Original Research
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Grafting biomaterials associated to topical glucocorticoid: effects on pre-osteoblastic cells (MC3T3-E1)

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Abstract: The topical glucocorticoid budesonide has been prescribed before and after sinus lift surgery as adjuvant drug treatment for maxillary sinus membrane inflammation. However, there is no study on the effects of budesonide on the regenerative process of bone grafting biomaterials. We investigated the effect of the association of budesonide with some biomaterials on the growth and differentiation capacity of pre-osteoblastic cells (MC3T3-E1 subclone 4). Xenogeneic (Bio-Oss and Bio-Gen) and synthetic hydroxyapatites (Osteogen, Bonesynth, and HAP-91) were tested in conditioned medium (1% w/v). The conditioned medium was then supplemented with budesonide (0.5% v/v). Cell viability was assessed using the MTT assay (48, 96, and 144 h), and mineralized nodules were quantified after 14 days of culture using the Alizarin Red Staining. Alkaline phosphatase activity was assessed through the release of thymolphthalein at day seven. All biomaterials showed little or no cytotoxicity. The Bio-Gen allowed significantly less growth than the control group regardless of the experimental time. Regarding differentiation potential of MC3T3-E1, the HAP-91-conditioned medium showed remarkable osteoinductive properties. In osteodifferentiation, the addition of budesonide favored the formation of mineral nodules when cells were cultured in medium conditioned with synthetic materials, whereas it weakened the mineralization potential of cells cultured in xenogeneic medium. Regardless of whether budesonide was added or not, Osteogen and Bio-Oss showed higher alkaline phosphatase activity than the other groups. Budesonide may improve bone formation when associated with synthetic biomaterials. Conversely, the presence of this glucocorticoid weakens the mineralization potential of pre-osteoblastic cells cultured with xenogeneic hydroxyapatites.

Keywords: Bone Substitutes; Hydroxyapatites; Glucocorticoids; Budesonide.

Introduction

Bone regeneration procedures can be performed with autogenous bone or bone substitutes, such as allografts, xenografts, and alloplastic materials or a mixture of autogenous bone and biomaterials.[1] Autogenous bone is the gold standard in guided bone regeneration due to its

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osteogenic potential.[2] Nevertheless, the drawbacks of autologous grafts have stimulated the search for potential synthetic and bioinert, or preferably bioactive materials, as substitutes for bone grafts.[3] Among the available synthetic bone graft substitutes, hydroxyapatite (HA) and beta-tricalcium phosphate (β-TCP) stand out. Although biocompatible and osteoconductive, HA has a low substitution rate.[3,4] β-TCP is also biocompatible and biodegradable, presenting a faster resorption rate than HA.[4,5] The association of HA and β-TCP may combine their biomedical properties, originating a material classified as biphasic calcium phosphate (BCP), which has enhanced osteoconductive potential.[4,5] Otherwise, osteogenic components, such as growth factors and glucocorticoids, are also used to optimize biomaterials to induce new bone formation.[6,7]

Sinus lift with biomaterials grafting is a routine procedure for alveolar ridge augmentation in the posterior region of the maxilla. Treatment of existing rhinosinusitis may be required before bone grafting in this region to avoid complications during or after surgery.[8] Furthermore, complications of sinus lift surgery can cause postoperative sinusitis.[9] In both situations, topical glucocorticoids such as budesonide have been considered as an optional adjuvant treatment to control chronic rhinosinusitis by ensuring patency of the maxillary ostium.[8,10]

Although the prescription of budesonide has been clinically indicated before and after graft surgeries, there is no study on the effects of budesonide on the regenerative process in bone grafting. We hypothesized that the use of budesonide, as a glucocorticoid, could influence bone neoformation. The aim of the present *in vitro* study was to assess the effect of budesonide on the osteogenic potential of MC3T3-E1 pre-osteoblastic cells grown in cultured media conditioned with two xenogeneic and three synthetic HA or BCP.

Methodology

Materials

The materials used are presented in Table 1.

Table 1. Description and composition of the biomaterials and drug used in the study.

