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Histological evaluation of natural coral skeleton as a grafting material in miniature swine mandible

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Natural coral skeleton (NCS) has recently been proposed as a bone graft substitute that enhances bone formation. The present paper describes the effects of implanting NCS in bone cavities prepared in the mandibles of miniature pig, and compares these with the effects of two alloplastic materials; a tricalcium phosphate (TCP) and a porous hydroxyapatite (PHA). On 11 pigs, 5×5 mm windows were created through alveolar bone of the four mandibular incisors. Three cavities were filled with the various materials and the fourth was left unfilled. The animals were slaughtered at 0, 1, 2, 4, 12, 26 and 52 weeks post-operatively and the tissues were examined histologically. Healing completed at 26 weeks for NCS and TCP, and at 52 weeks for PHA. NCS granules provided surface for cell attachment and deposition of a distinguishable organic matrix two weeks post-operatively. This matrix developed to bone after four weeks. The granules gradually resorbed and were replaced by bone at 52 weeks. The excellent properties of NCS, biocompatibility, porosity and osteogenic effect make us suggest that it might be a suitable replacement for bone grafting.

1. Introduction

In the past few years, various alloplastic grafting materials have been tested as bone implants to restore lost alveolar bone, mainly in periodontal osseous defects and for alveolar ridge augmentation [1–8]. Alloplastic implants are readily available, easy to manipulate, can be stored without any particular precaution and can be used immediately as needed. Furthermore, they are safe since there is no risk of cross contamination. However, the exact role played by these materials is still open to question. Recent reports have shed some light on their potential to restore bone, and on the histologic response to their implantation in man and in animal models [9–18].

Different surgical models for studying healing of bony defects have been proposed. The closed model of Bye *et al.* [19], where communication is prevented between the artificial bony defect and the oral environment, is useful in comparing healing in the presence and in the absence of different biomaterials placed under similar experimental conditions.

The natural coral skeleton (NCS) used in the present study is of genus porites, and presents porous architecture with pore sizes of 100–220 μm, all interconnected. NCS structurally resembles spongy bone. Chemically, NCS is made of 98% calcium carbonate, 1% oligo-elements, and 0.005% heavy metals, in the form of aragonite crystals [20]. Previous studies on animals have shown that NCS is well tolerated when implanted into intra-bony sites [20, 21]. In dog's femoral diaphysis, NCS is gradually resorbed and replaced by new bone formation [20]. Studies in man also showed similar results regarding the tolerance to NCS and the enhancement of new bone formation [22–24].

The purpose of the present investigation was to examine the effects of natural coral skeleton on healing of surgically created alveolar bone defects in the miniature swine's alveolar bone. The results were compared with those obtained by implanting two other different alloplastic grafts: a resorbable tricalcium phosphate (TCP), and a coralline-based porous hydroxyapatite (PHA). The two materials are calcium phosphate ceramics which have been widely investigated. The PHA tested is a replicate based on the structure of coral porites, made by hydrothermal conversion of the calcium carbonate of natural coral into hydroxyapatite [10].

2. Material and methods

Eleven two-year-old miniature pigs (SGC 70, CECAB, Cambremer, France) were used in this experiment. Clinically, the gingiva around the four lower incisors was healthy with no bone loss nor pocket formation.

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The animals were anesthetized with Nesdonal™ 20 mg kg, followed by Halothane. Local anesthesia (Xylocaine 2%, epinephrine 1/80000) was added to induce local vasoconstriction. A mucoperiosteal flap, extending from the lower right to the lower left canine, was reflected after two vertical releasing incisions and an intrasulcular incision were made. In each animal, four windows, 5 × 5 mm, were cut in the facial alveolar bone of the mandible adjacent to the four incisors teeth and 5 mm below the alveolar crest. The windows extended to the facial aspect of the root and upper and lower marker grooves were prepared on the root surface. An additional window was prepared 5 mm apical to that on the right central. The technique used to prepare all windows was as follows; a No.1 Oshenbein bone chisel delimitated the 5 × 5 mm window and the rest of the window was prepared with a No.0 round bur.

After thorough rinsing and drying with gauze under pressure, the prepared cavities were filled with the appropriate materials mixed with a few drops of saline solution. The two cavities on the right central incisors were filled with different size granules (300–450 μm and 450–600 μm) of NCS. The cavity prepared against the left lateral incisor was filled with 250 μm granules of tricalcium phosphate (TCP). The cavity over the left central incisor was filled with 1000 μm particles of porous hydroxyapatite (PHA), while the cavity on the right lateral incisor was left empty and used as a control. The flap was then sutured back into position.

