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Bone response to hydroxyapatites with open porosity of animal origin (porcine [OsteoBiol[®] mp3] and bovine [Endobon[®]]): a radiological and histomorphometric study

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Abstract

Purpose: To carry out a radiological and histomorphometric evaluation of bone response to two xenografts of animal origin, one porcine, and the other bovine, inserted in rabbits' tibiae.

Material and methods: Twenty New Zealand rabbits weighing 3900–4500 g were used. Twenty bovine bone grafts (Endobon[®]) in granulated form of 500–1000 µm granulometry were inserted in the proximal metaphyseal area of the animals' right tibia, and 20 porcine bone grafts (OsteoBiol[®] mp3) in granulated form of 600–1000 µm granulometry were inserted in the proximal metaphyseal area of the animals' left tibia. Following graft insertion, the animals were sacrificed in four groups of five, after 1, 2, 3 and 4 months, respectively. Anteroposterior and lateral radiographs were taken. Samples were processed for observation under light microscopy. Histomorphometric measurements were presented as mean values ± standard deviations.

Results: At 4 months after treatment, the bone defects displayed radiological images that showed complete repair of osseous defects. Histomorphometric evaluation showed that for the porcine xenograft, the study averages for newly formed bone represented 22.8 ± 1.8%, for residual graft material 23.6 ± 3% and for connective tissue 53.5 ± 2.5%, while for the bovine xenograft newly formed bone represented 23.1 ± 1.8%, residual graft material 39.4 ± 3% and non-mineralized connective tissue 37.5 ± 2.5%.

Conclusions: The biomaterials assessed in the study were shown to be biocompatible and osteoconductive. Collagenized porcine xenografts proved more resorbable than bovine xenografts. Both can be used as possible bone substitutes without interfering with normal reparative bone processes.

With the ongoing development of implant systems, current implantology methods are achieving increasing rates of success. At the same time, demand arising from complex cases requiring prior bone regeneration via the use of grafts is also rising (Esposito et al. 2006). The substitution of bone tissue constitutes an, as yet, unsolved problem requiring research into the diverse materials that may be used to repair defects and stimulate host bone growth in order to achieve repair (Aghaloo & Moy 2007). At present, this challenge has been partially met through the use of both autologous bone grafts and allografts (Barone & Covani 2007). Nonetheless, given the intrinsic disadvantages involved, including secondary surgical zones (Nkenke et al. 2002) (donor sites) and undesirable post-operative

stages (Sasso et al. 2005), many clinicians are looking to a new range of materials generically referred to as bone-graft substitutes, which, as well as offering a much-needed solution, also reduce surgical time (Arcuri et al. 2005). Xenografts (obtained from other species of animals) display osteoconductive properties (Santos et al. 2010) and numerous studies have demonstrated the effectiveness of these bone-substitute biomaterials (Norton et al. 2003; Worth et al. 2005; Orsini et al. 2006; Nannmark & Sennerby 2008), showing that these graft materials provide scaffolding for bone growth, a process that starts from the edge of the defect. These materials can be permanent or resorbable. Their osteoconductive properties imply that the material has a capacity to influence non-pluripotential cells from the

