**Molecular and structural patterns of bone regeneration in surgically
created defects containing bone substitutes**

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Abstract

Several biomaterials have been introduced for bone augmentation. However, information is lacking about the mechanisms of bone regeneration and/or integration of these materials in the recipient bone. This study aimed to determine the molecular and structural events in bone defects after augmentation with synthetic tetrapod-shaped calcium phosphate (Tetrabone; TetraB) compared with natural deproteinized bovine bone (DBB). Defects were created in the epiphyses of rat femurs and filled with TetraB or DBB or left empty (Sham). After 3, 6, 14 and 28d, samples were harvested for histology, histomorphometry, ultrastructure and gene expression analyses. At 3d, higher expressions of bone formation (ALP and OC) and remodeling (CatK) genes were detected in TetraB compared with DBB and Sham. Downregulation of bone remodeling genes (TRAP and CatK) was detected in DBB as compared to Sham after 14d. Histomorphometry at 6 and 14d demonstrated greater bone contact with the granules in TetraB. At 28d, a larger bone area per defect was found in TetraB. The present experiments show that a synthetic substitute, consisting of α -tricalcium and octacalcium phosphates, induces early osteogenic and osteoclastic activities and promotes bone formation in trabecular bone defects.

Keywords: α -tricalcium phosphate; octacalcium phosphate, deproteinized bovine bone, gene expression; in vivo; ultrastructure

Introduction

Over the last few years, the need for bone augmentation in skeletal surgical operations has increased. The application of bone grafts to promote bone regeneration has become a routine procedure, especially when the endogenous regenerative mechanisms are insufficient to achieve complete healing, e.g. large bone defects. In clinical practice, autografts have been regarded as a golden standard for bone repair and substitution, followed by allografts, where both have great osteoinductive properties. However, problems related to limitations, morbidity, rejection and/or infections are still drawbacks [1, 2]. Many efforts have been made to develop synthetic grafting materials as an alternative option for bone substitution.

Ceramic materials, based on calcium phosphate, are the most developed and clinically used materials, given their relative biocompatibility and similarity to the bone mineral composition. The state-of-the-art material is a natural deproteinized bovine bone (DBB) with good experimental and clinical documentation and follow-up [3-5]. On the other hand, several synthetic forms, showing variable biological behaviors and bone tissue response, have been suggested [6, 7]. One example of synthetic materials is a recently developed bone substitute consisting of alpha-tricalcium phosphate (α -TCP) and developed as tetrapod-shaped granules (Tetrabone; TetraB) [8]. The granules underwent additional treatment to recrystallize their surfaces into octacalcium phosphate (OCP) and, when implanted *in vivo*, promoted greater bone formation in comparison to beta-tricalcium phosphate [8]. Whereas data are available on the overall bone formation in conjunction with natural and synthetic substitutes, the molecular mechanisms evoked by these materials, and their relationship to the degree of bone formation, have not been characterized.

1 Bone healing and regeneration is a complex sequence of events that involves the
2 modulation of the local microenvironment with cytokines and growth factors, as well
3 as the recruitment and differentiation of different cell populations [9]. Inflammatory
4 cytokines, such as tumor necrosis factor-alpha (TNF- α) and interleukin-1 beta (IL-
5 1 β), are integral molecules during the early phase of bone healing and may positively
6 or negatively affect the regeneration process [10]. Osteogenic differentiation, on the
7 other hand, is determined with specific differentiation molecules, such as alkaline
8 phosphatase (ALP) and osteocalcin (OC). Other important biological molecules are
9 those involved in bone remodeling, such as cathepsin K (CatK) and tartrate-resistant
10 acid phosphatase (TRAP), which are important for bone maturation. It is hypothesized
11 that, during defect augmentation, these events are influenced by the presence, and the
12 properties, of the substitute, which may in turn affect the overall bone formation and
13 its structural and ultrastructural characteristics. In addition, it is also possible that
14 biological processes, such as bone remodeling, influence the substitute material, e.g.
15 persistence or degradation. Nevertheless, to the authors' knowledge, these hypotheses
16 have not yet been characterized *in vivo*. The main questions to be addressed are (i)
17 How do the different bone substitute materials influence the temporo-spatial gene
18 expression in cells in defects in comparison to unaugmented defects? (ii) What is the
19 relationship between molecular activities and the amount and structure of the newly
20 formed bone in response to the different substitutes? (iii) Does the expression of
21 genes denoting inflammation, bone formation and bone remodeling correlate and
22 coordinate in specific ways with and without the substitute material? Furthermore,
23 information about osteoclastic activities during bone augmentation may cast some
24 light on the controversy relating to the biodegradation and/or resorbability of natural
25 bovine and synthetic substitutes.

1 The general aim of the present study was to compare the differences in biological
2 response between synthetic α -tricalcium and octacalcium substitute and the clinical
3 golden standard, deproteinized bovine bone substitute. The following specific aims
4 were formulated: (i) to investigate the spatial and temporal pattern of gene expression
5 regulating inflammation, bone formation and remodeling in surgically created defects
6 with and without augmentation with natural or synthetic bone substitutes and (ii) to
7 relate the molecular patterns associated with the natural and synthetic substitutes and
8 unaugmented defects to the morphology and ultrastructure of the regenerated bone.
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10 **Materials and Methods**

11 ***Material preparation***

12 The fabrication process of tetrapod-shaped synthetic bone substitute has been
13 described in a previous study [8]. In short, the binder, a mixture of olefin resin (55%),
14 wax (30%) and plasticizing materials (15%), was prepared. α -TCP powder (Taihei
15 Chemical Industrial Co., Tokyo, Japan; 60%) and binder (40%) were mixed for 30
16 min and the α -TCP compound was injected into tetrapod-shaped injection molds with
17 a size of 1 mm using an injection-molding machine (J35AD; Japan Steel Works,
18 Tokyo, Japan). Molded α -TCP compounds were degreased at 500°C for 1h and
19 calcined at 700°C for 1h. They were soaked in 0.2 M succinic acid for 24h to form
20 OCP and rinsed twice with distilled water. They were dried under reduced pressure
21 and sterilized by electron beam irradiation at 25 kGy.

22 The natural substitute was purchased as anorganic deproteinized bovine bone with a
23 particle size of 250-1000 μ m (Bio-Oss; Geistlich Pharma, Wolhusen, Switzerland).
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25 ***Material characterization***

1 For X-ray diffraction analysis (XRD), TetraB and DBB were ground into powder and
2 analyzed using a Bruker D8 X-ray diffractometer (Bruker, Karlsruhe, Germany) with
3 a CuK α radiation source at 40kV and 40 mA, scanning from 3 to 80°.