The animals were given oxytetracycline (10 mg kg) for one week until the sutures were removed. The animals were fed their usual diet and no oral hygiene measures were taken.

Eleven miniature pigs were used in this experiment: one animal was slaughtered immediately after surgery, one after a week, one after two weeks, two after a month, two after three months, two after six months, and two after a year.

Immediately after slaughter, block sections of the experimental teeth, surrounding bone and gingival tissue were fixed in 10% buffered formalin solution for two weeks. Tissues from one animal/interval were processed undemineralized and those from the second animal, when available, were demineralized in EDTA solution. Demineralized tissues were embedded in paraffine and 5 μm thick sections were stained with hematoxylin and eosin. Undecalcified tissues were embedded in methylmethacrylate, then serially sawn into 100 μm thick slices (Sagemikrotom, Leitz 1600), finely ground and polished to a final thickness of 50–60 μm sections. The sections were then stained with paragon stain and basic fuchsin; nuclei, collagen and osteoid stained blue, and bone and cytoplasm stained pink. New bone formation was also monitored by microradiography and by oxytetracycline precipitate using fluorescence microscope on undecalcified sections.

The histological examination is based on evaluating the following.

1. Bone regeneration as evidenced by the formation of new bone trabeculae radiating centrally from the walls of the defects, and the formation of cortical bone, periosteum and endosteum.
2. The deposition of calcified tissue on the surfaces of the implants, which is not connected to regenerating bone, perigranular calcification.
3. The changes occurring in the roots of the teeth; resorption and deposition of calcified tissues.
4. Various soft tissue responses including inflammatory cell infiltration, regeneration of periodontal ligaments and epithelial changes.
5. The resorption and degradation of the implant materials.

3. Results

Throughout the experimental period no infection or other complications were noticed. All grafted materials were well tolerated with no clinical evidence of inflammation or rejection.

The histologic findings are described below and a summary is presented in Table I.

TABLE I Summary of the histological findings

Chronology of morphological changes in bony defects (values in weeks)	Empty	PHA	TCP	NCS
1. Defect closed by trabecular bone	4–12	26	4–12	4–12
2. Defect closed by cortical bone	12–26	26–52	12–26	12–26
3. Perigranular calcification within defect[a]	–	12	4	2
4. Perigranular calcification in gingiva	–	–	–	4
5. Perigranular calcification in periodontium	–	12	12	4
6. Material resorption[b] started, completed	–	26	2	12
	–	>52	26	52
7. Dental epithelium attached to particles[c]	–	4	–	4
8. Phagocytic activity[d]	–	4	4	–

With the exception of feature No. 8, which was registered for peak activity, all other features were registered for the time when they were first evidenced.

[a] Perigranular calcification describes the deposition of pink matrix on the surface of the implant.

[b] Resorption was considered when the material showed reduction in particle size and number.

[c] Reduced enamel epithelium extended to cover the adjacent particles.

[d] Phagocytic activity does not include osteoclastic activity.

3.1. Immediately after surgery, day 0

The sections showed the bony cavity surrounded by the alveolar bone on two sides, the root in one side and the gingival tissue on the other. The control cavity was filled with coagulated blood, and in the experimental cavities with the different filling materials. The small particles (TCP and NCS 450 µm) were more densely compacted than the larger ones (PHA and NCS 600).

3.2. One week

The three biomaterials showed the same healing pattern: small trabeculae of newly formed bone were visible at each edge of the cavity. This corresponded to the normal reparative process since the same features were visible in the control cavity. The cavities were filled with granulation tissue which showed mild to moderate inflammatory cell infiltration regardless to the presence or absence of the grafts. The infiltration was made mainly of neutrophils and some lymphocytes.

3.3. Two weeks

Some differences could already be noticed between the three materials. The particles of NCS were covered by thin organic matrix and cells which resemble osteoblasts (Fig. 1). The matrix appeared as pink rings around the particles, and was not related to the regenerative bone trabeculae which extended from the walls of the defects. No difference was marked in the presence of the two different sizes of NCS. In contrast, TCP already began to resorb (Fig. 2) with some particles appearing much smaller than those at one week post-operatively. PHA particles were surrounded by a fibro-cellular tissue with no evidence of resorption. In the control cavity bone regeneration took place at the edges.

