Hydroxyapatite and a New Fibrin Sealant Derived from Snake Venom as Scaffold to Treatment of Cranial Defects in Rats

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Biomaterials are used as a promising alternative to bone grafts, including bioceramics whose composition resembles that of bone and fibrin sealants due to their hemostatic properties. The objective was to evaluate the repair of cranial defects in 40 rats, grafted with hydroxyapatite and a new fibrin sealant derived from snake venom. The animals were divided into four groups: C (control, no graft); Ha (hydroxyapatite); FS (fibrin sealant), and HaFS (hydroxyapatite and fibrin sealant). The animals were euthanized 2 and 6 weeks after surgery and wound area were submitted to analysis. After 2 weeks, immature bone was formed from the borders of the defect and in groups Ha and HaFS, few hydroxyapatite particles were surrounded by new bone. After 6 weeks, the new bone was mature and surrounded several hydroxyapatite particles, without connective tissue interposition and the volume of new bone was higher in HaFS group. The hydroxyapatite in combination with the new fibrin sealant accelerates bone repair.

Keywords: bioceramic, fibrin sealant, bone regeneration

1. Introduction

Autologous bone grafts have been the most common option in orthopedics and dentistry for reconstruction of the craniomaxillofacial skeleton and for bone reconstruction after trauma. These grafts have found wide acceptance because of their osteoinductive potential, providing cells that immediately trigger bone regeneration. However, difficulties in obtaining these grafts and associated risks of injury have encouraged the development of new synthetic materials as bone graft substitutes. An acceptable biomaterial should be degradable and resorbable, should serve as a scaffold for the growth of regenerated bone tissue, and should not cause any complication. These features depend on both the physical and chemical properties of the material and should be compatible with the physiological reactions of bone. In this respect, calcium phosphate ceramics are the biomaterials that show maximum chemical resemblance to bone.

Hydroxyapatite is a suitable bioceramic for bone repair due to its similarity to the mineral apatite of human bone. In addition, this biomaterial is atoxic and has a controllable microstructure in terms of pore size. In view of its osteogenic properties, hydroxyapatite is widely used in bone reconstruction surgeries. Another material used mainly for the regeneration of soft tissues is fibrin sealant, which serves as a scaffold for cell adhesion and growth, in addition to presenting hemostatic properties. Other applications of fibrin sealants include healing induction, cavity sealing, and as drug delivery systems.

Commercial fibrin sealant is prepared as a mixture of human fibrinogen concentrate and reconstituted bovine thrombin in calcium chloride solution. Fibrinogen is converted into fibrin, producing a stable clot. This reaction is enhanced by the activation of factor XIII, which also participates in the synthesis of collagen, stimulating fibroblast proliferation and contributing to tissue healing. Factor XIII is also an excellent hemostatic agent. However, the use of this commercial fibrin sealant is limited by the...
possession of transmission of viruses such as parvovirus, hepatitis and HIV. In addition, patients may develop antibodies against bovine thrombin. In an attempt to overcome these difficulties, another sealant consisting of a thrombin-like enzyme extracted from snake venom and fibrinogen obtained from large animals to replace human components has shown excellent results in experimental studies on the regeneration of different tissues, in mesenchymal cell cultures, and in the healing of skin wound, venous ulcers and gingival graft sutures in humans. This new sealant is a biodegradable product that does not cause adverse reactions, does not contain human blood components, has good adhesive capacity, and does not transmit infectious diseases. Considering these favorable properties, this new sealant should be explored clinically in different regenerative therapies. Therefore, the objective of the present study was to evaluate the osteogenic potential of a combination of hydroxyapatite and this new fibrin sealant in accelerating bone regeneration.

2. Procedure

2.1. Hydroxyapatite

2.1.1. Synthesis

Hydroxyapatite was synthesized from 1 mol L\(^{-1}\) Ca\((NO_3)_2\), 4H\(_2\)O and 0.6 mol L\(^{-1}\) (NH\(_4\))\(_2\)HPO\(_4\) at pH 11 (NH\(_4\))OH in an atmosphere of N\(_2\). The suspension was shaken for 40 h, filtered, washed in deionized water, and dried at 90°C for 15 h. Granulometric sieves for particle size separation were used to obtain particles smaller than 200 nm. The chemical reaction of hydroxyapatite formation can be written as follows:

\[
\text{Ca}_{10} (\text{PO}_4)_{6} (\text{OH})_{2} + 2\text{NH}_4\text{NO}_3 + 6\text{H}_2\text{O} \rightarrow \text{Ca}_{10} (\text{PO}_4)_{6} (\text{OH})_{2} + 2\text{NH}_4\text{OH} + 2\text{NH}_3 + 6\text{H}_2\text{O}.
\]

2.1.2. Characterization

\(\text{Ca/P Ratio} \): Ca and P were determined by chemical conventional procedures by means of phosphomolybdate, sodium EDTA.

