BONE FORMATION IN THE MAXILLARY SINUS BY USING PLATELET-RICH PLASMA: AN EXPERIMENTAL STUDY IN SHEEP

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Recently, platelet-rich plasma (PRP) has been proven to be an effective regeneration adjunct when combined with autogenous bone in the reconstruction of mandibular defects. However, little is known about the effect of PRP when combined with a bone allograft in the maxillary sinus. The purpose of this study was to quantitatively evaluate the ability of PRP to enhance bone regeneration in the maxillary sinus of sheep when combined with demineralized freeze-dried bone allograft (DFDBA) and cortical cancellous freeze-dried bone allograft (CCFDBA). Ten sheep were selected for bilateral sinus augmentation DFDBA + CCFDBA + PRP (test) and DFDBA + CCFDBA (control). Five were sacrificed at 3 months and the other 5 at 6 months. Hematology tests were performed for platelet count, and histology slides were obtained for histomorphometric analysis taking 2 measures of interest: total area (square millimeters) and percentage of bone fill. Student t tests showed no significant difference between test and control groups for total area ($P > .25$) and percentage of bone fill ($P > .80$) at either 3 or 6 months. The control group showed no statistical difference for total area ($P < .095$) and percentage of bone fill ($P < .60$) between 3- and 6-month healing times. The test group, however, showed a significant increase in total area ($P < .025$) but not in percentage of bone fill ($P < .40$) for the 2 healing periods. When the treatments were compared for interactions within the animal model, no clear tendency was evident for the test group to perform in relation to the control group regarding total area ($r = .766$, $P < .01$). A moderate tendency existed between the percentages of bone filled ($r = .824$, $P < .005$). Platelet-rich plasma showed higher platelet count than did the whole blood (2 to 5 times). However, no correlation was found between the log ratio and the bone measures. Within the limitations of this study, PRP failed to enhance or accelerate bone regeneration in the maxillary sinus of sheep when combined with bone allograft.

Introduction

The posterior edentulous segment of the maxilla is often a problem for the insertion of dental implants. It is common to find the sinus floor close to the alveolar crest. Several authors relate this appearance to 2 phenomena: (1) the enlargement of the sinus at expense of the alveolus after tooth extraction because of the increased osteoclastic activity of the periosteum of the Schneiderian membrane,[1] and (2) increased pneumatization of the sinus simply because of the increase in positive intra-antral pressure.[2]

Pioneer implantologists have dealt with this problem by using different techniques. In 1977, Geiger and Pesh[3] placed ceramic implants penetrating the maxillary sinus without any postoperative complication. Also in 1977, Tatum[4,5] elevated and grafted the sinus membrane by using a modified Caldwell-Luc procedure. Boyne and James[6] first published this procedure in 1980 by using autogenous bone graft from the iliac crest. Vassos[7] described a blade implant with a broad flat surface to push the sinus floor superiorly. In 1987, Smiler and Holmes[8] reported on the use of nonresorbable material for the grafting of the maxillary sinus.

Since then, multiple regimens have been followed for the augmentation of the subantral sites, including autogenous bone grafts[9–13]; mineralized or demineralized bone grafts[14,15]; or xenogenic,[16] hydroxyapatite,[8,13,17] or composite bone substitutes.[18,19] According to a consensus in 1996, many bone-graft materials are suitable for the sinus graft procedure, but the autogenous bone graft had fewer complications.[20]

The survival rate of dental implants in the augmented maxillary sinus has been associated with several factors such as implant type,[21] implant placement with or after maxillary sinus grafting,[10,20,22] pretreatment bone height,[18,20] oral hygiene status, patient history of cigarette smoking,[20,23] and type of bone substitute.[20] The amount of new bone formation beneath the maxillary sinus in humans after sinus lift and grafting ranges between 5% and 49% over a 6- to 12-month healing period.[24–27] Studies have found that autologous bone alone or in combination with another type of bone-graft material has the highest percentages of new bone formation.[13,17,28]

Autogenous bone can be harvested extraorally (ileac crest, tibia) or intraorally (tuberosity, mandibular ramus, symphysis of the mandible), but the necessity of a second surgical intervention makes autogenous bone harvesting more costly and time consuming. The morbidity at the donor site also increases, and the amount of bone is usually insufficient when the intraoral approach is used.[29–31]

The type of bone that is commonly found in the posterior maxilla is type III and type IV, according to Lekholm and Zarb.[32] Implant failure rate as high as 35% has been reported in type IV bone.[33] The amount of bone volume that has been found in type III and IV bone is between 27% and 42%.[24,34,35] The percentages found in these studies are similar to the amount of new bone formation found after the grafting procedures in the maxillary sinus. Failures rates for loaded dental implants in the maxillary sinus range between 0% and 25% with autogenous bone grafts[6,10,11] and between 0% and 30% with allogenic and combined bone substitutes.[19,36] Despite apparently acceptable clinical outcome, histologic reports in humans suggest that bone formation after implantation of allogenic, xenogenic, or alloplastic bone substitutes is limited.[8,13,17,24]

To improve the rate of new bone formation without the use of autogenous bone graft in the sinus graft procedure, several authors have studied the feasibility of growth factors and cytokines for maxillary sinus augmentation such as BMP-2 and BMP-7 [OP-1] with positive results.[35,37–40] However, these materials are extremely expensive and are not currently approved by the Food and Drug Administration for dental purposes.[41]

Animal Model for Maxillary Sinus Augmentation

The growth factors and cytokines have been studied with different animal models. Such models described in the literature include rabbits, nonhuman primates, goats, and sheep.[40,42–45]

Rabbits are valuable models for bone research because they are easy to handle; however, the healing rate varies from animal to animal and the maxillary sinus is small.[46] Nonhuman primates, though closest to humans from the point of view of bone biology, are rarely considered as models for bone repair because of their high cost, low availability, and handling difficulty.[43] The goat and sheep models have been shown to be appropriate animal models, with an easy working disposition, adequate maxillary sinus size, and bone physiology and structure similar to that of humans.[46]

Platelet-rich Plasma

Recently, Marx et al[47] have shown promising results in new bone

formation, treating mandibular defects with a combination of autogenous bone graft with growth factors and cytokines that are contained in a concentration of platelets in a small volume of plasma called platelet-rich plasma (PRP). The radiographic maturation rate was 1.62 to 2.16 times faster than the group without the PRP. The histomorphometric analysis showed greater amount of trabecular density (75% ± 11%) in comparison with native posterior alveolar bone (38.9% ± 6%) and grafts without PRP (55.1% ± 8%).[47–49]

Platelet-rich plasma has been defined as the volume of autologous plasma that has a platelet concentration above baseline. The average normal platelet count is approximately 200 000/mL. Because the scientific proof of bone and soft tissue healing enhancement has been shown by using PRP with 1 000 000 platelets/mL, it is this concentration of platelets in 5-mL volume of plasma that is the working definition of PRP, according to Marx et al.[47]

This concentration of platelets is sequestered and concentrated from differential centrifugation of autologous whole blood. The whole blood is obtained from the patient before surgery and is then centrifuged with a gradient cell separator machine. This concentration of platelets is then mixed with thrombin and calcium chloride (CaCl). Platelets are activated in the presence of thrombin and release myriad factors that help in the development of the fibrin clot, the first step in the healing process.

