CLINICAL ORAL IMPLANTS RESEARCH

Arturo Martinez
Oscar Balboa
Iria Gasamans
Xose Luis Otero-Cepeda
Francisco Guitian

Deproteinated bovine bone vs. beta-tricalcium phosphate as bone graft substitutes: histomorphometric longitudinal study in the rabbit cranial vault

Authors' affiliations:
Arturo Martinez, Estomatología, Facultad de Medicina y Odontología, Instituto de Cerámica de Galicia, Universidad de Santiago de Compostela, Santiago de Compostela, Spain
Oscar Balboa, Iria Gasamans, Estomatología, Facultad de Medicina y Odontología, Universidad de Santiago de Compostela, Santiago de Compostela, Spain
Xose Luis Otero-Cepeda, Bioestadíastica, Facultad de Medicina y Odontología, Universidad de Santiago de Compostela, Santiago de Compostela, Spain
Francisco Guitian, Instituto de Cerámica de Galicia, Universidad de Santiago de Compostela, Santiago de Compostela, Spain

Corresponding author:
Arturo Martinez
Estomatología
Facultad de Medicina y Odontología
Universidad de Santiago de Compostela
15782 Santiago de Compostela
Spain
Tel.: 34 881816870
Fax: 34 981564242
e-mail: arturo.martinez@usc.es

Key words: biomaterials, bone regeneration, bone substitutes, guided tissue, morphometric analysis, regeneration

Abstract

Objectives: This article aims to study differences in the bone formation and the graft resorption of two bone graft substitutes (BGS). Besides, it is our attempt to observe possible qualitative and quantitative differences in the bone reparation of the outer layer covered by collagen membrane and the uncovered inner layer in close contact with dura mater.

Material and methods: Twelve rabbits were employed. Deproteinized bovine bone (DBB) and β-tricalcium phosphate (BTCP) were used as BGS. Four subcritical round defects (7 mm) were drilled in the cranial vault, removing both cortical walls. One of the holes was filled with DBB, and other was filled with BTCP. Each symmetrical position to DBB and BTCP was left empty. The whole defect set was covered with a collagen membrane. Histological and morphometric analysis was performed for 1, 4, 8, 16, 32 and 52 weeks. Morphometry measurements were carried out taking into account the whole defect and splitting inner and outer areas.

Results: In DBB sites, a rapid bone growth is observed, linking the remaining particles and integrating them into the bone matrix. Permanence of these DBB particles from week 16 onwards restrains the growth of bone fraction. A greater bone growth appears in areas repaired with BTCP than in those repaired with DBB, both in the outer layer (under-membrane) and the inner layer (over dura mater). In DBB sites, a slower growth is observed in the inner layer, with no significant differences in the final bone fraction at both strata.

Conclusions: Both materials favour the closure of the defects provoked. In both cases, a synergistic effect with the collagen membrane is observed. DBB remains integrated in the bone matrix, while BTCP displays a pattern of highly developed progressive resorption with an outstanding bone fraction development.

In the past years, development and characterization of bone graft substitutes (BGS) have become quite relevant. Availability of enough bone volume plays an important role not only because of aesthetics but also for functional purposes (Chen et al. 2010). Long-term behaviour of dental implants is conditioned by the available amount of alveolar bone (Lekholm et al. 1986). Even though auto grafts are still considered the "golden standard" by some authors (Misch & Dietsh, 1994; Schmitt et al. 2012), they exhibit two relevant drawbacks such as unpredictable resorption (up to a 40%) of the grafted volume (Johanson et al. 2001; Schlegel et al. 2003; Albert et al. 2006), or the fact that graft harvesting from other parts of the organism with the risk of morbidity appearance, as well as unexpected complications due additional surgery.

Within the BGS, the deproteinized bovine bone (DBB) has exhibited remarkable properties due to its excellent biocompatibility and well-known osteoconductive capability (Guizzardi et al. 1995; Artzi et al. 2004). Although its osteoinductive competence is not so evident (Buser et al. 1998; Busenlechner et al. 2008), this material exhibits long-term permanence inside the matrix of the host bone (Piatelli et al. 1999; Schlegel et al. 2003; Artzi et al. 2004). Although long-term stability goes against what is expected from

Date:
Accepted 19 January 2014

To cite this article:
Martinez A, Balboa O, Gasamans I, Otero-Cepeda XL, Guitian F. Deproteinated bovine bone vs. beta-tricalcium phospate as bone graft substitutes: histomorphometric longitudinal study in the rabbit cranial vault.
Clin. Oral Impl. Res. **00**, 2014, 1–10
doi: 10.1111/clr.12349

the ideal biomaterial, which should be totally replaced by host bone, this long-term stability might be an advantage because it can preserve bone volume given that bone remodelling is conditioned by its presence in the bone matrix (Araujo et al. 2011). DBB is a material which possesses a microhierarchical architecture where apatite crystals are organized in the same manner as in host bone, allowing this "nanotexture" and its pore distribution a good colonization by the host (Benezra Rosen et al. 2002) as well as an intimate apposition of new bone growth to residual DBB particles (Piatelli et al. 1999; Jensen et al. 2006). The overall result is a "composite" made of bone and the remaining particles (Hämmerle et al. 1998; Donos et al. 2004; Artzi et al. 2004) with higher density and equal or even better mechanical properties than bone itself (Haas et al. 1998; Orr et al. 2001). This composite is adequate for implant placement (Berglundh & Lindhe 1997). However, this long-term permanence of DBB particles creates some controversy, due to the fact that it has not been elucidated yet whether its presence constrains bone formation and later remodelling.

Another BGS which has also shown an excellent biocompatibility is β-tricalcium phosphate (BTCP). In contrast to DBB case, the synthetic origin of BTCP prevents disease transmission. Similarly, BTCP also shows good biocompatibility and osteoconductive capability (Busenlechner et al. 2008), as well as its possible competence in osteoinductivity (Knabe et al. 2000; Dalcusi et al. 2003; Cheng et al. 2013; Samavedi et al. 2013). BTCP is completely resorbed by host bone. The material resorption seems to be related to its solubility *in vivo* and its degradation by multinucleated cells (Yamada et al. 1997; von Doenrberg et al. 2006). The degradation rate of this material can be controlled by modifying the synthetic process, by changing grain size distribution, pore size and porosity, to obtain BTCP particles with controlled porosity, and grain size distribution as well as longer permanence in the host (Ghanaati et al. 2010). The longer permanence of this material will preserve the bone volume and make it less sensitive to bone remodelling. It is also possible that this more stable BTCP could possibly exhibit a porosity which can promote the interaction with bone forming cells and boost its development. Although it has extensively been studied and recognized as the best alternative possible, due to its potential osteogenic capability (Klijn et al. 2010), some of the questions that remain unanswered about this material are: 1. Long-term permanence along with its reduced solubility, 2. Its interaction with neo formed bone and the possible existence of stable interfaces (Artzi et al. 2008), 3. The interaction with collagen membranes (Artzi et al. 2004; Schulten et al. 2013).

Retention and surgical stabilization of these materials are commonly achieved by collagen membranes which favour bone regeneration by osteoprogenitor cells, preventing the graft site from being invaded by soft tissue (Tarnow et al. 2000; von Arx et al. 2005). Besides providing an excellent biocompatibility, collagen membranes seem to exhibit osteogenic capabilities (Donos et al. 2004; Taguchi et al. 2004), facilitated by the presence of BGS particles which preserve the volume and avoid membrane collapse (Hämmerle et al. 1998; Hammerle & Jung 2003; Donos et al. 2004). There are scarce works about guided tissue regeneration (GTR) where a bi-cortical defect reparation is studied having each side in contact with different interfaces: one in contact with encephalic membrane and the other side covered with collagen membranes. Nowadays, there are still some doubts about the need of use of collagen membranes along with BGS. Some studies support its synergistic effect (Tarnow et al. 2000; Wang et al. 2002), whereas others consider it as a limited contribution (Artzi et al. 2004), or other even inexistent as a positive interaction (Schulten et al. 2013).

