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A Comparison of Growth Factor Release Between Platelet Rich fibrin and Recombinant Human Platelet Derived Growth Factor BB (RHPDGF-BB) and Beta-Tricalcium Phosphate: An In Vitro Analysis

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A COMPARISON OF GROWTH FACTOR RELEASE BETWEEN PLATELET RICH FIBRIN AND RECOMBINANT HUMAN PLATELET DERIVED GROWTH FACTOR BB (RHPDGF-BB) AND BETA-TRICALCIUM PHOSPHATE : AN IN VITRO ANALYSIS

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A DISSERTATION

Submitted to the graduate faculty of the University of Alabama at Birmingham in partial fulfillment of the requirements of the degree of
Master of Science

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2022

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2022

A COMPARISON OF PDGF-BB RELEASE BETWEEN PLATELET RICH FIBRIN AND RECOMBINANT HUMAN PLATELET DERIVED GROWTH FACTOR BB (rhPDGF-BB) AND BETA-TRICALCIUM PHOSPHATE: AN IN VITRO ANALYSIS

MAGGIE ANN MISCH

PERIODONTICS

ABSTRACT

Patients presenting with significant alveolar bone loss can pose challenges for the clinician. Traditional bone augmentation techniques may be inadequate in achieving necessary gains to restore missing teeth with dental implants. The adjunctive use of growth factors may accelerate the healing process and enhance tissue regeneration. GEM21S is a product that includes a recombinant growth factor approved by the Federal Drug Administration for oral surgery. It is delivered as a two-component system: 1) recombinant human platelet-derived growth factor-BB (rhPDGF-BB) and 2) beta-tricalcium phosphate (β-TCP).[1]

The research on GEM 21S for ridge augmentation is mostly limited to case studies.[2-4] Biologically, PDGF-BB triggers chemotaxis and mitogenesis of cells of mesenchymal origin.[5,6] It also works synergistically with BMP-2 in bone formation.[7-9] These are ideal properties for wound and bone healing that should be investigated further for potential beneficial effects in ridge augmentation cases.

Today, platelet-rich fibrin (PRF) is commonly used as an adjunctive measure in ridge augmentation. PRF is a second-generation autologous platelet concentrate that contains a supraphysiologic concentration of molecular mediators, including a variety of growth

factors.[10] The concept of utilizing PRF to make a growth factor-enriched bone graft matrix ("sticky bone") has been described. The addition of PRF has been noted to improve handling properties and volumetric stability of the bone graft. Further, due to its natural fibrin clot formation, PRF may provide a scaffold to allow more gradual release of growth factors during healing. Although the use of PRF in oral surgery has the potential to enhance wound healing, its utility may have limitations as PRF has been shown to resorb within a 2 to 3-week period and no osteoinductive capabilities of PRF have been demonstrated.

The rationale for this study is that PDGF-BB is proven to play a critical role in wound healing. It is postulated that a combinatorial approach with PRF could provide clinicians the benefits of a clinically proven and consistent dose of rhPDGF-BB to improve wound healing and bone formation in conjunction with the benefits of the improved physical handling properties of PRF as well as the potential for a longer duration of growth factor delivery.

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LIST OF ABBREVIATIONS

ACS absorbable collagen sponge

β-TCP beta-tricalcium phosphate

BMPs bone morphogenetic proteins

DFDBA demineralized freeze-dried bone allograft

ELISA enzyme-linked immunosorbent assay

EGF epidermal growth factor

FDA Federal Drug Administration

FDBA freeze-dried bone allograft

rhPDGF-BB recombinant human platelet-derived growth factor-BB

IGF insulin growth factor

MSCs mesenchymal stem cells

PBS phosphate-buffered saline

PRF platelet rich fibrin

PRP platelet rich plasma

RhBMP-2recombinant human bone morphogenic protein-2

TGF-β transforming growth factor-β

RBC red blood cells

RPM revolutions per minute

INTRODUCTION

Bone grafting for implant placement

Adequate bone at the site of implant placement is essential for the long-term survival and success of dental implants. Insufficient alveolar bone for implant placement can be due to a number of reasons, including: congenitally missing teeth, trauma, pathology, chronic/acute infection, periodontal disease, other resorptive processes, or tooth loss. When there is inadequate available bone for implant placement, clinicians can perform bone augmentation surgery to enhance bone volume.[11,12] The goal of these procedures is to provide sufficient bone for prosthetically-driven dental implant placement with adequate hard-tissue support to allow for long- and short-term stability of the implant restoration and peri-implant tissues.

