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ORIGINAL PAPERS

Bone tissue engineering. Design and development of biologically active vitroceramic-based hybrid materials to be used as bone substitutes

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KEYWORDS
Glass-ceramic;
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Abstract
Purpose: To describe the development and characterisation of a vitroceramic material as well as the initial response of adult mesenchymal stem cells (MSCs-A) isolated from bone marrow.
Material and methodology: The material was obtained by heating glass with composition in mol% 55SiO_2-41CaO-4P_2O_5 by a sol gel method. Cells were isolated from direct iliac crest aspirates from young adult patients. An analysis was performed of the degree of adhesion, proliferation and osteoblastic differentiation of MSCs-A seeded onto the material. Cell differentiation was evaluated through the production of osteocalcin and the loss of the CD90 mesenchymal marker. Cell proliferation on the substrate was performed using the tetrazolium salt reduction method. The seeded material was implanted in a critical defect caused in a rabbit femur in order to determine its osteogenerating capacity; CT observations were carried out.
Results: MSCs-A se bound to the material, expanded, proliferated and produced mineralised extracellular matrix on the material during the culture period. At the same time, they showed an osteoblastic phenotype, increasing osteocalcin production and losing CD90 expression. The material was partially resorbed at the end of the study.

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Conclusion: The material is cytocompatible, osteoconductive, bioactive and has a capacity to promote osteoblastic differentiation of MSCs-A as well as new bone formation following its implantation in association with MSCs-A; an appropriate matrix for bone tissue regeneration.

PALABRAS CLAVE
Cerámica vítrea;
Andamiaje;
Células madre mesenquimales adultas;
Osteoblastos;
Defecto óseo

Ingeniería tisular del tejido óseo. Diseño y desarrollo de materiales híbridos biológicamente activos basados en vitrocerámicas para sustitución ósea

Resumen
Objetivo: Describir el desarrollo y caracterización de un material vitrocerámico y la respuesta inicial de células madre mesenquimales adultas (MSC-A) aisladas de la medula ósea.
Material y metodología: El material se obtuvo por calentamiento de un vidrio 55SiO_2-41CaO-4P_2O_5 (mol/%) por el método sol-gel. Las células se aislaron por aspirados directos de cresta ilíaca de pacientes adultos jóvenes. Se estudió el grado de adherencia, proliferación y diferenciación a osteoblastos de las MSC-A sembradas sobre el material. La diferenciación celular se evaluó mediante la producción de osteocalcina y la pérdida del marcador mesenquimal CD90. La proliferación celular sobre el sustrato se realizó mediante el ensayo de reducción de sales de tetrazolio. El material sembrado se implantó en un defecto crítico realizado en fémur de conejo para valorar su capacidad osteorregeneradora, y se observó mediante TAC.
Resultados: Las MSC-A se adhirieron, expandieron, proliferaron y produjeron matriz extracelular mineralizada sobre el material durante el tiempo en cultivo, al mismo tiempo que mostraron fenotipo osteoblástico, e incrementaron la producción de osteocalcina y la pérdida de expresión de CD90. El material se reabsorbió parcialmente al final del estudio.
Conclusión: El material es citocompatible, osteoconductor, bioactivo, con capacidad de promover la diferenciación de MSC-A a osteoblastos y la neoformación ósea después de su implantación en asociación con MSC-A; es una matriz adecuada para la regeneración del tejido óseo

Introduction

Bone substitutes are currently designed and developed from natural and synthetic materials, among which can be found calcium phosphate ceramics, bioglasses (Bg) and biologically active vitroceramics (Vc).[1-3] These materials are biocompatible, biologically active and reabsorbable to varying degrees, with the capacity to integrate into the receiving bone tissue without interpositioning of fibrous tissue. They are used in clinical practice, bone tissue replacement, filling of small bone defects, and to increase the volume of bone grafts. The possibility of isolating adult osteogenic progenitor cells (adult mesenchymal stem cells [MSCs-A]) has raised new strategies for regeneration, and both calcium phosphate ceramics (hydroxyapatite [HA], FTC, and biphasic compounds) and Vc and Bg compounds are suited to support adhesion, proliferation, and formation of extracellular matrices by differentiated cells of the osteoblast lineage[4-6] due to the modifications of the surface of the material. Thus it is no surprise that a high level of interest exists in improving the osteogenic properties of these materials by constructing hybrid materials that are more biologically active, and culturing different types of cells[4-8] in order to imitate bone structure and biology.

A hybrid compound or material that combines allogeneic MSCs-A and a new bioactive matrix in the SiO_2-CaO-P_2O_5 system could meet the precise requirements to be considered as a material with osteoconduction, osteoinduction, and osteogenesis capabilities. In order to demonstrate this, we have designed, developed, and characterised a matrix composed of 55-SiO_2; 41-CaO; 4-P_2O_5 mol/%in order to test *in vitro* whether or not MSCs-A are capable of adhering, proliferating, and expressing osteoblast phenotypic differentiation on the matrix.