This work intends to shed light over some of the most controversial aspects regarding GTR with these BGS. Therefore, so we have designed a longitudinal and transversal comparison of a DBB and a microporous BTCP with slow solubility. A defect of two walls in the cranial vault was chosen as a model of study this kind of defect, particularly aggressive, was balanced with a subcritical size of the defect. After removing both cortical layers, we presumably obtained a defect with two interfaces with different osteogenic capabilities.

Both BGS were employed alone with no addition of autologous bone to avoid bias in osteogenesis and osteoconductivity due to the presence of the autograft. Our first hypothesis (h0.1) sets the absence of differences in repairing and bone remodelling, neither qualitative nor quantitative, between BGS in the long term. According to our second hypothesis (h0.2), bone growth takes place in a similar manner in the inner and in the outer layer of the defect with independence of the BGS employed.

Material and methods

Study design

Twelve white New Zealand rabbits (Charles River Laboratories, France) weighting 2.5–3 kg were employed. The study protocol was approved by the Ethics Committee of the University of Santiago de Compostela. The animals were randomly distributed in six groups for the analysis of the defects after healing periods of 1, 4, 8, 16, 32 and 52 weeks.

Surgical protocol

All surgical procedures were done under strict aseptic protocol. The animals were premedicated with diazepam 0.2 ml/kg (Valium 10, Roche), and anaesthetized with ketamine 10 mg/kg (Imalgène 1000, Merial, France) and medetomidina (0.1 mg/kg) (Domtor, Orion. Finland) injected intramuscularly in the hind leg. The animals were shaved from the eyes to the occipital eminence and between the ears. Their skin was disinfected with a povidone-iodine solution 10% (Betadine, Medapharma). Infiltrative anaesthesia 1.8 ml (Ultracain 40/0.005, Normon, Spain,) was injected under the scalp. A midline sagital incision was made, and skin and periosteum were carefully raised, so vault surface was exposed. Using a bone trephine of 7 mm external diameter (Bontempi, Italy), at low speed rotation and under profuse physiological saline refrigeration (KaVo Intrasurg 300, Karlsruhe, Germany), four no-critical size defects (Hollinger & Kleinschmidt 1990) were prepared: two to each side of the sagital suture, being careful of not to invade the frontal or occipital sutures (Fig. 1). The tabula externa and tabula interna were carefully sectioned, and the bone discs were carefully removed with a Lucas Carver and a Freer periosteotome (Bontempi, Italy), preserving the integrity of the dura mater. Randomly, in

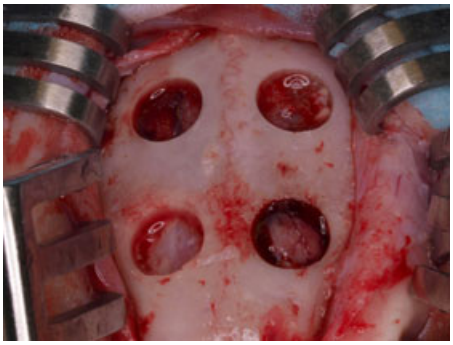

Fig. 1. Four circular defects of 7 mm diameter were prepared in the rabbit vault.

one side, one defect was filled with DBB Bio-Oss (0.25–1 mm Geistlich, Switzerland) and the other was not filled. Similarly, in the other side, and again randomly, one defect was filled with BTCP (KeraOs [0.25–1 mm], Keramat, Spain) while the other was not filled. All the defects were covered with a collagen membrane (Bio-Gide.25 × 25 mm, Geistlich, Switzerland). No additional device was employed to stabilize the membranes. The periosteum was sutured with continuous monofilament resorbable suture (Monocryl3-0, Ethicon, Edinburg, UK). The skin was sutured with interrupted threaded sutures (Vicryl 3-0, Ethicon, Edinburg, UK). Skin was again soaked with povidone solution. An intramuscular injection of benzathine ampicilin (50 mg/kg) and 0.01 ml of prednisone (Urbason 40, Sanofi, Barcelona, Spain) was administered in the immediate postoperative and within the 48 h after surgical procedure metamizol (500 mg/12 h) (Nolotil, Boehringer Ingelheim España) was employed as pain reliever.

Sacrifice

The animals were deeply anesthetized following the same protocol used in surgery and were killed with Nembutal (5 ml/kg). After sacrifice, the cranial vault was removed with a saw and a diamond disc saw under continuous cooling. The galea aponeurotica and the dura mater were not removed. Specimens were immediately immersed in a solution of 7% formaldehyde buffered with phosphate.

Histological sections

The blocks were rinsed in running tap water, and a radiograph was taken to determine the exact location of the defects. Specimens were trimmed and dehydrated in graded series of increasing ethanol concentrations and embedded in acrylic resin (Technovit 7200, Kulzer, Wehrheim, Germany). Sections were made, under copious cooling with ethanol, parallel to the sagital suture, following Donath and Breuner's method (Donath and Breuner, 1982) (cutting and grinding). Four undecalcified thin sections of about 30 μm were obtained from each defect. The slides were stained with Harris Hematoxiline (Merck, Germany) and Wheatley's modification of trichromic stain (Chromotrope 2R, Newcommer Supply, USA).

Histomorphometrical evaluation

Histological analysis and photomicrographs were obtained using a light-transmitted microscope (Nikon Optiphot 2pol, NIPPON KOGAKU K.K., Tokyo, Japan) equipped with a digital camera attached to the microscope (Olympus DP12, Olympus Optical Co. [Europe] GMBH, Hamburg, Germany). The obtained images were processed with ImageJ-1.46r software (Rasband 2012). Morphometric readings were performed at least from three preparations per each defect. When necessary, polarized light microscope was employed to determine the boundaries of the newly formed bone. To study the differences in bone formation in the outer layer versus the inner layer, each defect section was artificially divided into two halves, and morphometry was carried on in the whole defect and in the two halves. All measurements were taken by the same researcher, and boundaries were revised by a second. To determine the reproducibility and the measurement error, ten randomly selected slides were measured three times, in three different days (Bayley & Byrnes 1990). The measurement errors were as follows: bone 0.09%, biomaterial 1% and soft tissue 1.2%.

Statistical analysis

Given the relatively small size of the sample, a Kruskal–Wallis test was employed to make comparisons between the different grafted sites (bone, filler and soft tissue) within time. We have performed post hoc analyses to determine the signification levels of the analysed variables for each time. For the transversal study, taking into account the limitations of the sample, we have chosen a repeated measurement ANOVA due to its robustness. A post hoc of repeated measurements was also performed to analyse the differences between fillers at each time. A Wilcoxon signed rank test was employed to compare the morphometric values from the outer layer data versus the inner layer. The statistical packages employed were Statistix 10.0 (Tallahassee, FL, USA) and the IBM-SPSS 20.0 (Chicago, IL, USA). The level of significance was set to $P \leq 0.05$.

Results

Histological description

1 week

A persistent blood clot, mainly in the defect centre, is observed as a common histological characteristic in all the examined positions. BGS (Fig 2) are coated by a matrix of proteins, and an outstanding development of mesenchymal undifferentiated tissue between materials with a dense vascular proliferation could also be observed (Fig 3). Small projections of woven bone and a layer of new bone can be seen from the defect walls, especially in the BTCP samples (Fig 2b). Some multinucleated cells are also visible in close contact with the smooth surface of the BTCP particles. DBB surface is covered by mesenchymal cells; intense cell proliferation and mitosis are seen in the gap between bone walls and graft (Fig 3a). Vascular buds, fibroblasts and mesenchymal cells invade the different collagen membrane layers; some round mononuclear cells could be seen between the collagen bundles. There was no inflammatory reaction, and no inflammatory cell aggregates were observed. Control sites were occupied by granulation tissue in the defect centres, and new bone layers start to grow from the lateral walls.

