Experimental Model of Bone Response to Collagenized Xenografts of Porcine Origin (OsteoBiol® mp3): A Radiological and Histomorphometric Study

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ABSTRACT

Background: Adequate alveolar ridges are fundamental to successful rehabilitation with implants. There are diverse techniques for reconstructing atrophied ridges, of which bone substitute grafts is one possibility.

Purpose: The aim of this study was to carry out radiological and histomorphometric evaluations of bone response to collagenized porcine bone xenografts over a 4-month period following their insertion in rabbits' tibiae.

Material and Methods: Twenty New Zealand rabbits were used. Twenty collagenized porcine bone xenografts (Osteobiol® mp3, Tecnoss Dental s.r.l., Torino, Italy), in granulated form of 600 to 1,000 μm, were inserted in the proximal metaphyseal area of the animals' tibiae and 20 control areas were created. Following implantation, the animals were sacrificed in four groups of five, after 1, 2, 3, and 4 months, respectively. Radiological and histomorphometric studies were made.

Results: After 4 months, radiological images revealed bone defects with a decrease in graft volume and the complete repair of the osseous defect. No healed or residual bone alterations attributable to the presence of the implants were observed. Histomorphometric analysis at 4 months found mean values for newly formed bone, residual graft material, and non-mineralized connective tissue of 25.4 ± 1.8%, 36.37 ± 3.0%, and 38.22 ± 2.5%, respectively. There were no statistical differences in the length of cortical formation with collagenized porcine xenograft (98.9 ± 1.1%) compared with the control samples (99.1 ± 0.7%) at the end of the study period.

Conclusions: The biomaterial used proved to be biocompatible, bioabsorbable, and osteoconductive and as such, a possible bone substitute that did not interfere with the bone's normal reparative processes.

KEY WORDS: biomaterial, bone graft, collagenized porcine xenografts, hydroxyapatite

INTRODUCTION

In clinical practice, we often come across alveolar ridges that present varying degrees of resorption, which may be because of a variety of causes.[1] When it comes to prosthodontic rehabilitation, bone insufficiency in the maxilla presents an important problem. Adequate alveolar ridges are a fundamental condition for successful rehabilitation with implants, and patients with alveolar atrophy will require some sort of bone augmentation technique prior to implant insertion.[2,3]

There are various techniques for reconstructing atrophied ridges and one of these is the use of bone substitute grafts.[4] All the techniques available place limitations of one kind or another on an ideal clinical outcome.[5]

The ideal biomaterial must be biologically safe and the safety of a bone substitute material depends on reliable reproducibility, biocompatibility, and absence of

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toxicity. Clearly, all aspects of a substitute must be studied thoroughly in order to make predicative risk assessment possible.[6]

The different bone substitute products developed by the biomedical industry have had widespread clinical uptake and an analysis of the results of their use points to the overall superiority of bone substitutes of natural origin over derivative substitutes.[7] In particular, there is one animal species with a genotype close to human – the pig – and xenograft materials of porcine origin have provoked a great deal of research to assess their potential as a substitute for osseous grafts.[8] Various studies have shown that such materials provide an effective osteoconductive matrix.[9–11]

This study investigates one xenograft of porcine origin, as a possible substitute for bone grafting: OsteoBiol mp3® (Tecnoss Dental s.r.l., Torino, Italy), an antigen-free bone consisting of 90% granules of between 600 and 1,000 μm mixed with 10% pure Type-I porcine collagen.

MATERIALS AND METHODS

Animals, Surgery, and Treatment

A total of 20 collagenized porcine xenografts were placed in the proximal metaphyseal area of the left tibias of 20 albino New Zealand rabbits, of 30 to 35 weeks of age, weighing 3,900 to 4,500 g. The graft material used was OsteoBiol® mp3 (Tecnoss Dental s.r.l.), an antigen-free bone consisting of 90% granules of between 600 and 1,000 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. The α-1 chains were in turn made up of 338 consecutive Gly-Xaa-Yaa triplets, where Gly is glycine and Xaa and Yaa are different amino-acids (excepting tryptophan and cystein), these being proline and hydroxyproline, respectively, in one out of every three cases. The Tecnoss patented manufacturing process used to produce these materials achieves biocompatibility by avoiding temperatures higher than 130°C (that would cause ceramization of the granules), preserving part of the collagen matrix of the original animal bone. The result is a unique biomaterial consisting of mineral components and an organic matrix with a level of porosity very similar to autogenous bone that resorbs progressively as new bone formation takes place.