The alpha granules of platelets contain various growth factors and cytokines, which display activities known to be important in the healing process. Biologically active proteins from platelets include platelet-derived growth factor (PDGF), transforming growth factor beta-1 and beta-2 (TGF-β1, TGF-β2), as well as other less well-described angiogenic and differentitive protein factors. The alpha granules of platelets also contain extracellular matrix components such as fibronectin, thrombospondin, and vitronectin.[47–50]

Marx also showed that autogenous graft cells are programmed to respond to the growth factors in platelets by monoclonal antibody staining of hip and tibia donor sites, identifying cells bearing membrane receptors for the PDGF and TGF-β series of growth factors. Monoclonal antibody labeling also documented that the graft specimens harvested at 2 weeks showed a marked increase in bone-forming cells, indicating, according to Marx et al and Marx, that graft cells do indeed respond to PDGF and TGF-β from platelet in vivo.[47,48]

Several techniques for processing PRP showing different platelet enrichment have been described in the literature.[47,51–54] However, the maximum benefit possible from platelet concentration and the relationship of cellular responses at various platelet concentrations is currently unknown.

Clinically, PRP has been used in several bone regeneration procedures with different bone-grafting materials. However, clinical findings have been described without histomorphometric or statistical analysis.[55–57]

GROWTH FACTORS AND THE REGULATION OF BONE FORMATION

Bone remodeling is a complex process involving a number of cellular functions directed toward the coordinated resorption and formation of new bone. Two mechanisms have been postulated for maintenance of bone volume: (1) systematic regulation by calcium- and phosphate-regulating hormones and (2) local regulation. Local mechanisms are believed to involve the actions of growth factors,[58] which are polypeptides-signal molecules that increase cell replication and have important effects on differentiated bone function.[59] Frequently, these local factors are synthetized by bone-forming cells or by cells of the immune or hematology system, and, as such, they are present in the bone microenvironment.[60] Therefore, growth factors have effects on cells of the same class (autocrine factors) or on cells of another class within the tissue (paracrine factors). Growth factors are present in the circulation and may act as systemic regulators of skeletal metabolism. Locally, their activity can be modulated by changes in synthesis, activation, receptor binding, and binding proteins.

Growth factors involved in the local regulation of remodeling have been classified according to their origin by Canalis et al[60] in 1988. Transforming growth factor beta, bone-derived growth factor, insulin-like growth factor–1 (IGF-1), and PDGF are all synthetized by skeletal cells. Acidic fibroblast growth factor and basic fibroblast growth factor (bFGF) had been isolated from bone matrix. Insulin-like growth factor and bFGF had been synthesized as well by cells of adjoining tissue-like cartilage. Interleukines-1, tumor necrosis factor alpha, macrophage-derived growth factors, and PDGF are synthesized in blood cells.

A full description of the growth factors involved in the regulation of bone remodeling is not under the scope of this work, but the growth factors that may be participants and have been

quantified in the use of PRP will be discussed.[47,54]

Platelet-derived growth factor

Platelet-derived growth factor is a homo- or heterodimeric disulphide-linked polypeptide composed of 2 subunits, A and B, and may exist in 3 different combinations: PDGF-AA, PDGF-BB, and PDGF-AB. The A and B chains share approximately 56% sequence homology.[61] The role of different isoforms of PDGF may be important, but this area is relatively unexplored.[62] Platelet-derived growth factor has been isolated from blood cells and considered a potent mitogen for all cells of mesenchymal origin, including osteoblast and fibroblast, thus making this polypeptide important in the early phases of wound healing such as bone repair.[63,64] Platelet-derived growth factor stimulates bone-cell replication, and, as a consequence of an increased number of cells, PDGF stimulates bone-collagen synthesis. However, PDGF does not stimulate the differentiated function of the osteoblast, and it acutely inhibits bone-matrix apposition rates. It also stimulates bone resorption by increasing the number of osteoclasts. The synthesis of locally produced PDGF is regulated by other growth factors such as TGF-β.[65–67]

In vivo, PDGF in concentrations of 20 to 100 ng has been demonstrated to increase ectopic bone formation and alkaline phosphatase activity with demineralized bone matrix as a carrier in rats.[68] When locally applied, PDGF can also stimulate bone healing in rabbit osteotomies[69] as well as in rabbit calvaria defects in combination with barrier membranes.[70] Platelet-derived growth factor has been found to be expressed by many cell types during different stages of normal fracture healing.[71]

Transforming growth factor beta

Transforming growth factor beta is synthesized by many tissues, but bone and platelets are the major source for this molecule.[72] It is a polypeptide that stimulates the replication of precursors cells of the osteoblast lineage, and it has a direct stimulatory effect on bone-collagen synthesis. Therefore, TGF-β modulates bone-matrix synthesis by various mechanisms, including increasing in the number of cells capable of expressing the osteoblast genotype as well as acting directly upon the differentiated osteoblast. It also decreases bone resorption by inducing apoptosis of osteoclast. The levels of TGF-β can be modified by a variety of hormones and other factors which will change its synthesis and its activity.

In vivo, TGF-β failed to induce ectopic bone formation.[75] In the rabbit calvaria onlay model, TGF-β1 in a demineralized bone-matrix carrier resulted in bone formation,[76] and a single application of recombinant human TGF-β1 in 3% methyl-cellulose gel carrier resulted in a dose-dependent bone formation in a 12-mm diameter rabbit calvarial defect.[77,78] In contrast, a single application of various doses of TGF-β1 was not sufficient to induce bone regeneration in primates.[79] Exogenous administration of TGF-β to experimental fractures resulted in increased size of the callus and some improvements in different biomechanical parameters.[80] Both TGF-β and PDGF have shown positive effects in new bone formation when demineralized bone matrix is used in vivo in small animals. However, little is known about the ability of PRP, which has increased level of these polypeptides, to enhance new bone formation when combined with allografts.

Purpose

The purpose of this split-mouth experimental study was to evaluate the ability of PRP to enhance bone regeneration in the sinus graft procedure when combined with demineralized freeze-dried bone allograft (DFDBA) plus cortical cancellous freeze-dried bone (CCFDB).

Materials and Methods

The experimental design is a modification of an established animal model used to evaluate maxillary sinus augmentation.[73,76,77] Animal selection, management, and surgical protocol followed a standard laboratory protocol approved by the Institutional Animal Care and Use Committee, Loma Linda University, Loma Linda, Calif.

Animal Selection and Management

Ten young male western sheep (approximately 20 to 36 months old) were obtained from a licensed farm (Nebeker Ranch Inc, Lancaster, Calif). Upon arrival, the sheep were examined, weighed, and thereafter observed daily. They weighed approximately 80 kg, tested negative against Q fever, and had no evidence of any disease.

Housing

The sheep were individually housed in 2.7- × 1.1-m runs with raised floors, allowing them to exercise freely. They were not removed from the cages during

the first 3 months except for experimentation and evaluation, thereby minimizing animal handling and risk of infection. After the first group (5 sheep) was sacrificed, the remaining 5 sheep were sent to a farm for 10.5 weeks and were returned 15 days before a second sacrifice period for bone labeling. Room temperature at the runs was maintained at 22°C ± 2°C. The light intensity was approximately 30 fc at the level of the sheep, and fluorescent lamps were controlled by a timer delivering a light-dark cycle of 12:12 hours.