4 weeks

Deproteinized bovine bone sites: (Fig 2d) there is an increasing presence of trabecular woven bone which emerges from the lateral walls to the centre of the defect in close apposition to DBB particles. The bridging of newly formed bone takes place under the membrane surface in the outer layer of the defect. The newly formed trabeculae are covered by a periosteum-like tissue, with an outstanding vascular component. The intertrabecular space is filled with a primitive bone marrow. The former Haversian structures of DBB particles are occupied by vascular structures, and scattered osteoclasts can be seen in the biomaterial surface associated with osteoblast cells (Fig 4). The DBB lacunae are frequently occupied by osteocyte-like cells. Membrane thickness is observed to decrease, and it is vigorously colonized by capillars and fibroblasts. At the inner layer, over dura mater, bone formation is lesser and there is no bridging between the defect walls; furthermore, in this area, DBB particles are surrounded by vascularized stroma.

Beta-tricalcium phosphate sites: (Fig 2e) newly formed bone trabeculae bridge the defect walls with no differences between inner and outer layers. The BTCP particles are surrounded by woven bone linking particles. It seems that smoother surfaces tend to be more covered with bone while rougher zones seem to be more soluble and release more material particles. These material particles are frequently trapped by reticulo-endothelial system cells either placed on the material surface or in the adjacent soft tissue (Fig 5). The osteoblasts and osteocytes embedded into the bone matrix can be seen in close contact with the material surface. There is a periosteum-like layer that covers the new formed trabeculae underneath the membrane and over the dura mater. The

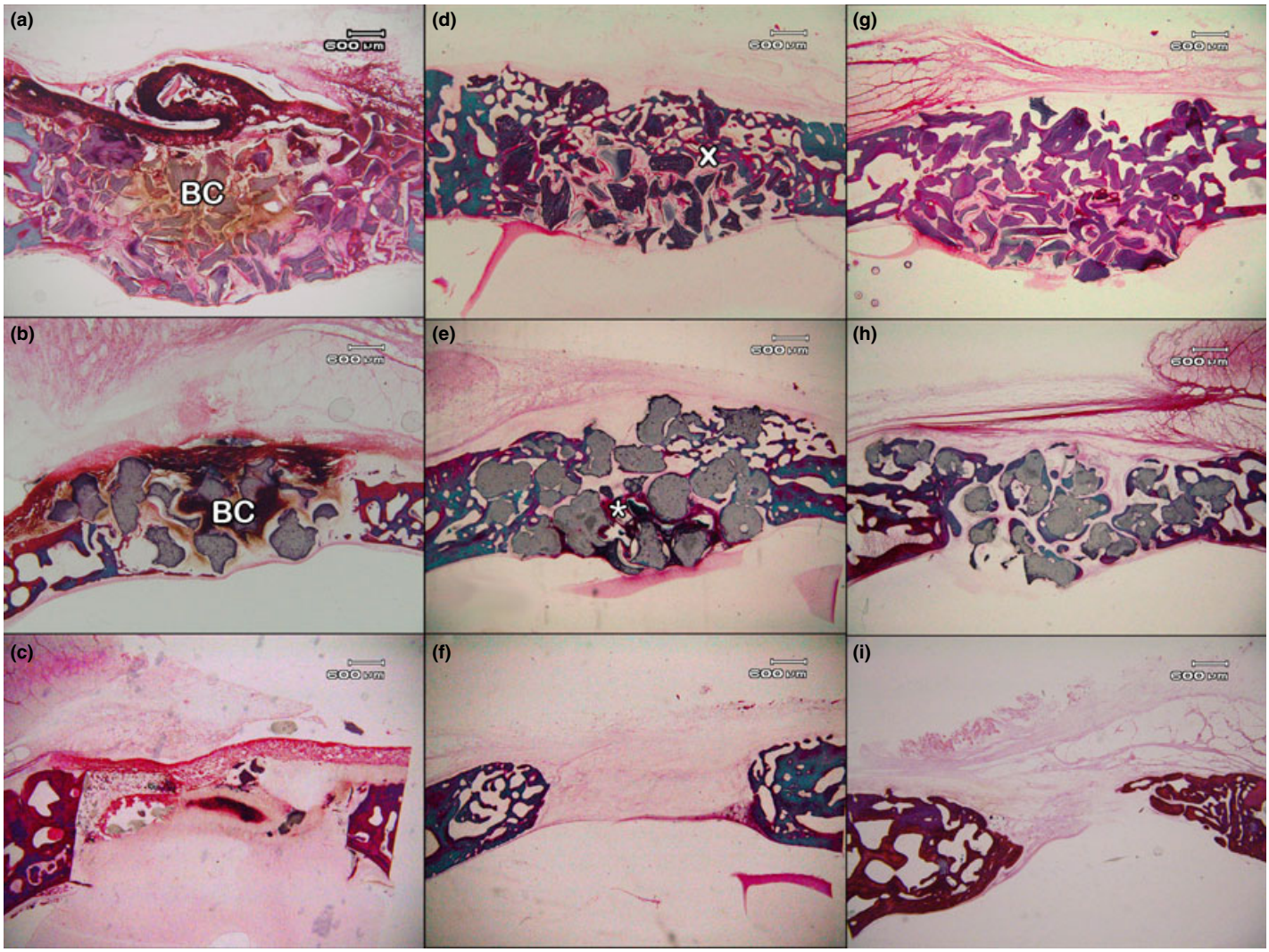

Fig. 2. Left column: Histological overview sections of defects after 1 week. (a) defect filled with deproteinized bovine bone (DBB); note the prevalence of the blood clot in the centre of the defect (BC). (b) void defect covered with membrane defect filled with beta-tricalcium phosphate (BTCP), note the fibrin and proteins surrounding the granules (detail in Fig 3b). (c) control, void defect covered with membrane. Middle column: Histological overview sections after 4 weeks. (d) DBB defect, note the woven bone reticulae formed linking the graft particles in the outer surface (cross). (e) BTCP defect, woven bone in the outer surface and granulation dense tissue in the centre and inner surface (asterisk). (f) control, the formation of woven bone starts on the walls of the defect. Right column: Histological overview sections after 8 weeks. (g) DBB defect, note the bone trabeculae bridging the defect in the outer zone, the DBB particles remain integrated in the bone structure. (h) BTCP, the woven bone trabeculae surround the remaining particulae that are resorbed. (i) control, note the formation of new bone mainly in the outer surface.

membrane is partially degraded as in DBB sites.

Control sites: (Fig 2f) the reparation of the defect starts in the base by forming a thin bone plate over the dura mater without bridging both walls. In the outer layer, there is a small formation in the tabula externa reparation. Membrane collapses in the vane that is now occupied by granulation tissue and mesenchymal stroma with small scattered nuclei of bone formation.

8 weeks

Deproteinized bovine bone sites: (Fig 2g) there is a whole trabecular bone, closing only the outer layer. Woven bone grows on the material surface linking the bone particles within a network. The periosteum covers inner and outer bone. There is mature bone marrow beside the outer layer and mesenchymal stroma in the inner zone. The membrane was replaced by a fibrous dense layer in the galea aponeurotica.

Beta-tricalcium phosphate sites: woven trabecular bone grows mainly on the surface of the material particles, surrounding them all, and establishing a bridge both in the inner and in the outer zone. Bone marrow is similar to DBB sites.

Control sites: there is reparation of the defect in the outer and inner layer, periosteum is covering new bone over dura mater and underneath the fibrous band that has replaced the membrane, and under this layer, there is a concavity of the newly formed bone and a thin layer of connective tissue.

16 weeks

Deproteinized bovine bone sites: (Fig 6a, Fig 7) images are similar to those taken after 8 weeks, evincing a greater consolidation of the outer bone bridge. Bone marrow is mature in the outer zone with abundant adipocytes. DBB particles are integrated into the new trabecular structure formed, and resorption is rare over their surface (Fig 7).

