Calscorbate Alloplastic Implants in Primates: A Sequential Histopathologic Study

Lawrence William Jenkins

Loyola University Chicago

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CALSCORBATE ALLOPLASTIC IMPLANTS
IN PRIMATES: A SEQUENTIAL
HISTOPATHOLOGIC STUDY

by

Lawrence William Jenkins, B.S., D.D.S.

A Thesis Submitted to the Faculty of the Graduate School
of Loyola University in Partial Fulfillment of
the Requirements for the Degree of
Master of Science in Oral Biology

May
1977
DEDICATION

To my father and mother, Alexander and Antoinette Jenkins, for their love, confidence, encouragement, and assistance.
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I wish to thank the members of my advisory committee: Dr. Patrick Toto, Dr. Anthony Gargiulo, and Dr. Joseph Keene, for their assistance and suggestions in the preparation of this thesis. Their recommendations and criticisms have been most valuable. In particular, I would like to thank Dr. Toto for his direction and guidance throughout the entire research project.

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CHAPTER I

INTRODUCTION

Osseous defects are often encountered in periodontal disease. Surgical elimination of infrabony osseous lesions are generally performed by flap procedures which permit access to the underlying bone. The infrabony lesion can be treated by the subtractive techniques of osteoplasty and osteoectomy in which bone is recontoured to an acceptable architectural norm. Other ways the infrabony lesion can be treated are by nongraft reattachment procedures, and by various implant or grafting techniques.

In the treatment of periodontal disease, it is generally accepted that pockets should be eliminated, shallow sulci obtained, and physiologic contours established in both the osseous tissue and the overlying mucogingival complex of tissue. The definitive elimination of a periodontal pocket is complicated by osseous defects. The tooth's ability to withstand functional stresses is compromised with loss of alveolar support. As stated, a number of procedures can be utilized to correct osseous defects, but only
reconstructive approaches have the potential to increase the alveolar supporting apparatus.

In advanced periodontal disease, the osseous defects may not lend themselves to corrective treatment by subtractive techniques or by nongraft reattachment procedures alone. In these situations osseous grafting assumes its role as a therapeutic modality.

An ideal graft or implant material having biological acceptability, clinical feasibility, minimum risk and maximal predictability has not yet been found. Many different graft materials have been tried. Viable osseous grafts include cortical autografts, bone blend, cancellous bone and marrow grafts (intraoral and iliac crest), variously prepared iliac allografts. These graft procedures require added patient procedures and discomfort, there is a limit of material available, some require special complicated storage techniques, and the degree of predictability is limited.

Other xenografts and alloplasts have been utilized as graft materials to offer a substitute to the disadvantage of autogenous graft material. Boplant, anorganic bone, and boiled cow bone powder were utilized. Also, plaster of paris, sclera, ceramics, and collagen
have been used as an alloplast material with some promise.

Mineralized materials acting as a calcium source were utilized as graft material in periodontal defects in an attempt to stimulate osteogenesis. A pepsin treated citrate extract of ox bone was found to be a powerful potentiator of new bone formation. It was also noted that ascorbic acid has a critical role in both the formation and maintenance of collagen in healing wounds.

It may be desirable to have both calcium and ascorbate within a graft material. A commercially available preparation, such as calscorbate, may make available both calcium and ascorbate if used as an alloplast. The purpose of this investigation is to determine, on a histologic level, the sequential healing phenomena of implanted calscorbate powder in two-walled osseous defects, and to observe any histomorphologic changes in the rhesus monkey periodontium.

*Cole Pharmacal Company
Ascorbic acid has a critical role in both the formation and maintenance of collagen in healing wounds. Proline and hydroxyproline enter the collagen molecule separately; both are derived from the proline pool which is available for this purpose. Ascorbic acid is probably involved in both the hydroxylation of proline to hydroxyproline, and lysine to hydroxylysine, and the subsequent incorporation of these amino acids into the collagen molecule.

In 1926, Wolbach described the lack of collagen formation in dentin, bone, and healing wounds during ascorbic acid deficiency states. And after the administration of ascorbic acid, a prompt appearance of argyrophilic fibers followed shortly by fibers having the typical staining characteristics of collagen.

As early as 1748, Richard Walter described how old wounds broke open under the influence of scurvy. Many other references exist in the older literature which show
not only that the healing of wounds was delayed but also that they were likely to break open again in scurvy. In 1923, Hojer suggested that vitamin C was essential for the maintenance of collagen.

In 1933, Lauber found that application of vitamin C to skin wounds in mice stimulated healing, but that injections of vitamin C into normal guinea pigs had no effect on the speed at which wounds healed. Thus, it may be possible that the increased local concentration of vitamin C as a result of its direct application to the lesion in animals such as mice (able to synthesize their own vitamin C) may have enabled the repair process to proceed faster than if the injured tissues, or tissues undergoing repair, had been dependent upon the slower diffusion from the blood. Mann and Pullinger (1940) could not demonstrate that local applications of vitamin C had a stimulatory effect on the regeneration of a cornea.

The increased concentration, and possible need, of vitamin C locally in an injured or repairing area had been shown by Lauber and Rosenfeld (1938). They demonstrated a mobilization of vitamin C from the tissues to the injured region. Also, the tensile strength of scar tissue was found to depend directly upon its vitamin C content by
Bartlett et al. (1942). In 1937, Lanman and Ingalls were the first to demonstrate a quantitative relationship of the rate of production of intercellular material to the amount of ascorbic acid administered. More recently Wolbach (1953) again demonstrated this quantitative relationship.