Energy Dispersive X-Ray Analysis (EDX): were obtained in a EDX equipment LEO 440 (LEO Electron Microscopy Ltd, Cambridge, England), with an Oxford detector (Oxford Instruments Inc., Concord, USA) with 112eV resolution and samples previously coated with carbon at a distance of 20 mm.

X-Ray Diffraction: Powder: X-ray diffractograms were performed in a Rigaku RU200B equipment, using CuK\(\alpha\) radiation, 50 kV, 80 mA, rate scanning 2\(^\circ\) min\(^{-1}\) and 20 between 5 to 80\(^\circ\).

Infrared Spectroscopy (FTIR): Infrared absorption spectrum was obtained from HA powder in KBr in a Bomen FTIR MB-120 spectrophotometer (Bomen Inc., Quebec, Canada), range from 400 to 4000 cm\(^{-1}\) with a resolution of 4 cm\(^{-1}\).

2.2. Fibrin sealant

The fibrin sealant was produced from a serine protease extracted from Crotalus durissus terrificus snake venom and fibrinogen was obtained from the cryoprecipitate of blood extracted from adult buffaloes. These components were reconstituted in a liquid state, generating a fibrin network. The fibrin sealant, under the scope of the Brazilian Patents BR 10 2014 01147 32 and BR 10 360 2014 0114, was kindly provided by the Centro de Estudos de Venenos e Animais Peçonhentos (CEVAP, Botucatu, Brasil).

2.3. Animals and study design

Forty male Wistar rats (Rattus norvegicus) aged 12 weeks and weighing 330 g were used. For surgery, the animals were anesthetized by intramuscular injection of 1 mg/kg body weight xylazine (Virbac Brasil Ind. E Com., São Paulo, Brazil) and ketamine (Sespo Ind. E Com., Jarcare, São Paulo, Brazil) at a proportion of 1:1\(^{19}\). The animals were placed in ventral decubitus and the skull was shaved. Next, an incision was made in the skin to expose the parietal bones. The periosteum was detached with an appropriate surgical instrument and a defect measuring 5 mm in diameter was created in the left parietal bone using a surgical bur in a micromotor (BELTEC LB-100, Araraquara, São Paulo, Brazil). The defect depth was 0.5 mm, avoiding injury to the dura mater in brain (Figure 1).

The animals were divided into four groups of 10 animals each: group 1 (C), animals with a parietal bone defect not submitted to any treatment; group 2 (Ha), animals with a parietal bone defect filled with 8 mg hydroxyapatite particles; group 3 (FS), animals with a parietal bone defect filled with 8 ml fibrin sealant; group 4 (HaFS), animals with a parietal bone defect filled with 8 mg hydroxyapatite and 8 ml fibrin sealant.

The fibrin sealant was removed from the freezer at the time of use and its constituents mixed in a microtube together with hydroxyapatite. This mixture was then applied to animals (group 4). In groups 2 and 3, hydroxyapatite and fibrin sealant were applied directly into the animals, respectively.

After surgery, the periosteum and skin were repositioned and sutured. Five animals of each group were sacrificed 2 and 6 weeks after surgery. The skullcaps were removed, photodocumented and radiographed for gross inspection of the bone defect. For morphologic analysis and quantification of newly formed bone at the defect site, histologic sections were obtained from the wound area and stained with hematoxylin-eosin. The study was approved by the Ethics Committee on Animal Experimentation of Centro Universitário Padre Anchieta, Jundiaí, São Paulo, Brazil (Protocol 01/2012).

2.4. Analysis stereological and statistical

The volume of a newly bone in 2 and 6 weeks in histologic images (magnification 100X) was calculated using a 100-point quadrilateral grid system coupled to the eyepiece of a light microscope. Bone neoformation was quantified based on the principle of Delesse using the formula \(V_v = P_p/P_t\) (%), where \(V_v\) is the volume density or relative volume, \(P_p\) is the number of points (line intersection) on new bone, and \(P_t\) is the total number of points of the system. The results were analyzed by Anova and the Tukey test (p<0.05) using the Bioestat 5.0 software.
3. Results

3.1. Hydroxyapatite (HA)

Synthesis of HA resulted in a white powder and in its FTIR spectrum (Figure 2) peaks were observed at 3568 cm⁻¹ (OH⁻), 1095, 1032, 962, 603 e 565 cm⁻¹ (PO₄⁻³), 634 cm⁻¹ (OH⁻), similar to values described for HA. X-ray diffraction (Figure 3) main peaks were detected with d values of 2.81, 2.72, 3.43 and 1.84Å, as described for HA [HA, JCPDS 9-0432]. The crystallite size of the synthetized HA was measured by the (002) peak broadening, using Scherrer’s equation:

\[ L_{002} = \frac{K \lambda}{\beta \cos(\theta)} \]  

where \(L\) is the mean crystallite size, \(\lambda\) the wavelength of X-ray radiation (\(\lambda = 0.154056\) nm for CuKα radiation), \(K\) a constant related to the crystallite shape approximately equal to unity, \(\beta\) the broadening of the 002 diffraction peak measured at half of its maximum intensity (in radians) and \(\theta\) the Bragg diffraction angle (°). The estimate crystallite size of the hydroxyapatite crystals was 40 nm.