Beta-tricalcium phosphate sites: (Fig 6b) disaggregation and resorption of the material contained between trabeculae are very significant. Trabecular bone starts to be lamellar in

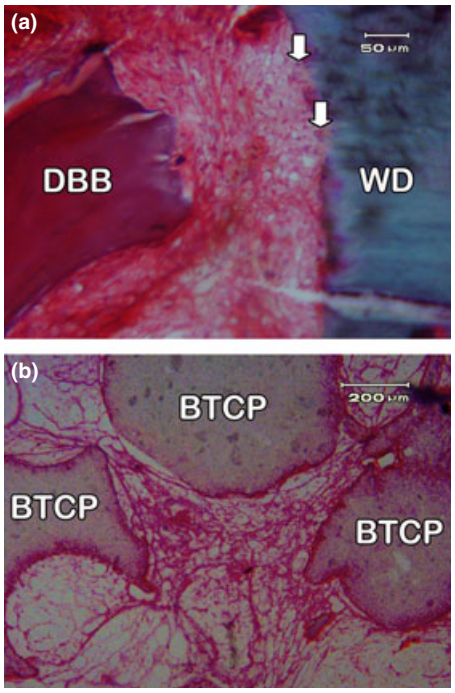

Fig. 3. One week details. (a) jumping space between deproteinized bovine bone surface and the wall defect (WD), notice the dense granulation tissue with abundant mitosis, (white arrows). (b) Beta-tricalcium phosphate particles at 1 week surrounded by protein network and prevascular structures.

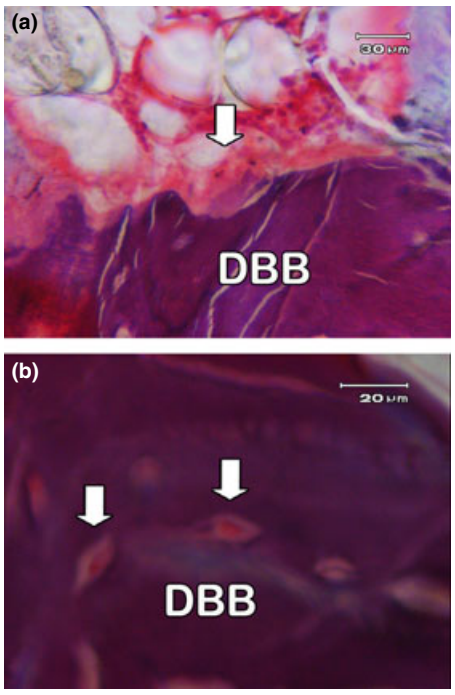

Fig. 4. Detail of deproteinized bovine bone (DBB) particles after 4 weeks. (a) osteoclastic-like activity in the surface of the particle (arrow). (b) DBB particle: former DBB lacunae occupied by osteocite-like cells (arrows).

the inner layer and near the defect walls. There is complete bone bridging both in the inner and outer layer.

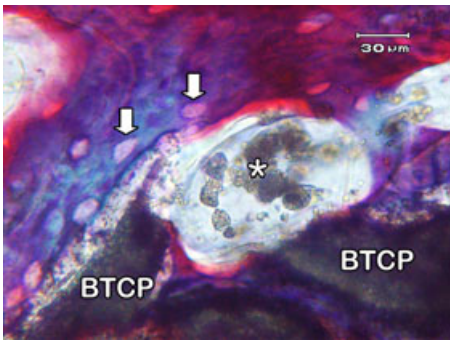

Fig. 5. Detail of beta-tricalcium phosphate particles in close contact with woven bone after 4 weeks. Observe the vehiculization of the granules of material captured by reticulo-endothelial system cells (asterisk). Osteocyte lacunae (arrows) can be observed in the newly formed bone trabeculae.

Control sites: (Fig 6c) a thin structure formed by an irregular tabula externa, small diploe and thin tabula interna bridges the defect walls. Bone trabeculae are formed by woven bone, and bone marrow is mature only in the proximity of the defect walls.

32 weeks

Deproteinized bovine bone sites: (Fig 6d) there is complete integration of the material, and a thin lamellar bone surrounds the remaining DBB particles bridging particles together, closing both the tabula externa and, more weakly, the tabula interna. Some remodelling basic multicellular units (BMU) could be observed in the new formed trabeculae. There is periosteum covering the ectocraneum and the endocraneum.

Beta-tricalcium phosphate sites: (Fig 6e) there is close apposition of woven bone on the particle surfaces. Those remaining areas of woven bone are surrounded by mature lamellar bone forming a thick trabecular network. Material disaggregation is still present, with material components in the bone marrow. There is no lack of continuity in any of the tabulae.

Control sites: (Fig 6f) there is a complete closure of the defect and there is a small concavity in the outer surface. Trabecular bone is almost completely lamellar with residual small areas of residual bone mainly in the inner closure.

52 weeks

Deproteinized bovine bone sites: (Fig 6g) very similar situation to 32nd week. The remaining DBB particles are integrated into a trabecular structure. Bone remodelling is mainly in the composite of bone and particles formed on the dura mater.

Beta-tricalcium phosphate sites: (Fig 6h) persistence of some thick woven bone areas; these structures are surrounding BTCP fragments showing an advanced resorption degree. The trabecular structure is formed by mature lamellar bone. Bone marrow is mature with great proportion of adipocytes.

Control sites: (Fig 6i) slight concavity on the tabula externa outer surface. The defect is completely closed. Trabecular structure is formed by mature lamellar bone, and bone marrow is mature with great proportion of adipocytes.

Morphometry

Kruskal–Wallis analysis shows significant differences for bone growth between BGS and control group within this study.

Longitudinal study

Bone: although a remarkable bone growth was observed between 1 and 4 weeks, even more intense in CTRL and BTCP sites, due to the great dispersion of the obtained data, these differences are not statistically significant. No significant increments of bone growth were observed, in consecutive periods, for any group. The first statistically significant difference is found for the bone growth between 1 and 16 weeks in the three groups. Mention should be made to the fact that this increase in the bone proportion for DBB sites grew from 23.9% (week 1) to 46.8% (week 16). Even though bone growth increased after the 16th week, this increment was not significant in DBB sites. In BTCP sites, there are significant differences between week 1 and week 16. Bone fraction in BTCP sites increased progressively from 24.9% (week 1) until it reached 63.2% in week 52 (Fig 9). All the obtained values, along with the average evolution curves, for the bone growth are depicted in Fig. 10. Note the different dispersion in the values exhibited by the three groups and its evolution within the time.

In sites filled with both BGS, bone fraction is bigger in the outer layer than in the inner layer, but it is significant in BTCP sites. In contrast, the control sites do not show differences between inner and outer layer. Bone fraction is also significantly larger in BTCP sites both for inner and outer layers (Fig 11).