Biomaterial Batt	Manufacturer	Composition	Particle size
Bonesynth® BOAN14-C (BS)	Neomatrix® Materiais Avançados, Belo Horizonte, Brasil	Synthetic Hydroxyapatite (90%) Beta-Tricalcium phosphate (10%)	150–425 μm
Osteogen® C0805-1598 (OG)	Intralock System, São Paulo, Brasil	Synthetic Hydroxyapatite	300–400 μm
HAP-91® 1604003-3M -91	JHS Biomateriais, Sabará, Brasil	Synthetic Hydroxyapatite (95%) Beta-Tricalcium phosphate (5%)	595 μm
BIO-OSS® 81700362 (BO)	Geistilich Biomaterials®, São Paulo, Brasil	Inorganic Bovine Hydroxyapatite	250–1000 μm
BIOGEN® 152006 (BG)	Bioteck®, Lohcus Health Technology, São Paulo, Brasil	Deantigenated Equine Hydroxyapatite	500–1000 μm
BUSONID® Budesonide 32 mcg 1706084 (BUD)	Biosintética Farmacêutica Ltda, São Paulo, Brasil	Microcrystalline cellulose, carmellose sodium, glucose, polysorbate 20, calcium edetate disodium dihydrate, purified water and hydrochloric acid	

Scanning electron microscopy (SEM)

The morphology and microstructure of the HA powder were observed by scanning electron microscopy (JSM-IT300, Jeol, Tokyo, Japan). Samples were assembled on aluminum stubs and sputter-coated with a layer of gold (approximately 45 μm).

Cell culture

MC3T3-E1 (ATCC Subclone 4 CRL-2593) pre-osteoblastic cells were thawed and grown in regular medium containing α-MEM supplemented with 1% antibiotic Penicillin-Streptomycin (10.000 U/ml) and 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher, Waltham, USA). Cells were kept at 37°C in a humid atmosphere with 5% CO_2. Culture media were replaced every 2–3 days.

Study design and conditioned media

First, the maximal inhibitory concentration of budesonide (Busonid, Biosintética Farmacêutica Ltda, São Paulo, Brazil) to inhibit 50% of cell growth was determined (IC50), as described in the following section. Next, cell viability and osteodifferentiation assays were performed using a conditioned medium.[11] Briefly, each hydroxyapatite was prepared at a concentration of 1% w/v of culture medium and incubated for 24 h at 37°C, prior to the experiments. Cells were then cultured in association with or without the determined IC50 glucocorticoid dose. For cell viability/proliferation and differentiation assays, cells cultured under regular conditions (no conditioned medium or budesonide) served as internal negative control. Cells grown under inductive conditions (no conditioned medium or budesonide) were used as positive control in the differentiation assays.

Determination of glucocorticoid dose (IC50)

For the determination of the maximum topical glucocorticoid dose that preserves at least 50% of cell viability, budesonide (32 μg) was first tested in eight serial dilutions: 0.0156, 0.03125, 0.0625, 0.125, 0.25, 0.5, 1.0, and 5.0%. Cells were plated at a density of 2 x 10^4 cells/well in 96-well microplates and, 24 h later, incubated with each dilution for 24 h. Next, a cell viability assay (MTT) 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (Gibco, Thermo Fisher, Waltham, USA)] was performed according to the manufacturer's recommendations. Briefly, a 5 mg/mL MTT solution was prepared and added to the cell cultures for 4 hours in the dark, at 37°C, in humid atmosphere containing 5% CO_2. Then, formazan crystals were dissolved with 50 μL in each well of the DMSO solution (Sigma Aldrich, St. Louis, USA). The absorbance was determined by optical density in a spectrophotometer Spectra Max 190 (BioTeK, Winooski, USA) with 540 nm filter. The readings were performed using the Software Soft Max (Molecular Devices, San Jose, USA).

In this experiment, cell survival remained above 50% (IC50) up to a concentration of 0.5%. Above 0.5%, the percentage of cell death approached 90%. Therefore, the concentration of 0.5% budesonide was selected for this study, which is equivalent to 0.8 μg in each 125 mL of culture medium (volume used in a well of 96-wells microplates).

Cell viability and proliferation analysis (MTT)

Cells were plated at a density of 2 x 10^4 cells/well in 96-well microplates 24 h before the contact with the biomaterials. Cell proliferation was analyzed at 48, 96, and 144 h by using MTT assay, as previously described.

Cell differentiation analysis

For the cell osteodifferentiation assay, cells were plated at 5 x 10^4 cells/well in 48-well microplates. Twenty-four hours later, osteogenic/inductive media containing α-MEM supplemented with 1% antibiotic Penicillin-Streptomycin (10.000 U/ml), 10% FBS (all from Gibco), 180 mM KH_2PO_4, and 10^{-8} of dexamethasone (both from Sigma-Aldrich) and conditioned with each biomaterial were added to the cells in association or not with 0.5% budesonide for 14 days. The same groups but in regular media were also added to the cells in association or not with 0.5% budesonide for 14 days. Media were changed every 2–3 days during the experiments. Internal negative and positive controls were performed as aforementioned.

