In Vitro and In Vivo Osteoinductive and Osteoconductive Properties of a Synthetic Bone Substitute

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Purpose: *The present study tested a recently introduced bone substitute material (BSM) with a novel structure to determine its osteoinductive and osteoconductive properties in vitro and in vivo. The specific aims were to determine the microstructure of the as-manufactured BSM, as analyzed with scanning electron microscopy, and to characterize different cellular interactions.* ***Materials and Methods:*** *Human bone marrow stromal cells were cultured in the presence of the BSM. In vitro, attachment of osteoblastlike cells (SAOS-2) to the BSM was observed with the scanning electron microscope. The expression of genes related to osteogenic differentiation (alkaline phosphatase, bone sialoprotein, type I collagen, and osteocalcin) was determined by reverse-transcriptase polymerase chain reaction. In vivo, bone formation was examined with a murine model of ectopic bone formation through histology and computed tomographic scanning by using tissue-engineered constructs with the BSM and ovine bone marrow stromal cells.* ***Results:*** *Early cellular attachment could be detected as early as 6 hours. Cellular morphology developed in the following 66 hours toward a starlike appearance. Human bone marrow stromal cells cultured in the presence of the BSM showed no reduction in their viability. Osteocalcin was up-regulated during cell culturing, demonstrating an osteoinductive effect of BSM. Histologic and computed tomographic analyses showed the formation of new bone surrounding BSM particles, and a vascular meshwork was observed in the porosity of the particles.* ***Conclusion:*** *The analyzed bone substitute of synthetic origin presented osteoinductive properties that may exert a differentiative stimulus upon osteoprogenitor cells. The tested material allowed cellular adhesion of osteoblastlike cells and, following tissue construct implantation in vivo, supported the formation of new bone.* Oral Craniofac Tissue Eng 2011;1:244–251

Key words: *beta-tricalcium phosphate, bone augmentation, bone marrow stromal cells, ectopic bone formation, gene expression, hydroxyapatite, reverse-transcriptase polymerase chain reaction*

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Bone substitute materials (BSMs) have been employed extensively in oral surgery to supplement guided bone regeneration (GBR), filling critical-size defects,[1] particularly as it is often difficult to harvest significant amounts of autologous bone, which is the gold standard.[2] Several studies have stated that natural bone graft materials are superior to BSMs in their osteogenic and osteoinductive properties.[3–6] On the other hand, synthetic BSMs eliminate the risk of infection, including the transmission of spongiform encephalopathy, in contrast to animal-derived bone substitutes.[7,8] A recent study refuted such concerns for a specific BSM[9]; regardless, the safety of industrial processes that might reduce such risks has not yet been fully disclosed.

Scaffolding materials with cubic, cylindric, or even individual designs (typically created via computer-aided design/computer-assisted manufacture) are currently

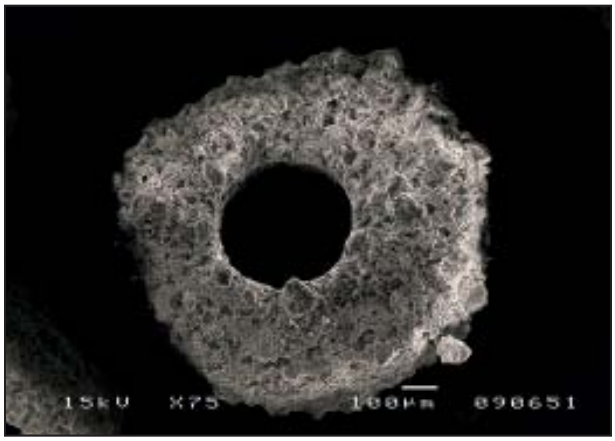


Fig 1 Microphotograph at low magnification showing the general macrostructure of BonePlus Eagle Eye bone substitute material. Each particle of this synthetic β-TCP/HA composite has a ringlike structure with surface microporosities offering channels into the core of the material.