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7 To evaluate the material shape and surface morphology, scanning electron
8 microscopy (Leo Ultra 55 FEG SEM) was performed operating at 5 kV using a
9 secondary electron detector. Magnifications between 50 and 100,000 times were used
10 to assess the macroscopic geometry of the materials and the micron and submicron
11 surface morphology.
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19 *Animal surgery*

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21 The animal experiments were approved by the University of Gothenburg, local ethics
22 committee for laboratory animals (Dnr 301-2009). A total of 84 two-month-old
23 female Sprague-Dawley rats underwent surgery in the present study. All the rats were
24 allowed a one-week acclimatization period and free movement with food and water
25 *ad libitum* before surgery. At surgery, the animals were anesthetized using an
26 isofluorane (Isoba Vet, Schering-Plough, Uxbridge, UK) gas inhalation with a
27 Univentor 400 anesthesia unit (Univentor, Zejtun, Malta). Anesthesia was maintained
28 by the continuous administration of isofluorane via a mask. The distal aspect of the
29 femur was cleaned with 5 mg/mL chlorhexidine and 70% ethanol. Subsequently, the
30 site was shaved and a longitudinal incision was made, followed by skin and periosteal
31 reflection with a blunt instrument. A defect was made in each femoral epiphysis
32 (trabecular bone region) using a trephine with a 2.3 mm internal diameter and 3 mm
33 penetration depth under profuse irrigation with NaCl 0.9%. Each defect was filled
34 with either tetrapod-shaped bone substitute (TetraB) or deproteinized bovine bone
35 substitute (DBB) or left empty (Sham). Substitute and Sham sites were randomized,
36 ensuring equal distribution and rotation among the animals and between right and left
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locations. The granules from either type were installed one by one in each defect until the defect was completely filled. The subcutaneous layer of the wound was closed with resorbable polyglactin sutures (4-0, Vicryl) and the skin was closed with intracutaneous, resorbable monocryl sutures (4-0). Postoperatively, each rat received an analgesic (Temgesic 0.03 mg/kg, Reckitt & Coleman, Hull, UK) by subcutaneous injection. The retrieval procedure was performed at 3, 6, 14 and 28d for gene expression, histology, histomorphometry and ultrastructural analyses. Animals were sacrificed using an overdose of barbiturate (Mebumal, ACO Läkemedel AB, Solna, Sweden). The skin was reopened and the bone defects filled with the TetraB or DBB or the unfilled Sham ones were identified. Sites intended for gene expression analysis were retrieved using a 2.3 mm trephine. The samples were immediately preserved in tubes containing RNAlater (n=8). Sites designated for histology and ultrastructure were harvested *en bloc* using dental disc. The harvested samples were then immersed in formalin (n = 6).

Quantitative polymerase chain reaction

Total RNA from the trephine-retrieved samples was extracted using an RNeasy® Mini kit (QIAGEN GmbH, Hilden, Germany). Prior to extraction, samples were homogenized in phenol/guanidine-based Qiazol lysis reagent using 5 mm stainless steel beads (QIAGEN GmbH, Hilden, Germany) and TissueLyser (QIAGEN GmbH, Hilden, Germany). Following the addition of chloroform, the samples were centrifuged at 12,000 g for 15 min and the aqueous phase was used for subsequent RNA extraction. DNase treatment was performed in order to eliminate any contamination by genomic DNA. Reverse transcription was carried out using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, USA). RNA was normalized to 50 ng/μl before the reverse transcription. The design of primers for six target genes,

tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), alkaline phosphatase (ALP), osteocalcin (OC), tartrate resistant acid phosphatase (TRAP) and cathepsin K (CatK), as well as five putative reference genes, was performed using the Primer3 web-based software [11]. The expression profiles of reference genes were evaluated by geNorm [12] and Normfinder [13] software, in order to determine the best reference gene for normalization. Design parameters were adjusted to minimize the formation of artifact products and to be able to use an annealing temperature in the PCR of about 60°C. Primers were designed to yield short amplicons (preferably shorter than 200 bp) and to function well with SYBR Green I fluorescent dye for the detection of the PCR products in real time. Real-time PCR was performed in duplicate using the Mastercycler ep realplex (Eppendorf, Hamburg, Germany) in 10 μ l reactions. The cycling conditions were 95°C for 10 min, followed by 45 cycles of 95°C for 20 s, 60°C for 20 s and 72°C for 20 s. The fluorescence was read at the end of the 72°C step. Melting curves were recorded after the run by a stepwise temperature increase (1°C/5s) from 65 to 95°C. Both geNorm and Normfinder identified the genes hypoxanthine phosphoribosyltransferase 1 (HPRT1) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ) as the most stable ones in the present experimental study. Quantities of target genes were normalized using the mean Ct values of HPRT1 and YWHAZ. The normalized relative quantities were calculated using the delta Ct method and 90% PCR efficiency ($k \cdot 1.9^{\Delta Ct}$) [14].

Histology and histomorphometry

After fixation, the blocks were dehydrated in ascending series of ethanol, followed by embedding in acrylic resin (LR White) (London Resin Company Ltd, Berkshire, UK). The embedded specimens were cut along the long axis of the defect using a diamond

1 saw. Subsequently, one half of each specimen was used to produce ground sections
2 prepared by sawing and grinding (Exakt Apparatebau GmbH & Co, Norderstedt,
3 Germany). The sections were made with a thickness of 10-20 µm and stained with 1%
4 toluidine blue. The sections were then coded and evaluated blindly under a light
5 microscope (Nikon Eclipse E600) for histological and histomorphometric analyses.
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7 The qualitative histological evaluation was made in an optical microscope (Nikon
8 Eclipse E600; Nikon Ltd., Tokyo, Japan), using image software (ACT-1; Nikon Ltd.,
9 Tokyo, Japan). A quantitative histomorphometry analysis was performed in the same
10 microscope using analytical software (Easy Image Measurement 2000; Technooptik
11 AB, Huddinge, Sweden).

