Evaluation of platelet-rich plasma in combination with freeze-dried bone in the rabbit cranium

A pilot study

Key words: animal study, bone grafting, cranial defects, freeze-dried demineralized bone, freeze-dried mineralized bone, histomorphometry, platelet-rich plasma

Abstract: Platelet-rich plasma (PRP) offers a new and potentially useful adjunct to allograft materials in oral and maxillofacial bone and implant reconstructive surgery. This study compares bone healing in four cranial defects in the rabbit grafted with freeze-dried mineralized bone (FMB) alone, FMB + PRP, freeze-dried demineralized bone (FDDB) alone, and FDDB + PRP. Fifteen New Zealand white rabbits were included in this randomized, blind, prospective pilot study. Four equal 8 mm diameter defects were created in each rabbit cranium and immediately grafted with the above materials. Five rabbits were evaluated at 1, 2, and 4 months. Radiographically, FMB + PRP showed a tendency toward increased bone density over FMB alone, but was not statistically significant (P > 0.05), and FDDB + PRP showed a tendency toward increased bone density over FDDB alone, but was not statistically significant (P > 0.05). Histomorphometrically, FMB + PRP showed a tendency toward increased bone area over FMB alone at 1 and 4 months, but was not statistically significant (P > 0.05), and FDDB + PRP showed a tendency toward increased bone area over FDDB alone, at 1 and 2 months, but was not statistically significant (P > 0.05). This study failed to show a radiographic or histomorphometric increase in bone formation with the addition of PRP to either FMB or FDDB in non-critical-sized defects in the rabbit cranium.

Freeze-dried bone is a well-documented bone-grafting material, utilized for oral bone grafting in periodontal bony defects, extraction sockets, maxillary sinus grafts, and around dental implants [Hurt 1968; Lane et al. 1972; Mellonig et al. 1976; Sanders et al. 1983; Mellonig & Tripplett 1993; Rominger & Tripplett 1994; Valentini & Abensur 1997; Groeneveld et al. 1999a, 1999b; Tal 1999; van den Bergh et al. 2000; Haas et al. 2001; Karabuda et al. 2001]. It has also been successfully used to regenerate bone in rabbit critical-sized skull defects [Shermak et al. 2000; Clokie et al. 2002]. Freeze-dried bone is isolated from cadavers, sterilized, lyophilized, or freeze-dried, and stored in tissue banks [Burchardt et al. 1978; Mellonig 1999]. Freeze-dried bone can be mineralized or demineralized. The demineralization process, in removing the mineral phase, exposes the collagen and growth factors, including bone morphogenetic proteins (BMPs) [Mellonig 1999], and may activate them [Schwartz et al. 1996]. Freeze-dried bone, especially the demineralized type, may stimulate bone formation through osteoinduction [Urist 1965; Yeo- mans & Urist 1967; Urist et al. 1968; Urist & Dowel 1970; Reddi & Huggins 1972] or osteoconduction [Piatelli et al. 1996; Groeneveld et al. 1999]. However, human clinical trials fail to show osteoinductive properties [Pinholt et al. 1992, 1994; Becker et al. 1994, 1996; Paul et al. 2001], and
osteoc conductive properties are also questioned (Pinholt et al. 1992; Schwartz et al. 1996; Block & Kent 1997). Some early studies show fibrous connective tissue surrounding freeze-dried demineralized bone (FDDB) particles and no new bone formation (Wetzel et al. 1995), and other studies show incorporation of FDDB particles with new bone and healthy osteocytes (Brugnami et al. 1996). FDDB has even been compared to autogenous bone and was found to be similar in union of graft and host bone, mechanical strength, porosity, and new bone formation (Burchardt et al. 1978; Quintero et al. 1982; Mellonig 1984; Haas et al. 2001). When freeze-dried bone was compared to other non-autogenous grafting materials, such as hydroxyapatite and deproteinized bovine bone granules, it resorbed faster in sinus grafts and was able to successfully support functioning implants (Karabuda et al. 2001). Other studies show that FDDB is comparable to deproteinized bovine bone material in preserving alveolar ridge height after extractions, and may be able to support underlying tissue vascularization (Tal 1999). And still other studies show promising results for FDDB for periodontal therapy (Sepe et al. 1978; Mellonig 1984; Gher et al. 1994).