3.4. Four weeks

3.4.1. Control

The cavity was not completely closed by regenerating thin bone trabeculae extending from the existing walls of the defects. The newly formed bone was easily distinguished by the fluorescence microscopy. At the time of cavity preparation, the root surfaces were marked by upper and lower grooves. These appeared histologically as denuded enamel surface and exposed dentin. These denuded root surfaces started to show regenerative/reparative calcification by cement-like tissue. The soft tissue within the cavity was fibrocellular with little inflammatory cell infiltration.

3.4.2. NCS

Similar to the control, bone regeneration took place from existing bone edges. However, the bony trabeculae seemed to follow the distribution of the granules. The perigranular matrix observed at two weeks post-operatively was clearly developed to osteoid tissue with readily distinguishable osteoblasts at the surface and osteocytes in lacunae. This perigranular osteoid was not related to the presence or absence of regenerating bony trabeculae. This was clear in some granules located labial to the alveolar bone, i.e. in the alveolar mucosa, which were also surrounded by osteoid. Similarly, particles situated in the periodontal space were covered with cement-like tissue. Some particles in the periodontal space were covered by epithelium originating from the reduced enamel epithelium. There was no apparent difference in healing response between the two cavities filled with particles of different sizes.

3.4.3. TCP

The quantity of granules had already been reduced due to resorption, possibly by phagocytic cells. These were mainly mononucleated foam cells and some multinucleated giant cells. This phagocytic activity extended also to the denuded root surface. Similar to NCS, TCP showed the formation of perigranular calcification in the bony defect. Often, however, these rings were incomplete and sometimes phagocytic cells, mainly mononucleated foam cells, were seen acting on the exposed parts. No epithelial proliferation around the particles in the periodontal space was observed.

3.4.4. PHA

The cavity was not closed yet by regenerative bone. The particles near to the cavity walls were partly embedded in the developing bone. This incorporation was often incomplete, leaving part of the particle exposed to the surrounding soft tissue. Some multinucleated giant cells were associated with the particles particularly in the centre of the cavity (Fig. 3). Some particles in the periodontal space were covered by epithelium originating from the reduced enamel epithelium. Reparative cement covered the denuded root surface.

3.5. 12 weeks

3.5.1. Control

The cavities were closed with well formed bony trabeculae. Cortical bone was not completely formed and the periodontal ligament started to regenerate.

3.5.2. NCS

Bone regeneration proceeded as described for the control. The granules were completely embedded in the newly formed trabecular bone. It was rare to find a granule or part of a granule exposed to the developing marrow. Evidence of NCS resorption was the reduction in quantity and in size of the granules. The staining of the bone around resorbing granules was always dark purple.

3.5.3. TCP

The cavity was closed by trabecular bone. The particles were mostly covered with bone but some were partly exposed to soft tissue. Particles exposed to