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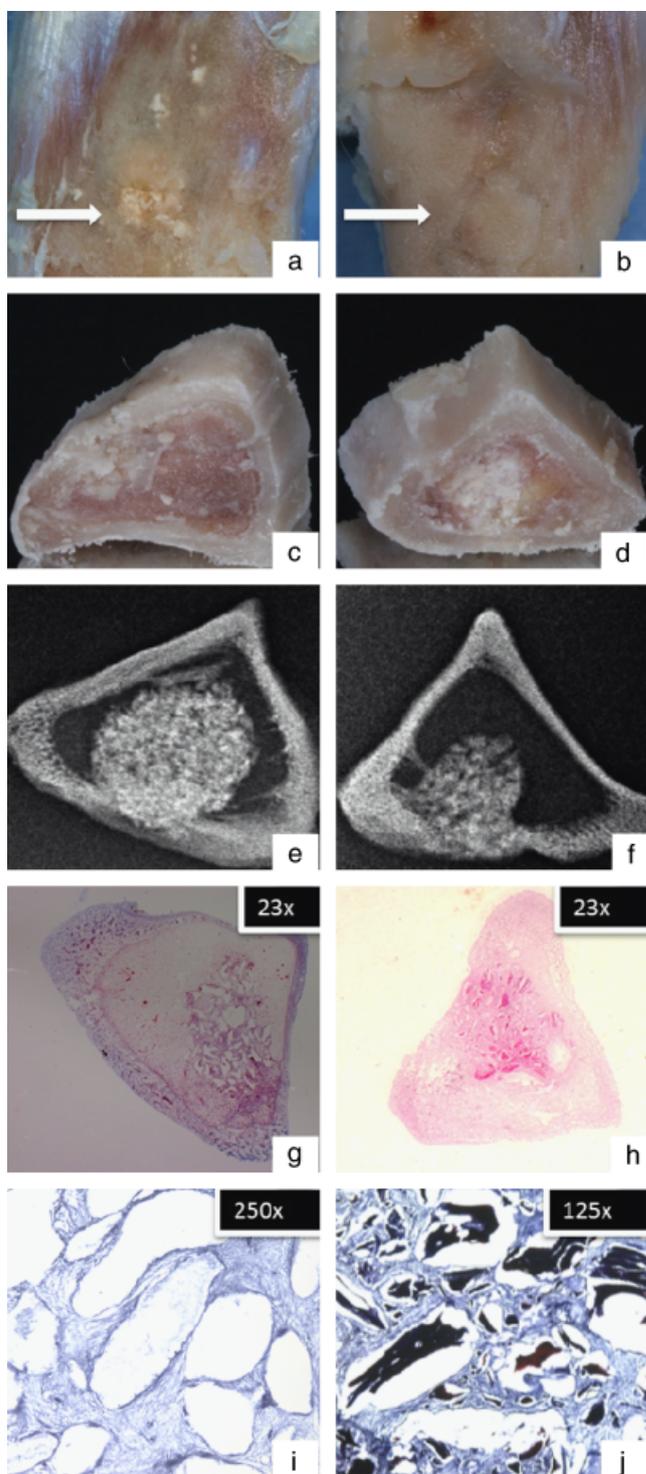


Fig. 1. One month: (a) macroscopic image of right tibia. (b) Macroscopic image of left tibia. (c) Macroscopic image of right tibia sectioned to show bovine graft (Endobon[®]). (d) Macroscopic image of left tibia sectioned to show porcine graft (OsteoBiol[®] mp3). (e) Radiovisigraph of transversal section of right tibia showing bovine graft (Endobon[®]). (f) Radiovisigraph of transversal section of left tibia showing porcine graft (OsteoBiol[®] mp3). (g) Panoramic × 23 hematoxylin–eosin stain image of bovine graft material (Endobon[®]). (h) Panoramic × 23 hematoxylin–eosin stain image of porcine graft material (OsteoBiol[®] mp3). (i) Microscopy detail, × 250 stained with Masson's trichrome, of bovine graft material (Endobon[®]). (j) Microscopy detail, × 250 stained with Masson's trichrome, of porcine graft material (OsteoBiol[®] mp3).

“bedding” to convert into osteoblasts, necessary for bone regeneration (Kin et al. 2004).

The aim of the present study was, therefore, to investigate two xenografts of animal origin, one

porcine (OsteoBiol[®] mp3), and the other bovine (Endobon[®]) in order to ascertain that both are biocompatible, osteoconductive and thus may be used as possible bone substitutes.

Materials and methods

A total of 40 grafts were placed in the proximal metaphyseal areas of both rear tibiae of 20 albino New Zealand rabbits of 30–35 weeks of age and weighing 3900–4500 g. Two different xenografts were evaluated: 20 porcine xenografts made up of 90% granulated bone particles of 600–1000 μm in size and 10% pure collagen, in the form of a bone granulate (OsteoBiol[®] mp3, TECNOSS s.r.l., Giaveno, Italy) were placed in the proximal metaphyseal area of the left tibiae, while 20 bovine xenografts with bone granules of 500–1000 μm in size (Endobon[®], RegenerOss[™], BIOMET3i, Palm Beach Gardens, FL, USA) were placed in the right tibiae. All experiments were approved and performed according to 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 ketamine plus chlorbutol, 5–8 mg/kg intravenously, 0.5–1 mg/kg acepromazine maleate as a coadjuvant and 0.05 mg/kg atropine. Amoxicillin was administrated at the end of surgery (0.1 ml/kg intramuscularly).

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 concave defects approximately 4 mm in diameter was carried out with spherical surgical drills at a low rotation speed and constant irrigation. These were then filled with the granulate xenografts. The five rabbits in each group were sacrificed with an intracardiac overdose of thiopental at 1, 2, 3 and 4 months following surgery.

