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Experimental model of bone response to xenografts of bovine origin (Endobon[®]): a radiological and histomorphometric study

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Abstract

Objective: To carry out a radiological and histomorphometric evaluation of bone response to bovine bone implants inserted in rabbits' tibiae.

Materials and Methods: Twenty New Zealand rabbits weighing 3900–4500 g were used. Twenty bovine bone implants (Endobon[®]) in granulated form of 500–1000 µm granulometry were inserted in the proximal metaphyseal area of the animals' right tibia and 20 control areas were located in the proximal metaphyseal area. Following implantation, the animals were sacrificed in four groups of five, after 1 month, 2 months, 3 months and 4 months, respectively. Anteroposterior and lateral radiographs were taken. Samples were sectioned at 5 µm and stained using Hematoxylin-Eosin and Masson's trichromic.

Results: After 4 months, radiological images showed complete repair of the bone defects. No healed or residual bone alterations attributable to the presence of the implant were observed. Histomorphometric analysis at 4 months showed the presence of a higher density of newly formed bone with mean values for new bone, residual graft material and non-mineralized connective tissue of $22.8 \pm 1.5\%$, $39.4 \pm 2.3\%$ and $37.7 \pm 2.5\%$. There were no statistically significant differences in the length of cortical formation with bovine bone, $98.8 \pm 1.1\%$, compared with the control group, $99.1 \pm 0.7\%$, at the end of the study period.

Conclusions: The biomaterial used in the study was shown to be biocompatible, osteoconductive and non-resorbable and as such a possible bone substitute that does not interfere with normal reparative bone processes.

Bone tissue substitution or replacement has been a subject of widespread research for many years due to its importance in buccal and maxillofacial surgery, given the obvious need to substitute bone tissue in certain situations (McAllister & Haghighat 2007; Esposito et al. 2010). Current therapies both in traumatology and tissue regeneration dentistry are based on the use of artificial or natural materials, which produce stimulating signals that trigger physiological regeneration (Ersanli et al. 2004; Esposito et al. 2006; Aghaloo & Moy 2007), a process dependent on three mechanisms: osteogenesis, osteoinduction and osteoconduction. The ideal bone substitute that offers these three is autologous bone, the "gold standard" for regeneration (Schlegel et al. 2003; Barone & Covani 2007; Johansson et al. 2010). Nevertheless, given the intrinsic difficulties involved in both autologous bone substitution and allografts (Nkenke et al. 2002; Cricchio & Lundgren 2003; Sasso et al.

2005), the quest for other alternatives is a necessity. Xenografts, defatted and deproteinated in order to reduce immune response, offer one such alternative. These would appear to be biocompatible and are presently a component of various bone graft preparations (Arcuri et al. 2005). Their source may be bovine (Piatelli et al. 1999; Norton et al. 2003; Worth et al. 2005), porcine (Barone et al. 2005; Orsini et al. 2006; Nannmark & Sennerby 2008) or equine (Di Stefano et al. 2009).

The grafting material used in this study was RegenerOss[™] BIOMET3i Endobon[®]. This is a porous ceramic hydroxyapatite that has been used successfully for bone replacement in clinical applications including orthopedic and maxillofacial procedures since 1989 (Hing et al. 1997; Briem et al. 2002). Endobon[®] has a wide range of applications including the repair of bone defects arising from fractures, bone cysts, arthrodeses and bone tumors (Kehr & Gosset 2000; Werber et al. 2000;

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Grimm et al. 2001; Motomiya et al. 2007). Based on these premises, and given that a number of previous studies have demonstrated the effectiveness of xenografts as osteoconductive matrices (Wiltfang et al. 1996; Hing et al. 1997; Gierse & Donath 1999; Bareille et al. 2000; Kehr & Gosset 2000; Baer et al. 2002; Tamai et al. 2002; Tadic & Epple 2004), the present study investigates this xenograft of bovine origin, as a possible substitute for bone grafting.

Materials and methods

Animals, surgery and treatment

A total of 20 Endobon (RegenerOss, Biomet 3i, Palm Beach Gardens, FL, USA) implants were placed in the proximal metaphyseal area of the right tibiae of twenty albino New Zealand rabbits of 30–35 weeks of age and weighing 3900–4500 g. All experiments were approved and performed according to the Spanish Government Guidelines and European Community Guidelines for animal care.

Fifteen minutes before general anesthesia, the animals received an intramuscular injection of 0.5–1 mg/kg acepromazine maleate, an anxiolytic. General anesthesia included 5–8 mg/kg ketamine plus chlorbutol administered intravenously, 0.5–1 mg/kg acepromazine maleate as coadjuvant and 0.05 mg/kg atropine. Amoxicillin (0.1 ml/kg intramuscularly) was administered at the end of surgery.