All experiments were approved by the Murcia University Ethical Committee and performed following Spanish Government and European Community Guidelines for animal care.

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

The surgical approach was performed in the proximal metaphyseal-diaphyseal area of the tibia, several millimeters below the anterior tibial tuberosity. Bone tissue was removed with spherical surgical drills of 5 mm in diameter at a low rotation speed, with constant irrigation to form two concave defects reaching the spinal canal. The first was filled with the collagenized xenograft and the second was used as a control site. Following implantation, the animals were sacrificed in four groups of five, after 1 month, 2 months, 3 months and 4 months, by means of an intracardiac overdose of thiopental.

Radiological Study

Two x-rays, anteroposterior and lateral, were taken of the sections of bone containing implants using the Kodak RVG 6100 Digital Radiography System (Kodak DS, Rochester, NY, USA); x-rays were taken at 32 Kv, 40 mA, using an automatic exposure meter. After sacrifice, radiovisiographs were taken of each transversal section of tibiae containing the implants. Radiographs of all groups (experiment and control) were taken.

Optical Microscopy

The samples were fixed in 10% neutral buffered formalin and decalcified. The decalcification method utilized Osteomol® (Merck KbaA, Whitehouse Station, NJ, USA) containing H Cl (10%) and CH_2O (4%), immersing samples for 17 days, renewing the solution every 24 hours.

Subsequently, all samples were paraffin embedded, sectioned at 5 μm and stained using hematoxylin-eosin, a useful and convenient stain method, and

Masson's trichromic (used to reveal young lamellar bone). All samples were examined under light microscopy.

Morphometric Analysis

The central portion of each core was selected in order to avoid any potential bias; in this way, both the coronal (remaining native host bone) and the apical portion (using a safe margin of 1.5–2 mm) were excluded from analysis. The entire circumference of each section (containing bone, grafted particles, and connective tissue) was traced manually to create an individual region-of-interest.

Histomorphometric measurement of the samples was conducted using Image J software, developed by the National Institute of Health (NIH) of the United States of America. Evaluation consisted of measuring the area of bone and area of porcine particles in relation to the total measurement area. Values for the total percentage of newly formed bone, residual graft material, and nonmineralized connective tissue were then calculated.

Examinations were performed under a Nikon Eclipse 80i microscope (Teknooptik AB, Huddinge, Sweden) equipped with an EasyImage 2000 system (Teknooptik AB) using ×10 to ×40 lenses for descriptive evaluation and morphometric measurement.

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, enlarged ×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 QWin V3 image analysis software.

Statistical Analysis

Factors such as individual difference and position of the implant could be excluded as not significant. Normality tests (Kolmogorov–Smirnov and Shapiro–Wilk) and equality of variance tests (Mann–Witney and Wilcoxon) were applied and no assumption violations were observed. The analysis of variance was used to identify significant mean differences and standard deviations using SPSS 15.0 for Windows software (SPSS Inc., Chicago, IL, USA). Mean values and standard deviations among animals were calculated for each variable. The signification level established was $p < .05$.

RESULTS

One 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. (Figure 1A)

X-rays revealed the implanted material as a cylindrical element with a 5×5-mm rectangular structure with great radiopacity, allowing its identification within the trabecular bone structure in which it was implanted (Figure 1B).

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 (Figure 1C,E,G).

At the implant sites, anatomopathological study highlighted the cortical bone; the surgical orifice was formed by a fine layer of fibrinohematic tissue interrupted mainly by granules of graft material. Optical microscopy of the implant and cortical defect site revealed the substitution of bone by granulated tissue extending toward the implant and invading the grafted 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 (Figure 1D,F,H).

Morphometric Analysis. Histomorphometry showed that the newly formed bone represented $19.7 \pm 1.5\%$, residual graft material represented $39.8 \pm 3.2\%$, and connective tissue represented $40.5 \pm 3.1\%$. The length of cortical formation at porcine bone implant sites was $34.6 \pm 3.6\%$ compared with $90.1 \pm 0.7\%$ at the control sites.