utilized in orthopedics.[10–12] Oral GBR surgeries usually deal with infrabony defects, dehiscences, and horizontal resorptions, which are less clearly defined compared to long bone fractures.[13] Oral surgeons must apply particulate material, which needs to be adapted during the surgical procedure (ie, to fully cover exposed implant threads or distribute a BSM over an atrophic mandible).[14] The advantage of a scaffolding material is in its better adaptation to a precise defect measured with imaging (eg, a computed tomographic [CT] scan).[15] Nevertheless, bulky scaffolding materials raise concerns regarding the development of a vascular meshwork in the scaffold and the colonization of osteogenic cells.[16–18] For this reason, structural modifications have been developed with different microstructures, porosities, and chemical compositions.[18] Microporosities were introduced to create scaffolds that would be more favorable for vascular ingrowth and subsequent bone formation.[19] Particulate bioceramics and bone substitutes may enhance neovascularization in the implanted BSM thanks to the spaces already present between the packed particles. Additional porosities may be present in the particles themselves to encourage vascular supply.[20]

BSMs are often introduced into clinical use without knowledge of their in vitro and in vivo performance.[21] A new synthetic BSM made up of composite ceramic (60% beta-tricalcium phosphate/40% hydroxyapatite [β-TCP/HA]) has been introduced. Novel materials ought to be extensively tested for biocompatibility and osteogenic properties prior to clinical application. To determine the biocompatibility and the osteoinductive and osteoconductive effects of this new BSM, osteoprogenitor cells (bone marrow stromal cells [BMSC]) were cultured in its presence. In addition, the expression patterns of genes related to osteodifferentiation were analyzed. Although the studied material is a traditional β-TCP/HA, it is novel in its ringlike configuration and its microcribrose structure in its thickness. Therefore, the in vitro adhesion of SAOS-2 osteoblastlike cells was evaluated via scanning electron microscopy (SEM) to confirm the ability of the selected BSM to allow bone formation. Finally, BSM+BMSC tissue bioconstructs were implanted to evaluate bone formation through histology and microCT.

MATERIALS AND METHODS

In Vitro Culturing of SAOS-2 Cells on the BSM

Early cellular adhesion onto the BSM was studied with SEM at 6, 24, and 72 hours. The selected synthetic bone graft material (BonePlus Eagle Eye, MegaGen) comes in particles with a doughnut-like shape with a diameter of 1 mm; it is made of synthetic β-TCP/HA composite (60%/40%) (Fig 1). Each particle presents with a cribrose structure made of interconnected channels with an average diameter of 50 µm that open on the external surface. SAOS-2 osteoblastlike cells (5 × 104 cells/well) were cultured at 37°C and 100% humidity with 5% carbon dioxide in standard medium (Coon's modified Ham's F12 supplemented with 10% fetal calf serum, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin) in 24-well plates in which 1 mg per well of the BSM had been placed. After the selected time periods, cells were fixed for 20 minutes in buffered 4% glutaraldehyde/0.2 mol/L sodium cacodylate solution at 4°C, dehydrated in a graded series of alcohols (70%, 80%, 95%, and 100% for 20 minutes each), dried, and gold sputtered (Sputter Coater, SPI). Samples were observed at 2,000× magnification with SEM (JEOL 5200, JEOL).

Table 1 SYBR Green Quantitative PCR Pairs of Primers for Selected Genes Related to Osteogenic Differentiation

Target gene	Forward sequence	Reverse sequence
Alkaline phosphatase	GGGAACGAGGTCACCTCCAT	TGGTCACAATGCCCACAGAT
Bone sialoprotein	GCCTGCTTCCTCACTCCAGG	TTCCCAAAATGCTGAGCAAAA
Type I collagen	CAGCCGCTTCACCTACAGC	TTTTGTATTCAATCACTGTCTTGCC
Osteocalcin	CGGTGCAGAGTCCAGCAAA	TCTCTTCACTACCTCGCTGCC

Two-dimensional BMSC/BSM Cultures

BMSCs were isolated from bone marrow aspirates from healthy donors after ethical committee approval, according to previously described methods.[22] A preliminary cell expansion phase was carried out in standard culture medium (Coon's modified Ham's F12 supplemented with 10% fetal calf serum, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin) supplemented with fibroblast growth factor-2 (1 ng/mL) to confirm comparable numbers of colony-forming units.