Further studies illustrated the relationship between vitamin C and collagen production. In 1936, Jeney and Toro found that in culture media in which fibroblasts were growing showed a more rapid production with the addition of vitamin C. Studies on the effect of vitamin C on skin wounds of guinea pigs showed that the animals receiving the least amount of vitamin C had a greater amount of reticular (precollagen) fibers than in those receiving the greatest amount. Ham and Elliott (1938) have illustrated that vitamin C is needed to produce new collagen fibers but not necessarily to maintain those already formed.

There is a definite relationship between wound healing and vitamin C; and, as discussed, a definite relationship between collagen production and vitamin C. It is uncertain, though, as to the exact mechanism in which vitamin C deficiency affects collagen production.
Wolbach and Howe\textsuperscript{18} implied that the intercellular ground substance produced collagen fibers by some sort of "jellying" process; and, that in scurvy, it was this "jellying" process which was affected leaving the cells more or less normal. But the lack of understanding of the biochemical function of the vitamin, makes any interpretation of vitamin C deficiency difficult.

With vitamin C deficiency, it has been shown biochemically and histochemically that there is a decrease in the activity of oxidative enzymes (cytochrome oxidase and succinic dehydrogenase), and a decrease in phosphatase and esterase activities.\textsuperscript{36,37} Also, there are two major metabolic defects which occur in connective tissue and cartilage in scurvy: (1) a defect in chondroitin sulfate production,\textsuperscript{38} and (2) a defect in galactosamine formation.\textsuperscript{39}

Bates et al. (1969)\textsuperscript{40} noted the effects upon mucopolysaccharide synthesis in ascorbic acid deficiencies. His study revealed that the granulation tissue formed contained less sulfated glycosaminoglycans and hydroxyproline compared to the controls. This suggested that an intimate relationship existed between these two molecules. The decrease in sulfated glycosaminoglycans was attributed to a lack in galactosamine or the inhibition of glycosamino-
glycan synthesis at the protein or polysaccharide level.

Another change in connective tissue in scorbutic animals is the failure of hydroxylatation of proline to hydroxyproline. Experiments in tissue culture by a mouse fibroblast line (T6) showed that ascorbic acid deficiency resulted in half the amount of hydroxyproline-containing material being laid down; thus, supporting the hypothesis that ascorbic acid affects the intermolecular cross-linking of collagen through certain hydroxylysine residues as well as affecting the hydroxylation of proline. Peck et al. (1967) indicated that ascorbic acid promotes collagen synthesis in isolated bone cells by directly stimulating the hydroxylation of a proline-rich peptide. How vitamin C facilitates the hydroxylation of proline is not known, but it appears to be required for the formation of hydroxyproline and hydroxylysine which are essentially found only in collagen.

Hertz (1936) illustrated that following bone injury in a completely scorbutic animal, there was no periosteal hyperemia; that it is difficult for a scorbutic animal to produce an inflammatory reaction in response to injury; and the normal process of healing could not be induced.

The work of Persson (1953) further demonstrated
the changes in connective tissue with a deficiency of vitamin C. He noted that there was an increased amount of ground substance mucopolysaccharides in scurvy, that the ground substance is in an abnormal state of organization, and that the water binding capacity of the skin is increased. Persson also suggested that local treatment of skin wounds with ascorbic acid and dehydroascorbic acid may be effective when the treatment is extended over several days.

Ostergaard and Loe (1975) demonstrated that ascorbic acid is a prerequisite for the maintenance of the collagen pool in the tissues.

It was also noted that there is a distortion of ribosomes in scorbutic fibroblasts. Stassen et al. in 1973, noted a loss of normal configuration in the rough endoplasmic reticulum. Addition of ascorbic acid restored their normal architecture; thus, suggesting that ascorbate may exert its action at the proline hydroxylase level. The addition of ascorbate to cultured fibroblasts induced a five fold increase in enzymatic activity which was independent of RNA or protein synthesis.

Thus, since vitamin C deficiency is associated with changes in the connective tissue, and since the principle elements of connective tissue are also present in bone;
it might, therefore, be expected that bone would also be affected.

It has been shown that ascorbic acid is required for normal wound healing. Golub (1973) concluded that ascorbic acid increased bone collagen remodeling \textit{in vitro} by favoring the accumulation of newly synthesized bone collagen and the removal or degradation of the older preformed material. Embryonic chick tibias grown in tissue culture displayed a marked dependence upon the presence of ascorbic acid for normal growth.

A citric acid soluble fraction of normal human and ox bone collagen influences the rate of hydroxyapatite formation \textit{in vitro}. A later study by Solomons and Gregory found that \textit{in vitro} ability of citrate-soluble collagen to modify the rate of hydroxyapatite formation was supported \textit{in vivo}; and it was concluded that citrate-soluble collagen accelerated bone mineral deposition. A study by Hiatt \textit{et al.} found a citrate extract of ox bone treated with pepsin, placed in surgically created periodontal pockets in dogs, resulted in rapid proliferation of repair cells with accelerated healing in connective tissue, bone, and cementum. In addition there was greater amounts of new bone formation in the healed lesion.
Bourne\textsuperscript{54} noted that when calcium ascorbate was injected subcutaneously into rats the amount of bone healing was statistically increased over that of animals injected with calcium gluconate. This may be associated with the availability of calcium with the ascorbate. Ramp and Thornton (1971)\textsuperscript{55} showed that ascorbic acid increased calcium turnover in bone, possibly through increased metabolic activity of the bone cells.

