Bone Formation Following Implantation of Titanium Sponge Rods into Humeral Osteotomies in Dogs: A Histological and Histometrical Study

Paulo Esteves Pinto Faria, DDS, MD;* Alexandre Leite Carvalho, DDS, MD;†
Daniela Nair Borges Felipucci, DDS;‡ Cui'e Wen, Dr;§ Lars Sennerby, DDS, PHD;‖
Luiz Antonio Salata, DDS, PHD**

ABSTRACT

Background: Titanium (Ti) is widely proven to enhance bone contact and growth on its surface. It is expected that bone defects could benefit from Ti to promote healing and to increase strength of the implanted area.

Purpose: The present study aimed at comparing the potential of porous Ti sponge rods with synthetic hydroxyapatite (HA) for the healing of bone defects in a canine model.

Material and Methods: Six mongrel dogs were submitted to three trephined osteotomies of 6.0 × 4.0 mm in one humerus and after 2 months another three osteotomies were performed in the contralateral humerus. A total of 36 defects were randomly filled either with Ti foam, particulate HA, or coagulum (control). The six animals were killed 4 months after the first surgery for histological and histometrical analysis.

Results: The Ti-foam surface was frequently found in intimate contact with new bone especially at the defect walls. Control sites showed higher amounts of newly formed bone at 2 months – Ti ($p = 0.000$) and HA ($p = 0.009$) – and 4 months when compared with Ti ($p = 0.001$). Differently from HA, the Ti foam was densely distributed across the defect area which rendered less space for bone growth in the latter's sites. The use of Ti foams or HA resulted in similar amounts of bone formation in both time intervals. Nevertheless, the presence of a Ti-foam rod preserved defect's marginal bone height as compared with control groups. Also, the Ti-foam group showed a more mature bone pattern at 4 months than HA sites.

Conclusion: The Ti foam exhibited good biocompatibility, and its application resulted in improved maintenance of bone height compared with control sites. The Ti foam in a rod design exhibited bone ingrowth properties suitable for further exploration in other experimental situations.

KEY WORDS: bone healing, hydroxyapatite, osteoconductivity, titanium scaffold

INTRODUCTION

The development of materials engineering over the past decades led to the improvement of polymers and ceramics as osteoconductive materials. These materials can guide bone ingrowth by providing cells with a microstructured scaffold that promotes sequential cell maturation. This process starts with tissue migration of predifferentiated cells from the host tissue, followed by cells proliferation and differentiation to create bone within the scaffold.[1] However, polymers and ceramics have proved poor strength-to-weight ratio and toughness

*Doctorate Fellow, Department of Oral & Maxillofacial Surgery, Faculty of Dentistry of Araçatuba, University of São Paulo, São Paulo, Brazil; †Master in Science, Department of Oral & Maxillofacial Surgery and Periodontics, Faculty of Dentistry of Ribeirão Preto, University of São Paulo, São Paulo, Brazil; ‡Department of Dental Materials and Prosthodontics, Faculty of Dentistry of Ribeirão Preto, University of São Paulo, São Paulo, Brazil; §Doctorate Fellow, Department of Engineering and Technology, Deakin University, Geelong Campus, Geelong, Victoria, Australia; ‖Professor, Department of Biomaterials, Sahlgrenska Academy, Gothenburg University, Göteborg, Sweden; **Professor, Department of Oral & Maxillofacial Surgery and Periodontics, Faculty of Dentistry of Ribeirão Preto, University of São Paulo, Ribeirão Preto, Brazil

Reprint requests: Prof. Luiz A. Salata, The University of São Paulo, Faculty of Dentistry of Ribeirão Preto, Department of Oral & Maxillofacial Surgery and Periodontics, Avenida do Cafe s/n, Campus USP 14040-904 Ribeirão Preto, São Paulo, Brazil; e-mail: lasalata@forp.usp.br