Material and methods

The 55%SiO_2; 41%CaO; 4%P_2O_5 (mol %) precursor glass was obtained using the sol-gel method.[9] The dried gel was then ground and sieved until obtaining particles between 32 and 68mm. These particles were created by compacting 0.1g of dry gel by uniaxial compression (55 MPa) followed by isostatic pressure (150 MPa) in steel casts. The material was then heated to 1,100° C for 3 hrs. The final product was discs of 4.8mm diameter and 1.3mm length for the *in vitro* studies and 4.8mm x 6mm cylinders for the *in vivo* studies. The crystalline phases present were pseudowollastonite (54%), wollastonite (38%), and cristobalite (4%). All of the pieces were cleaned using pressurised air, washed several times in PBS, and individually packed, followed by gas-plasma sterilisation.

The pieces were characterised by X-ray diffraction (Microanalysis-Link-ISIS software JEOL 6400, New Brunswick, Canada), scanning electron microscopy (SEM) (JEOL 6400, Microscope-Oxford Pentafet, New Brunswick, Canada), and mercury porosimetry (Autopore III 9420, Micromeritics Instrument, Norcross, GA, USA).

In vitro bioactivity was evaluated by submerging the material in a simulated body fluid (SBF) during 3 to 7 days. The morphology of the material was analyzed before and after the SBF immersion using SEM, energy-dispersive spectroscopy (EDS), and Fourier transform infrared spectroscopy (Nicolet NEXUS spectrometer, Oxford, UK).

Preparation and culture of adult mesenchymal stem cells

The MSCs-A were isolated from bone marrow directly aspirated from the iliac crests of volunteer donors who had previously signed an informed consent. For isolation of the cells, the aspirated material was deposited in a tube containing heparin sodium (20 U/ 1 ml of aspirate) for transport to the laboratory. Ficoll was then added (1:1ml) and the solution was centrifuged at 1,800g for 30 min. The buffy coat was then aspirated and deposited in a tube along with a phosphate buffer (PBS, pH 7.4) and was centrifuged at 2,400g for 10 min. (this procedure was repeated 3 times). Next, the product was resuspended in an alpha-minimal essential medium (alpha-MEM) (Gibco, Invitrogen Corporation, Carlsbad, CA, USA) supplemented with foetal calf serum (FCS) (Gibco) at 15% and routine antibiotics (100 U/ ml penicillin and 100µg/ ml streptomycin) (Gibco). At the end of the process, following a viability test using trypan blue, the cells were seeded at a density of 1.0×10^6 in 75cm^2 culture flasks supplemented with 10ml of alpha-MEM, and were incubated at 37° C in an atmosphere of 5% CO_2 and 95% relative humidity.

Seven days later, the culture medium was renewed; this eliminated the unadhered haematopoietic cells and the MSCs-A were selected based on their capacity to affix to the plastic of the culture flasks. One week later, the confluent layer of cells was subcultivated in a ratio of 1:3 and the culture flask was treated with trypsin at 0.25% and EDTA (0.02%) in PBS for 5 min. Some of the cells were frozen in a culture medium with 15% FCS and 10% DMSO for later use in different assays, and others were used for cell characterisation, the study of interactions with the material, and possible differentiation into osteoblasts (OB).

Isolation and culture of osteoblasts

The OBs used as controls in this study were obtained by enzymatic digestion. Small samples of spongy bone 1–2mm were washed several times in PBS with routine antibiotics. These were then incubated for 15 min. at 37º C with type xi collagenase (1.25mg/ ml in Hank solution). The digestion product was then filtered through a 100µm mesh and centrifuged at 200g for 10 min. The cells obtained were then resuspended in a culture medium (DMEM and Ham's F12 1:1, FCS at 10%, and routine antibiotics), and stained with trypan blue in order to assess their number and viability. The medium was renewed every 3 or 4 days. The incubation was performed at 37º C in a 7.5% CO_2 atmosphere and 95% relative humidity. The first subculture (Po) was performed following 7 days of incubation in 25cm^2 flasks after trypsinisation, and the first two subcultures were used for cell identification.

The isolated OBs were identified and characterised by their morphology and growth patterns using a phase contrast microscope and SEM, and by their mineralisation capacity (Von Kossa stain).

The MSCs-A were characterised and differentiated using a growth medium (GM) composed of alpha-MEM supplemented with heat deactivated FCS at 10% and routine antibiotics, and were incubated in standard conditions. Subsequently, the cells were separated from the flasks by trypsinisation and seeded for characterisation by optical microscope and SEM, and expression of CD90, CD45, and osteocalcin (OC).