In 1923, Robison\textsuperscript{56} first described a local mechanism of calcification by deposition of the bone mineral from supersaturated solutions. Later, in 1934, he stated that the concept of a local mechanism as an enzyme system did not preclude the possibility that factors of a different type, such as surface forces in the colloidal matrix, might also assist in the deposition of calcium salts.\textsuperscript{57}

The ionic composition of intercellular fluid is normally supersaturated with respect to calcium and phosphate in the sense that these fluids will lose ions to a crystal or precipitate of calcium phosphate.\textsuperscript{58} Most calcific deposits consist of crystalline calcium phosphates in the form of apatite. But whether crystal formation is the initial event, that is amorphous calcium phosphates form first with subsequent crystallization, is still un-
settled. As in bone and cartilage, there are only two generally considered theories of calcium salt formation. One states that a high enough local ionic concentration of calcium and phosphate somehow must accumulate for precipitation to occur. The other states that some organic component, collagen in the case of bone, has properties mimicking those of apatite crystals. Since the extracellular fluid is supersaturated and metastable with respect to calcium and phosphate, such an organic structure could act as a seed crystal and thus as a starting point for a self-propagating process. Possibly, both situations may concurrently occur.

Murry concluded that calcium in the graft was necessary for the calcification of newly forming bone and cementum. He also stated that there is an affinity by the fibrin clot for calcium and may thus aid in the retention of the bone powder.

Beube using heterografts of boiled cow bone powder, stated that the bone powder may help supplement the calcium concentration available from the circulatory system.

Linghorne and O'Connell (1951) stated that the presence of resorbing calcified tissues--the autogenous implanted bone chips--appeared to be a factor in the dif-
ferentiation of osteoblasts.

A number of investigations utilized mineralized materials acting as a calcium source to stimulate osteogenesis. Stallard and Randall (1959), Toto et al. (1961), and Glickman and Patur (1962) utilized "anorganic bone"; and Radentz and Collings (1965) utilized plaster of paris. The materials offered inconsistent results in relation to osteogenesis stimulation.

Wadkins et al. (1974) has shown that exposure of ordinarily noncalcifying matrices in vivo to an unphysiologically high concentration of calcium can produce a matrix which catalyzed mineral formation.

Ramp and Newman (1973) observed that the ratio of Ca:P0₄ in the culture media had a direct effect upon mineralization of explanted chick embryo tibias; thus, showing that mineralization was more dependent on calcium than on phosphate ion concentration. Heeley and Irving (1973) showed that mineralization begins with formation of a calcifiable matrix and calcium is the first ion to be bound. Groer and Marshall (1973) have shown that the process of bone surface exchange of calcium takes place in a layer of bone one to four microns thick. De Luca (1967) suggested that vitamin D acts by facilitating
the transport of calcium from bone fluid to bone cell.

Better (1972)\textsuperscript{70} grafted calcium phosphate particles and tricalcium phosphate as ceramic implant materials. He noted that both materials were well tolerated. Tricalcium phosphate resorbed the slowest and calcium phosphate the most rapidly.

In the literature, the present state of knowledge cannot attribute any single factor to the property of calcifiability. The postulate exists of a local mechanism involving both physical and chemical components acting together in the living organism to bring about calcification.

The source of the osteoblasts have been proposed as three possible origins: Bruns\textsuperscript{71} suggested that they may arise from pre-existing osteoblasts; Ham and Harris\textsuperscript{72} suggested they arise from the endosteal lining of the marrow cavities; and Bloom\textsuperscript{73} suggested they arise from the previous two sources and also from the perivascular undifferentiated mesenchymal cells.

Goldhaber (1961)\textsuperscript{74} introduced a concept of an osteogenic induction factor. He implanted allogenic neonatal skull bone subcutaneously in a millipore diffusion chamber and found that new bone formation was elicited on the host side of the filter. Thus, new bone was derived
from the host tissue in response to a diffusible osteogenic inductor.

Urist\textsuperscript{75} termed the "bone induction principle," in which a cellular differentiation phenomenon of a tissue substrate exerts a physio-chemical effect upon competent mesenchymal cells to stimulate their differentiation into osteoblasts capable of both osteogenesis and further induction (autoinduction).

Host site cells may themselves be competent of bone induction. Klingsberg (1974)\textsuperscript{76} using scleral grafts in the repair of periodontal osseous defects in humans, illustrated an advantageous method to repair destroyed bone. Re-entry after two years showed a lasting restoration of the bone defects after scleral grafting. The sclera persisted, but along its edges osteoblasts were present, suggesting that the sclera was being transformed into bone. Thus, the host site cells may themselves be competent of induction. The graft may just occupy space allowing the prepared host site to react in a positive manner.

In the investigation of various graft materials, a number of experimental designs were utilized. One experimental design, used repeatedly in the past and was selected for use in this study, utilized surgically created chronic,
two-walled periodontal defects in the rhesus monkey. 77, 78, 79, 80

There is substantial evidence in the literature of the critical role played by ascorbic acid in the formation and maintenance of collagen. Also, calcium ascorbate has been shown to increase the speed of healing. As was mentioned, an increased calcium concentration may stimulate osteogenesis. If a graft material does not itself induce bone formation, it may offer benefit by occupying space allowing for host induction.

In view of the evidence provided in the literature, a study of calscorbate * (containing both calcium and ascorbic acid) used as an alloplast was undertaken. The study concerns itself with the histomorphologic changes in the rhesus monkey periodontium in which an experimental surgical defect was created and implanted with calscorbate.

*Cole Pharmacal Company, St. Louis, Mo.

Calscorbate Tablets Contain:
- Calcium Ascorbate (100mg)
- Dibasic Calcium Phosphate (Hydrous) (1.08gm)
- Vitamin D₂ (Calciferol) (50 U.S.P. Units)

Calscorbate Chemically:
- Two Moles Ascorbic Acid
- One Mole Calcium
  \((C_6H_7O_6)₂Ca\)

(Calcium Ascorbate)
CHAPTER III

MATERIALS AND METHODS

A. EXPERIMENTAL DESIGN

Two adult male rhesus monkeys (*Macaca mulatta*) were utilized as experimental models in this study. The experiment covered a one-hundred and seven day interval utilizing the first twenty-one days as a quarantine and tuberculosis inoculation period. This period allows the monkeys to acclimate to the Loyola animal care facility and a definite parameter of health to be established. Throughout the experimental period, the animals maintained their physical parameters as recorded during their quarantine, and appeared to remain in good health during the remaining eighty-six days of the experimental period.