Material: DBB exhibits a decrease: from week 8 (28%) to week 16 (23.3%) and from that moment on, there is no significant decrease in DBB presence. BTCP decreases significantly between week 1 (23%) and week

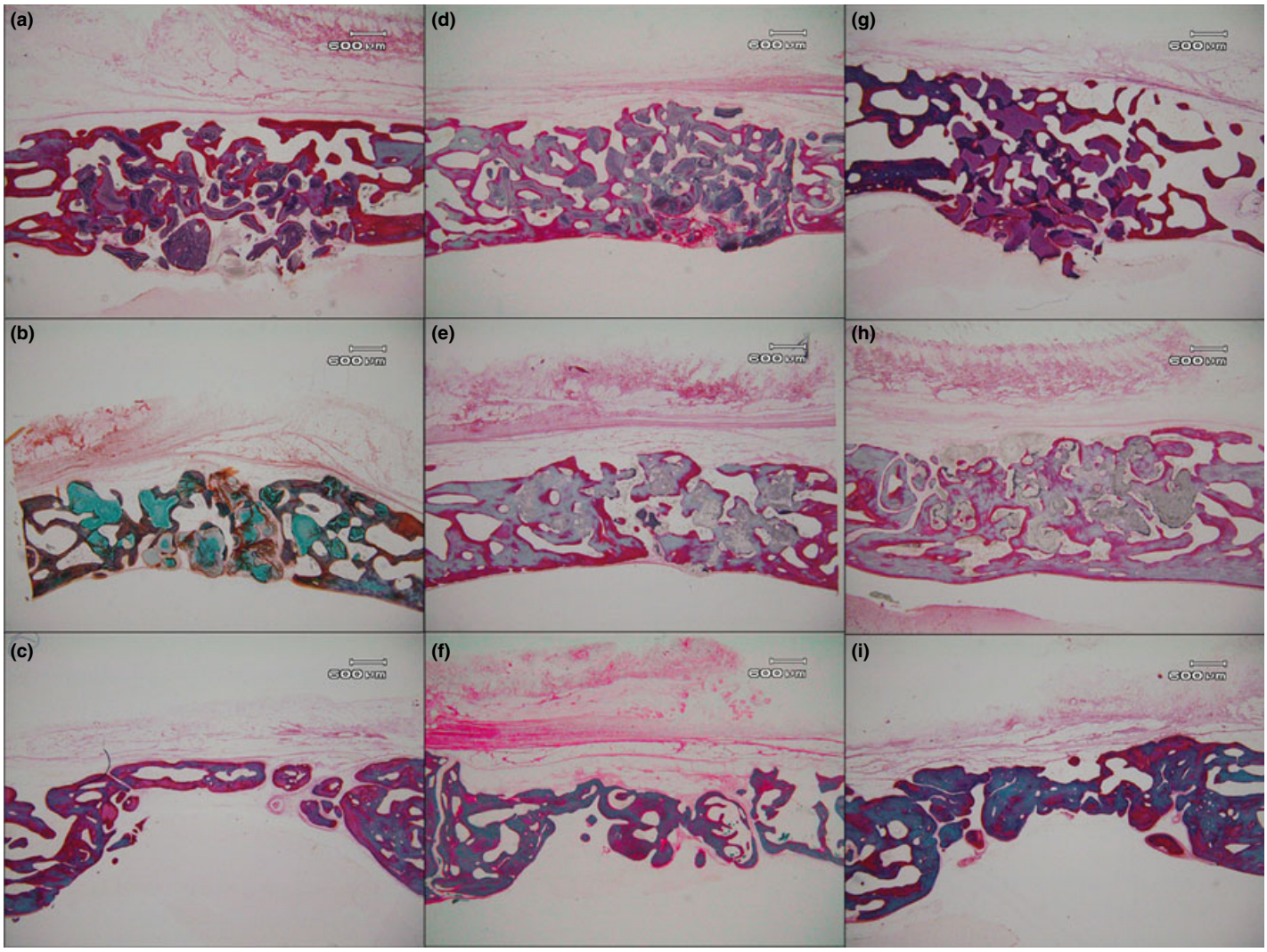

Fig. 6. Left column: Histological overview sections after 16 weeks. (a) Deproteinized bovine bone (DBB) defect, stronger consolidation in the outer zone and lower bone formation in the inner zone. (b) Beta-tricalcium phosphate (BTCP) defect, there is a quite complete bridging in the outer and in the inner layer. (c) control, observe the thin plate of new bone in the outer zone, collagen membrane is completely reabsorbed. Middle column: Histological overview sections at 32 weeks. (d) DBB defect, thin lamellar layer of new bone bridges the remaining DBB particles. (e) BTCP defect, particles appear to be quite reabsorbed. (f) control, closure of bone in the defect, there is a volumetric collapse. Right column: Histological overview sections at 52 weeks. (g) DBB defect, note the thin trabeculae surrounding the particles, and the lower integration in the material placed in the supra dura mater zone. (h) BTCP defect, note the close integration of the remaining material in the new formed trabecular structure. (i) control, closure of the defect; note the volumetric shrinkage.

16 (10%), slowing its resorption down in the following weeks (Fig 9).

Soft tissue: a ca. 20% significant reduction in soft tissue appears in every site from week 1 to week 4, which could be attributed to the reduction in postoperative oedema and to the organization of the wounded sites that underwent an initial shrinking.

Transversal study

Bone: bone fraction becomes significantly larger after week 32 in BTCP sites (61.6%) when compared to DBB sites (44%) and to the control sites (53%), there is a borderline signification ($P = 0.06$). Bone fraction in DBB sites exhibits smaller standard deviation than BTCP sites or control sites within the duration of this study (Fig 10). It is also worth to mention that the deviation of the data gets reduced within the time.

Material: after week 16, BTCP proportion (10%) becomes smaller than DBB proportion (20%), and this difference lasts until week 52.

Soft tissue: in sites filled with BGS, the smaller proportion of soft tissue becomes significant after week 4, due to the sum of BGS and new bone (mineralized area) in contrast to the absence of BGS in the control sites. The lowest values for soft tissue after week 8 are found in BTCP sites (28.1–33.4%) with no significant difference to DBB sites (30.7–35.5%).

In the sight of the findings above described, both hypotheses are rejected.

Discussion

This study shows that bone growth pattern is different for both BGS. Bone formation is faster in places grafted with DBB, where bone fraction is significantly smaller in the long term compared to BTCP sites and also than in control sites. The sites grafted with BTCP exhibit faster rebuilding of endocraneal layer that sites grafted with DBB. Bone growth is faster in the outer layer than in the inner layer for both materials.

In BTCP sites, bone fraction is significantly larger than bone fraction grown around DBB. This is in agreement with previous reports (Buser et al. 1998; Artzi et al. 2004; Jensen et al. 2006) and introduces the suspicion that

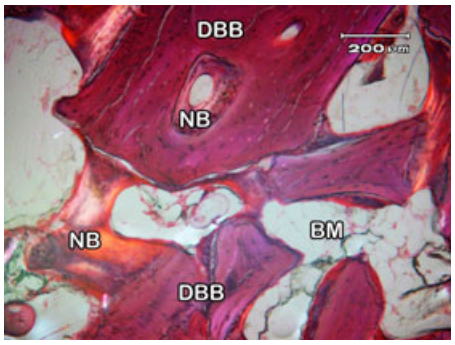

Fig. 7. Defect filled with deproteinized bovine bone (DBB) after 16 weeks. Polarized light allows to distingue the graft remaining particles to the thin surrounding new formed trabeculae (orange colour NB). Few trabeculae presented parallel-fibred structure. The main part of new bone is woven structured. Hawersian-like structures of new bone grow inside the old DBB vascular structures. Mature bone marrow (BM) can be observed between the bone and the particle structure.

