Calcium sulfate and PTFE nonporous barrier for regeneration of experimental bone defects

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Abstract
The aim of the study was to evaluate the possibility to obtain guided bone regeneration with two types of physical barriers (calcium sulfate and PTFE nonporous barrier) in surgical defects created in rat parietal bones. In the right parietal bone the calcium sulfate barrier filled out the whole defect and in the left parietal bone the barrier of PTFE was positioned in the floor and externally to the surgical defect. After 7, 14, 30 and 45 days four animals were sacrificed in each period and the bone containing the defects were submitted to the microscopic analysis. The results of the study revealed that the PTFE barrier was more effective for bone regeneration in shallow transcortical defects compared to the calcium sulfate. However, additional experiments are necessary to determine if calcium sulfate would be successful in other bone defects types or the use of the material under another consistence could complement the results obtained in this work.

Key words: *Guided tissue regeneration, bone regeneration, artificial membranes, bone substitutes, polytetrafluoroethylene, calcium sulfate.*

Introduction
Several experimental studies have been performed in order to evaluate the behavior of different types of cells involved in the process of tissue regeneration in surgical wounds. The guided bone regeneration (GBR) principles are applied in the rebuilding of periodontal tissues, damaged by the periodontal inflammatory process (1-3). A new insertion takes place by means of a selective repopulation of the radicular surface by cells originated from the periodontal ligaments, and possibly, from the osseous alveolar tissue (4,5). This is made possible by putting a physical barrier in the space between the mucoperiosteal flap and radicular surface, so that nondesirable types of tissue cells can be prevented from migrating into the wound area and therefore, jeopardizing the reparation process.
Bone regeneration is prevented from happening because of a faster fibroblast proliferation inside the defects caused by inflammatory process in the periodontal tissues. Bone defects resulting from various causes, such as, infection, trauma, tumor resection, endodontic problems and localized alveolar bone reabsorption, are invaded by connective tissue, stopping the bone defect from sealing completely.

The non osteogenic connective tissue will keep the surgical wound from being healed by osteoblastic cells (6-8), compromising the osteogenesis. The physical barrier hinders the area of the bone defect, creating a space where only osteogenic cells can participate of the natural biological process of bone neoformation.

The results of experimental studies, both clinical and case report, have motivated the great applicability of GBR in many periodontal and bone situations (9,10). Since the introduction of GBR biological principles, a wide range of materials have been tested and used as a physical barrier in order to accomplish the adequate desired formation of bone tissue. Therefore, materials like cellulose acetate, expanded polytetrafluorethylene (e-PTFE), polyglycolic acid, polyurethane, titanium mesh, polylactic acid, polyglactin 910, collagen and other rubber based materials were employed in experimental and clinical procedures (1,2,11,12).

The nonporous PTFE barriers have demonstrated to be biocompatible and have showed excellent results in experimental studies and clinical trial (9). The applicability of the GBR has brought tests to interesting results when used in smaller bone defects. On the other hand, the membrane exposure and consequent local infection prevents the bone from regenerating (13-17).

At present, the autogenous material continues to be considered the best choice when reconstruction of bone defects is intended (18,19). But this type of material graft implicates in additional surgical trauma to the patient, and occasionally, cannot be obtained in enough amounts to fill out the whole defect. Besides this, not all patients accept to go under surgery for the removal of bone from the iliac bone, calvaria and tibia.

The calcium sulfate barrier is a rapid absorption biocompatible material and has been employed for many years in the medical and dental area in treatments of bone and periodontal defects. Calcium sulfate can work as a completion material, space maintainer, vehicle for a controlled release of certain drugs, associated with other graft materials (20-22).

The aim of this experimental study was to evaluate the tissue behavior of calcium sulfate and PTFE nonporous barrier in bone repair in rat parietal bone, observing the GBR biological principles.

Material and Method

For the accomplishment of this work sixteen male rats (Rattus norvegicus, albinus, Wistar), weighing approximately 300 g were used. All animals received human care according to the National Research Council's criteria and the study protocol had been previously approved by the Committee for Animal Use of the São José dos Campos Dental School of the São Paulo State University – UNESP.

Two different types of material were used in this study as physical barriers according to GBR biological principles. One is the nonporous PTFE barrier with thickness of 0.13 mm (Tecnoflon & Brasflon, São Paulo, SP, Brazil), with suitable properties and biocompatibility to be used in surgeries.