Radiological study

Two X-rays, anteroposterior and lateral, of the sections of bone containing xenografts were taken using the Kodak RVG 6100 Digital Radiography System with X-rays taken at 32 Kv, 40 mA, and automatic light metering. Radiovisigraphs of each transversal tibia section containing graft materials were taken. Radiographs of all groups were taken.

Optical microscopy

The surgically acquired samples were fixed in 10% neutral-buffered formalin and decalcified by 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 or Masson's trichrome. All samples

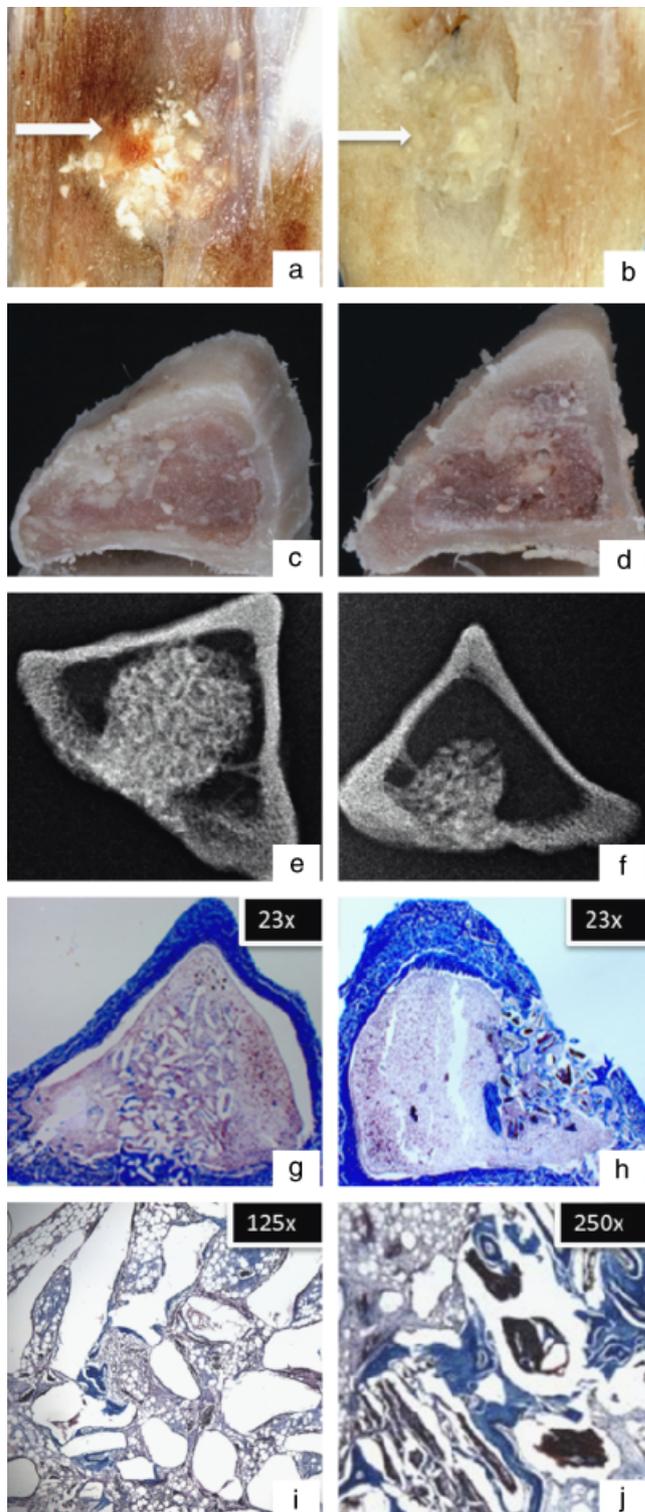


Fig. 2. Two months: (a) macroscopic image of right tibia. (b) Macroscopic image of left tibia. (c) Macroscopic image of right tibia sectioned to show bovine graft (Endobon[®]). (d) Macroscopic image of left tibia sectioned to show porcine graft (OsteoBiol[®]mp3). (e) Radiovisigraph of transversal section of right tibia showing bovine graft (Endobon[®]). (f) Radiovisigraph of transversal section of left tibia showing porcine graft (OsteoBiol[®]mp3). (g) Panoramic image $\times 23$ stained with Masson's trichrome of bovine graft material (Endobon[®]). (h) Panoramic image $\times 23$ stained with Masson's trichrome of porcine graft material (OsteoBiol[®]mp3). (i) Microscopy detail, $\times 125$ stained with Masson's trichrome, of bovine graft material (Endobon[®]). (j) Microscopy detail, $\times 250$ stained with Masson's trichrome, of porcine graft material (OsteoBiol[®]mp3).

were examined under light microscopy (Microphoto FXA, Nikon, Tokyo, Japan).

