Abstract: The aim of this study was to evaluate the physico-chemical and biocompatibility characteristics of two different hydroxyapatites. Physical and chemical properties were analyzed using granulometric analysis, scanning electron microscopy (SEM), X-ray energy-dispersion (EDX), X-ray fluorescence (XRF) and X-ray diffraction (XRD). Biomaterials were implanted into the subcutaneous tissue on the dorsum of 36 Wistar rats, divided into the following groups: Group 1 – GeneOx™ (natural); Group 2 – HA-U (synthetic) and Group 3 – Control (Sham). After 15 and 30 days, 6 animals/period were sacrificed and the subcutaneous tissue was taken for histological and histometric analysis, giving consideration to inflammatory reaction and granule area. The granulometric test results showed a mean granule diameter of 161.6 µm (min = 19.0 µm; max = 498.0 µm) and 48.7µm (min = 7.0 µm; max = 256.0 µm) for groups 1 and 2 respectively. Analysis with SEM demonstrated irregular and sharp-edge particles in group 1 (3332.8 ± 274.3 µm²) and irregular and rounded particles in group 2 (1320.8 ± 83.0 µm²) (P < 0.0001; Student’s t test). EDX and XRF revealed calcium, carbon, oxygen, sodium and phosphorus in both groups. XRD indicated that both biomaterials were pure and crystalline. There was a statistically significant difference in granule area between the two groups after 15 days (P = 0.022; Student’s t-test). After 15 days, an increased inflammatory response was seen in group 2 (P < 0.0001; ANOVA and Tukey’s post hoc test) whereas it was more pronounced in group 1 after 30 days (P < 0.0001; ANOVA and Tukey’s post hoc test). It was concluded that these biomaterials have similar physical, chemical and biocompatibility characteristics. (J. Oral Sci. 48, 219-226, 2006)

Keywords: hydroxyapatite; biomaterials; biocompatibility.

Introduction

A number of researchers have been trying to find an ideal biomaterial for use as a bone substitute (1-3). Autogenous bone grafts (ABGs) are most widely used by surgeons for ridge augmentation and the reconstruction of osseous defects, since these grafts contain viable cells such as bone marrow osteoprogenitor cells, collagenous and noncollagenous extracellular matrix and growth/differentiation factors. Therefore, ABGs are still considered the “gold standard” therapy for bone repair, because they induce osteogenesis, osteoinduction, and osteoconduction. The major disadvantages of ABGs are donor site morbidity, limitations on the quantity of grafted materials, and high cost (4-7). Other complications of allografts and xenografts, such as viral transmission and immunogenicity, are of serious concern. Therefore, there is a critical need for the development of bone substitute materials that match the properties of bone without the drawbacks of autografts or allografts, being available at any time, in any amount and at lower cost (7-10).
Considerable attention has been directed towards the use of synthetic grafts including hydroxyapatite (HA), tricalcium phosphate and bioactive glass (1,4,6,7,11,12). Hydroxyapatite, Ca₁₀(PO₄)₆(OH)₂ (HA), is the most well known and studied calcium phosphate (Ca-P) material, obtained from natural sources (coralline hydroxyapatite and bovine bone) or synthesized by precipitation using chemical reagents (6,12). Synthetic Ca-P grafts may be the material of choice, especially when large defects need to be filled. It is generally accepted that these bioceramics are only osteoconductive (have the ability to serve as a scaffold for ingrowth and bone formation) and not osteoinductive (stimulate mesenchymal cells to differentiate into bone-forming cells) (4-7,12).

Bone graft materials may be either resorbable or nonresorbable, this classification being related to the extent of dissolution of Ca-P materials (12,13). Among several factors, chemical composition, particle size, porosity (microporosity and macroporosity), surface area and crystallinity are likely to affect the solubility of ceramics, which may be adjusted for the desired purpose. Reabsorbed (dissolution and degradation) calcium phosphate materials are usually unsintered Ca-P materials. Resorption rate increases as crystallinity decreases, and also with an increase in surface area. Crystallinity is highly dependent on sintering temperature: the higher the sintering temperature, the more perfect the crystal and thus the lower the degradation rate of Ca-P materials (12,13-16). Different applications require materials with different resorption rates, which can be regulated by the mixture of several calcium phosphate phases (6,12,17,18).

