JOURNAL OF TISSUE ENGINEERING AND REGENERATIVE MEDICINE
J Tissue Eng Regen Med 2012;**6:** 497–504.
Published online 2 August 2011 in Wiley Online Library (wileyonlinelibrary.com) **DOI:** 10.1002/term.455

RESEARCH ARTICLE

Evaluation of a multilayered chitosan–hydroxyapatite porous composite enriched with fibronectin or an *in vitro*-generated bone-like extracellular matrix on proliferation and diferentiation of osteoblasts

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Abstract

The use of extracellular matrix (ECM) molecules from tissues is an interesting way to induce specific responses of cells grown onto composite scaffolds to promote adhesion, proliferation and differentiation. There have been several studies on the effects on cell proliferation and differentiation of osteoprogenitor cells cultured onto composites, either adding some ECM molecules or grown in the presence of growth factors. Other studies involve the use of osteoblasts cultured on a three-dimensional (3D) matrix, enriched with ECM molecules produced by the same cells grown previously inside the composite. Here, the effect of enrichment of a novel multilayered chitosan–hydroxyapatite composite with ECM molecules produced by osteoblasts, or the addition of 25 or 50 μg/ml fibronectin to the composite, on proliferation and differentiation of osteoblasts cultured on these composites was studied. The results showed an increase in the number of osteoblasts from day 1 of culture, which was higher in the group grown onto composites enriched with the highest concentration of fibronectin or with ECM molecules produced naturally by osteoblasts cultured previously on them, when compared with the control group. However, this increment tended to decline in all groups after day 7 of culture, the day when they reached the highest peak of proliferation. Differentiation expressed as alkaline phosphatase activity followed the proliferation pattern of the cells cultivated on the scaffolds. The results demonstrate the potential offered by these enriched 3D multilayered composites for improving their ability as bone grafting material. Copyright © 2011 John Wiley & Sons, Ltd.

Received 7 December 2010; Accepted 26 May 2011

Keywords chitosan–hydroxyapatite; composite; fibronectin; alkaline phosphatase; extracellular matrix; scaffold

1. Introduction

Bone tissue remodelling and regeneration is one of the major concerns of tissue engineering and regenerative medicine (Caplan, 2009). The basic organization of bone tissue requires the design and fabrication of a porous 3D structure or 'scaffold' for homing the bone-forming cells which induce bone regeneration *in vivo*. This kind of scaffolds should be formulated from biocompatible, osteoconductive and non-immunoreactive materials. 3D scaffolds must provide the necessary support for cells to proliferate and maintain their ability to differentiate. Scaffold materials include natural or synthetic polymers, ceramics and composites. Some scaffolds have shown a natural ability to induce bone formation. However, under certain circumstances, hydroxyapatite promotes differentiation and affects the growth of osteoblast cells

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(Shu *et al.*, 2003). Therefore, enrichment of this scaffold with some enhancer of the osteoinductive capabilities is desirable (Shin *et al.*, 2003). In fact, it is expected for a scaffold not only to serve as a 3D framework but also to enhance or promote some cellular responses from the cells cultured on it. In this regard, molecules present in bone extracellular matrix have been used (Roehlecke *et al.*, 2001; Ong *et al.*, 1999; Hidalgo-Bastida and Cartmell, 2010). Some of these enhancers include molecules present in native bone extracellular matrix (ECM), with the inorganic filler hydroxyapatite (Moursi *et al.*, 2002; Ma *et al.*, 2001) and cell-adhesive proteins or polypeptides (Shin *et al.*, 2003; Zheng *et al.*, 2009). Among these, fibronectin plays important roles in cell adhesion, migration, proliferation and differentiation (Pankov and Yamada, 2002). In addition, bone ECM previously synthesized *in vitro* on osteoblastic differentiation of marrow stromal cells has been evaluated (Datta *et al.*, 2005; Pham *et al.*, 2008). On the other hand, different kinds of hydroxyapatite–chitosan scaffolds have been evaluated for osteo- and chondrogenic differentiation but none of them could be considered an ideal scaffold for all the desired purposes (Malafaya *et al.*, 2005; Oliveira *et al.*, 2006; Teng *et al.*, 2009).