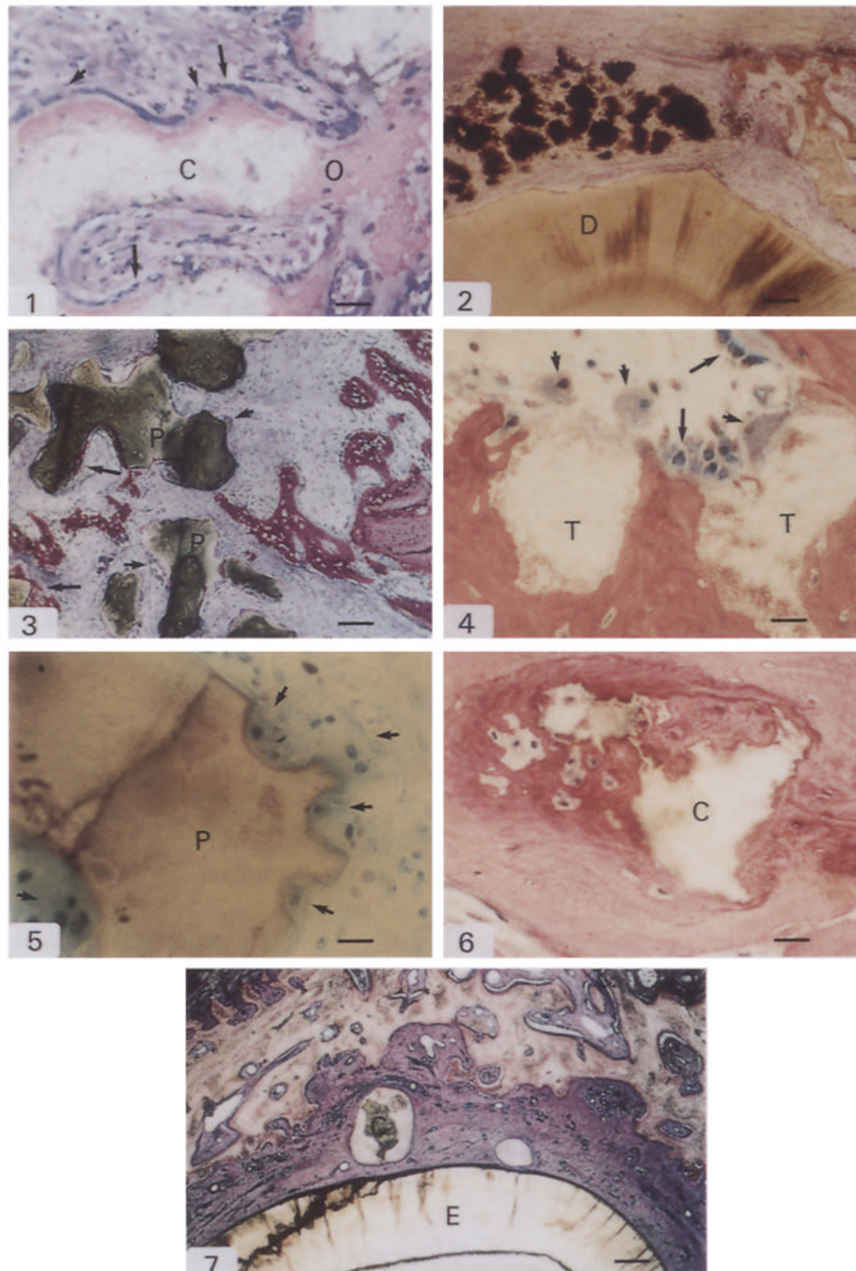

Figure 1 At 15 days post-operative, coral particles (C) were surrounded by osteoid matrix with evidence of ossification (O). The figure shows the mineralizing front (red) containing osteocytes and osteoblasts (arrows) depositing osteoid. Insertions of collagen fibres in the osteoid are indicated by arrow heads. Paragon stain of undecalcified section, bar = 60 μm.

Figure 2 Tricalcium phosphate implanted cavities showed rapid resorption of the material as early as 15 days post-operatively. The cavity walls show regeneration by trabecular bony extensions. The dentin of the root (D) was exposed during cavity preparation. Within the defect, the resorbing material is incorporated in fibrocellular tissue containing macrophages. Paragon stain of undecalcified section, bar = 650 μm.

Figure 3 Porous hydroxyapatite implanted defect one month post-operative. The figure shows regenerative trabecular bone extending from the walls of the defect. The great majority of the particles (P) are present in a fibrocellular tissue without perigranular calcification. Those present near the cavity walls are partly embedded in the regenerative bone. Multinucleated giant cells (arrow heads) are seen around some particles. Paragon stain of undecalcified section, bar = 220 μm.

Figure 4 Tricalcium phosphate implanted cavity three months post-operatively. The material (T) are partly covered by bone. The uncovered parts are associated with mononucleated macrophages (arrow heads), one of these attached to the material surface. The cytoplasm of these cells is vacuolated (foam cells). Osteoblasts are seen on the bone surface (arrows). Paragon stain of undecalcified section, bar = 35 μm.

Figure 5 Porous hydroxyapatite implanted defect three months post-operative. High magnification of a particle (P) attacked by multinucleated giant cells (arrow heads). The cells are present in Howship's lacunae. There is no evidence of calcification around the particle. Paragon stain of undecalcified section, bar = 35 μm.

Figure 6 Coral implanted cavity six months post-operative. The particles became smaller in size and fewer in number suggesting resorption. The figure shows one small particle (C) surrounded by bone. The bone around the material stains dark pink which distinguishes the perigranular calcification. Paragon stain of undecalcified section, bar = 35 μm.

