Influence of Platelet-Rich Plasma Added to Xenogeneic Bone Grafts in Periimplant Defects: A Vital Fluorescence Study in Dogs

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ABSTRACT

Background: The use of platelet-rich plasma (PRP) has been suggested in order to increase the rate of bone deposition when sites are augmented prior to or in conjunction with dental implant placement.

Purpose: The goal of this study was to investigate whether the addition of PRP to xenogeneic bone grafts would increase the rate of bone formation in dogs.

Materials and Methods: Ninety endosseous dental implants were inserted in the mandibles of nine hound dogs. Subsequently, mesial and distal three-wall periimplant defects were surgically created. Defects were randomly assigned to three groups: demineralized freeze-dried bone graft plus platelet-rich plasma (DFDBG plus PRP), demineralized freeze-dried bone graft alone (DFDBG), and no treatment. Postsurgically each dog received a series of three fluorescent labels for estimation of bone cell activity at baseline and during different stages of healing, with particular attention to the bone formation rate per tissue volume (BFR/TV). Animals were sacrificed at 1 month, 2 months, and 3 months, and specimens were subjected to analysis by fluorescence microscopy. Treatment effects were evaluated with analysis of variance models.

Results: Overall, the average BFR/TV differed by treatment although this difference approached only minimal statistical significance ($p = .057$). The largest difference occurred between periimplant defects treated with DFDBG only and defects that were not treated (mean percentage BFR/TV, 0.0720% vs 0.0994%). There was no evidence of an overall treatment effect ($p = .27$) for the mineral apposition rate (MAR) values. The data also suggest a consistent variability in the bone formation parameters among the three groups at different healing points.

Conclusion: In this animal model the addition of PRP to xenogeneic bone grafts did not demonstrate evidence of faster bone formation during healing. However, limitations of the histologic technique possibly played a negative role in the assessment of bone formation parameters.

KEY WORDS: animal study, dental implants, growth factors, platelet-rich plasma, vital bone labeling

In implant dentistry numerous techniques have been studied for promoting and accelerating the osseous healing of dental implants and bone grafts by increasing the bone regenerative potential. These techniques include the application of platelet-rich plasma (PRP),[1–3] bone morphogenetic protein,[4–7] and growth factors.[8–11]

The addition of PRP to bone grafts has been suggested to accelerate the rate and degree of bone formation.[1,3,12] However, the experimental design of these studies has been criticized, and more studies are therefore required.[13,14]

Vital bone labeling with fluorochromes is a well-established technique for monitoring the new bone formation.[15–25] Fluorochromes are calcium-binding substances that are preferentially taken up at the site of active mineralization of bone (known as the calcification front),[26–28] thus labeling sites of new bone formation.

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They are detected by using fluorescence microscopy on an undecalcified section.[26] The rate and extent of bone deposition and resorption can be determined with double or triple fluorochrome labeling sequences.[29,30] Therefore the sequential use of fluorochromes of clearly contrasting colors permits a more detailed record of events relating to calcification.[27]

To the best of our knowledge, no studies have used vital fluorescence analysis to evaluate the rate of bone formation when PRP is used as an enhancement growth factor in bone regenerative therapy. The purpose of this study was to investigate whether the addition of PRP to xenogeneic bone grafts would increase the rate of bone formation in dogs.

MATERIALS AND METHODS

Study Design and Surgical Procedures

This study used nine fully mature (older than 2 years) adult male hound dogs, divided into three groups. The groups differed in study duration (1 month, 2 months, and 3 months). Nine sites on each side of the mandible were studied, namely, the mesial and distal aspect of the five implants per side placed in the premolar/molar region. To maintain the balance in the treatments, the distal aspect of the most anterior implant was not studied. Each dog within a group was studied on a separate treatment randomization schedule. The defect sites of adjacent mesial and distal aspects of adjacent implants were assigned the same treatment in order to avoid contamination or carryover effects. The Institutional Animal Care and Use Committee at the Mayo Clinic, Rochester, reviewed and approved this study.

Surgical extraction of the mandibular right and left premolars and molar teeth was accomplished at the beginning of the study by using a previously described technique.[31] All surgical procedures were performed with the dogs under general anesthesia.