We have developed a novel chitosan–hydroxyapatite, 3D, multilayered, porous composite scaffold (Arias, 2008; Arias *et al.*, 2009) with a reduced swelling behaviour suitable for bone tissue engineering. We already know that this composite supports the adhesion of chicken calvaria osteogenic cells (Arias, 2008). However, we do not know whether already committed osteoblast cells adhere well to the composite, proliferate and keep their osteoblast phenotype. We hypothesized that if the composite alone does not allow attachment, proliferation or differentiation of osteogenic cells, the use of fibronectin or bulk bone ECM components would improve these cell properties. Here we report the effect of this novel composite enriched with fibronectin, or with an *in vitro* generated bone-like ECM, on osteoblast proliferation and differentiation.

2. Materials and methods

2.1. Scaffold preparation

A multilayered chitosan–hydroxyapatite composite designed previously (Arias *et al.*, 2009) was prepared as follows. A solution of 2% low molecular weight chitosan (20–200 cps; Aldrich), 86% deacetylated, in 5% acetic acid was shaken for 24 h at room temperature and then filtered to remove non-dissolved chitosan and impurities. This solution was divided in two halves. 5% nanometric hydroxyapatite (HAP; Sigma-Aldrich No. 693 863 nanopower <200 nm particle size BET) was added to one half (solution A). 0.1% glutaraldehyde was added to the other half (solution B). The multilayered chitosan material was fabricated according to a modification of the method described by Deville *et al.* (2006). Briefly, it consisted of sequential immersions of a glass stirring rod in solution A and B, intercalated by an immersion in liquid nitrogen, until 10 layers were reached. The stirring rod, with the chitosan layered material at its end, was lyophilized for 48 h and then the material was manually removed. Samples of this multilayered composite were chemically phosphorylated by functionalization of chitosan with phosphate groups for 3 h at 70 °C, according to a variation of the Wan *et al.* (2003) method. Additional HAP was deposited on the phosphorylated chitosan composite samples by a double diffusion (DD) method adapted from Falini *et al.* (2001). The composite scaffolds were then cut into pieces of 4 mm^3 and sterilized with ethylene oxide gas before osteoblasts were seeded.

2.2. Scanning electron microscopy (SEM) and X-ray diffraction (XRD) characterization

Surface morphology, pore size and structure of the scaffolds were examined under a TESLA BS 343A SEM, operating at 15 kV. The specimens were coated with a 20 nm thick gold layer, using an EMS-550 automated sputter-coater with the current set at 25 mA for 4 min.

An X-ray diffractometer (Siemens D-5000X) employing Cu–K_α radiation (40 kV and 30 mA; graphite monochromator, wavelength 1.5418 ´Å at 298 K) was used to analyse crystal diffraction peaks and the occurrence of HAP. Data were collected from 5–60° 2θ values.

2.3. Fibronectin scaffold enrichment

Although the concentration of fibronectin most frequently used in the literature is 100 µg/ml when 4–6 × 10^4 cells are seeded (Dennis *et al.*, 1992, 1998; Dennis and Caplan, 1993), here we used concentrations of 25 and 50 µl/ml fibronectin (Gibco) in Hanks' balanced salt solution (HBSS) because we seeded only half or one-third of the cells (2 × 10^4 cells), and also because a lower concentration of fibronectin is used when it is deposited directly on the scaffold, as it was in this case (von Walter *et al.*, 2005). The composites were immersed in each solution in a sterile tube, where vacuum was applied with a 5 ml sterile syringe to incorporate the solution into the composites. They were allowed to dry at room temperature for 24 h before being used.

2.4. Extracellular matrix molecules scaffold enrichment

The multilayered composites were seeded with 35 µl complete α-MEM (Hyclone) culture medium [15% fetal bovine serum (FBS; Hyclone), 1% antibiotic–antimycotic solution (Hyclone), 1 M β-sodium glycerophosphate (Sigma), 10^{-7} M dexamethasone (USP)], containing 2 × 10^4 osteoblasts from the MC3T3-E1 (ATCC) cell line. After 1 h, to allow cell adhesion, 1 ml complete medium was added to each composite. The medium was changed

DOI: 10.1002/term

every other day and the cells were cultured in an incubator (VWR) with 5% CO_2 and 95% humidity at 37 °C. To stimulate cell differentiation, 50 μg/ml L-ascorbic acid was added. After 12 days of culture, the composites were decellularized by three freeze–thaw cycles for 10 s each in liquid nitrogen, followed by a 37 °C water bath (10 min each) (Datta *et al*., 2005; Pham *et al*., 2008). After 24 h, new osteoblasts were seeded onto them. Scaffolds without fibronectin or ECM derived from decellularized composites were used as control specimens.