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under loading conditions.[2,3] As an alternate, metallic scaffolds became attractive because their mechanical strength and fatigue resistance over extended periods. Given the excellent overall biocompatibility, commercial-pure titanium (Ti) and alloys (Ti_4Al_6V) have been widely tested for joint replacement and spinal-fusion procedures.[4–6]

Wen and colleagues[7,8] developed a Ti foam using a novel powder metallurgical process to render a bimodal pore distribution (macropores and micropores) resembling natural bone architecture. Previous in vitro studies have shown that osteoblast-like cells grow into Ti scaffolds micropores. These cells are not only able to attach and spread well on the inner surface of Ti scaffolds but are also able to form an extracellular matrix.[9,10] The factors related to the cells' attachment seem to be the optimal pores' size between 100 and 500 μm[11] and the pores' interconnectivity to guarantee the maintenance of the vascular system required for the continuing bone development.[7,12]

The only *in vivo* study on the use of Ti foam was recently published by Sargeant and colleagues.[13] The authors tested a hybrid implant material by assembling Ti–6Al–4V foam and peptide-amphiphile nanofiber matrix using a rat femora model. The histological examination at 4 weeks revealed new-bone deposition from the cortical bone toward the implants and inside the pores, indicating that Ti foam promoted anchorage from the surrounding bone tissue to increase the mechanical stability of the implanted material. Given that Ti foam is a shapable material makes it a potential candidate for applications in different fields, such as craniofacial reconstruction and implantology.

The aim of this study was to assess the osseous conductivity of a Ti foam as compared with synthetic porous hydroxyapatite (HA) for the healing of bone defects in a canine model.

MATERIALS AND METHODS

Ti-Foam Fabrication

Pure Ti powder developed in the Deakin University, Department of Engineering and Technology at Geelong Campus, Australia, (purity ≥ 99.9%, powder size ≤ 45 μm) was used as starting material. Ammonium bicarbonate particles (purity ≥ 99.5%, particle size 200 ~ 500 μm) were sieved as space-holding particles. The fabrication process consisted of three steps: mixing, compacting, and sintering, as shown in Figure 1. At first, Ti powder and space-holding particles were mixed together in an agate mortar. After the ingredients were homogeneously mixed, the mixture was uniaxially pressed into green compacts in steel dies under a pressure of 200 MPa. The green compacts were then sintered in a high-temperature vacuum furnace. The sintering was carried out in two steps: the first step was performed at low temperature of 200°C to remove the space-holding particles, and the second step was performed at high temperature of 1,200°C for 2 hours to sinter the Ti powder into a porous structure. For this in vivo testing, the Ti-foam rods sizing 5.0 mm wide and 4.0 mm long with porosity of 80% and macropore size of 200 ~ 500 μm were fabricated and prepared by wire cut from larger samples. The scanning electron microscopy's micrograph of the Ti foam is shown in Figure 2.

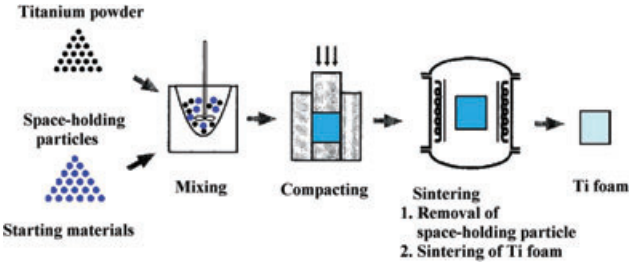

Figure 1 Schematic illustration of Ti-foam fabrication. Published in Wen et al. (2002).[7]