The Ca/P ratio of HA determined by chemical procedures was 1.50. Compared to an expected value of 1.67 for stoichiometric HA, it suggests the formation of calcium deficient HA. EDX technique showed that Ca and P were the only chemical elements present in the calcium phosphate ceramic. Although this technique is not quantitative the calculated value for Ca/P ratio was 1.50, similar to the chemical procedure.

3.2. Histological analysis of the surgical area

Macroscopic and radiologic analysis showed good definition and normal morphology of the wound area in all groups. There were no signs of pathological reactions indicating immune rejection of the biomaterials (Figures 4 and 5).

Histologic analysis of animals sacrificed 2 weeks after surgery revealed the presence of immature trabecular bone characterized by disorganized arrangement of several lacunae harboring osteocytes. New bone projected from the borders of the bone defect, a finding that was more pronounced in group HaFS-2. Also after 2 weeks, some hydroxyapatite particles were surrounded by new bone in groups Ha-2 and HaFS-2, but most of the bioceramic was still covered with connective tissue (Figure 4). Mature newly formed bone projecting from the borders of the defect was observed 6 weeks after surgery. In group Ha-6, hydroxyapatite particles located on the surface were surrounded by new bone. Bone neoformation was intensified in HaFS-6, in which several hydroxyapatite particles were completely surrounded by new bone in the absence of connective tissue interposition (Figure 5).

Significant difference was observed between all groups as shown in Figure 6. The relative volume of new bone in the defect area after 2 weeks was 5.66 ± 0.57, 6.66 ± 0.57, 20 ± 1.0 and 21 ± 1.0 in groups C-2, Ha-2, FS-2, HaFS-2, respectively. After 6 weeks, the relative volume of new bone was 10.66 ± 0.57, 20.66 ± 1.15, 29.66 ± 1.52 and 53.66 ± 0.57 in groups C-6, Ha-6, FS-6, HaFS-6, respectively.

4. Discussion

The synthesis of HA resulted in the formation of calcium phosphate whose FTIR and X-ray diffraction spectra proved to be similar to HA. The crystal size of synthetized HA was similar to that found for cortical bone being about 50 nm. However, the Ca/P ratio suggested the formation of a calcium-deficient HA.

The objective of biomaterials is not only to fill the space of a defect, but also to stimulate a specific biological response that triggers tissue regeneration. This capacity depends on some intrinsic properties of the material, such as electron distribution, three-dimensional arrangement, molecular conformation, piezoelectric properties, porosity, and specific physicochemical properties. In addition, the material should serve as a scaffold that mimics
Figure 2. FTIR for synthetic hydroxyapatite.

Figure 3. X-ray diffraction spectrum for synthetic hydroxyapatite.
Figure 4. (A) Macroscopic, (B) radiologic and (C) histologic images obtained for animals of the four groups sacrificed 2 weeks (C-2, HA-2, FS-2, HAFS-2) after surgical creation of a parietal bone defect. In A and B, observe the absence of inflammatory signs and good definition of the bone defect area. In C, a higher quantity of new bone (*) projecting from the borders of the original bone (ob) was observed in groups FS-2 and HAFS-2. Hydroxyapatite particles (h), connective tissue (ct).

Figure 5. (A) Macroscopic, (B) radiologic and (C) histologic images obtained for animals of the four groups sacrificed 6 weeks (C-6, Ha-6, FS-6, HaFS-6) after surgical creation of a parietal bone defect. Note the absence of inflammatory signs. Microscopically, new bone was formed (*) from the borders of the original bone (ob) in all groups. This bone formation was more pronounced in group HaFS-2 in which most hydroxyapatite particles (h) were surrounded by new bone without connective tissue (ct) interposition.
extracellular matrix and permits cell adhesion, proliferation and differentiation. The biomaterials tested so far have advantages and disadvantages, as well as indications and contraindications, and the ideal material to stimulate bone reconstruction therefore needs to be determined.