12 The histomorphometric analysis was performed using 10x objective. In each defect,
13 the area percentages of granules (TetraB or DBB), as well as the newly formed bone
14 (TetraB, DBB or Sham), were calculated with respect to the total defect area.
15 Furthermore, the percentage of bone-granule contact (TetraB or DBB) was calculated
16 with respect to the total circumference of the individual granules. This was achieved
17 by performing the measurement using a software grid consisting of nine zones, which
18 covered the total area of defect. The area of the newly formed bone, the area of
19 granules or the bone-granule contact were separately traced in each zone and
20 percentages of the areas (bone or granules) and the contacts were then calculated from
21 the sum of the zones. The discrimination of the newly formed bone from the natural
22 bovine bone substitute was judged by the absence of osteons/Haversian system, the
23 relatively darker staining and the presence of relatively round and viable osteocytes.
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58 *Ultrastructural analysis*

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1 The other halves of the specimens were prepared for electron microscopy. The cut
2 side was polished and the specimen was glued to a regular SEM stub and
3 subsequently coated with a 10 nm thick conductive coating (AuPd). Back-scatter
4 SEM (BS-SEM) was performed in a Leo Ultra 55 FEG SEM (blab la) operating at 20
5 kV. A quadrapole detector using Compo mode was used to obtain optimal Z-contrast.
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7 Magnifications between 50 and 50,000 were used to evaluate the overview bone
8 response and resolve the interfacial reactions over time. For the 28d time point,
9 transmission electron microscopy (TEM) samples across the material-bone interface
10 were prepared using focused ion beam (FIB, FEI Strata DB235) *in-situ* lift out [15].
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12 The samples were thinned to a thickness of approximately 100 nm using a decreasing
13 ion beam current. TEM analyses were performed in a FEI Tecnai TEM/STEM, using
14 both bright-field TEM and high-angle annular dark-field STEM (HAADF-STEM).
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29 ***Statistics***

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31 For histomorphometry and gene expression analyses, statistical comparisons were
32 made between the three groups (Sham, DBB and TetraB) and between the time
33 periods for each group. A non-parametric Kruskal-Wallis test was used to identify
34 statistical differences between the groups or time periods. Whenever a statistical
35 difference was found, the Mann-Whitney test was used to determine the statistical
36 difference between two groups. Spearman's correlation analysis was performed
37 between the expression levels of the analyzed genes in each defect type and at the 6,
38 14 and 28d time points. Analyses were made using SPSS Version 10 software (SPSS,
39 Inc., Chicago, USA) and the significance was set at $p < 0.05$. The data are presented
40 as the mean $\pm$ standard error of the mean.
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60 **Results**

Material characterization

The SEM evaluation of the materials showed that the DBB had an irregular shape, with dimensions varying between a few hundred μm to one mm (Fig. 1A), while the TetraB showed a highly ordered four-legged shape, with dimensions of the struts of a couple of hundred μm in diameter and a length of 600 μm , creating a global size of one mm (Fig. 1B). At higher resolution, smaller granules, 5-20 μm in size, were present on the DBB surface (Fig. 1C), which was otherwise characterized as a solid material on this length scale. At similar magnification, TetraB also showed a solid material with a surface composed of flake-like structures ranging in size from 5-10 μm (Fig. 1D). At high resolution (Fig. 1E, F), a rougher surface was observed for both materials; the DBB was composed of aggregates of particles ranging in size from 400-500 nm, with individual particle sizes of less than 100 nm in the smallest dimension. The TetraB showed a porous surface between the unordered flake structure. The dimensions of the flakes showed a smallest dimension of approximately 100 nm, with fairly sharp edges.

The XRD analysis (Fig. 2) revealed that the prominent phase of DBB was mainly hydroxyapatite, whereas TetraB revealed several α -TCP peaks and, to a lesser extent, peaks that correspond to OCP and hydroxyapatite.

Histology and histomorphometry

At 6d, both DBB and TetraB granules appeared well contained within the created defect boundaries (Fig. 3B, C). At this time point, there was no major evident bone formation in the central region of any of the defect groups (Fig. 3A, B, C). The central regions were mainly occupied by soft tissue in the Sham defects (Fig. 3A), whereas the augmented defects were mainly occupied by the DBB (Fig. 3B) or TetraB (Fig. 3C) granules. Peripherally, newly formed osteoid was observed in all defect types. No

major inflammatory infiltrates were detected in any of the defects. Evaluation at higher magnification (Fig. 4) showed that osteoid was formed in association with cells in the intergranular space. Two major observations distinguished the DBB and TetraB defects from one another. Firstly, the osteoid in the TetraB group (Fig. 4D, E, F) revealed greater proximity and contact with the granule surfaces than that formed in the DBB group (Fig. 4A, B, C). Secondly, areas of partially mineralized bone, indicated by slightly lighter staining than the osteoid, were mainly detected in the TetraB-treated defects (Fig. 4D). Osteoblast seams were commonly observed adjacent to the osteoid areas in all defect groups. Histomorphometry at 6d revealed no major difference in the area percentage of the newly formed bone between the three groups, which ranged between 9-11% of the total defect area (Fig. 7). On the other hand, the analysis revealed a higher percentage of bone-granule contact in the TetraB (37.4 ± 10.7) compared with the DBB (5 ± 1.8 ; $p < 0.05$) (Fig. 7). Measurements of the area percentages occupied by the granules of each material revealed slightly higher values in the TetraB group ($35.4\% \pm 1.3$) compared with the equivalent for DBB (28 ± 4.5), but the difference was not statistically significant.

At 14d, a substantial amount of bone had formed, extending from the periphery of the defect and filling the central region in all groups (Fig. 3D, E, F). In the augmented sites (Fig. 3E, F), the newly formed bone displayed a trabecular pattern and appeared to fill the intergranular spaces. In comparison with 6d, a higher degree of mineralization of the newly formed bone was observed for all groups, as judged by the decreased intensity of the toluidine blue staining and the presence of osteocytes (Fig. 5). Moreover, as judged by the staining, the newly formed bone in the TetraB group appeared more mineralized than that observed in the DBB group (Fig. 5A, E). In the TetraB group, the newly formed bone showed a greater degree of contact with