Tubes containing preweighed 1-mg aliquots of the BSM were sterilized with a 25-KGy dose of gamma irradiation. One aliquot was layered aseptically in each well of a 24-well plate. BMSC cultures were supplemented with fresh osteogenic medium every 3 days for 4 weeks. Monolayer cultures were established with 5 × 104 cells/well. Positive control cells were supplied with osteogenic medium in the absence of BSM, whereas negative control cells were cultured with standard medium and without BSM. Each experiment was repeated three times. Cultures were further processed for gene expression analysis at 2 and 4 weeks.

Evaluation of Gene Expression

Total RNA was isolated from cultured cells using Trizol (Gibco) according to the manufacturer's protocol. Complementary DNA was synthesized from 1 µg of total RNA using SuperScript II Reverse-Transcriptase reagents following the manufacturer's protocol (18064-014, Invitrogen).

Genetic expression of mRNA was analyzed and normalized to the housekeeping gene by quantitative real-time polymerase chain reaction (PCR) (ABI PRISM 7900 Sequence Detection System, Applied Biosystems) with primer pairs specific for four osteogenic genes coding for osteogenic protein: alkaline phosphatase, bone sialoprotein, type I collagen, and osteocalcin (Table 1). This was done with Primer Express (version 1.5, Applied Biosystems) with sequences spanning separate exons to prevent random genomic DNA amplification. Thermal cycling was done as follows: 10 minutes at 95°C, 20 minutes at 95°C, and 1 minute at 60°C; this was repeated for 40 cycles with a final melting curves analysis. Raw data counts were collected in a digital spreadsheet, and relative gene expression was determined after normalization to glyceraldehyde phosphate dehydrogenase (GAPDH). Linear regression of standard curves and the *t* test were calculated using SPSS version 13.0 (SPSS, IBM). Differences of $P < .01$ were considered statistically significant.

Implantation of BMSC/BSM Constructs

The osteogenic potential of the BSM was evaluated through an in vivo ectopic/unloaded bone formation experiment.[23] Sheep BMSCs were previously expanded for 1 week in standard medium that also included fibroblast growth factor-2, then trypsinized (0.05% trypsin/0.01% ethylenediaminetetraacetic acid) when confluent, collected from petri dishes, washed in serum-free medium, and resuspended at a concentration of 2 × 106 cells/30 µL of Tissucol (human fibrin glue, Baxter). The cells, after being resuspended in Tissucol, were seeded onto the BSM (50 mg). After 20 minutes, 20 µL of thrombin (Zimotrombina Baldacci) were added to polymerize the fibrin. Six tissue-engineered constructs were implanted subcutaneously in the dorsum of anesthetized CD-1 nu/nu mice (Charles River Laboratories). The mice were sacrificed after 1 and 2 months and implanted constructs were retrieved and processed for hematoxylin and eosin histologic examination. Implants were fixed with 4% formaldehyde in phosphate-buffered saline for 3 hours at 4°C, decalcified in Osteodec (Bio Optica), and embedded in paraffin. Four-micron sections were cut and stained with hematoxylin-eosin and observed under transmitted light to observe bone formation. Histomorphometric analysis was conducted at the aforementioned time points (1 and 2 months) to evaluate the formation of new bone. Tracing imaging software was utilized, which had been calibrated with the photomicrograph scale bars (Image J, US National Institutes of Health). One sample was also analyzed, prior to histologic analysis, with microCT with synchrotron radiation at the SYRMEP beamline of Elettra, Trieste, Italy, to determine the internal structure of the BSM.