Prosthetically-driven dental implant placement requires adequate bone height and width to insert the implant in an ideal position to facilitate restoration and function. Based on the findings from Spray et al.[13], a minimum of 2 mm buccal bone thickness is recommended around the implant to reduce incidence of biologic complications and progressive bone loss. However, improved implant designs, such as platform-switching and conical connections, may better maintain marginal bone.[14] Adequate bone height for dental implant placement is largely based on the anatomic region. In the posterior maxilla, the maxillary sinus can limit the available bone height. Sinus bone grafting can be used to augment the bone height for implant placement. In the posterior mandible, the

mandibular canal and lingual cortex can limit the bone height for dental implant placement. Vertical bone augmentation using a number of techniques may be required to achieve adequate implant length and reduce prosthetic space challenges. As an alternative, short implants may be a viable alternative to bone augmentation and the placement of longer implants in some clinical situations.[15]

The importance of adequate bone for implant placement is further emphasized by the emergent literature on biologic complications, such as peri-implantitis. Peri-implantitis is an inflammatory condition that results in progressive loss of supporting marginal bone.[16] A review showed the prevalence of peri-implantitis ranged from 28-56% of patients and 12-43% of implants.[17] One risk factor for peri-implantitis is exposure of the rough implant surface to the oral cavity. In these cases, there is greater plaque accumulation and mucosal inflammation, which can progress to peri-implantitis resulting in progressive bone loss and, ultimately, loss of the implant.[18] This demonstrates the importance of adequate bone volume for ideal implant placement and to establish an environment that facilitates implant success.

Bone grafting materials

Bone graft materials may be classified by their origin and include autografts, allografts, xenografts, and alloplasts. Autogenous bone grafts have been considered the gold standard in bone regeneration procedures due to their well-documented osteogenic, osteoinductive, and osteoconductive properties.[19] However, there are limitations associated with the use of autogenous bone as a grafting material. These include the added time and effort to harvest bone, the morbidity and potential complications at the donor site, bone availability, and the required training and skills of the surgeon to harvest

bone. As such, bone substitutes, with or without the addition of adjunctive materials to enhance healing, have been used to reduce the need for autogenous bone. Bone substitutes are used to reduce the morbidity associated with autogenous bone harvest and to potentially enhance the amount of materials available for use. While they are able to provide unlimited quantity of materials, decreased donor site morbidity, and decreased intrasurgical time, the disadvantages of their use may include potential antigenicity, cost, and extended healing time.

Bone substitutes include allografts, xenografts, and alloplasts. Allografts are derived from genetically different individuals of the same species. These grafts are harvested from cadaveric donors, decontaminated, and processed. Allografts may be processed in their mineral form as freeze-dried bone allograft (FDBA) or demineralized freeze-dried bone allograft (DFDBA). DFDBA is demineralized with hydrochloric acid. This process removes the need for osteoclastic resorption to release the growth factors, particularly bone morphogenetic proteins (BMPs), which are osteoinductive. A major disadvantage of using DFDBA for ridge augmentation procedures is the rapid resorption of the graft material, lack of long-term space maintenance, and limited scaffolding properties. For this reason, FDBA is more commonly used in ridge augmentation procedures. Although this graft does not possess osteoinductive properties, advantages include a lack of donor site morbidity, unlimited quantity of materials, and decreased surgical time. Disadvantages may include potential antigenicity, cost, and longer healing time when compared to some autogenous grafts.

Xenografts are derived from animal origin, primarily porcine or bovine. The main advantage of xenografts in alveolar ridge augmentation is their volumetric stability due to

slow resorptive properties. Xenograft-specific disadvantages include longer resorption rates and the lack of viable cells and biological components.[20]

Alloplasts are synthetic bone substitutes made in a laboratory. They are typically derived from different combinations of calcium and phosphate formulated with sintering processes. These substitutes are osteoconductive only and act as a scaffold for bone replacement. These grafts may be used for patients that object to other materials, including cadaver or animal products, for personal or religious reasons. Another advantage is that over time many alloplasts are resorbed and completely replaced with new bone leaving no residual particles.

With advances in biotechnology, growth factors have become another tool in an implant surgeon's armamentarium, generally as adjunctive bone augmentation materials. Currently, there are two recombinant growth factors approved to promote bone regeneration in oral surgical procedures: 1) recombinant human bone morphogenic protein-2 (rhBMP-2) and 2) recombinant human platelet derived growth factor-BB (rhPDGF-BB).