Diet

The western sheep were fed a standard sheep diet consisting of alfalfa pellets (Miller Inc, Landcaster, Calif). The food ration (approximately 30 cm^3) was delivered each morning and adjusted on an individual basis, and the sheep were free to drink ad libitum.

Anesthesia

Surgical procedures were performed in a strict sterile environment with general anesthesia. After the sheep were brought to the surgical room, both sides of the neck were shaved to locate the carotid arteries. Once the carotid arteries were found, a dose of penthotal 5% (1 g) 20 mL was administrated for anesthesia induction. The sheep were then intubated, followed by inhalation of 2.5% halothane for general anesthesia maintenance.

Bone-graft processing

One adult pregnant western sheep and its fetus were selected to serve as donors for the mixture of demineralized freeze-dried bone and freeze-dried bone. The sheep served as experimental subjects for an unrelated research project conducted by the department of perinatology. The tissue harvesting was performed within 4 hours of sacrifice. The carcasses were refrigerated in a –4°C freezer from the time of sacrifice to time of tissue harvest. The long bones of each sheep were extracted by aseptic techniques, and all tissue fragments and periosteum were removed. The bones were stored within a –70°C freezer for approximately 12 hours, packed on dry ice, and shipped overnight to a commercial tissue-processing center for preparation (Osteotech Inc, Shrewsbury, NJ).

Specific formulation was requested to the commercial tissue bank consisting of demineralized bone fibers in a puttylike consistency in which nondemineralized cortical particles (500–1000 μm) were added. This technique is identical to routine allograft procurement for human use. Once the tissue bank received the bone, the shafts were treated to remove the lipid, blood, and cellular material and were stored at –70°C until use. To produce fibers, the shafts were milled to 1 to 60 mm in length and approximately 0.5 mm in width with a custom device. To yield the desired particles, remnant pieces of the shafts were freeze dried and pulverized, and the particles were sieved to a range of 500 to 1000 μm. The fibers were decalcified in 0.6% N HCl (to approximately less than 0.5% residual calcium), rinsed in sterile water, and soaked in sterile ethanol. Fibers then were mixed with glycerol to produce malleably putty. The demineralized fiber putty and cortical bone powder were mixed together in a 1:1 ratio and packaged in 3-mL vials.

Platelet-rich plasma

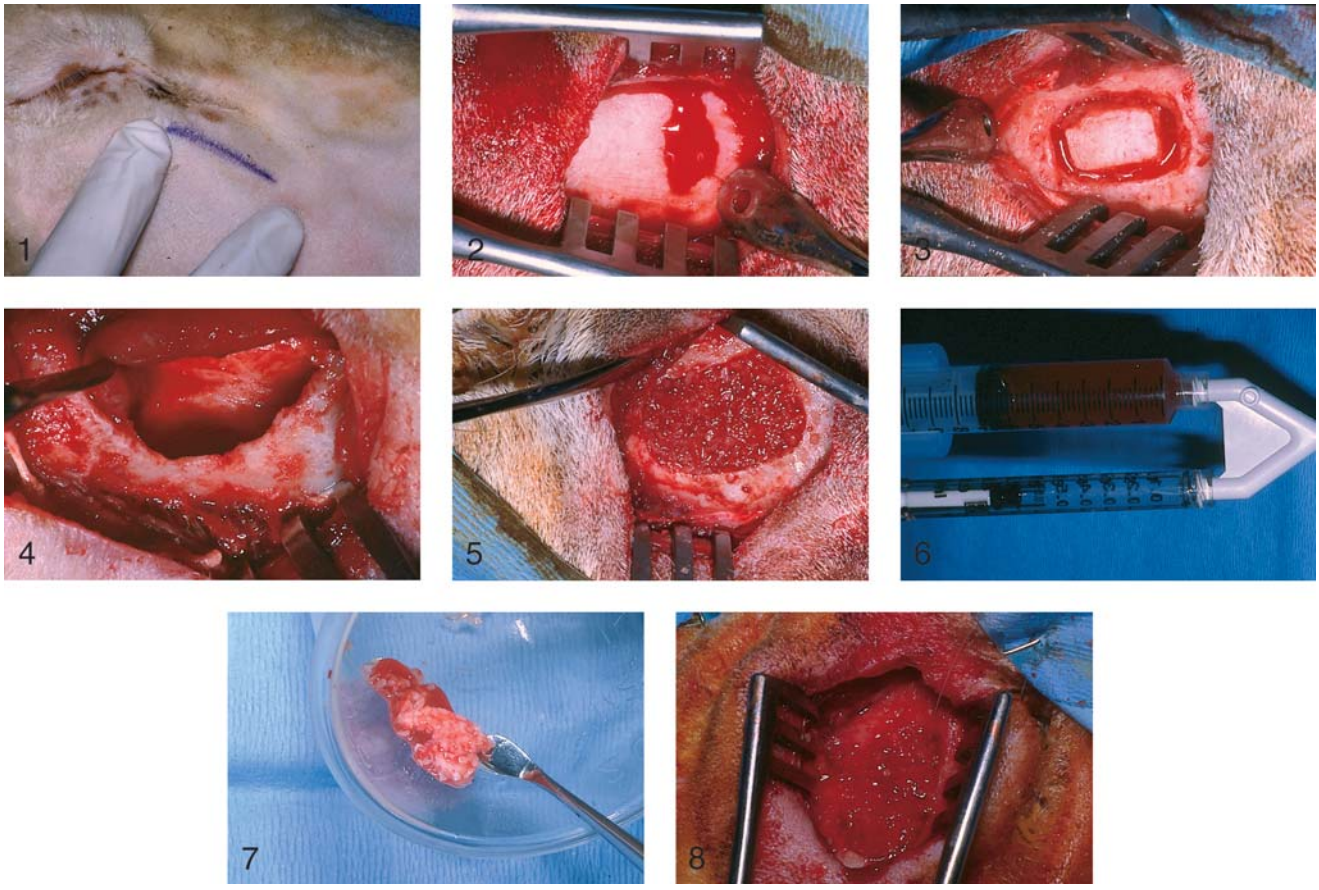
The PRP was processed with the Harvest Smart PreP Processing unit (Plymouth, Mass). Seven milliliters of citrate-anticoagulant (ACD-A) was drawn into 60-mL syringes. Then, 2 mL of the 7 mL of ACD-A was injected into the plasma chamber of the processing disposable. This left 5 mL of ACD-A within each of the 60-mL syringes for the whole-blood draw, which was incorporated within the whole-blood samples drawn from the sheep. Apheresis of the sheep's blood into the 60-mL syringes was performed immediately after the animal was under general anesthesia to ensure adequate blood collection and preparation of the PRP.

Once the blood was collected from the carotid artery and adequately mixed with ACD-A, the syringes were immediately transferred to the blood-chamber side of the processing cup. The filled cup was placed into the centrifuged bucket in a vertical position, and the PRP and platelet-poor plasma (PPP) were separated by a double-centrifugation technique. The cup was removed once the PRP fraction was separated, and a small amount of PPP was removed from the top of the chamber by using a blunt cannula with a sterile 10-mL syringe.