The internal approach was performed in the proximal metaphyseal–diaphyseal area of each tibia, several millimeters below the anterior tibial tuberosity. The removal of bone tissue to form two concave defects approximately 4 mm in diameter per tibia was carried out using spherical surgical drills at low speed with constant irrigation. The first was filled with the granulate-like xenograft and the second was used as a control site (Fig. 1).

The five rabbits in each group were sacrificed by means of an intracardiac overdose of thiopental at: 1 month (Group I), 2 months (Group II), 3 months (Group III) and 4 months (Group IV) following implantation.

Radiological study

Two X-rays, anteroposterior and lateral, were taken of the section of bone containing implants using the Kodak RVG 6100 Digital Radiography System (Kodak DS, Rochester, NY, USA) with X-rays taken at 32 kV, 40 mA, using an automatic light meter. Radiovisiographs were taken of each transversal tibia section containing implants. Radiographs were taken of all groups.

Optical microscopy

The surgically acquired samples were fixed in 10% neutral buffered formalin and decalcified by

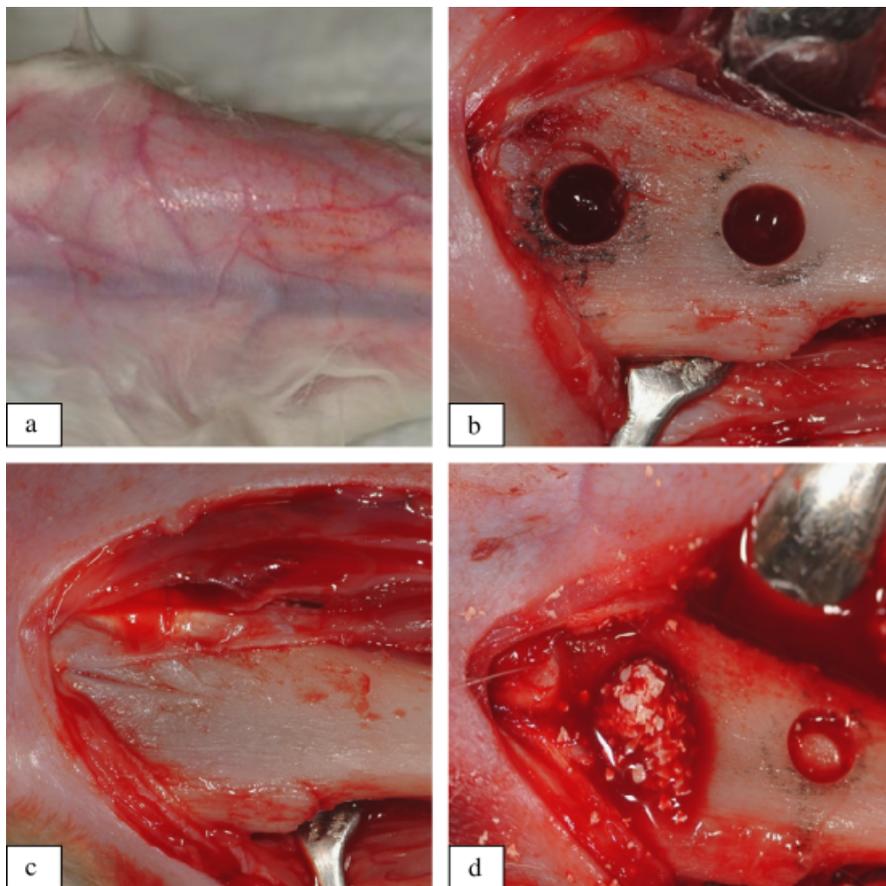


Fig. 1. (a) Tibiae anterior interior proximal zone. (b) Surgical incision taking proximal and distal protuberances of tibial tuberosity as reference points. (c) Cortical Perforation with a 4 mm trephine bur as far as the left tibial medullar canal. (d) Placement of Endobon[®] xenograft of porcine origin in upper orifice; a second unfilled orifice acting as control sample.

means of immersion in Osteomol[®] (Merck KbaA, Whitehouse Station, NJ, USA) containing HCl (10%) and CH₂O (4%) for 17 days, renewing the solution every 24 h. Subsequently, all samples were embedded in paraffin via the usual method, sectioned at 5 µm and stained using Hematoxylin–Eosin, Masson's trichromic, which is the most effective and convenient stain for revealing young lamellar bone. All samples were examined under light microscopy (Microphoto FXA, Nikon, Tokyo, Japan). The entire circumference of each section (containing bone, grafted particles and connective tissue) was traced manually to create individual regions of interest.

Morphometric analysis

Histomorphometric evaluations comprised measurements of the areas of bone and bovine particles in relation to the total measurement area. The central portion of each core was selected in order to avoid any potential bias; in this way, both the coronal (native host's remaining bone) and the apical portion (using a safe margin of 1.5–2 mm) were excluded from analysis. Histomorphometric measurement of the samples was conducted using Image J software, developed

by the National Institute of Health of the United States of America. Values for the total percentage of newly formed bone, residual graft material and non-mineralized connective tissue were then calculated.