The animals demonstrated a slight marginal gingivitis with varying amounts of materia alba, plaque and calculus. The gingiva was firm in consistency and pink in color. The sulcus depths were examined and noted to be within acceptable limits, one to three millimeters (Figure 1).
The maxillary and mandibular quadrants were utilized as experimental sites. Thus, four maxillary and four mandibular quadrants were included in the study. Two, two-walled surgically created osseous defects per quadrant were created. Created defects distal to the second bicuspids were utilized as the control area, and the created defects distal to the first molars were corrected by calscorbate. The control site preparation and correction (by surgical curettage without placement of calscorbate implants) was accomplished at the same time as the preparation of the analogous implanted defects within the same quadrant.

The one-hundred and seven day schedule of the experiment was planned to allow for a 21 day quarantine and a 86 day experimental period. This allowed the two-walled osseous defects to be created thirty days before the initial implantation. The predetermined schedule was so designed to allow the sacrifice of the control and the calscorbate implants on 0, 3, 7, 14, 21, 28, 42, and 56 days, postoperatively. Each time sequence allowed a control experimental defect and a defect corrected by calscorbate.

Twenty minutes prior to the surgical procedures, the monkey received an intramuscular injection of 8 mg Sernylan*.

*Parke, Davis, and Co., Detroit, Michigan
for sedation. When long procedures were encountered, an additional dose of 5 mg Sernylan was given intramuscularly as needed for sedation. Prior to the surgical intervention, a local anesthetic of Xylocaine 2% with 1:100,000 epinephrine was used in the area of surgical intervention.

Throughout the course of each surgical procedure, strict conditions of asepsis were maintained for the operator and the monkeys' protection. All pertinent clinical observations were recorded.

B. GENERAL PREPARATION

Full mucoperiosteal flaps from the distal of the cuspid to the distal of the second molar were utilized for both the creation and correction of the defects. An intrasulcular incision was carefully performed with a #15-C Bard-Parker blade and reflected with a small periosteal elevator (#7 wax spatula). Care was taken so the tissue would not be perforated, and the incision was scalloped for better primary closure. Tissue tags on the inner surface of the flap were removed with a small tissue scissors. Gracey curettes were used to remove all interproximal granulation tissue and to thoroughly plane the exposed root surfaces.

C. PREPARATION OF THE DEFECTS

The sites chosen for the creation of the osseous defects were the interproximal osseous septae distal to the second premolar and the first molar. These areas offered a sufficient amount of bone present to allow for the creation of the two-walled defects, and these areas were easily accessible and readily visible. This experimental area also affords protection postoperatively due to the interproximal contacts between adjacent teeth, and due to the contour of the crowns. The control defect was placed distal to the second premolar, and the defect created distal to the first molar received the calscorbate implant.

Two-walled, surgically created, osseous defects were made in the selected sites. A 701 tapered fissure bur was used in a slow speed dental handpiece to remove 3 mm of alveolar crest bone, along the distal root surface. The depth and width of the defect (3 mm) was carefully monitored with the use of a calibrated periodontometer.* The resulting osseous defects consisted of a lingual and distal wall of bone, a mesial wall of cementum and/or dentin and, no buccal wall (Figure 2). While removing the bone during the creation of the defects, isotonic saline

*HU-FRIEDY--Michigan probe
was used as a coolant to avoid temperature increases which could damage the bone.

D. INTRODUCTION OF CHRONIC IRRITANTS

A wooden toothpick approximately 6 mm in length was introduced into each osseous defect to serve as a chronic irritant, and the excess afforded a wedging effect for retention of the toothpick. The wood acted as a route of direct communication from the oral cavity to the defect, allowing bacteria and their toxins ingress. This was performed in an attempt to simulate a chronic defect seen in human periodontitis.

The full mucoperiosteal buccal flap was repositioned and secured close to its original position with interrupted vertical mattress, interproximal 5-0 Ethaflex* sutures, and were tied on the lingual surface. This suturing technique provided additional stabilization of the chronic irritant.

E. POSTOPERATIVE CARE

The monkey received a prophylactic injection of an antibiotic, prior to the initiation of any surgical pro-
cedure. An intramuscular injection of 600,000 units (3 cc) of Combiotic* (Penicillin and dihydrostreptomycin) was given. The Combiotic gives a 72 hour antibiotic coverage to the monkey. Post-operative instructions specified that the animals receive no food for the first 24 hours, a soft diet for the following 2 days, and a normal diet thereafter.

F. REMOVAL OF IRRITANTS

Seven days following the creation of the osseous defects, the animal was sedated, as before with Sernylan, and the sutures and wooden irritants were removed. Clinically inflamed gingiva was noted at this time.

G. PROCUREMENT OF IMPLANT MATERIAL

Calscorbate** tablets were crushed until a fine, even consistency powder was obtained. This Calscorbate powder without any other modification was utilized as the implant material.

*Pfizer Company
**Cole Pharmacal Company, Inc.
H. CORRECTION OF THE DEFECTS

As outlined previously, the same surgical procedures were performed. Exactly 30 days postoperative to the creation of the two-walled osseous defects, the osseous defects were exposed for surgical implantation or curettage (in the case of the control). All chronic inflammatory tissue was carefully removed from the previously created two-walled osseous defects, and the root surfaces were thoroughly planed.