2.5. Osteoblast seeding

Enriched multilayered composites were seeded with 35 μl complete α-MEM medium containing 2×10^4 osteoblasts from the MC3T3-E1 (ATCC) cell line. After 1 h, to allow cell adhesion, 1 ml complete medium was added to each composite. The medium was changed every other day and the cells were cultured in an incubator (VWR) with 5% CO_2 and 95% humidity at 37 °C. Proliferation and cell differentiation were evaluated at days 0, 1, 7 and 14 of culture.

2.6. Cell proliferation assay

For cell proliferation estimation, a colorimetric MTT method (Invitrogen) was used. Yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), a yellow tetrazole, is reduced to purple formazan in the mitochondria of living cells. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable (living) cells. Briefly, cell/scaffold constructs were washed with HBSS (Hyclone) to remove unattached cells, then 1 ml culture medium plus 100 μl 0.5% MTT was added; the constructs were incubated for 3 h, and after removal of the MTT reagent a lysis solution consisting of 10% SDS in DMSO/HCl was added. After 5 min incubation to allow cell lysis, the samples were vortexed for 5 min, and then squeezed to obtain all the lysed cells. Absorbance was read at 550 nm on an ELISA plate reader to determine the cell number per construct by comparison with a standardized cell proliferation curve.

2.7. Cell differentiation assay

Alkaline phosphatase (ALP) is an enzyme expressed by cells and is an early marker of osteoblastic differentiation. ALP enzyme activity of the construct was measured using a *p*-nitrophenyl phosphate assay (Calbiochem). The enzyme modifies a substrate (*p*-nitrophenol phosphate) to produce a yellow product (*p*-nitrophenol), which corresponds to the amount of enzyme in solution (Gomes *et al*., 2003). Briefly, the cell–scaffold constructs were treated for 20 min with 0.2% Triton X-100 (Sigma) at 37 °C, sonicated for 10 min, and vortexed for 10 s to incorporate the enzyme into the solution. Then 50 μl of this solution was mixed with 100 μl alkaline phosphate solution containing 78 μl distilled water, 2 μl 15 mM *p*-nitrophenyl phosphate and 20 μl 10% *p*-nitrophenyl phosphate buffer. The plate was covered and incubated for 1 h at 37 °C, after which 100 ml 0.3 M NaOH was added to each well to stop any further reactions. Absorbance was read at 415 nm on an ELISA plate reader to determine the enzyme concentration per construct. Samples were run in triplicate; as a positive control, a known concentration (0.05 UI/100 μl) of alkaline phosphatase (Biolabs) was used, and as a negative control distilled water plus 2% Triton X-100 was used.

2.8. Statistical analysis

All assays were performed in triplicate and the results expressed as mean ± standard deviation (SD) for $n = 5$. Statistical analysis was performed on the three treatments and the control results along the different incubation times using factorial analysis of variance.

3. Results

3.1. Scaffold

The phosphorylated chitosan scaffold consisted of a multilayered construct in which the glutaraldehyde-crosslinked layers appeared more dark yellow than the non-crosslinked ones (Figure 1a). Under the stereoscopic microscope, HAP deposition appeared to be more intense in the non-crosslinked layers (Figure 1b). The multilayered phosphorylated chitosan scaffold, previous to HAP double diffusion deposition, was a highly porous construct. The layers were 0.5–0.9 mm thick and showed multiple open and interconnected pores with a size diameter in the range 30–200 μm, delimited by thin transverse septa (Figure 2). SEM observations did not allow the differentiation of glutaraldehyde-crosslinked layers from non-crosslinked ones.

After double diffusion, the chitosan–HAP scaffold showed deposition of material on the pore surface (Figure 2), and the strongest deposition was found on the non-crosslinked layers. The crystalline material deposited appeared as butterfly-like plates, which corresponds to HAP (Figures 3,4).

3.2. Cell proliferation

The addition of 25 μg/ml fibronectin to the composite produced a significant increase in the number of osteoblasts cultured onto the composite until day 7 (Figure 5), reaching a two-fold amount compared with the starting cell number. After day 7, the cell number of both the control and fibronectin-added composites started to decrease, but it remained higher than that of the fibronectin-free control.