Two 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

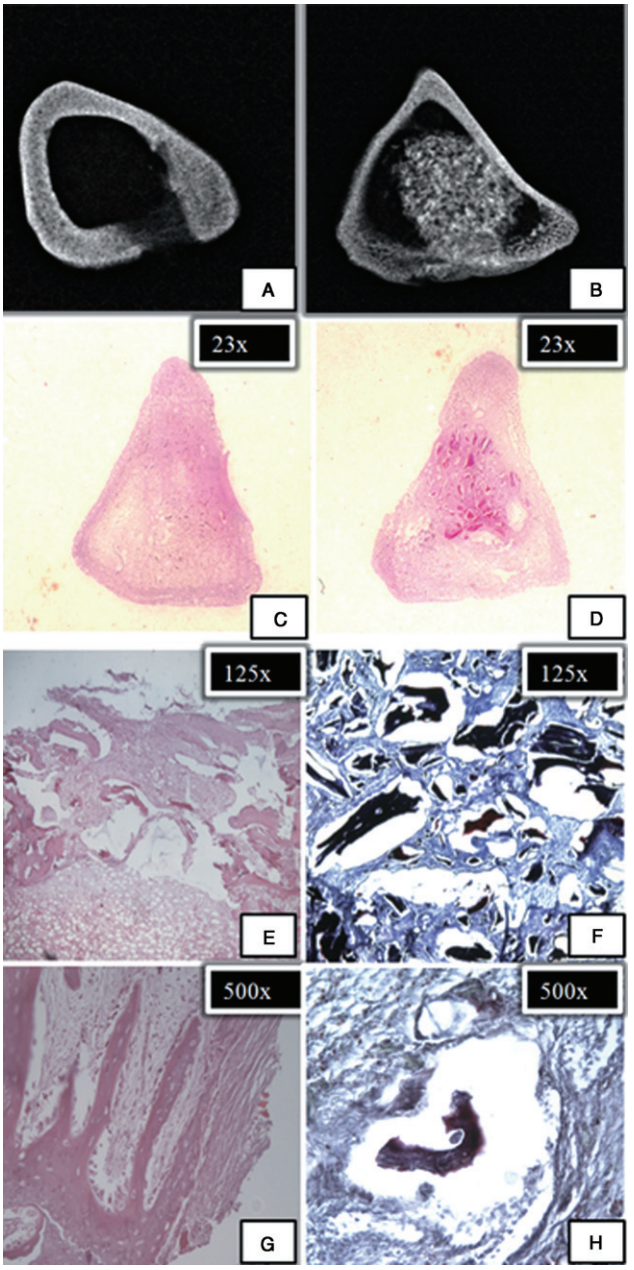

Figure 1 The most relevant morphological changes to the bone defect area in the control group were at the cortical level where an outer layer of fibrinohematic tissue covered the orifice. At the implant sites, anatomopathological study highlighted the cortical bone; the surgical orifice was formed by a fine layer of fibrinohematic tissue interrupted mainly by granules of graft 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 grafted 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 (Figs. 1c, 1e, 1g, 1d, 1f, 1h).

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 (Figure 2A).