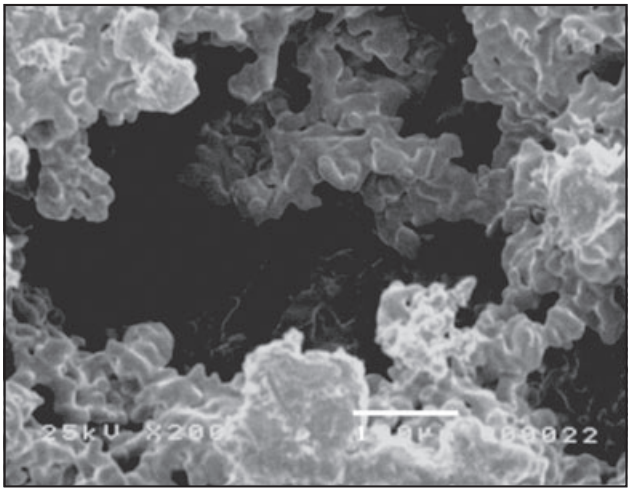

Figure 2 Scanning electron microscopy showing Ti-foam architecture.

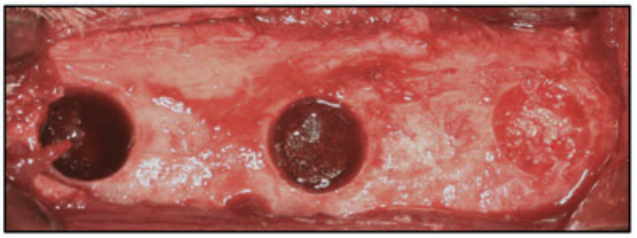

Figure 3 Distribution of trephined bone defects in the humerus. View of the defects after materials were inserted. From left to right: coagulum, Ti foam, and hydroxyapatite.

HA

The highly crystalline synthetic HA with particles' size ranging from 150 to 300 μm, as described by Carvalho and colleagues,[14] was used as a reference control.

Surgery

Six male mongrel dogs, aged 4–5 years old, supplied by the University of São Paulo's breeding house, weighing between 20 and 25 kg, were included in the study. The experimental protocol used in the study was approved by the local ethic committee. Before being admitted for surgeries, all the animals were vaccinated and put into quarantine for clinical observation.

The animals were pre-anaesthetized with xylazine (Ronpum®, Bayer, São Paulo, Brazil, 20 mg/kg I.M.) and 1 g ketamine (Dopalen®, Vetbrands, São Paulo, Brazil, 0.8 g/kg I.M.), and anaesthetized with 1 g thionembutal (Tiopental®, Cristália, Itapira, Brazil, 20 mg/kg I.V.). During the entire surgery the animals inhaled O_2 and were kept in intravenous infusion of saline. The animals' legs were shaved and the skin moistened with iodine alcohol (Povidine®, Rio Química, S.J. Rio Preto, Brazil). A linear incision was carried out over the anterior eminence of humeral epiphysis. Three osteotomies of 6.0 mm in diameter by 4.0 mm long were trephined (BIOMET 3i, Palm Beach Gardens, FL, USA) in one humerus of all six dogs. The defects were either randomly filled with 55.0 mg of particulate synthetic HA and Ti-foam rod measuring 5.0 mm in diameter by 4.0 mm long or left untreated (Figure 3). The wounds were sutured in layers with 3-0 Vicryl™ (Ethicon, Johnson & Johnson, Brussels, Belgium) for the periosteum and 4-0 Nylon™ (Johnson & Johnson, São Paulo, São José dos Campos, Brazil) for the skin using interrupted stitches. After 2 months another three bone defects were drilled in the contralateral humerus and treated similarly to the protocol described earlier. Just after surgeries the animals were given vitamin compounds (Potenay®, Fort Dodge Animal Health, Campinas, Brazil), anti-inflammatory/analgesic drugs (Banamine®, Schering-Plough Animal Health, São Paulo, Brazil), and antibiotics (Pentabiotico®, Fort Dodge Saúde Animal, São Paulo, Brazil).

The animals were kept in cages at the university's laboratory with free access to water and fed with moistened, balanced dog chow. Postoperatively, the animals were daily inspected for clinical signs of complications or adverse reactions. Four months after the first surgery, all six dogs were euthanized through overdose of Thiopentax® (Cristalia Ltd., Campinas, Brazil), and samples containing the experimental sites and surrounding soft tissues were explanted (Figure 4) and submitted to laboratory post-processing.