Examinations were performed under a Nikon Eclipse 80i microscope (Teknoptik AB, Huddinge, Sweden) equipped with the EasyImage 2000 system (Teknoptik AB) using $\times 1.0$ to $\times 4.0$ lenses for descriptive evaluation and for taking morphometric measurements. To calculate the percentage of cavity defect covered, images were generated using a Leica Z6 APO macroscope connected to a Leica DC 500 (Barcelona, Spain) digital camera and enlarged $\times 23$. These were used to digitalize and calibrate images of the defect cavity zone and then interactive measurements of the areas of interest were obtained using Leica Q Win V3 (Barcelona, Spain) image analysis software.

Statistical analysis

Firstly, factors such as individual difference and position of the implant could be excluded as not significant. Normality tests (Kolmogorov–Smirnov and Shapiro–Wilk) and tests for equality of

variances (Mann–Whitney and Wilcoxon) were applied without any observed violation assumptions. Variance analysis (ANOVA test), a parametric test, was used to identify significant mean differences and standard deviations using specialized software (SPSS 15.0 for Windows, Chicago, IL, USA). Mean values and standard deviations between samples were calculated for each variable. The significance level established was $P < 0.05$.

Results

1 month

Radiological study

Control bone defects showed radiotransparent concave depressions of round or rectangular morphology depending on the image studied. They had clear and regular outlines showing a homogenous density that clearly defined their boundaries (Fig. 2a).

X-rays revealed the characteristics of the implanted material as being a cylindrical element, with a 4×6 mm rectangular structure, whose radiological density allowing its identification within the trabecular bone structure in which it was implanted (Fig. 2b).

Optical microscopy

The most relevant morphological changes to the bone defect area in the control group were at the cortical level where an outer layer of fibrinohematic tissue covered the orifice (Fig. 2c, e and g).

At the implant sites, anatomopathological study highlighted the cortical bone; the surgical orifice had re-formed with a fine outer layer of fibrinohematic tissue interrupted mainly by granules of implant material. Optical microscopy of the implant and cortical defect site revealed the substitution of bone by granulated tissue extending towards the implant and invading the implanted material, which it partially furnished. This tissue was made up of numerous endothelial sprouts and capillary blood vessels, as well as abundant mesenchymal cells of irregular morphology with ample cytoplasm and numerous fibroblasts arranged randomly in a matrix of abundant fundamental substance, collagen fibers, macrophages and scattered lymphocytes (Fig. 2d, f and h).

Morphometric analysis

Histomorphometry showed that newly formed bone represented $18.5 \pm 1.5\%$, residual graft material $40.7 \pm 3.2\%$ and connective tissue $40.8 \pm 3.1\%$. The length of cortical formation at bovine bone implant sites was $63.7 \pm 3.7\%$ compared with $90.1 \pm 0.7\%$ at the control sites.