1 the granule surface than that in the DBB (Fig. 5 B, F). Furthermore, the interface
2 between the newly formed bone and the TetraB granules frequently revealed a blade-
3 like appearance (Fig. 5 H). Osteoblast seams and osteoclast-like cells were frequently
4 detected, in many cases in connection with the granule surfaces of either material
5 (Fig. 5 C, D, G, I). In addition, surface depressions and irregularities, indicating
6 resorption areas, were frequently detected in association with the osteoclast-like cells.
7 These resorption areas were observed on the surfaces of both bone and granules in the
8 TetraB defects (Fig. 5 G), whereas a similar finding was only made for bone surfaces
9 in the DBB defects (Fig. 5 C). In comparison with 6d, histomorphometry revealed a
10 significant increase in the proportion of newly formed bone for all defect types (Fig.
11 7). In this period, albeit not statistically significant, Sham sites revealed a slightly
12 higher bone area percentage ($31.8\% \pm 2.2$) compared with DBB (24.6 ± 1) and TetraB
13 (26.6 ± 3.4) (Fig. 7). After 14d, the bone contact with the TetraB granules (90%) was
14 significantly higher than that with DBB (60%) (Fig. 7). For both defect types, the
15 percentage of bone-granule contact revealed a significant increase from that observed
16 after 6d. No major difference in the granule area percentages was observed between
17 the TetraB and DBB (31.3 ± 2 and 26 ± 1.5 respectively). No significant differences
18 in granule area percentage between 6d and 14d were detected.

19 At 28d, the bone formed in the Sham site showed considerable resorption, leaving a
20 few trabeculae of mature bone, bordered endosteally by the bone marrow and
21 periosteally by the overlying soft tissue (Fig. 3G). In the Sham defects, the original
22 bone contour was not achieved. The soft tissue on the periosteal side of the Sham
23 defects displayed downgrowth into the defect area (Fig. 3G). In contrast, the
24 augmented sites appeared to maintain a high degree of mature bone after 28d. In both
25 the DBB- and TetraB-augmented defects, the mature bone extended to the top of the

defects, regaining the original level of the bone at these sites (Fig. 3H, I). A larger amount of mature bone appeared to fill the intergranular spaces in the TetraB group compared with the DBB (Fig. 3H, I, Fig. 6A, D). Active remodeling sites were encountered, characterized by newly formed osteoid, osteoblast seams and osteoclast-like cells. This observation was more commonly made in the TetraB group (Fig. 6E, F). Histomorphometric analysis at 28d revealed a significantly higher bone area percentage in TetraB defects (37.5 ± 3.9) compared with the Sham (21.2 ± 4) and the DBB (24 ± 2.2) (Fig. 7). On the other hand, the bone-granule contact was significantly decreased in the TetraB sites and unchanged in the DBB sites, resulting in a 76.7 ± 4.5 and 67 ± 1.5 contact percentage, respectively. In this period, there was no significant difference in the percentage of bone-granule contact between the TetraB and DBB. Like the previous time periods, no major changes in the percentages of the granule areas were detected, in either TetraB (29.3 ± 4) or DBB (27.9 ± 4.6) defects.

Ultrastructure

At the early time points, 3 and 6d, limited mineralized tissue had formed within the defects in the Sham (data not shown) and DBB (Fig. 8A, C) groups. For TetraB, some mineralization had occurred in association with the surface (Fig. 8B, D). After 14d, bone formation around and in direct contact with both materials was evident (Fig. 9). Less mature tissue was observed towards the center of the defect, as judged by less organization and less contrast. Osteocyte lacunae were frequently seen close to both materials. At higher resolution, bone had formed around the flakes on the TetraB surface, but, further into the surface coating, low contrast was observed, indicating a porous zone (Fig 9D). At 28d (Fig. 10), the bone tissue had matured around both materials as compared to the 14d time point. Osteocytes were present close to the

1 materials, at a distance of 2-5 μm from the surface. The bone contoured the surface
2 irregularities and topography, while a porous zone of a couple of μm was still present
3 in the subsurface of the TetraB samples. This was further confirmed by TEM, where a
4 hollow zone around the flakes was present between the bone tissue and the bulk
5 grains of the material (Fig. 10F). The DBB showed intimate contact with bone at all
6 the different resolutions within the study. The collagen fibers ran parallel to the
7 surfaces, indicated by the collagen banding (TetraB; Fig. 10F) or the transversely cut
8 collagen fibers (DBB; Fig. 10E).

19 *Gene expression*

21 Comparisons were made of gene expression in bone samples harvested from the
22 defects after different healing periods. In this way, a selection of targeted genes
23 involved in inflammation, bone formation and bone remodeling was analyzed at 3, 6,
24 14 and 28d after the defect creation with and without augmentation. The results of
25 these gene expression analyses are summarized in Fig. 11. In addition, the
26 relationships between the expressions of the targeted gens in Sham, DBB or TetraB
27 defects, in the different time periods, were evaluated and the data are presented in
28 Table I.

41 *Gene expression of proinflammatory cytokines*

43 At 3d, the expression levels of $\text{TNF-}\alpha$ and $\text{IL-1}\beta$ were downregulated about two-fold
44 in the TetraB sites compared with the Sham, although the differences were not
45 statistically significant. Furthermore, no major difference between DBB and Sham
46 was detected at this time. At 6d, all the defects revealed comparable levels of these
47 proinflammatory cytokines. Later, at 14d, a two-fold lower expression of $\text{IL-1}\beta$ was
48 demonstrated in the DBB in comparison to the Sham ($p < 0.05$) and to the TetraB ($p >$

0.05). Similar to 6d, no major differences between the three groups were detected after 28d with respect to the expression of the proinflammatory cytokines.

The temporal expression pattern of both cytokines revealed high peaks for all groups at 3d. Thereafter, there were general downregulations in all groups from 3d to 6d. The downregulations were statistically significant in all groups with respect to TNF- α , whereas they were only significant in the DBB defects with respect to IL-1 β . From 6d to 14d, there was a slight increase in the expression of TNF- α , mainly in the Sham defects. On the other hand, IL-1 β revealed temporal increases from 6d to 14d in the Sham ($p < 0.05$) and the TetraB ($p > 0.05$) but not in the DBB. No major temporal changes were observed in the expression of both cytokines from 14d to 28d, in all the groups.

Gene expression of bone formation markers

Gene expression analysis at 3d revealed significantly higher levels of ALP and OC in the TetraB group. In this early period, TetraB revealed about a 2-fold higher expression of ALP in comparison to Sham ($p < 0.05$) and to DBB ($p > 0.05$). At the same time, the expression of OC was 3- and 13-fold significantly higher in TetraB compared with Sham and DBB, respectively. Apart from the slightly lower expression of OC in the DBB group at 14d, there were no major differences between the groups at the remaining time points with respect to the osteogenic gene expression.

