



Testosterone improves the osteogenic potential of a composite in vitro and in vivo

Kelen J. R. da Costa¹ · Alfonso Gala-García¹ · Joel J. Passos² · Vagner R. Santos³ · Ruben D. Sinisterra² · Célia R. M. Lanza³ · Maria E. Cortés¹

Received: 16 July 2018 / Accepted: 22 November 2018
© Springer-Verlag GmbH Germany, part of Springer Nature 2019

Abstract

Testosterone (T) has been suggested as a promising agent in the bone osteointegration when incorporated in a bioceramic/polymer combination for the local application. The objective of this study was to evaluate the activity of a testosterone composite of poly (lactic-co-glycolic acid) (PLGA), polycaprolactone (PCL), and biphasic calcium phosphate (BCP) as a strategy for enhancing its osteogenic effect and to evaluate tissue response to the composite implantation. PLGA/PCL/BCP/T and PLGA/PCL/BCP composites were prepared and characterized using thermal analysis. Composite morphology and surface characteristics were assessed by SEM and EDS. The evaluations of in vitro effects of testosterone composite on osteoblasts viability, alkaline phosphatase activity, collagen production, osteocalcin concentration, quantification of mineralization, and nitric oxide concentration, after 7, 14, and 21 days. Testosterone was successfully incorporated and composites showed a homogeneously distributed porous structure. The PLGA/PCL/BCP/T composite had a stimulatory effect on osteoblastic activity on the parameters evaluated, except to nitric oxide production. After 60 days, the PLGA/PCL/BCP/T composite showed no chronic inflammatory infiltrate, whereas the PLGA/PCL/BCP composite showed mild chronic inflammatory infiltrate. Angiogenesis, cellular adsorption, and fibrous deposit were observed on the surfaces of implanted composites. The composites in combination with testosterone can be exploited to investigate the use of this scaffold for bone integration.

Keywords Testosterone · Bone formation · Calcium phosphate · Ceramic composite · Mineralization

Introduction

In regenerative medicine, the scaffold serves as a three-dimensional template for cell adhesion, proliferation, and formation of an extracellular matrix, as well as a carrier

for growth factors, drugs, or other biomolecular signals (Mei et al. 2005). Among the number of scaffold materials reported thus far, poly (lactide-co-glycolide) (PLGA) is the most common biodegradable polymer, due to its advantages such as adjustability of degradation rates, good mechanical properties, particularly toughness, and easy handling (Yu and Ding 2008; Shen et al. 2008). Polycaprolactone (PCL) is a semi-crystalline and biodegradable polymer, commonly used as biomaterial for tissue engineering that has been used in the medical field for the past 30 years (Chen and Boccaccini 2006).

Over recent years, synthetic calcium phosphate ceramics have been widely used in bone surgery (Naidoo et al. 2008). Among these ceramics, biphasic calcium phosphate (BCP) composed of hydroxyapatite (HA) and tricalcium phosphate (TCP) has received attention due to its reliable and controllable bioabsorption rate. The bioceramics HA and TCP may have superior bone ingrowth into BCP ceramics (Victor and Kumar 2008), although they are too brittle to carry considerable dynamic loads.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s00441-018-2970-3>) contains supplementary material, which is available to authorized users.

✉ Maria E. Cortés
mecortes@ufmg.br

- ¹ Restorative Dentistry Department, Dentistry Faculty, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, Belo Horizonte CEP 31270901, Brazil
- ² Department of Chemistry, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, Belo Horizonte, MG CEP 31270901, Brazil
- ³ Department of Dental Clinic, Pathology and Surgery, Dentistry Faculty, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, Belo Horizonte, MG CEP 31270901, Brazil

Androgens have also been playing an important role in the regulation of bone metabolism by influencing bone cell function via local and systemic growth factors and cytokines (Benghuzzi et al. 2004). Androgens have been demonstrated to influence bone cells in a complex fashion. Mostly, its effect on osteoblasts proliferation is biphasic in nature, showing growth enhancement following short or transient treatment and significant inhibition after a longer treatment (Wiren et al. 2004; Cheng et al. 2013; Steffens et al. 2014).

Testosterone (T) is a steroidal male hormone that regulates cellular calcium transport. The natural androgen testosterone has been used to prevent osteoporosis and coronary artery diseases, suppressing diverse immune reactions, and with prescriptions in gerontology. Both estrogen (E) and T sex hormones have receptors on all bone cells, with androgen dominance on osteoblasts and osteocytes.

The activity of the sex steroids, influenced by various enzymes found in bone, is reflective of the hormone ligand before its binding to the bone cells (Notelovitz 2002). As a result, T acts both directly and via its aromatization of estradiol. The activity of the androgens also varies with the bone surface; periosteal cells, for example, do not have 5 α -reductase activity, indicating that T is the active metabolite at this clinically important site. Androgens enhance osteoblastic differentiation by regulating bone matrix production, organization, and mineralization as well as regulating the recruitment and activity of osteoclasts. The sustained delivery of T or its metabolite can stimulate osteoblastic activities eventually causing an increase in cortical bone density while maintaining a healthy femur with a normal architecture (Callewaert et al. 2010). Furthermore, *in vivo* studies showed that testosterone delivered with a scaffold was effective in promoting the healing of a critical-size segmental defect of the femoral bone in mice (Cheng et al. 2013).

Attempts to enhance bone ingrowth have been focused on modifying or coating surfaces to promote osteogenic conditions for regeneration. Osteoblastic cells appear to be stimulated by androgens *in vitro*. However, their use *in vivo* is controversial due to the virilizing side effects as well as alterations in the lipoprotein profiles.

A preliminary study showed that the PLGA/PCL/BCP/T scaffold exhibited desirable characteristics for bone tissue applications (i.e., adhesion, growth, and cellular proliferation) and the absence of cytotoxic effects (Da Costa et al. 2012). This research proposed the use of testosterone incorporated into biodegradable polymers to form a bioactive ceramic composite in the form of porous matrices, developed with the aim of accelerating bone ingrowth at osseous defect sites. Thus, this study evaluated the osteogenic cellular response of biodegradable PLGA/PCL/BCP/T composite scaffolds after 7, 14, and 21 days of *in vitro* culture and the subcutaneous tissue response of the composite implanted in rats after 15, 30, and 60 days.

Materials and methods

Materials

The testosterone propionate (Pharma Mostra, MG, Brazil), PLGA (50:50 lactide/glycolide molar ratio (MW) = 60,000 g/mol), and PCL (MW = 10,000 g/mol) were obtained from Birmingham Polymers, Inc., Durect Corporation, Pelham, USA. Other materials used included silver nitrate PA, sodium thiosulfate, glacial acetic acid (Synth, SP, Brazil), and safranin (Panreac, Barcelona, Spain). The BCP (Osteosynt®), with 65% hydroxyapatite (HA) [$\text{Ca}_5(\text{PO}_4)_3\text{OH}$] and 35% β -tricalcium phosphate (β -TCP) [$\text{Ca}_3(\text{PO}_4)_2$] (w/w), was kindly donated by Einco Biomaterial, MG, Brazil. Dichloromethane (DCM) from Quimex, SP, Brazil).