The significance of growth factors in bone regeneration biology

Bone regeneration is an intricate series of biologic events that involves complex molecular interactions to promote the migration, proliferation, and differentiation of mesenchymal stem cells followed by maturation and induction of both osteoclast and osteoblast cells.[21] This well-orchestrated process requires several different cell types and molecular-signaling pathways.[22] Many osteoinductive factors regulate bone regeneration, including pro-inflammatory cytokines, proteins in the transforming growth factor-β (TGF-β) superfamily, and angiogenic factors.[23] Growth factors are critical to

this process as they regulate this bone regeneration so that the events mirror events that occur during embryonic bone development, rather than promoting fibrous scar tissue formation. Appropriate growth factors can increase the ability of bone to regenerate by enhancing cellular chemo-attraction, differentiation, and proliferation. Growth factors act by binding to surface receptors of specific target cells, which in turn induce intracellular signaling pathways and activate genes that alter cellular activity.[24] Each growth factor is part of a sophisticated process of feedback loops, which may up- or down-regulate various other growth factors, enzymes, and binding proteins which have various functions throughout the stages of wound healing.[25] BMPs, insulin growth factor IGF)I and -II, transforming growth factor β-1 (TGF β-1), PDGF, and fibroblast growth factor – 2 (FGF-2) are examples of growth factors that actively participate in bone healing.[26]

Growth Factors for Tissue Regeneration in Dentistry

Healing after injury or surgical therapy requires the complex interactions of bioactive molecules and inflammatory growth factors. As such, the adjunctive use of growth factors to induce differentiation and upregulate function of specific cellular components integral to the healing process has been a common practice in oral regenerative procedures. Furthermore, innovations in biomedicine and recombinant protein technology have shown promise in improving outcomes for the regeneration of advanced alveolar defects. Recombinant growth factors and biologic materials may allow for the implementation of more minimally invasive surgical procedures as well as improved clinical outcomes and decreased healing times in complex oral regenerative procedures.

To date, there are two recombinant growth factors approved by the Federal Drug Administration (FDA) for oral surgery applications.[27] Recombinant BMP-2 is a

morphogen that is a well-known regulator of bone formation[28] and has been widely studied for bone reconstruction.[29-33] Commercially-available rhBMP-2 is currently delivered as a two-component system: 1) rhBMP-2 (1.5mg/mL) and 2) absorbable collagen sponge (ACS) as a carrier for the growth factor. This recombinant growth factor is approved by the US FDA as an alternative to autogenous bone grafting in sinus augmentation and localized alveolar ridge augmentation for defects associated with extraction sockets. [34] It has also been reported to enhance the results of oral vertical bone augmentation procedures.[35, 36] Recombinant PDGF-BB is another commercially available growth factor, under the name GEM 21S, that has demonstrated potent mitogenic, angiogenic, and chemotactic effects on bone cells *in vitro* and in animal studies.[5, 6] GEM 21S is currently delivered as a two-component system: 1) recombinant human platelet-derived growth factor-BB (rhPDGF-BB) and 2) synthetic beta-tricalcium phosphate (β-TCP) as a carrier for the growth factor. Recombinant PDGF-BB was first approved in a gel formation by the US FDA in 1997 for the treatment of recalcitrant neuropathic dermal ulcers in diabetic patients. It was later approved in 2005 for bone and periodontal regeneration as well as for the treatment of gingival recession.

The biology of PDGF

PDGF is naturally found in the bone matrix and is released locally during clotting by platelets during the wound healing process. There are five biologically active ligands included in the PDGF signaling network. These include four homodimers: PDGF-AA, PDGF-BB, PDGF-CC, and PDF-DD and one heterodimer: PDGF-AB (Alvarez 2006). There are three dimeric combinations (PDGFR-aα, PDGFR-bβ, and PDGFR-aβ) of two receptors (PDGFR-α and PDGFR-β).[37] PDGF-BB is the most recognized of these and is

considered the universal PDGF due to its ability to bind to all known receptor isotypes and potent physiologic capabilities.[38-40] Boyan et al. studied the various isoforms of PDGF on the mitogenic and chemotactic responses of PDL fibroblasts and found that rhPDGF-BB was the most potent of the isoforms.[41]