For the scanning electron microscope assessment of the MSCs-A, $1x10^5$ cells were seeded in Leighton tubes and cultivated in GM for 15 days. The medium was renewed every 3 or 4 days. Subsequently, the cultivated cells were washed with PBS and fixed with 3% glutaraldehyde in a 0.1 M cacodylate buffer for 30 min at 4° C. The cells were then washed and postfixed in osmium tetroxide for 1 hr and dehydrated using sequentially higher concentrations of ethanol (30, 50, 70, 90 [v/v]) with a final dehydration in pure alcohol, and dried using the critical point method. Samples were then coated by gold or carbon (for EDS) and observed under the scanning electron microscope. The surface of the material was also analyzed to evaluate cell adhesion and proliferation using the same process.

Determination of the expression of CD90 and CD45 by adult mesenchymal stem cells

We used CD90 and CD45 antibodies coupled with fluorescein isocyanate (BD Pharmingen, BD-Biosciences Europa, Brussels, Belgium). The cells were separated from the culture flask with trypsin/ EDTA (0.25/ 0.25%) and adjusted to a concentration of $5x10^6$ cells/ ml. 100µl were taken from the suspension, to which the antibodies were added and incubated for 15 min; the samples were then analyzed in a Becton-Dickinson flow cytometer (FACSort model, Oxford, UK) with an argon laser at 488nm and 15mw. The primary human OB cell line obtained in our laboratory and the Kato III human gastric carcinoma cell line (ECACC, Salisbury, UK) were used as the negative controls.

Osteocalcin production

OC was produced in the MSC-A and OB cultivated cell supernatants, and the FCS was removed from the culture medium 5 days prior to the determinations. The presence of OC was detected using a Gla-type osteocalcin EIA kit (Takara cat# MK111) (Reactiva SA, Barcelona, Spain); the assay was applied to all of the samples under the kit standards in triplicate, using the OB line as a positive control.

Study of the proliferation of adult mesenchymal stem cells on the material

The cells were seeded on plates with 96 wells, and the bottom was covered by a 0.6% agarose upon which the discs

were placed for cell seeding. This ensured that the increase in cell number that would be detected during the study would result from only those cells that grew adhered to the material. $5x10^3$ cells were seeded on each disc, and adhesion and growth of the cells were measured on days 1, 7, 14, 21, and 28, as well as measuring the level of coating using SEM and the increase in cell number by a tetrazolium salt reduction assay (XTT). XTT was briefly added to each well (final concentration: 0.2mg/ml) with menadione (final concentration: 0.02mM), and incubation lasted 4 hrs. The absorbance (OD) of the culture medium of cells growing on the material and controls (grown on plastic) was measured at 450nm in a Lab System plate reader (mod. Multiskan MCC 340, Fisher Scientific, Pittsburgh, PA, USA).

After one month, the cultivated cells' production of OC and expression of CD90 and CD45 was measured to evaluate possible osteoblastic differentiation of the MSCs-A. Measurements of cellular proliferation on the material and enzymatic determinations were performed in triplicate.

Design of the *in vivo* study

We used a total of 12 animals (New Zealand albino rabbits), adults of both sexes, with an approximate weight of 3.5 – 4kg, performing critical segmental resections of the right femurs. The defect was filled with hybrid material and stabilised using an external fixator. The same defect was created in the contralateral femur but without filling as a control.

The animals were randomly assigned to 3 established study groups of different time periods (1, 3, and 6 months), 4 to each group. The rabbits were kept in individual cages, eating and drinking *ad libitum*, with environmental conditions controlled according to the protocol established by the Commission of Ethics, Use, and Care of Laboratory Animals of the University of Murcia.

The implant was prepared by combining the MSCs-A with the study material. The cultured cells corresponding to the second phase (P2) were separated from the flask using trypsin-EDTA incubation at 25% for 5 min, followed by centrifugation at 1,000g/10 min and resuspended in GM; at the same time, the material was immersed in FCS and incubated for 30 min. The cells were seeded on the material surface by direct pipetting (1×10^4 cells/cm^2) and cultivated in static conditions for one hour before adding the culture medium. The hybrid implant was kept in incubation for one week before its implantation in the bone defect.

Anaesthesia was applied following the established protocol (50mg/kg ketamine, i.m.; 10mg/kg chlorpromazine, i.m.) with antibiotic prophylaxis (20mg/kg amoxicillin, i.m.) in a single dose. Postoperative pain was controlled by administration of tolfenamic acid (0.1mg/kg, s.c.) every 12 hrs for 2 days. The right femur was exposed through a direct lateral approach over the intermuscular septum of the quadriceps and the femoral biceps of the hamstring; the first was set anteriorly and the following posteriorly. The periosteum was removed from the shaft between 1cm distal to the greater trochanter and 1cm proximal to the lateral epicondyle. We then performed a careful haemostasis by electrocauterisation. Proximal and distal nails were then implanted in the base of the greater trochanter near the epicondyle. The template was then implanted with 2 remaining nails. Next, a critical 1.5cm bone segment including the periosteum was resected from the middle third of the shaft using a circular minisaw and continuous irrigation. The resulting space was washed repeatedly with saline solution. Next, the fixator was adapted to the leg and the hybrid material was implanted in the defect; the fixator components were then fixed, ensuring alignment and stability. Finally, the surgical wound was closed in layers with a reabsorbable suture material (Vicryl® 3/0 and Vicryl-Rapid® 3/0). The animals were sacrificed following sedation (ketamine at 10mg/kg, i.m.) with a thiopental intracardiac overdose.