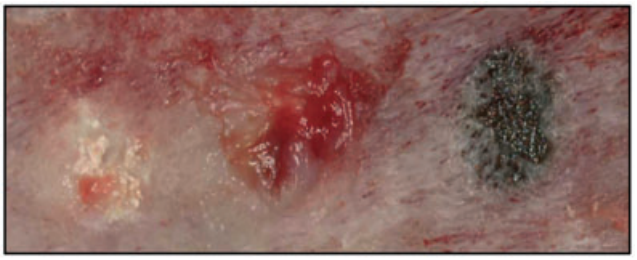

Figure 4 Clinical view of the bone defects' sites after a 4-month healing. From left to right: hydroxyapatite, coagulum, and Ti foam.

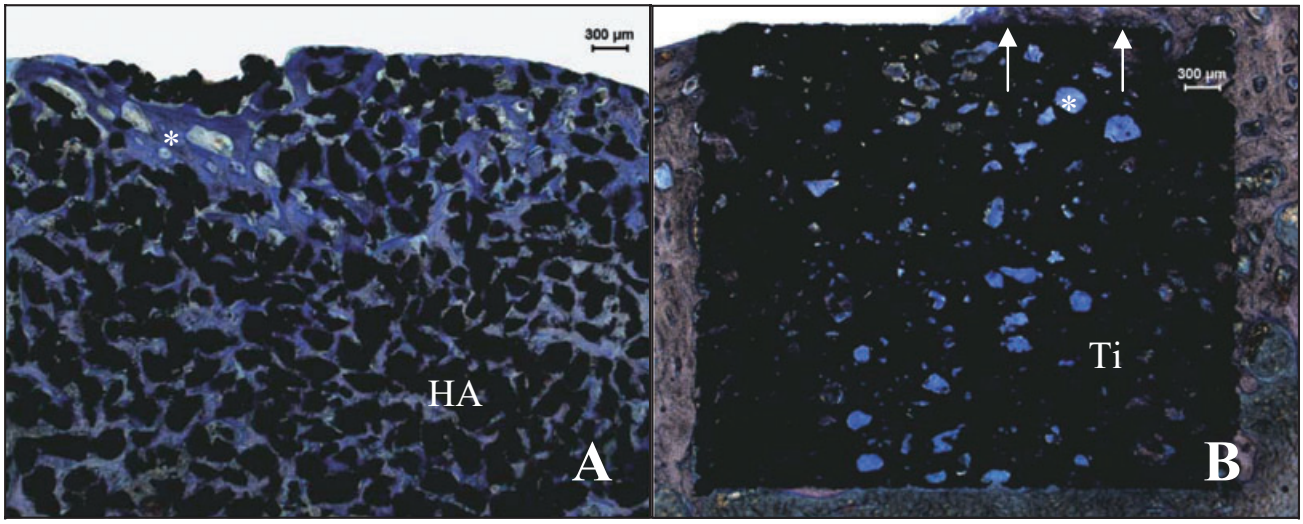

Figure 5 Photomicrograph showing in (A) the upper section of a defect site treated with hydroxyapatite (HA). (*) Note soft tissue presence among granules. In (B) the pores of Ti foam are filled with new bone (*); the arrows indicate bone growth on the top of the material. The bars indicate magnification. Staining: toluidine blue-pyronine.

Ground Sections' Preparation

The samples were fixed in 4% formaldehyde solution that was changed every 48 hours for 10 days, followed by dehydration in a series of graded ethanol for 15 days, and finally, embedded in resin (LR White® hard grade, London Resin Company Ltd., Berkshire, UK) over the period of up to 15 days. The blocks were cut across the long axis of the humerus using a diamond band saw fitted in a precision-slicing machine (Microslice 2™, Ultratec, Santa Ana, CA, USA). The thickness of the histological slides was 20–25 µm, stained with toluidine blue-pyronine, and examined twice in double-blind controlled method by the same investigator (PEPF) under standard light microscope (Leica Microsystems™, Wetzlar GmbH DMLB, Solms, Germany). The histometrical assessment – intended to measure the percentage of bone tissue, materials, and soft tissue occurring inside each defect area – was carried out twice with the QWin Plus® software (Leica Microsystems™, Wetzlar GmbH). The same equipments and software were used to determine the proximal, central, and distal height of each defect after 2 and 4 months.