At the appropriate time, with the special double syringe, the PRP and the CaCl–bovine thrombin (Jones Pharma Inc, St Louis, Mo) were mixed for activation and incorporated within the DFDBA + CCFDB. The DFDBA + CCFDB was mixed with the PRP gel and the CaCl–bovine thrombin mixture, creating a homogenized mixture.

Hematology test

Approximately 2 mL of the whole blood, PRP, and PPP of each sheep was saved in a tube containing buffered Cit Na (9:1) and sterile ethylenediamine tetraacetic acid (K2) liquid, respectively,



FIGURES 1–8. FIGURE 1. Surgical site and the incision line located and prepared for surgery. FIGURE 2. The maxillary periosteum. FIGURE 3. The lateral wall of the sinus showing a rectangular surface antrostomy. FIGURE 4. Maxillary sinus membrane elevation. FIGURE 5. Maxillary sinus grafted with demineralized freeze-dried bone allograft (DFDBA) and cortical cancellous freeze-dried bone allograft (CCFDBA) (control). FIGURE 6. Double syringe containing platelet-rich plasma (PRP) and bovine thrombin–calcium chloride. FIGURE 7. Allograft + PRP. FIGURE 8. Maxillary sinus grafted with DFDBA + CCFDBA + PRP (test).

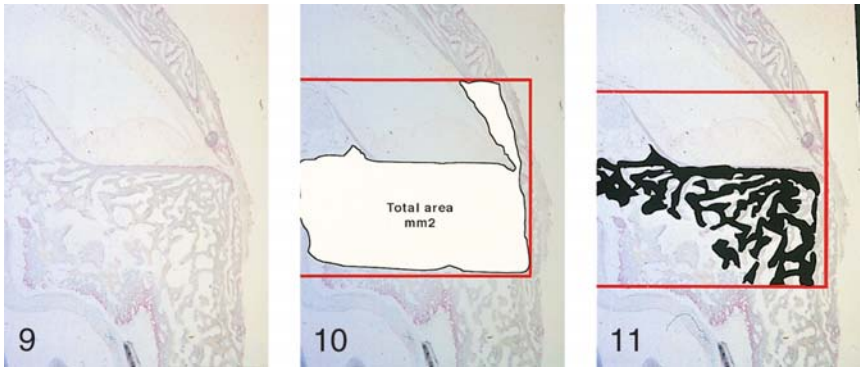
and sent within 6 hours to a commercial veterinary laboratory (ANTECH Diagnostics, Irvine, Calif) for a complete blood count and platelet count test.

Surgical protocol

The surgical field was prepared by shaving the skin to visualize main landmarks, namely, the angular vein of the eye and the transverse artery of the face. The sheep were prepped and draped in a customary manner for a sterile surgical procedure. The surgical site and the incision line were located and prepared with iodine (Figure 1). Approximately 3.6 mL of local anesthesia (Polocaine 2%, 1:20 000 levonordefrin; Astra Pharmaceuticals, Westborough, Mass) was administered in the surgical site. An oblique caudodorsal, rostroventral, extraoral incision approximately 5 cm in length was made over the most ventral aspect of the maxillary sinus with a #15 scalpel blade. Subcutaneous tissue and the masseter muscle were divided to expose the maxillary periosteum, which was incised and elevated dorsally (Figure 2). The lateral wall of the sinus was approached with a #6 surgical rotating tungsten bur to perform a rectangular surface antrostomy under abundant irrigation with saline solution (Figure 3). The antrum window was removed by fracturing along the osteotomy with a chisel instrument. The sinus lining and floor were meticulously evaluated to remove any remaining soft tissue (Figure 4). Maxillary sinus elevation was performed bilaterally in each sheep. One sinus was randomly selected to receive the experimental graft material, PRP + DFDBA + CCFDB, whereas the contralateral side received the DFDBA + CCFDB alone, serving as the positive control (Figures 5 through 8). An equal volume of graft material was used (3 cm^3) within each sinus cavity. Deep and superficial

fasciae of the masseter muscle were reapposed with 3–0 vicryl in a simple continuous pattern. Subcutaneous tissue and the skin were closed separately in a similar manner with 3-0 vicryl.

In 1 sheep, a broken tooth was detected at the test site with periapical infection communicating to the sinus cavity. The tooth was extracted and surgery of the test site was performed 1 month later. The sheep was sacrificed at 6 months, allowing 6 months of healing at the test site, and 7 months of healing at the control site.



FIGURES 9–11. FIGURE 9. Microphotograph of the specimen. FIGURE 10. Area of 16 mm^2 that was used for histomorphometric analysis. The area was located by using the neurovascular bundle and the external surface of the specimen as the fix internal and external reference points, respectively. FIGURE 11. Bone-containing region within the square area measured by planimetry and the threshold system to estimate the area of bone and the marrow space of the total traced area.

Bone labeling

Polyfluorochrome sequencial labeling was carried out to assess the time course of new bone formation and remodeling. The sheep received tetracycline hydrochloride (Sigma Chemical Co, St Louis, Mo) 15 mg/kg intravenously 3 weeks postoperatively. Tetracycline was made into a solution by adding 40 mL of sterile water. Seven weeks later and 2 weeks before sacrifice, 20 mg/kg of calcein green and Alizarin complexon (Sigma) were administrated intravenously. Both were made into a solution by adding 19.04 mL of $NaHCO_3$ (Abbot Laboratories, North Chicago, Ill) and 200 mL of sterile water.

HISTOLOGIC AND MORPHOMETRIC ANALYSIS

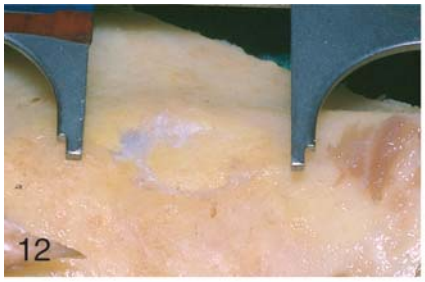
Five experimental sheep were sacrificed each time at 12- and 24-weeks postsurgery. Euthanasia was induced with intravenous overdose injection of concentrated sodium pentobarbital (100 mg/kg). Perfusion was performed by introducing 2 gal of sterile water and 2 gal of 10% formalin into the carotid artery. Hard and soft tissues of the maxillary sinuses were examined and photographed. Each maxilla was separated from the skull, and gross sectioning of the specimen was performed. The specimens were fixed in 10% buffered formalin for 15 days. Six coronal sections were cut within the confines of the original osteotomy, and 3 of them were randomly chosen for morphometric evaluation.