PDGF-BB plays a central integrating role in the pathways of angiogenesis, osteogenesis, and mesengenesis, all of which are critical processes for bone regeneration and repair.[42] In response to an injury to the bone, a local inflammatory response is initiated and pro-inflammatory mediators are secreted. There is an influx of platelets, monocytes, macrophages, and other cells of the inflammatory cascade. The platelets and macrophages release bioactive molecules, including PDGF. When the platelets aggregate at the wound site, the released PDGF diffuses into the surrounding tissue and acts as a chemo-attractant for other cells involved in bone and soft tissue regeneration. The most important actions of PDGF include increasing the population of reparative cells, angiogenesis, and macrophage activation, which allows for the debridement of the wound site and a second phase source of growth factors for continued bone regeneration. In a mechanically stable environment, new blood vessels incorporate into the tissues via both angiogenesis and vasculogenesis and mesenchymal stem cells differentiate into osteoblasts that make new bone. Despite the majority of the PDGF being present in the wound for less than 48 hours, the effects are long-standing due to this biologic cascade effect and the activation of downstream biologic mediators and cells.

The development and clinical application of rhPDGF-BB in dentistry

There are several *in vitro* studies demonstrating the extensive biological effects and mechanisms of rhPDGF-BB activity.[43] For dental applications, Lynch et al. was the

first to demonstrate the regenerative potential of periodontal structures using rhPDGF in the 1980s.[44] Since then, there have been many studies exploring the efficacy of rhPDGF in periodontal and peri-implant bone regeneration.[45, 46] A large multi-center randomized clinical trial comparing rhPDGF + β-TCP to β-TCP alone at intrabony periodontal defects found a significant increase in the rate of clinical attachment gain, reduced gingival recession, and improved bone fill and defect resolution in the rhPDGF + β-TCP group.[46] Due to the significant regenerative potential demonstrated in periodontal defects, rhPDGF has been further studied other oral surgical procedures, including alveolar ridge preservation[47], maxillary sinus augmentation[48], guided bone regeneration[49, 50], and soft tissue augmentation and root coverage for gingival recession.[51, 52] rhPDGF has also been investigated in combination with different bone graft materials, including autografts, xenografts, and alloplasts.[45, 53, 54] These studies have shown that rhPDGF can be used safely and effectively with various bone grafting materials to treat osseous defects.

Use of rhPDGF for ridge augmentation

The research on rhPDGF-BB has primarily focused on periodontal regeneration; however, its use has also been reported in sinus augmentations, horizontal bone augmentation, and ridge preservation applications, mostly limited to case studies.[5, 6, 55, 46] To date there have been twenty-two clinical studies, including 281 patients and 425 sites, studying the use of rhPDGF-BB in combination with various bone grafts for horizontal and vertical guided bone regeneration.[56] Many of these case studies have looked at the use of rhPDGF-BB particularly in vertical ridge augmentation cases.[5, 55] Their rationale for the use of rhPDGF-BB in these procedures is to provide a less invasive surgical

technique that decreases morbidity, is less technique sensitive, and can promote faster bone regeneration.[5]

The effect of rhPDGF-BB on bone regeneration in alveolar ridge augmentation procedures was first studied in a dog model using rhPDGF-BB and a deproteinized bovine block for vertical ridge augmentation.[57] These histological results demonstrated a significant amount of bone regeneration, high bone-to-implant contact, and intense osteoblastic activity.[57] Remodeling of the bovine block was found, with graft particles embedded in the new bone and a large number of resorption lacunae.[57] The study also suggested that better results may be seen when rhPDGF-BB is used without a fully cell-occlusive barrier membrane for guided bone regeneration.[57] The rationale for this conclusion was that the barrier membrane may obstruct the chemotactic effect of the growth factor on periosteal pluripotent mesenchymal cells.[57] Despite these findings, it is important to point out that animal studies are lower level of evidence and bone regeneration in a dog model is not necessarily identical to humans. It can be easier to regenerate bone in an animal model due to higher human bone turnover and remodeling activity.[58] Although some animal studies have suggested that bone growth can occur from the periosteal surface, in adult humans, periosteum elevated from bone does not seem to play a significant role in bone regeneration.[59, 60] Funato et al. published a case series report using a combined surgical approach to vertical ridge augmentation using titanium mesh, a resorbable collagen membrane, and rhPDGF-BB.[61] Their rationale for the use of a collagen membrane was that it may help the pharmacologic effect of rhPDGF-BB on the osteogenic cellular migration and angiogenesis from bone marrow. Herford et al. used a porcine model to study the use of rhPDGF-BB on a collagen

membrane covering titanium mesh grafts.[62] They found that the rhPDGF-BB group showed a strong increase in hard and soft tissue healing and favored bone formation, reducing bone resorption even if the mesh was exposed. This could be another strategy for use of the growth factor to decrease the complication of wound dehiscence. Additional higher-level studies are needed to elucidate the benefits of using rhPDGF in bone augmentation procedures for dental implants.