Statistical Analysis

The descriptive analysis of the raw data was performed using the *SPSS*® software (SPSS Inc., Chicago, IL, USA). The pertinent comparisons between relevant variables in the groups were calculated. The analysis of variance test was used for comparison of treatment groups with respect to the sacrificed time. In connection with statistical evaluations, a difference was considered significant if $p \leq 0.05$.

RESULTS

Clinical Observations

The postoperative period occurred uneventful. Gross examination of the animals' skin revealed no signs of tattooing associated with sites treated with Ti foam, as assessed at the euthanasia.

Histological Observations at 2 and 4 Months

The histological evaluation aimed at determining the nature of the bone-material interface. Differently from synthetic HA, the Ti foam was densely distributed into the defect area (Figure 5, A and B) that did not impair bone formation on the surface and the inner compartments of the Ti foam during the experiments.

Two Months. A common finding in the control group was new-bone deposition at the central portion of the defect featured by some trabecular bone formation whereas the upper third of the defect was frequently invaded by fibrous tissue (Figure 6). In sites treated with synthetic HA, the granules were partially embedded in new bone and marrow tissue in the center (Figure 7) and at the margins of the defects. The Ti foam always promoted direct bone contact at the margins what rendered

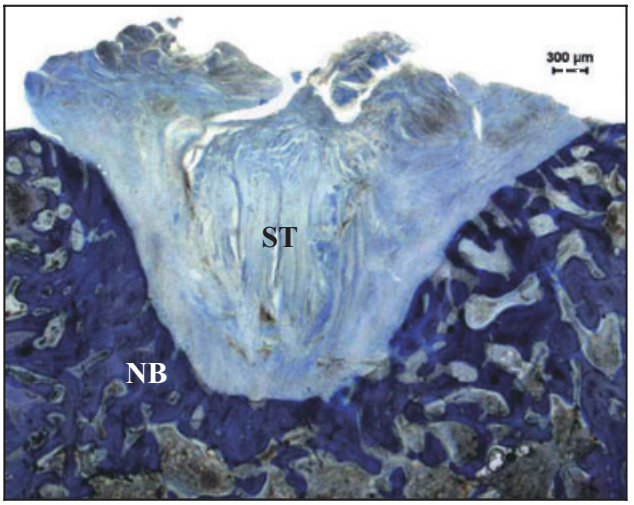

Figure 6 Photomicrograph showing the upper section of a control site at 2 months. The defect has been healed with new bone (NB). Note the bone depression (arch-shaped) of the newly formed cortical which is accompanied by soft-tissue (ST) presence. The bar indicates magnification. Staining: toluidine blue-pyronine.