After 2 months of the healing phase, a second surgical intervention was performed under the same operating conditions that were described for the dental extractions. Thirty minutes before surgery the PRP was procured with a commercially available platelet concentrator centrifuge system (SmartPrep PCCS, Harvest Technologies Corporation, Plymouth, MA, USA). The technique used for obtaining the PRP has been previously described.[14]

Subsequently a midcrestal incision was made, and both buccal and lingual full-thickness flaps were reflected to expose the alveolar bone. After implant osteotomies were done, a three-wall bony defect was created surgically in the mesial and distal aspects of the osteotomy sites with the use of a surgical-fissure bur in a high-speed air-driven handpiece, with sterile saline irrigation. The size of the defect was standardized to 2 mm in a mesiodistal direction, 3 mm in a buccolingual direction, and 5 mm in a corono-apical direction. All these measurements were accomplished by using a standard periodontal probe (PCP UNC15, Hu-Friedy Mfg. Co., Chicago, IL, USA). After that, endosseous titanium implants (Brånemark System®, Nobel Biocare, Westmond, IL, USA) were placed in the mandible, in accordance with a standard implant insertion technique. The prepared defects received one of the three treatment modalities according to the randomization schedule. Each defect received (1) demineralized freeze-dried bone graft plus platelet-rich plasma (DFDBG plus PRP), (2) DFDBG alone, or (3) no treatment. No membrane barriers were placed. The bone grafts were reconstituted with sterile saline a few minutes before application. The PRP was activated to achieve coagulation and release of growth factors with the use of a special double syringe (LK/2 Applicator, Harvest Technologies Corporation, Plymouth, MA, USA) that equally mixed the PRP and 1 mL of 10% calcium chloride per 5,000 units of topical bovine thrombin (Thrombin-JMI®, Jones Medical Industries, Inc., Saint Louis, MO, USA) while these solutions were applied to the xenograft.

After PRP application, the coagulated grafting material was transferred to the defect sites. The surgical flap was repositioned and closed with 4–0 polyglactin 910 suture (Vicryl®, Ethicon, Inc., Johnson & Johnson Co., Sommerville, NJ, USA). Postsurgical care for these animals was also described previously.[31]

Postsurgical Administration of Fluorochromes

Postsurgically each dog received a series of three fluorescent labels for estimation of bone cell activity at baseline and during the different stages of healing, with particular attention to the bone formation rate. Each label was administered in a way similar to the intervals used by other studies,[11,24] in which each group of animals had different administration schemes (Table 1).

Two fluorochrome labels were used: calcein green (Sigma Chemical Co., St. Louis, MO, USA) at a dose of 8 mg/kg intravenously (IV) and tetracycline

TABLE 1 Vital Fluorescence Analysis: Fluorochrome Administration Schemes

Group	1st Dose	2nd Dose	3rd Dose	Healing Time (mo)
1	Day 0, CG	Day 15, TNC	Day 23, CG	1
2	Day 0, CG	Day 30, TNC	Day 45, CG	2
3	Day 0, CG	Day 60, TNC	Day 75, CG	3

CG = calcein green; TNC = tetracycline hydrochloride.

hydrochloride (TNC) (Sigma Chemical Co.) at a dose of 30 mg/kg IV.

Specimen Preparation

At 1 month, 2 months, and 3 months after osseous regenerative therapy, three animals, respectively, were euthanized with an overdose of sodium pentobarbital (Sleepaway, Fort Dodge Laboratories, Dodge, IA, USA). Following the sacrifice, the mandibles were resected with a band saw (Horbart, Horbart Co., Troy, OH, USA) and fixed in 10% formol for 24 to 48 hours.

Treatment sites (dental implant and surrounding bone) were subsequently retrieved and sectioned in a buccolingual (coronal) direction with the use of a small hand hacksaw. Then the individual specimens were placed in labeled containers containing 70% ethanol and sent to the hard-tissue laboratory for histologic analysis slide preparation according to the cutting-grinding technique.[32] The region of interest was divided into several blocks parallel to the long axis of the implants. These blocks were prepared with ascending grades of alcohol and were embedded in light-curing resin (Technovit® 7200 VL + BPO, Heraeus Kulzer, Wehrheim, Germany). With these blocks, different cuts of 200 μm thickness were made with EXAKT® cutting and grinding equipment (EXACT Apparatebau, Norderstedt, Germany; EXAKT Medical Instruments, Oklahoma City, OK, USA). The cutting-grinding technique was performed until a thickness of 30 to 50 μm was obtained.