Platelet rich fibrin (PRF)

Wound healing involves a cascade of complex events that includes four main phases: hemostasis, inflammation, proliferation, and remodeling. When a blood clot forms, platelets release biomolecules critical to wound healing, including growth factors, coagulation factors, adhesion molecules, and angiogenic factors.[63] These biomolecules stimulate the proliferation and activation of cells, including fibroblasts, neutrophils, macrophages, and mesenchymal stem cells (MSCs).[63] Because platelets play an important role in tissue regeneration by promoting angiogenesis and other important stages of wound healing, the use of platelet concentrates may be advantageous in regeneration procedures.

Platelet rich plasma (PRP) was the first platelet concentrate introduced to oral surgery by Whitmann in 1997.[64] The goal of PRP was to isolate the highest quantity of platelets, and ultimately growth factors associated with their collection, and to use them during surgery. Despite the initial success and use of PRP, there were limitations that prevented its full potential. The technique to prepare PRP was lengthy and required the additional use of exogenous anti-coagulant factors to prevent clotting using bovine thrombin and $CaCl_2$. These factors are both known inhibitors of wound healing and

antigenic reactions to bovine thrombin have been reported.[65] These drawbacks encouraged the development of a second-generation platelet concentrate, termed platelet rich fibrin (PRF).

PRF is a second-generation autologous platelet concentrate derived from whole blood that has been utilized for many applications in regenerative medicine and dentistry.[66] The preparation time for PRF less cumbersome than that of PRP and does not require the use of anticoagulants. PRF contains a supraphysiologic concentration of molecular mediators, including growth factors, that promote angiogenesis and soft tissue healing.[15] These include TGF-β, PDGF and vascular endothelial growth factor (VEGF), IGF, and epidermal growth factor (EGF).

The use of PRF for ridge augmentation

PRF is used as an adjunctive material in alveolar ridge augmentation procedures. It is employed as a method to enhance both healing and handling characteristics of bone replacement graft materials. A novel concept of making a growth factor-enriched bone graft matrix (also known as “sticky bone”) using PRF has been reported. The PRF is cut into small fragments and mixed with the bone graft material of choice. The rationale for its use is that the PRF can facilitate angiogenesis, which is a critical step in bone regeneration. Sticky bone also improves handling properties of the bone graft, which can aid in stabilization of the bone graft at the site of the alveolar defect and provide volumetric stability. This may potentially accelerate tissue healing and minimize bone loss during the healing period. Although the use of PRF in oral surgery has the potential to enhance wound healing and improve postoperative patient reported outcomes, the effects on bone formation are less clear as it resorbs within a 2 to 3-week period and it

does not possess osteoinductive or osteogenic capabilities.[67]

Although PRF is utilized in guided bone regeneration procedures, there are only two RCTs published on the topic.[68, 69] These trials explored the use of PRF for a barrier membrane in GBR procedures as an alternative to a collagen membrane. There are currently no studies comparing the addition of PRF into a bone grafting material in a comparative study, making it difficult to determine if PRF influences new bone formation during GBR procedures.

There is one proof-of-concept study available that prepared fragmented PRF membranes and mixed with a bone graft in a 50:50 ratio for horizontal bone augmentation in the maxilla.[70] Pre-operative and post-operative cone beam computed topography (CBCT) scans demonstrated improvements in average linear horizontal bone gains of 4.6 mm (+/- 2.3), 5.3 mm (+/- 1.2), and 4.4 mm (+/- 2.3), measured at 2, 6, and 10 mm from the alveolar crest, respectively.[70] The average resorption rate of 15.6% (+/- 6.7) at 5-8 months post-operatively.[70] Randomized controlled clinical trials and histological analysis are needed to further explore the benefits of PRF as an adjunctive to bone graft materials in guided bone regeneration procedures.

It is also important to consider that it is challenging to evaluate the effectiveness of the different platelet concentrates because there is a wide variability in study designs, inconsistent materials used (graft, membrane, or combination), and surgical techniques.[71] There is also great variability in the protocols used to make PRF and the centrifugation devices used and any future studies may also be limited by the potential influence of patient-related characteristics (e.g. overall growth factor content in blood,

coagulation potential, etc.) that may lead to heterogeneity of materials between patients. There is currently a need for improved controlled clinical studies on the topic.