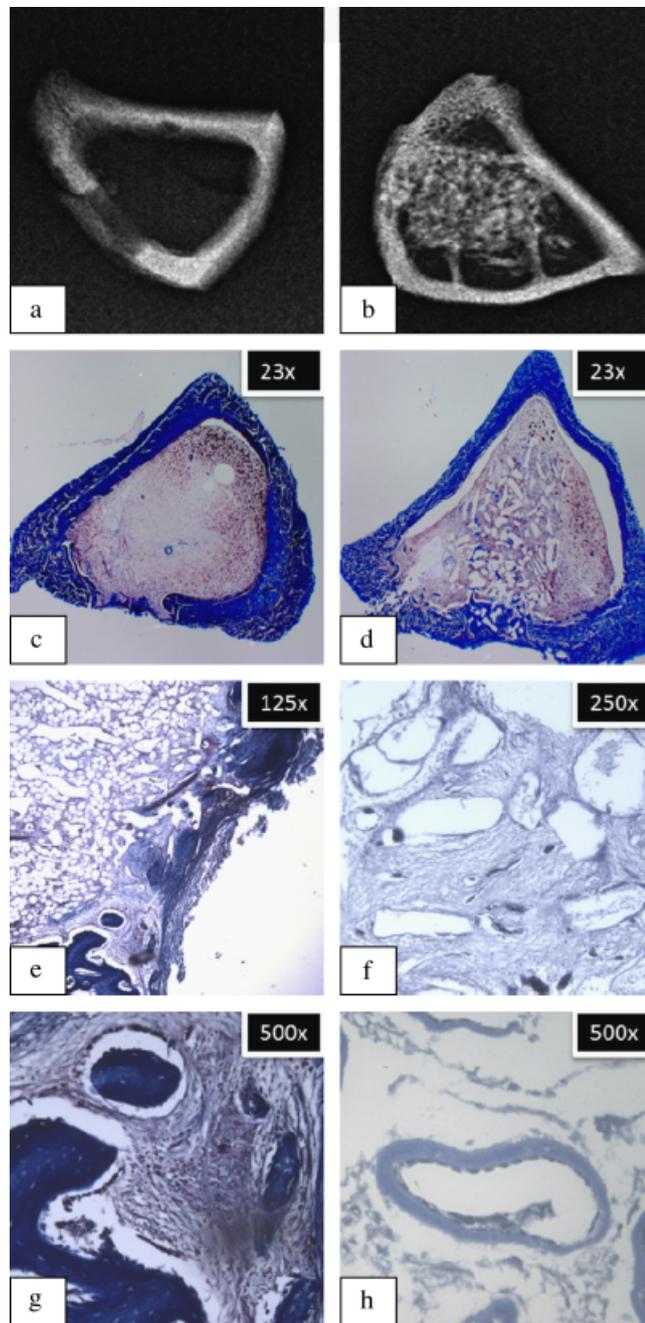


Fig. 2. 1 month: (a) Transversal section radiovisiograph of surgical specimen (control). (b) Transversal section radiovisiograph of surgical specimen (implanted material Endobon®). (c) Panoramic image $\times 23$ - HE Endobon®. (d) Panoramic image $\times 23$ - HE Endobon®. (e) Microscopy detail $\times 125$ - HE control. (f) Microscopy detail $\times 125$ - TM Endobon®. (g) Microscopy detail $\times 500$ - HE control. (h) Microscopy detail $\times 1000$ - TM Endobon®. HE, hematoxylin-eosin; TM, Masson's trichrome.

2 months

Radiological study

In the bone defects taken as control sites, some significant differences were observed when compared with the previous evaluation period. X-ray images revealed linear elements representing irregular trabecular lines that did not follow the axes or load forces of adjacent bone trabeculation. Some of these images were framed inside areas of greater radiotransparency, which at this point in time did not show the same

concave bone defect morphology as the study sites (Fig. 3a).

In the xenograft-filled bone defects X-rays highlighted the cortical-osteoblastic line as being completely repaired, albeit with less density than that of the adjacent cortical bone. The radiological density of this material was lower than that observed for the previous time-period, although a reinforced radiological density could imply the formation of bone around the implant. The spherical form of the implanted material