The present study demonstrated the biocompatibility of the new fibrin sealant with bone tissue since no macroscopic, radiologic or histologic signs of inflammation were observed that would characterize immune rejection of this biomaterial. An ideal scaffold should not only maintain, induce and restore biological functions, but should also have the right characteristics with respect to degradation, cellular uptake, cell binding, mechanical strength, and particularly non-immunogenicity.

The promising results showing favorable bone growth in the groups treated with the new fibrin sealant are directly related to the spatial configuration of the sealant, which consists of a network of interposed fibrils and a porous structure that promotes the integration between live tissue and the implant. These properties are essential for cell growth and proliferation. In this respect, research demonstrated in vitro the migration and adhesion of mesenchymal cells to the new sealant. It permits the preparation of differentiated scaffolds that are suitable for every need.

Okamoto et al. concluded that conventional fibrin sealant does not present adequate osteogenic activity when applied to bone defects created in rats. In contrast, in the present study significant growth of new bone in the defect area was observed in groups FS and HaFS, in which the bone defect was filled with the new fibrin sealant obtained from snake venom. This new sealant exhibited biofunctionality, causing no adverse biological reactions and favoring bone healing. In addition, osteogenesis at the bone defect site was more pronounced in group HaFS which received the new sealant combined with a bioceramic, especially at 6 weeks after surgery (HaFS-6). In this case, bone growth started from the border of the original bone and adjacent to the hydroxyapatite particles, with most particles being surrounded by new bone. These events significantly reduced the amount of connective tissue, with new bone occupying a greater volume in the defect area. This biological response is essential for bone regeneration.

According to Konig Junior, it is important that the bioceramic is not resorbed too quickly so that the material can exert its osteoconductive and osteoinductive activity during bone repair. Schliephake and Kage concluded that the balance between degradation of the biomaterial and bone neoformation is very delicate and that chemical and cellular reactions during degradation may counteract bone formation. In this study, observed the presence of hydroxyapatite after 6 weeks of surgery with a proportional increase in bone formation and that the morphometric value of bone formed in group HaFS-6 was 5 times higher compared to C-6. These results demonstrate the osteoconductive capacity of hydroxyapatite and the excellent affinity of fibrin sealant in bone cell adhesion and proliferation.

Gasparotto et al. used a new fibrin sealant (FS) derived from snake venom to evaluated the in vitro growth and cell viability of mesenchymal stem cells (MSCs) and showed potential as a three-dimensional scaffold, maintaining cell survival without promoting differentiation. The ability of FS to capture and promote cell adhesion on its surface and the presence of cellular extensions into the interior of fibrin scaffold was demonstrated using transmission electron microscopy showing a uniform surface, forming an intensive 3D network of fibrin. With these observations of Gasparotto et al., it suggests that good results obtained in this study regarding bone formation in the skull defect of the animals, is directly related to bone cell affinity in the new sealant.

The new fibrin sealant is a three-dimensional scaffolding candidate capable of maintaining cell survival without interfering with differentiation. It might also be useful in drug delivery. The fibrin sealant has a low production cost, does not transmit infectious diseases from human blood and has properties of a suitable scaffold for stem cells because it permits the preparation of differentiated scaffolds that are suitable for every need.

An inflammatory response and clot formation occurs during the first weeks of repair of bone defects grafted with porous biomaterials. Next, the proliferation of osteoprogenitor cells promotes the formation of trabecular bone 4 weeks after implantation of the biomaterial, thus completing the first stage of bone growth. The second stage is characterized by the remodeling of new bone and is influenced by the properties of the biomaterial. In the present study, immature trabecular bone was observed in animals sacrificed after 2 weeks. Furthermore, there was no significant difference in the volume of new bone between groups C-2 and Ha-2 or between FS-2 and HAFS-2. This finding demonstrates the lack of significant osteoconductive capacity of the bioceramic in the early stage of bone repair, in contrast to what was observed in the groups receiving the new fibrin sealant. Cortical morphology and greater volume of new bone were observed in animals sacrificed after 6 weeks. In addition, during this period the volume of new bone increased gradually from groups C-6 to HaFS-6. This finding indicates that the osteogenic activity of the bioceramic is higher during the late stage of bone repair, but is still exceeded by that of the new fibrin sealant. The combination of these materials as done in group HaFS-6 resulted in the intensification of bone formation and a marked reduction in connective tissue, characterizing the principle of osseointegration and osteoregeneration.
5. Conclusion

The new fibrin sealant derived from snake venom exhibited biofunctional and osteoconductive properties during bone repair. Its combination with hydroxyapatite provided osteogenic stimulation during the late stage of bone defect repair. The combination of these specific biomaterials is therefore an interesting option that should be explored in alternative therapies for treatment of bone lesions. However, 6 weeks were not sufficient for the complete repair of the bone defect, requiring more time for regeneration of the skull due to its intramembranous source and osteogenic slow action.

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References