The temporal expression pattern showed the upregulation of both ALP and OC from 3d to 6d in the Sham and the DBB groups, whereas no significant change was found for TetraB. From 6d to 14d, there was a general upregulation in the expression of both ALP and OC in all the groups and these upregulations were statistically significant in TetraB (for ALP expression) and in Sham and TetraB (for OC expression).

Thereafter, from 14d to 28d, there was a significant downregulation in the expression of ALP in all the groups, whereas the expression of OC did not change significantly.

Gene expression of bone resorption markers

In general, a similar expression profile was observed for TRAP and CatK. At 3d, both osteoclastic genes were expressed at higher levels (1.5-2.5-fold) in TetraB defects compared with Sham and DBB defects. However, these higher levels only attained statistical significance with respect to CatK. Both genes were expressed at similar levels among the three defect types at the 6d time point. At 14d, the expression of CatK and TRAP showed similar levels for Sham and TetraB defects. At this time point, both genes revealed about a 1.5-fold lower expression in DBB defects compared with Sham and TetraB, but the difference was only statistically significant for TRAP expression when compared with Sham. No major differences between the groups were detected at 28d.

The temporal expression of CatK and TRAP revealed a general upregulation from 3d to 6d in all the groups. From 6d to 14d, the temporal expression of both genes was further increased in Sham and TetraB, whereas it was maintained at similar levels in DBB. Thereafter, from 14d to 28d, both genes demonstrated a downregulation in all defect types.

Correlation analysis of gene expression

The results from the correlation analysis of the different genes are presented in Table I.

At 3d, the Sham defects showed a significantly negative ($r = -0.94$; $p = 0.004$) and positive ($r = 0.83$; $p = 0.04$) correlation for TNF- α with OC and TRAP, respectively. On the other hand, in the DBB and TetraB defects, a significant association was only

1 registered as a positive correlation between the osteoclastic/remodeling genes, TRAP
2 and CatK, ($r = 0.83$; $p = 0.04$) and ($r = 0.96$; 0.0004), respectively.
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4 At 6d, a significantly positive correlation between osteoclastic/remodeling genes
5 (TRAP and CatK) was detected in the three defects (Sham, DBB and TetraB). At this
6 time point, TetraB defects revealed significantly positive relationships between the
7 bone formation gene (OC) and the remodeling genes, TRAP and CatK, with
8 correlation coefficients of $r = 0.89$ ($p = 0.006$) and $r = 0.79$ ($p = 0.04$), respectively.
9 Furthermore, the bone formation gene (ALP) in the TetraB defects demonstrated a
10 positive correlation with the inflammatory cytokine (TNF- α) at a level of $r = 0.79$ ($p =$
11 0.04).
12

13 At 14d, a positive correlation between inflammatory cytokines (TNF- α and IL-1 β)
14 was detected in all defect types. At this time, whereas this was the only correlation
15 detected in the Sham defects, there were several correlations between the different
16 genes in the DBB and TetraB defects. In both defects, there was a positive correlation
17 between the osteoclastic/remodeling genes (TRAP and CatK). Moreover, positive
18 correlations were demonstrated for both osteoclastic/remodeling genes (TRAP and
19 CatK) with the inflammatory cytokines (TNF- α and IL-1 β), in both defects. The
20 highest level of significance was registered between IL-1 β and CatK, with $r = 1$ and r
21 $= 0.96$ ($p < 0.0005$) in the DBB and TetraB, respectively.
22

23 At 28d, significantly positive correlations were found between the proinflammatory
24 cytokines (TNF- α and IL-1 β) and between the osteoclastic/remodeling genes (TRAP
25 and CatK) in all defects. At this time, the Sham defects showed two types of
26 significant correlation. The first was a positive correlation for the bone formation
27 gene (ALP) with the inflammatory cytokine (TNF- α , $r = 0.89$; $p = 0.02$) and the
28 second was the positive correlation for the other bone formation gene (OC) with both
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osteoclastic/remodeling genes (TRAP and CatK, $r = 0.89$; $p = 0.02$). In the DBB and TetraB defects, significantly positive correlations were registered between the bone formation genes (ALP and OC) and for the bone formation gene (ALP) with the osteoclastic gene (CatK). In addition, the TetraB defects demonstrated significantly positive correlations for the inflammatory cytokine (TNF- α), with the bone formation gene (ALP, $r = 0.93$; $p = 0.002$) and the osteoclastic/remodeling gene (CatK, $r = 0.79$; $p = 0.04$) and for the other inflammatory cytokine (IL-1 β) with the osteoclastic remodeling gene (TRAP, $r = 0.82$; $p = 0.02$) and (CatK, $r = 0.79$; $p = 0.04$).

Discussion

The present study describes the relationship between the molecular determinants of bone regeneration and the kinetic changes in the amount and structure of the regenerated bone during defect augmentation with apatite-based bone substitutes. The main findings were that the presence of the bone substitute, irrespective of type, significantly modulates the level and the coordination of the molecular activities, resulting in the different level and distribution of bone from that seen in unaugmented defects. Furthermore, the molecular and structural events of bone regeneration are substantially affected by the differences in the crystallinity, structure and/or surface morphology of the two substitute materials.

During the early phase of bone defect healing, the trigger of osteogenic differentiation and bone formation corresponds to the diminution of the acute inflammation induced by the surgical trauma. The present findings relating to the early global downregulation of proinflammatory gene expression, in the presence or absence of the substitute materials, indicate that both natural and synthetic substitutes do not disrupt the biological switch from inflammation to bone regeneration or, at least, do not prolong the inflammatory phase after implantation.

1 One major observation in the present study was the early, increased level of bone
2 contact with the surface of the synthetic substitute in parallel with the higher
3 expression of bone formation and remodeling genes and the downregulation of
4 proinflammatory genes. This relationship has been previously reported in similar
5 experimental designs, but during titanium implant osseointegration [16, 17]. Oxidized
6 implants associated with higher bone formation and remodeling and lower
7 proinflammatory gene expressions induced a higher level of bone contact than
8 equivalent implants with machined surfaces [17]. Apart from the differences in the
9 design and the bulk materials, both findings illustrate a rapid, intense and confined
10 pro-osteogenic microenvironment established between the material surface and the
11 adherent cells. In contrast to osseointegration, the consequence of increased bone-
12 material contact during augmentation with the particulate materials remains to be
13 determined.