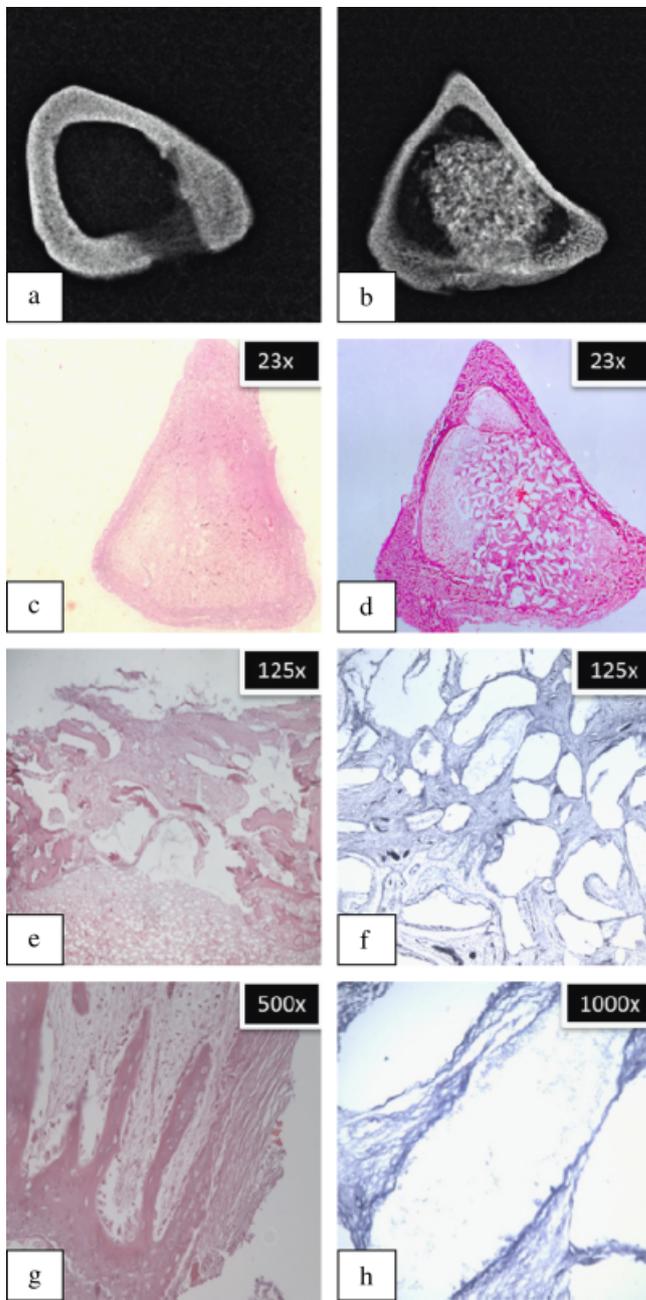


Fig. 3. 2 months: (a) Transversal section radiovisiograph of surgical specimen (control). (b) Transversal section radiovisiograph of surgical specimen (implanted material Endobon®). (c) Panoramic image $\times 23$ - TM control. (d) Panoramic image $\times 23$ - TM Endobon®. (e) Microscopy detail $\times 125$ - TM control. (f) Microscopy detail $\times 250$ - TM Endobon®. (g) Microscopy detail $\times 500$ - TM control. (h) Microscopy detail $\times 500$ - TM Endobon®. HE, hematoxylin-eosin; TM, Masson's trichrome.

had given way to a more oval and irregular shape (Fig. 3b).

Optical microscopy

In this study period, the control group showed noticeable bone repair phenomena around the defects' peripheries and around adjacent bone marrow (Fig. 3c, e and g).

The anatomopathological study highlighted the perforation made in the cortical bone in order to perform the xenograft as being almost completed by neoformed or immature osseous tissue.

Likewise, the implanted xenograft was surrounded by osseous trabeculae, which were more extensive, and thicker than those observed for the previous time-period, giving the implant zone a reticular appearance; no inflammatory response of note was observed (Fig. 3d, f and h).

Morphometric analysis

Histomorphometry showed that newly formed bone represented $21.7 \pm 1.5\%$, residual graft material $39.7 \pm 3.1\%$ and connective tissue $38.6 \pm 2.1\%$. The length of cortical formation

at bovine bone implant sites was $84 \pm 3.7\%$ compared with $92.3 \pm 1.9\%$ at the control sites.

3 months

Radiological study

After three months, X-ray images of control sites showed similar characteristics to the previous study period (Fig. 4a).

X-rays revealed the external cortex of the artificial osseous lagoons into which the bone-granulate implant had been introduced as having a calcium density similar to that of the adjacent cortex, making it difficult to identify the surgical orifice. At the level of the cortex, the implant area displayed a decreased radiological density with respect to the previous group as well as a more oval shape with a lower calcium density within. Well-defined borders could not be distinguished radiologically. In some areas there was continuity between the osseous cortex and the implanted material as manifested by linear images of osseous trabeculae (Fig. 4b).

Optical microscopy

Complete repair of the external cortex was observed with extensive bone regeneration phenomena which spread beyond the lower edge of the adjacent cortex (Fig. 4c, e and g).

Anatomopathological study showed complete bone reparation of the cortex at the implant orifice, manifested as well-organized trabecular bone with an increase in osseous remodeling. We also observed, albeit to a lesser degree, the formation of osseous trabeculae as well as a marked increase in haematopoietic and adipose bone marrow in the center, which had partially replaced the granulated tissues (Fig. 4d, f and h).

Morphometric analysis

Histomorphometry showed that newly formed bone represented $23.6 \pm 2.2\%$, residual graft material $38.9 \pm 3.5\%$ and connective tissue $37.5 \pm 1.5\%$. The length of cortical formation at bovine bone implant sites was $95.7 \pm 2.3\%$ compared with $96.5 \pm 2.1\%$ at the control sites.

4 months

Radiological study

X-rays of the control bone defects at the end of the experiment, showed characteristics similar to those described for the previous group with one or more rectilinear lines that could be observed traversing the bone perpendicularly (Fig. 5a).

X-rays of the implanted material produced images of an undefined geometric structure and a decrease in graft volume. Complete repair of the osseous defects was also observed. Trabeculae reaching the implant were greater in number