SPECIFIC AIMS OF THE STUDY

Platelet-derived growth factor BB (PDGF-BB) is known to be a potent mitogen and chemotactic agent for cells important in wound healing and bone regeneration. PDGF-BB is also a strong angiogenic agent. These properties provide a solid biological mechanism of action and rationale for the widespread use of PDGF-BB in dental and orthopedic surgery, as well as in the treatment of difficult soft tissue wounds. Clinically, two sources of PDGF-BB have been proposed: 1) Platelet concentrates [including platelet rich plasma and platelet rich fibrin (PRP/PRF)]; and 2) GEM 21S, which contains rhPDGF-BB. The objectives of the present study are to determine:

1. The rate of release of PDGF-BB from PRF preparations at designated time points (1 hour, 8 hours, 1 day, 3 days, 10 days)
2. The rate of release of PDGF-BB from β-TCP saturated with PRF at designated time points (1 hour, 8 hours, 1 day, 3 days, 10 days)
3. The rate of release of PDGF-BB from β-TCP saturated with rhPDGF-BB at designated time points (1 hour, 8 hours, 1 day, 3 days, 10 days)
4. The rate of release of PDGF-BB from β-TCP saturated with rhPDGF-BB and PRF being added to the saturated bone graft (1 hour, 8 hours, 1 day, 3 days, 10 days)
5. Qualitative analysis to determine if the addition of rhPDGF-BB to β-TCP prior to making “sticky bone” with PRF alters the physical handling properties of the bone graft

MATERIALS AND METHODS

Patient selection

Five volunteer donors were screened for participation in this study based upon the following inclusion/exclusion criteria:

Inclusion Criteria	Exclusion Criteria
English speaking	Non-English speaking
Able to read and understand informed consent document	Smokers/tobacco users
Systemically healthy, non-smoker, no medications	Patients with systemic pathologies or conditions contraindicating oral surgical procedures or adversely affecting wound healing

Table 1: Inclusion and exclusion criteria utilized at the screening visit

Whole blood samples

Blood samples were collected with the informed consent from five volunteer donors. Six plastic vacutainer tubes (three 9mL red cap tubes with serum clot activator and three 9mL white cap tubes with no additives) of whole blood were collected from each donor.

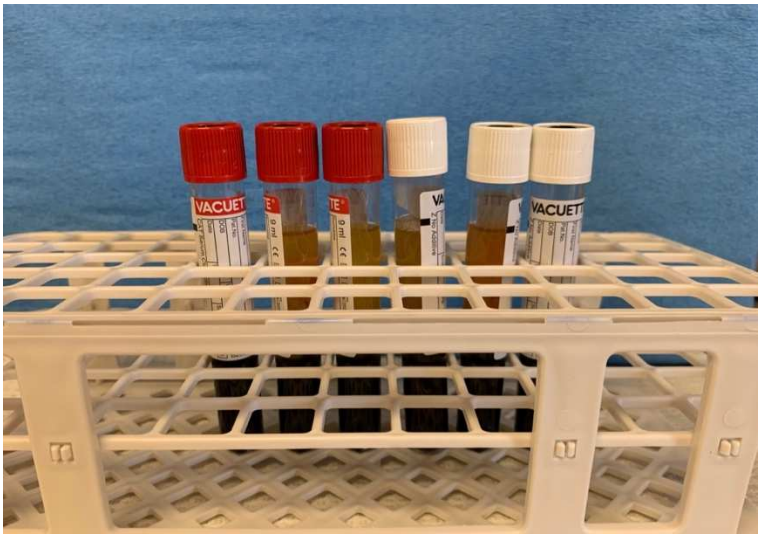

Figure 1: Three red tubes and three white tubes collected from patient

Preparation of PRF

Two vials of blood (one 9mL red cap tube and one 9mL white cap tube) were used to prepare a PRF clot. The tubes were placed in the IntraSpin® (BioHorizons Inc.; Birmingham, AL) system centrifuge and PRF was prepared using the recommended manufacturer protocols. Briefly, the tubes were immediately placed in the centrifuge and centrifuged at 2700 rpm. The white tube was removed after 3 minutes and the red tube was centrifuged for a total of 12 minutes with a balancing tube with distilled water. The liquid fibrinogen was aspirated from the white cap tube with a sterile syringe. After removal of the PRF clot from the red cap tube and careful separation from the red corpuscle portion of the sample, the PRF clot was compressed in the manufacturer's weighted Xpression® box (BioHorizons Inc.; Birmingham, AL). Upon completion, the PRF clot was mixed with 1 cc of liquid fibrinogen and was transported to a 12 well culture dish.