Vital Fluorescence Analysis

Bone formation rates were assessed by fluorescence in unstained sections viewed with an ultraviolet microscope (Nikon Eclipse E400™, Nikon Corporation, Japan). The distance between the fluorescent bands was measured where the vital labels were seen by using the OsteoMeasure™ analysis system (OsteoMetrics, Inc., Atlanta, GA, USA). The system consists of a personal computer coupled with a photomicroscope and image analysis. There is a high-resolution color video camera (Sony DXC-970 MD, Sony Corporation, Ichinomiya, Japan) that records the specimen through the microscope and displays the image on a sonic video monitor that registers the movement of a digitizing pen on a graphics tablet (OsteoTablet™, OsteoMetrics). The average mineral apposition rate (MAR), expressed as micrometers per day, and the bone formation rate per tissue volume (BFR/TV), expressed as percent per day, were determined by measuring each of the foci of stains found within two fields ($2 \times 1{,}750\ \mu m^2$) along the five most coronal threads at the mesial and distal sites of each implant. The BFR/TV referent was calculated according to the following formula:

$$\%BFR/TV = MAR \times [dLPm/(TAr - VdAr)]$$

where *dLPm* means double label perimeter, *TAr* is total area, and *VdAr* is void area.

Statistical Analysis

The fluorescence microscopic measurements were taken from either the buccal or the lingual site of the periimplant defects, depending on which slide showed the more distinct fluorochrome deposition. Analysis of variance (ANOVA) models were fit to assess treatment differences in the bone formation rates and the MARs per day, respectively. Each ANOVA model included effect terms for duration of healing or sacrifice time period (1 month, 2 months, or 3 months), animal within healing time, side of jaw within animal (left or right), treatment (DFDBG plus PRP, DFDBG, and no treatment), and the treatment by healing time interaction. Contrast statements were used to evaluate differences in the presence of a statistically significant treatment by healing time interaction effect. If the interaction effect was not significant, contrast statements were also used to evaluate treatment differences in the

presence of either a significant overall treatment effect or to evaluate healing period differences in the presence of a significant overall healing period effect. The ANOVA models assume that the outcome measurements follow a gaussian (normal) distribution; natural logarithmic transformations were applied to both measurements to satisfy this assumption. All calculated *p*-values were two-sided, and *p*-values of less than .05 were considered statistically significant. No adjustments were made for multiple comparisons. Statistical analyses were performed with the SAS® software package (SAS Institute Inc., Cary, NC, USA).

RESULTS

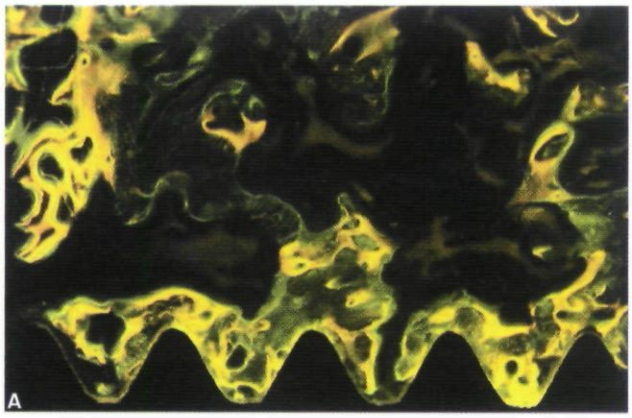
Descriptive Fluorescence Microscopy

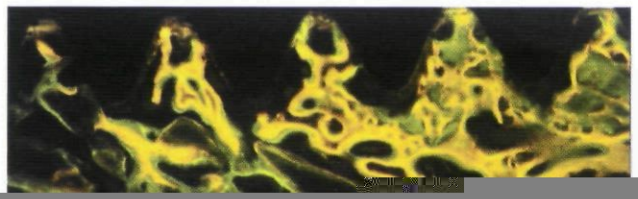
There was distinct incorporation of calcein green and tetracycline hydrochloride, except for the first dose of calcein green, which was inconsistently incorporated into the bone and was therefore eliminated from the analysis.