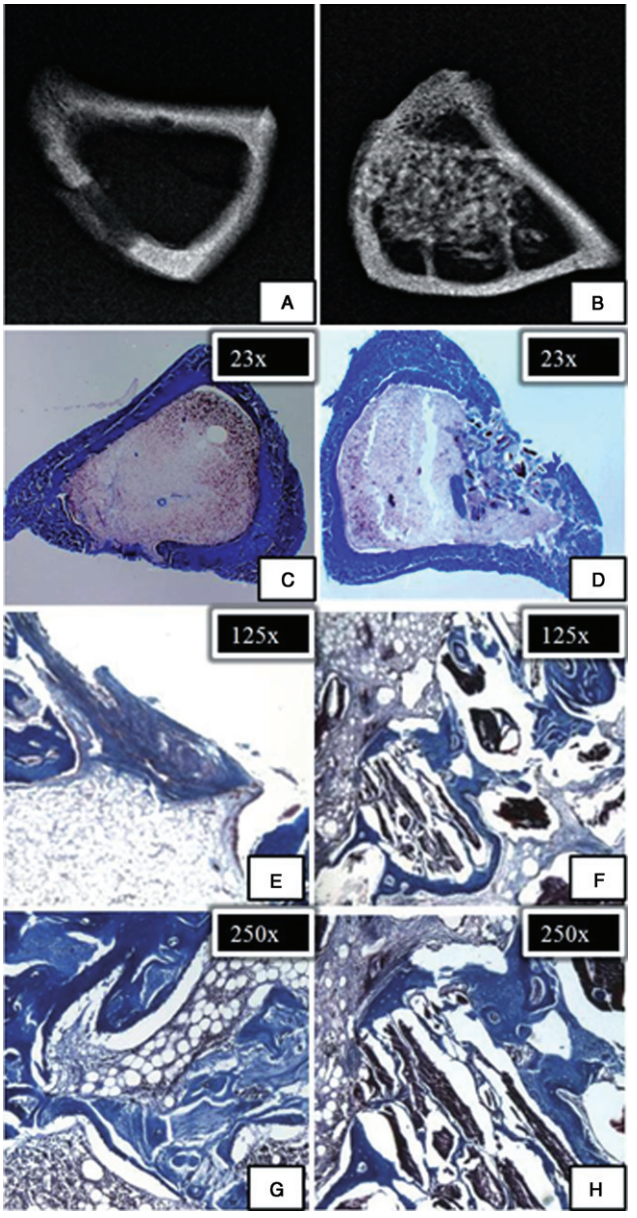

Figure 2 In this study period, the control group showed noticeable bone repair phenomena around the defects' peripheries and around adjacent bone marrow. The anatomopathological study highlighted the perforation made in the cortical bone in order to perform the xenograft as being almost completely repaired by neoformed or immature osseous tissue. Likewise, the implanted xenograft had been substituted by osseous trabeculae, which were more extensive and thicker than those observed for the previous time-period, thus giving the implant zone a reticular appearance. No inflammatory response of note was observed (Figs. 2c, 2e, 2g, 2d, 2f, 2h).

In the collagenized xenograft-filled bone defects, x-rays revealed the cortical-osteoblastic cell line as being completely repaired, albeit with less density than that of the adjacent cortical bone. Although a reinforced radiological density could imply the formation of bone around the xenograft, the radiological radiopacity of this material was lower than that observed for the previous time period; the spherical form of the grafted material had given way to a more oval and irregular shape (Figure 2B).

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Morphometric Analysis. Histomorphometry showed that the newly formed bone represented $22.5 \pm 1.4\%$, residual graft material represented $37.4 \pm 3.1\%$, and connective tissue represented $40.1 \pm 2.1\%$. The length of cortical formation at porcine bone implant sites was $59.7 \pm 3.7\%$ compared with $92.3 \pm 1.9\%$ at the control sites.

Three Months

Radiological Study. After 3 months, X-ray images of control sites showed similar characteristics to the previous study period (Figure 3A).

X-rays revealed the external cortex of the artificial osseous lagoons into which the bone-granulate implant had been introduced; this showed a radiopacity similar to that of the adjacent cortex, making it difficult to identify the surgical orifice. At the level of the cortex, the xenograft area displayed a decreased radiological radiopacity with respect to the previous time period, as well as a more oval shape with a lower radiopacity 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 the linear image of osseous trabeculae (Figure 3B).