Radiographic study

Simple radiographs and CT scans were taken of the specimens devoid of any adhered soft tissue at the different time periods of the study.

Anatomopathological study

The samples obtained were initially fixed in a 10% neutral formol solution; these were then decalcified (TBD-1) and dehydrated in alcohol at progressively greater concentrations, and finally embedded in paraffin and stained with haematoxylin-eosin and Masson trichrome.

Statistical analysis

We used an analysis of variance (ANOVA) with repeated means to evaluate the statistical significance of the results. Values of $p < 0.05$ were considered significant for *in vitro* studies. After applying a natural logarithmic transformation, the data complied with the conditions for homoscedasticity and sphericity of the analysis.

Results

Characterisation of the material

The crystalline phases of the material were pseudowollastonite, wollastonite, tricalcium phosphate, and cristobalite. The formation of an apatite layer over the surface of the material following the immersion in SBF was an indicator of *in vitro* bioactivity. After 3 days of immersion, the surface of the material was covered by a newly formed layer of small crystalline aggregates that increased in size at 7 days immersion. The EDS analysis showed an increase in the calcium and phosphorous contents, and a decrease in the content of silicone, while the infrared spectral analysis showed new bands corresponding to phosphate groups (apatite phase) and a decrease in the bands corresponding to pseudowollastonite due to the solubilisation during this phase.

Characterisation and identification of the adult mesenchymal stem cells

The adhered cells isolated by aspiration of the bone marrow and initially considered to be MSCs-A at first (24 – 48 hrs)

showed a variable fusiform morphology that was bipolar or tripolar when grown at low densities, and matured with time until forming a monolayer. Following the first subculture and in the proceeding ones, the cells returned to growing in a uniform manner at the bottom of the plate without forming colonies.

The OBs isolated from the spongy bone tissue that were used as a control for differentiation were of a larger size, with a generally tripolar morphology and long cytoplasmic projections and a characteristic multilayered growth that formed nodules of radial growth, in the centre of which accumulations of a refracting material could be observed. This material was identified later as calcium deposits (Von Kossa stain). The MSCs-A showed a flattened and elongated, occasionally polygonal aspect under the scanning electron microscope, with several long cytoplasmic projections and spicules and small nodules on the surface. Later, once the culture reached confluency, the cells grew in multilayers and adopted a fusiform morphology with the tendency to orient themselves in the same direction with abundant filopodia and intercellular connections.

After placement in GM for 15 days, the MSCs-A barely showed levels of OC. However, the OBs used as controls produced appreciable levels of OC in the same medium.

The adhering cells obtained from the bone marrow aspiration that were initially considered to be MSCs-A were positive for CD90 (CD90+) and negative for the haematopoietic CD45 antibody (CD45–), indicating that these cells presented membrane antigens characteristic of mesenchymal stem cells (MSC). Both Kato III and OB cells were CD90–.

Growth of mesenchymal stem cells on the material

In the XTT assay, the absorbance values detected at 24 hrs were very low, indicating that the number of cells that