When the composites were enriched with 50 μg/ml fibronectin, a significant increase in osteoblast number

DOI: 10.1002/term

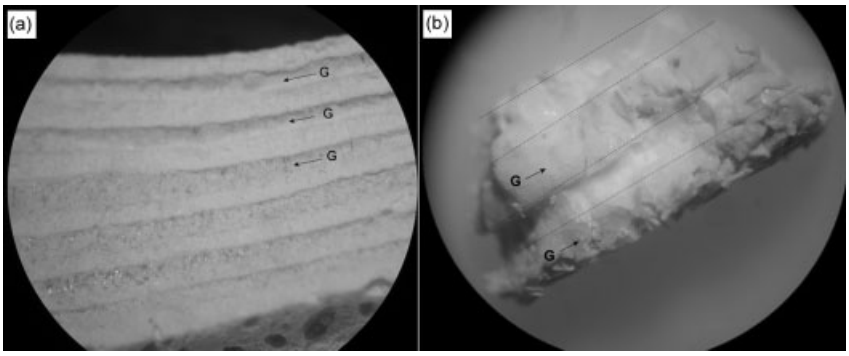

Figure 1. Stereoscopic microscope observations of the multilayered phosphorylated chitosan scaffold: (a) previous to HAP double diffusion deposition (×20); (b) a four-layered scaffold after HAP deposition (×40). G, glutaraldehyde-crosslinked layers

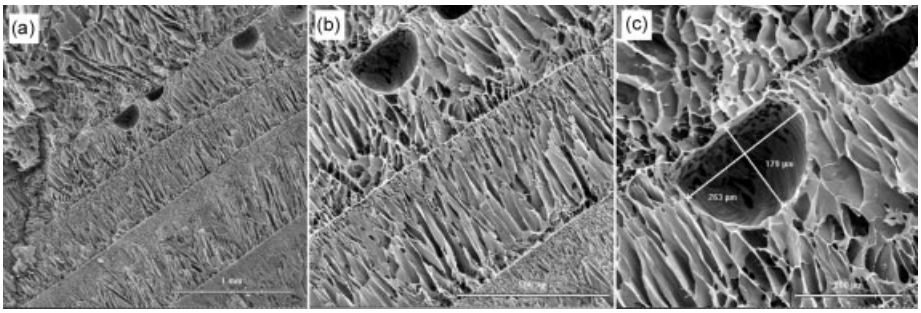

Figure 2. SEM micrographs of the phosphorylated chitosan scaffold at three magnifications, showing the multilayered structure (a), transverse septa delimiting pores (b) and details of the pore structure (c)

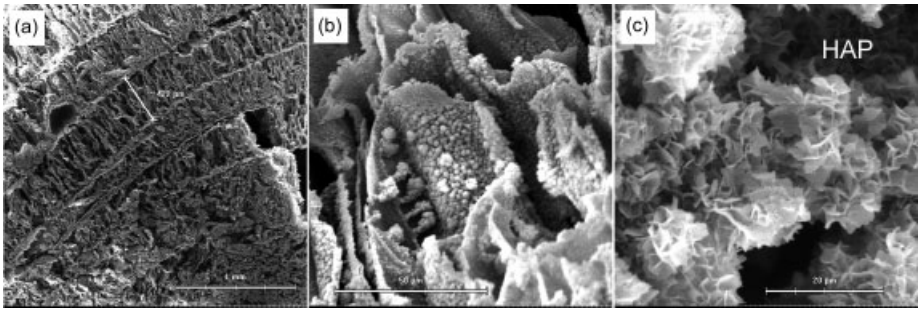

Figure 3. SEM micrographs of the phosphorylated chitosan scaffold after HAP double diffusion deposition at three magnifications. Deposited material on the pore surface (a, b), in the form of butterfly-like plates (c), was observed

cultivated onto the composite was observed until day 7, when the cell number was almost three-fold the initial number (Figure 5). As was observed for 25 μg/ml fibronectin-enriched composites, again after day 7 the cell number of both the control and the fibronectin-added composites started to decrease, but it remained higher than that of the fibronectin-free control.