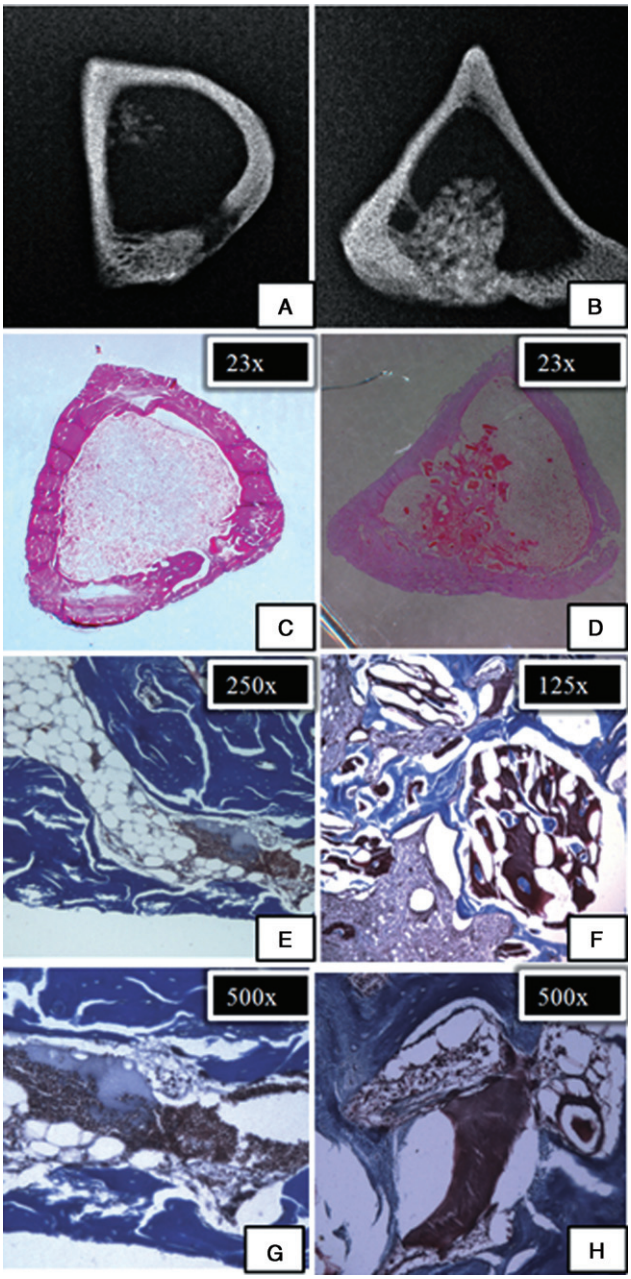

Figure 3 In the control group extensive bone regeneration was observed spreading beyond the lower edge of the adjacent cortex. The anatomopathological study showed complete bone reparation of the cortex at the implant orifice, manifested as well-organized trabecular bone at the cortex with an increase in osseous remodeling. We also observed, albeit to a lesser degree, the formation of osseous trabeculae, as well as a marked increase in hematopoietic and adipose bone marrow in the center, which had partially replaced the granulated tissues (Figs. 3c, 3e, 3g 3d, 3f, 3h).

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Morphometric Analysis. Histomorphometry showed that the newly formed bone represented 27.9 ± 2.2%, residual graft material represented 35.2 ± 3.5%, and connective tissue represented 36.9 ± 1.5%. The length of cortical formation at porcine bone implant sites was 96.5 ± 2.1% compared with 96.7 ± 2.3% at the control sites.

Four Months

Radiological Study. X-rays of the control bone defects at the end of the experiment showed characteristics similar to those described in the previous, with one or more rectilinear lines that could be observed traversing the bone perpendicularly (Figure 4A).

The bone defects, into which the collagenized xenografts had been placed, displayed radiological images of an undefined geometric structure, with a decrease in graft volume. Complete repair of the osseous defect was also observed. Radiological images showed those trabeculae that reached the implant as being greater in number and radiopacity than those for the previous time period, giving the grafted area a slightly reticular appearance. No healed or residual bone alterations attributable to the presence of the xenograft were observed. Nor did we observe any osseous malformations or structural changes to bone development over the study period (Figure 4B).

Optical Microscopy. Complete bone remodeling was observed at the control sites. The anatomopathological study found the presence of mature osseous bone in the cortex, which was not differentiable from the adjacent cortex (Figure 4C,E,G).

In this last period, the anatomopathological study was characterized by the presence of mature osseous bone in the cortex of the implant insertion site so that it was not differentiable from the adjacent cortex. We also observed the osseous remodeling of osseous trabeculae around the implant, which was more pronounced in the proximity of the cortex (Figure 4D,F,H).