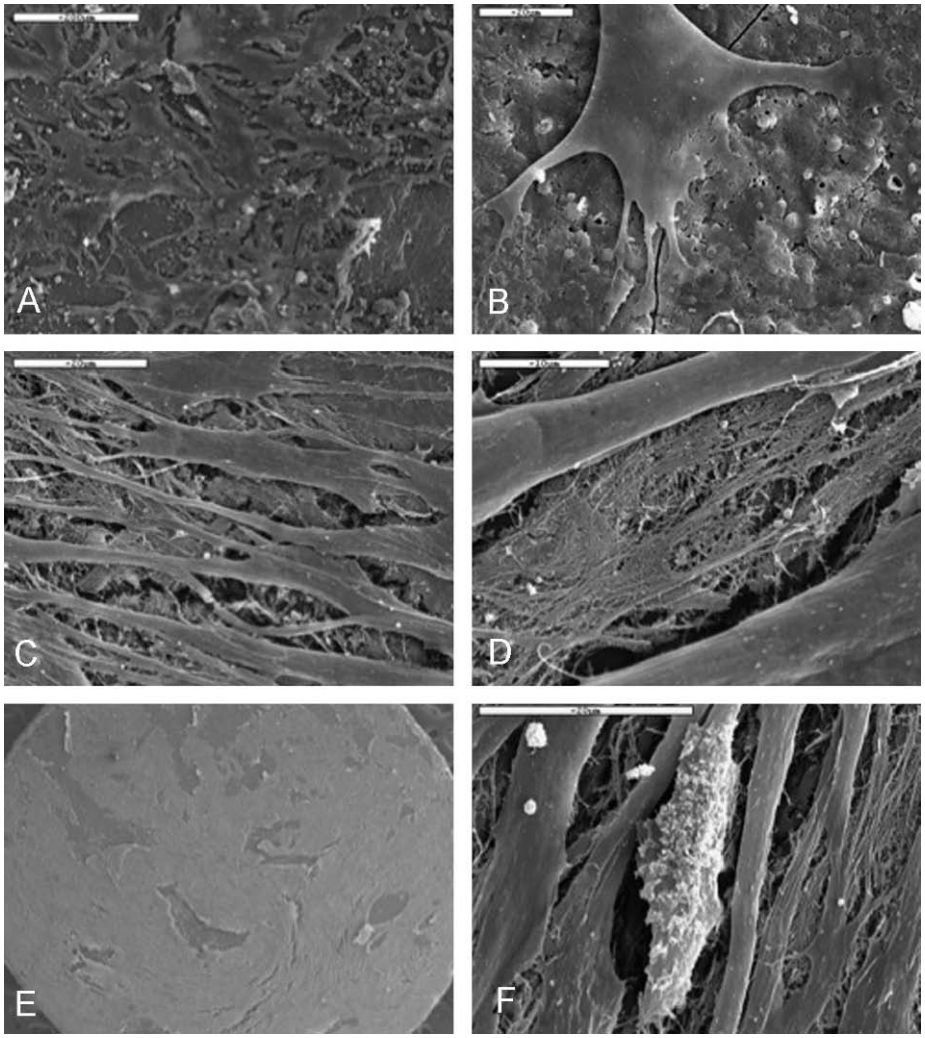

Figure 1 Microphotographs from a scanning electron microscope (SEM) of the cells growing on the material surface showing: A) progress at 24 hrs in culture; B) firm adhesion of the cells using their cytoplasmic extensions; C) progress at 7 days; D and E) progress at 21 days, occupation of the intercellular spaces; F) granular aspect at 21 days (scale bars: 200, 20, 20, 10, 300, and 20μm, respectively).

initially adhered to the material were scarce. However, the growth of those cells that were able to affix progressed exponentially. Using the SEM (fig. 1) we observed various cells that had adhered in the first 24 hrs of culture in an isolated manner or in small groups dispersed along the surface of the material giving it a rough appearance. This adhesion was strong due to the multiple cytoplasmic digitations (filopodia) that extended over the surface of the material, increasing the area of contact. At 7 days, the cells expanded and propagated, forming accumulations dispersed along the surface; some intercytoplasmic connections were observable between them. After 2 weeks in culture, the cells continued to proliferate. As they increased in number, small deposits of extracellular fibrillar matrices became apparent in certain areas. At 21 days, the cell layer became confluent: the cells adopted a fusiform morphology and on occasion formed multilayers and displayed a greater number of interconnections as well as an abundant extracellular matrix; they eventually covered the entire surface of the discs. Furthermore, the cellular surface showed a granulated aspect due to the presence of abundant whitish nodules that emerged to the surface.

At the termination of the growth assay of the MSCs-A on the material, we determined OC, CD90, and CD45 concentrations to discover if any functional or differentiating effect in the cells was apparent.

The cells that grew on the material presented greater levels of OC than those that grew on the plastic using the same culture medium, for which we intuit that the material induced the production of OC in the MSCs-A.

The results obtained by flow cytometry showed that the expression of CD90 was considerably reduced (18.9%) in cells that grew on the material compared to the cells that grew on the plastic under the same conditions.

At 16 days, the MSCs-A cultivated in Leighton tubes produced extracellular matrices in a fibrillar network that occupied the intercellular spaces; also, we identified deposits of a whitish granular material included in the fibrillar plots.

These findings, together with the OC expression, the presence of the mineralised matrix, and the progressive loss of the CD90 markers could be sufficient data to affirm that the MSCs-A had undergone a differentiation process and were exhibiting an osteoblastic phenotype.