Alizarin red staining (AZR S)

The formation of mineralized nodules was detected using the AZR S staining. Cell cultures were washed twice in PBS, fixed with 75% isopropanol, rehydrated in distilled water, and stained with alizarin red (Sigma-Aldrich) 1% (pH 4.2) for 5 min. The cultures were washed three times in PBS and left to dry. The wells were photographed using a multimode reader (Cytation 5, Biotek Multiplate Image Reader, software Gen 5 Image 3.3). For quantitative analysis, a solution of 10% acetic acid and methanol (4:1 v/v) was added to the wells for 30 min for the dissolution of mineralized nodules. The absorbance was read in a spectrophotometer (Biotek) using the 490 nm filter.

Alkaline phosphatase activity (APA)

APA was evaluated through the release of thymolphthalein by hydrolysis of the substrate of thymolphtaleinmonofosfate, according to the manufacturer's recommendations (Labtest, Lagoa Santa, Brazil). The assay was performed in the supernatant of cells cultured under regular and inductive conditions collected at seven days. The absorbance was measured at 590 nm and the APA was calculated from the standard solution. Data were expressed as U/L.

Statistical analysis

Data were analyzed using GraphPad Prism 9 software (GraphPad, San Diego, USA). Differences among groups were tested by ANOVA followed by the Tukey post hoc. The Student's *t* test was used to assess differences between groups. In all cases, $p < 0.05$ was considered statistically significant.

Results

SEM characterization

The granules of the biomaterials are shown in Figure 1. SEM analysis showed that the synthetic HA are mostly microparticulate, whereas the xenogeneic ones have a larger particles size. The latter two are composed of non-uniform polygonal granules with a smooth and dense surface texture. Among the synthetic HA, Osteogen (biphasic) presented qualitatively more heterogeneous particle sizes. Small particle clusters were observed in the HAP-91 (biphasic) and Bonesynth (pure HA) biomaterials.

Determination of 50% cytotoxicity glucocorticoid dose

Before testing the budesonide in association with each biomaterial, we have preliminarily identified the glucocorticoid dose with around 50% of cytotoxicity (IC 50) considering a range of eight initial concentrations (Figure 2). The dose of 0.5% was the closest budesonide concentration of the IC 50 limit and was then selected to be tested in association with the biomaterials.

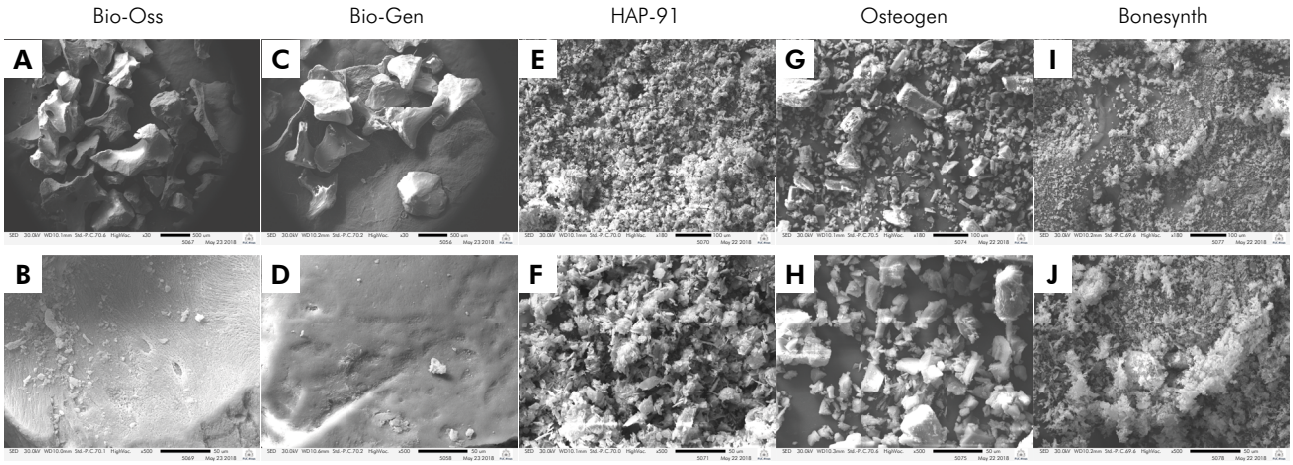

Figure 1. SEM analysis showing biomaterials morphology at 30x (a, c), 180x (e, g, i), and 500x (b, d, f, h, j) magnifications.