The entire circumference of each section (containing new 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 xenograft 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 bone) and the apical portion (using a safe margin of 1.5–2 mm) were excluded from analysis. Examinations were performed under a Nikon Eclipse 80i microscope (Teknooptik AB, Huddinge, Sweden) equipped with the EasyImage 2000 system (Teknooptik AB) using $\times 1$ to $\times 4$ lenses for descriptive evaluation and for taking morphometric measurements. Histomorphometric measurement of the samples was conducted using Image J software, developed by the National Institute of Health of the United States. Values for the total percentage of newly bone formed, residual graft material, and non-mineralized connective tissue were then calculated.

Statistical analysis

Firstly, factors such as individual difference and graft position 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.

Results

Histomorphometric evaluation showed that there were no significant differences in newly formed bone between the biomaterials at the end of the study. However, there were significant differences in the residual graft material and non-mineralized connective tissue between the two biomaterials (Table 1).

One month

Endobon[®]

X-rays revealed the characteristics of the grafted material as being a cylindrical element, with a 4×6 mm rectangular structure adapted to the

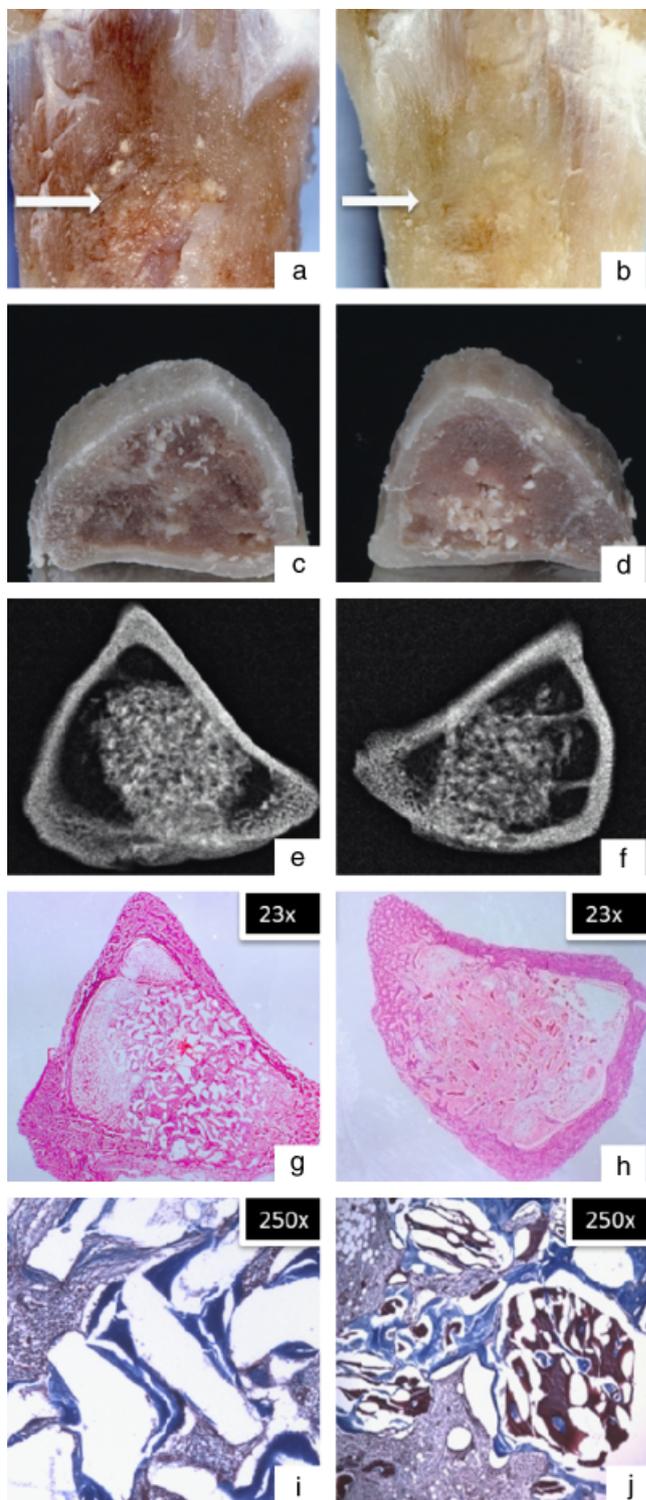


Fig. 3. Three months: (a) macroscopic image of right tibia. (b) Macroscopic image of left tibia. (c) Macroscopic image of right tibia sectioned to show bovine graft (Endobon[®]). (d) Macroscopic image of left tibia sectioned to show porcine graft (OsteoBiol[®] mp3). (e) Radiovisiograph of transversal section of right tibia showing bovine graft (Endobon[®]). (f) Radiovisiograph of transversal section of left tibia showing porcine graft (OsteoBiol[®] mp3). (g) Panoramic × 23 hematoxylin-eosin stain image of bovine graft material (Endobon[®]). (h) Panoramic × 23 hematoxylin-eosin stain image of porcine graft material (OsteoBiol[®] mp3). (i) Microscopy detail, × 250 stained with Masson's trichrome, of bovine graft material (Endobon[®]). (j) Microscopy detail, × 250 stained with Masson's trichrome, of porcine graft material (OsteoBiol[®] mp3).

morphology of the artificially created bone defect. The grafts showed very high radiopacity, greater than that of adjacent bone structures, which made them easily distinguishable. The xenograft