Specimens at 1 Month of Healing. For all groups, fluorescence microscopy showed diffuse tetracycline- and calcein-stained bone after 4 weeks, reflecting woven bone formation (Figure 1A–C). This bone was characterized by an accelerated bone deposition with thick trabeculae. Both experimental and control groups demonstrated various degrees of mineralized tissue surrounding DFDBG particles. In general, experimental sites showed more bone deposition in the grafted areas. There was important evidence of tetracycline-labeled bone next to the implant surfaces (days 15–23) (see Figures 1A and B).

Specimens at 2 Months of Healing. During this period woven bone was being replaced by lamellar bone, and MARs decreased. There was a clear distinction between tetracycline- and calcein-stained bone (Figure 2A and C). In general the DFDBG group showed limited bone deposition in the grafted areas (see Figure 2B).

Specimens at 3 Months of Healing. These specimens clearly showed more tetracycline-labeled bone (days 60–75) than calcein-labeled bone (days 75–90) (Figure 3A–C). There was also more evidence of bone mineralization next to graft particles.





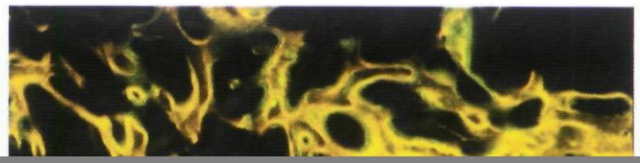


Figure 1 Fluorescence microscopy images of different treatments at 1 month of healing (×5 original magnification; unstained sections). *A*, specimen treated with demineralized freeze-dried bone graft plus platelet-rich plasma. *B*, Specimen treated with demineralized freeze-dried bone graft. *C*, Specimen with no treatment.

TABLE 2 Vital Fluorescence Analysis, by Healing Time and Treatment*

	Treatment		
Period of Healing	DFDBG + PRP	DFDBG	No Treatment
Month 1			
BFR (%/d)	0.0657 (0.0533)	0.0617 (0.0361)	0.0496 (0.0256)
MAR (μm/d)	2.333 (0.4265)	2.4094 (1.1536)	2.3291 (0.5293)
Month 2			
BFR (%/d)	0.1031 (0.1731)	0.0636 (0.1010)	0.1195 (0.1126)
MAR (μm/d)	1.8729 (2.0929)	1.3369 (0.5494)	1.8248 (0.9449)
Month 3			
BFR (%/d)	0.1060 (0.0982)	0.0911 (0.0704)	0.1274 (0.0789)
MAR (μm/d)	2.7043 (1.0405)	2.9784 (1.1871)	2.9463 (0.9253)

BFR = bone formation rate; DFDBG = demineralized freeze-dried bone graft; MAR = mineral apposition rate; PRP = platelet-rich plasma.
*Data shown are mean values (standard deviations are in parentheses).

Bone Formation Parameters

Bone formation rates and MARs per day as assessed by fluorescence are summarized by healing time and treatment in Table 2. There was no evidence that the effect of the three treatments on bone formation rates differed by healing time ($p = .12$ for the healing time by treatment interaction). There was evidence that average bone formation rates differed by treatment across all 3 months although this difference only approached statistical significance ($p = .057$). However, the largest difference occurred between periimplant defects treated with DFDBG only and defects that were not treated (mean bone formation rates per day of 0.0720 vs 0.0994) (Table 3). The average bone formation rates were significantly different depending on the duration of healing ($p < .001$). In particular, defects allowed to heal for 3 months had a significantly higher average bone formation rate compared to defects allowed to heal for either 1 month (0.1088 vs 0.0590; $p < .001$) or for 2 months (0.1088 vs 0.0962; $p = .002$).

There was no evidence that the effect of the three treatments on MAR per day differed by healing time ($p = .55$ for the month by treatment interaction). In addition, there was no overall treatment effect for MARs ($p = .27$) (see Table 3). However, the average MAR per day was significantly different, depending on the duration of healing ($p < .001$). In particular, defects allowed to heal for 3 months had a significantly higher average compared to defects allowed to heal for either 1 month (2.88 vs 2.36; $p = .032$) or for 2 months (2.88 vs 1.69; $p < .001$); also, the average MAR per day was significantly different between defects allowed to heal for 1 month and those allowed to heal for 2 months ($p < .001$). In addition a great amount of variability among the different treatments was observed during this study.