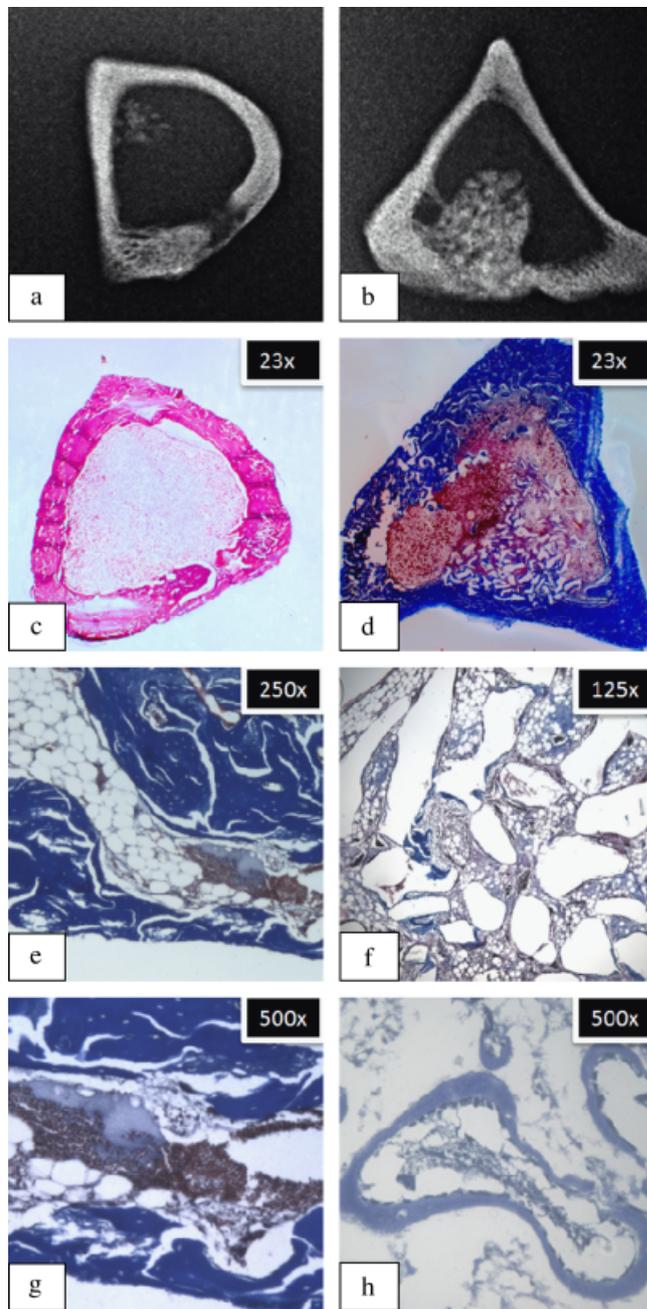


Fig. 4. 3 months: (a) Transversal section radiovisograph of surgical specimen (control). (b) Transversal section radiovisograph of surgical specimen (implanted material Endobon®). (c) Panoramic image $\times 23$ - HE control. (d) Panoramic image $\times 23$ - TM Endobon®. (e) Microscopy detail $\times 250$ - TM control. (f) Microscopy detail $\times 125$ - TM Endobon®. (g) Microscopy detail $\times 500$ - TM control. (h) Microscopy detail $\times 500$ - TM Endobon®. HE, hematoxylin-eosin; TM, Masson's trichrome.

and density than those of the previous time-period, giving the implanted area a slightly reticular appearance. No healed or residual bone alterations attributable to the presence of the implant were observed. Nor were any osseous malformations or structural changes to bone development observed over the study period (Fig. 5b).

Optical microscopy

Complete bone remodeling was observed at the graft sites. The anatomopathological study found

the presence of mature osseous bone in the cortex of the implant insertion site, so that it was not differentiable from the adjacent cortex (Fig. 5c, e and g).

We also observed osseous remodeling of osseous trabeculae around the implant, which was more pronounced in the proximity of the cortex. Morphometric measurements revealed increasing amounts of mineralized bone as time went on, with significant differences between the periods of evolution. Simultaneously, a parallel decrease in the area of bovine bone was observed in all the

groups. No statistical differences were found regarding resorption (Fig. 5d, f and h).

Morphometric analysis

Histomorphometry showed that newly formed bone represented $27.5 \pm 2.4\%$, residual graft material $38.3 \pm 2.3\%$ and connective tissue $34.2 \pm 3.4\%$. The length of cortical formation at bovine bone implant sites was $98.8 \pm 1.1\%$ compared with $99.1 \pm 0.7\%$ at control sites.

Table 1 shows the mean values for the total percentage of newly formed bone, residual graft material and non-mineralized connective tissue for all study periods. Cortical formation length increase values are shown in Table 2.

Discussion

Autologous grafts continue to be the best option because they contain viable cells that include bone marrow osteoprogenitor cells, collagenous and non-collagenous extracellular matrix and growth differentiating factors (Yamamichi et al. 2008). However, many clinicians seek alternatives (Cutter & Mehrara 2006) in order to avoid both secondary surgical zones (donor sites) (Nkenke et al. 2002) and undesirable post-operative stages (Cricchio & Lundgren 2003), and also to reduce surgical time. Nevertheless, studies carried out by (Barone & Covani 2007) of cortico-spongy autologous block grafts showed very low morbidity with patients experiencing slight pain up until the third day after surgery.

For many years now, bovine cortico-spongy bone has been the first choice for buccal and maxillofacial surgery. There are numerous studies that have shown the effectiveness of a variety of xenografts that incorporate biomaterials derived from bovine bone (Van Steenberghe et al. 2000; Hising et al. 2001; Norton et al. 2003; Worth et al. 2005). However, there are possible complications associated with their use such as viral transmission (Lang et al. 2000; Wenz et al. 2001).

