EFFECTS OF GRANULE SIZE ON THE OSTEOCOCONDUTIVITY OF BOVINE AND SYNTHETIC HYDROXYAPATITE: A HISTOLOGIC AND HISTOMETRIC STUDY IN DOGS

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Two bovine hydroxyapatites (BHAs), one with granule size of 150 to 200 μm and one with granule size of 300 to 329 μm, and 2 synthetic hydroxyapatites (SHAs), with granule size of 150 and 300 μm, respectively, were compared for effectiveness in repairing circumferential bone defects in dogs. The hydroxyapatites (HAs) were characterized through powder x-ray diffraction (XRD) analysis and scanning electron microscopy (SEM). Three trephined bone defects (5.0 mm wide × 4 mm long) were created in the humeruses of 8 dogs. In a random manner, the defects on each side were treated with either BHA with small granules (BHA(S)), BHA with large granules (BHA(L)), SHA with small granules (SHA(S)), SHA with large granules (SHA(L)), or left to heal unaided (bilateral control). Four dogs were sacrificed after 6 and 12 postoperative weeks, respectively. Ground sections of each defect were submitted to histologic and histomorphometric analysis (percentage of area occupied by bone, bone marrow, and biomaterial). As a rule, the HA granules exhibited direct bone contact, regardless of the origin and the size of the granules. Control sites were related and had an increased amount of connective tissue infiltration. At 12 weeks, BHA(S) exhibited improved bone formation compared with SHA(S) and SHA(L). The SHA(S) delivered reduced amounts of bone compared with the remaining groups (control included). The area of bone measured in BHA(S) sites was significantly higher at 12 weeks than 6 weeks. The XRD revealed the tested HA samples to be highly crystalline, while BHA appeared with rougher surface at SEM analysis. The BHA(S) performed better than the SHA(S) and SHA(L), as assessed by the amount of bone measured in both implantation sites at 12 weeks. The BHA’s material characteristic itself rather than granules size accounted for the distinctive biological behavior. The increased roughness of the BHAs’ surface, as assessed through SEM, seemed to benefit the osteoconduction process.

Key Words: synthetic hydroxyapatite, bovine hydroxyapatite, granule size, crystallinity, surface roughness, osteoconduction
INTRODUCTION

The spontaneous alveolar ridge healing that follows teeth extractions frequently leads to bone deformities that result in unsuitable sites for titanium implant placement. Several granulated biomaterials have been tested as therapeutic adjuvant to obviate the formation of bone defects in postextraction dental sockets as well as for sinus floor augmentation procedures, either used alone or mixed with autogenous bone. Among these biomaterials, bovine and synthetic hydroxyapatite (HA)—Ca₁₀(PO₄)₆(OH)₂—granules have gained particular interest over the recent decades because of the multidisciplinary clinical application.

The mechanisms behind tissue response to HAs have not as yet been comprehensively established in the literature. A number of studies have demonstrated that osseointegration and osteoconduction processes are influenced by physical and chemical properties of the material, including granule size, granule morphology, crystallinity and porosity, surface roughness, and ratio of calcium to phosphate (Ca:P) in the composition. Nevertheless, the requirements the best performance for these biomaterials have not been thoroughly addressed.

Oonishi et al. evaluated HA with granules of 1 to 3 μm, 10 μm, and 100 to 300 μm in diameter and noticed that a minimal size of 10 μm was necessary to enable a direct contact between bone and the particles. Sun et al. studied the effect of different sizes of HA granules (from 0.5 to 841 μm) in osteoblast cultures and reported inhibitory effects for the 0.5 to 3 μm group. Kuroda evaluated bone defects filled with HA granules of 100 to 2000 μm and observed improved osteoconductive activity in the group with granules of 100 to 300 μm.

Misiek et al. compared sharp-edged and rounded HA granules and observed that although a mild inflammatory response was seen at the implant sites with both particle shapes, inflammation resolved faster in sites implanted with rounded granules.

Yang et al. stated that the dissolution and crystallinity of HAs were related in a negative manner in vitro. The Ca:P ratio in the composition of HAs seems to directly affect crystallinity. Thus, highly crystalline HA—Ca₁₀(PO₄)₆(OH)₂—has a Ca:P molar ratio of 1.67, whereas less crystalline bioceramics, such as tricalcium phosphate and tetracalcium phosphate, are characterized by lower or higher ratios of 1.50 and 2.0, respectively.