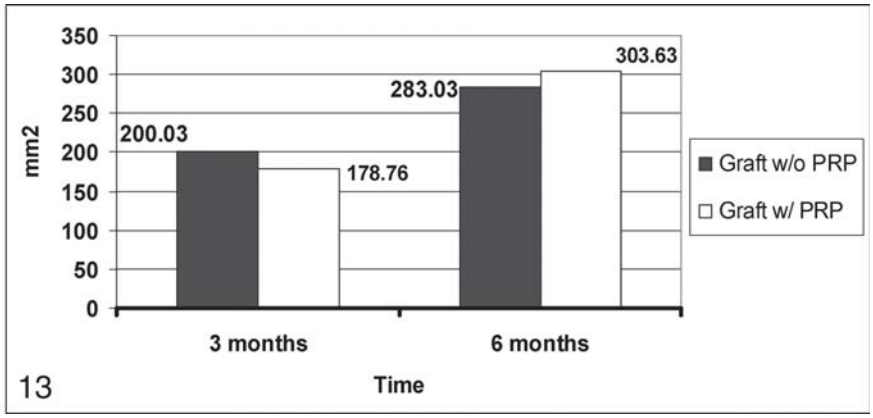
The specimens were dehydrated in different concentrations of alcohol (70%, 80%, 90%, and 100%) and then embedded in specialized ultraviolet-light–activated resin (GMA 2-hydroxyetilmetacrilate; Technovit 7200 VCL, Kulzer, Wehrheim, Germany). After polymerization, the preparation of the slides was performed via the cutting-grinding technique described by Donath and Breuner.[81] Briefly, the specimens were sectioned with a high-precision diamond saw at approximately 300 μm in thickness and were then ground down to approximately 120 μm in thickness with a lapping and polishing machine (Exakt Medical Instruments, Oklahoma City, Okla).

For the morphometric analysis of the 3 slides, a sampling protocol was chosen to provide a nonbiased analysis. The protocol used a macrolens video camera with low magnification. The video camera was used to take a photograph of the specimen, and a 16-mm^2 area was used for the morphometric analysis. The square area was located by using the neurovascular bundle and the external surface of the specimen as the fixed internal and external reference points, respectively. The top of the neurovascular bundle was used as the highest point of the square and the external surface of the specimen was used as the anterior limit of the square. The bone-containing region within the square area was measured by planimetry, and the threshold system from the ImagePro Plus (v1.3; Media Cybernetics Inc, Silver Spring, MD) was used to estimate the area of bone and the marrow space of the total traced area. The area of bone of the 3 slides of each specimen was added and computed as a mean percentage of the total traced area of the 3 slides (Figures 9 through 11).

DYNAMIC HISTOMORPHOMETRIC ANALYSIS

For the dynamic histomorphometric analysis, the slides indicating the area of interest were marked and sent to SkeleTech





FIGURES 12 AND 13. FIGURE 12. Clinical picture of the sinus wall after 3 months. FIGURE 13. Mean total tissue traced area of demineralized freeze-dried bone allograft (DFDBA) and cortical cancellous freeze-dried bone allograft (CCFDBA) vs DFDBA + CCFDBA + platelet-rich plasma.

Inc (Seattle, Wash). The slides were mounted on a microscope and viewed on a monitor with a ×1 objective (model of scope: Nikon Eclipse E400 with Sony DXC-950 color camera). Three parameters were measured by using the OsteoMeasure software V. 4.00c (OsteoMetrics Inc, Atlanta, Ga): total tissue area, total bone area, and total fluorochrome label area (tetracycline given at 3 weeks after surgery, and calcein green given 10 weeks after surgery). No measurements were done for the alizarin complexon because it was not very distinctive on the specimens. All these parameters were calculated according to the American Society for Bone and Mineral Research histomorphometry nomenclature committee[82] and represented as a percentage according to the following formulae: (1) % of the new bone area = total bone area ÷ total tissue area × 100, (2) % of tetracycline area = total tetracycline area ÷ total bone area × 100, (3) % of calcein green area = total calcein area ÷ total bone area × 100.

STATISTICAL ANALYSIS

To test any interaction between treatments and sacrifice time, Student *t* tests were performed to determine if the mean differences of PRP + DFDBA + CCFDBA vs DFDBA + CCFDB was significantly different for the 2 sacrifice times.

The main effect of treatment was evaluated by paired Student *t* tests, and associations between DFDBA + CCFDBA + PRP and DFDBA + CCFDB treatments were evaluated by Pearson correlation coefficients.

To ensure that an increase in blood platelet count was observed, a 1-sample Student *t* test was performed to confirm that the mean log ratio was significantly greater than 0 ($P < .015$).

Pearson correlation coefficients were used to determine any correlation between PRP platelet count and sinus cavity measures. No statistical analysis was done for the dynamic histomorphometry.

RESULTS

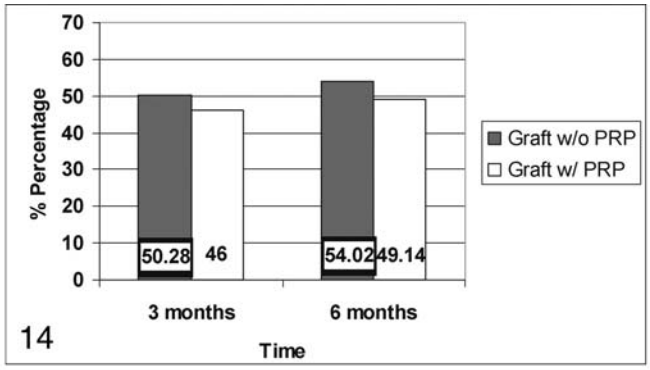
Clinical analysis

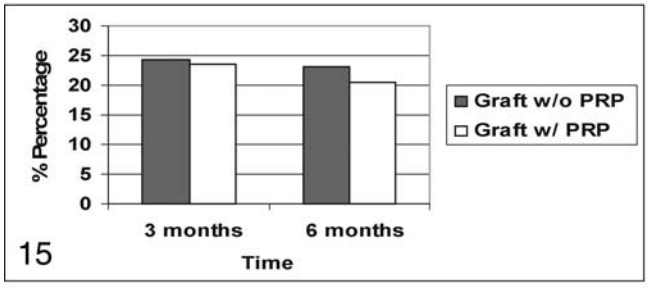
Healing was uneventful in all sheep. The test site showed slightly more inflammation than in the control group during the first week after surgery, and no difference in skin healing was observed during the healing interval.

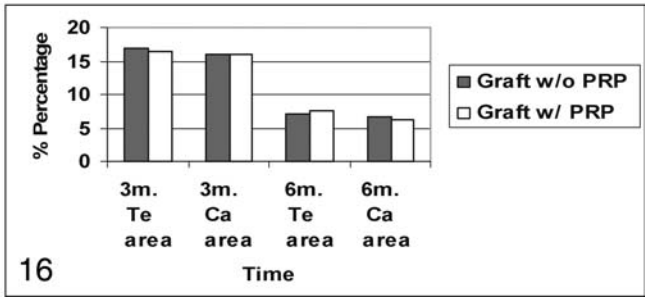
The sinus wall in all groups presented a centripetal bone healing, and most of the time the wall was regenerated from the coronal margin to the apical margin of the defect. The healing of the sinus wall ranged from complete to partial regeneration and was independent of the treatment. However, most of the osteotomies presented 60% at 3 months and 90% at 6 months of sinus wall regeneration, leaving just a thin layer of connective tissue between the margins (Figure 12).

Morphometric analysis

Mean total tissue area within the square was 200.03 mm^2 (SD ± 54.82) at 3 months and 283.03 mm^2 (SD ± 81.11) at 6 months for the control group and 176.78 mm^2 (SD ± 20.65) at 3 months and 303.63 mm^2 (SD ± 98.09) at 6 months for the experimental group. Mean total tissue area within the square for both sacrifice times was 241.53 mm^2 (SD ± 78.57) for the control group and 240.21 mm^2 (SD ± 94.53) for the experimental group (Figure 13). Mean percentage of trabecular bone fill of the total tissue area was 50.28% (SD ± 7.06) at 3 months and 54.02% (SD ± 14.36) at 6 months for the control group







FIGURES 14–16. FIGURE 14. Mean percentage of trabecular bone fill demineralized freeze-dried bone allograft (DFDBA) and cortical cancellous freeze-dried bone allograft (CCFDBA) vs DFDBA + CCFDBA + platelet-rich plasma. FIGURE 15. Dynamic histomorphometry measurements for total tissue area and total bone area. FIGURE 16. Dynamic histomorphometry measurements for the total label area (tetracycline and calcein green).