It is often desirable to have a graft material that will eventually be reabsorbed, leaving space for new bone formation. Furthermore, despite the fact that bone growth can occur in porous and dense HA particulates, the bone conductive effect is limited (3,4,7). These biomaterials generally bond to surrounding osseous tissue and enhance bone tissue formation (7,14-18). The chemical, physical and mechanical properties of these biomaterials favor their use in humans (12,13,19-21).

The purpose of this study was to investigate the physical-chemical characterization and biocompatibility evaluation of natural and synthetic hydroxyapatites.

Materials and Methods

Hydroxyapatite powders

The biomaterials tested were divided into two groups: Group 1 (G1) Gen-Ox™ (lyophilized mineral bovine bone, Baumer S.A., Mogi Mirim, SP, Brazil) and Group 2 (G2) HA-U (experimental synthetic hydroxyapatite - State University of Ponta Grossa). HA-U was made by neutralization of Ca(OH)₂ solution (2.0M/L) by H₃PO₄ (1.2M/L) under controlled temperature (80°C) at pH 8. HA-U was obtained after the solution gel had been dried (90°C), and ground in an agate mortar with a pestle, followed by passage of the powder through a 100-mesh (154 µm) sieve. Afterwards, the powder was sintered at 700°C for 2 h, and the sample was sterilized in an oven at 170°C for 2 h.

Particle characterization

Physico-chemical characterization

Physico-chemical characterization was done to establish the micro-architecture, phase purity, crystallinity, composition and functional groups of the hydroxyapatites.

Granulometric analysis

The granule size of the samples was automatically measured (µm) using a laser scanner particle size analyzer (Cilas 920™, Cilas, Marseille, France).

Scanning electron microscopy (SEM)

The hydroxyapatite powders were gold-coated in an ion sputter apparatus (Shimadzu™ C-50) and their microstructure (shape and surface) was examined in a scanning electron microscope (JSM T330™ JEOL, Tokyo, Japan). Electron micrographs were obtained at ×100 and ×5000 magnification. Particle area was measured with Image Tool™ version 3.0 image analysis software (University of Texas Health Science Center, San Antonio, TX, USA).

Energy-dispersive X-ray (EDX) analysis

Qualitative information about the chemical elements in the samples was obtained from the EDX spectra at 20 kV for 300 s (JEOL 8400™, Jeol, Tokyo, Japan).

X-ray fluorescence (XRF)

An XRF spectrometer (XRF 700™, Shimadzu, Kyoto, Japan) was used to quantify the chemical composition (Ca and P) ratio of the samples. The data were acquired with an axial wavelength-dispersive XRF unit.

X-ray diffraction (XRD)

XRD (XRD-6000™, Shimadzu, Kyoto, Japan) spectra were measured by the powder diffraction method. The materials were scanned from 5° to 80° in 2 Theta (θ) and then the diffraction peaks of G1 (Gen-Ox™) and G2 (HA-U) were used to determine the phase purity and crystallinity.

Biocompatibility analysis

Thirty six male rats (Rattus norvegicus - Wistar),
weighing about 300 - 400 g (3 - 4 months old) were randomly divided into three groups (12 animals for each group); G1: Gen-Ox™, G2: HA-U and G3: Sham. The study was carried out following the guidelines of the Ethics Committee for Teaching and Research on Animals (protocol n.1049/02).