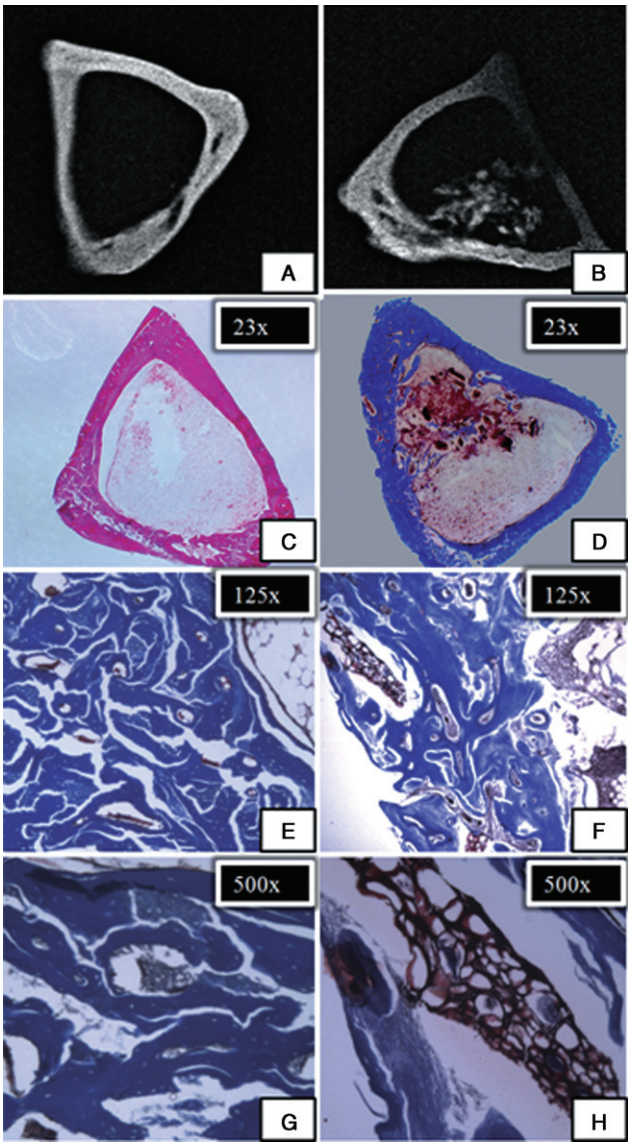

Figure 4 Complete bone remodeling was observed at the control sites. The anatomopathological study found the presence of mature osseous bone in the cortex that was not differentiable from the adjacent cortex. In this last period the anatomopathological study was characterized by the presence of mature osseous bone in the cortex of the implant insertion site so that it was not differentiable from the adjacent cortex. We also observed the osseous remodeling of osseous trabeculae around the implant, which was more pronounced in the proximity of the cortex (Figs. 4d, 4f, 4h, 4c, 4e, 4g).

Morphometric Analysis. Histomorphometry showed that newly formed bone represented 31.5 ± 2.4%,

TABLE 1 Mean-SD Values for Newly Formed Bone, Residual Graft Material, and Nonmineralized Connective Tissue for Each Evaluation Period

	New Bone (%)	Residual Graft Material (%)	Connective Tissue (%)
1 month	19.7 ± 1.5	39.8 ± 3.2	40.5 ± 3.1
2 months	22.5 ± 1.4	37.4 ± 3.1	40.1 ± 2.1
3 months	27.9 ± 2.2	35.2 ± 3.5	36.9 ± 1.5
4 months	31.5 ± 2.4	33.1 ± 2.3	35.4 ± 3.4
Media-SD	**25.4 ± 1.8**	**36.3 ± 3.0**	**38.2 ± 2.5**

residual graft material represented 33.1 ± 2.3%, and connective tissue represented 35.4 ± 3.4%. The length of cortical formation at porcine bone implant sites was 98.9 ± 1.1% compared with 99.1 ± 0.7% at the control sites.

Table 1 shows mean values for the total percentage of newly formed bone, residual graft material, and nonmineralized connective tissue at each evaluation period. Cortical formation length increase values are shown in Table 2.

DISCUSSION

For many years, bone tissue substitution or replacement has been the subject of widespread research because of the necessity of bone tissue substitution in certain situations in buccal maxillofacial surgery. The current demand in clinical dentistry is for materials which will help to accelerate bone regeneration processes.

There are three mechanisms that govern the success of bone regeneration: osteogenesis, osteoinduction, and osteoconduction.[12] The ideal substitute, which combines all three of these characteristics, is autologous bone, the "gold standard" for regeneration.[13,14] Studies of cortico-spongy autologous block grafts[15] show very low morbidity, with patients experiencing only slight pain up until the third day after surgery. However, many clinicians seek alternatives that avoid both secondary surgical zones (donor sites) and undesirable postoperative stages, and which also reduce surgical time.[16–18] Several studies demonstrate the effectiveness of different xenograft biomaterials of porcine origin,[8] in particular, their efficacy as osteoconductive matrices.[9–11]

TABLE 2 Mean-SD Values for Length of Cortical Formation for Each Evaluation Period

	Length of Cortical Formation	
Month/s	Control (%)	Porcine Xenograft (%)
1	90.1 ± 0.7	34.6 ± 3.6*
2	92.3 ± 1.9	59.7 ± 3.7*
3	96.7 ± 2.3	96.5 ± 2.1
4	99.1 ± 0.7	98.9 ± 1.1

*$p < .05$.