However, when the composites were enriched with the bone-like matrix produced by osteoblasts cultured onto them for 12 days, and then seeded with new osteoblasts cultured for 14 additional days, an increasing number of cells was observed until day 14 of culture (Figure 6).

3.3. Cell differentiation

Cell differentiation was estimated by ALP activity, which was determined for each experimental group using a plate ELISA reader to determine the relation between absorbance and the amount of enzyme activity. Absorbance was read at 450 nm. The addition of both concentrations of fibronectin (25 and 50 μg/ml) to the composites did not increase ALP activity compared with control constructs (Figure 7). However, at days 7 and 14 of culture, ALP activity was higher than that of the non-enriched scaffolds, although a decrease was observed at day 14. Contrarily, a significant increase of ALP activity by bone-like matrix-enriched composites was observed until day 14 of culture (Figure 8).

4. Discussion

In order to regenerate or repair bone, not only is the occurrence of different kinds of osteogenic cells and growth factors needed, but also the availability of an appropriate scaffold is of equal importance. There have

DOI: 10.1002/term

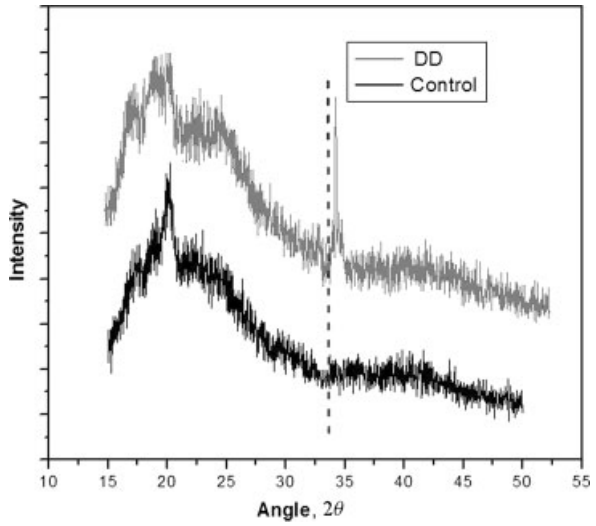

Figure 4. XRD pattern of the chitosan–HAP scaffold (upper grey spectrum), showing a 33° angle crystalline peak corresponding to HAP, as compared with the chitosan scaffold pattern (lower black spectrum)

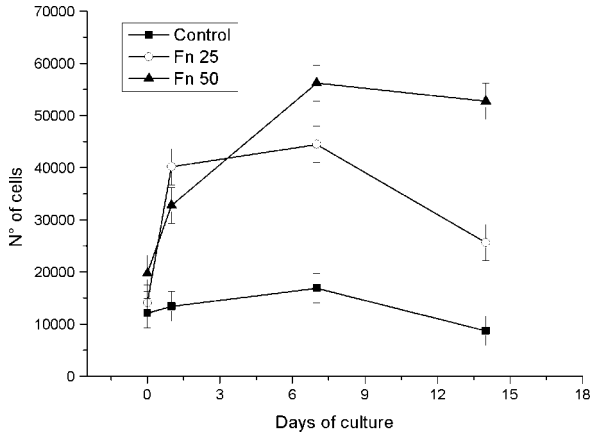

Figure 5. Cell proliferation, estimated by the MTT method, of 25 and 50 μg/ml fibronectin-enriched composites (Fn 25 and Fn 50) compared with non-enriched composites of osteoblasts cultivated for 14 days

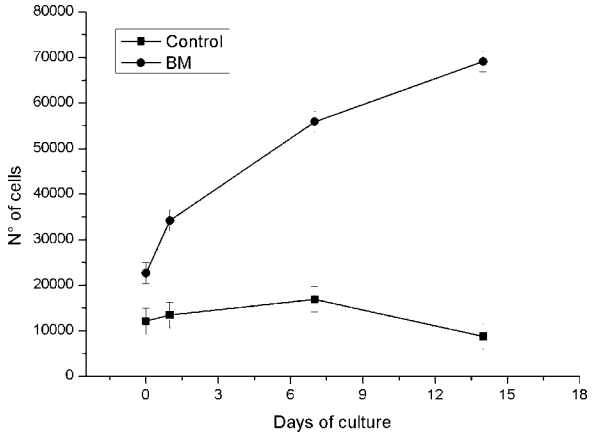

Figure 6. Cell proliferation estimated by the MTT method of bone-like matrix-enriched composites (BM) compared with non-enriched composites (control) seeded with new osteoblasts and cultivated for 14 days