Preparation of the composites

The composites were prepared by the solvent evaporation method at 1:1:8 (PCL/PLGA/BCP) (Kim et al. 2004) and T/(BCP + PCL + PLGA) = 0.1 w/w ratio. One gram of each polymer (PLGA and PCL) was dissolved in 50 mL of DCM and 8 g of BCP was added to the polymer solution and stirred for 2 h at room temperature (PLGA/PCL/BCP). A fixed concentration (10^{-9} mol/L) of T was added to the solution [T/(BCP + PCL + PLGA) = 0.1 w/w]. The composites were placed into a 4-mm-diameter cylindrical mold and sliced to yield 1-mm-thick disks weighing 150 mg. The disks were then sterilized using the ethylene oxide process, with a maximum of 50.6 °C.

Characterization

In order to determine the thermal profile of the starting materials, the composite's thermogravimetric measurements (TG) and differential thermal analysis (DTA) were performed using a thermo-balance SDT Q600 (TA Instruments, Inc.). An average of 5 mg of the sample was used for each analysis.

The morphology was evaluated by scanning electron microscope and energy-dispersive X-ray spectrometer (SEM/EDS). Three samples each from PLGA/PCL/BCP/T and PLGA/PCL/BCP composites were examined for qualitative assessment by SEM for the presence of pores, before and after 21 days in a simulated body fluid (SBF) (Kokubo and Takadama 2006). EDS analyses were performed to study the *in vitro* bioactivity through the formation of bone-like apatite on the surface of the samples before and after 21 days in SBF. The composites were immersed in 2 mL SBF for 21 days at 37 °C and the SBF solutions were replaced every 3 days with fresh solution. An FEG scanning electron microscope, FEI Quanta 200, from the Microscopy Center of the Federal University of Minas Gerais was used for evaluation.

The pH of the composites with and without hormone was evaluated. The samples were placed in Eppendorf tubes containing 2 mL PBS with pH 7.4 on an incubator bench (CTM45, Contemp) for 21 days. The supernatant was collected and replaced with fresh PBS solution every 24 h, and the pH was measured using a digital pH meter (HI 221 Hanna Instruments®).

In vitro studies

Cell attachment, viability, differentiation, and mineralization resulting from the interaction between composites and osteoblasts were studied in hexaplicate ($n = 6$) for each experimental time in three different experiments.

Osteoblasts were isolated from the calvaria of newborn Wistar rats at 1–5 days of age (Declercq et al. 2004). The procedures performed on animals complied with the standards of the Ethics Committee on Animal Use of UFMG. The calvaria was dissected, removed all soft tissue, cut into fragment, and washed in sterile buffered PBS without calcium and magnesium. Calvary fragments were digested in 1% trypsin-EDTA for 5 min, and the precipitates following centrifugation at 1000 rpm for 5 min were treated with four sequential incubations with 2% collagenase II at 37 °C for 30 min each. Supernatants from the first two incubations were discarded. Supernatants from the last three collagenase incubations were centrifuged at 1000 rpm for 5 min, and the pellet of cells with a high proportion of osteoblasts was resuspended in 5 mL of DMEM medium supplemented with 10% FBS, 1% antibiotic-antimycotic, and ascorbic acid 100 µg/mL and transferred to cell culture flasks. Cells adhered to the flask characterized the first passage. When these reached 80% of subconfluence, they were trypsinized and transferred to culture flask until the second passage and cultured for 2–4 days in a humidified atmosphere of 5% CO₂–95% air at 37 °C.

After the second passage, osteoblasts (5×10^4 cells/cm²) seeded on the composite disks placed onto 24-well plates in a CO₂ incubator. After 7, 14, and 21 days of incubation, they were tested for viability, production of alkaline phosphatase and total protein content, collagen production, osteocalcin levels, mineralization, and measurement of nitric oxide (NO). Cells cultured in polystyrene were used as a positive control and the assays were performed in hexaplicate.

The viability of osteoblasts cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich). After each experimental time point, the cells were incubated for 4 h with 40 µL of MTT solution (5 mg/mL) at 37 °C in a humid atmosphere. After complete conversion of the MTT to formazan crystals, 400 µL of 10% dimethyl sulfoxide (SDS)/0.01 N HCl solution was added to each well to extract the formazan crystals under gentle shaking. After an overnight incubation, the optical

density was measured at 570 nm in a spectrophotometer (Multiskan Spectrum Thermo Scientific, Finland).

To determine the alkaline phosphatase (ALP) activity, the culture medium was first removed and 1 mL of deionized water was added. The cells were then lysed in five freeze/thaw cycles (–20/37 °C). The cell lysates were placed in a 96-well plate for measurement of ALP activity using the 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) in conjunction with nitro blue tetrazolium (NBT) assay (Invitrogen/Vybrant, OR, USA). The amount of total protein in the cell lysates was determined using a Micro-BCA Protein Assay. ALP activity was normalized to total protein concentration and expressed as units per milligram of protein.

The collagen production of osteoblasts was analyzed by Sircol Collagen Assay Invitrogen® (Invitrogen/Vybrant, OR, USA). This method is based on the selective binding property of the Sirius Red dye to the [Gly-X-y] tripeptide end sequence of mammalian collagen. The solubilized collagen was evaluated by optical density analysis at 555 nm. The amount of collagen was calculated using a standard curve representing the relationship between collagen concentration and optical density.

The quantification of Rat Osteocalcin EIA Kit® (Biomedical Technologies Inc., USA) was used to evaluate osteocalcin synthesis. The supernatants of each experimental group and controls were collected at the experiment time points. Samples of 25 and 100 µL osteocalcin antiserum were transferred to a pretreated plate with a monoclonal antibody directed against the N-terminal region of osteocalcin and incubated at 37 °C for 2.5 h. The plate was washed and then incubated with a second antibody (goat polyclonal) of high specificity against the C-terminal of rat osteocalcin. Detection was achieved using a horseradish peroxidase conjugate of a donkey anti-goat IgG antibody and subsequent enzyme assay. The osteocalcin concentration was proportional to color development. Standards of highly purified rat osteocalcin were used to generate a standard curve. The absorbance was measured at 450 nm using the Multiskan spectrophotometer.

The quantification of matrix mineralization was evaluated by the mineralization nodule formation where cells of each experimental group and the control were submitted to staining by the Von Kossa technique, after each experimental time point. The culture medium was removed, and cells were washed three times with PBS and fixed with 4% paraformaldehyde for 5 min. The solution was discarded and the cells washed with deionized water (DI-PAK®, Milli-DI, Millipore). A solution of silver nitrate 5% was added and plates exposed to ultraviolet light for 60 min. The cells were again washed with deionized water three times, followed by the addition of 5% sodium thiosulfate solution for 3 min. The cells were counterstained with 1% safranin for 30 s. The nodules were counted in five random fields per well using an inverted optical microscope (×10).

Finally, the nitrite concentration (NO) was measured in the culture supernatant of osteoblasts as an indirect measure of NO synthesis using the Griess Reagent (G-7921) (Invitrogen Corporation, USA). The culture supernatants from each group and the control group were collected at specific time points and 50 μ L of each sample was incubated with 50 μ L of reagent for 1 h at room temperature, protected from light. The optical density was measured at 548 nm in a spectrophotometer. The nitrite concentration was calculated by averaging the values of the standard curve of NaNO₂ and values were expressed as millimoles per liter of nitrite (Green et al. 1982).