Figure 2: Fibrin clot extracted from the red tubes prior to separation from the red blood cell sample component.

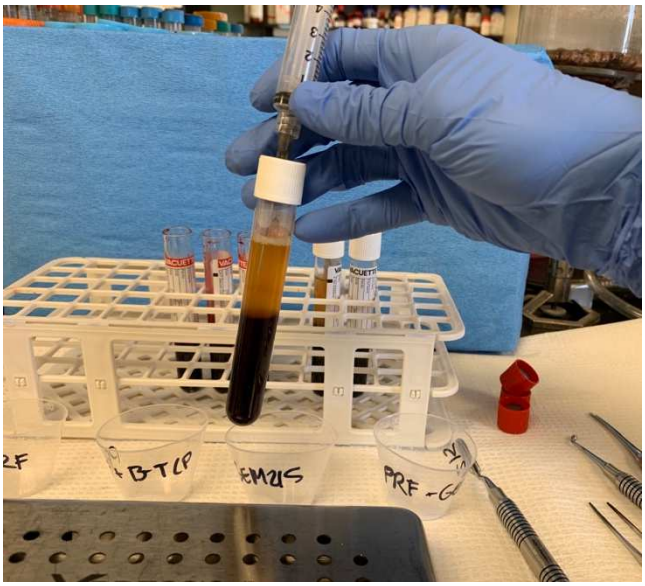

Figure 3: Liquid fibrinogen extracted with a 18G 1.5-inch needle

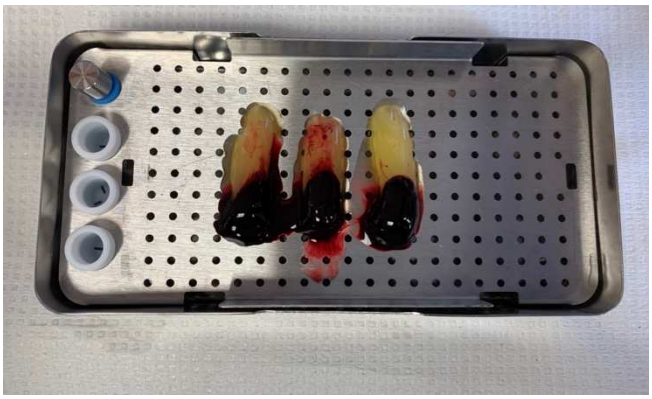

Figure 4: Three fibrin clots placed in the weighted Xpression® box prior to PRF compression.

Preparation of PRF + β-TCP

Two vials of blood from each of the five donors were used for each "sticky bone" preparation of PRF + β-TCP. Briefly, the tubes were immediately placed in the centrifuge and centrifuged at 2700 rpm. The white tube was removed after 3 minutes and the red tube was centrifuged for a total of 12 minutes. The liquid fibrinogen from the white cap tube was aspirated with a sterile syringe. The PRF clot was compressed in the IntraSpin Xpression® box. One membrane was cut into pieces approximately $5mm^2$ and mixed with .5 cc of β-TCP. Using an 18G 1.5-inch needle, the liquid fibrinogen was taken and mixed with the bone graft complex to make "sticky bone". Upon completion, these "sticky bone" preparations were transported to a 12 well culture dish (Corning Inc; Kennebunk, ME).

Preparation of rhPDGF-BB + β-TCP

0.5 cc rhPDGF-BB was added to 0.5 cc β-TCP and placed in a bowl. After 15 minutes of hydration, the rhPDGF-BB/β-TCP graft complex was transported to a 12 well culture dish.

Preparation of PRF + rhPDGF-BB + β-TCP

Two vials of blood from each of the five donors were used for each "sticky bone" preparation. Briefly, the tubes were immediately placed in the centrifuge and centrifuged at 2700 rpm. The white tube was removed after 3 minutes and the red tube was centrifuged for a total of 12 minutes. The liquid fibrinogen from the white cap tube was aspirated with a sterile syringe. The PRF clot was compressed in the IntraSpin box. 0.5 cc rhPDGF-BB was added to 0.5 cc β-TCP and placed in a bowl. This was allowed to sit for 15 minutes. One membrane was cut up and mixed with the β-TCP saturated with rhPDGF-BB. Using an 18G 1.5-inch needle, the liquid fibrinogen was taken and mixed with the bone graft complex to make "sticky bone". Upon completion, these "sticky bone" preparations were transported to a 12 well culture dish.