appeared to be formed of small highly radiopaque granules or particles, small in size, and of rounded but heterogeneous appearance. For this reason, the edges of the graft area appeared

irregular, formed of these particles, which were clearly outlined due to their high radiopacity. A break in the cortex of the epiphysary zones, where the xenograft had been introduced, was observed. In some external marginal areas, the xenograft made contact with the absent cortical bone line and showed a more radiopaque morphology of granular aspect.

Under optical microscopy, the graft material was characterized by small accumulations of fine granular material, found to be intensely basophilic, given that only a slight inflammatory response of little relevance was observed. At the center of the granulation tissue in contact with the grafted material, numerous deposits of osteoid material were observed. The surgical orifice had re-formed with a fine layer of fibrinohematic tissue interrupted mainly by granules of graft material (Fig. 1a, c, e, g and i).

OsteoBiol[®] mp3

X-rays revealed the characteristics of the grafted material as being a cylindrical element, with a 4 × 6 mm rectangular structure adapted to the morphology of the artificially created bone defect. The xenografts showed very high radiopacity, greater than that of adjacent bone structures, but not as high as with the bovine xenograft. In some external marginal areas, grafts made contact with the missing cortical bone line.

Under optical microscopy, a large quantity of porcine xenograft particles were observed, distributed throughout the whole sample area, without granuloma being formed. There were some neo-formed trabeculae between the fragments of graft material with numerous dots of young granulation tissue (Fig. 1b, d, f, h and j).

Two months

Endobon[®]

At the 2-month point, the xenografts showed a lower radiopacity. The bone defect was still partly occupied by graft material. Transversal radiological images of the pieces showed complete repair of the cortex where the xenograft had been inserted, although the biomaterial's radiopacity was clearly lower and more heterogeneous than the rest of the bone cortex.

Under optical microscopy, there were numerous deposits of osteoid material and even small neo-formed osseous trabeculae within the body of the granulated tissue in contact with the cortex; the trabeculae were irregular in size and distribution and more abundant in areas adjacent to the cortex. Between the particles, it could be seen that osseous medulla was being replaced by osteoid material, which took on a trabecular form (Fig. 2a, c, e, g and i).