The same surgical procedure was used for all animals. The rats were anesthetized with an intraperitoneal injection (ketamine 75 mg/kg and xylazine 10 mg/kg). The dorsum of the animal, following the sagittal line, was subjected to trichotomy for exposure of the skin, followed by sterilization with gauze soaked in iodated alcohol. A straight 18-cm incision was performed on the skin with a #15 blade (Surgi-Blade, Sunshine International, Miami, Fl, USA) in a lateral direction between the front legs of the animal, exposing the subcutaneous connective tissue. The margins of the incisions were then retracted and the connective tissue was dissected for placement of 30 mg of biomaterial (each animal received only one biomaterial). After material implantation, the margins of the wound were joined and closed with interrupted sutures (3-0 silk sutures; Ethicon™, Johnson & Johnson S/A, São José dos Campos, Brazil) for a perfect coaptation, distant from the material. Asepsis was performed again after suturing. All animals were given a normal diet and water during the entire study period.

The animals were anesthetized and a specimen of reactive tissue containing the material was removed 15 and 30 days after implantation. Thereafter, the animals were sacrificed by cervical displacement, according to the guidelines of the Brazilian College of Animal Experimentation (COBEA). The biopsy samples were fixed in 10% phosphate-buffered formalin for 24 hours. After histological processing, 5-µm-thick alternate sections were taken from each specimen and stained with hematoxylin-eosin.

Qualitative histological analysis was carried out at ×100 magnification (Leica™, Leica do Brasil, São Paulo, Brazil). The biological response was evaluated for inflammatory (presence of edema, vascular alterations and inflammatory infiltration) and reparative (degree of fibrosis, angioblastic and fibroblastic proliferation) alterations of the tissues around the material.

Inflammatory response was also measured from the first cell layer in contact with the material to the first muscle cell layer. In the Sham group the inflammatory response was measured in muscle tissue (×40 magnification). Particle area (reabsorbed) was measured in the same section (×100 magnification).

All the measurements were made by one blinded, previously trained operator using digital analysis software (Image Tool™).

Statistical analysis

Intra-examiner reproducibility (particle area and inflammatory response) was tested with intraclass correlation coefficient (ICC). Comparisons among groups considering particle area (in vitro), inflammatory response and granule degradation after the experimental periods were tested using Student’s t-test and analysis of variance (ANOVA) with Tukey’s post hoc test. An alpha value of ≤ 0.05 was used to indicate statistically significant differences among groups. All analyses were performed using the GraphPad Prism™ version 3.00 software program for Windows (GraphPad Software, USA).

Results

Reproducibility

The intra-examiner intraclass correlation coefficient (ICC) was 0.89 for particle area (in vitro study) and 0.81 for inflammatory response (in vivo study).

Particle characterization

The results of granulometric analysis of G1 (Gen-Ox™) and G2 (HA-U) powders are presented in Table 1. In the G1 powder, 90% (in volume) of the particles were smaller than 345.6 µm, with an average diameter of 161.6 µm. In the G2 powder, 90% of the particles were smaller than 123.7 µm and the average diameter was 48.7 µm.

Table 1 Granulometric analysis of HA samples in granular form

<table>
<thead>
<tr>
<th>Biomaterials</th>
<th>Mean</th>
<th>Percentiles</th>
<th>Min</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1 (Gen-Ox™)</td>
<td>161.6</td>
<td>123.0 - 200.0</td>
<td>19.0</td>
<td>498.0</td>
</tr>
<tr>
<td>G2 (HA-U)</td>
<td>48.7</td>
<td>30.0 - 84.0</td>
<td>7.0</td>
<td>256.0</td>
</tr>
</tbody>
</table>

Comparison between particle size (µm) was determined by a laser scanner particle size analyzer.
Biocompatibility analysis

The rats were healthy and did not present signs of edema or suppuration during the entire postoperative period.

15 days: In G1 (Gen-Ox™) the presence of edema and vascular proliferation was evident, together with some foreign body reaction involving polymorphonuclear leukocytes (PMNs) and a large degree of fibrosis and fibroblastic proliferation around the granules (Fig. 5-A1). G2 (HA-U) showed more severe inflammation than G1 (Fig. 5-B1). In G3 (Control - Sham) a few inflammatory cells were evident and the tissue exhibited a normal aspect (Fig. 5-C1).