Takeshita et al. observed that nonporous HA granules grafted into bone defects surrounding titanium implants resulted in fibrous encapsulation during the early healing stages.

Deligianni et al. used a bone marrow cell-culture model to demonstrate that cell adhesion, proliferation, and detachment strength increased as the roughness of HA increased.

To date, a wide source of HA has been made available for clinical application. Synthetic hydroxyapatites (SHAs) are the most frequently used, but they do not completely match the chemical composition of bone. Bovine hydroxyapatites (BHAs) have recently been used as an interesting alternative to SHA for bone regeneration procedures because of the lower production cost and because they naturally derive directly from the bone itself. In a rare in vivo comparison between SHA and BHA, the former delivered higher elasticity modulus than both BHA and the resident bone after 6 and 26 weeks of implantation in rabbit femur. The BHA equalized the compressive strength of natural bone. It is expected that biomaterials with high modulus of elasticity can adversely influence the remodeling of the surrounding bone, as the mechanical stimulus is impaired from evenly dissipating through both sites.

The distinctive biological and mechanical properties exhibited by these biomaterials have raised the attention of many investigators. While widely studied in the literature, to our knowledge the influence of the granule size of synthetic and bovine bone substitutes has not been addressed by in vivo studies. The purpose of the present study is to compare the performance of BHA and SHA tested in 2 different granule sizes in a bone-healing model in the dog humerus.

MATERIAL AND METHODS

Materials

The BHAs were supplied by the Department of Industrial Engineering, University of Marmara, Turkey. The BHAs were produced according to the method described by Oktar et al. The bovine bone obtained from diaphyseal tibia was irrigated with tap water and soaked in a 1% concentration of an antiseptic solution. Thereafter, samples were reirrigated with tap water and calcined at 5°C/min to 850°C for 5 or 6 hours. The matter was sintered at 1200°C for 4 hours, ground in a ball-grinder, and sieved into particle sizes of 150 to 200 μm and 300 to 329 μm. The SHAs were prepared from apatites obtained by hydrolysis and subsequently sintered at 1200°C for 4 hours. The
matter was ground in a ball-grinder and sieved into particles sizes of 150 to 300 μm. The HA portions allotted to each bone defect (55 mg) were placed in polymer sterilizing bags and subjected to ethylene oxide sterilization (IPEN—Nuclear Power Research Institute, São Paulo, Brazil).

**Surgery**

Eight young male mongrel dogs weighing between 15 and 20 kg were used in this study. The research protocol as described subsequently was approved by the local Animal Research Ethics Committee at the University of São Paulo at Ribeirão Preto.

The animals were sedated with intramuscular 5.0 mg/kg xylazine (Dopaser, Calier Laboratories, Barcelona, Spain) and 20 mg/kg ketamine (Ketamine Agener, National Pharmaceuticals Chemistry Union, Embu-Guaçu, Brazil) just before surgery. Afterwards, they were anesthetized with 1 mL/kg of endovenous thiopental sodium (Anental, 20 mg/kg thiopental diluted in 50 mL saline solution, Strides Arcolab Limited, Secunderabad, India).

After the legs were shaved and the skin was disinfected with iodine tincture, an incision was made at the midshaft of the humerus intended for full-flap reflection. Using a trephine (3i, Implant Innovations Ltd, Palm Beach Gardens, Fla) (5.0 mm wide × 4.0 mm long), 3 perforations separated by 10 mm distance were created in the humeral bone (Figure 1) so that every animal had 6 bone defects each.

The defects were divided into 5 groups according to type of treatment: BHA with small granules (BHA[S]) (150 to 200 μm); BHA with large granules (BHA[L]) (300 to 329 μm); SHA with small granules (SHA[S]) (150 μm); SHA with large granules (SHA[L]) (300 μm); and control (sham defect). The treatments were randomly allocated.

Each perforation was filled with 55 mg of granules mixed with 0.1 mL blood to facilitate manipulation and insertion into the defect (Figure 2). In all cases the amount of material implanted was sufficient to fill the bone defect.

After the materials were implanted, the flap was repositioned using separate sutures for periosteum and muscular fascia (Vicryl 4–0, Ethicon, Johnson & Johnson, São José Dos Campos, Brazil). The skin was closed with nylon stitches (Nylon Monofilament 5–0, Brasuture, São Sebastião da Grama, Brazil).