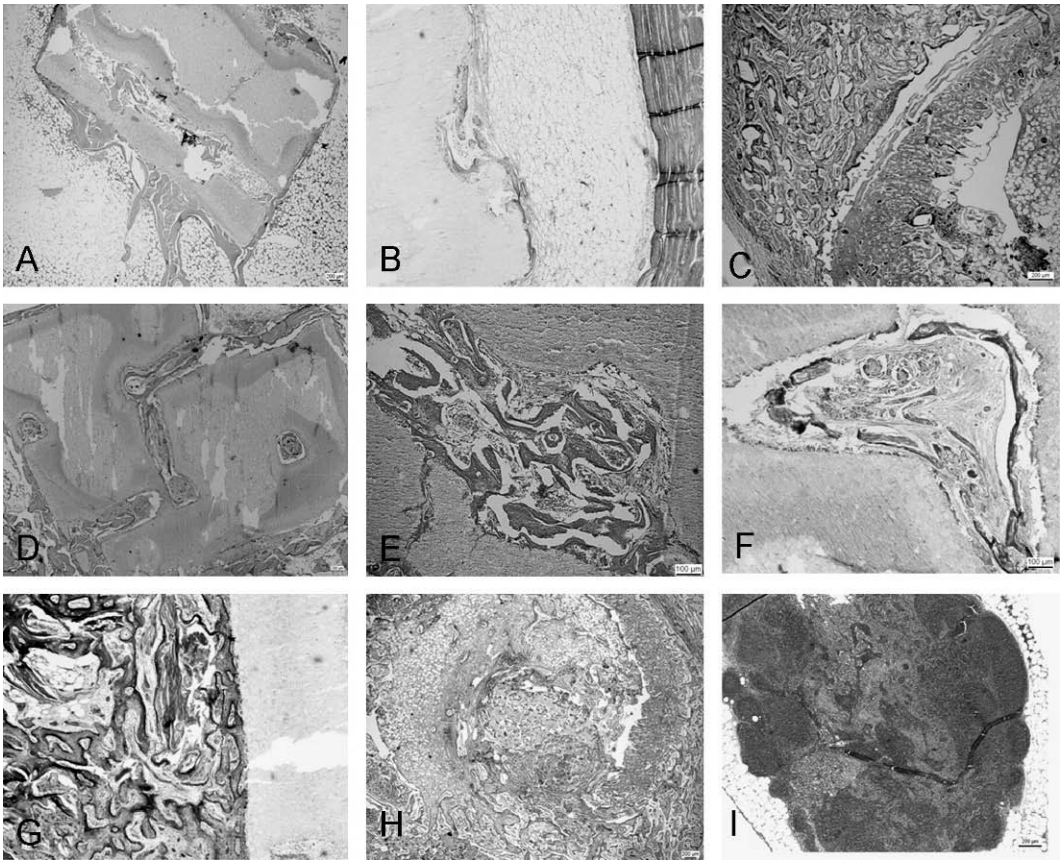

Figure 2 Microphotographs from an optical microscope: A) at one month (Haematoxylin-eosin [HE], 200×); B) cortical bone, bone marrow, and the peripheral portion of the implant (Masson trichrome, 312.5×); C) peripheral callus in formation (Masson trichrome, 250×); D) progress at 3 months (HE, 400×); E) bone tissue from the interior of the implant (HE, 500×); F) newly formed trabeculae inside the pore (T. Masson, 400×); G) progress at 6 months (Masson trichrome, 200×); H) reabsorption of the implanted material and partial replacement by bone tissue at one end (HE, 500×); I) regional lymphatic ganglion with chronic reactive adenitis (HE, 125×).

In vivo experimental model

All of the surgical wounds healed without complications, and we observed no signs of infection, even in those specimens that died without apparent cause or were sacrificed due to other complications.

Histologically (fig. 2), we observed the conservation of implanted material at one month of evolution that showed signs of colonisation of the haematopoietic bone marrow towards the interior of the implanted material, which in some zones came into contact, forming a reticulated pattern. Around the implant, the adjacent bone marrow showed multiple centres of bone neoformation with osteoid deposits and newly formed bone trabeculae that occupied virtually the entire perimeter of the material. In the area of the bone defect, we observed signs of bone regeneration characterised by the accumulation of granulated tissues with irregular deposits of osteoid material and endochondral ossification that delimited the perimeter of the implanted material, along with signs of partial remodelling.

At 3 months, the phenomena of colonisation were more extensive, as were the areas of bone neoformation with the presence of abundant osteoid deposits and newly formed bone trabeculae upholstered by numerous osteoblasts. These signs were observed in greater detail when assisted by the Masson trichromic technique.

Finally, at the 6 month period, the defect was practically filled by newly formed bone tissue, although not completely remodelled. As far as the material, the areas of reabsorption and replacement by newly formed bone tissue was especially notable, reaching its maximum intensity around the borders.

We did not register any adverse inflammatory responses, or the formation of a fibrous capsule that would delimit the implanted material from the adjacent bone tissue, nor any type of associated pathological alteration. We did observe regional adenopathies that corresponded microscopically to the chronic reactive adenitis characterised by cortical follicular hyperplasia with marked sinus histiocytosis.

In the CT scans after 4 weeks, the implanted material appeared as a high-density cuboid element in the interior of the bone defect, surrounded by a radiolucent line. At 3 months, the implanted material could be observed with a density similar to that of the bone tissue. We also observed voluminous peripheral bone neoformation that surpassed the borders of the defect.

At 6 months, we saw a hypertrophic bone callus that filled the entire defect with no evidence of any ectopic bone formation. In contrast, in the control bone defect we observed only a limited formation of new bone around the borders and a total absence of bone repair at the interior. The transversal CT slices showed trabecular lines of calcium density that ran from the adjacent bone tissue to the interior of the implant. The implant in the interior of the bone defect showed changes in its shape and a decrease in size that eliminated the reticular appearance.