The present study confirms the biocompatibility of OsteoBiol® mp3,[10,19] an antigen-free bone consisting of 90% granules of between 600 and 1,000 µm mixed with 10% pure Type-I porcine collagen. The inflammatory response observed in the initial stages of the study was of little relevance and after 1 month was only recognizable as a low number of scattered lymphocytes and macrophages, without the formation of granulomas. Furthermore, in an earlier rabbit study, no fibrosis was observed at the borders between graft and host bone.[11]

Our results have also demonstrated the osteoconductive capacity of OsteoBiol® mp3, which acted as "scaffolding" for bone cells.[20] The presence of collagen within each granule confers hygroscopic characteristics that facilitate successive mixing with pure collagen. Previous studies of hydroxyapatite particles and collagen fibers[21,22] have shown the capacity of osteoblasts for proliferating, differentiating, and mineralizing the matrix, a fact that the present study confirms. Collagen has a fundamental role in the osteoconduction process, acting as a valid substratum for platelet activation and aggregation. It also attracts and stimulates the mesenchymal stem cells present in bone marrow,[23] and can augment osteoblast proliferation levels by as much as two or three times.[24]

Another important factor influencing osteoconduction is the biomaterial's granule size. This study confirms the findings of an earlier study that tabulated the relation between pore size/granulometry and the quantity of neoformed osseous tissue.[25] This led the authors to the observation that with hydroxyapatites of a pore size of between 100 and 160 µm, a 17% degree of bone formation occurs, and this rises progressively to 96% for pore sizes greater than 276 µm. They concluded that pore size and granulometry should not be overly reduced, as both pore diameter and interporotic connections have a significant effect on the type and quantity of newly formed bone tissue.

Other authors[26] have shown that interporosity facilitates bone ingrowth into particles. This fact was verified in the present study by optical microscopy and x-rays taken at 3 and 4 months – the two final study periods – that showed bone growth not only in surface pores but also into pores within the body of the implant. An appreciable decrease in the total volume of biomaterial was observed which, together with optical microscopy findings, points to the replacement of osteoid tissue by adipose and hematopoietic bone marrow, demonstrating the bone grafts' partial and progressive resorption. A study of human subjects[19] found that this process became more accentuated in the fourth month and that after 5 months, partial resorption of the material had taken place. After 4 months, trabecular bone and wide marrow spaces were present and there was a higher density of newly formed bone growth at the surface and in pores. Mean values for newly formed bone, residual graft material, and nonmineralized connective tissue at 4 months were $31.5 \pm 2.4\%$, $33.1 \pm 2.3\%$, and $35.4 \pm 3.4\%$, respectively.

Other authors have described this characteristic in studies of hydroxyapatite. When a bone porcine paste composed of 80% granulated mix and 20% pure collagen[6] was examined at 3 months, complete resorption and substitution with trabecular bone tissue were observed.

Five months after sinus elevation, biopsies of implants consisting of a mixture of autologous bone and cortico-spongy porcine bone,[19] revealed signs of resorption of the xenogaft particles.

Other research[10] with transmission electron microscopy of human biopsies following maxillary sinus elevation with porcine bone showed direct contact between newly formed bone and material particles, but found no signs of resorption.

A study carried out by Nannmark and Sennerby[11] compared porcine bone xenografts with and without added collagen and did not find significant differences with regard to the resorption process. According to the said authors, the mixing of collagen and porcine bone particles facilitated clinical handling, but did not influence bone responses to the material, which exhibited osteoconductive properties and was eventually resorbed. Studies made with porcine xenografts covered by membranes[27] to preserve postextraction alveoli also showed a low percentage of residual graft material (24.5%) at 4 months after implant insertion. However, a recent study using cortical porcine bone augmentation material[20] showed that, after 4 and 6 months, no evidence of graft resorption could be seen; only a few osteoclasts were observed in specimens examined at 6 months.

CONCLUSION

Our results suggest that OsteoBiol® mp3, an antigen-free bone consisting of 90% granules of between 600 and 1,000 μm mixed with 10% pure Type-I porcine collagen, may be a biocompatible material, causing only a minor, early-stage inflammatory response. It also has osteoconductive properties, with the material acting as "scaffolding" for bone cells, leading to progressive increases in bone growth in and around the xenograft. We also observed the substitution of osteoid tissue by adipose and hematopoietic bone marrow, a process which points to the partial and progressive resorption capacity of this collagenized porcine xenograft material.

The said biomaterial may be considered a satisfactory substitute for bone tissue, a material that does not interfere with the bone's normal reparative processes.

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