At the end of the surgery the dogs received a single dose of intramuscular flunixin meglumine analgesic (Banamine, 1 mL/10 kg, Schering-Plough, Rio de Janeiro, Brazil) and 24 000 IU of penicillin/kg along with 10 mg/kg of streptomycin (Pentabiotics, 0.5 mL/5 kg, Ford Dodge, Campinas, Brazil) antibiotics. The dogs were allowed to consume water and dog chow ad libitum and were checked daily for postoperative complications.

After 6 and 12 postoperative weeks, respectively, 4 animals were sacrificed using a lethal dose of thiopental. One bone block was removed from each humerus, which encompassed the entire region with the 3 defects.

**Histologic processing**

The bone blocks were fixed in 4% formalin for 10 days, dehydrated in increasing alcohol grade concentrations up to 100%, and finally embedded in LR White resin (London Resin Company, Berkshire, England). The resin blocks were sectioned axially to the defect using the ground sectioning technique described by Donath and Breuner19 for hard tissue. The histologic sections were stained with Stevenel’s blue and Alizarin red S.

**Histomorphometric analysis**

Histologic slides nearly 100 μm thick from each defect were divided into thirds (apical, mid, and cervical) and the images were captured using LEICA DC 300F video camera (Leica Microsystems GmbH Nussloch, Germany) coupled to a LEICA MZFL III stereomicroscope (Leica Microsystems GmbH Nussloch, Germany), at ×100 magnification. The images were analyzed using Image J software (National Institutes of Health, Bethesda, MD) through the point-counting method described elsewhere.24,5,20,21 A grid with 56 intersection points was superimposed on each third, 168 points for the entire defect (Figure 3). The number of points was converted into percentage of the area for bone, biomaterial, and bone marrow.

**X-ray diffraction and scanning electron microscopy analysis**

These two analyses were aimed at characterizing the tested HAs so as to correlate biomaterial composition and morphology with the respective biological behavior. The x-ray diffraction (XRD) spectra were taken using Cu (kα1) radiation (Siemens D5000 diffractometer, Germany). The spectra were recorded from 29 = 6 to 60 at a step size of 0.02°. A Zeiss DSM 940A (Germany) microscope was used for the scanning electron microscopy (SEM) analysis.

**Statistical analysis**

At 6 and 12 weeks, the 2 types of HAs in 2 different granulation each and the control were compared in relation to the amount of material (HA groups), bone, and bone marrow occurring in the defects. The
The Friedman test was used for multiple comparisons and Wilcoxon’s test was applied when statistical differences were identified. When the data were compared, focusing on the differences between 6 and 12 weeks, the values of material, bone, and bone marrow were compared in pairs using the Mann-Whitney U test. Finally, the Friedman test (for multiple comparisons) and the Wilcoxon test (for paired observations) were applied for data of bone density at the apical third of the defects comparing the HAs and the control groups at 12 weeks. A \( P \) value of \( \leq .05 \) was considered significant.

**RESULTS**

All surgical sites healed uneventfully, and there were no signs of clinical reaction to the treatments used for this investigation.

**Histologic analysis**

The humerus cross sections at both 6 and 12 weeks showed tightly packed HA particles all over the area of experimental defects. Invariably, the bone tissue was in intimate contact with the particles, drawing a mosaic-like arrangement (Figure 4). Several trabeculae could be seen spanning the cortical-cancellous bones from contiguous areas into the defect. Osteoblast layers were lining the woven bone at both 6 and 12 weeks (Figure 5) suggesting that the process of bone deposition was yet in its course. Osteoclasts in Howship’s lacunae at bone surface indicated evidences of bone remodeling in 6 weeks (Figure 4). The comparison among the 5 groups presented significant difference in relation to the bone formation (\( P = .009 \)). The bone amount of control group was statistically lower in relation to BHA(s), BHA(l), and SHA(s) (\( P < .05 \)). Besides that, BHA(s) was higher than the 2 synthetic ones and BHA(l) was higher than SHA(l) (\( P < .05 \)). Considering the levels of bone marrow, the control group presented considerably higher amounts (\( P = .05 \)) compared with the remaining materials, and SHA(l) was related with larger areas than SHA(s) (\( P = .05 \)).

**Histomorphometric analysis**

In control groups the new bone formation process resembled an attempt to restore the original structure. Nevertheless, the bone formed at the cortical region showed characteristics of cancellous bone, characterized by fine bone trabeculae. Moreover, the bone at the open edge of the defect was filled with dense connective tissue that, in some cases, extended into the mid third creating a concave surface (Figure 7).