Cell viability and proliferation (MTT)

All synthetic HA tested as conditioned medium presented cell viability/proliferation above or close to that of the control cells cultured under ideal conditions throughout the experimental time (Figure 3). A decrease in cell growth was observed at the 96h-time interval, but with no statistical difference from the control. The Bio-Oss, in turn, showed significantly lower cell viability/proliferation at the first experimental time point (48 h) regarding the synthetic bone grafts. The subsequent experimental times (96 and 144 h) showed recovery of cell growth after culturing in the Bio-Oss conditioned medium. Bio-Gen, otherwise, showed moderate cytotoxicity during the entire experimental period.

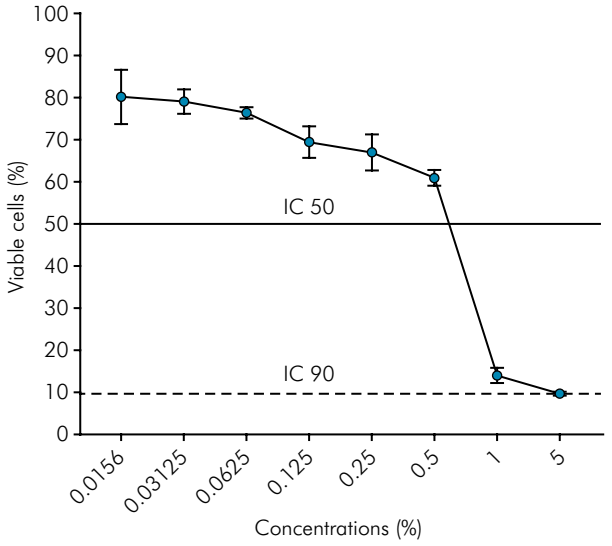

Figure 2. The maximum glucocorticoid dose that preserves at least 50% (IC 50) and 90% (IC 90) of cell viability of MC3T3-E1 cells was first evaluated using eight doses of budesonide (0.0156, 0.03125, 0.0625, 0.125, 0.25, 0.5, 1.0, and 5.0%).

Osteogenic differentiation

Figures 4a, 5a, and 6a show the results of cells cultured with bone grafts conditioned media without budesonide in regular and inductive conditions. In regular conditions, cells cultured with HAP-91 conditioned medium showed remarkable formation of mineralized nodules similarly to the positive control grown in osteogenic medium. Among the other biomaterials, only Bio-gen lacked osteoinductive properties (Figure 4a). Cells cultivated under inductive conditions showed improved mineralization potential (Figure 4a). HAP-91 conditioned medium showed higher mineral nodule formation than all the other bone grafts groups, but similar to the positive control. Bonesynth, Bio-Oss, and Bio-Gen conditioned osteogenic media showed improved mineralization compared to the regular medium conditioned with the same biomaterials. Osteogen, in turn, remained similar to the regular condition in terms of differentiation potential.

Figures 4b, 5b and 6b show the results of cells cultured with HA conditioned media containing budesonide in regular and inductive conditions. Comparing regular and inductive conditions, the

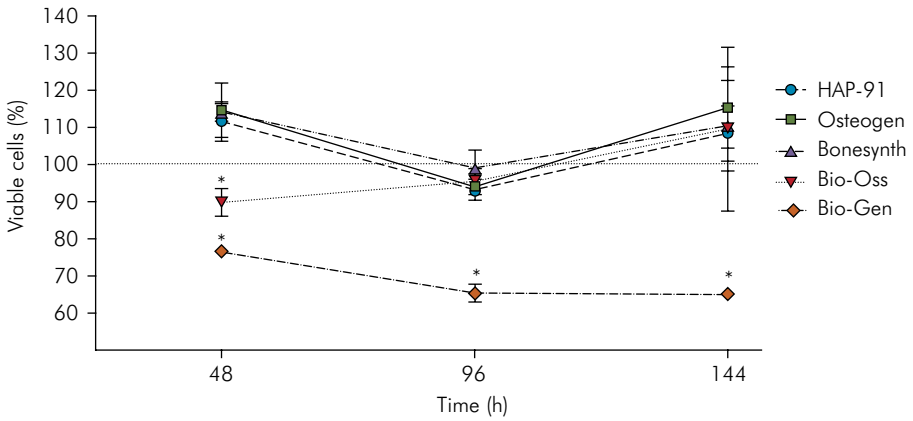

Figure 3. Cell viability/proliferation of MC3T3-E1 cells cultured in media conditioned with each HA and evaluated at three-time intervals (48, 96, and 144 h). *Significant differences between natural and synthetic materials.

osteogenic medium improved the mineralized nodule formation of MC3T3-E1 cultured in synthetic biomaterials conditioned media. The inductive ingredients of xenogeneic HA showed no improvement in the mineralized nodule formation.