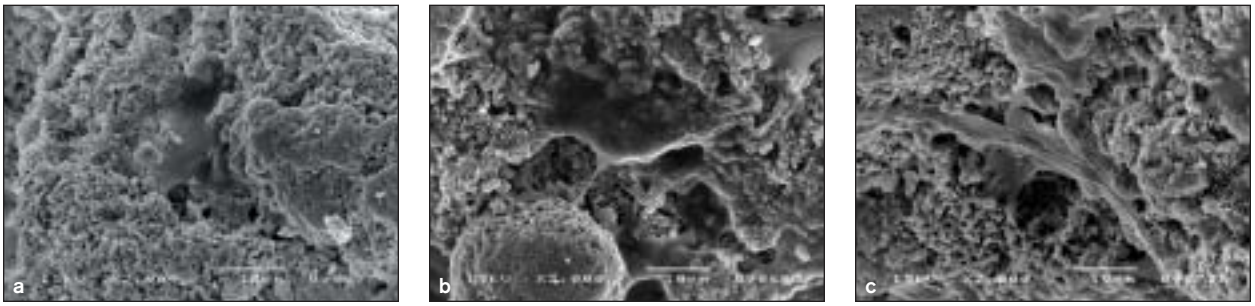
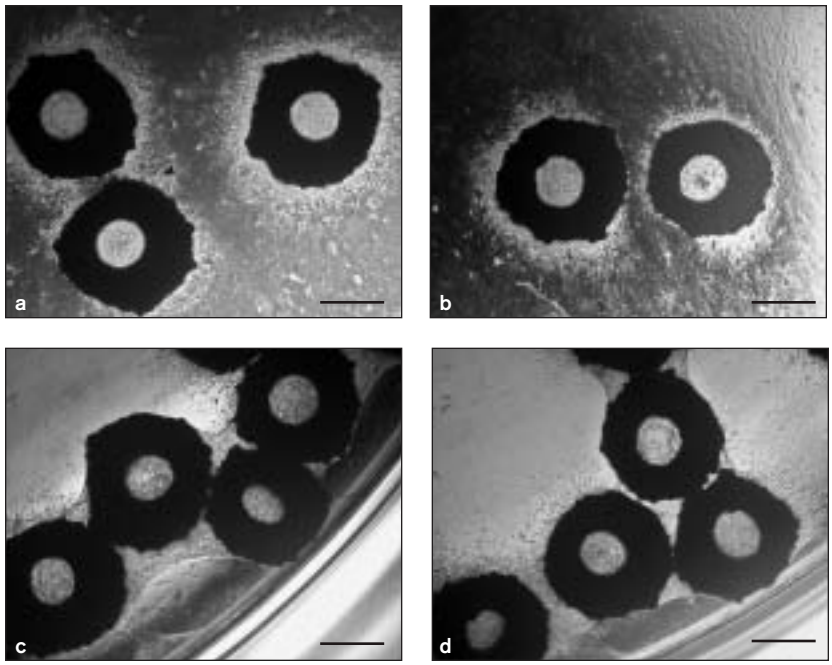


Fig 2 Microphotographs (magnification ×2,000) showing early adhesion after *(a)* 6, *(b)* 24, and *(c)* 72 hours of osteoblastlike cells (SAOS-2) onto synthetic β-TCP/HA composite bone substitute. Cellular adhesion was already evident at 6 hours. Subsequently, the cellular morphology improved, with a starlike spreading morphology developing after 72 hours, as is typical of mature osteoblastic cells.

Fig 3 Optical microscope microphotographs (scale bar = 500 μm) showing two-dimensional BMSC/BSM cultures at *(a and b)* 14 and *(c and d)* 28 days. The cellular monolayers remained intact throughout the experiment. Extracellular matrix was observed adjacent to the BSM particles.