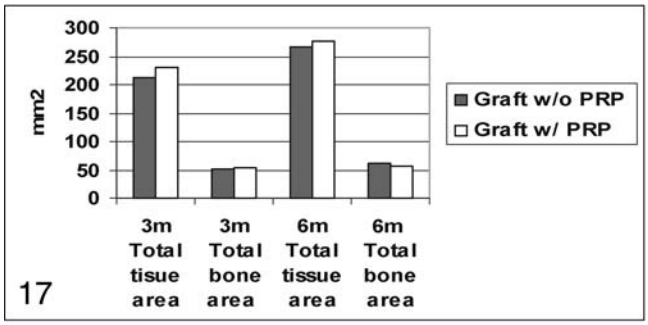
and 46% (SD ± 3.71) at 3 months and 49.14% (SD ± 7.65) at 6 months for the experimental group. Mean percentage of trabecular bone fill of the total tissue area of both sacrifice times was 52.15% (SD ± 10.85) for the control group and 47.57% (SD ± 5.90) for the experimental group (Figure 14).

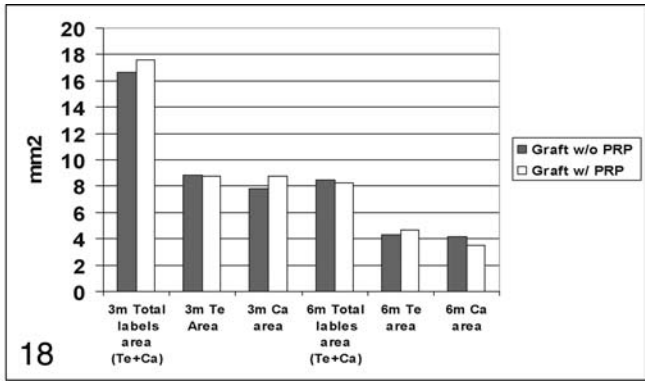
Dynamic histomorphometric analysis

Mean total tissue area was 213.70 mm^2 (SD ± 32.59) at 3 months and 267.52 mm^2 (SD ± 36.45) at 6 months for the control group and 230.25 mm^2 (SD ± 41.32) at 3 months and 276.54 mm^2 (SD ± 36.34) at 6 months for the experimental group. Mean of total bone area was 51.07 mm^2 (SD ± 12.5) at 3 months and 61.27 mm^2 (SD ± 14.08) at 6 months for the control group and 54.35 mm^2 (SD ± 3.76) at 3 months and 56.5 mm^2 (SD ± 22.81) at 6 months for the experimental group (Figure 15). Mean tetracycline area was 8.80 mm^2 (SD ± 3.82) at 3 months and 4.29 mm^2 (SD ± 1.73) at 6 months for the control group and 8.79 mm^2 (SD ± 3.64) at 3 months and 4.68 mm^2 (SD ± 3.72) at 6 months for the experimental group. Mean calcein green area was 7.84 mm^2 (SD ± 2.10) at 3 months and 4.19 mm^2 (SD ± 2.10) at 6 months for the control group and 8.77 mm^2 (SD ± 5.15) at 3 months and 3.54 mm^2 (SD ± 2.14) at 6 months for the experimental group. Mean total label area (tetracycline + calcein green) was 16.64 mm^2 (SD ± 4.51) at 3 months and 8.48 mm^2 (SD ± 3.17) at 6 months for the control group and 17.56 mm^2 (SD ± 8.38) at 3 months and 8.22 mm^2 (SD ± 5.66) at 6 months for the experimental group (Figure 16). Percentage of total bone area was 24.29% (SD ± 6.80) at 3 months and 23.02% (SD ± 5.09) at 6 months for the control group and 23.58% (SD ± 8.04) at 3 months and 20.42% (SD ± 7.42) at 6 months for the experimental group (Figure 17). Percentage of tetracycline was 17% (SD ± 4.87) at 3 months and 7.06% (SD ± 2.58) at 6 months for the control group and 16.43% (SD ± 4.98) at 3 months and 7.56% (SD ± 4.03) at 6 months for the experimental group. Percentage of calcein green was 16.01% (SD ± 4.98) at 3 months and 6.06% (SD ± 2.53) at 6 months for the control group and 16.10% (SD ± 5.41) at 3 months and 6.24% (SD ± 2.75) at 6 months for the experimental group (Figure 18).

Statistical analysis

To test any interaction between treatment and sacrifice time, Student *t* tests were performed to determine if the mean differences of experimental vs control





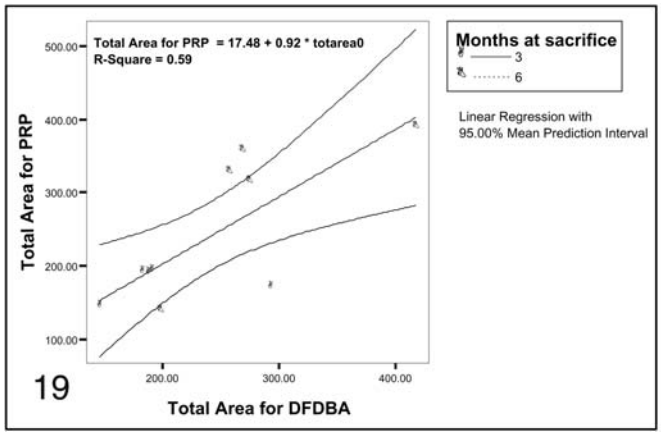
FIGURES 17 AND 18. FIGURE 17. Dynamic histomorphometry measurements for the percentage of trabecular bone within the total tissue area. FIGURE 18. Percentage of tetracycline and calcein green area at 3 and 6 months for both groups.