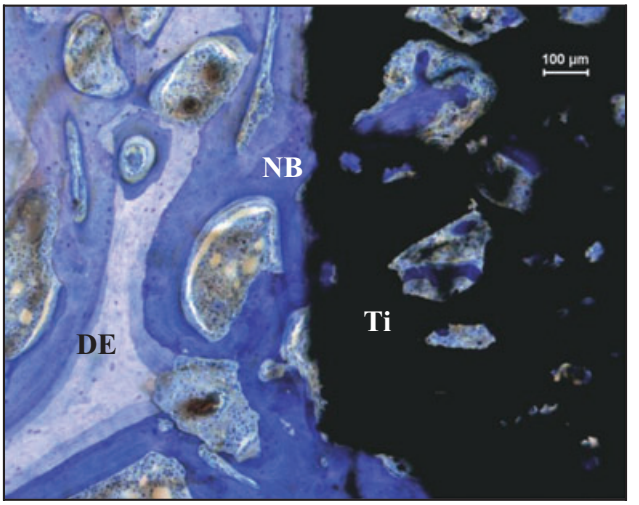

Figure 8 Photomicrograph showing the margins of a defect treated with Ti foam after 2 postoperative months. The Ti surface is paved with new bone (NB) grown from the defect's edge (DE). Further into the defect, NB can be seen penetrating pores of the material (Ti). The bar indicates magnification. Staining: toluidine blue-pyronine.

bone to grow into the pores of the material (Figure 8). The former empty spaces within the foam structure were occupied by scarce marrow tissue and woven bone at this stage.

Four Months. The bone walls in the control group appeared lined with new bone, which contrasted with the center of the defect where the soft tissue predominated. With regard to the HA group, the granules seemed increasingly surrounded by a new bone thoroughly the defect (Figure 9) except at the upper third where the granules were partially immersed in soft tissue. Compared with the histological features at 2 months, the bone at the margins of the defect and within pores, in the Ti-foam group, showed a more mature pattern at 4 months (Figure 10).

Histometrical Observations at 2 and 4 Months

The data on histometrical analysis are shown in Figure 11. Overall, the statistical significance between

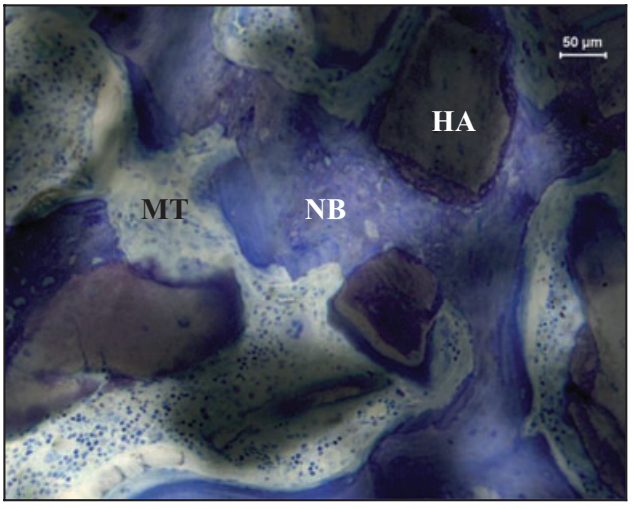

Figure 7 Photomicrograph showing the center of a defect treated with HA after 2 months. The granules (HA) presented immerse in marrow (MT) and new bone (NB). The bar indicates magnification. Staining: toluidine blue-pyronine.

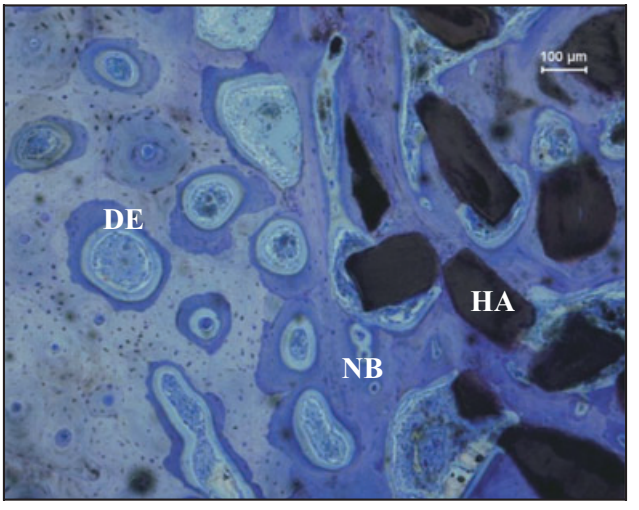

Figure 9 Photomicrograph showing the margins of a defect treated with hydroxyapatite (HA) after 4 postoperative months. The granules (HA) appear involved in new bone (NB) from the defect's edge (DE). The bar indicates magnification. Staining: toluidine blue-pyronine.