Discussion

The replacement of bone tissue by tissue engineering implies the use of biocompatible, osteoconducting, and bioactive matrices that favour adhesion and *in vitro* cellular growth, allowing transport into the defect to be repaired. Some materials have demonstrated a highly variable capacity to influence the adhesion, proliferation, and differentiation of cells due to the dynamic and reactive characteristics of the material surfaces that permit the release of Ca, P, Si, Na, and Mg ions when they come into contact with biological fluids and that can affect cellular response.[10,11]

The material developed for this study has a composition of 55 SiO; 41 CaO; 4 P_2O_5 (mol/%) and showed a rapid *in vitro* bioactivity related to the formation of an apatite layer on the surface following 3 days of immersion in SBF.[9,12,13] This layer is essential for the primary chemical bonding of the material in the receiving bone tissue following implantation. Similarly, the apatite layer formed along with the ion release of Si, Ca, P, and Na into the medium can affect the cellular response[11,14-16] as well can the alkalisation of the medium, caused by the freeing of Na.

OC production acts as a biochemical marker frequently used to determine OB functionality, as is the production of alkaline phosphatase and type i collagen. However, the production of OC is an exclusive characteristic of OB, and this procedure is considered to be much more simplified and specific than the production of alkaline phosphatase and type i collagen, which are also produced by fibroblasts and other cell types. For this reason, the production of OC was the criterion used in our study in order to control the process of differentiation of MSCs-A isolated in bone marrow to OBs, using the primary OB cell line isolated from spongy bone tissue[17] and the Kato III line as positive controls. In order to evaluate their production, the isolated MSCs-A and OBs were cultivated in GM. At this point, the OBs spontaneously produced appreciable quantities of OC; on the contrary, the MSCs-A were incapable of doing this in the first subcultures even after 2 months in culture. But when the MSCs-A were cultivated on the material, we observed an increase in OC production, indicating the expression of an osteoblastic phenotype. This result was accompanied by the gradual loss of expression of the CD90 marker characteristic of MSCs-A. Based on these findings, we can assume that the material itself exerts a certain inducing effect on the osteoblastic differentiation of MSCs-A. This effect could be favoured by the release of Si, Ca, and P ions into the medium and by the increase in pH,[12] which influences intracellular enzymatic activity, similar to the genetic control that some Bg exert on the cellular cycle of OBs, which favours the expression of genes that regulate osteogenesis and the production of growth factors.[18,19] The mechanism by which the material studied induces the differentiation of the MSCs-A to OBs will be researched in future studies.

The capacity of the cells to produce a mineralised matrix and nodules of mineralisation is one important aspect to highlight in the design and development of materials for bone regeneration.

In our study and the study performed by Vrouwenvelder et al,[20] the cells that grew in the presence of the material were able to produce, although not in a uniform manner, small mineral deposits with Ca-P content, identified by Von Kossa histochemical staining and EDS-microanalysis, both

on the interior of the matrix and near the cells. The mineralised matrix produced by the MSCs-A cultivated on the material, associated with OC and the loss of the CD90 marker, supports the interpretation that the MSCs-A were differentiated to the point of presenting an osteoblastic phenotype induced by the material. The formation of nodules of mineralisation is basically considered to be the final process of differentiation into OBs.[20,21]

The cells that grew on the material initially held a flattened shape with cytoplasmic digitations and a homogeneous cellular membrane. With longer time spent in the culture, some of these cells started to show irregularities in the cellular membrane in the form of small granules. Furthermore, the cells developed abundant filopodia and intercytoplasmic connections, characteristics similar to those observed by Vrouwenvelder et al,[20] who described these as being characteristic of OBs. The cells exhibited good adhesion to the surface of the material, aided by their expanded shape and abundant filopodia. These observations are similar to those described by various authors for seeded OBs on HA ceramics, titanium, and Vc.[17,20-22]

For evaluation of proliferation capacity, the MSCs-A were seeded on the material and agarose was added to the bottom of the wells in order to ensure precise data by preventing adhesion and growth of cells in the spaces between the disc and the well walls. The finding of the different periods of the study showed good adhesion of the cells to the surface of the material, facilitated by their spread out shape. This finding, coinciding with those by Vitale-Brovarone et al,[22] takes place at an early stage prior to proliferation/differentiation. The cells grew exponentially, even though initial growth was slow due to a period of adaptation to the medium.

The observed cell adhesion and OC production were important results indicating cytocompatibility of the material. In our study, the relevant observations of excellent cellular adhesion and proliferation, as well as the increase in OC production, constitute indicators of biocompatibility.