The use of budesonide was also compared in regular and inductive conditions between each bone graft (Figure 7). When cultured in regular medium, no difference was found by adding budesonide in association with the conditioned

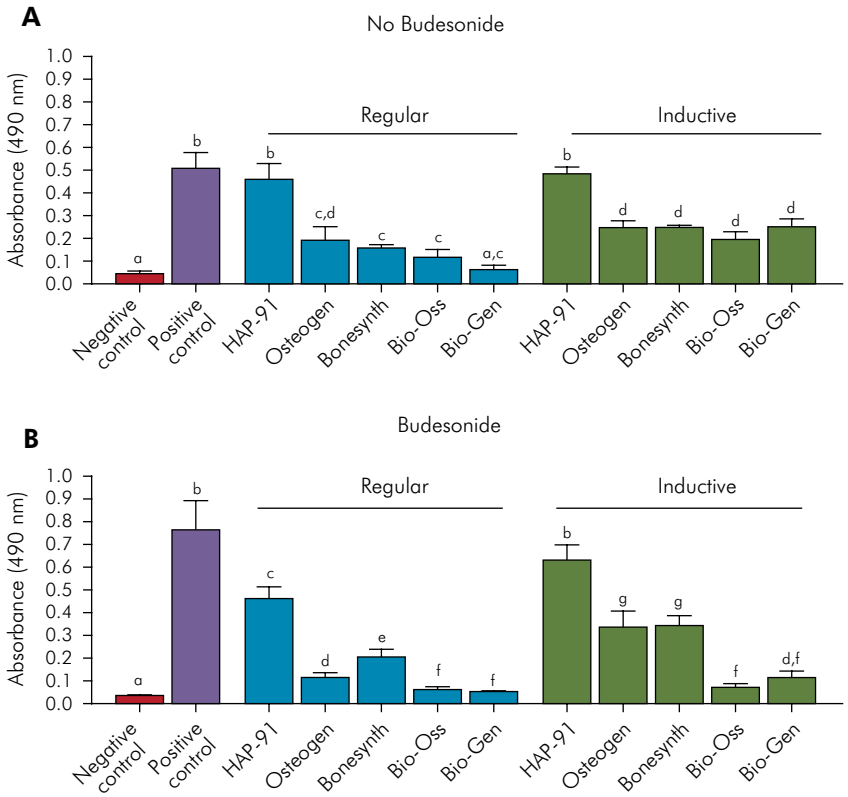

Figure 4. Bar graphs (quantitative AZR S) representing the mineralization potential of MC3T3-E1 cells cultured in media conditioned with each HA comparing the regular and inductive conditions, considering the absence (a) or addition of 0.5% v/v budesonide (b).

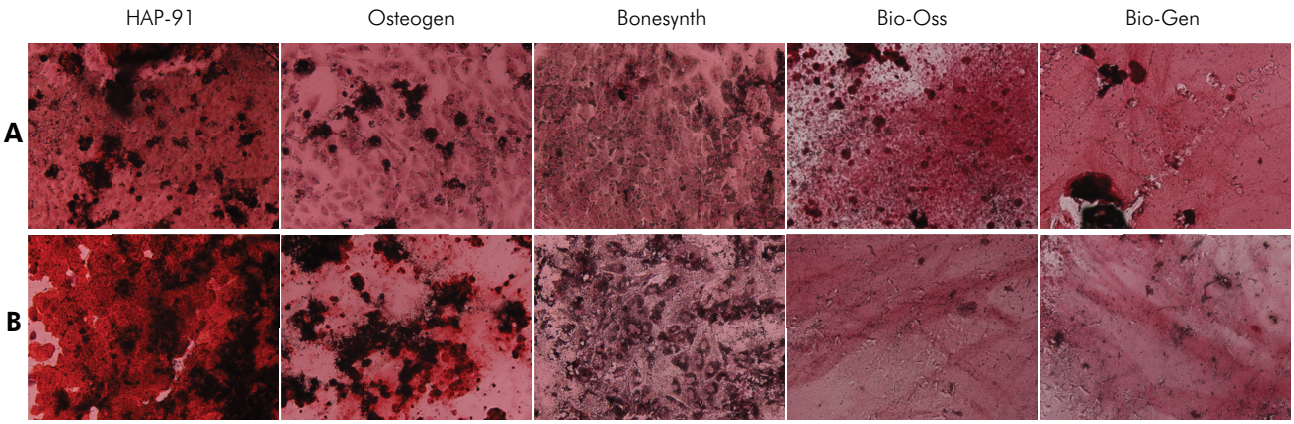

Figure 5. Representative bright-field images of alizarin red-stained MC3T3-E1 cells cultured in media conditioned with each HA under regular conditions, considering the absence (a) or addition of 0.5% v/v budesonide (b).