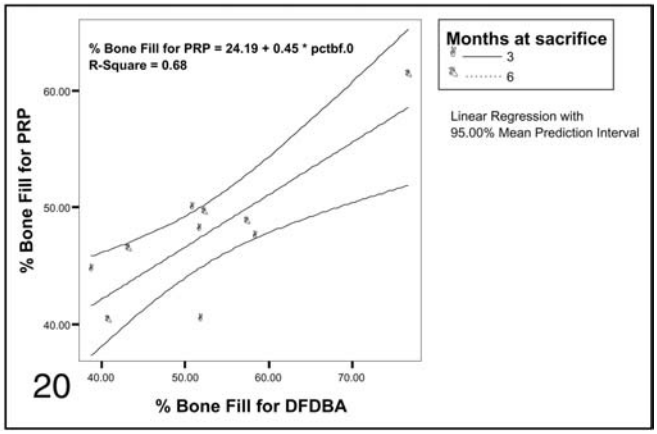
group were significantly different for the 2 sacrifice times. Both of the mean PRP + DFDBA + CCFDBA and DFDBA + CCFDB differences of total tissue area ($P > .25$) and percentage of trabecular bone fill ($P > .80$) were not significantly different for sacrifice at 3 weeks vs 6 weeks. Because no interactions were observed for the model, the main effects of sacrifice time and treatment were evaluated by unpaired Student t tests, and associations between experimental and control treatments were evaluated by Pearson correlation coefficients. Mean total area of bone within the square for control treated sinuses was marginally greater ($P = .095$) for the sacrifice time of 6 weeks vs 3 weeks, but mean percentage of bone fill for control treated sinuses was not significantly different ($P > .60$) for sacrifice times. Mean total tissue area for experimental treated sinuses was significantly greater ($P < .025$) for the sacrifice time of 6 weeks vs 3 weeks, but mean percentage of bone fill for experimental treated sinuses was not significantly different ($P > .40$) for sacrifice times. Mean total tissue area was not significantly different ($P > .90$) for treatments, but mean percentage of bone fill was marginally greater ($P = .064$) for control vs experimental groups. Experimental and control treatments had significant Pearson correlation coefficients for total area ($r = .766$, $P < .01$) and percentage of bone fill ($r = .824$, $P < .005$) (Figures 19 and 20).

Hematology test

Complete blood count showed normal ranges in all the sheep, indicating that the animals were not experiencing any hematology disorder at the time of the surgical procedure. Mean platelet count baseline was $261.5 \times 1000/\mu L$ (SD $118.7 \times 1000/\mu L$), mean platelet count for PRP was $588.7 \times 1000/\mu L$ (SD $358 \times 1000/\mu L$), and mean platelet count for PPP was $62 \times 1000/\mu L$ (SD $48 \times 1000/\mu L$) (Table 1).

Blood platelet count before and after centrifugal stimulation was also measured. The hypoth-





FIGURES 19 AND 20. FIGURE 19. Correlation graph for total area. FIGURE 20. Correlation graph for percentage of trabecular bone fill.

esis to be tested was whether the increase in blood platelets (expressed as a ratio) was correlated with total area or percentage of bone fill. A 1-sample Student *t* test was performed to ensure that an increase in blood platelet count was observed, and it confirmed that the mean log ratio was significantly greater than 0 ($P < .015$). After applying the exponential function to the mean and 95% confidence limits of the log ratios, the mean ratio was determined to be 2.035 (95% CI 1.234, 3.354). However, no significant correlations ($P > .15$) were observed between the log ratio and the sinus cavity measures.

TABLE 1

Platelet count (baseline, platelet-rich plasma [PRP], and platelet-poor [PPP])

Sheep No. (Time of Sacrifice)	Baseline (×1000 μL)	PRP (×1000 μL)	PPP (×1000 μL)
348 (3 mo)	400	760	155
458 (3 mo)	70	335	35
364 (3 mo)	171	286	20
459 (3 mo)	194	472	102
386 (3 mo)	381	889	91
445 (6 mo)	169	804	55
409 (6 mo)	371	234	18
481 (6 mo)	346	225	25
389 (6 mo)	440	1300	104
343 (6 mo)	271	662	15
Mean	261 (SD 118.7)	588.7 (SD 358)	62 (SD 48)

DISCUSSION

A variety of bone grafts have been used in craniofacial surgery. In reconstructive surgery, osteoinductive agents (cytokines and growth factors) are frequently required either to repair continuity or discontinuity defects or for bone augmentation. Human studies have evaluated the efficacy of PRP as a bone-graft enhancer when used in combination with autogenous bone in continues mandibular defects.[47] However, little is known about the effect of PRP when used with other type of bone grafts and as a bone enhancer in the maxillary sinus procedures.

The present study did not show statistical difference in percentage of bone fill between DFDBA + CCFDBA and PRP + DFDBA + CCFDBA at 3- and 6-month sacrifice times in the maxillary sinus of the sheep. This study also failed to demonstrate that PRP in combination with allograft accelerates the bone healing, for no significant statistical difference was seen between both sacrifice times.

This study disagrees with some in vitro and in vivo studies that have shown an increase in osteoblast activity and bone formation in the animal model when mineralized and demineralized bone matrix were used as a carrier for TGF-β1, PDGF, and IGF-1. Results by Mott et al[83] demonstrated that DFDBA used as a carrier and supplemented with various combinations of growth factors such as IGF-1, PDGF, and TGF-β1 enhance murine osteoblast activity and proliferation at 7 days in vitro.

Howes et al[68] studied the effect of supplemental growth factors on bone formation induced by subcutaneous implantation of demineralized bone matrix in rats. The authors suggest that bone induction under some conditions is submaximal and can be increased by local supplement of PDGF because they observed an increase of alkaline phosphatase activity, calcium content, and production of messenger ribonucleic acid for collagen II content. Kiebblewhite et al,[76] using various animal models, demonstrated better bone formation when demineralized bone matrix was supplemented with TGF-β1 in a critical size defect of the rabbit calvaria. Other studies by Nash et al[69] and Vikjaer et al,[70] which investigated the effect of PDGF-BB on critical size defects in rabbit calvaria and long bone fractures, have shown that administration of single doses of this growth factor stimulates bone formation in rabbits.

Supplementation of TGF-β or PDGF has shown positive effects in new bone formation when demineralized bone matrix is used as a carrier in vitro and in vivo in small animals. These previous results may lead to the hypothesis that the use of PRP, which has an increased level of these polypeptides, may have the ability to enhance new bone formation when combined with allografts. However, when PRP has been used in combination with nonliving graft materials, the data are not as promising. Our study is in agreement with Wironen et al,[84] who failed to demonstrate the osteoinductivity capacity of PRP when combined with ground mineralized bone matrix plus demineralized bone-matrix gelatin powder.

Other studies and case reports using different bone-grafting materials other than allografts have demonstrated the same unfortunate results. Furst et al[85] examined the value of autogenous rich plasma and xenogenic hydroxiapatite as grafting materials for the maxillary sinus. The results indicate that PRP and xeno-

TABLE 2

Increment in platelet counts and most significant results in each animal*

Sheep No. (Time of Sacrifice)	% Increment	Morphometry: % Total Bone		Dynamic Histomorphometry: % Total Bone		Dynamic Histomorphometry: Total Label Area (mm2) (TE + CA)	
		Control	PRP	Control	PRP	Control	PRP
348 (3 mo)	190	38	44	20.6	23.5	13.0	12.0
364 (3 mo)	167	51	40	29.9	19.5	22.3	14.0
459 (3 mo)	243	50	49	20.6	24.9	16.2	24.3
458 (3 mo)	478	51	48	29.8	24.4	17.1	14.8
386 (3 mo)	233	58	47	20.3	25.3	14.3	22.4
343 (6 m)	244	46	46	24.1	23.2	7.7	12.5
389 (6 m)	295	57	48	20.8	26.2	10.8	12.3
445 (6 m)	475	52	48	18.1	12.1	6.7	3.1
481 (6 m)	0	43	40	25.5	17.5	7.7	4.5
409 (6 m)	0	76	71	26	24	7.2	8.9

*PRP indicates platelet-rich plasma, TE indicates tetracycline, CA indicates calcien green.