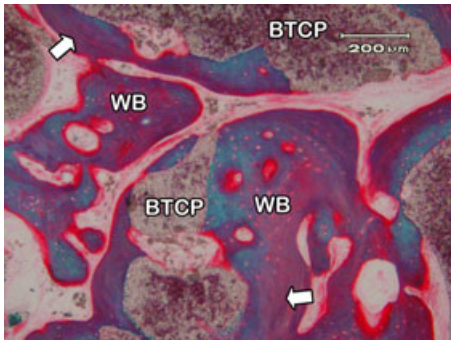

Fig. 8. Newly formed bone surrounding the remaining β-tricalcium phosphate particles after 16 weeks, a close interface is developed; notice the osteoblastic activity in the purple border surrounding the bone trabeculae. Parallel fibred bone (arrows) starts to develop near the predominant woven bone (WB).

bone formation is intimately related to the degradation of BTCP particles (Samavedi et al. 2013). This bone formation takes place while those particles are still present (Cheng et al. 2013) and this bone fraction being even larger than in the control sites. As a consequence of the coexistence of BGS and bone fraction, these sites exhibit a significantly smaller fraction of soft tissue (Artzi et al. 2004; Jensen et al. 2006). The increment of the mineralized areas in BTCP sites would reinforce the hypothesis that BTCP acts positively over osteoblastic activity (Knabe et al. 2000; Dalcusi et al. 2003; Cheng et al. 2013; Samavedi et al. 2013). Unlike in DBB sites, the bone growth in areas next to dura mater takes place at early stages in BTCP sites. It is worth to highlight that in this work, we have observed that early bone formation in DBB sites takes place only under collagen

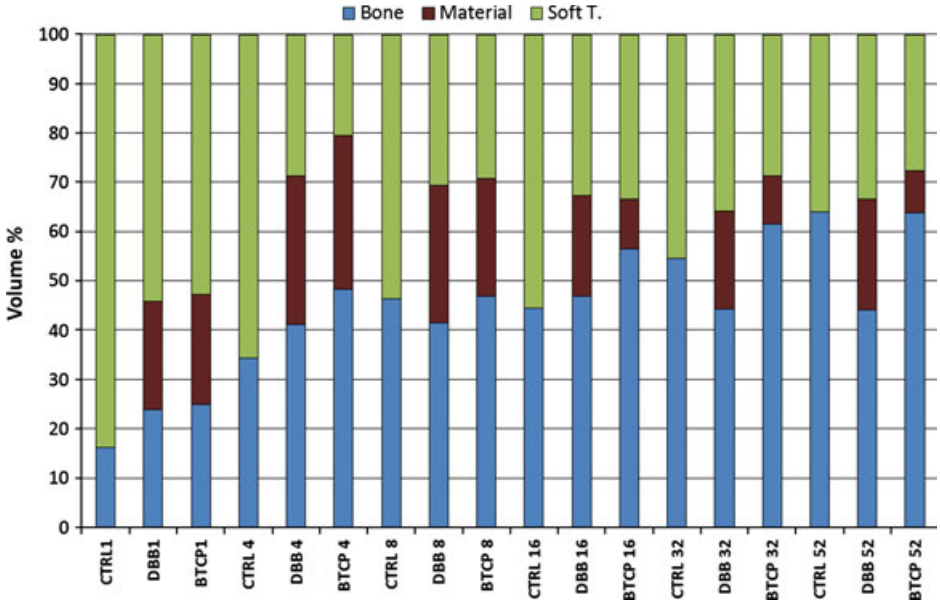

Fig. 9. Histomorphometrical study of the mean volume fractions of bone, grafting material and soft tissue occupying the defects: from 1 week to 52 weeks. CTRL: control, DBB: deproteinized bovine bone, BTCP: beta-tricalcium phosphate.

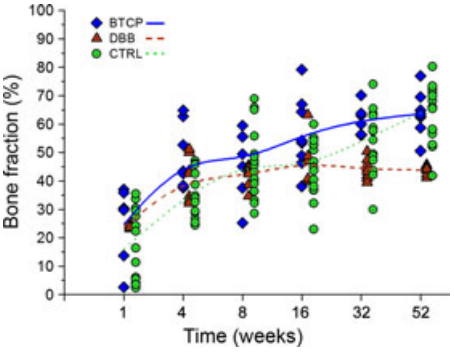

Fig. 10. Scattered plot graphic representation for the whole data of the bone fraction for beta-tricalcium phosphate (BTCP), deproteinized bovine bone (DBB) and control (CTRL) sites vs. time. The mean variations are represented by the lines (BTCP: continuous line, DBB: dashed line and CTRL: dotted line).

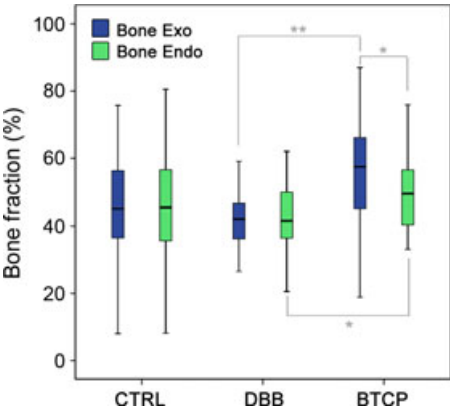

Fig. 11. Box and whisker plots for bone growth in the outer zone (Bone Exo) vs. the inner zone (Bone Endo) for the three sites.*:$P < 0.05$ and **:$P < 0.001$.

membrane at the outer layer, and bone growth at the inner layer takes place in a later period, being the bone growth at last term the same in both layers. No presence of fibrous growth has been detected around DBB particles which are not covered by collagen membrane and are not involved in the bone growth (Donos et al. 2004). Previous works refer to the positive interaction of collagen membrane over bone growth (Wang et al. 2002; von Arx et al. 2005) which would act, mainly at early stages, as an osteopromotive agent (Tarnow et al. 2000; Artzi et al. 2004), by transforming undifferentiated stroma into osteogenous tissue and therefore promoting cellular migration (Taguchi et al. 2004). The action of membrane as a booster of the bone growth is inferred by the complete closing of the defect in control sites. Even though there is a partial collapse of the collagen membrane (Hammerle & Jung 2003), it induces reparation of the bone network in the submembrane gap and, for some authors, it is the principal element in the defect bone healing (Donos et al. 2004). The absence of BGS to prevent membrane collapse ends in an incomplete restoration of the bone volume, being detectable a concavity in the outer layer (Fig 6i) (Hämmerle et al. 1998; von Arx et al. 2005; Artzi et al. 2004) and, as presented in this work, volumetric restorations not *ad integrum*.

Bone growth around DBB particles seems to be more regular and homogeneous, exhibiting smaller standard deviations in the

measurements than BTCP. It is worthwhile pointing out that rapid bone growth rates measured in DBB sites get stabilized after week 8. From that moment on, bone maturation processes and qualitative changes in the organization of the trabecular network take place, but not any significant increase in bone fraction. Both BGS exhibit a different bone growth pattern. DBB eases bone growth, creating a bone network around particles. In the long term, there are DBB particles linked by lamellar bone (Piatelli et al. 1999; Jensen et al. 2006). As previously stated by Jensen et al. (2005), the presence of bovine bone particles reduces bone growth when this DBB-bone composite material is created (Donos et al. 2004). Due to their micro-architecture, DBB particles are completely integrated into bone matrix, becoming part of the intrinsic neo formed trabecular structure. As our work has shown, their role is not completely passive, because in the periferic osteocitarian lacunae of DBB, there is colonization by host cell with morphology similar to osteocytes (Piatelli et al. 1999; Martinez et al. 2010). Vascular structures are frequently observed to colonize Havers channels found in DBB particles. Therefore, DBB particles could have become integrated as an active part of the mecano-receptor system of host's bone integrated by osteocytes (Turner 2004). The low resorption of DBB particles after 16 week supports such possibility. There is a preliminary stage where there are erosion processes over DBB particles, with the presence of resorptive cells (Fig 4a); this process has been defined as an initial cleaning of the graft surface before a bone deposition process (Piatelli et al. 1999; Jensen et al. 2006); however, after this initial cleaning, DBB particles remain as a not resorbed element which maintains bone fraction below the values exhibited by control group, giving the impression that contains bone formation.

Long-lasting presence of DBB particles in neo formed bone, even though preserve the initial volume, also has the capability of blocking the bone remodelling process, as it has already pointed out (Araujo et al. 2011). In the present work, the impairing of block remodelling in the long term can be seen in the protrusion of the inner tabula in the encephalic cavity (Fig 6a,g). Resorption zones or bone remodelling areas are hardly attested in DBB sites in the long term.