The control defect on the distal of the second premolar was carefully debrided and irrigated with isotonic saline, in order to leave the control defect free of any material.

The powdered calscorbate was placed in the debrided defect distal to the first molar and overfilled. The implant material was allowed to mix with the blood in the defect area. The implant material was placed easily, hemorrhage in the area was well controlled. (Figure 3).

The control area and the implant area were covered by the full mucoperiosteal flap which was repositioned and held firmly in place with sutures (as previously described). The same post-operative antibiotic and diet instructions
as previously described were given.

Seven days after the surgical correction of the osseous defects, the animal was again sedated for suture removal. The monkey was then maintained until the next procedure or until the scheduled sacrifice.

I. COLLECTION OF SPECIMENS

The animals were sedated as previously described and sacrificed by giving a intra-arterial injection of a lethal dose of Totaltox.*

A Stryker saw was used to obtain the block sections. The specimens were washed with distilled water and placed in pre-labeled jars containing 10% formalin for fixation. The volume of formalin was 20 times that of the specimen.

J. PREPARATION FOR HISTOLOGIC EXAMINATION

After two weeks of fixation, each specimen was de-calcified in formic acid and sodium citrate (50%/50% solution), trimmed, embedded in paraffin, sectioned at 10 microns in a transverse buccolingual plane, and stained with hematoxylin and eosin (H&E). The degree of decalc-

*Chicago Veterinary Supply, Chicago, Illinois
fication was determined by radiographs taken at weekly intervals after an initial four week period. The slides from each experimental site were stained with hematoxylin and eosin, and a representative histologic section was selected for a detailed histologic analysis.
CHAPTER IV

FINDINGS

A. CLINICAL OBSERVATIONS

Both monkeys presented with a marginal chronic gingivitis at the initiation of the study (Figure 1). The papillary and marginal gingiva appeared somewhat edematous and magenta in color. The attached gingiva was generally firm and pink in color. The sulcus depths were within acceptable limits of one to three millimeters. Seven days following the creation of the two-walled osseous defects and placement of the irritants the gingiva demonstrated an increased degree of inflammation. The marginal and papillary gingiva was edematous, erythematous, and displayed hemorrhage upon probing. Thirty days following the creation of the osseous defect, a decrease in the degree of gingival inflammation was noted as compared to seven days following the creation of the osseous defect, but greater than at the initiation of the study.

Thirty days after creation, the osseous architecture of the defects demonstrated additional bone resorption.
The two-walled osseous defects on the buccal cortical plate showed additional resorption mesially and distally giving a larger V-shaped defect.

One week after the correction of the defects, the marginal gingiva was edematous, boggy and erythematous as compared to adjacent areas. The tissue inflammation decreased by the fourteenth post-operative day and appeared close to normal.

B. HISTOLOGIC OBSERVATIONS

1. INTRODUCTION

Changes in the epithelium were inflammatory in nature. The "normal" chronic gingivitis and the result of the surgical insult can be the contributing factors.

The connective tissue initially demonstrated inflammation, followed by repair. Inflammatory changes included vasodilation, and inflammatory cell infiltration. Repair consisted of fibroblastic and endothelial proliferation with re-organization, approaching a normal fibrous connective tissue.
2. TWO-WALLED CONTROL
   a. Epithelium

   0 Day

   Just prior to sacrifice, the area was surgically flapped and curetted as were all control defects (3-56 days). Keratinized stratified squamous epithelium was present but not adherent to the tooth. A normal, thin sulcular epithelium was discernable with some expected degree of inflammation.

   3 Day

   Keratinized stratified squamous epithelium was present over the non-defect area. The sulcular epithelium exhibited some intercellular edema, some degeneration, and a fibrinopurulent exudate. The full mucoperiosteal flap over the defect area was probably lost in the histologic preparation.

   7 Day

   Keratinized stratified squamous epithelium was present. Sulcular epithelium of normal thickness and expected inflammatory condition.
**14 Day** (Figure 7)

Keratinized stratified squamous epithelium was present. The epithelial attachment was present (separated in histologic processing).

**21 Day** (Figure 10)

The keratinized stratified squamous epithelium and the non-keratinized sulcular epithelium appeared generally normal with the expected inflammation especially adjacent to the sulcular epithelium.

**28 Day**

Keratinized stratified squamous epithelium was noted. The section, as cut, could not demonstrate the epithelial attachment.

**42 and 56 Day** (Figure 12)

A keratinized stratified squamous epithelium was noted. The "col" area epithelium was thin as normally expected.
b. Connective Tissue

0 Day

There was a plasmocytic infiltration and some edema. A few small spicules of bone were noted embedded within the collagen fibers adjacent to the defect. Hemorrhage was seen near the detached flap.

3 Day (Figure 5)

The full mucoperiosteal flap was absent from the sections and probably lost in histologic preparation. Loosely arranged tissue fibers were present. There is a fibrinopurulent exudate clot filling the defect; also, polymorphonuclear leukocytes were noted.

7 Day

The connective tissue showed some plasma cell infiltrate, proliferating fibroblasts, and young capillaries.

14 Day

"Old" collagen and "newly formed" collagen
were noted especially as the defect was approached. Inflammatory changes and cellular infiltrate, especially adjacent to the sulcular epithelium, were noted. A fibrous attachment is forming, getting a reattachment. New cementum is forming near the defect.

21 Day

The connective tissue was unremarkable. The connective tissue repair by fibrogenesis was generally completed.

28 Day

The lamina propria was composed of mature connective tissue. Some degree of inflammation was noted and expected especially near the epithelium.