RESULTS

Attachment of SAOS-2 Cells to BSMs

The bone substitute particles (Fig 1) represented a suitable substrate for osteoblast adhesion after 6 hours. For bioceramics, cellular adhesion capacity is a desirable characteristic, since it is needed to allow further osteoblast colonization of implanted particles and formation of new bone. The SAOS-2 osteoblast-like cells developed during the attachment phase from a round shape to a more spread-out shape with a starlike appearance (Fig 2).[24]

Two-dimensional BMSC/BSM Cultures

Cells were successfully cultured in monolayers throughout the experimental period (14 and 28 days) in the presence of the BSM. The cells reached confluency and their phenotype progressed, particularly close to the surface of the BSM, toward an organized matrix (Fig 3).

Gene Expression Patterns

Quantitative real-time PCR determined the expression of genes related to osteogenic differentiation of the BMSC. BMSCs cultured in the presence of the β-TCP/HA synthetic composite ceramic showed up-regulation in the expression of alkaline phosphatase and osteocalcin throughout the in vitro experiment, with a significantly higher peak of osteocalcin expression at day 28 for the BSM experimental group, as compared to the positive and negative controls ($P < .01$) (Figs 4a to 4d). Bone sialoprotein and type I collagen showed earlier peaks in expression at day 14, in agreement with their role as early osteogenic markers, compared with osteocalcin.