In vivo study

The toxicity of the PLGA/PCL/BCP/T and PLGA/PCL/BCP composites was assessed by subcutaneous tests in vivo. Thirty male *Wistar* rats were used (aged 4 weeks and weighing 150–210 g). For this experiment, the animals were randomly distributed each into the control group ($n = 2$), PLGA/PCL/BCP/T group ($n = 4$), and PLGA/PCL/BCP group ($n = 4$) and evaluated after 15, 30, and 60 days of the surgery by using one implant per animal. The animals were anesthetized with an intramuscular injection of 10% ketamine (Cetamin®, Syntec, São Paulo, Brazil) mixed with 10% xylazine (Anasedam®, Vetbrands®, São Paulo, Brazil) at a ratio of 2:1 ketamine to xylazine *v/v* (0.1 mL/100 g of body weight). The skin in the dorsal region of the animals was shaved and disinfected with iodine, a perpendicular incision was made, and a subcutaneous pocket was created by blunt dissection. The composite was then inserted and the incision was sutured with a 4–0 nylon monofilament. The animals were housed in pairs and maintained under controlled conditions of room temperature, light–dark periods of 12 h. After the experimental time, the animals were anesthetized and sacrificed by cervical dislocation. The implants and the surrounding tissue removed were processed using a standard procedure for removing histopathological analysis described elsewhere (Gala-García et al. 2012). Thin sections (5 μ m) were prepared using a microtome and three sections of each specimen were stained with hematoxylin and eosin.

The parameters of inflammation (presence of cell infiltrate, polymorphonuclear leukocytes, macrophages, and lymphocytes), fibrosis, granulation tissue, and necrotic tissue were qualitatively analyzed by a pathologist in a blinded manner. For SEM, the specimens were fixed in 5% buffered glutaraldehyde for 1 h, washed with PBS, and post-fixed in 1% osmium tetroxide, followed by dehydration with graded water-ethanol series of solutions (30, 50, 70, 90, and 100%) for 10 min each. The samples were critically point dried and sputter coated with gold. Samples were examined using SEM (FEG Quanta 200 FEI). To the histological examination, scores were attributed as 0 (absence of inflammation), 1 (slight), 2 (moderate), and 3 (intense inflammation).

Statistical analysis

Statistical evaluation of data was performed, to in vitro study, using ANOVA two-way and Bonferroni's post-test. Quantitative data were obtained from repeated experiments in hexaplicate ($n = 6$) for all in vitro studies and reported as the mean standard deviation, as indicated. To in vivo study, the Kruskal-Wallis test and Dunn's post-test were used to evaluate the data of non-parametric samples. The P values ≤ 0.05 were considered statistically significant. For both data analyses, the software GraphPad Prism 5 was used.

Results

Thermal properties

The testosterone demonstrated thermal stability until 340 °C after which a continuous weight loss was observed (Fig. 1a). Analyzing the T DTA curve, two endothermic events were also observed: the first approximately at 110 °C, associated with the T melting point, and the second at 340 °C, associated with the complete degradation of the drug (Fig. 2a). TG/DTA data for bioceramic showed a weight loss of 1.6% up to 100 °C, after which thermal stability was observed until 700 °C (Figs. 1b and 2b). This first process could be associated with the water loss from the ceramic matrix. PCL showed thermal stability up to 330 °C, with a continuous weight loss up to approximately 450 °C (Fig. 1c). The DTA curve of PCL showed two endothermic events (Fig. 2c), the first around 50–60 °C associated with the polymer melting point, and the

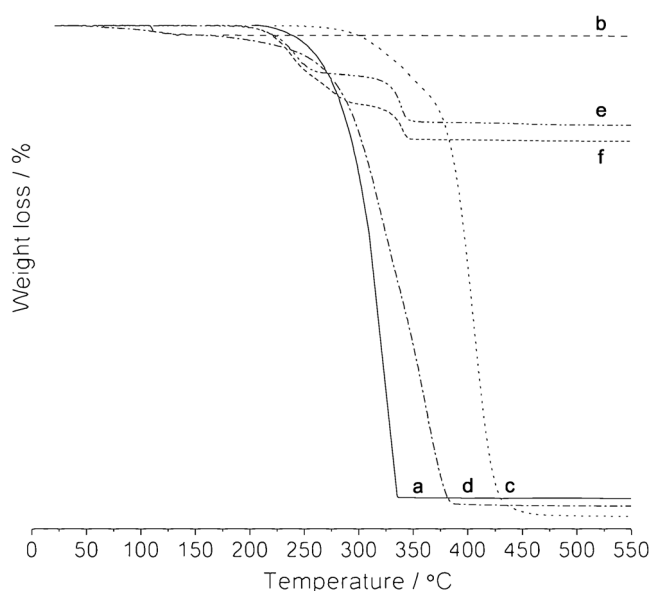


Fig. 1 TG curves of **a** testosterone, **b** biphasic calcium phosphate, **c** polycaprolactone, **d** poly (lactic-co-glycolic acid), **e** PLGA/PCL/BCP scaffold, and **f** PLGA/PCL/BCP/T scaffold

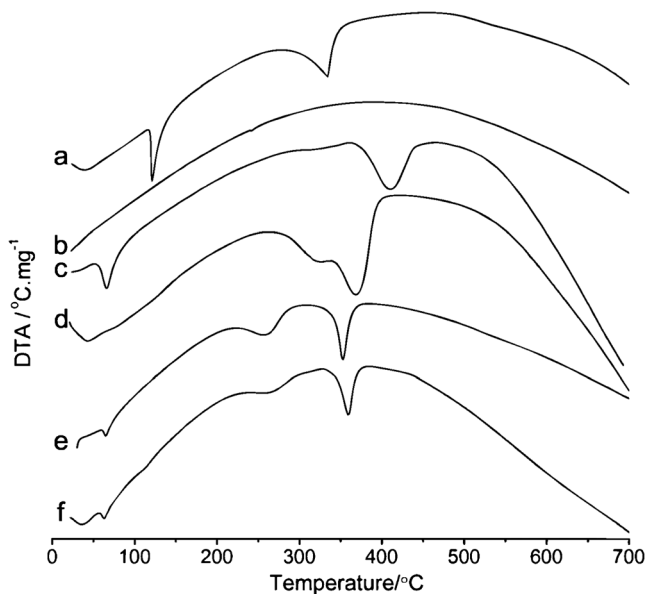


Fig. 2 Differential thermal analysis (DTA) of **a** testosterone, **b** biphasic calcium phosphate, **c** polycaprolactone, **d** poly (lactic-co-glycolic acid), **e** PLGA/PCL/BCP scaffold, and **f** PLGA/PCL/BCP/T scaffold

second endothermic peak at approximately 400 °C, representing the total degradation of the polymer (Barbanti et al. 2008). PLGA showed a thermal stability up to 250 °C, followed by a single thermal event at approximately 375 °C, referring to the complete decomposition of the polymer (Fig. 1d). The PLGA DTA curve (Fig. 2d) showed an endothermic event at approximately 45–50 °C associated with the polymer glass transition (T_g) and the second at 300–370 °C associated with the thermo-decomposition (Barbanti et al. 2008).