42 and 56 Day

The lamina propria shows mature collagen with some inflammatory changes of slight edema and some plasma cell infiltration (normal). There is newly forming periodontal ligament.
c. Alveolar Bone and Defect

0 Day (Figure 4)

The area was surgically flapped and curetted. The defect was noted in alveolar bone. Some small spicules of bone were noted among the fibrin clot debris.

3 Day (Figure 5)

Alveolar bone apical to the created defect showed the periodontal ligament to be attached to the cemental surface of the root. The defect was filled with a fibrinopurulent exudate, loosely arranged fibrous connective tissue, and polymorphonuclear leukocytes. Active osteogenesis with osteoblasts was seen within the marrow spaces adjacent to the defect area. Along non-defect bone, osteoblastic activity was noted.

7 Day (Figure 6)

The defect area had many proliferating fibroblasts, young capillaries, and polymorphonuclear leukocytes. There was residual
necrotic bone and new bone forming on the surface of old bone. Thus, some repair is noted at seven days.

14 Day
Cementum formed on the root dentin surface adjacent the defect, allowing for a re-attachment. Within the defect there are proliferating and maturing fibroblasts with collagen fiber bundles being formed. Some new bone was forming in the defect, showing active osteoblast activity.

21 Day (Figure 10)
Some bone repair in the defect is noted with osteoblastic activity.

28 Day (Figure 11)
A small amount of bone regeneration and osteoblastic activity was noted adjacent to the more mature bone. The section, as cut, reveals a new periodontal ligament fiber attachment to the newly forming cementum of the tooth.
42 and 56 Day (Figures 12, 13, and 14)

A small amount of new bone apposition is noted over the old alveolar bone with active osteogenesis. Young collagenous fibers extend from the new bone to newly formed cementum. A fibro-periosteum is noted over new bone.

3. CALSCORBATE TWO-WALLED IMPLANT

a. Epithelium

0 Day

Just prior to sacrifice, the area was surgically flapped and curetted and calscorbate implanted into the osseous defect. Stratified squamous keratinized epithelium demonstrating a slight intercellular edema was noted.

3 Day

Stratified squamous keratinized epithelium was noted. Over the defect area the specimen did not show epithelium, probably due to the histologic processing and existance of a dense fibrinopurulent exudate below making a weaker attachment to the underlying connective tissue.
7 Day

There was keratinized stratified squamous epithelium demonstrating slight intercellular edema.

14 Day

The specimen showed tooth root material only. The osseous and soft tissues were lost during the histologic preparation.

21 Day

A stratified squamous keratinized epithelium was noted. The "col" area epithelium was found in the section and was thin and not keratinized as expected.

28 Day

A stratified squamous keratinized epithelium was noted.

42 and 56 Day

A keratinized stratified squamous epithelium was noted. "Col" areas were demonstrated with normal thin, non-keratinized epithelium.
b. Connective Tissue

**0 Day**

The full mucoperiosteal flap is detached. The connective tissue shows a plasmacytic infiltration and slight edema. Extravasated blood was present between the tooth and flap.

**3 Day** (Figures 17, and 18)

The full mucoperiosteal flap was detached. A fibrinopurulent exudate was present in the wound site. Loosely arranged collagen fibers were noted as well as more mature collagen. Pigment granules were noted within the lamina propria at and near the graft site which may represent hemosiderin pigment or calscorbate granules or both. Polymorphonuclear leukocytes are present and some appear to be phagocytizing the granular material.

**7 Day** (Figure 19)

There is a fibrinopurulent exudate residue on the surface. A fibrous connective tissue is present supporting the epithelium. There is
a proliferation of fibroblasts among collagenous fibers and new capillaries; thus, a granulomatous reaction within the connective tissue.

14 Day

The specimen showed tooth root material only. The osseous and soft tissues were lost during the histologic preparation.

21, 28, 42, and 56 Day (Figure 20)

The connective tissue was repaired. New, young collagenous fibers were noted among mature collagenous fibers. Many dilated capillaries and fibroblasts were noted. In some sections it was able to demonstrate the extension of collagenous fibers to newly formed cementum.

c. Alveolar Bone and Defect

0 Day (Figures 15 and 16)

A defect in the "old" bone is noted. A fibrin clot debris, red blood cells, portions of connective tissue and bone spicules were found in the prepared defect. Pigment material which
may possibly represent hemosiderin and/or
calscorbate implant granules were also noted—
this may be described as an amorphous yellow-
black pigment in H&E.

3 Day (Figures 17, and 18)

The pigment granules (possibly hemosiderin
and/or calscorbate) are also seen in the defect
area with red blood cells, polymorphonuclear
leukocytes, and a fibrinopurulent exudate.
The immediate marrow spaces show hyperplasia
with mitosis. The surviving marrow adjacent to
the wound site shows proliferation.

7 Day (Figure 19)

There was new bone formation in the defect.
Remodeling is occurring in the area of the
defect and implant. An acute inflammatory
process, proliferation of fibroblasts and new
capillaries (granulation tissue) exists. "Old"
bone shows remodeling and resorption with osteo-
clasts and the surface shows new bone formation
similar to an involucrum. The bone surface
shows some osteoclastic resorption. New bone
is found forming around some amorphous debris (unknown fragments of bone or possibly the calscorbate granular implant).

**14 Day**

The specimen showed tooth root material only. The osseous and soft tissues were lost during the histologic preparation.

**21 Day** (Figures 20, and 21)

The section demonstrates very active formation of new bone with active osteogenesis on the surface of the new bone. The granular product is most probably calscorbate at this time.

**28 Day** (Figures 22, and 23)

Active osteogenesis is still noted. The periodontal ligament formation is noted from newly forming bone to newly formed cementum; thus, getting a periodontal ligament attachment.