media to any biomaterial (Figure 7a). However, the supplementation with corticosteroid in the inductive media favored higher mineral nodule formation in the synthetic bone graft groups – HAP-91, Osteogen, and Bonesynth (Figure 7b). The supplementation with budesonide 0.5% also increased mineral nodules formation in the positive control group (data not shown). Conversely, the addition of

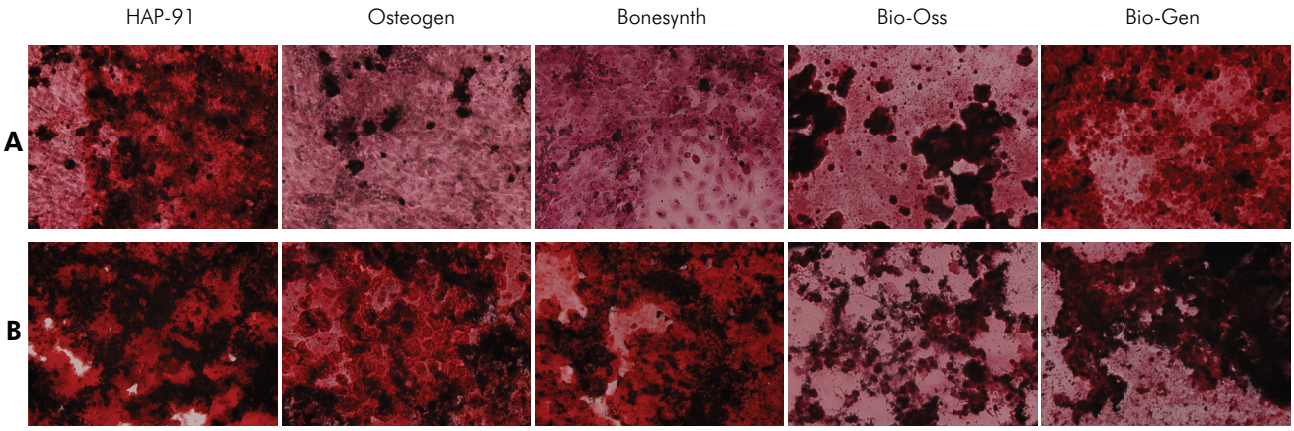

Figure 6. Representative bright-field images of alizarin red-stained MC3T3-E1 cells cultured in media conditioned with each HA under inductive conditions, considering the absence (a) or addition of 0.5% v/v budesonide (b).

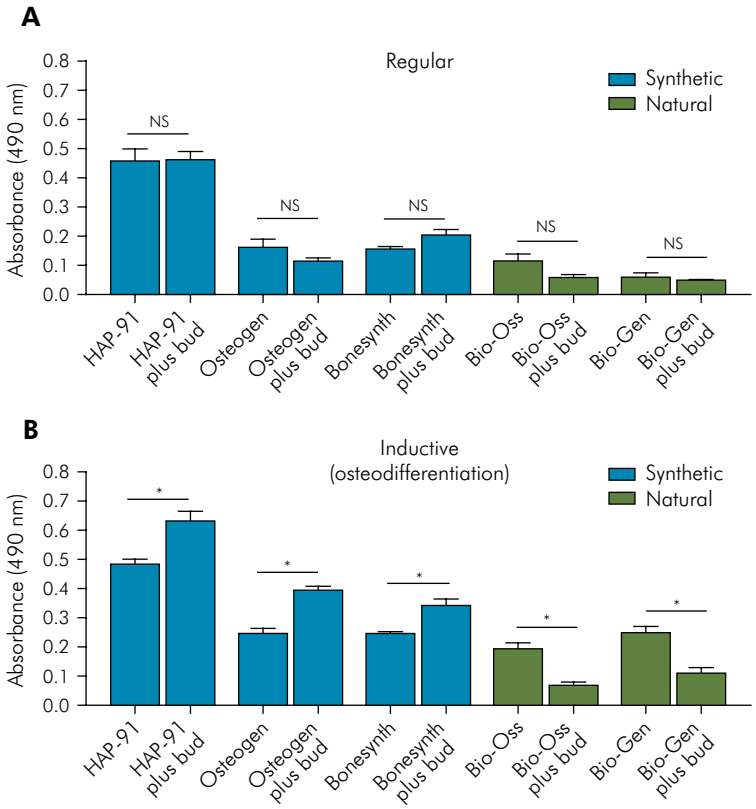

Figure 7. Bar graphs comparing the mineralization potential of each HA conditioned media after the addition of 0.5% v/v budesonide in regular (a) and inductive (b) conditions.

the glucocorticoid weakened the mineralization potential of MC3T3-E1 cells grown in xenogeneic HA conditioned inductive media (Figure 7b).