30 days: G1 showed slight inflammation with a foreign body reaction, a higher degree of fibrosis and blood vessels among the biomaterial particles (Fig. 5-A2). G2 demonstrated fibroblastic activity, but a more discrete inflammatory response than in G1, with organized collagens fibers among the granules (Fig. 5-B2). G3 exhibited normal connective tissue, and an absence of inflammatory response with intact muscle fibers (Fig. 5-C2).

Fig. 2 Particle area (µm²) before implantation. G1 (Gen-Ox™) and G2 (HA-U): Statistically significant difference (P < 0.0001; Student’s t-test). Mean and standard error.

Measurement of the inflammatory response demonstrated a reduction from 15 to 30 days in the same group (P < 0.0001; Student’s t-test) and among the groups (P < 0.0001; ANOVA and Tukey’s post hoc test). Statistically significant differences were observed in terms of groups, periods and interaction (P < 0.0001; two-way ANOVA) (Fig. 6). All biomaterials tested exhibited resorption (particle area reduction) from 15 to 30 days. A statistically significant difference between G1 and G2 was detected at 15 days (P = 0.022; Student’s t-test) (Fig. 7).

Discussion

Autogenous bone has several restrictions, such as donor site morbidity and limitations on the availability of graft materials (1-3). Therefore, researchers are trying to develop artificial bone substitute materials. However, artificial materials implanted into bone defects do not have the same properties as autogenous bone. Therefore, the development of new materials for use in surgical procedures is desirable (4-10).

Calcium phosphate ceramics such as hydroxyapatite (HA) have been investigated as bone substitutes because of their close chemical and crystal resemblance to bone minerals. HA exhibits osteoconductivity and biocompatibility, but close proximity to the host bone is
essential to achieve osteoconduction. Moreover, this biomaterial shows no osteoinduction (4,7,16,18).

Several studies (6,12,14-16) have investigated the use of hydroxyapatite as a bone replacement material, but it is becoming increasingly obvious that there is significant variability in the physical and chemical characteristics of the material. HA can be used in block form or as particles (dense or porous). The biological properties may vary depending on differences in processing, composition, size and shape of the particles and their surface area (7-9,12,13).

The present study was designed to investigate the powder characteristics and biocompatibility of natural (Gen-Ox™) and synthetic (HA-U) hydroxyapatites. Size, chemical components, morphology and surface characteristics of the particles were evaluated. Biocompatibility analysis was done by implantation in rat subcutaneous tissue, considering the inflammatory response and particle resorption (dissolution and degradation) after 15 and 30 days.

HAs analyzed in this study showed irregular and non-uniform particle size (MEV: G1 = 3332.8 ± 274.3 µm² and G2 = 1320.8 ± 83.0 µm²; granulometric findings: G1 = 161.6 µm and G2 = 48.7 µm). A smaller particle size was observed in G2 than in G1. Particle size and size range are important, as they directly affect the surface area available to react with cells and biological fluids. If the distribution is large, the smaller particles tend to obstruct the spaces among the larger particles, thus reducing vascularization (7,12). Conversely, if distribution is narrow, HA resorbability will increase with small particles (2,6,7). The largest granules are reabsorbed slowly. In guided bone regeneration procedures it is important that the biomaterial is reabsorbed simultaneously with osseous formation; therefore, these materials should act as a scaffold for osteogenesis (2,12,18). Some studies have suggested that the inflammatory response is directly associated with particle size, shape and irregularity (4,18). Sharp-edge small particles (1-30 µm) tend to induce a greater inflammatory response, with increased production of

Fig. 3 XRD spectrum patterns in A: G1 (Gen-Ox™) and B: G2 (HA-U), both biomaterials were considered pure and crystalline, since similar peaks were evident.

Fig. 4 EDX spectrum of granules in A: G1 (Gen-Ox™) and B: G2 (HA-U), demonstrating the presence of O (oxygen), Na (sodium), P (phosphorus) and Ca (calcium).
tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6). The presence of irregularly shaped particles results in no difference in inflammatory and anti-inflammatory cytokine expression in comparison with regularly shaped (spherical) particles (17).