Especially at the apical region, the defects filled with HA were mostly occupied with thick trabecular bone, contrasting with a less trabecular pattern in areas adjacent to the defect (Figure 4).

**Analysis of XRD**

Given that the XRD is a granule-size independent method to measure phases of compounds (crystalline or amorphous), the diffractograms were presented for BHA and SHA irrespective of granule size (Figure 11). The diffractograms showed that only the HA phase...
was present in the samples. The BHAs and the SHAs presented the sharpening of the main reflections located between $2\theta = 30^\circ - 35^\circ$. This is compatible to HA with highly crystalline structure, according to the 09–0432 form$^{22}$ and to Deligianni et al.$^{15}$

**Analysis of SEM**

The photomicrographs obtained from HA powders dramatized the differences in relation to the size of the granules, according to the one established in the samples, as well as a similarity in the format. When the granules were evaluated in larger magnification (Figure 12), it was possible to observe a difference in relation to the surface roughness of the granules. In all samples, the granules had micropores ($<10 \mu m$),$^{23,24}$ but the surface area of the granule was clearly larger in the BHAs because of its greater surface roughness.

**DISCUSSION**

This study compared BHAs and AHAs with different granules size as bone substitutes for the healing of cortico-cancellous bone defects in the dog humerus. The HAs' composition characterization through XRD analysis as well as the relative standardization of granule size of SHA and BHA used in this study ensured an overall understanding about the role played by both origin and particles size on the osteoconductivity of these bone substitutes. Moreover, this investigation gains further importance because of the scarce scientific documentation in the literature over the comparison between BHA and SHA either in vitro or in vivo.

With regard to bone formation, the outcomes of HA implantation after 6 weeks showed no consistent effect in the comparisons. At this experimental time the HA granules were captured paving the whole extent of the defect with no evidence of inflammatory cells infiltrate. At 12 weeks, the amount of bone tissue found in defects treated with BHA(s) was significantly higher than at 6 weeks. Compared with SHA(s) and SHA(L), BHA(s) sites were related with significantly larger bone areas at 12 weeks. Also, the amount of bone formed in BHA(L)-treated defects was consistently higher than in the SHA(s)-treated defects at this experimental time. In fact, the SHA(s) delivered the
There is some scientific controversy about the size of HA granules that would not come to harm bone-forming cells differentiation and proliferation. In an in vitro study, Evans\textsuperscript{25} found increased fibroblasts mitotic rate in the presence of synthetic HA particles between 3.7 and 99 µm. Oonishi et al,\textsuperscript{8} in an in vivo study, demonstrated that particles larger than 10 µm were required for successful bone augmentation procedure. Sun et al\textsuperscript{9} tested different sizes of synthetic HA particles (0.5 to 3.0, 37 to 63, 177 to 250, and 420 to 841 µm) in osteoblast culture and demonstrated that this cell population grew at a similar rate on the HA 37 to 841 µm particle size range, contrasting with the poor results obtained with 0.5 to 3.0 µm granules. Weissenboeck et al\textsuperscript{26} examined mesenchymal stem cells in relation to the ability for osteoblastic differentiation and the output of proteins related to bone formation cultured on algae-derivative HA granules (10 to 100, 200 to 500 and 600 to 1000 µm). The authors concluded that the smallest granules (10 to 100 µm) were more effective. In our study, the BHA\textsuperscript{(s)} granule size ranged from 150 to 200 µm and the outcomes in terms of bone formation at 12 weeks (47.22%) were superior (23.10%) to those reported by Artzi et al\textsuperscript{20} with BHA granules ranging from 250 to 1000 µm in defects made on the mandible of dogs using the same size bone defect as in our study. When it comes to clinical application, BHA is commercially available in granules ranging from 250 to 1000 µm.\textsuperscript{2}

All these data together may indicate a lack of consensus in the literature toward the most suitable HA granule size for bone substitution. The present study...
study showed that BHA(s) performed better than SHA(s) and SHA(L) at 12 weeks, but not against BHA(L). Intriguingly, SHA(S) exhibited the poorest results even when compared with SHA(L) and the control. One can raise the question as to whether granules size within a determined range or the biomaterials themselves were responsible for the observed effects.