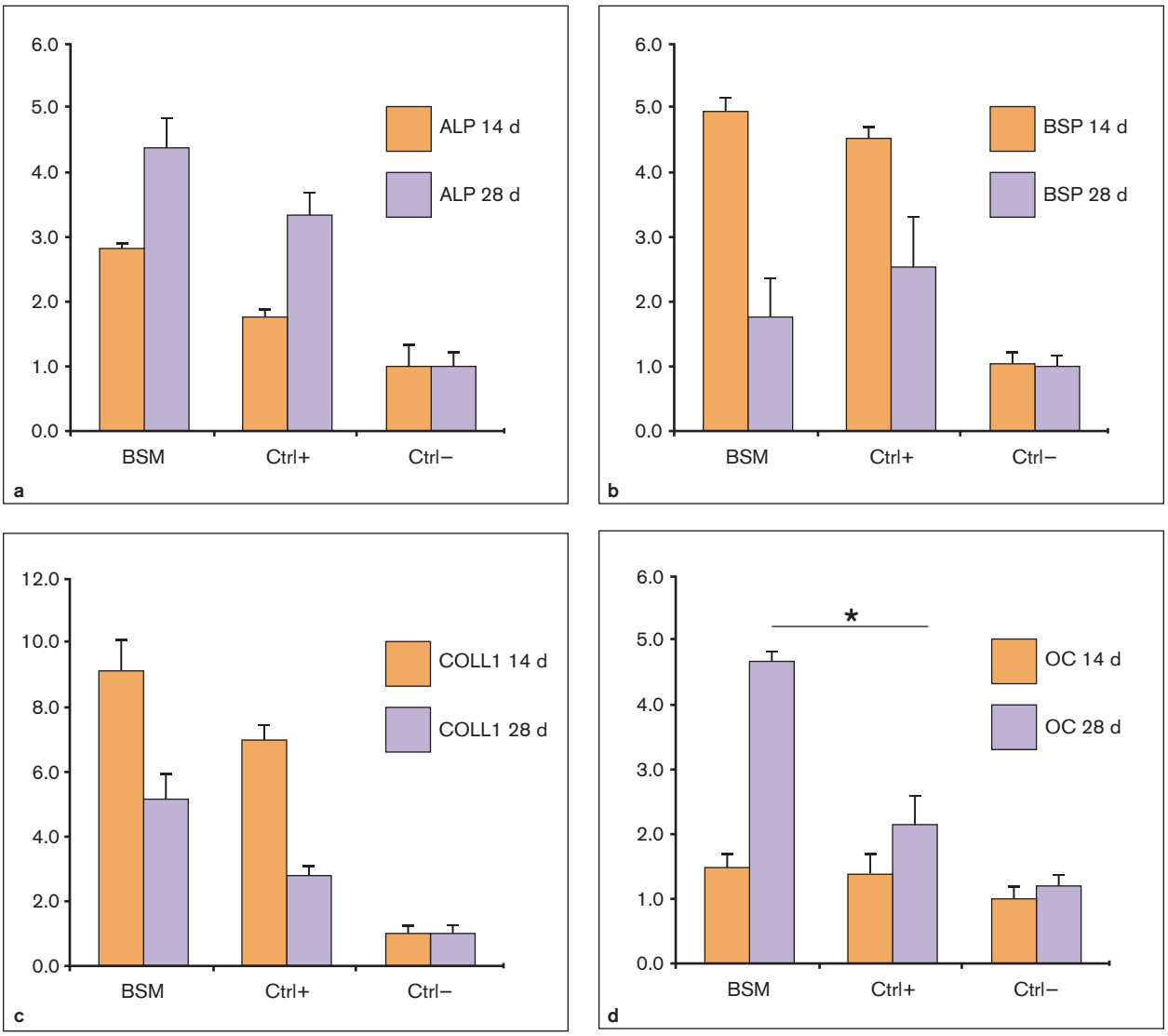


Fig 4 Relative expression, normalized to GAPDH, of four genes representative for osteogenic differentiation at day 14 and 28 of bone marrow stromal cells cultured in the presence of a synthetic bone substitute with osteogenic medium. *(a)* Alkaline phosphatase, *(b)* bone sialoprotein, *(c)* type I collagen, and *(d)* osteocalcin. Osteocalcin was expressed significantly more in the presence of the BSM compared to the positive control (*$P < .01$). Positive control cultures were supplemented with osteogenic medium but grown in the absence of the BSM material. The negative control received neither osteogenic medium nor the BSM.

In Vivo Findings of BMSC/BSM Constructs

Tissue-engineered constructs were implanted in vivo to mimic the biologic behavior of implanted BSM particles. Newly formed bone was observed on the BSM surface and partially bridging the separate particles.

Two months after implantation, the amount of new bone deposition was similar to the quantity observed after 1 month (Figs 5a to 5d). Gaps and voids between the particles and around the implanted tissue-engineered constructs had been filled by cells. Histomorphometric analysis demonstrated, on average, an increase in the active bone-forming surface of 18% between 4 and 8 weeks. Bone deposition involved both the external surface of the BSM and the main central pore. MicroCT slices showed the inner structures and the microchannels branching into the core of the BSM (Fig 6).

DISCUSSION

The search for a synthetic material endowed with osteoinductive and osteoconductive properties is of paramount importance for bone augmentation and repair in oral surgery. Autologous bone remains the gold standard in GBR procedures.[25] Nevertheless, autologous sources may be scarce,