The PLGA/PCL/BCP composite showed a similar decomposition profile of the polymers in which thermal overlapping phenomena was observed (Fig. 1e). The high residual mass (79.2%) observed is attributed to the presence of BCP, as this showed a higher thermal decomposition profile compared with the PLGA, PCL polymer, and T drug. The PLGA/PCL/BCP composite DTA curve showed two endothermic peaks: one at 56 °C, associated with the melting point of PCL, and the second at 250–350 °C, associated with the thermal decomposition of both polymers (Fig. 2e) (Abd El-Mohdy 2012; Barbanti et al. 2008). In addition, the PLGA/PCL/BCP/T composite showed thermal stability up to approximately 160 °C, at which point a mass loss began until 340 °C when a second continuous mass loss was observed until 360 °C (Fig. 1f). The presence of T in the composite changed the thermal decomposition profile, which may be due to the stronger interaction between the T hormone and the polymers, especially the PCL. The DTA curve of the composite showed three endothermic events associated with some of the raw materials (Fig. 2f). The event observed at 47 °C can be attributed to the T_g temperature of PLGA, the second at 56 °C

suggests the melting point of PCL, and the third at approximately 250–350 °C corresponds to the complete degradation of polymers and T.

pH variation

During the first 24 h of the pH evaluation, a slight pH decrease was observed in all samples (6.5). However, at 48 h, the pH stabilized at approximately 7.4.

In vitro bioactivity (SEM/EDS)

The SEM and EDS results of the PLGA/PCL/BCP/T composite revealed a porous structure and a higher prevalence of calcium and phosphate, respectively (Fig. 3a and Suppl. 3a, respectively). SEM-EDS of the same composite after soaking in SBF for 21 days showed an increased exposure of pores and higher intensity of the calcium and phosphate peaks, respectively (Fig. 3b and Suppl. 3b). Examination of the composite without hormone showed the same characteristics before and after 21 days in SBF (Fig. 3c and Suppl. 3c). EDS analysis further confirmed the Ca/P molar ratio of the composite surface was 1.63.

Viability study

Under normal conditions, osteoblasts showed significantly higher values of viability increasing over the time (7, 14, and 21 days). However, in the presence of the hormone (T), this was more notorious even when compared to the bioceramic group. In this group, a higher number of viable cells were observed after 7 and 14 days, compared to the group without hormone. The difference was maintained at 21 days ($P < 0.001$) also and statistically significant (Fig. 4a).

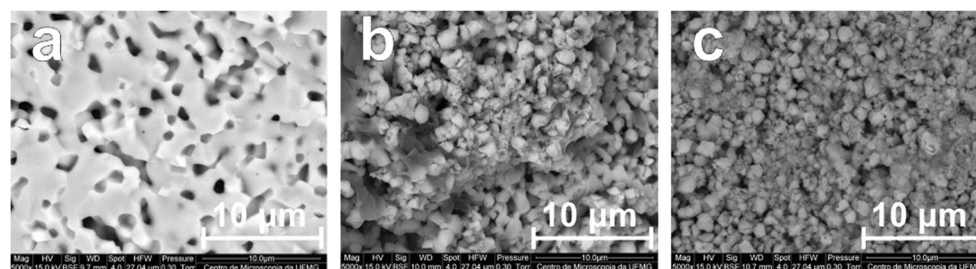
Assay of alkaline phosphatase

In agreement with viability data, alkaline phosphatase assay was used to evaluate osteoblasts cells activity. Figure 4b presents the alkaline phosphate activity for each group in the three experimental times.

Collagen production

To examine the effects of composites with T on collagen production, the Sircol assay was performed (Fig. 4c). The collagen production in the control group increased over time ($P < 0.05$). The production of collagen by the osteoblasts by PLGA/PCL/BCP/T was $P < 0.05$ than the collagen production when compare with control group and composite without the hormone until the 14th day of stimulation. After 21 days of stimulation, the production of collagen in the presence of T was statistically lower than the collagen production in the

Fig. 3 SEM images of **a** PLGA/PCL/BCP/T and **b** PLGA/PCL/BCP/T after soaking in SBF for 21 days and **c** PLGA/PCL/BCP after soaking in SBF for 21 days



control group and similar with the composites without hormone at the same stimulation period.

Quantification of osteocalcin

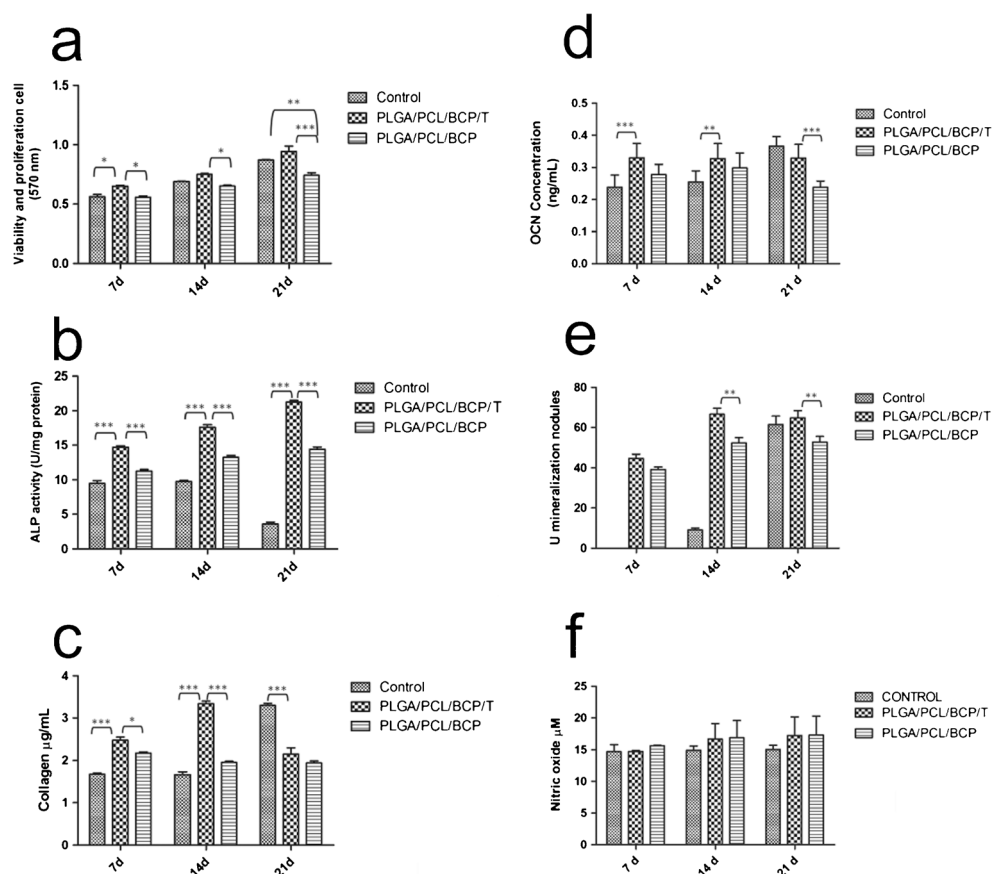
Osteocalcin (OCN) is a non-collagenous protein synthesized by osteoblasts, and its expression is linked to mineralization of the extracellular matrix. The concentration of osteocalcin was statistically higher in the composite with T compared to the respective controls of 7 and 14 days of stimulation (Fig. 4d). These findings demonstrate that cells seeded with the PLGA/PCL/BCP/T composite are at a more advanced stage of maturation compared with the cells seeded with the composite without hormone and control cells (Xynos et al. 2000; Shor et al. 2007). The cells with the composite with T had

higher concentrations of OCN after 21 days (0.35 ng/mL) compared with the composite group without hormone (Fig. 4d; $P < 0.001$). This result is consistent with the MTT, alkaline phosphatase, and collagen results, which showed that the T enhances the viability and osteoblastic cell response, increasing the production of osteocalcin.