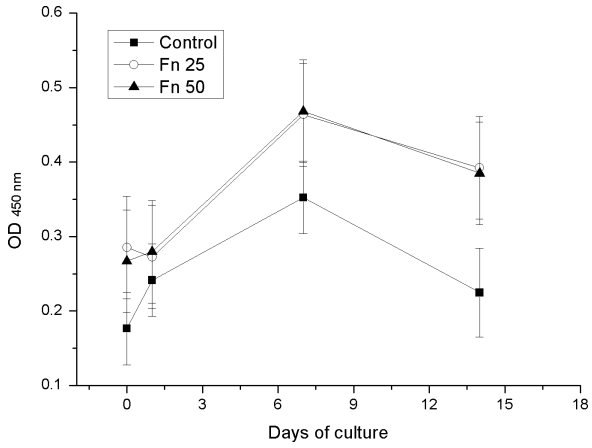

Figure 7. ALP activity of 25 and 50 μg/ml fibronectin-enriched composites (Fn 25 and Fn 50) and non-enriched composites seeded with osteoblasts for 14 days

been efforts to combine chitosan with calcium phosphate salts as a scaffolding material for bone regeneration (Lee *et al*., 2000). However, it is since the pioneering and independent studies of E. Khor and R. Reis laboratories (Ge *et al*., 2004; Malafaya *et al*., 2005; Oliveira *et al*., 2006) that several kind of chitosan–hydroxyapatite composites have been prepared and tested as suitable scaffolds for tissue engineering (Chesnutt *et al*., 2009; Su *et al*., 2009; Chen *et al*., 2009, 2010; Lu *et al*., 2010; Weir and Xu, 2010; Wu *et al*., 2010). One of the main physical features for a good tissue-engineering scaffold is to have high porosity, large surface area, a suitable pore size and high connectivity among the pores. Several preparation methods have been used to produce porous chitosan scaffolds, including porogen leaching (Ma *et al*., 2001) and thermally induced phase separation (Ho *et al*., 2005; Hsieh *et al*., 2007). However, freeze-drying and freeze-gelation methods produce membranes or few-layered homogeneous scaffolds. Finally, one of the main problems of chitosan-based scaffolds is the chitosan wettability and swelling behaviour, which can produce a volume increase of up to 300% (Yazdani-Pedram *et al*., 2003).

The alternate immersion of layers of crosslinked and non-crosslinked chitosan solutions in liquid nitrogen reported here produces a novel 3D multilayered scaffold which, through a phase-separation mechanism, forms suitable and interconnected pores. In addition, the phosphorylation of the chitosan covering the scaffold pores surface stimulates the biomimetic precipitation of hydroxyapatite onto the surface of its interconnected pores, and reduces the chitosan swelling behaviour down from 300% to 25%, as has been shown elsewhere (Arias, 2008; Arias *et al*., 2009). In addition, the final addition of ECM components improved the cellular responses to the scaffold. In this regard, it has been shown that the immobilization of RGD, the cell-binding region of fibronectin and other cell adhesive proteins,

DOI: 10.1002/term

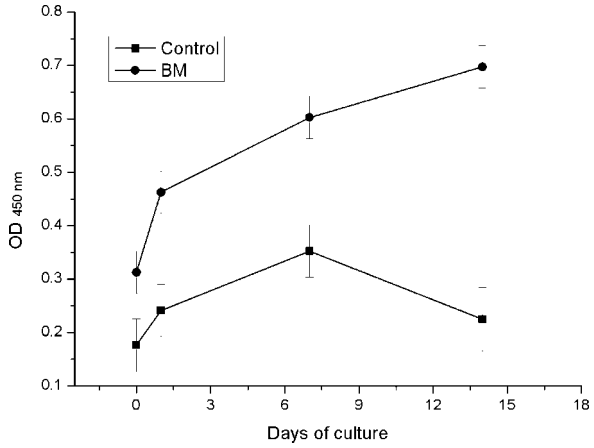

Figure 8. ALP activity of bone-like matrix-enriched composites (BM) and non-enriched composites seeded with osteoblasts for 14 days

