric phase of the composites, is expected that TC release drops with the advance of the degradation. Nevertheless, presumably cationic Ca^{2+} groups of the soluble bioceramic phase, corresponding to β-TCP, would neutralize partially or totally the polymer degradation products and, at a minor scale, TC acid groups. Although, Ca^{2+} ions released by bioceramic degradation could enhance tissue adherence to the composites. These ions serve as mediators to the bind of some integrins to the RGD sequence (arginine, glycine and aspartic acid) of fibronectin and other adhesive proteins, e.g., thrombospondin, vitronectin and osteopontin (Ignjatovic and Uskokovic, 2004).

Although discovered at 1948 as natural fermentation products of *Streptomyces aureofaciens*, the tetracyclines continues been studied. The results founding in this study contribute to demonstrate the TC anti-inflammatory activity suggesting that it acts at the critical period of the inflammatory process resolution, which is an important factor with bone tissue engineering. This system reveals quite a significant delay in the release of the active substance, which is dependent on the amount of the tetracycline used and published studies have documented elsewhere (Popovic et al., 2002; El Attar et al., 1998; Kalish and Koujak, 2004). Nevertheless, the association of calcium phosphate with polymers such as PLG could intensify the activity of alkaline phosphatase, which is important for the differentiation of osteoblasts, which regulate the regeneration process within the organism (Ignjatovic et al., 2007).

The clinical implications of the low TC concentration addition in the composite are based on the intrinsic TC properties described above. It is well known that the osteoclast activity is increasing by the collagenase presence. Since hydrogen ions are pumped against a high concentration gradient by proton pumps, specifically a unique vacuolar-ATPase. In addition, collagenase and cathepsin K, hydrolytic enzymes are released to digest the organic components of the bone matrix. These enzymes are released by lysosomes. Nevertheless, the tetracyclines may stimulate the new bone formation and prevent the losing in mineral density (osteoporosis) by a reduction in osteoclast's activity (Ryan et al., 1996). The anti-collagenase activity of the tetracyclines could contribute to the success of the composite device implant.

In addition, angiogenesis, cellular adsorption and the deposition of fibrous tissue surrounding the implantation area are strong signs of material biocompatibility. Angiogenesis may provide a temporally regulated flow of cell populations capable of expression of the osteogenic phenotype (Ripamonti, 1999; Ripamonti et al., 1999).

The composites obtained from bioceramic and polymers rendered biomaterials with high plastic properties. These composites showed excellent biocompatibility, permitting angiogenesis, cellular adsorption and fibrous deposit. PLGA:BC composites presented the higher degradation rate. PLA:BC with tetracycline composites showed the best in vivo performance. Tetracycline addition optimized composites biocompatibility and contributed to anti-inflammatory response during the early phases of the wound healing process. These materials promise possible application in bone tissue engineering. Although, future bone-defect studies of heterotrophic or orthotropic material applications should be done to elicit the biological response in vivo.

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