The grafting material used in this study was RegenerOss™ BIOMET3i Endobon®, a hydroxyapatite ceramic derived from cancellous bovine bone, fully deproteinated by a two-step high temperature manufacturing process to avoid safety risks from bacteria, viruses and prions. This process consists of two stages: firstly pyrolysis at a temperature of $>900^\circ$ that eliminates the organic element and secondly a ceramization process at temperatures $>1200^\circ$ that creates a crystalline structure. The process ensures the absence of immunological reactions and guarantees biocompatibility (Gierse & Donath 1999).

Endobon® is available in the form of blocks, cylinders and granules in different sizes. The Endobon® used in this study consisted of granules

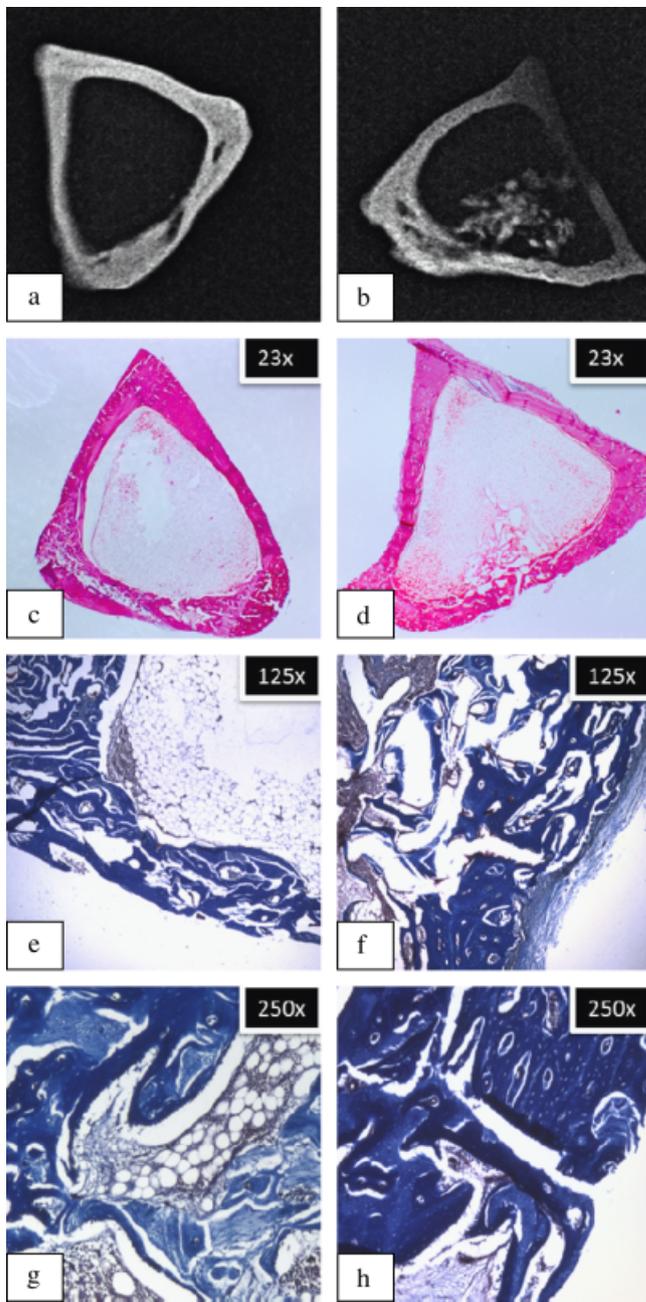


Fig. 5. 4 months: (a) Transversal section radiovisiograph of surgical specimen (control). (b) Transversal section radiovisiograph of surgical specimen (implanted material Endobon®). (c) Panoramic image × 23 - HE control. (d) Panoramic image × 23 - HE Endobon®. (e) Microscopy detail × 125 - TM control. (f) Microscopy detail × 125 - TM Endobon®. (g) Microscopy detail × 250 - TM control. (h) Microscopy detail × 500 - TM Endobon®. HE, hematoxylin-cosin; TM, Masson's trichrome.

Table 1. Mean ± SD values for newly formed bone, residual graft material and non-mineralized connective tissue for each evaluation period

	New bone (%)	Residual graft material (%)	Connective tissue (%)
1 month	18.5 ± 1.5	40.7 ± 3.2	40.8 ± 3.1
2 months	21.7 ± 1.4	39.7 ± 3.1	38.6 ± 2.1
3 months	23.6 ± 2.2	38.9 ± 3.5	37.5 ± 1.5
4 months	27.5 ± 2.4	38.3 ± 2.3	34.2 ± 3.4
Mean ± SD	22.8 ± 1.8	39.4 ± 3.0	37.7 ± 2.5

of between 500 and 1000µm, with porosity >50%. Thanks to its structure and characteristics, the biomaterial displays exceptional malleability and plasticity, facilitating application; it is

also of low radiopacity. It has a similar structure to bone with good pore interconnection as the pores, generated by heat treatment, are derived from natural bone (Liebendörfer & Tröster 1997;