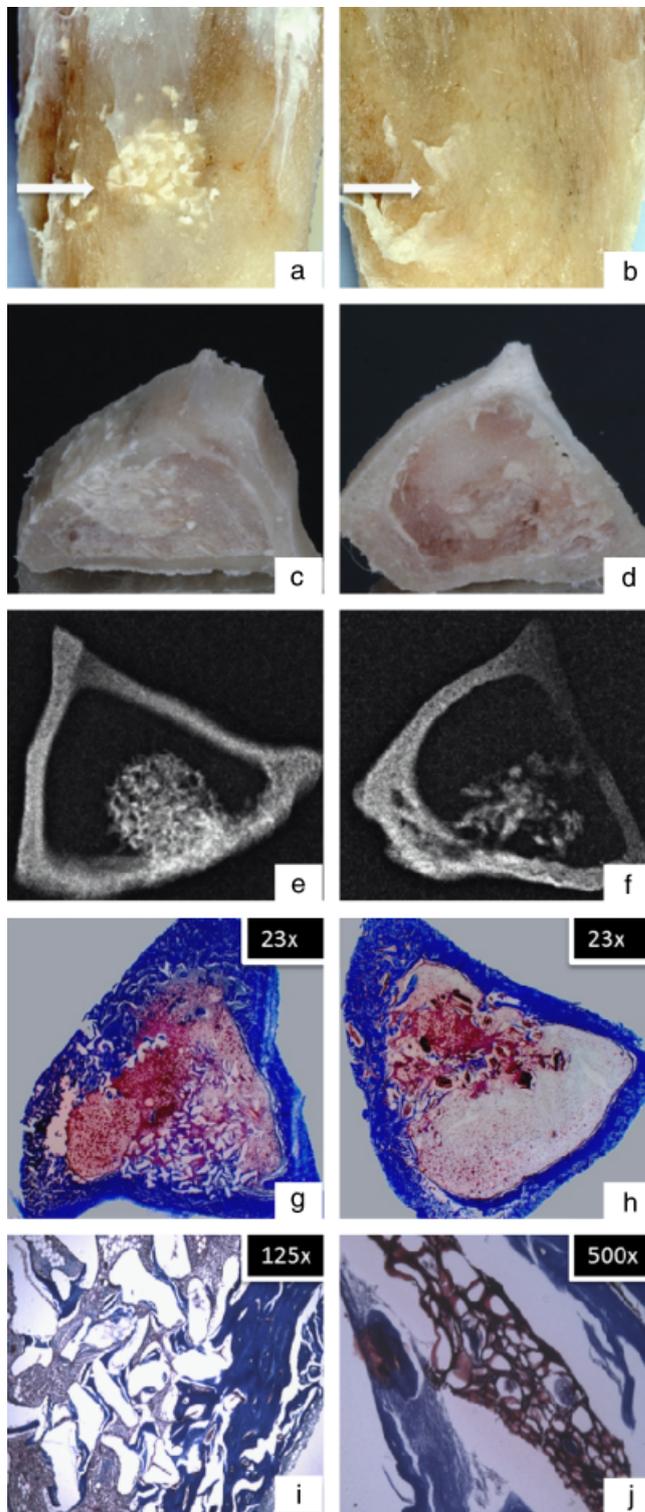


Fig. 4. Four months: (a) macroscopic image of right tibia. (b) Macroscopic image of left tibia. (c) Macroscopic image of right tibia sectioned to show bovine graft (Endobon®). (d) Macroscopic image of left tibia sectioned to show porcine graft (OsteoBiol®mp3). (e) Radiovisiograph of transversal section of right tibia showing bovine graft (Endobon®). (f) Radiovisiograph of transversal section of left tibia showing porcine graft (OsteoBiol®mp3). (g) Panoramic image $\times 23$ of bovine graft material stained with Masson's trichrome (Endobon®). (h) Panoramic image $\times 23$ of porcine graft material (OsteoBiol®mp3) stained with Masson's trichrome. (i) Microscopy detail, $\times 125$ stained with Masson's trichrome, of bovine graft material (Endobon®). (j) Microscopy detail, $\times 500$ stained with Masson's trichrome, of porcine graft material (OsteoBiol®mp3).

OsteoBiol®mp3

The radiopacity of the graft material had decreased, showing irregular edges in contact with

the surrounding bone. The bone cortex was observed to be incomplete, still containing graft material.

Under optical microscopy, there was abundant granulation tissue between islands of graft material with specks of osteoid material at the border zone between cortex and graft area. In this way, resorption of the biomaterial was evident. Likewise, the xenograft had been substituted by osseous trabeculae, which were now more extensive and thicker than those observed for the previous time period, giving the graft area a reticular appearance. No inflammatory response of note was observed (Fig. 2a, d, f, h and j).

Three months

Endobon®

At the third month, the xenografts showed a slight decrease in external volume, manifested as an increase in radiotransparent areas between particles. In the area where the xenograft had been introduced, the cortex was found to be completely repaired showing radiological characteristics similar to the surrounding bone cortex.

Under optical microscopy, no internal resorption phenomena of the bovine xenograft were observed although osseous regeneration phenomena could be observed, characterized by neoformed trabeculae of an irregular character in continuity with osseous medulla and only sparse deposits of graft material (Fig. 3a, c, e, g and i).

OsteoBiol®mp3

At the third month, xenografts showed characteristics similar to the previous time period. Radiographs revealed an increased granular aspect in the interior of the graft and the complete repair of the external cortex.

Microscopy revealed signs of resorption in the interior, with lagoons of osteoid material at the center. There were also signs of neo-formation in the outer areas, with the biomaterial surrounded by bone material in a phase of resorption and substitution (Fig. 3b, d, g, h and j).

Four months

Endobon®

The grafted material showed a reduced size and decreased radiopacity but remained perfectly individualized in the interior of the bone without showing signs of internal resorption.