A considerable number of different lines of research exist that focus on the field of biomaterials. These have spurred the development and application of diverse segmental bone defect models for different species of animals in preclinical evaluations of possible materials. In order to be able to compare the different studies and use them as a base for further development, especially with clinical uses in mind, it is essential that the animal model, the fixation device, the surgical technique, the methods for quantification, etc. be standardised. In the emerging discipline of tissue engineering, the general principles are based on the association of cells with a scaffold or matrix (natural or synthesised) in order to obtain a more biological hybrid material that can be implanted into the defect to be repaired. However, a review of the medical literature demonstrates that the preclinical models that have been used for the study of critical bone defects are not sufficiently described or standardised, since the selection of an animal model (rats, rabbits, sheep, dogs, micro pigs, etc.) does not take into account the various factors related to physiological and physiopathological analogues in humans (age, sex, bone microstructure, metabolic rates, etc.). Although these all provide a number of advantages and disadvantages, we believe that the decision of which model to use should be tailored to give a response to a concrete question without undervaluing other factors such as cost, maintenance, manageability, skeletal size, viability, social acceptance, tolerance to captivity, housing, etc. For our study we chose to use the New Zealand albino rabbit, since, after the rat, it is the most often used for studies of bone repair and regeneration in the most prominent publications from our specialty.[23] Recently, different preparations (FTC, hormones, MSC, polymers, etc.) have been developed for tissue engineering and used for the regeneration of diverse bone defects (tibia, femur, ulna, radius) in different species, making it difficult to compare these with our results.[24-26] However, all experts on the subject presently agree that for the formation of bone tissue, the following is needed: a) a source of multipotent MSCs capable of differentiation into OBs; b) growth factors (osteoinducing proteins) that impulse these cells to disperse over the defect, proliferate, and differentiate into OBs; c) a reabsorbable matrix that supports the adhesion and migration of these cells within the defect, and d) the neoformation of a vascular network (angiogenesis).

Regarding the type of cells used in this study, Arinzed et al[26] and De Nicola et al[27] demonstrated that allogeneic MSCs-A do not provoke an adverse immune response when they are implanted in bone defects, and do not require immunosuppressive treatments; these studies indicate that MSCs are capable of suppressing the proliferation of T cells and class ii molecule expression. These characteristics make these cells immune response regulators; on occasion, they have been used in experiments to improve the survival rates of allografts.[28] These results confer a level of certainty when designing this type of hybrid material. Histologically, we have observed a substantial amount of bone neoformation in the peripheral portions of the implant as in the interior of the implant pores in quantities superior to those found in defects without this treatment. Similar to the results from Bruder et al,[24] we observed that the implanted defect had practically filled with bone tissue at the 3 month period of the study, which was growing hypertrophically and surpassing the borders of the defect. There was no evidence of ectopic bone formation, nor were there any adverse inflammatory reactions during the entire study period.

The placement of the implants in broad areas of bone marrow and the > 4 week survival of the MSCs-A in the implant zone, as in results demonstrated by Gazit et al,[29] is evidence that the material promoted the process of differentiation of the MSCs-A into OB bone tissue producers and does not exclude the possibility that the material exerts a direct effect on other peripheral osteoprogenitor cells included in the bone marrow cell populations. Our experimental design did not permit us to quantify the number of cells that finally grew over the material before its implantation in the defect, although the type of growth was exponential as time passed.

Apparently, newly formed bone tissue originated along the borders of the defect as well as in the interior, in definite contact with the material, which corroborates the osteoinducing effect on the MSCs-A and, probably, on other progenitor cells of the haematopoietic bone marrow. The process of reabsorption of the material commenced along its surface, although this process was not completed by the

end of the study; this was one of the inconveniences of this material.

The choice of an adequate matrix for supporting adhesion and proliferation of MSCs continues to be a subject of debate. Materials based on Ca-P systems and with a close parallel of bone tissue mineral ratios (1:67) correspond to the most often utilised matrix types. However, these demonstrate dubious osteoinducing activity capable of initiating differentiation into cells of the osteoblast lineage. On the other hand, the low rates of reabsorption, above all with HA, could limit the long-term success of these and biphasic materials, principally if these maintain a 65:35 relationship in favour of HA. In this respect, an expansion of studies centred on other proportions more in favour of FTC would be required.

In our work we have developed a different strategy for the repair of bone defects, which includes direct implantation of allogeneic MSCs-A seeded in a porous bioactive and osteoconducing matrix, this last property being what makes this a novel study.

The material that we have tested in this study has shown excellent bioactivity and histocompatibility properties. Furthermore, it has been able to provide an adequate environment for adhesion, expansion, and proliferation of MSCs-A. During the time spent in culture with the material, the MSCs-A expressed an osteoblastic phenotype characterised by the production of OC and a mineralised extracellular matrix accompanied by falling levels of expression of the CD90 marker. The differentiation of allogeneic MSCs-A into OBs took place because of the osteoinducing effect of the material on the cells.

Following implantation in the *in vivo* study, the developed hybrid material was able to regenerate the lost bone matter in a critical segmental defect of the rabbit femur.

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Conflict of interest

The authors affirm that they have no conflicts of interest.

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