onto chitosan membranes improves cell attachment (Ho *et al*., 2005; Tigli and Gumusderelioglu, 2008). However, the use of the whole protein may provide additional important actions which regulate cell proliferation or differentiation, especially mediated by the binding to different integrin receptors (Garcia and Reyes, 2005; Marletta *et al*., 2005; Amato *et al*., 2007; Martino *et al*., 2009; Tsutsumi *et al*. 2009). On the other hand, it has been shown that osteoblastic cells cultured onto different substrates, including HAP, and grown in the presence of osteogenic supplements do form bone–matrix molecules *in vitro* (Ozawa and Kasugai, 1996) and that this bone-like matrix, after decellularization, can support the differentiation of marrow stromal cells towards osteogenic cells (Datta *et al*., 2005; Pham *et al*., 2008).

The attachment of the osteoblast cells to the chitosan–hydroxyapatite construct can be inferred from the MTT assay tested on cells recovered by lysis just after the composite scaffolds had been seeded. On this regard, we observed that at the beginning of the experiment only 65% (13 000 of 20 000) of the seeded cells remained in the composites non-enriched with ECM components. However, the capability of the construct to support cell attachment increases with the concentration of fibronectin added to the scaffold, reaching a 100% attachment of the seeded cells when the scaffold was enriched with bone-like matrix produced by cells cultivated previously onto the scaffold.

It is known that the proliferation rate of cells can be measured using the MTT assay. Although fibronectin coating of metal or tissue culture polystyrene did not have any effect on osteoblast-like cells proliferation measured by this assay (Muhonen *et al*., 2009), it has been shown that fibronectin immobilized on chitosan substrates improves cell adhesion and proliferation (Custodio *et al*., 2010), and coating glass or hydroxyapatite with fibronectin resulted in stronger cell adhesion, proportional to fibronectin concentration (Garcia *et al*., 1997). Our results showed that cells did not proliferate on scaffolds not enriched with ECM molecules. However, they did proliferate during the first 24 h in scaffolds containing fibronectin; the population is maintained or increased at day 7 and then decreases, in both cases, inversely proportional to the initial concentration of fibronectin added. Comparatively, in scaffolds coated with ECM components derived from osteoblasts previously seeded, a continuous increase in cell proliferation was observed up to the end of the experiment. Although we do not have experimental evidence, one plausible explanation for these results could be related to the decrease in cell number observed elsewhere after 4 and 7 days after coating ceramics with fibronectin (Dennis and Caplan, 1993), or the desorption of fibronectin occurring during incubation time when it is adsorbed, instead of covalently immobilized, to chitosan substrates, as has been recently described by Custodio *et al*. (2010). Contrarily, if the ECM components left by the osteoblasts previously seeded onto the scaffold are responsible for the progressive cell proliferation observed, it is safe to suggest that these components remained well attached to the scaffold throughout the incubation.

The role of fibronectin in the control of differentiation of osteogenic cells has been established (Globus *et al*., 1998; Gronthos *et al*., 2001; Stephanson *et al*., 2002; Keselowsky *et al*., 2007; Sogo *et al*., 2007; Petrie *et al*., 2008; Hamidouche *et al*., 2009). ALP is an early marker for osteoblast differentiation and a crucial enzyme for bone mineralization, and its expression level depends on the stage of cellular differentiation. The MC3T3-E1 osteoblast-like cell line used here always expresses ALP if ascorbic acid and β-glycerophosphate are provided. Although we do not know the exact nature of the ECM molecules deposited on the scaffold, our results showed that ALP expression of these cells, during the time they were cultivated on scaffolds enriched and non-enriched with ECM molecules, followed the proliferation pattern of the cells. This means that neither fibronectin nor bone-like matrix molecules improve the expression of the osteogenic cells cultivated onto the chitosan–hydroxyapatite scaffolds, but they do not interfere with an even expression of the cells under these experimental conditions.

Therefore, it is safe to conclude that the different constructs used here not only do not change the differentiation status of the MC3T3-E1 cells, but also the scaffold enriched with osteogenic cells for 12 days, and then decellularized, better supported the attachment and proliferation of the MC3T3-E1 cells. The main physicochemical, mechanical and *in vivo* biological properties of these constructs are presently under investigation.

Acknowledgements

This research was supported by Project Nos FONDECYT 1080185 and 3100021, granted by the Chilean Council for Science and Technology (CONICYT).

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DOI: 10.1002/term