RESULTS AND DISCUSSION

In the present study the influence of PRP on MAR and bone formation rate (BFR) in a dog model was

TABLE 3 Vital Fluorescence Analysis by Grouped Healing Times

	Mean (SD)		
Overall Healing Time	DFDBG + PRP	DFDBG	No Treatment
Bone Formation Rate (%/day)	0.0918 (0.1194)	0.0720 (0.0733)	0.0994 (0.0866)
Mineral Apposition Rate (μm/day)	2.2956 (1.4061)	2.2448 (1.2017)	2.3773 (0.9332)

DFDBG = demineralized freeze-dried bone graft; PRP = platelet-rich plasma; SD = standard deviation.

investigated. Clinical healing was eventful, and all dental implants used in the study showed evidence of osseointegration, both clinically and microscopically.

The results of this investigation showed no statistically significant differences in the regenerative response to PRP-treated defects in terms of the MAR and BFR parameters. However, the histologic appearance of PRP plus DFDBG sites only showed evidence of increased bone formation at early healing (1 month) when compared to the control groups, but this observation was not supported by the quantitative analysis. These findings are in agreement with those of a recent study in rats, wherein the authors concluded that the qualitative analysis of fluorochrome labels suggested that PRP and recombinant bone morphogenetic protein-7 accelerate bone growth.[33] The results of the quantitative analysis showed a great amount of variability, evidenced by large standard deviations and denoting technical difficulties when bone formation parameters are measured by immunofluorescence microscopy and specimens prepared with the cutting-grinding technique.[32] As previously mentioned, this histologic technique is one of the most commonly used techniques when healing parameters in implant dentistry are studied, and it requires a minimum thickness of 30 to 50 μm, which could be the source of these limitations. This variability could also be associated with other factors. For example the immunofluorescence analysis was limited to the periimplant defect area where de novo bone formation was observed. This new bone formation showed an irregular pattern of deposition that made the identification of sites with active mineralization more difficult.

The rationale of the use of PRP as a bone regeneration–enhancing agent lies in the possibility of concentrating the growth factors contained in platelets and applying them to the healing site. During the early bone healing process, growth factors play an important function, regulating cellular processes such as mitogenesis, chemotaxis, differentiation, and metabolism.[2] Growth factors work together with one another, forming a cascade of different signal proteins with multiple pathways, ultimately leading to the activation of gene expression and then protein production. This initial release of growth factors is thought to accentuate the reparative response.[14] The results of our study are consistent with those of previous studies on the application of single or combination growth factors (platelet-derived growth factor [PDGF] or PDGF combined with insulin-like growth factor I) that also showed an enhanced early healing response both in vitro[34,35] and in vivo.[8,36] It is also theorized that once the healing cascade is amplified by the growth factors contained in PRP, the regeneration process is able to maintain a high proliferative and metabolic level through the synthesis of the same growth factors by osteoblasts and macrophages involved in the process.[10,34,37] This theory has not been confirmed by our study, perhaps because after a period of only a few hours of activity, the initial advantageous influence of growth factors stored in PRP decrease markedly. Recently Weibrich and colleagues,[38] in a rabbit study, suggested that the platelet concentration required for a positive PRP effect on bone regeneration seems to span a very limited range. Furthermore, the authors stated that advantageous biologic effects seem to occur when PRP with a platelet concentration of approximately 1,000,000/μL is used. It seems that at lower concentrations, the effect is suboptimal whereas higher concentrations might have an inhibitory effect. Of importance, a limitation of our study is that we did not calculate platelet concentrations prior to adding the PRP solution to the graft. However, the platelet concentration reported by the manufacturer of the platelet cell separator system used in our study is similar to the optimal concentration recommended by Weibrich and colleagues.

CONCLUSION

This animal study found that the addition of PRP to xenogeneic bone grafts did not promote faster bone formation during healing. However, the limitations of the histologic technique used possibly played a negative role in the assessment of bone formation parameters. More basic science research on PRP in implant dentistry, perhaps using a different model, is needed before we can recommend the clinical application of this treatment modality.

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