Matrix mineralization

Matrix mineralization was evaluated by von Kossa staining using a short- and long-term exposure of osteoblasts to composites. Interestingly, nodules of mineralization were observed with osteoblasts seeded with composites loaded with T after 7 days, while no mineralization was observed with the control group (Fig. 4e). Higher amounts of mineralization

Fig. 4 In vitro assays of osteoblasts cells with polymer-bioceramic composites incorporated with testosterone. **a** Cell viability (MTT assay), **b** alkaline phosphatase activity, **c** collagen concentration, **d** osteocalcin concentration, **e** nodules of mineralization, and **f** nitric oxide concentration after 7, 14, and 21 days of stimulation. Data analyzed represent mean \pm SD of optical density measurement of each test of hexaplicate from three separate experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$



nodules were observed with the composite with T compared to hormone-free composites at 14 days. However, after 21 days, there was no significant difference between the number of mineralization nodules in the control and the PLGA/PCL/BCP/T composites (Fig. 4e).

NO concentration

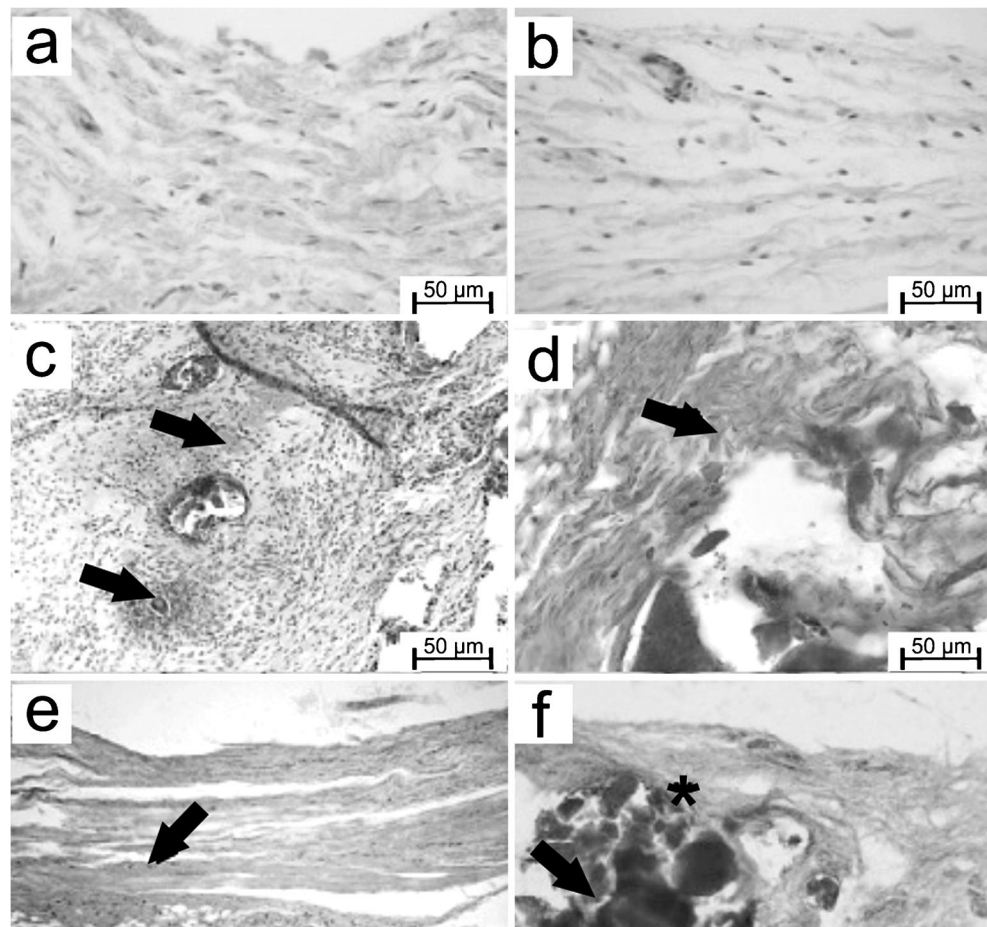
Figure 4f shows the NO concentrations at the three experimental time points in cell cultures with the composites. The NO concentration was low in all groups at all experimental times studied (Fig. 4f). No significant differences in the concentration of NO were observed among groups. NO has been shown to interfere with bone metabolism (Van't Hof and Ralston 2001). Although its role is still somewhat unknown, studies have shown that it has a biphasic effect in osteoblastic and osteoclastic activities. In vitro studies indicate that low concentrations of NO produced constitutively by osteoblasts might act as an autocrine stimulator in osteoblasts proliferation (Riancho et al. 1995; Lin et al. 2008), whereas high concentrations inhibit both activity and proliferation of these cells (Lin et al. 2008).

Histopathological analysis

During the experimental period, the animals showed no post-operative complications. They quickly returned to their normal diet and showed no loss of body mass. Furthermore, no animal died during the experiment and infection was not detected in the injured area.

The control group presented inflammation compatible with the physiological process at all times evaluated (Fig. 5a, b). In the PLGA/PCL/BCP/T group, chronic inflammatory infiltrates with lymphocytes, plasma cells, and macrophages in connective tissue, capillary neoformation, and fibroblasts with collagen fibers were observed after 30 days (Fig. 5c). The presence of chronic inflammatory infiltrate, multinucleated giant cells, dilated vessels and hyperemia, fibroblasts, and collagen fibers were observed after 60 days (Fig. 5d). Similarly, the PLGA/PCL/BCP group showed chronic inflammatory infiltrate, foreign body cells around the amorphous material, giant cells, dilated vessels, and hyperemia, capillary neoformation, fibroblasts, and collagen fibers in the chronic inflammatory infiltrate after 30 and 60 days (Fig. 5e, f). In summary, the PLGA/PCL/BCP/T or PLGA/PCL/BCP scaffolds did not induce tissue necrosis. Therefore, these results

Fig. 5 Histological evaluations of composite/tissue interaction after implantation in the dorsal subcutaneous of rat. Control group after **a** 15 days, **b** 30 days, **c** and **d** 60 days of surgical procedure. PLGA/PCL/BCP/T composite after **d** 15 days, **e** 30 days, and **f** 60 days of implantation. PLGA/PCL/BCP composite after **g** 15 days, **h** 30 days, and **i** 60 days of implantation



indicate that PLGA/PCL/BCP/T scaffolds are biocompatible materials in this setting.

Over time, SEM analyses of composite scaffold showed superficial bioerosion. No difference between the composites with or without T related to biodegradability and biocompatibility was observed by macroscopic and SEM analyses. However, a strong tissue-composite interaction was observed until 60 days of implantation, independent of T addition. Furthermore, the composites stimulated microvasculature (Fig. 6).

Discussion

In the present work, physicochemical and biological properties were examined since those mean an important role in physiological and pathological processes including tissue remodeling and inflammation. Previous assembly procedures using biodegradable polymer and calcium phosphate scaffolds allowed the production of scaffolds with characteristics suitable for the growth of osteoblasts (Da Costa et al. 2012).

Initially, it was demonstrated that the biodegradable scaffolds were thermally stable up to approximately 100 °C, which guarantees the use of the composite with presence or absence of T in physiological and storage conditions. The addition of T to the composite did not alter its degradation temperature and this result is consistent with the literature (Barbanti et al. 2008; Dorozhkin 2011; Abd El-Mohdy 2012).