BTCP exhibits a completely different pattern for bone growth. Even though we have employed a low resorption BTCP in this study (Ghanaati et al. 2010), material disintegration has been observed. According to the literature, BTCP degradation occurs due to the activity of reticulo-endothelial system (RES) and material disintegration itself (Fig 5) (Yamada et al. 1997; Artzi et al. 2004; von Doenrberg et al. 2006; Jensen et al. 2006). There are parts of the material which are presumably more stable due to a better crystallization within sintering process. Such areas possess a suitable microporosity, and bone matrix is easily attached to their surface (Martinez et al. 2010). Other areas in the particles exhibit fracture surfaces due to fabrication process and result more soluble. This vehiculization of the material makes that the bone in direct contact with BTCP be mainly woven bone. It becomes completely a mature bone when BTCP fraction is smaller than 10% volume and the material disaggregation stabilizes (week 32). Bone remodelling and BMU presence are more evident in BTCP sites than in DBB ones. Quite likely, BTCP will be completely resorbed (Artzi et al. 2004), and bone will be completely remodelled when considering longer periods (Bain & Gross 2005). Besides, it is remarkable that the low solubility exhibited by the employed BTCP in this work, compared to other reported results (Artzi et al. 2004), which favours the establishment of fairly stable interfaces (Fig. 8). BTCP exhibits a high capability to stimulate bone formation, having even a higher bone fraction of the mineralized area than control sites. As already shown in this study, this capability promotes the bone formation both in the outer layer (under the membrane) and in the inner layer (over dura mater), allowing the complete closure of inner and outer cortical tabula sooner than in other sites. In the present work, BTCP shows a great osteogenic capability for new bone formation, even in those areas with lower osteogenic potential.

Due to the subcritical subcritical size, the model followed in our work allows a complete closure of the defects within the examined time (Hollinger & Kleinschmidt 1990). The removal of the inner cortical layer, exposing the dura mater membrane, creates a defect that provides osteogenic supply only from lateral walls, resulting equivalent to a two defect walls. Covering the defect with collagen, the membrane eases the defect closure (von Arx et al. 2005) allowing to make a comparison of osteogenic activity under the collagen membrane vs. inner layer over dura mater. One pole of the defect is under the collagen membrane which acts as a bridge favouring the jump of osteoprogenitor cells from bone walls, whereas the other pole is only limited by dura mater and encephalic membranes. Even though the collagen membrane lasts for only 4 and 8 weeks, it exhibit an early activity which conditions the defect bone healing (Wang et al. 2002; Artzi et al. 2004; von Arx et al. 2005) This seems to be a definitive element for bone regeneration. However, the relevant role played by the collagen membrane is possibly conditioned by many other factors, not fully clarified, for example the nature of the employed BGS. BTCP is less sensitive to coverage with collagen membrane (Schulten et al. 2013). It is worth remarking that the bone metabolism in the rabbits is faster than in humans (Pearce et al. 2007), so the timeline described in this work will probably be longer when grafting in human bone, which reinforces the importance of this long-term studies in animals.

Bearing in mind the limitations of the present study, more precisely the reduced size of the sample, it is noteworthy that the response trends exhibited by these BGS are different along the time.

Conclusions

Collagen membrane plays an important role in the defect bone healing because bone growth occurs earlier in the layer covered by a membrane. However, the nature of the graft substitute also plays a relevant role in the amount of bone formed. Bone growth is significantly faster in DBB sites, but, in opposition to BTCP sites, the presence of the DBB remnant particles seems to diminish the bone fraction in the long term. In the defect sites grafted with BTCP, more bone was observed in comparison with DBB site, and endocraneal layer is restored faster.

Acknowledgements: The authors wish to thank Dr Jaime Franco for his outstanding assessment and advice in the characterization and properties of the BGS. We are also grateful to Mr. Ruben Corral for his valuable assistance preparing the histologic sections and the two anonymous reviewers which have enriched this manuscript with their valuable comments and advice. The study was supported by grants from the Regional Government of Galicia (Xunta de Galicia PGIDT09TMT003239PR) and the Spanish Government (CENIT INTELIMPLANT, CDTI). None of the authors have any financial or commercial interest in any of the investigated biomaterials or products employed in this study.

References

Albert, A., Leemrijse, T., Druez, V., Delloye, C. & Cornu, O. (2006) Are bone autografts still necessary in 2006? A three-year retrospective study of bone grafting. *Acta Orthopaedica Belgica* **72**: 734–740.

Araujo, M.G., Linder, E. & Lindhe, J. (2011) Bio-Oss collagen in the buccal gap at inmediate implants: a 6-month study in the dog. *Journal of Clinical Oral Implants Research* **22**: 1–8.

Artzi, Z., Weinreb, M., Carmeli, G., Lev-Dor, R., Dard, M. & Nemcovsky, C.E. (2008) Histomorphometric assessment of bone formation in sinus augmentation utilizing a combination of autogenous and hydroxyapatite/biphasic tricalcium phosphate graft materials: at 6 and 9 months in humans. *Journal of Clinical Oral Implants Research* **19**: 686–692.

Artzi, Z., Weinreb, M., Givol, N., Rohrer, M.D., Nemcovsky, C.E., Prasad, H.S. & Tal, H. (2004) Biomaterial resorption rate and healing site morphology of inorganic bovine bone and β_tricalcium phosphate in the canine: a 24-month longitudinal histologic study and morphometric analysis. *The International Journal of Oral & Maxillofacial Implants* **19**: 357–368.

von Arx, T., Broggini, N., Jensen, S.S., Bornstein, M.M., Schenk, R.K. & Buser, D. (2005) Membrane durability and tissue response of different bioresorbable barrier membranes: a histologic study in rabbit calvarium. *The International Journal of Oral & Maxillofacial Implants* **20**: 843–853.

Bain, S.D. & Gross, T.S. (2005) Structural aspects of bone resorption. In: Bronner, F., Frach-Carson, M.C. & Rubin, J., eds. Bone Resorption, 58–66. London:Springer-Verlag

Bayley, R.C. & Byrnes, J. (1990) A new, old method for assessing measurement error in both univariate and multivariate morphometric studies. *Systematic Zoology*, **39**: 124–130.

Benezra Rosen, V., Hobbs, L.W. & Spector, M. (2002) The ultraestructure of anorganic bovine bone and selected synthetic hydroxyapatites used as bone graft substitute materials. *Biomaterials* **23**: 921–928.

Berglundh, T. & Lindhe, J. (1997) Healing around implants placed in bone defects treated with Bio-Oss. *Journal of Clinical Oral Implants Research* **8**: 117–124.

Busenlechner, D., Tangl, S., Mair, B., Fugger, G., Gruber, R., Redl, H. & Watzek, G. (2008) Simultaneous in vivo comparison of bone substitutes in a guided bone regeneration model. *Biomaterials* **29**: 3195–3200.

Buser, D., Hoffmann, B., Bernard, J.P., Lussi, A., Mettler, D. & Shenk, R.K. (1998) Evaluation of filling materials in membrane-protected bone defects A comparative histomorphometric study in the mandible of miniature pigs. *Journal of Clinical Oral Implants Research* **9**: 137–150.

Chen, F.M., Zhang, J., Zhang, M., An, Y., Chen, F. & Wu, Z.F. (2010) A review on endogenous regenerative technology in periodontal regenerative medicine. *Biomaterials* **31**: 7892–7927.

Cheng, L., Shi, Y., Ye, F. & Bu, H. (2013) Osteoinduction of calcium phosphate biomaterials in small animals. *Materials Science and Engineering: C* **33**: 1254–1260.

Dalcusi, G., Laboux, O., Malard, O. & Weiss, P. (2003) Current state of the art of biphasic calcium phosphate bioceramics. *Journal of Materials Science: Materials in Medicine* **14**: 195–200.

von Doenrberg, M.C., von Rechenberg, B., Bohner, M., Grünenfelder, S., van Lenthe, G.H., Mueller, R., Gasser, B., Mathys, R., Baroud, G. & Auer, J. (2006) *In vivo* behavior of calcium phosphate scaffolds with four different pore sizes. *Biomaterials* **27**: 5186–5198.