Comparisons of freeze-dried mineralized bone (FMB) and FDDB exist, and again, varied results have been shown. Since FMB is mineralized, it may calcify faster than FDDB. Sinus lifts where FMB was utilized resulted in harder bony substance when compared to FDDB, which resulted in cartilage formation after 6 months (Meffert 1998). FMB has been well studied in adult periodontitis, showing 50% bone fill in more patients when it was added to autogenous bone, especially in furcation defects (Mellonig 1991). FMB also regenerated new bone, cementum, and periodontal ligament in adult male baboons when compared to control (Mellonig 1994). FMB may be more effective for fenestrations, minor ridge augmentation (Piatelli et al. 1996), fresh extraction sockets, and sinus lifts (Meffert 1998), but FDDB is more widely used (Mellonig 1999). However, more studies exist with the use of FDDB as a grafting material both alone and in combination with autogenous bone (Boeck-Neto et al. 2002).

The use of platelet-rich plasma (PRP) offers a potentially useful adjunct to auto-genous, allograft, and xenograft materials in oral and maxillofacial bone and implant reconstructive surgery. Some authors suggest that the addition of PRP to osteoc conductive grafting materials can potentiate osteoinduction (Kim et al. 2002a, 2002b). Platelets are very important in the wound healing process. They arrive quickly to the wound site and begin the coagulation process. They release multiple wound-healing growth factors and cytokines, including platelet-derived growth factor (PDGF), transforming growth factors beta 1 and 2 (TGF-β1 and TGF-β2), vascular endothelial growth factor (VEGF), platelet-derived endothelial cell growth factor (PDEGF), interleukin-1 (IL-1), basic fibroblast growth factor (bFGF), and platelet activating factor-4 (PAF-4) (Linder et al. 1979; Jones et al. 1992; Harrison & Cramer 1993; Miyadera et al. 1995; Mohle et al. 1997). These growth factors are thought to contribute to bone regeneration and increased vascularity, vital features of a healing bone graft. Questions exist whether PRP can be utilized with allografts, xenografts, or allograft materials without the incorporation of autogenous donor bone to create a bone graft, which is comparable or superior to autogenous bone.

There are very few studies where PRP was added to allograft or allograft bone (Kassolis et al. 2000; Kim et al. 2001, 2002a, 2002b; Shanaman et al. 2001; Froum et al. 2002; Rodrigue et al. 2003; Wiltfang et al. 2003; Wojtowicz et al. 2003). In many of these studies, few cases were evaluated and limited statistical testing was performed to confirm the validity of the results. Specifically with allografts, few scientific conclusions were reached.

Since both FMB and FDDB show such varied results in previous studies, this study aimed to determine whether FMB or FDDB either alone or in combination with PRP would result in bone regeneration in rabbit cranial defects. Further scientific testing of PRP in combination with allograft materials such as FDDB and FMB is obviously necessary. The present study was designed to test the effectiveness of PRP when added to an allograft.

Material and methods

Animal surgical procedure

Fifteen New Zealand white male rabbits between 2.8 and 4 kg were included in this randomized, blind, prospective pilot study. The UCLA Animal Research Committee and in accordance with staff in the UCLA Department of Laboratory and Animal Medicine approved all animal protocols. Each rabbit was anesthetized with ketamine (10 mg/kg) and acarpromazine (1–1.5 mg/kg), and given preoperative antibiotics (enrofloxacin 5 mg/kg, Bayer, Shawnee Mission, KS, USA). Ten milliliters of autologous blood was drawn from each rabbit to prepare the PRP. The rabbits underwent general anesthesia with 1–2% isoflurane with standard monitoring. The fur was shaved over the cranium and prepped and draped in a sterile fashion. An incision was made to the bony cranium and the periosteum was reflected. Four 8 mm diameter defects were created with a trephine bur with copious irrigation (Fig. 1). The four defects were randomly grafted with FMB (LifeNet Transplant Services, Virginia Beach, VA, USA) alone, FMB mixed with PRP, FDDB (LifeNet Transplant Services) alone, and FDDB mixed with PRP. The wound was closed with 3-0 Dexon (Owens and Minar, Irvine, CA, USA), first closing the dura mater to contain the grafting materials and prevent overflow of the different grafting materials, and 3-0 Dexon in a subcuticular fashion.