Under microscopy, areas in which osseous regeneration phenomena still predominated could be seen around small islands of granular material although osseous medulla with normal characteristics was seen to predominate; this alternated with other areas, particularly in the cortical area, in which osseous medulla with irregular anastomosed trabeculae of a lace-like appearance was observed (Fig. 4a, c, e, g, and i).

Table 1. Histomorphometric parameter values for newly formed bone, residual graft material, and non-mineralized connective tissue for each study period

	Newly formed bone		Residual graft material		Connective tissue	
	Bovine graft material (%)	Porcine graft material (%)	Bovine graft material (%)	Porcine graft material (%)	Bovine graft material (%)	Porcine graft material (%)
1 month	18.5 ± 1.5	21.7 ± 1.5	40.7 ± 3.2	31.8 ± 3.2	40.8 ± 3.1	46.5 ± 3.1
2 months	21.7 ± 1.4	22.9 ± 1.4	39.7 ± 3.1*	27.4 ± 3.1	38.6 ± 2.1	49.7 ± 2.1
3 months	23.6 ± 2.2	23.4 ± 2.2	38.9 ± 3.5*	20.2 ± 3.5	37.5 ± 1.5	56.4 ± 1.5*
4 months	28.5 ± 2.4	23.5 ± 2.4	38.3 ± 2.3*	15.1 ± 2.3	33.2 ± 3.4	61.4 ± 3.4*
Mean ± SD	23.1 ± 1.8	22.8 ± 1.8	39.4 ± 3*	23.6 ± 3	37.5 ± 2.5	53.5 ± 2.5*

The data are expressed as mean values ± SD.
*Statistically significant: $P < 0.05$.

OsteoBiol[®]mp3

At the end of the fourth month, a significant reduction in xenograft size together with a considerable reduction in radiopacity were observed. There was an increase in xenograft surface irregularities in the areas in contact with the surrounding bone, in which dense trabecular lines could be seen running towards the grafts' interior.

Under optical microscopy, osseous regeneration phenomena, characterized by numerous neoformed trabeculae of irregular characteristics, could be observed in the cortical area in continuity with the osseous medulla and occasional deposits of graft material. The xenografts showed signs of resorption in their interiors, which had been almost entirely replaced by bone (Fig. 4b, d, f, h and j).

Discussion

The present experiment confirmed the biocompatibility of OsteoBiol[®]mp3 and Endobon[®]. OsteoBiol[®]mp3 is an antigen-free bone consisting of 90% granules of between 600 and 1000 µm, mixed with 10% pure Type-I porcine collagen made up of a heterotrimer with two identical α -1 chains and one α -2 chain and a homotrimer with three identical α -1 chains. Endobon[®] is a hydroxyapatite ceramic derived from cancellous bovine bone, in granulated form of 500–1000 µm granulometry, fully deproteinated by a high-temperature manufacturing process for safety from bacteria, viruses and prions. This process consists of two steps: firstly, pyrolysis at a temperature of >900°C that eliminates the organic element, and secondly, a ceramization process at temperatures >1200°C that creates a crystalline structure. The process ensures the absence of immunological reactions and guarantees biocompatibility. This characteristic was confirmed by the present study, as there was only a slight inflammatory response in the initial stages of the study, which was of little relevance, and which, after the first month, was only recognizable by a low number of scattered lymphocytes and macrophages, with no granuloma being

formed. Neither was any fibrosis observed on the border between grafts and host bone, which, together with the fact that bone growth was observed around both xenograft types, confirms the osteointegrative capacity of both materials.

The biocompatibility and osteointegrative capacities of other forms of hydroxyapatite have been defined previously in a range of classic studies (Kehr & Gosset 2000; Werber et al. 2000; Briem et al. 2002; Cardaropoli & Cardaropoli 2008; Scarano et al. 2009). In agreement with other authors (Norton et al. 2003; Kin et al. 2004), our results have demonstrated the osteoconductive capacity of porcine and bovine xenografts, which acted as scaffolding for bone cell generation. Others authors (van Blitterswijk et al. 1986) have already established a table relating pore size and granulometry with the quantity of neo-formed osseous tissue. These studies observed that with hydroxyapatites of a pore size from 100 to 160 µm, a 17% degree of bone formation is observed, which rises progressively to 96% for pore sizes of over 276 µm. They have concluded that pore size and granulometry should not be overly reduced, because both have a significant effect on the type and quantity of newly formed bone tissue. The physical properties of different products, including pore architecture, vary widely and there have been a number of investigations into the effect of pore structure on repair process. As a result of the many studies that have expressed the dependence of the degree of osteointegration on pore size (Motomiya et al. 2007), 100 µm has come to be regarded as a minimum pore size for guaranteed bone growth. However, other studies stress the importance of other parameters such as pore morphology, pore percentage, or the connection between pores (Hing et al. 2004).