genic hydroxyapatite for sinus augmentation in minipigs was not superior to xenogenic hydroxyapatite alone during the 12-week observation period with respect to new bone formation and bone-implant contact. Neukam[86] also failed to demonstrate in a critical size defect in pigs any difference in osteoinductive capacity of PRP when it was used with allograft and nonliving materials, but difference was found when autogenous bone graft was used. Terheyden[87] compared rhOP-1 and PRP in bilateral sinus grafts that used 100% inorganic bovine bone as a grafting material. The PRP was not effective in producing bone regeneration, whereas in the contralateral sinus the rhOP-1 was effective. Froum et al[88] tested the efficacy of PRP in 3 bilateral sinus graft cases with grafts of inorganic bovine bone that contained minimal or no autogenous bone. Histomorphometric analysis indicated that the addition of PRP to the graft did not make a significant difference either in vital bone production or in interfacial bone contact of the test implants. Shanaman et al[57] showed a series of clinical cases in which PRP was used to enhance bone regeneration in alveolar ridge defects. Three defects were treated: 1 defect received DFDBA alone and the other 2 received a mixture of DFDBA and autogenous bone. Horizontal and vertical gains were observed clinically. The histologic evaluation revealed the presence of residual allograft particles surrounded by connective tissue as well as newly formed bone within the grafted areas. Yet according to the authors, the addition of PRP did not appear to enhance the quantity or quality of new bone formation over that reported in comparable guided bone regeneration. However, the results of the present study are in contrast with the results by Okasaki et al[89] in which more volume fraction of bone was observed in the maxillary sinus of 5 rabbits when augmented with PRP plus beta-tricalcium phosphate vs beta-tricalcium phosphate alone.

The literature indicates that PRP may be effective only when used in combination with autogenous bone graft. However, the combination of PRP and autogenous bone has also failed to be osteoinductive in some small animal models. Using a critical size defect in 15 rabbits, Aghallo et al[90] did not show a significant increase in bone formation with the addition of PRP to cortical membranous autogenous bone histomorphometrically or radiographically. In addition, the PRP group alone had a tendency to have less new bone formation. In a histologic study, Galliani and Arrascue[91] found a better pattern of regeneration in a critical size defect of a rabbit when PRP and autogenous bone was used as a bone-grafting material, but no significant difference was found in bone healing.

Quantitative platelet counts verified that the PRP used in this study consisted of an increased number of platelets in the range of 0.7 to 5 times in relation to the whole-blood baseline platelet count. Two PRP samples of 2 different sheep demonstrated a decreased platelet count than the whole-blood baseline. These results may be because the PRP samples of these 2 sheep were not measured by the commercial veterinary laboratory the first time the samples were sent but were evaluated 3 days later because of communication problems with the laboratory. The commercial veterinary laboratory was asked to measure the platelet count manually, but different technicians received the samples, and the measurement could have been made by laser point. A laser-point counter is common and faster than the manual technique in commercial laboratories, but it cannot count platelets that are aggregated and therefore gives less quantification. The results of this study suggest no correlation between the increased numbers of blood platelets (expressed as a ratio) and the percentage of trabecular bone fill (Table 2).

According to the literature, the amount of growth factors available to tissues is directly propor-

tional to the concentration of platelets.[49,92] Landsberg et al[54] found 137 ng of TGF-β1 and 123 ng of PDGF-AB in 3 mL of PRP gel preparation by using 2 different methods for gelling agents (ITA vs 5000 units of bovine thrombin/10% CaCl). Wong et al[92] found that the level of TGF-β1 in the PRP preparation with bovine thrombin and CaCl was approximately twice as that of the control group in human serum. Weibrich et al[93] compared the growth factor levels in PRP produced by 2 different contrifugation machines: the Curasan-type PRP kit vs the Platelet Concentrate Collection System (PCCS) 3i PRP system (Implant Innovations, Palm Beach, Fla). The amount of TGF-β1 was approximately 467.1 ng/mL for the PCCS method and 79 ng/mL for the Curasan method. The amount of PDGF-AB was 251 ng/mL for the PCCS method and 314 ng/mL for the Curasan method, and the amount of IGF-1 was 91.0 ng/mL for the PCCS method and 60.5 ng/mL for the Curasan method.

There is lack of information in the literature regarding which will be the biological significance of the different proportions of growth factors within the PRP preparations in humans and large animals. Using baboon insoluble collagenous bone matrix as a carrier with a different concentration of human TGF-β1 (5,30,100 μg), Ripamonti et al[79] showed no therapeutic implications for the healing of large cranial wounds in primates independent of the dosage of human TGF-β1. One limitation of the present study was that, for economic reasons, we were not able to measure the amount of growth factors in the samples, which is an issue that needs to be taken in consideration for subsequent PRP research protocols.

Because of the difficulty to maintain therapeutic concentrations in the bone defect, growth factors are frequently incorporated into biologically inert materials to reduce the rate of clearance. Time of growth factor activity needs to be elucidated. Some authors have postulated that release of growth factor occurs within 3 to 5 days after degranulation and that the time of growth factor activity may end in 7 to 10 days. Lynch et al[94] demonstrated that the short-term exposure of periodontal tissues to a combination of human recombinant PDGF-B and IGF-1 can stimulate a cascade of wound healing. In this study, Lynch et al found that less than 4% of the growth factors were present for more than 96 hours after application, and none could be detected after 2 weeks. The combination of growth factors in this study resulted in increased bone metabolism for 4 weeks after application and increased bone and periodontal regeneration at 2 and 5 weeks. Lynch et al[94] concluded that their findings might suggest that these growth factors stimulate a cascade of wound-healing events that continue even in the absence of the growth factors.

Recently, a heterogeneous in vitro study by Rose et al[95] failed to establish a relationship between the platelet concentration and the cytokines release. Most cytokines release occurred within the first 2 hours and continued to a lesser degree up to 50 hours postactivation. This study by Rose et al also showed no significant difference between rat bone marrow cells (RBMCs) proliferation in the presence or absence of PRP and no evidence of bone nodule growth production within the clots at 18 days cultured; however, RBMCs effectively migrate in the PRP clots.

The disappointing results combining DFDBA + CCFDBA + PRP in the present study may be because of the low number of platelets in the PRP preparation if we assume that the statement by Marx is correct: ''The more growth factors that can be delivered to the injury site, the greater the potential to enhance the healing process.'' However, this statement needs to be elucidated. Another possible explanation of the disappointing results may be the hypothesis suggested by Marx[49] that PRP might not produce the desired stimulatory effect with allograft because vital bone cells are not present for this stimulation to occur. To support this hypothesis, and on the basis of what is known about the pattern of bone formation in the maxillary sinus originated from the bony walls (proposed by Boyne and James[6]), we want to postulate the hypothesis that when the allograft is placed in the maxillary sinus and is impregnated with PRP, bovine thrombin and CaCl activate the PRP to form the coagulum, which may restrict the flow of the vascular content inside the graft and decrease the number of bone cells. This question has been partially answered by the results of Rose et al[95] in which PRP attracted the bone cells via the chemotactic proteins, but no bone nodules were inside the clot by day 18.

CONCLUSIONS

Within the limitations of this study, the addition of PRP to allograft fails to demonstrate more bone fill in the maxillary sinus of sheep. No correlation is between the number of platelets within the PRP and the percentage of bone fill when PRP is added to an allograft mixture in the sheep maxillary sinus.

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