Additionally, the pH is a critical parameter for cell viability since the majority of cells requires an optimal pH of 7.4 for normal growth and begins to lose viability when the pH reaches

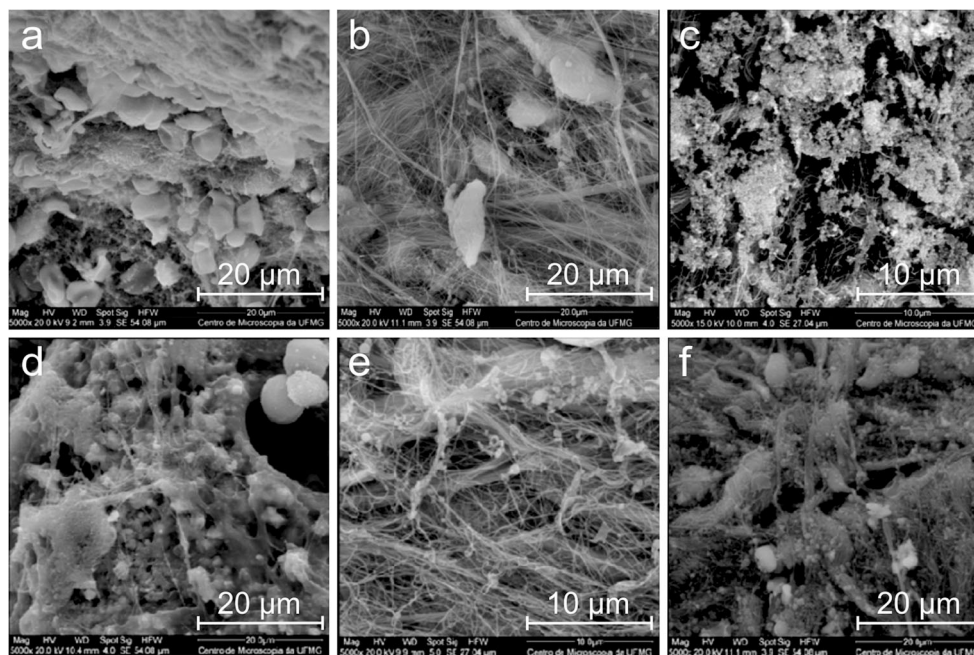
6.0–6.5 (Freshney 1994). The pH measurement was taken from all composite samples, with or without hormone and the slight acidification (6.4) of the medium recorded in the first 24 h did not interfere with the adhesion and viability of osteoblasts. This fact is related to the degradation of the PLGA polymer and PCL that when in the form of polymer blend feature faster degradation (Zhao et al. 2011; Galow et al. 2017).

Since the addition of T to the composite would affect the apatite-inducing ability of BCP, the bioactivity of scaffolds was investigated in parallel. The present work verified *in vitro* by EDS examination the formation of like-hydroxyapatite on the surfaces, which showed a similar characteristic as those reported in the literature (Zhao et al. 2011).

The EDS analyses showed that the molar ratio of calcium/phosphate on the composite surface was approximately 1.63, which is approximated to the data reports in the literature. In this work, it was evaluated whether the PLGA/PCL/BCP/T composite would affect the capacity of induction of apatite of BCP. The results demonstrated that BCP still has this capability to induce the formation of bone-like apatite and that this activity was independent of the incorporation of T to the composite.

Biphasic calcium phosphate bioceramic was shown to induce the formation of bone-like apatite on the surface of the materials *in vitro* and *in vivo* when implanted in an animal, which reflects the potential of the materials to bond with bone tissue (Legeros 2008; Lobo et al. 2015). These results demonstrate that BCP still has the ability to induce the formation of bone-like apatite and that this activity was independent of T incorporation in the composite and was in accordance with those of the cell viability demonstrated by MTT assay.

Fig. 6 SEM images of evaluation in rats subcutaneous connective tissue treated with **a** PLGA/PCL/BCP/T after 15 days, **b** PLGA/PCL/BCP/T after 30 days, **c** PLGA/PCL/BCP/T after 60 days, **d** PLGA/PCL/BCP after 15 days, **e** PLGA/PCL/BCP after 30 days, and **f** PLGA/PCL/BCP after 60 days



The androgens directly stimulate osteoblasts proliferation *in vitro* and induce the expression of alkaline phosphatase in osteoblastic cells (Kasper et al. 1989). These actions are mediated by androgen receptors on osteoblasts that increase DNA synthesis and differentiation or by aromatization of androgens.

The overall process of mineralization can be attributed to the cell metabolism process including proliferation, matrix maturation, and mineralization. For that reason, the alkaline phosphatase was measured, which is associated with the mineralization process of the extracellular matrix and is responsible for the hydrolysis of organophosphates, increasing the concentration of phosphate ions at the sites of mineralization, as together with calcium ions promote the formation of deposits of calcium phosphate in mineralization matrix (Müller et al. 2011). The osteoblasts seeded with composites with the hormone showed higher alkaline phosphatase activity, representing the largest cell differentiation in the three time points when compared to their respective controls and to the composite without T ($P < 0.05$).

An interesting finding was that the cultured cells with composites with testosterone addition maintaining the activity of alkaline phosphatase production up to 14 days, with the release of osteocalcin and extracellular matrix mineralization. Also, the scaffold-T presence favored highest collagen production of osteoblasts after 7 and 14 days in comparison with the other experimental and control group ($P < 0.001$). The collagen type I is the most present component of the non-calcified bone matrix that will act as a framework for apatite and bone crystals. Hormones, as well as growth factors and cytokines, can act as signaling molecules during the organic phase (Nanci and Ten Cate 2008, Lin et al. 2008), and the presence of testosterone may have contributed to this increased production of collagen in the initial periods.

Moreover, the results further proved that T could improve osteoblasts cell differentiation to greater levels ($P < 0.001$) than the composite without T during the long-term (Fig. 4b). Simultaneous and enhanced cell differentiation would provide an ideal situation for bone growth and repair.

Dihydrotestosterone increases bone formation on the periosteal surface of the cortical bone, whereas estrogens reduce bone formation (Turner et al. 1990). Studies have demonstrated that systemic administration of T in primary regimens for osteopenia and hypogonadism replacement therapy could help not only ameliorate the symptoms but also to increase bone mineral density. Since androgens might help promote the proliferation and differentiation of osteoblasts as well as inhibit osteoclastic activity decreased bone density may result. Here an attempt has been made to exploit this property of T could bring a potentially high value designed for tissue engineering in support of enhancing bone repair.

According to Notelovitz (2002), testosterone seems to play an important role in regulating the organization and

production of the bone matrix. These results agree with the results of Da Costa et al. (2012) which showed the presence of this hormone in composites of biodegradable polymers and biphasic calcium phosphate increases the viability of osteoblasts. In contrast, Wiren et al. (2004) showed that androgen stimulates osteoblasts and osteocyte apoptosis even in anabolic settings.

It is well-established that not only the number but also the activity of osteoblasts is critical for normal bone growth and maintenance. Androgens play a key role in the regulation of bone matrix production and organization, stimulating osteoblast proliferation, differentiation, and synthesis of extracellular matrix proteins, such as type I collagen, osteocalcin, as well as mineralization (Benghuzzi et al. 2004).