Chemical element composition and ratio can define the compound's properties and its interaction with biological systems. The photomicrographs in Fig. 5 illustrate the histological changes induced by the implanted materials in rat subcutaneous tissue. G1 at A1 (15 days) and A2 (30 days) particles associated with polymorphonuclear leukocytes. G2 at B1 (15 days) and B2 (30 days) particles with different sizes and reduction of the inflammatory infiltrate. G3 (Control - Sham) at C1 (15 days) and C2 (30 days) connective tissue with normal characteristics. (*) Particles. Original magnification ×100, H-E staining.

Fig. 5 Photomicrographs of the materials implanted in rat subcutaneous tissue. G1 at A1 (15 days) and A2 (30 days) particles associated with polymorphonuclear leukocytes. G2 at B1 (15 days) and B2 (30 days) particles with different sizes and reduction of the inflammatory infiltrate. G3 (Control - Sham) at C1 (15 days) and C2 (30 days) connective tissue with normal characteristics. (*) Particles. Original magnification ×100, H-E staining.
resorption grade (dissolution), crystallinity, osteoblast proliferation and many mechanical properties (11-13). Solid materials can be either crystalline or non-crystalline (amorphous). Crystalline structures characterize the spatial form and atomic organization of the material. Highly crystalline hydroxyapatites tend to be very insoluble, while poorly crystalline hydroxyapatites have higher relative solubility (4,18,19). In the present study, all biomaterials were crystalline.

XRF spectrometry analysis showed a Ca/P ratio of 1.667 for G1 and G2, and the XRD spectrum indicated the phase purity and crystallinity of the tested materials. Studies have shown that the rate of degradation is inversely related to this ratio. Therefore HA with a Ca/P ratio of 1.667 will degrade slowly than similar implants made of tricalcium phosphate with a Ca/P ratio of 1.50 (12,14,20). EDX revealed the presence of oxygen, sodium, phosphorus and calcium in all groups. These results were similar to those of other studies (7,12).

Synthetic grafts placed in the subcutaneous tissue of animals can be used to evaluate the in vivo compatibility of biomaterials in contact with connective tissues. The results of this method can be used as a preliminary source of information on the biocompatibility of hydroxyapatites (13,18,21).

The inflammatory response at 15 days was more intense in G2 (HA-U) with the presence of edema, vascular proliferation, some foreign body reaction with PMNs, presence of marked fibrosis and fibroblastic proliferation around the granules, but was reduced at 30 days, showing slight inflammation and foreign body reaction, probably due to the wide variation of particle size. After 30 days the results were similar for G1 and G2 in terms of foreign body reaction. Fibrosis was better organized in this period and exhibited capsule formation around the material (G1 and G2). G3 (Sham) exhibited normal connective tissue, and absence of an inflammatory response with intact muscle fibers. These results were similar to those of a previous study with calcium phosphate cementum (21).

The smaller particles were reabsorbed, but the larger ones remained and influenced the inflammatory response. It was possible to observe a reduction in particle area throughout the experimental period for G1, where the particle size decreased from 15 to 30 days (481.3 µm² to 367.7 µm² respectively). In G2, however, particle size increased (236.6 µm² to 375.5 µm² respectively). Biomaterials may be reabsorbed by either a solution-mediated process (i.e., dissolution of grafts in physiologic solution by enzymatic hydrolysis) or by a cell-mediated process (i.e., physiologic bone remodeling by macrophage phagocytosis) (4). Others studies showed that smaller particles produce more inflammatory response (5,16,21), but some research has concluded that HA is well tolerated by tissue, evoking a mild and transitory inflammatory response. These results were similar to those of other studies (4,5,18).

In conclusion, the materials analyzed showed similar physical and chemical characteristics and were considered biocompatible, being partially reabsorbed in the subcutaneous tissue.
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References