Figure 7 Coral implanted cavity was closed by well-formed trabecular and cortical bone 12 months post-operative. The material resorbed completely in the bony defect. Coral granules (C) in the periodontal space did not resorb and were covered by calcified tissue which resembles cellular cement. Part of the root is seen covered with enamel (E) and the reduced enamel epithelium. Widening of the periodontal space correlates with the presence of the calcifications. Paragon stain of undecalcified section, bar = 650 μm.

developing marrow were attacked by phagocytic cells mainly mononucleated histiocytes. Multinucleated giant cells were less often found (Fig. 4). The cytoplasm of these cells was vacuolated and contained material resembling TCP (foam cells). In the periodontal space, a few granules were covered with cement-like tissue.

3.5.4. PHA

The cavities were partly closed by trabecular bone formation. Although some granules were completely covered by the developing bone, others were not covered or were incompletely covered by the bone. Others in the periodontal space were surrounded by a cement-like tissue. Multinucleated giant cells were acting on exposed particles (Fig. 5).

3.6. 26 weeks

3.6.1. Control

The defect was no more visible, the bone resembled the normal bone around, and periodontal ligament fibres were re-established between the newly formed bone and the root surface.

3.6.2. NCS

The cavities were closed, with a well-formed cortex. The particles were small in size and fewer in number. All the particles were completely surrounded by bone which was deeply stained pink compared with the adjacent trabecular bone (Fig. 6). A few particles were present in the periodontal space. These were also surrounded with calcification that resembles cellular cement.

3.6.3. TCP

The bony defect healed completely with cortex covering the newly formed alveolar bone. The particles were not visible any more. One particle, however, was seen near to the root surface surrounded by a cement-like tissue.

3.6.4. PHA

The cavity was closed by trabecular bone and the cortex started to reform. The particles were partly covered by bone. Macrophages appeared acting on uncovered particles.

3.7. 52 weeks

The control cavity was totally reconstituted and it was not possible to distinguish it from surrounding bone. NCS and TCP were totally resorbed within newly formed alveolar bone. PHA was embedded into well-formed bone that was already covered with cortex with no signs of resorption. However, the particles showed size variation which suggested some degree of resorption. In the periodontal ligament, few particles from the three materials were covered with cement-like with no evidence of resorption (Fig. 7).

4. Discussion

Healing of non-grafted cavities started by the development of new bone trabeculas extending from the cavity walls towards the centre of the defect. These trabeculas closed the defect with spongy bone. Formation of cortical bone followed and gave insertion for the regenerating periodontal ligaments. The control cavities demonstrated complete healing earlier than cavities grafted with PHA. Bone remodelling was also better in the controls than in the grafted defects. Similar results were also reported in previous papers when sterile surgically created bony defects were prepared in various animals [16, 21]. The authors explained the delayed healing by tissue irritation induced by implant rough edges. Nevertheless, the control cavities served as a model to follow the normal healing within the site under investigation, and allowed for comparisons to be made with grafted defects. By using TCP and PHA as controls we were able to distinguish differences which characterize healing in the presence of NCS. Complete healing occurred within six months in the presence of NCS, TCP and after one year in the presence of PHA. Both NCS and to a lesser extent TCP induced the deposition of perigranular calcification that soon changed to bone-like tissue and was then integrated into the developing alveolar bone. For NCS this started as early as two weeks post-implantation and at one and three months all particles were incorporated in bony tissue. Conflicting results concerning TCP bone growth properties have been reported in human trials, as well as in animal experiments [1, 6, 9, 12, 16, 17]. Levin *et al.* [16], in dogs, also showed eosinophilic rings around TCP particles which was interpreted as being osteoid tissue. Bye *et al.* [19] observed direct bone apposition against TCP implant surface, but questioned the osteogenic capability of this material.

In the present study perigranular calcification around TCP occurred after invasion by macrophages and drastic degradation of the material, thus reducing both particle size and number that may contribute to healing.

In cavities grafted with PHA, perigranular calcification was not observed. When some particles were partly covered by bone, it appeared to be an extension from adjacent regenerating bone trabeculas originating from the defect walls. Similar observations were previously described [25]. Previous studies on the effects of PHA have shown that this material is highly biocompatible, does not stimulate bone formation and can be regarded as an inert bone filling material [3, 4, 10, 14].