The other material is a hemi hydrated calcium sulfate (CAPSET® - LifeCore, Biomedical. Chaska, MN, USA). Water was added to the dry powder resulting in an exothermic reaction and crystallization and hardening of the preparation. When powder and liquid were combined in order to become the reabsorbing barrier upon the bone defect, the resulting paste could be easily adapted and shaped. It was displayed in a sterilized packing and was used following the manufacture's recommendations.

The animals were anesthetized intramuscularly with Anasedan® 33 mg/kg (Bayer SA, São Paulo, SP, Brazil) as a preanesthesitic solution and Dopalen® 13 mg/kg (Agribands do Brasil Ltda., Paulínia, SP, Brazil) for complete anesthesia.

After trichotomy and asepsis of the operative field, an incision was made in the sagittal plane of the head, followed by muscular dissection, plane to plane. Subsequently, a surgical bone defect was created in each parietal bone, with the aid of 4 mm trephine and irrigated with saline solution. The bone defect had a circular form, with its depth equal to the thickness of the removed cortical bone.

In the PTFE group, the barrier was placed on the floor of the defect and on the surface of the surgical bone defect. In the calcium sulfate group (CS group), the barrier was prepared at the moment of use and was adapted into the bone defect. Subsequently, the periosteum and muscle were sutured as well as the skin.

Histological and histomorphometric evaluation

Four animals in each period were sacrificed with a high dose of the anesthetic at 7,14,30 and 45 days postoperatively. The bone containing the surgical defect was removed in bloc, fixed in 10% formalin for 48 h, decalcified in Plank-Rychlo solution and embedded in paraffin. The histological sections were cut approximately with 5 µm of thickness and were stained with Hematoxylin-Eosin.

The central point of histological section randomization and selection for histomorphometric analysis was accomplished randomly, eliminating the occurrence of sampling bias (23). A Zeiss II reticule was placed over a compensation ocular 10X Zeiss microscope (W-PI, Carl Zeiss, Gottinger, Germany) to evaluate the bone density. The reticule image was superimposed on the desired histological fields. The reticule points and the total number of points over the bone defect were counted. The chosen bone defect was submitted for examination with serial microscopic sections, from which approximately 100 sections were obtained. From these sections, 4 were randomly chosen for histomorphometric analysis. Subsequently, 8 histological fields from each section, in the surgical bone defect region, were analyzed. At this step, a 20X objective (A-Plan, Carl Zeiss, Gottingen, Germany) and an ocular 10X (W-PI,

Carl Zeiss, Gottingen, Germany) of an optical microscope (Axioskop 40, Carl Zeiss, Gottingen, Germany) were used. The objective showed a 100-point reticule corresponding to 7840μ2 for measuring the bone tissue area.
The histomorphometric results were submitted to analysis of variance ANOVA (Statistix 8.0 for Windows; Analytical Software; Tallahassee, FL, USA). The level of significance used was $p<0.05$.

Results

- PTFE

7 days

Microscopic analysis of the treated area with PTFE barrier demonstrated bone defects with evident margins of cuts, exhibiting a necrotic basophilic line. Some fragments of bone close to the internal portion of the defect and remodeling areas containing some giant cells were also evidenced. The bone defect was fulfilled by blood clot and granulation tissue mainly present next to the margins area. Next to the cutaneous area and the internal surfaces of the defect, a clear space could be observed, corresponding to the space previously occupied by the barrier, which had been removed before the processing. Such space was surrounded by granulation tissue with discreet fibrosis, containing some focal area of bone deposition. In the cortical next to the defect a slight bone formation could be noticed.

14 days

In this period, microscopic analysis of bone defects still showed well defined margins, with a slight bone formation in the defect inner space, such as in the cortical area of the defect. In some specimens, bone tissue could be observed in the central part of the defect. The bone defect was filled with granulation tissue and newly formed blood vessels. Some of them were obstructed and demonstrate some hemorrhagic focuses. Along with the fibrous membrane that involved the space previously occupied by the barrier, new bone formation could be noticed, facing mainly the encephalic area.

30 days

The area of the defect was partially filled with bone tissue characterized by thick trabeculae and wide medullar spaces containing newly formed blood vessels. In some specimens a bone bridge was noticed binding both defect ends, however, without rebuilding the total thickness of the bone in that area, that still possesses fibrous conjunctive tissue. Bone formation could be seen in the capsule around the membrane space.

45 days

The area of the defect presents incomplete bone bridge, which was thicker than the one observed in the previous period (Figure 1). This bridge was comprised by a mature bone tissue with smaller medullar spaces. Fibrous connective tissue was observed, close to newly formed bone and at the place where it became discontinuous.