14 Another interesting finding was the relationship between the early gene expression
15 and the ultrastructure of the bone interface and the synthetic granules. The early,
16 coupled upregulation and the strong correlation of bone formation and bone
17 remodeling gene expression were associated with the early, ultrastructural detection
18 of, at least partially, mineralized bone in response to the synthetic substitute.
19 Remodeling is an essential process for the maturation of the bone that forms early
20 during healing; however, this process has traditionally been regarded as a late
21 maturation stage, which takes place over several weeks. The present results suggest
22 that an accelerated remodeling phase takes place in conjunction with early bone
23 formation in response to specific properties of the synthetic substitute.

24 The impact of proinflammatory cytokines during bone regeneration *in vivo* is highly
25 controversial. For instance, there are many indications that TNF- α plays different

1 roles during different periods of bone healing and regeneration [18-20]. They include
2 effects on mesenchymal stem cell migration and osteogenic and osteoclastic cell
3 differentiation [21-23]. During the 3d period, even though there were no significant
4 differences in the expression of TNF- α in the three defects, the higher trend in the
5 expression of TNF- α in the unaugmented Sham defects was in parallel with a negative
6 correlation with the expression of osteogenic genes. This observation suggests a
7 negative role for TNF- α at the high level of expression detected in the unaugmented
8 defect at the 3d period. Interestingly, at the late time period (14d and 28d), the lower
9 level of expression of TNF- α was positively related to the osteogenic genes. Although
10 no direct conclusions can be derived from these relationships, possible explanations
11 can still be suggested. Firstly, TNF- α at a high level of expression may negatively
12 affect the osteogenic activity and bone formation *in vivo*. This assumption is
13 supported by previous *in vitro* data, showing that TNF- α inhibits mineralization and
14 osteogenic gene expression [24, 25]. Secondly, TNF- α at a low level of expression
15 may positively influence the osteogenic gene expression during the late period of
16 bone regeneration *in vivo*. This latter assumption is supported by previous *in vitro*
17 studies showing that TNF- α increases the matrix mineralization and osteogenic gene
18 expression [23]. Whether the level of expression or the healing time period or both are
19 the most important factor when it comes to determining the osteogenic impact of
20 TNF- α would require further investigation.

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Histologically, the bone response to deproteinized bovine bone has been previously
investigated [26, 27]. The material has been considered to be osteoconductive [28].
However, there is limited information about the plausible mechanism of bone
regeneration in association with DBB. One major debate relates to the resorbability of
the natural material when introduced into the biological environment of bone defects.

Whereas several studies described the presence of osteoclasts and osteoclast-like cells on the surface of the DBB [29, 30], other studies revealed that DBB is persistent and undergoes minimum resorption [5, 31, 32]. Clinically retrieved samples showed the presence of DBB granules after 11 years in a volume comparable to that when it was implanted [33]. One main finding in the present study was the downregulation of osteoclastic genes, which may provide a plausible explanation for the low resorptive capacity of the DBB. The histological findings in the present study are in line with this assumption. At the 14d period, osteoclast-like cells were detected on the surfaces of both materials, but, after 28d, the appearance of these cells was more evident in association with the surface of the synthetic substitute. Furthermore, at the 14d period, a sign of resorption was mainly observed on the bone side facing the osteoclast-like cells associated with DBB, whereas the cells at TetraB were associated with resorption areas, on surfaces of both bone and granules. Moreover, at 28d, the osteoclast-like cells in TetraB-augmented defects were commonly accompanied by osteoblast seams, suggesting active remodeling sites at the immediate contact with the synthetic substitute.

The XRD data showed that the main crystallinity phase of DBB is hydroxyapatite, which is generally assumed to be the chemically most stable phase of calcium phosphates. Furthermore, the process of deproteinization of bovine bone, including the heat and chemical treatment, may result in increased HA crystallinity, which may make it further inclined towards lower solubility. On the other hand, the surface of the TetraB was chemically treated to produce octacalcium phosphate (OCP) crystallinity, as indicated by the XRD and the ultrastructural surface analyses. The present findings that the osteoclast-like cells produced signs of resorption on both the bone and the material sides, in conjunction with the OCP-containing substitute, and only on the

bone side in the HA-containing DBB substitute are in accordance with previous findings reported by Kamakura and co-authors [34]. In the latter ultrastructural study *in vivo*, multinucleated cells associated with OCP were able to form a clear zone and ruffled border in the interface between bone and the granule surface, whereas only a clear zone developed in association with HA. These findings were associated with the frayed appearance of both bone and granule sides in the OCP group and only the bone side in the HA. The present study indicates that the resorptive activity of the osteoclast is down-tuned at the molecular level, where the expressions of major osteoclastic enzymes are significantly downregulated in response to the HA-dominated DBB.

Using histology, the appearance of cells with an osteoclastic phenotype in association with OCP granules has been previously reported [35, 36]. The present morphological and molecular findings show the upregulation of osteoclastic activity at 3 and 14d and the frequent appearance of osteoclast-like cells in association with the OCP-containing substitute at 14 and 28d. Taken together, these observations suggest major interactions between the osteoclasts and the surface of the OCP granules, leading to the prompt mineralization of the early formed bone in direct contact with the granules and ultimately to an increased amount of mature bone forming in the defects. The pro-osteogenic potential of osteoclasts may involve different mechanisms: the delivery or activation of latent growth factors released from bone sites during the resorption process [37-39]; the provision of pro-osteogenic signals via cell-to-cell contact [40] or via the release of active pro-osteogenic factors [41-43]. Furthermore, additional mechanisms can be considered based on the present *in vivo* findings of increased osteoclastic activity at the surface of the synthetic substitute and on previous *in vitro* findings, showing the ability of osteoclastic cells to resorb different calcium

phosphate substrates [44-47]. Mechanisms of this kind imply that it is possible that osteoclasts are able to condition the surface of granules or expose the surface features that have been biologically coated after implantation in the recipient sites. This surface conditioning and/or exposure may influence the osteoprogenitors and osteoblasts, upregulating osteogenic gene expression and increasing the level of bone formation and degree of mineralization.