Donath, K. & Breuner, G.A. (1982) A method for the study of undecalcified bones and teeth with attached soft tissues. The Säge-Schliff (sawing and grinding) technique. *Journal of Oral Pathology* **11**: 318–326.

Donos, N., Lang, N.P., Karoussis, I.K., Bosshardt, D., Tonetti, M. & Kostopoulos, L. (2004) Effect of GBR in combination with deproteinized bovine bone mineral and/or enamel matrix proteins on the healing of critical size defects. *Journal of Clinical Oral Implants Research* **15**: 101–111.

Ghanaati, S., Barbeck, M., Orth, C., Willesrshausen, I., Thimm, B.W., Hoffmann, C., Rasic, A., Sader, R., Unger, R.E. & Peters, F. (2010) Influence of β-tricalcium phosphate granule size and morphology on tissue reaction *in vivo*. *Acta Biomaterialia* **6**: 4476–4487.

Guizzardi, S., Raspanti, M., Martini, D., Scandroglio, R., Govoni, P. & Ruggeri, A. (1995) Low-temperature heat-deproteinated compact bone to heal large bone defects. *Biomaterials* **16**: 931–936.

Haas, R., Mailath, G., Dörbudak, O. & Watzek, G. (1998) Bovine hydroxyapatite for maxillary sinus augmentation: analysis of interfacial bond strength of dental implants using pull-out tests. *Journal of Clinical Oral Implants Research* **9**: 117–122.

Hämmerle, C.H., Chiantella, G.C., Karring, T. & Lang, N.P. (1998) The effect of a deproteinized bovine bone mineral on bone regeneration around titanium dental implants. *Journal of Clinical Oral Implants Research* **9**: 151–162.

Hammerle, C.H. & Jung, R.E. (2003) Bone augmentation by means of barrier membranes. *Periodontology 2000* **33**: 36–53.

Hollinger, J.O. & Kleinschmidt, J.C. (1990) The critical size defect as an experimental model to test bone repair materials. *Journal of Craniofacial Surgery* **1**: 60–68.

Jensen, S.S., Broggini, N., Hjörting-Hansen, E., Schenk, R. & Buser, D. (2006) Bone healing and graft resorption of autograft, anorganic bovine bone and β-tricalcium phosphate. A histologic and histomorphometric study in the mandibles of minipigs. *Journal of Clinical Oral Implants Research* **17**: 237–243.

Jensen, S.S., Broggini, N., Weibrich, G., Hjörting-Hansen, E., Schenk, R. & Buser, D. (2005) Bone regeneration in standardized bone defects with autografts or bone substitutes in combination with platelet concentrate: a histologic and histomorphometric study in the mandibles of minipigs. *The International Journal of Oral & Maxillofacial Implants* **20**: 703–712.

Johanson, B., Grepe, A., Wannfors, K. & Hirsch, J.M. (2001) A clinical study of changes in the volume of bone grafts in the atrophic maxilla. *Dentomaxillofacial Radiology* **30**: 157–161.

Klijn, R.J., Meijer, G.J., Bronkhorst, E.M. & Jansen, J.A. (2010) A meta-analysis of histomorphometric results and graft healing time for various biomaterials compared to autologous bone used as sinus floor augmentation material in humans. *Tissue Engieneering Part B Reviews* **16**: 493–507.

Knabe, C., Driessens, F.C., Planell, J.A., Gildenhaar, R., Berger, G., Reif, D., Fitzner, R., Radlanski, R.J. & Gross, U. (2000) Evaluation of calcium phosphates and experimental calcium phosphate bone cements using osteogenic cultures. *Journal of Biomedical Materials Research* **52**: 498–508.

Lekholm, U., Ericsson, I., Adell, R. & Slots, J. (1986) The condition of the soft tissues at tooth and fixture abutments supporting fixed bridges. *Journal of Clinical Periodontology* **13**: 558–562.

Martinez, A., Franco, J., Saiz, E. & Guitian, F. (2010) Maxillary sinus floor augmentation on humans: packing simulations and 8 months histomorphometric comparative study of anorganic bone matrix and B-ticalcium phosphate particles as grafting material. *Materials Science and Engineering: C* **30**: 763–769.

Misch, C.E. & Dietsh, F. (1994) Endosteal implants and iliac crest grafts to restore severely resorbed totally edentulous maxillae. A retrospective study. *Journal of Oral Implantology* **20**: 100–110.

Orr, T.E., Villars, P.A., Mitchell, S.L., Hsu, H.-P. & Spector, M. (2001) Compressive properties of cancellous bone defects in a rabbit model treated with particles of natural bone mineral and synthetic hydroxyapatite. *Biomaterials* **22**: 1953–1959.

Pearce, A.I., Richards, R.G., Milz, S., Schneider, E. & Pearce, S.G. (2007) Animal models for implant biomaterial research in bone: a review. *European Cells and Materials* **13**: 1–10.

Piatelli, M., Favero, G.A., Scarano, A., Orsini, G. & Piatelli, A. (1999) Bone reactions to anorganic bovine bone (Bio-Oss) used in sinus augmentation procedures: a histologic long-term report of 20 cases in humans. *The International Journal of Oral & Maxillofacial Implants* **14**: 835–840.

Rasband, W.S. (2012) ImageJ. U. S. National Institutes of Health:Bethesda, MD, USA, http://imagej.nih.gov/ij/, 1997–2012.

Samavedi, S., Whittington, A.R. & Goldstein, A.S. (2013) Calcium phosphate ceramics in bone tissue engineering: a review of properties and their influence on cell behavior. *Acta Biomaterialia* **9**: 8037–8045.

Schmitt, C., Karasholi, T., Lutz, R., Wiltfang, J., Neukam, F.W. & Schlegel, K.A. (2012) Long-term changes in graft height after maxillary sinus augmentation, onlay bone grafting, and combination of both techniques: a long-term retrospective cohort study. *Journal of Clinical Oral Implants Research* **25**: 38–46.

Schlegel, K.A., Fichtner, G., Schultze-Mosgau, S. & Wilfang, J. (2003) Histologic findings in sinus augmentation with autogenous bone chips versus a

bovine bone substitute. *International Journal of Oral and Maxillofacial Implants* **18**: 53–58.

Schulten, E.A.J.M., Prins, H.J., Overman, J.R., Helder, M.N., Bruggenkate, C.M. & Klein-Nulend, J. (2013) A novel approach revealing the effect os a collagenous membrane on osteoconduction in maxillary sinus floor elevation with β-Tricalcium Phosphate. *European Cells and Materials* **25**: 215–228.

Taguchi, Y., Amizuka, N., Nakadate, M., Ohnishi, H., Fujii, N., Oda, K., Nomura, S. & Maeda, T. (2004) A histological evaluation for guided bone regeneration induced by collagenous membrane. *Biomaterials* **26**: 6158–6166.

Tarnow, D.P., Wallace, S.S., Froum, S.J., Rhorer, M.D. & Cho, S.-C. (2000) Histologic and clinical comparison of bilateral sinus floor elevations with and without barrier membrane placement in 12 patients: part 3 of an ongoing prospective study. *The International Journal of Periodontics and Restorative Dentistry* **20**: 116–125.

Turner, C.H. (2004) Biomechanical aspects of bone formation. In: Bronner, F. & Farach-Carson, M.C., eds. Bone Formation, 79–105. London. Springer-Verlag

Wang, H.L., Miyauchi, M. & Tanaka, T. (2002) Initial attachments of osteoblasts to various guided bone regeneration membranes: an *in vitro* study. *Journal of Periodontal Research* **37**: 340–344.

Yamada, S., Heymann, D., Bouler, J.M. & Dalcusi, G. (1997) Osteoclastic resorption of calcium phosphate ceramics with different hydroxyapatite/beta-tricalcium phosphate ratios. *Biomaterials* **18**: 1037–1041.