![Graft sites prepared](Image)

Fig. 1. Surgical sites prepared with 8 mm trephine burr.
The rabbits recovered from anesthesia without complications. They were given postoperative narcotic pain medication and antibiotics.

**PRP preparation**

The 10 ml of autologous blood drawn from each rabbit was combined with 1.1 cm$^3$ of anticoagulant citrate dextrose phosphate (ACD-A) to prevent coagulation. The blood was centrifuged at 1500 rpm (215 g) for 10 min to separate the plasma containing the platelets from the red cells [Sorvall, Irvine, CA, USA]. The plasma was drawn off the top, mixed with 0.4 cm$^3$ of ACD-A anticoagulant, and centrifuged for an additional 10 min at 3000 rpm (863 g) to separate the platelets. The platelet-poor plasma (PPP) was separated from the PRP along with the buffy coat. The buffy coat and PRP, approximately 1–1.5 cm$^3$, were resuspended and used within minutes to add to the grafting materials. Topical bovine thrombin powder 5000 U [Jones, St Louis, MO, USA] were reconstituted with 5 cm$^3$ of 10% calcium chloride [American Regent Laboratories, Shirley, NY, USA]. The ratio of PRP to calcium chloride activator was 10:1. This protocol is similar to those utilized in clinical practice with some of the commercially available machines and the original scientific article [Marx et al. 1998]. Platelet counts were performed on each sample, including a peripheral blood count, PPP count, and PRP count. A Unopette microcollection system (Becton Dickinson, Franklin Lakes, NJ, USA) was used to lyse the leukocytes and erythrocytes as well as provide a standard dilution of 1:100 for platelet counts. The platelets were hand counted with a standard hemocytometer, and the total was calculated for each sample.

**Sample evaluation**

Rabbits were sacrificed with Pentobarbital (Western Medical Supply Inc., Arcadia, CA, USA) 100 mg/kg IV at time periods 1, 2, and 4 months. There were five rabbits in each group. The entire cranium was removed with a reciprocating saw, without encroaching upon the grafted areas.

Radiographs were taken of the rabbit cranium in its entirety before histologic sections were performed. A calcium carbonate step wedge was used in each radio-
particles, which decrease over time and are replaced by woven bone. When PRP is added, bone formation is slightly increased. In all groups, most of the bony ingrowth is from the edges of native bone and from the minimal osteoconductive activity that occurs throughout the 4-month study period. By 4 months, many of the freeze-dried bone particles exist, and minimal bone formation is taking place by osteoconduction by this time. There was no difference in the method of bony ingrowth between the grafting materials, whether PRP was added or not.

Histomorphometric evaluation
Figure 6 shows the histomorphometric percent bone area as a function of time for each grafting material. When histologic sections were evaluated with histomorphometry, all grafting materials showed an increase in bone area over the study period (1–4 months). FDDB + PRP showed a tendency toward increased bone area over FDDB at 1 and 2 months, but this was not statistically significant ($P > 0.05$). FMB + PRP showed a tendency toward increased bone area over FMB at 1 and 4 months, but was not statistically significant ($P > 0.05$).