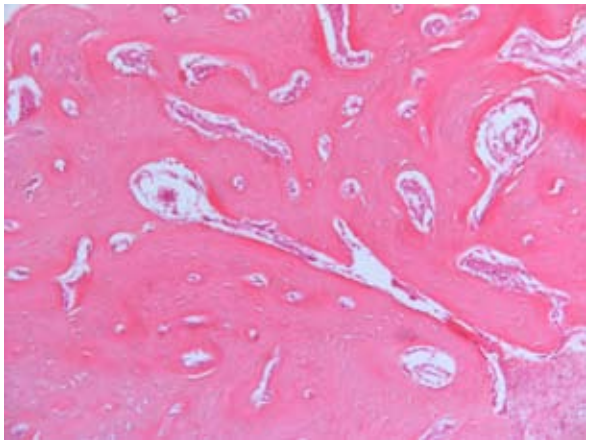

Fig. 1. Photomicrographs of the surgical defect in the PTFE group after period of 45 days showing newly formed bone tissue (HE; x100).

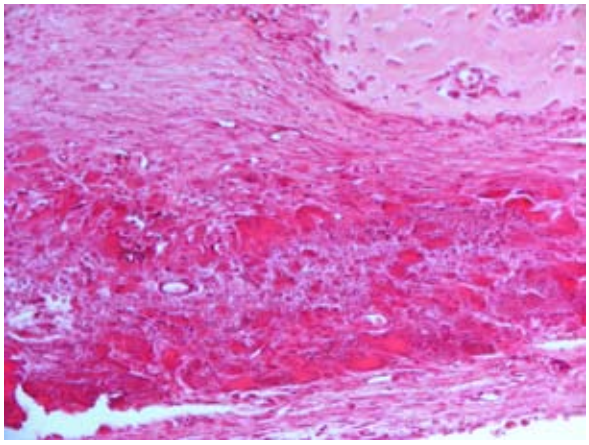

Fig. 2. Photomicrographs of the surgical defect region of the CS group in the 14 days period. Minimal bone formation, some giant cells and granular basophilic material (HE; x200).

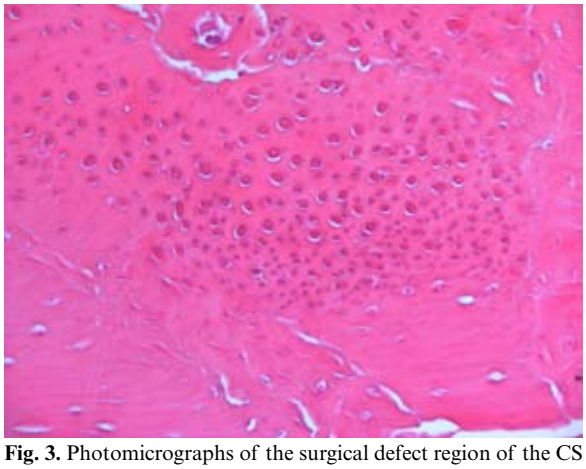

Fig. 3. Photomicrographs of the surgical defect region of the CS group after period of 45 days. Limits of the surgical bone defect and granular basophilic material inside the newly formed bone tissue (HE; x200).

- Calcium sulfate

7 days

Microscopic analysis in this group showed a bone defect with well defined margins, presenting a basophilic necrotic line and some bone fragments facing a more internal area of the defect. Some remodeling areas, containing several giant cells were also observed. The bone defect was fulfilled with blood clot, which was formed by a fibrin net and red blood cells in a smaller amount when comparison with the PTFE group. The clot was partially substituted by granulation tissue and contained several giant cells and sparse leukocytes. In the cutaneous and internal surfaces of the defect a slight fibrosis could be observed. In the cortical, facing the encephalon, a new bone formation that was more discreet close to the margins was noticed.

14 days

The defect was filled out by granulation tissue, containing several giant cells and some leukocytes, that was continued by fibrosis in its external parts. Occasionally, giant cells containing granular basophilic material could be seen. This material and bone fragments were also observed along with the giant cells (Figure 2). It was also possible to observe blood clot and new bone formation close to the margins of the defect.

30 days

Through this period was possible to notice bone formation in the extremities, surrounded by a line of osteoblasts, containing wide medullar spaces. However, most part of the defect was filled with fibrous connective tissue. Giant cells associated with bone fragments and granular basophilic material were still present.

45 days

At this period the bone assembled in the margins presents a more mature aspect. Granular basophilic material could be seen into the newly formed bone tissue (Figure 3). Nevertheless, the defect showed in most parts of its extension fibrosis tissue.