The alkaline phosphatase (AP) activity after culturing MC3T3-E1 cells in the biomaterial's conditioned media, with and without budesonide, is shown in Figure 8. In regular conditions, AP activity did not show significant variations among the bone graft conditioned media, except for higher AP activity for Bonesynth compared to HAP-91 and negative control. Osteogen, Bio-Oss, and Bio-Gen showed increased AP activity under osteogenic induction, whereas Bonesynth showed the lowest values for AP activity compared to all the other groups. The inductive media increased AP activity compared to the regular group within the HA comparisons, except for the Bonesynth and negative control. After budesonide addition, AP activity was similar between Osteogen and Bio-Oss and between HAP-91 and Biogen. The inductive media increased AP activity compared to the regular group within the different biomaterials, except for the Bonesynth and negative control. The addition of budesonide did not improve the AP activity in the regular and inductive media comparisons.

Discussion

Budesonide is a well-known corticosteroid anti-inflammatory drug prescribed to alleviate inflammatory symptoms. Considering that topical budesonide has been prescribed in maxillary surgeries aiming at bone alveolar ridge augmentation, our preliminary study has investigated whether

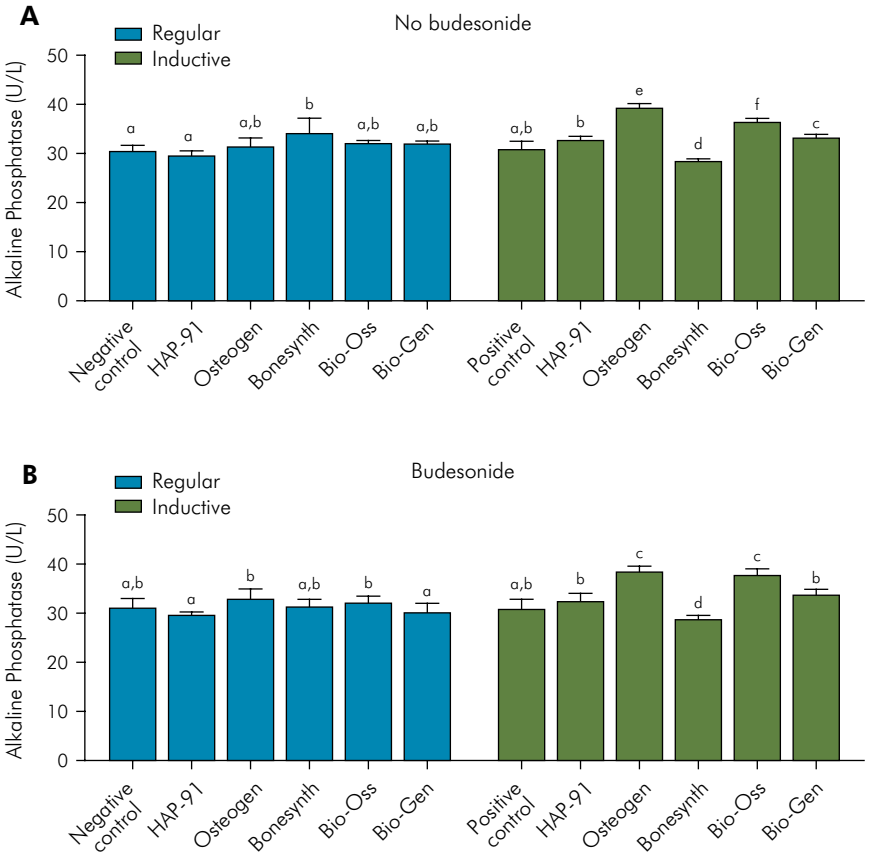

Figure 8. Bar graphs showing the results of the APA assay representing the mineralization potential of MC3T3-E1 cells cultured in media conditioned with each HA comparing the regular and inductive conditions, considering the absence (a) or addition of 0.5% v/v budesonide (b).

this drug could potentially interfere with bone regeneration processes using xenogeneic and synthetic hydroxyapatites *in vitro*. Upon induction, MC3T3-E1 cells cultured in media conditioned with synthetic bone grafts (HAP-91, Osteogen, and Bonesynth) and with the addition of 0.5% v/v budesonide presented higher mineralization potential compared to those grown without the glucocorticoid. Conversely, the same effect was not observed when pre-osteoblastic cells were cultured with xenogeneic hydroxyapatites (Bio-Oss and Bio-Gen) conditioned media. Under inductive conditions, budesonide reduced the mineralization potential of both xenogeneic materials.