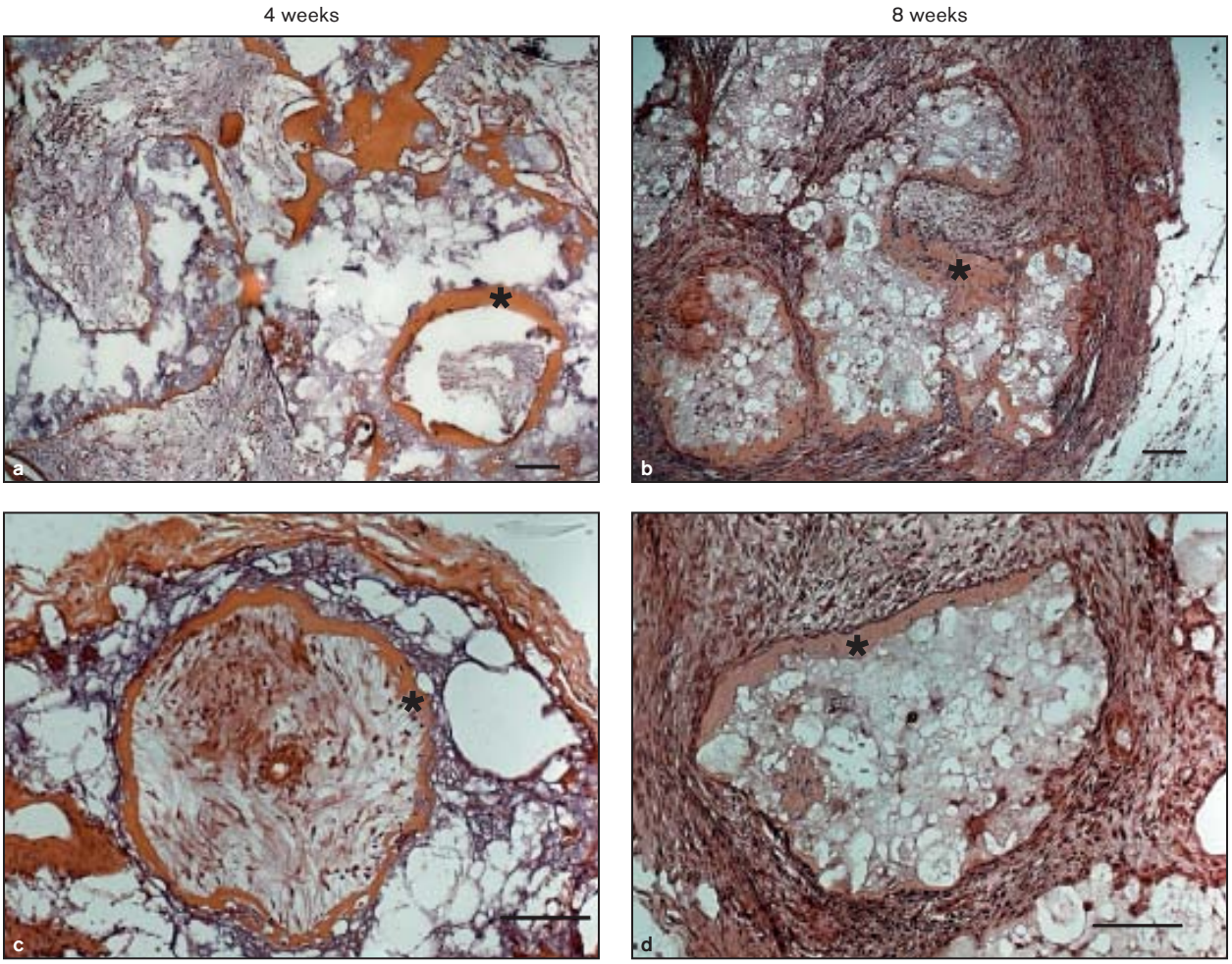


Fig 5 The BSM/BMSC tissue-engineered construct was implanted in vivo to determine new bone formation at *(left column)* 4 weeks and *(right column)* 8 weeks. Histologic sections were stained with hematoxylin-eosin. Newly formed bone *(asterisk)* was detectable already at 1 month after implantation in dorsal murine pouch. Scale bar = 100 μm.

and their harvesting may lead to donor site morbidity.[26] Heterologous bone sources, particularly those of animal origin, pose risks of prion and viral cross-infection, despite standard sterilization procedures.[27] Furthermore, intense gamma irradiation may affect the properties of these natural bone–derived materials.[28] The development of synthetic ceramics may overcome these limitations and concerns.

In the past, these synthetic BSMs were proven to be inferior to other bone grafting materials.[29] Continuous modifications and improvements in composition and structure led to a significant finding of osteoconductive/osteoinductive properties. Early ceramics were characterized by a solid structure, and they used a single component (HA). The desire to provide a scaffolding material that can provide controlled resorption coupled with a wave of newly formed bone led to modifications of the chemical composition of bone substitutes. It was found that pure HA did not resorb, instead remaining unmodified in the implanted area.[18]