- Histomorphometric analysis

The histomorphometric analysis aimed at measuring the volume density of the newly formed bone matrix in the bone defects of the PTFE and CS groups, as well as to provide the necessary data for the statistical analysis of these measurements. The mean values and standard deviation of the bone defect histomorphometry of the studied groups for the different periods are shown in Table 1. Based on the statistical analysis, the CS group presented less bone formation than the PTFE group.

Table 1. Volume density of newly formed bone matrix (Mean + SD).

Days after surgery	PTFE	CS
7	0.00±0.00	0.00±0.00
14	0.25±0.09	0.04±0.02*
30	0.67±0.09	0.24±0.04*
45	0.89±0.03	0.33±0.09*

*p<0.05 (interaction between PTFE and CS)

Discussion

This study was designed to histologically analyze the calcium sulfate and PTFE physical barriers on bone healing in surgically created defects in rat parietal bone. The newly formed bone in the cortical region of the surgical defect was evaluated by both histological and histomorphometric analyses.

The main aspect that prevents the bone regeneration of being successful is that the connective tissue is produced in a faster way, compromising the desired osteogenesis. Therefore, the invasion of soft tissue, located in the adjacencies, can harm or hinders the bone from being settled in the desired area (1-3,6-8).

The biocompatible and occlusive physical barrier creates a compartment that enable the osteogenic and angiogenic cells originated from the adjacent medullar spaces to repopulate and regenerate those defects with new bone tissue (4,5). The defects without physical barrier usually show an incomplete bone formation and are characterized by the presence of a connective fibrous tissue in its interior (9, 10). At the present study, this occurrence was confirmed, in which PTFE group showed larger bone formation.

The PTFE group showed notably quantitative and qualitative superiority of new bone formation when compared to the CS group. The best structural bone quality among the PTFE group is characterized for having larger degree of bone tissue organization, with numerous thick trabecular covering most of the extension and depth of the surgical defect.

Calcium sulfate is one of the first materials even to be used as a bone substitute. It has been investigated by several authors who have demonstrated its biocompatibility and its rapid rate of resorption (20-22). Its use has been advocated to repair bony defects because of its unique capability of stimulating osteogenesis (20).

In the present study, we did not observe that material contributed to stimulating osteoblasts in CS group in the osseous healing process. Most of the upper material was resorbed before bone apposition. We speculated that it was just a space filler or stabilizing agent for particulate materials.

Although many variables, including type and size of defect and time of healing response, as well as differences in host response, make comparisons and conclusions difficult, the result of the present study suggest that calcium sulfate is readily available, resorbable, biocompatible, and well tolerated by the tissues. However, it was not effective in regeneration of transcortical defects, probably due to the morphology of the defect, which prevented the material maintenance in position, avoiding the creation of a local environment that would be able to guide a new bone formation. This suggests that the use of calcium sulfate barrier is only effective in space maintainers bone defects, circumscribed by enough bone tissue. A confined cancellous defect, one that does not have access to the encephalon,

may also inhibit the clearance of the dissolving calcium sulfate. It is less probable that calcium sulfate has behaved as a barrier to the additional bone infiltration, once the literature shows positive results when using this material in other types of bone defects (21,24,25).

The in vivo mechanism of calcium sulfate in bone formation has not been elucidated, although it has been used for more than a century clinically (20-22,25). Calcium sulfate bone substitutes may not only act as space maintaining material but also as an accelerator of the healing process through a pH-dependent pathway or other unknown mechanisms (26). The negative results in this study may be related to a local environment that could not support new bone formation or the calcium sulfate acting as a barrier to additional bone infiltration.

Another point to consider is the consistence of the calcium sulfate, which might have allowed the material displacement. In addition, there was a more accelerated degradation and the invasion of undesirable cells among the granules of the sulfate, obstructing its differentiation in the osteogenic tissue. Therefore, a steady consistency is desirable for the maintenance of the material in position.

Regarding the absorption of calcium sulfate, until the period of 45 days, granulations suggested as being derived from the material were observed, either dispersed in the fibrous conjunctive tissue or into the newly formed bone tissue. The present study also attested the safety and biocompatibility of calcium sulfate, based on the reduced inflammatory reaction in the grafted area (21, 22,24,25).

Conclusions

The findings of this study demonstrated that PTFE barrier showed to be more effective when compared to calcium sulfate during bone regeneration, involving transcortical shallow defect. However, additional experiments are necessary to determine if calcium sulfate would be successful in other bone defects types or the use of the material under another consistence could complement the results obtained in this work.

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