The exact mechanism by which DBB downregulates osteoclastic activity is unclear. The main downregulation of osteoclastic gene expression in the DBB appeared to take place during the 14d period. This period in the present animal model corresponds to the phase of active bone remodeling where the early-formed woven bone is remodeled into mature bone. Proinflammatory cytokines, such as TNF- α and IL-1 β , are considered important for this phase of bone healing. Studies of a similar animal model showed that, after the downregulation of the initial proinflammatory phase, the expressions of proinflammatory cytokines increase again during the remodeling phase about two weeks after injury [10, 48]. This was confirmed in the unaugmented Sham defects, where the levels of both cytokines showed a temporal increase after 14d. Whereas this temporal upregulation was maintained in the TetraB-augmented defects, there was a major downregulation, especially for IL-1 β , in the DBB-augmented defects. TNF- α and IL-1 β bind to respective receptors on osteoclasts and augment their differentiation and resorptive potential [21, 49, 50]. Possible positive roles for these cytokines in osteoclastic activity are also indicated by the strongly positive correlations between the gene expression of these cytokines and the expression of osteoclastic genes at 3d, in the unaugmented Sham defects, and at 14d, both in the unaugmented Sham and in the augmented defects.

1 The bone that formed after 28d appeared as highly mineralized mature bone, indicated
2 by the high contrast at ultrastructural level. The maturation of bone was not limited to
3 any specific substitute type, as the ultrastructural analysis showed well-organized
4 bone, with collagen fibers and numerous osteocytes, in direct connection with the
5 surfaces of either type of the substitutes. On the other hand, the TetraB-augmented
6 defects displayed a larger amount of newly formed bone than the DBB-augmented
7 defects. In fact, it was the unaugmented sham defects that underwent a massive
8 reduction in bone area percentage, underscoring the beneficial effect of the substitute
9 materials.

10 In the 28d time period, no major differences were observed between the defects with
11 respect to the magnitude of gene expression for any of the biological processes. In
12 contrast to the Sham and DBB defects, which showed positive correlations between
13 genes involved in bone formation and remodeling, the TetraB-treated defects
14 exhibited multiple, strong correlations between the genes denoting the different
15 processes (inflammation, bone formation and remodeling). The increased correlations
16 between the different genes may reflect a state of steadiness and coordination between
17 different cells at the site of bone regeneration.

41 **Conclusion**

42 Under experimental conditions, in contrast to empty defects, both natural and
43 synthetic bone substitute materials promote bone regeneration and the restitution of
44 the surgically created defect. The present gene expression, morphological and
45 ultrastructural data demonstrate that the bovine substitute (DBB) is osteoconductive
46 and elicits low resorption activity. Further, the synthetic substitute (TetraB) induces
47 early osteogenic and osteoclastic activity, resulting in greater bone formation in
48 comparison to the natural substitute (DBB).

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Figure legends

Figure 1: Scanning electron microscopy images of deproteinized bovine bone (DBB) (A, C and E) and synthetic tetrapod-shaped calcium phosphate (TetraB) (B, D and F) at increasing magnification; x100 (A and B), x1,000 (C and D) and x50,000 (E and F).

Figure 2: X-ray diffraction analysis of deproteinized bovine bone (DBB) (A) and synthetic tetrapod-shaped calcium phosphate (TetraB) (B). The XRD peaks show that the main phase of DBB is hydroxyapatite, whereas TetraB mainly consists of α -TCP (peaks around 20-35°) and part of OCP (main peak at 4.70°) and hydroxyapatite. (* = hydroxyapatite; # = α -TCP; O = OCP).

Figure 3: Survey light micrographs of undecalcified toluidine blue-stained sections of bone defects created in the trabecular region of the rat femoral epiphysis. The defects were unaugmented Sham site (A, D and G) or augmented with either deproteinized bovine bone (DBB) (B, E and H) or synthetic tetrapod-shaped calcium phosphate (TetraB) (C, F and I). The images show the pattern of new bone formation (NB) within the three defects at 6d (A, B and C), 14d (D, E and F) and 28d (G, H and I). At 6d (A, D and G), there was no evident bone formation in the central region of any of the defect groups. The central regions are mainly occupied by soft tissue (ST) in the Sham sites (A), whereas those in the augmented defects appear to be mainly occupied by the DBB (B) or TetraB (C) granules. Peripherally, newly formed bone (NB) is observed in all defect types extending from the peripheral original bone (OB). The NB reveals dark staining assuming an unmineralized osteoid appearance. No major signs of inflammatory infiltrate can be detected in any of the defects. At 14d (D, E and F), a substantial amount of bone has formed, extending from the periphery of the

defects and filling the central region in all groups. In the augmented sites (E and F), the NB in a trabecular pattern appears to fill the intergranular spaces and connect between the individual granules. In this period, the NB staining appears to be slightly lighter than at 6d, whereas the TetraB (F) is even lighter, assuming a certain degree of mineralization. At 28d in the Sham site (G), the NB, observed earlier, is considerably remodeled, leaving few trabeculae of lightly stained bone bordered by bone marrow (BM) and soft tissue. The soft tissue on the top of the Sham defects reveals a downgrowth inside the defect area. In this period in the augmented sites (H and I), the NB is maintained, filling the major area of the defects, together with the granules of either substitute material. In both the DBB and TetraB, the NB extends to the top of the defects, retaining the original level of the bone at those sites. A larger amount of lightly stained mineralized bone appears to fill the intergranular spaces in the TetraB group compared with the DBB.

Figure 4: Light micrographs of undecalcified toluidine blue-stained sections of bone defects created in the trabecular region of the rat femoral epiphysis. The images show the augmented sites at 6d. The defects were augmented with either deproteinized bovine bone (DBB) (A, B and C) or synthetic tetrapod-shaped calcium phosphate (TetraB) (D, E and F). In this period, the newly formed bone is primarily unmineralized osteoid (Os) formed in the bone marrow (BM) in the close vicinity of granules of either type. The Os in the TetraB group reveals higher proximity and contact with the granule surfaces than that formed in the DBB group. In addition, areas of partially mineralized bone (PMB), lightly stained, are mainly detected in the TetraB-treated defects. Osteoblast seams are also observed adjacent to the Os areas in both defect groups (some are indicated by white arrowheads).

Figure 5: Light micrographs of undecalcified toluidine blue-stained sections of bone defects created in the trabecular region of the rat femoral epiphysis. The images show the augmented sites at 14d. The defects were augmented with either deproteinized bovine bone (DBB) (A, B, C, D) or synthetic tetrapod-shaped calcium phosphate (TetraB) (E, F, G, H, I). In this period, the bone filling the intergranular spaces in both groups appears as a mixture of partially mineralized (PMB) and mineralized bone (MB), indicated by the lighter degree of staining and the presence of osteocytes. In the TetraB group, the MB shows a higher degree of contact with the granule surface (F) than that in the DBB (B). Furthermore, the interface between the MB and the TetraB granules frequently reveals a blade-like appearance (indicated by white arrows in H). Osteoblast seams and osteoclast-like cells are detected, in many cases in connection with the granule surfaces of either material (indicated by white and black arrowheads, respectively). Surface irregularities (resorption-like sites; indicated by black arrows), facing the osteoclast-like cells, appear in both the MB and the granule surface in the TetraB defects and only in the MB in the DBB defects.