Similarly, Cheng et al. (2013) evaluated the use of scaffolds, containing testosterone or bone morphogenetic protein-2 or the combination of both, for the treatment of critically sized bone defects in wild-type rats and knockout for the androgen (Arko). The results showed that BMP-2 induced bone formation within 14 days in both analyzed groups wild and Arko rats. Testosterone treatment also induced bone formation within 14 days in wild rats but not in Arko rats. This fact is suggesting that the androgen receptor is required for testosterone initiate bone formation. These results demonstrate that testosterone is as effective as BMP-2 to promote healing of critical-size bone defects and that the combination therapy of testosterone and BMP-2 is superior to single therapy. In the present work, the mass of testosterone used to prepare the scaffolds was not cytotoxic, since its release into the medium occurs slowly.

Another important parameter in relation to the structure of the scaffolds is the presence and size of the pores. The porosity of the 3D supports used in bioengineering of the bone tissue has a considerable importance, as it influences several processes related to physicochemical properties (such as surface area, ion release by the matrix, mechanical properties, among others) and biological processes (cell colonization, diffusion of gases and nutrients, formation of new blood vessels, etc.). Porosity is a critical factor for cell migration and the elaboration of a bone matrix. Yamasaki and Sakai (1992) reported having found heterotopic bone formation around porous ceramic hydroxyapatite granules but not around dense granules. The porous granules had a size between 200 and 600 μm and a continuous and interconnected network, with a microporosity ranging from 2 to 10 μm in diameter. Boyan et al. (1996) reported that osteoblasts prefer to adhere to pores with sizes ranging from 200 to 400 μm in diameter to facilitate migration, adhesion, and proliferation.

In this work, the pore size of the composites was verified through the MEV; it can be said that they were favorable for cell adhesion and migration, because to support the growth and invagination of trabecular bone tissue, the pores must have the size, minimum, between 40 and 100 μm . The size

of the trabeculae in the bone tissue that varies from 20 to 100 μm allows the trabeculae to conduct its own blood vessel, in the same way as the compact bone (Nasr et al. 1999).

EDS analysis showed that the Ca/P molar ratio of the composite surface is approximately 1.63, which is close to the hydroxyapatite results reported in the literature (Zhao et al. 2011). Biomass of biphasic calcium phosphate has the ability to form bone-like apatite on its surface both in vitro and in vivo, which reflects the potential of the material to bond with bone tissue (Legeros 2008). In this work, it was evaluated whether the composite PLGA/PCL/BCP/T would affect the capacity of induction of apatite by BCP and that this activity was independent of the incorporation of T to the composite. The composites loaded with T could increase the rate of bone mineralization.

Despite the subcutaneous tissue inflammatory response observed in the histopathological study, there was no difference among the groups. The effect of testosterone on the inflammatory process is still not well-established. Studies which evaluated this relationship showed varying results, depending on the tissue and cells evaluated the model of study used and the dosage of T employed. For example, Steffens et al. (2014) suggested that both low and high testosterone levels increase inflammatory bone loss in male rats. While low testosterone predominantly increases the inflammatory response, high testosterone promotes a higher osteoblast-derived RANKL/OPG ratio.

The in vivo results corroborate that the angiogenesis, cellular adsorption, and the deposition of fibrous tissue surrounding the implantation area stimulated with bioceramics and T incorporation in the scaffold testosterone did not alter this response. Contrary, Engeland et al. (2009) related that T activity increased mucosal healing rates and that the testosterone may impact upon the proliferative phase of healing which involves re-epithelialization and angiogenesis. Angiogenesis may provide a temporally regulated flow of cell populations capable of osteogenic phenotype expression. Those are strong parameters of material biocompatibility. Nevertheless, more in vivo studies are necessary in order to know other systemic effects that may appear after the use of scaffolds containing testosterone.

Conclusions

The data from the present study demonstrated that the testosterone in combination with polymeric composite is able to increase osteoblast viability with enhanced production of extracellular matrix and mineralization. Additionally, this scaffold was biocompatible causing cellular adhesion and angiogenesis, which are crucial to increasing bone regeneration and can be exploited to investigate this promising scaffold for bone integration.

Acknowledgements The authors are grateful to the Chemistry Department of UFMG for the use of their Thermal Spectroscopy and Center of Microscopy (UFMG) for providing the equipment.

Funding This study had financial support by the *Fundação de Amparo à Pesquisa do Estado de Minas Gerais* (FAPEMIG), *Instituto Nacional de Ciência e Tecnologia Nanobiofar* (INCT-Nanobiofar) CNPq Brazilian agencies.

Compliance with ethical standards

Conflict of interest The authors declare that they do not have any conflict of interests.

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

References

- Abd El-Mohdy HL (2012) Controlled release of testosterone propionate based on poly N-vinyl pyrrolidone/2-acrylamido-2-methyl-1-propanesulfonic acid hydrogels prepared by ionizing radiation. *J Polym Res* 19:9931. <https://doi.org/10.1007/s10965-012-9931-4>
- Barbanti SH, Zavaglia CAC, Duek EAR (2008) Effect of salt leaching on PCL and PLGA (50/50) resorbable scaffolds. *Mater Res* 11(1):75–80. <https://doi.org/10.1590/S1516-14392008000100014>
- Benghuzzi H, Tucci M, Tsao A, Russell G, England B, Ragab A (2004) Stimulation of osteogenesis by means of sustained delivery of various natural androgenic hormones. *Biomed Sci Instrum* 40:99–104 ISSN: 0067-8856
- Boyan BD, Hummert TW, Dean DD, Schwartz Z (1996) Role of material surfaces in regulating bone and cartilage cell response. *Biomaterials* 17(2):137–146
- Callewaert F, Boonen S, Vanderschueren D (2010) Sex steroids and the male skeleton: a tale of two hormones. *Trends Endocrinol Metab* 21(2):89–95. <https://doi.org/10.1016/j.tem.2009.09.002>
- Chen QZ, Boccaccini AR (2006) Poly (D,L-lactic acid) coated 45S5 Bioglass®-based scaffolds: processing and characterization. *J Biomed Mater Res A* 77:445–457. <https://doi.org/10.1002/jbm.a.30636>
- Cheng BH, Chu TMG, Chang C, Kang HY, Huang KE (2013) Testosterone delivered with a scaffold is as effective as bone morphologic protein-2 in promoting the repair of critical-size segmental defect of femoral bone in mice. *PLoS One* 8:e70234. <https://doi.org/10.1371/journal.pone.0070234>
- Da Costa KJR, Passos JJ, Gomes ADM, Sinisterra RD, Lanza CR, Cortés ME (2012) Effect of testosterone incorporation on cell proliferation and differentiation for polymer-bioceramic composites. *J Mater Sci Mater Med* 23:2751–2759. <https://doi.org/10.1007/s10856-012-4733-0>
- Declercq H, Van den Vreken N, De Maeyer E, Verbeeck R, Schacht E, De Ridder L, Cornelissen M (2004) Isolation, proliferation and differentiation of osteoblastic cells to study cell/biomaterial interactions: comparison of different isolation techniques and sources. *Biomaterials* 25:757–768. [https://doi.org/10.1016/S0142-9612\(03\)00580-5](https://doi.org/10.1016/S0142-9612(03)00580-5)
- Dorozhkin SV (2011) Calcium orthophosphate occurrence, properties, biomineralization, pathological calcification and biomimetic applications. *Biomaterials* 1(2):121–164. <https://doi.org/10.4161/biom.18790>