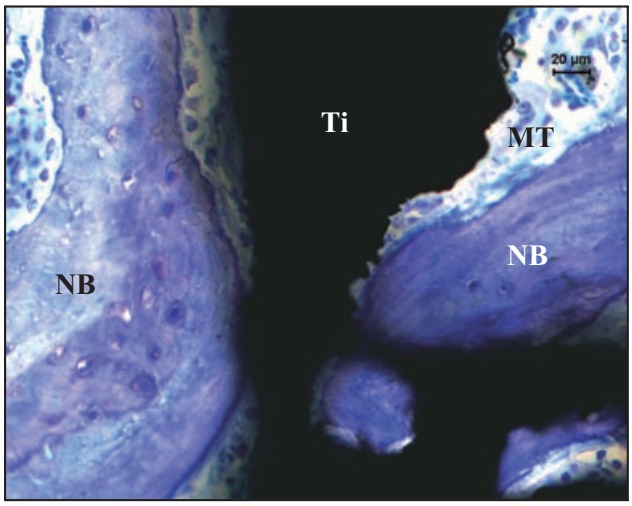

Figure 10 Photomicrograph showing a defect treated with Ti foam after 4 postoperative months. The Ti pores are predominantly filled with new bone (NB) and marrow tissue (MT). The bar indicates magnification. Staining: toluidine blue-pyronine.

treatments and experimental time points was detected when the amount of bone measured in HA, Ti foam, and control sites was compared. The treatment with HA significantly rendered more bone formation than Ti foam in both 2 months ($p = 0.016$) and 4 months ($p = 0.001$), postoperatively. The control sites presented higher amounts of bone formation as compared with Ti foam in both 2 and 4 months ($p = 0.001$ and $p = 0.002$, respectively). When the percentage of area occupied by the biomaterials was compared, the Ti foam was statistically more concentrated than HA in both 2 months ($p = 0.031$) and 4 months ($p = 0.003$), postoperatively.

The data on bone-height preservation showed that Ti foam was more effective than control ($p = 0.017$ and

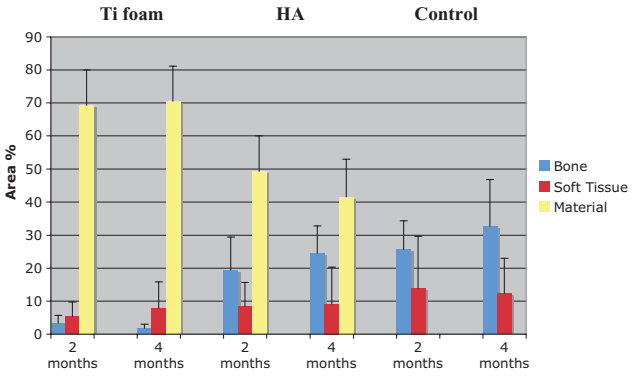

Figure 11 Variation in percentage of bone tissue, soft tissue, and materials in bone defects treated with Ti foam, hydroxyapatite (HA) granules, and control along the experimental time point. Vertical bars indicate the +SD.

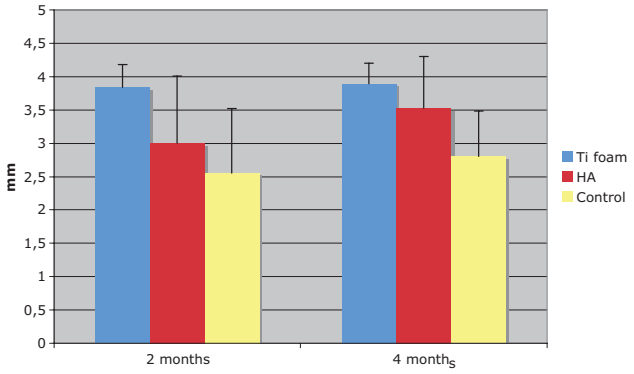

Figure 12 Variation in millimetres of bone defects height according with treatment and experimental time. Vertical bars indicate the +SD. HA = hydroxyapatite.