Table 2. Mean ± SD values for length of cortical formation for each evaluation period

	Length of cortical formation	
	Control (%)	Bovine xenograft (%)
1 month	90.1 ± 0.7	63.7 ± 3.7*
2 months	92.3 ± 1.9	84.8 ± 3.7*
3 months	96.5 ± 2.1	95.7 ± 2.3
4 months	99.1 ± 0.7	98.8 ± 1.1

*P<0.05

Tadic & Eppe 2004). Animal bone composition is morphologically more similar to human bone than any synthetic product and an analysis of the results of clinical testing and the clinical take-up of the different products developed by the biomedical industry show the overall superiority of bone substitutes of natural origin over derivative substitutes (Santos et al. 2010).

The present experiment has confirmed the biocompatibility of RegenerOss™ BIOMET3i Endobon® given that little inflammatory response was observed in the initial stages of the study and that this was of minimal relevance (Baer et al. 2002). At 1 month, this was only recognizable by a low number of scattered lymphocytes and macrophages and no granuloma were formed. Neither was any fibrosis observed at the border between the graft and the host bone which, together with the fact that bone growth was observed around the implants, confirms the osteointegrative capacity of RegenerOss™ BIOMET3i Endobon® (Gierse & Donath 1999; Khodadadyan-Klostermann et al. 2002). Likewise, previous studies have shown that following adequate implantation Endobon® osteointegrates perfectly and neoformed bone is deposited directly over the trabecular ceramic structure without a fibrous tissue interface (Briem et al. 2002).

Our results have also demonstrated the osteoconductive capacity of this xenograft material (Wiltfang et al. 1996), which acted as scaffolding for bone cells (Bareille et al. 2000). One of the main factors influencing osteoconduction is the biomaterial's granule size. This study confirms the findings of previous studies that have established a relationship between pore size and granulometry and the quantity of neoformed osseous tissue (Hing et al. 2004), concluding that pore size and granulometry should not be overly reduced, since both pore diameter and interporotic connections have a significant effect on the type and quantity of newly formed bone tissue. In the same way, other studies of hydroxyapatites of differing degrees of porosity (Motomiya et al. 2007) have reached the conclusion that hydroxyapatite of high porosity osteointegrates better in terms of newly formed bone compared with non-interconnected porous hydroxyapatite. The

present study also agrees with results obtained by other authors (Tamai et al. 2002) who felt that the existence of interconnected interporosity facilitated bone growth. Interconnecting micro and macro pores for bony integration lead to graft stability and vascular ingrowth. This material has a system of interconnected pores whose structure is similar to that of human spongy bone which may contribute to promoting this level of intimacy between the hydroxyapatite and the bone ingrowth (Schnettler et al. 1998).

Osteoconductive properties enable bone growth directly on the ceramic implant surface (Bareille et al. 2000). This fact has been ratified in the present study, which observed surface bone growth, this having been verified by optical microscopy. X-rays from the last two time periods, at 3 and 4 months, revealed an appreciable decrease in total volume, which together with the descriptions derived from optical microscopy, indicated the replacement of osteoid tissue by adipose and hematopoietic bone

marrow. However, this demonstrates an incomplete bone graft resorption phenomena. This characteristic of bovine hydroxyapatite has been described previously (Jensen et al. 1996); there are no histological signs of resorption or degradation as particles of the biomaterial can be found 18 months after insertion (Briem et al. 2002). In spite of its being non-resorbable, as in other studies (Liebendörfer & Tröster 1997), we did observe a widening of intergrain boundaries as well as the partial dissociation of superficial hydroxyapatite crystallites on the implant surface. According to other studies (Tadic & Epple 2004), calcined Endobon[®] samples do not show much loss of mass due to the calcinations of the bovine source material at high temperatures. As a result, the hydroxyapatite obtained is highly crystalline, containing small quantities of calcium oxide resulting from the decomposition of the carbonate content of the original bone mineral. Although the material is not resorbable, hydroxyapatite granules did promote

bone formation in the cortical defects; this did not happen in the same way at the control sites where no implant material was placed, a fact that agrees with other morphometric studies (Liljensten et al. 2003).

Conclusions

Our results suggest that:

1. RegenerOss[™] BIOMET3i Endobon[®] is a biocompatible material, causing only minor, early-stage inflammatory responses, that do not interfere with normal bone repair processes.
2. RegenerOss[™] BIOMET3i Endobon[®] is an osteoconductive material, which acts as "scaffolding" for bone cells, accompanied by progressive increases in bone growth in and around the implants.
3. RegenerOss[™] BIOMET3i Endobon[®] is a non-resorbable material over the time period covered by this study.

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