Dexamethasone – another glucocorticoid – is used as a supplement in osteogenic differentiation media to induce pre-osteoblastic cells to complete differentiation at the early stages of development in a dose-dependent manner, which may be demonstrated by increased cellular mineralization and AP activity.[12] However, excess glucocorticoids or glucocorticoids applied at late stages of osteoblast differentiation may decrease osteoblast proliferation and osteoblast-specific protein production.[12,13] The effects of budesonide and dexamethasone on the growth and differentiation of cultured human bone-derived osteoblast-like cells were compared and showed similar cell proliferation inhibition, increased AP activity, and decreased osteocalcin secretion.[14] Budesonide, in turn, presented more pronounced overall effects than dexamethasone in that previous study. In this study, the addition of budesonide did not improve the AP activity in any experimental condition. Otherwise, it led to a divergent response of mineral nodule formation depending on the category of bone grafts investigated.

Osteoinductivity was assessed by quantifying mineral nodule formation upon cultivating MC3T3-E1 cells for 14 days in regular conditioned media. The HAP-91 showed remarkable osteoinductive properties, whether budesonide was present or not. This material is biphasic and consists of synthetic HA with 5% β-TCP. It has been suggested that calcium release and greater protein adsorption may play a role in the responsiveness of cells to β-TCP-based ceramics.[15] Even at a small percentage, β-TCP might have shared its bioactivity with HA, thus contributing to the better performance of bone graft HAP-91 on the MC3T3-E1 cells. It is noteworthy that although the other biphasic hydroxyapatite Bonesynth showed osteoinductivity, it occurred mainly upon the addition of budesonide. Further studies on crystallinity and dissolution profiles of the synthetic or xenogeneic bone grafts might explain their different bioactivity behavior.[16]

Our primary data revealed that all media conditioned with the biomaterials showed little or no cytotoxicity. Of the different biomaterials tested in this study, the xenogeneic HA showed lower cell viability/proliferation than the synthetic HA, but Bio-Oss recovered cell growth over time. Notwithstanding, both cytocompatibility and osteoconductivity of Bio-Oss and Bio-Gen have been verified in previous studies, although cells were seeded directly on the biomaterials' granules.[17,18] Our results are consistent with the current literature. We also found that Osteogen, Bio-Oss, and Bio-Gen showed the highest AP activity values of osteodifferentiation at 7 days. Nonetheless, these findings were not determinant for the global data on osteodifferentiation at 14 days.

The biological performance of bone substitutes depends on space-making capability, biocompatibility, bioabsorption, and volume maintenance over time.[2] Clinical studies comparing xenogeneic and synthetic HA for maxillary sinus augmentation reported greater new bone formation using bovine HA than synthetic HA and β-TCP.[5,19-21] Compared with autogenous bone, xenografts presented less bone formation in healing times of less than six months after sinus lift, but similar results in more extended periods, indicating a slower resorption rate in xenografts.[22] Alloplastic materials, in turn, presented less bone regeneration than autogenous bone at each healing time, which may be associated with the higher resorption rates, especially in biphasic biomaterials.[22]

In this study, we used a mimicked milieu to test different bone substitutes on MC3T3-E1 cell monolayers to represent a sinus lift procedure followed by the administration or not of budesonide. Accordingly, the spontaneously immortalized murine calvarial cell line was selected because of its natural commitment to the osteogenic fate.[23] At

the transcriptional level, in turn, the commercially available MC3T3-E1 subclones bear little resemblance to murine calvarial pre-osteoblastic cells, which means that data derived from them should be interpreted cautiously.[23] Although our *in vitro* design has limited translation to the clinical setting, the topical glucocorticoid might interact with the bone regeneration process. Considering the success of xenografts in sinus lift procedures and their routine use by clinicians, the poor performance of the inorganic bovine HA tested here is a warning sigh for the prescription of topical budesonide in the treatment of patients who underwent bone grafts with these bone substitutes. On the other hand, although the influence of budesonide association on the osteoinduction potential of synthetic biomaterials seems beneficial, further confirmatory studies need to be conducted in randomized clinical trials.

Conclusions

The addition of budesonide to culture media conditioned with xenogeneic hydroxyapatites impaired osteoinductivity and cellular osteodifferentiation of MC3T3-E1 cells. However, regarding the synthetic HA, the presence of the glucocorticoid in the conditioned media favored the formation of mineral nodules.

Acknowledgments

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