$p = 0.009$) at 2 and 4 months, respectively (Figure 12). Although lacking statistical significance, the experiments showed a clear trend toward improved bone preservation in sites treated with Ti foam as compared with HA sites in both experimental time points.

DISCUSSION

The advantage of porous materials is their ability to provide biological anchorage for surrounding bone tissues.[15–19] Therefore, the microarchitecture of osseoconductive-like materials plays an important role in the process of bone formation[20] as the inner surfaces of pores favor for cells' seeding, attachment, proliferation, differentiation, and finally, tissue ingrowth.[21]

Both materials, Ti foam and HA, tested for comparisons in the present study, were porous. The pores' size ranged from 200 to 500 µm[7] in the former and sized always smaller than 10 µm[14] in the latter. Despite the ideal, porous size has been reported to vary between 100 and 500 µm[11], and that porous connectivity could only be found in the Ti foam; histologically, these structural differences have not unbalanced the excellent osseoconductivity-like observed in the present study in both groups. With regard to Ti foam, the cells' attachment occurred as described in in vitro studies,[3,7,9–11,22] that is, the process of new-bone deposition on the porous surface as well as adjacent canals at 2 months, while after 4 months, osteoblast-like cells were colonizing the pores' surface which coexisted with mineralized bone in materials bulk.

The histometrical analysis revealed that Ti foam presented the lowest percentage values over bone formation as compared with control and HA groups, in

both experimental time points. This occurred as a consequence of the significantly denser distribution of the Ti foam in the defect area which resulted in diminished space for bone growth in these sites. As a compensatory effect, the well-structured architecture of Ti foam led to a better bone-growth distribution in the implanted sites, even in places where HA and control failed, for example, at the crestal marginal bone. It has been reported that the mechanical properties of the Ti foams with relatively low densities – approximately 0.20–0.30 – match the human cancellous bone, whereas Ti foams with higher densities such as 0.50–0.65 are closer to the human cortical bone.[8] The presence of the Ti foam tended to preserve defect's marginal bone height as compared with HA and control groups, which may have occurred because of the efficiency of Ti to attract bone-forming cells and physical structure of the foam. Furthermore, the extent of bone formation into the foam demonstrates that the pores were sufficiently interconnected to allow for cellular migration between pores and nutrient diffusion.[12] Considering the HA was used in granular form, the maintenance of density was probably hindered by granules' micromotion at open margin of the defect, which explains the soft-tissue infiltration between granules into the defect. The fact that Ti foam was dense-packed into the defect led to more efficient bone-height preservation compared with HA implantation.

The outcomes of this *in vivo* study confirm the findings of previous in vitro studies[6,23,24] that Ti foam allows for bone ingrowth through interconected porous. While the biological properties of Ti are widely acknowledged, this is the first time Ti foam alone is tested for bone repair *in vivo*. Potentially, this system could serve as an interlocking system between bone tissue and implant (made of Ti or others) devices when increased stability of the latter is sought. Ongoing studies by this group are looking to the application of Ti foam of bimodal porous architecture welded onto dental implant surface in the attempt of improving osseointegration at marginal walls of bone defects.

CONCLUSIONS

According to the specific model used in this study, it can be concluded that the Ti foam exhibited good biocompatibility and its application resulted in bone formation within the whole Ti structure. The less material occupying a defect, the more newly regenerated bone was detected. For purposes of restoring and maintaining bone-rim height in defects, Ti rods and HA particles were superior to empty defects. Ti-foam group showed a more mature bone pattern at 4 months. Differently than Ti foam, the HA granules at the upper third of the osteotomitized area were partially immersed in soft tissue. Taken together, the results show that Ti foam in a rod design exhibited bone ingrowth properties suitable for further exploration in other experimental situations.

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