**42 Day** (Figure 24)

New bone formation is still occurring with active osteoblastic activity. Repair is continuing.
56 Day (Figures 25, and 26)

There is a dense new bone formation and normal periosteum. The created defect is filling with new bone with an active periosteum (the amount of new bone formation appeared greater than the control). Wide marrow spaces are seen in the new bone. There is a dense lamellated bone.
CHAPTER V

DISCUSSION

The primary significance of this study demonstrated that the implant of calscorbate powder is acceptable to the host. Calscorbate powder was successfully implanted in osseous defects of the rhesus monkey with no rejection phenomena elicited by the host. Osseous regeneration, re-attachment, and cementogenesis were noted in the calscorbate implanted defects. The control defects also showed regeneration. Although a quantitative comparison cannot be made in this study, the amount of bone regeneration in the calscorbate implanted defect appeared comparable or increased to that of the control. Important is the fact that the implanted defects did seem to show a somewhat delayed sequence of healing as compared to the control.

It should be pointed out that the experimental defects created in this study attempted to simulate chronic periodontitis both clinically and histologically. But the created defects may not be identical to those found in the
naturally occurring periodontal disease process. Naturally occurring periodontal disease can have very complex and often unknown etiologic factors that cannot be incorporated in the experimental animal model.

The study demonstrated a rapid regeneration of the gingival epithelium and connective tissue. As was already pointed out, ascorbic acid has a critical role in both the formation and maintenance of collagen in healing wounds, and may actually stimulate healing.

The dominant cells of the wound are fibroblasts and endothelial cells. Epithelium is repaired by mobilization of cells from the margin of the wound. The connective tissue of the wound repairs by fibroblastic proliferation and migration of these fibroblasts into the wound area, and there is a formation and remodeling of the wound's extracellular material. Also, there are definite proliferative response of the capillaries. A possible relationship may exist between the roles of epithelium and connective tissue in wound healing since the connective tissue response of endothelial and fibroblastic proliferation is stimulated by epithelial proliferation. Also, as healing progresses the new connective tissue appears to
have some inhibitory effect on the proliferation of the epithelium.

In the repair of bone, the osteoblasts are induced to produce new bone. As suggested, they may arise from pre-existing osteoblasts, from the endosteal lining of the marrow cavities, and from the perivascular undifferentiated mesenchymal cells. The bone induction properties of an implant is of interest, but the initiating factor of bone formation is not yet known. It had been reported that calcium in the graft was necessary for calcification of newly forming bone. It may help supplement the calcium concentration available from the circulatory system. And presence of resorbing calcified material may actually help in the differentiation of osteoblasts. An unphysiologically high concentration of calcium can, in fact, produce in vivo a matrix which catalyzes mineral formation. It has been shown that mineralization may be dependent upon calcium and that calcium is the first ion to be bound. Also, the rate of bone healing was increased with calcium ascorbate.

Thus, calscorbate as a graft material may benefit healing by both the calcium and ascorbate components. The collagen formation and mineralization may both be
favorably affected by the calscorbate implants in relation to bone repair.

Urist\textsuperscript{75} pointed out the possibility of a diffusible osteogenic inductor for new bone formation. He also presented the hypothesis that collagenolytic activity by histocytes or other cells can cause dissolution of the organic matrix, yielding an inductor substance for the formation of new bone which can calcify rapidly. Also, the host site has cells, which given the chance, may be competent of induction of bone. Further, it might be stated that any host compatible implant material can occupy space and delay healing of a defect. This would allow a greater proliferation of granulation tissue with its increased numbers of undifferentiated mesenchymal cells when compared to control defects. Thus, there could be a greater potential for osteogenesis. This point was brought out by Klingsberg\textsuperscript{76} in the repair of osseous defects in humans. He used collagen from sclera as a graft material. Periodic re-entry, at intervals up to two years, had shown the sclera graft persisted and active osteogenesis was present at the graft's edges. This may represent the sclera graft was being transformed into new bone. Thus,
the scleral graft just "occupied space" and allowed the host site cells to become competent of induction themselves.

If the calscorbate implant material does not itself help induce osteogenesis, it may act to occupy space and delay healing of the defect, thus allowing the chance for a greater proliferation of granulation tissue with its undifferentiated mesenchymal cells, allowing the host site cells to become competent of induction themselves. Therefore, the fact that the calscorbate implant material was well tolerated by the host, and that the implanted defects did seem to show a somewhat delayed sequence of healing as compared to the control, becomes significant.

Periodontal support includes not only bone but the periodontal ligament and cementum as well. It is not only important to restore bone but also to repair and restore cementum and periodontal ligament. The implanted calscorbate did not inhibit cementum formation or the formation of a periodontal ligament, in fact, its formation appeared somewhat more profound than the control. And, as mentioned, calscorbate does not interfere with the induction of osteogenesis and may potentiate it.

It must be recalled that the experimental model used in this study cannot duplicate the chronic periodontitis
found in man. The possible benefit that calscorbate has been shown within this investigation may not be, therefore, directly applied to human periodontal therapy (by design of this investigation). But a graft material such as calscorbate would be especially desireable in periodontal treatment because it is readily available, would involve a single surgical procedure, and is not believed to be immunologically sensitive as other osseous grafting techniques may be.
CONCLUSIONS

1. The two-walled osseous defects corrected by calscorbate were accepted by the host site and did not impede osteogenesis.

2. The experimental defects utilized in this study attempted to simulate chronic periodontitis. But the experimental animal model may not allow for a direct relationship to the chronic periodontitis in man.

3. Possibly, the calscorbate material implant acts as a "space occupier" allowing for a greater number of undifferentiated mesenchymal cells and host induction of osteogenesis.