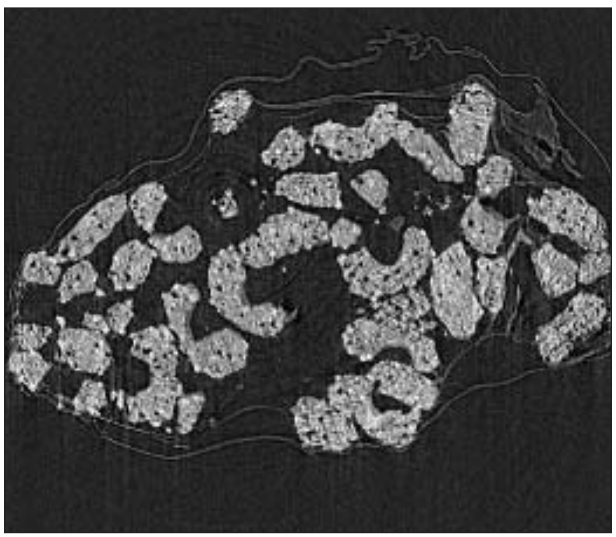


Fig 6 BSM slice as seen with microCT with synchrotron radiation. High-resolution tomography shows the inner structure of the BSM; channels branch in the core of the material and pores are present on the surface.

For this reason, β-TCP was added to the HA to induce progressive resorption.[30] Also, the microstructure of scaffolds has been improved through the creation of bonelike three-dimensional characteristics; channels and microporosities were developed to allow tissue ingrowth and facilitate nutrient supply in the core of the material filled with cells.[31]

Although the use of biomaterials is still controversial in self-contained defects to improve the osseointegration of titanium implants,[32] cellular seeding of scaffolds may provide further enhancement. A tissue-engineering approach could further enhance new bone formation, providing a direct cellular supply to the implanted bioconstruct rather than relying solely on the host cellular supply.[18]

In the present study, cells were successfully cultured in the presence of the BSM. The selected synthetic BSM was biocompatible, and it allowed survival of a single layer of cells for an experimental time of 4 weeks. The development of an organized extracellular matrix around the surface of the BSM particles represents the prelude to mineralized matrix deposition.[33]

In the present study, cells of different origin were employed. This approach may hinder the comparison of the results between different studies; however, the selected cell lines have been previously referred to in the literature as benchmarks for selected tests involving early attachment (SAOS-2), gene expression (hBMSC), and swift in vivo bone deposition (ovine BMSC).[18]

Gene expression analysis through quantitative real-time PCR revealed that the investigated BSM exerted a direct osteoinductive effect compared to a positive control culture treated with osteogenic medium; ie, osteocalcin, which is related to late ossification, was up-regulated in the experimental cultures cultivated in the presence of the BSM.

Further investigations must determine the loading resistance of bone-engrafted material. Microhardness tests would show whether the newly formed bone is hard as native bone.[34] Possibly the addition of BMSC to a scaffolding material would produce stiffer bone compared to an acellular scaffold.[35]

Also, recent studies have proposed longer follow-up periods to evaluate the resorption and rearrangement of implanted tissue constructs. Apparently, β-TCP/HA may undergo further modification after 6 months as a result of its slower resorption rate compared to pure β-TCP ceramic. A recent study suggests that cells seeded on the scaffold may survive for as long as 11 weeks after implantation in the host.[36]

The next challenge would be to develop a bioactive scaffold that is able to release drugs and target osteoprogenitors to be induced on the molecular level.[37] A recent study evaluated a modified tissue construct, which consisted of a biodegradable membrane rolled around a cell-loaded scaffold, to establish a vessel bundle to obtain vascularized bone with a good blood supply.[38]

Tissue-engineered constructs made of BMSC-seeded natural composite scaffold represent a promising possibility for dental implant anchorage and might be useful for clinical jaw reconstruction as well.[39] Two recent studies determined that the addition of platelet-rich plasma to BMSC/HA scaffolds in jaw defects and augmented sinuses led to enhanced bone formation after 3 months.[40,41] Fractions of platelet-rich plasma, such as platelet lysate, may be even more effective in inducing BMSC, thereby replacing the use of animal-derived sera in culture medium.[42]

Future applications of tissue engineering to oral surgeries and bone augmentation procedures look promising and may provide enhancements of osteoinductive/osteoconductive properties of β-TCP/HA composite ceramic.

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