Discussion
In implant dentistry, surgeons strive to improve upon current bone-grafting techniques and provide a faster and denser bony regenerate. In addition, alternative grafting materials are continuously studied to avoid autogenous donor site morbidity [Kalk et al. 1996]. Often extensive grafting material is required and only iliac crest bone gives adequate volume. This donor site has many potential complications including chronic pain, sensory loss, hematoma, seroma, wound breakdown, contour defect, hernia through the donor site, gait disturbance, instability of the sacroiliac joints, pathologic fracture, adynamic ileus, and ureteral injury [Kalk et al. 1996]. An ideal bone-grafting material should be able to produce bone by osteogenesis, osteoinduction, or osteoconduction, remodel the initial graft material to mature lamellar bone, maintain bone volume in function over time, have a low risk of infection, ease of availability, low antigenicity, and high
reliability (Block & Kent 1997). Freeze-dried bone is an allograft material widely used in localized ridge augmentation, periodontal bony defects, and grafting of small defects around implants, which may possess these characteristics of an ideal graft material. This material may possess osteoinductive, or more likely, osteoconductive properties (Pinholt et al. 1992; Groeneveld et al. 1999b). The most effective use for freeze-dried bone thus far, has been in regeneration of periodontal defects where 78% of defects responded with greater than 50% or complete bone repair (Melloni 1984), and potential for use in implant site development has shown some promising results [Haas et al. 2001]. Other studies have shown negative results for the use of FDDB, including lack of bone formation and results showing less bone regeneration than control sites when non-resorbable membranes are used (Becker et al. 1995). Since studies regarding freeze-dried bone are conflicting and it is difficult to conclude if it can predictably form bone as a solo grafting material, perhaps adding a mixture of growth factors can aid in increasing the graft vascularity and ultimately, its success. It has been shown that growth factors are a plausible way to improve and expedite bony wound healing, and may support osteoinduction of osteoconductive materials [Kim et al. 2002a, 2002b].

Platelets contain angiogenic, mitogenic, and vascular growth factors in their granules (Banks et al. 1998; Maloney et al. 1998). VEGF is a powerful angiogenic growth factor, with an important role in wound healing (Thomas 1996). TGF-β1 and TGF-β2 have been shown to inhibit bone resorption, osteoclast formation, and osteoclast activity, as well as trigger rapid maturation of collagen in early wounds [Bonesew & Mundy 1990; Steenfos 1994]. PDGF increases the population of wound-healing cells, and recruits other angiogenic growth factors to the wound site [Steenfos 1994]. It is therefore a reasonable hypothesis that increasing the concentration of platelets in a bone defect may lead to improved and faster healing. However, little evidence exists evaluating the effect of these growth factors to improve bone healing when added to osteoconductive materials [Kim et al. 2001, 2002a, 2002b].

Quantitative platelet counts verified that PRP was used in this study, consisting of 800,000–1,465,000 platelets in the concentrate [Marx et al. 1998]. Digital subtraction radiography with step-wedge calibration showed that all grafting materials increased in bone density over the study period [1–4 months]. FDDB + PRP showed a radiographic tendency toward increased bone density over FDDB alone at 1 and 2 months, but was not statistically significant (P > 0.05). FMB + PRP also showed a radiographic tendency toward increased bone density over FMB alone at 1 and 2 months, but was not statistically significant (P > 0.05).

When histologic sections were evaluated with histomorphometry, all grafting materials showed an increase in bone area over the study period [1–4 months]. FDDB + PRP showed a tendency toward increased bone area over FDDB at 1 and 2 months, but this was not statistically significant (P > 0.05). FMB + PRP showed a tendency toward increased bone area over FMB at 1 and 4 months, but was not statistically significant (P > 0.05). These data are not in agreement with Kim et al., who showed a significant histomorphometric increase in bone–implant contact in the dog iliac crest when PRP was added to FDDB simultaneously with placement of implants [Kim et al. 2002a]. Preliminary human case series’ have shown that FDDB in combination with PRP forms numerous areas of osteoid and bone without inflammatory infiltrate or soft-tissue epithelialization, and osseous trabeculae surround connective tissue (Kassolis et al. 2000; Shanaman et al. 2001). However, in these studies, no quantitative analysis was performed and the grafted sites were not standardized or randomized.

This study failed to show a significant increase in bone formation with the addition of PRP to FMB or FDDB radiologically or histomorphometrically in non-critical-sized defects in the rabbit cranium.