4. Possibly, the calscorbate may influence the bone collagen, and provide a calcium source to the healing defect.

5. The control areas treated by surgical curettage showed osseous healing analogous to the calscorbate corrected defects. Perhaps a larger sampling might show accelerated bone deposition by calscorbate.
CHAPTER VII

SUMMARY

This study was conducted to examine histologically the sequential healing phenomena in chronic two-walled periodontal osseous defects following their correction with calscorbate implants.

Two male adult rhesus monkeys were utilized as experimental models providing specimens from 0 to 56 days postoperatively. Both control and graft specimens were taken at 0, 3, 7, 14, 21, 28, 42, and 56 days. The control surgical defect was treated by surgical curettage only.

Commercially available calscorbate tablets were uniformly crushed to a powder and utilized as the experimental implant into the surgically created defects.

Histologic sections from each specimen were stained with H and E, a description of the sequential healing events were recorded, and the results discussed.

The implanted calscorbate was compatible with osteogenesis. The material was well accepted by the host.
site and did not impede osteogenesis. A quantitative relationship of the implant material to the control cannot be made, though the implant material did appear to have a more profound amount of bone repair over the control.
CHAPTER VIII

ILLUSTRATIONS
Figure 1. The preoperative appearance of the experimental area. Note the slight papillary and marginal gingivitis.
Figure 2. Experimentally created two-walled osseous defects (arrows) distal to the second premolar (control) and the first molar (area to receive the graft.)
Figure 3. Placement of calscorbate implant (overfilled) in the defect area distal to the first molar and the control area is distal to the second premolar.
Figure 4. 0 day flapped control, 40x.

Surgically created defect with fibrin clot debris showing pieces of connective tissue and spicules of bone (arrows).
Figure 5. 3 day control, 40x.  
The defect area is filled with a fibropurulent exudate (a).
Figure 6. 7 day control, 40x.

The defect has many proliferating fibroblasts.
New bone (n) is forming over old bone (o).
Figure 7. 14 day control, 10x.
Stratified squamous epithelium (e), epithelial attachment (a), and new collagen (c) as defect is approached.
Figure 8. 14 day control, 25x.
Cementum formed on the dental surface (arrow).
New bone formation (b).
Figure 9. 14 day control, 40x.
Active osteoblastic activity (o).
Figure 10. 21 day control 40x.
Keratinized stratified squamous epithelium (e). Some new bone repair (b), and osteoblastic activity (o).
Figure 11. 28 day control, 40x.
Newly formed periodontal ligament (1), to newly formed cementum (c), osteoblastic activity (o) adjacent the more mature bone.
Figure 12. 42 day control, 25x.
Keratinized stratified squamous epithelium (e), new bone formation (b).
Figure 13. 42 day control, 40x.

New bone with active osteogenesis (arrow).
Figure 14. 56 day control, 40x.
Defect area with some new bone (b), a fibro-periosteum is noted over the new bone (arrow).
Figure 15. 0 day calscorbate implant, 25x.
Prepared defect in old bone. Fibrin clot debris (a).
Figure 16. 0 day calscorbate implant, 40x.
Prepared defect with fibrin clot debris.
Portion of connective tissue remnant (a), spicule of bone (b), and pigmented material (arrow).
Figure 17. 3 day calscorbate implant, 25x. Fibrinopurulent exudate (a), loosely arranged collagen fibers (b).
Figure 18. 3 day calscorbate implant, 40x.
Fibrinopurulent exudate (a), fibrous connective tissue (b), and pigment granules (arrow).
Figure 19. 7 day calscorbate implant, 25x.
New bone formation (a) in the defect, and fibrous connective tissue (b).
**Figure 20.** 21 day calscorbate implant, 25x. Active formation of new bone (a), and fibrous connective tissue (b).
Figure 21. 21 day calscorbate implant, 40x.
Newly formed bone and osteoblastic activity (a), "old" bone (b).
Figure 22. 28 day calscorbate implant, 25x. Periodontal ligament (l), from newly formed bone (b), to new cementum (c).
Figure 23. 28 day calscorbate implant 40x.

Newly forming bone and osteogenesis (a).
Figure 24. 42 day calscorbate implant, 40x.
New bone formation and osteoblastic activity (arrow).
Figure 25. 56 day calscorbrate implant, 40x. Note dense new bone filling defect (a).
Figure 26. 56 day calciobrate implant, 40x.

Note dense new bone filling the defect (a), and osteoblastic activity (arrow).
REFERENCES


## APPENDIX

### GRAFT TERMINOLOGY

<table>
<thead>
<tr>
<th>Old</th>
<th>New</th>
<th>Definition</th>
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<tbody>
<tr>
<td>Autograft</td>
<td>Autograft</td>
<td>same individual</td>
</tr>
<tr>
<td>Isograft</td>
<td>Synograft</td>
<td>identical twins</td>
</tr>
<tr>
<td>Homograft</td>
<td>Allograft</td>
<td>within same species</td>
</tr>
<tr>
<td>Heterograft</td>
<td>Xenograft</td>
<td>different species</td>
</tr>
<tr>
<td></td>
<td>Alloplast</td>
<td>bone substitute</td>
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THESIS/DISSertation

COnIMITTEE MEMBERS

1. Dr. Anthony W. Gargiulo
   Professor and Chairman
   Department of Periodontics
   Loyola Dental School

2. Dr. Patrick D. Toto
   Professor and Chairman
   Departments of Pathology and Oral Pathology
   Coordinator of Advanced Education
   Loyola Dental School

3. Dr. Joseph Keene
   Associate Professor
   Coordinator of Graduate Periodontics
   Loyola Dental School
This thesis, submitted by Lawrence W. Jenkins, has been read and approved by three members of the faculty of the Department of Oral Biology.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements of the Degree of Master of Science.

5-4-77
Date

Signature of Advisor