Figure 6: Light micrographs of undecalcified toluidine blue-stained sections of bone defects created in the trabecular region of the rat femoral epiphysis. The images show the augmented sites at 28d. The defects were augmented with either deproteinized bovine bone (DBB) (A, B and C) or synthetic tetrapod-shaped calcium phosphate (TetraB) (D, E and F). In this period, the bone in the intergranular spaces appears to be mineralized, with numerous osteocyte lacunae indicating mature bone type (MB). The DBB and TetraB particles show a comparable degree of contact with the MB. Active remodeling sites are encountered, characterized by osteoblast seams (white arrowheads) and osteoclast-like cells (black arrowheads). The latter observation is more commonly detected in the TetraB group.

Figure 7: Histomorphometric analysis of defects created in the trabecular region of the rat femoral epiphysis. The defects were unaugmented Sham site or augmented with either deproteinized bovine bone (DBB) or synthetic tetrapod-shaped calcium phosphate (TetraB). The analysis was performed at 6, 14 and 28d. Measurements were made of the granule area percentages, bone area percentages and the percentages of bone-granule contact. The level of statistical significance was set at $p < 0.05$. Statistically significant differences are indicated by symbols or small letters: * = significant difference between the Sham site versus any of the augmented sites (DBB or TetraB); # = significant difference between DBB and TetraB; a = significant difference between 6d and 14d (for each group); b = significant difference between 14d and 28d (for each group). The results are presented as the mean percentages $\pm$ SEM.

Figure 8: Backscatter SEM micrographs of defects created in the trabecular region of the rat femoral epiphysis. The defects were augmented with either deproteinized bovine bone (DBB) (A and C) or synthetic tetrapod-shaped calcium phosphate (TetraB) (B and D). The images show selected areas of the augmented sites at 3d (A and B) and 6d (C and D). The insert shows a higher magnification of a selected area in (D). PMB = partially mineralized bone; OB = original bone; The white arrows (B and insert in D) refer to direct contact between PMB and TetraB.

Figure 9: Backscatter SEM micrographs of defects created in the trabecular region of the rat femoral epiphysis. The defects were augmented with either deproteinized bovine bone (DBB) (A and C) or synthetic tetrapod-shaped calcium phosphate (TetraB) (B and D). The images show selected areas of the augmented sites at 14d. White arrowheads refer to the interface between the particle surface and the formed bone. (OC) indicates osteocyte lacunae.

Figure 10: Backscatter SEM (A-D) and HAADF-STEM (E and F) micrographs of defects created in the trabecular region of the rat femoral epiphysis. The defects were augmented with either deproteinized bovine bone (DBB) (A, C and E) or synthetic tetrapod-shaped calcium phosphate (TetraB) (B, D and F). The images show selected areas of the augmented sites at 28d. White arrowheads refer to the interface between the particle surface and the formed bone. (OC) denotes osteocyte lacuna; (C) denotes canaliculus.

Figure 11: Gene expression analysis of tissue harvested from defects created in the trabecular region of the rat femoral epiphysis. The defects were unaugmented Sham site or augmented with either deproteinized bovine bone (DBB) or synthetic tetrapod-shaped calcium phosphate (TetraB). The analysis was performed at 6, 14 and 28d. The level of statistical significance was set at $p < 0.05$. Statistically significant differences are indicated by symbols or small letters: * = significant difference between the Sham site versus any of the augmented sites (DBB or TetraB); # = significant difference between DBB and TetraB; a = significant difference between 3d and 6d (for each group); b = significant difference between 6d and 14d (for each group); c = significant difference between 14d and 28d (for each group). The results are presented as the mean $\pm$ SEM.

	Sham			DBB			TetraB		
	Genes	(r)	(p)	Genes	(r)	(p)	Genes	(r)	(p)
3d				TRAP/CatK	0.83	0.04	TRAP/CatK	0.96	0.0004
	TNF- α /OC	-0.94	0.004						
	TNF- α /TRAP	0.83	0.04						
6d	TRAP/CatK	0.77	0.04	TRAP/CatK	0.94	0.004	TRAP/CatK	0.89	0.006
							TNF- α /ALP	0.79	0.04
							OC/TRAP	0.89	0.006
							OC/CatK	0.79	0.04
14d	TNF- α /IL-1 β	0.89	0.02	TNF- α /IL-1 β	0.89	0.006	TNF- α /IL-1 β	0.86	0.01
				TRAP/CatK	0.96	0.0004	TRAP/CatK	0.96	0.0004
				TNF- α /CatK	0.89	0.006	TNF- α /CatK	0.79	0.04
				TNF- α /TRAP	0.82	0.02	TNF- α /TRAP	0.86	0.01
				IL-1 β /CatK	1	1E-10	IL-1 β /CatK	0.96	0.0004
				IL-1 β /TRAP	0.96	0.0004	IL-1 β /TRAP	0.93	0.003
28d	TNF- α /IL-1 β	0.83	0.04	TNF- α /IL-1 β	0.82	0.02	TNF- α /IL-1 β	0.82	0.02
	TRAP/CatK	1	1E-10	TRAP/CatK	0.89	0.007	TRAP/CatK	0.93	0.002
				ALP/OC	0.89	0.007	ALP/OC	0.75	0.049
	TNF- α /ALP	0.89	0.02				TNF- α /ALP	0.93	0.002
							TNF- α /CatK	0.79	0.04
							IL-1 β /CatK	0.79	0.04
							IL-1 β /TRAP	0.82	0.02
				ALP/CatK	0.89	0.007	ALP/CatK	0.79	0.03
	OC/TRAP	0.89	0.02						
	OC/CatK	0.89	0.02						

Table I: Correlation analysis of genes expressed in samples retrieved from unaugmented Sham defects or from defects augmented with either deproteinized bovine bone (DBB) or synthetic tetrapod-shaped calcium phosphate (TetraB). The analysis was performed at 3, 6, 14 and 28d after augmentation. Spearman correlation coefficients (r) and level of significance (p-values) are presented.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10

Figure 11