The sample size was small, consisting of only five rabbits at each time period, which may have contributed to the results seen. A true critical-sized cranial defect in the rabbit model is 15 mm [Vikjaer et al. 1997]. Therefore, four critical-sized defects cannot be created in the rabbit cranium due to the small size of the cranium. We chose a non-critical-sized defect to evaluate the early healing, and the potential ability of PRP to improve this early healing when it was added to freeze-dried bone-grafting materials. Further studies are needed to evaluate the potential benefits of PRP in combination with various autogenous, allograft, and alloplast grafting materials.

Conclusion

This study evaluated grafting materials in rabbit cranial defects, and did not show a significant improvement with the addition of PRP to FMB or FDDB at 1-, 2-, and 4-month time periods.

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Résumé

Le plasma riche en plaquettes (PRP) offre un apport nouveau et potentiellement utile aux allogreffes dans la chirurgie reconstructrice implantaire et osseuse buccale et maxillofaciale. Cette étude compare la guérison osseuse dans quatre lesions cranienes chez le lapin greffé avec de l’os mineralise congele sec (FMB) seul, de l’os demineralise congele et sec (FDDB) seul FMB + PRP, et FDB + PRP. Quinze lapsins blancs de nouvelle-Zelande ont été inclus dans cette etude pilote prospective, aveugle et randomisee. Quatre lesions d’un diametre de 8 mm ont ete creees dans chaque cran de lapins et immediatement greffees avec les materiaux mentionnes ci-dessus. Cinq lapsins ont ete evaluees a un, deux et quatre mois. Radiographiquement FMB + PRP montre une tendance d’accroissement de la densite osseuse plus importante que FMB, mais cette difference etait non significative (P>0,05), FDDB + PRP accusait une tendance d’augmentation de la densite osseuse plus importante que FDDB mais également non-significative (P>0,05). Histomorphometricment, FMB + PRP montrait une tendance (P>0,05) a un accroissement de l’aire osseuse plus importante que FMB a un et quatre mois, FDDB + PRP montrait une tendance a un accroissement de l’aire osseuse plus importante que FDDB mais a un non-significative (P>0,05). Cette etude n’a donc pas demontré d’augmentation radiographique ou histomorphometric dans la formation ossee lorsque le PRP etait ajouté soit au FMB soit au FDDB dans des lesions de grandeur non-critique dans le cran du lapin.

Zusammenfassung

Die Evaluation von plättchenreicherem Plasma in Kombination mit gefriergetrocknetem Knochen am Kaninchenschädel: Eine Pilotstudie
El plasma rico en plaquetas (PRP) ofrece un nuevo y potencialmente útil acceso para materiales de aloinjerto en cirugía reconstructiva oral y maxilofacial de hueso e implantes. Este estudio comparó la cicatrización ósea en cuatro defectos craneales en el conejo injertado con hueso mineralizado crío-deseado (FMB) solo, hueso crío-deseado desmineralizado con PRP (FFDB) solo, y hueso crío-deseado desmineralizado con PRP y FMB. Se incluyeron quince conejos blancos de Nueva Zelanda en este estudio piloto aleatorio, ciego, prospectivo. Se crearon cuatro defectos iguales de 8 mm de diámetro en el cráneo de cada conejo y se injertaron inmediatamente con los materiales antes citados. Cinco conejos se evaluaron a uno, dos y cuatro meses. Radiográficamente, FMB + PRP mostró una tendencia hacia una área mayor de hueso sobre FMB solo, pero no fue estadísticamente significativa ($P > 0.05$), y FFDB + PRP mostró una tendencia hacia una mayor densidad ósea sobre FFDB solo, pero no fue estadísticamente significativa ($P > 0.05$).

Histomorfometricamente, FMB + PRP mostraron una tendencia hacia una área mayor de hueso sobre FMB solo a 1 y 4 meses, pero no fue estadísticamente significativa ($P > 0.05$), y FFDB + PRP mostraron una tendencia hacia una mayor área de hueso sobre FFDB solo, pero no fue estadísticamente significativa ($P > 0.05$). Este estudio fracasó en mostrar un incremento radiográfico o histomorfométrico en la formación de hueso con la adición de PRP a hueso crío-secano tanto mineralizado como desmineralizado en defectos de tamaño no crítico en el cráneo del conejo.

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