The present study also confirms results obtained by other authors (Wang et al. 2009), who felt that the existence of interporosity facilitated bone growth within. This fact has been ratified in our experiment, which observed bone growth not only in surface pores but also in pores within the graft. X-rays of the hydroxyapatite from the last two time periods indicated an appreciable de-

crease in total volume, which, together with the descriptions derived from optical microscopy indicating the replacement of osteoid tissue by adipose and hematopoietic bone marrow, demonstrate the existence of partial and progressive resorption phenomena, which were seen to become more accentuated by the fourth month following insertion. This characteristic of hydroxyapatite has been described previously (Santos et al. 2010).

Previous research has shown that the initial response and mechanical properties of a porous hydroxyapatite bone graft substitute are highly sensitive. In spite of the fact that higher porosity promotes rapid growth through the interconnectivity of pores, the substitute material needs to be sufficiently dense to maintain the integrity and stability of the graft. An open macroporous structure similar to cancellous bone will promote a complete infiltration by bone tissue, bone marrow and blood vessels, as occurs when autografts and allografts are used. For some authors (Tamai et al. 2002), differences in bone ingrowth volume depend initially on pore interconnectivity rather than pore size. Variations in pore connectivity will have an effect on osteointegration through the number of osteogenic cells that penetrate the porous structure. The importance of pore connectivity has also been shown in a study of osteointegration in hydroxyapatite grafts of varying porosity. This study supported the theory that pore connectivity, rather than pore volume, played a role in controlling the rate of osteointegration during the initial stages. This observation would support the theory that the differences in the quantity of ingrowth during early stages are not mediated by pore volume alone. A variation in the degree of angiogenesis (Yumada et al. 2008) resulting from the different degrees of pore connectivity would influence integration through a restriction of nutrient and oxygen availability in a less vascular environment. A decrease in porosity, both in terms of size and the frequency of pore interconnections, will lead to a subsequent reduction in the flow of nutrients throughout the structure. Interconnected microporosity may be a contributing fac-

tor in promoting levels of intimacy between the hydroxyapatite and bone ingrowth. Studies of hydroxyapatites of varying degrees of porosity (Motomiya et al. 2007) concluded that interconnected porosity integrates better in terms of new bone generation compared with non-interconnected porous hydroxyapatite. In the present study, both materials had a similar pore size and both are defined as being of open porosity. However, OsteoBiol[®] mp3 does have a higher degree of porosity and showed greater bone growth in the interior of the material. This did not occur with Endobon[®], in which bone formation was observed in direct contact with surrounding bone but not to such an extent in the interior of the graft. This could also be explained by the greater degree of resorption seen with OsteoBiol[®] mp3.

According to earlier studies (Briem et al. 2002), bovine xenografts are non-resorbable but in the present study, the biomaterial was seen to de-

crease partially. This seems to correspond to an other study (Liebendorfer & Tröster 1997), which observed a widening of intergrain boundaries as well as partial dissociation of superficial hydroxyapatite crystallites on the graft surface. However, we agree with other studies (Tadic & Epple 2004) that show how Endobon[®] samples do not show much loss of mass due to the calcinations of the bovine source material at high temperatures. If the size of hydroxyapatite ceramic crystals is very small or if carbonates are incorporated into them, degradation is observed to increase due to high solubility. This explains why grafts such as OsteoBiol[®] mp3 that contain a collagen matrix are seen to undergo greater degradation.

In spite of this, a morphometric study (Liljensten et al. 2003) showed that after 3 months, there was more newly formed bone in the two groups that used both resorbable and non-resorbable hydroxyapatite compared with a control group. Both resorbable and non-resorbable HA

granules promoted new bone formation in rabbit cortical defects but this did not occur in control defects. We agree that xenografts can be considered substitute bone materials that promote bone tissue regeneration in bone defects, as they do not interfere with normal reparative bone processes.

Conclusion

Our results prove that the bovine xenograft studied (Endobon[®]) was biocompatible and osteoconductive, acting as "scaffolding" for bone cells, to promote the progressive increase in bone growth around the grafts. The collagenized porcine xenograft used (OsteoBiol[®] mp3) proved to be biocompatible, osteoconductive and more resorbable than bovine bone. Both can be used as possible bone substitutes without interfering with normal reparative bone processes.

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