- Engeland CG, Sabzehei B, Marucha PT (2009) Sex hormones and mucosal wound healing. *Brain Behav Immun* 23:629–635. <https://doi.org/10.1016/j.bbi.2008.12.001>
- Freshney RI (1994) Culture of animal cells: a manual of basic technique, Wiley-Liss, New York. 3rd ed. Wiley-Liss, Inc: New York. XXIV, 486 pages (1994). <https://doi.org/10.1002/cbf.646>
- Gala-García A, Carneiro MB, Silva GA, Ferreira LS, Vieira LQ, Marques MM (2012) In vitro and in vivo evaluation of the biocompatibility of a calcium phosphate/poly (lactic-co-glycolic acid) composite. *J Mater Sci Mater Med* 23(7):1785–1796. <https://doi.org/10.1007/s10856-012-4657-8>
- Galow A-M, Rebl A, Koczan D, Bonk SM, Baumann W, Gimsa J (2017) Increased osteoblast viability at alkaline pH in vitro provides a new perspective on bone regeneration. *Biochem Biophysics Rep* 10:17–25. <https://doi.org/10.1016/j.bbrep.2017.02.001>
- Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR (1982) Analysis of nitrate, nitrite and [¹⁵N] nitrate in biological fluids. *Anal Biochem* 126:131–138. [https://doi.org/10.1016/0003-2697\(82\)90118-X](https://doi.org/10.1016/0003-2697(82)90118-X)
- Kasper CH, Wergedal JE, Farley JR (1989) Androgens direct stimulate proliferation of bone cells in vitro. *Endocrinol* 124(3):1576–1578. <https://doi.org/10.1210/endo-124-3-1576>
- Kim HW, Knowles JC, Kim HE (2004) Hydroxyapatite/poly (ε-caprolactone) composite coatings on hydroxyapatite porous bone composite for drug delivery. *Biomaterials* 25(7–8):1279–1287. <https://doi.org/10.1016/j.biomaterials.2003.07.003>
- Kokubo T, Takadama H (2006) How useful is SBF in predicting in vivo bone bioactivity? *Biomaterials* 27(15):2907–2915. <https://doi.org/10.1016/j.biomaterials.2006.01.017>
- Legeros RZ (2008) Calcium phosphate-based osteoinductive materials. *Chem Rev* 108:4742–4753. <https://doi.org/10.1021/cr800427>
- Lin IC, Smartt JM, Nah HD, Ischiropoulos H, Kirschner RE (2008) Nitric oxide stimulates proliferation and differentiation of fetal calvarial osteoblasts and dural cells. *Plast Reconstr Surg* 12:1554–1566. <https://doi.org/10.1097/PRS.0b013e31816c3bd7>
- Lobo SE, Glickman R, da Silva WN, Arinze TL, Kerkis I (2015) Response of stem cells from different origins to biphasic calcium phosphate bioceramics. *Cell Tissue Res* 361:477–495
- Mei N, Chen G, Zhou P, Chen X, Shao ZZ, Pan LF, Wu CG (2005) Biocompatibility of Polycaprolactone scaffold modified by chitosan-the fibroblasts proliferation in vitro. *J Biomater Appl* 19: 323–339. <https://doi.org/10.1177/0885328205048630>
- Müller WEG, Wang X, Diehl-Seifert B, Kropf K, Schlossmacher U, Lieberwirth I, Glasser G, Wiens M, Schröder HC (2011) Inorganic polymeric phosphate/polyphosphate as an inducer of alkaline phosphatase and a modulator of intracellular Ca²⁺ level in osteoblasts (SaOS-2 cells) in vitro. *Acta Biomater* 7(6):2661–2671. <https://doi.org/10.1016/j.actbio.2011.03.007>
- Naidoo K, Rolfes H, Easton K, Moolman S, Chetty A, Richter W, Nilen R (2008) An emulsion preparation for novel micro-porous polymeric hemi-shells. *Mater Lett* 62:252–254
- Nanci A., & Ten Cate AR (2008). Ten Cate's oral histology: development, structure, and function. St. Louis, Mo, Mosby. 7th Ed pages. 411
- Nasr HF, Aichelmann-Reidy ME, Yukna RA (1999) Bone and bone substitutes. *Periodontol* 2000:74–86
- Notelovitz M (2002) Androgen effects on bone and muscle. *Fertil Steril* 77(4):S34–S41. [https://doi.org/10.1016/S0015-0282\(02\)02968-0](https://doi.org/10.1016/S0015-0282(02)02968-0)
- Riancho JA, Salas E, Zarrabeitia MT, Olmos JM, Amado JA, Fernández-Luna JL, González-Macías J (1995) Expression and functional role of nitric oxide synthases in osteoblast-like cells. *J Bone Miner Res* 10:439–446. <https://doi.org/10.1002/jbmr.5650100315>
- Shen H, Hu XX, Bei JZ, Wang S (2008) The immobilization of basic fibroblast growth factor on plasma-treated poly(lactide-co-glycolide). *Biomaterials* 29:2388–2399. <https://doi.org/10.1016/j.biomaterials.2008.02.008>
- Shor L, Güçeri S, Wen X, Gandhi M, Sun W (2007) Fabrication of three-dimensional polycaprolactone/hydroxyapatite tissue scaffolds and osteoblast-scaffold interactions in vitro. *Biomaterials* 28:5291–5297. <https://doi.org/10.1016/j.biomaterials.2007.08.018>
- Steffens JP, Herrera BS, Coimbra LS, Stephens DN, Rossa CJ, Spolidorio LC, Kantarci A, Van Dyke TE (2014) Testosterone regulates bone response to inflammation. *Horm Metab Res* 46:193–200. <https://doi.org/10.1055/s-0034-1367031>
- Turner RT, Wakley GK, Hannon KS (1990) Differential effects of androgens on cortical bone histomorphometry in gonadectomized male and female rats. *J Orthop Res* 8:612–617. <https://doi.org/10.1002/jor.1100080418>
- Van't Hof RJ, Ralston SH (2001) Nitric oxide and bone. *Immuno* 103: 255–261. <https://doi.org/10.1046/j.1365-2567.2001.01261>
- Victor SP, Kumar TSS (2008) BCP ceramic microspheres as drug delivery carriers: synthesis, characterization and doxycycline release. *J Mater Sci Mater Med* 19:283–290. <https://doi.org/10.1007/s10856-006-0044-7>
- Wiren K, Toombs A, Zhang XW (2004) Androgen inhibition of MAP kinase pathway and Elk-1 activation in proliferating osteoblasts. *J Mol Endocrinol* 32:209–226. <https://doi.org/10.1677/JME-07-0021>
- Xynos ID, Buttery LDK, Hench L (2000) Ionic products of bioactive glass dissolution increase proliferation of human osteoblasts and induce IGF II mRNA expression and protein synthesis. *Biochem Biophys Res Commun* 276:461–465. <https://doi.org/10.1590/S1516-14392002000300004>
- Yamasaki H, Sakai H (1992) Osteogenic response to porous hydroxyapatite ceramics under the skin of dogs. *Biomaterials* 13(5):308–312
- Yu L, Ding J (2008) Injectable hydrogels as unique biomedical materials. *Chem Soc Rev* 37:1473–1481. <https://doi.org/10.1039/B713009K>
- Zhao L, Lin K, Zhang M, Xiong C, Bao Y, Pang X, Chang J (2011) The influences of poly(lactic-co-glycolic acid) (PLGA) coating on the biodegradability, bioactivity, and biocompatibility of calcium silicate bioceramics. *J Mater Sci* 46:4986–4993. <https://doi.org/10.3233/BME-2012-0719>