Indeed, in our study the implantation of BHA resulted in significantly more bone formation in the defects than SHA implantation, when the data were taken irrespective of the granule size ($P = .03$). These findings are supported by an ultrastructural comparative study between bovine and synthetic bone substitutes using 2- and 3-dimensional images derived from SEM and transmission electron microscopy. The authors remarked that the BHA after deorganification still conserves a template reflecting mineral-collagen interaction and crystallites. This structural characteristic of the bone mineral phase is not demonstrated by SHA materials, which are formed without a guiding organic template. Others suggested, based on an in vivo study, that BHA supports the maturation of collagen type I acting like a mineral deposit, which delivers ions for the new osteoid and promotes mineralization.

The surface texture is regarded as an important factor in the osseoconduction by the HAs. The SEM analysis showed that all samples tested in this investigation presented micropores. Andrade et al studied dense and porous HA cylinders and observed the fibrous tissue development surrounding dense implants and the direct contact of the new bone formed in the porous implants. Takeshita et al used dense HA granules (300 to 600 μm) in bone defects created surrounding osseointegrable implants and reported fibrous encapsulation of the granules. They concluded that dense HA granules negatively interfered with bone formation. Many other studies have reported improved bone-HA integration when the particles presented micro- and macropores. Recent studies have demonstrated that the increased HA surface roughness positively affected osteoblast adhesion and proliferation. In our study the BHA exhibited a rougher surface than SHA, which may also have cooperated for the best results in terms of bone formation in sites implanted with BHA.

Although bone formation was evident in all tested groups, the present study failed to demonstrate significant resorption of BHA and SHA granules from 6 to 12 weeks of the experiment. Ideally, the bone substitute should conduct or induce bone formation at the same time it is completely resorbed and substituted by bone tissue. Evidence from previous studies suggests that HA resorption can be mediated by cells (degradation by macrophages and osteoclasts) or by disintegration through the action of the extracellular fluids (chemical dissolution). Briefly, in our study the histologic analysis showed the presence of foreign body-like multinuclear giant cells in close contact with the HA surface and bone formation adjacent to the particles. Liljensten et al stated that even for HAs considered absorbable, the
resorption process is slow and the finalization is not well determined. The same authors, when comparing absorbable and nonabsorbable HAs over 12 weeks, did not observe statistical differences between granule areas either. Artzi et al., who evaluated BHA in dog mandibles using the same defect size as in our study, reported that despite the excellent osseoconduction of the material it was present at the experimental sites with no substantial resorption after up to 2 years of remodeling. A great deal of studies have reinforced similar behavior of HAs. In the context of osseoconduction output, the higher the crystallinity of the HAs the less likely that the granules will break down into fine bits and the better will be the ossointegration. The likely explanation for the poor resorption rate of HAs in our study might be found in the XRD outcomes. Both BHA and SHA were highly crystalline and, therefore, less soluble and more resistant to granular disintegration by extracellular fluids. This finding must be balanced with the trend that compounds formed with little crystalline and much amorphous (ie, HA/tricalcium phosphate at 30:70 ratio) bioceramics leads to increased bone regeneration and biomaterial replacement by bone tissue than crystalline HA alone.

The finding that test and control groups failed to present significant differences at 6 and 12 weeks can pose doubts whether or not 5.0-mm diameter defects in the dog humerus were actually of critical size. The previous studies reported that 20% to 30% of the mineral content in the defect, the use of bone substitutes can potentially jeopardize the osseointegration of titanium implants. Considering the high crystallinity of these biomaterials and, consequently, their diminished resorption rate, one can expect complex titanium-HA interface. This sort of interaction tends to result in biomechanical instability given the combination between resilient (bone) and rigid
(biomaterial and titanium surface) materials. Well-controlled experimental studies are required to test this hybrid interface under implants in load-bearing condition.

CONCLUSION

To our knowledge, this is the first in vivo study to address the comparison between BHA and SHA materials focusing on the influence of granule size on osseoconduction. The outcomes indicate that BHA performed better than SHA as osteoconductive biomaterial, as assessed by the amount of bone measured in the implantation sites at 12 weeks. The BHA's distinctive characteristics, such scaffold structure and surface roughness, rather than the granule size itself, accounted for this improved biological behavior. Neither BHA nor SHA exhibited signs of resorption until 12 weeks of implantation, which may relate to the high crystallinity presented by these biomaterials.

ACKNOWLEDGMENTS

We would like to thank Eliana Cristina da Silva Rigo for her assistance with the scanning electron microscopy and x-ray diffraction analysis and Sebastiao Carlos Bianco for histology slides preparation. This study was partly funded by the Turkish Republic Government Planning Organization in the framework of the project, “Manufacturing and Characterization of Electro-Conductive Bioceramics,” #2003 K120810.

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