Early events after grafting the materials included the appearance of mild inflammatory cell infiltration, made of polymorphonucleated leucocytes and lymphocytes. This infiltration was transient and soon disappeared. However, macrophages increased in cavities implanted with TCP and PHA not with NCS. Phagocytic activity on the surfaces of these materials

was reported by several investigators cited in this paper, and is sought to play a role in the resorption of the material. The only report which describes phagocytic activity in coral grafted tissue was that of Guillemin *et al.* [21], who implicated osteoclasts in coral resorption. It is important to note that we strictly use "Osteoclasts" to describe cells located in a concavity on the bone surface. That is to distinguish them from macrophages which are located on the implant surface. Hott *et al.* [26] did not identify phagocytes on the coral surface in rats. Whether this absence of phagocytic cells relates to site or animal differences is not clear. The resorption of coral must thus be largely enzymatic rather than phagocytic. *In vitro* studies have shown that phagocytes preferentially attach to rough surfaces, and fibroblasts attach to smooth surfaces [27]. In our laboratory, scanning electron microscopic examination of coral implanted in three-dimensional fibroblast culture showed that fibroblasts preferentially attach to the external unfractured surface rather than to the fractured surface of the particles [28]. It is then possible that the detection of phagocytes depends on the proportion of fractured to unfractured surfaces in the particles.

In the present study, resorption of NCS was much slower than TCP material and faster than PHA which showed little if any resorption during the experimental period. Only traces of NCS remained after 12 months post-implantation. The resorption of NCS was peculiar since the granules resorbed after being embedded in a newly formed bone or perigranular calcification. Guillemin *et al.* [21] suggested that osteoclasts and carbonic anhydrase, an enzyme present in osteoclasts [29], are responsible for this resorption and the replacement of calcium carbonate by calcium phosphate.

The rapid rate of TCP resorption was associated with dense infiltration made mainly of mononucleated phagocytic cells having clear cytoplasm (foam cells). Rapid degradation of TCP occurred when phagocytes surrounded the particles, and TCP crystals were visible within these phagocytes.

Previous studies have shown that PHA is biodegradable in dog's mandible [30] and long bones [31]. The present results suggest that this process is slow and phagocytic activity mainly by multinucleated giant cells is involved. The few reports on humans do not mention resorption [4, 32]. These observations need further investigations to establish the role of macrophages in material resorption.

It is interesting to note that all materials located in the periodontal space were covered by what appears like cement and were the least to resorb. The cause for this delayed resorption is not clear yet. However, this might be related to the nature of the covering cement-like tissue, or the nature of the periodontal tissue.

PHA was shown to stimulate connective tissue proliferation in humans [15] and in dogs [18]. Certain tissue responses to NCS and PHA were similar in that vascular elements and connective tissue cells invaded the porous structure of both materials. Furthermore, when placed near by the reduced enamel epithelium, they provided surface for epithelial cell attachment and proliferation alongside the particle surfaces. This may be explained by the fact that these two materials share similar architecture. It was shown that surface texture influence cell attachment and behaviour, for review see [27].

In the present study it was not possible to compare the healing of the periodontal ligament. The data were not complete, since some cavities were located in the level of enamel where there is no attachment. It was, however, possible to find insertions of periodontal fibres in the newly formed bone and, where found, in the reparative cement on the root surface.

NCS showed perigranular calcification, which acted as a nidus for bone formation, and thus can be regarded as potentially osteogenic. All the particles of NCS and some particles of TCP were rapidly surrounded by eosinophilic rings. Soon after, this perigranular rings developed to osteoid, characterized by typical osteocytes in bone lacunas and osteoblasts on the surface. In the periodontal space the perigranular rings developed to cement-like tissue, thus the perigranular calcification is influenced by the surrounding tissue and cells.

In contrast to differences between the three materials regarding the perigranular calcification within the bony defect, it is within the periodontal space that all materials were completely covered by cement-like deposits which were histologically similar to reparative cement covering the denuded root surface.

5. Conclusions

In the present study, the three biomaterials tested were clinically well tolerated. NCS and TCP were, to a variable extent, covered by perigranular calcification which we believe enhanced the healing of the bony defects in comparison with the effects of PHA which showed delayed healing.

The direct deposition of bone on the surfaces of coral particles makes us suggest that NCS is biocompatible material with osteogenic effect. NCS provided surface for the attachment of osteoblast-like cells and the deposition of organic matrix that rapidly mineralized. This osteoid tissue was then integrated with the surrounding bone. Enzymatic transformation of calcium carbonate of coral to calcium phosphate then follows and the material gradually disappears. The properties of natural coral skeleton might make it an excellent substitute for bone grafting in alveolar bone defects.

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