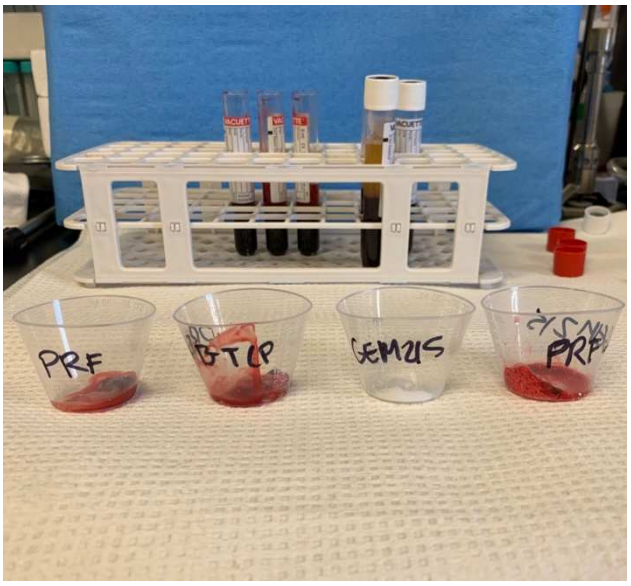

Figure 5: Samples placed in sterile plastic cups

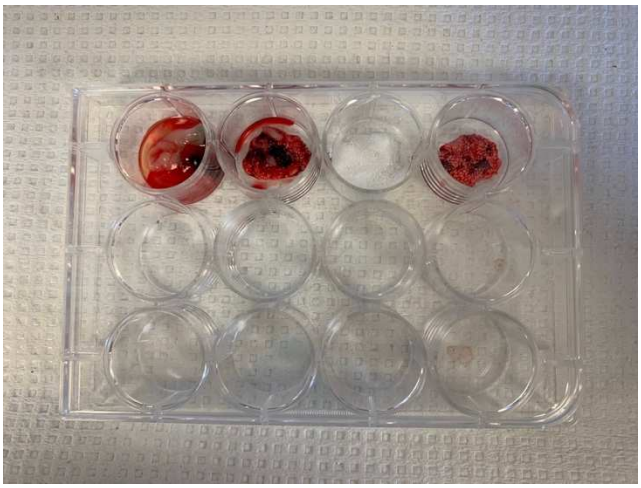

Figure 6: Samples placed in a 12 plate well with 2 mL of phosphate buffer saline

Qualitative Analysis

Photographs were taken to demonstrate the handling properties of this "sticky bone" preparation.

Protein Quantification with ELISA

The quantification was performed for all samples at 1 hour, 8 hours, 1 day, 3 days, and 10 days. All samples were placed in a cell incubator at 37°C to allow for growth factor release into a phosphate-buffered saline at physiologic body temperature to best recreate the *in vivo* conditions. At each desired time point assessment (1 hour, 8 hours, 1 day, 3 days, and 10 days), 2 ml of the sample was collected, frozen, and replaced with 2 ml of additional phosphate-buffered saline. The protein quantification was carried out using ELISA assay according to manufacturer's protocol (R&D Systems Human PDGF-BB Quantikine ELISA Kit #DBB00).

Statistical analysis

The data were analyzed for using a linear mixed effects model with a Tukey's test. Statistical significance for these data sets was set a $p<0.05$.

RESULTS

PDGF-BB growth factor release from PRF alone vs PRF + β-TCP over time

Analysis of PDGF-BB growth factor release from all groups at each designated time interval is displayed in Figure 7. There was no statistically significant difference in the amount of PDGF-BB released from PRF preparations compared to the PRF + β-TCP preparations at all time points (1 hour, 8 hours, 1 day, 3 days, and 10 days). Both groups demonstrated a similar trend in the release of PDGF-BB over time.

PDGF-BB growth factor release from PRF alone and PRF + β-TCP over time compared to rhPDGF-BB + β-TCP and PRF+ rhPDGF-BB + β-TCP groups

PRF alone and PRF + β-TCP groups had significantly lower levels of PDGF-BB at all time points compared to the groups with rhPDGF-BB. The average starting concentration, at one hour, of PDGF-BB in PRF was 662 pg/ml while the average starting concentration of PDGF-BB in rhPDGF + β-TCP was 9,071,593 pg/ml. The PRF groups appeared to exhibit a slower release of PDGF-BB over time; however, after 10 days the rhPDGF groups still contained significantly greater PDGF-BB than the PRF groups.

PDGF-BB growth factor release from rhPDGF-BB + β-TCP vs PRF+ rhPDGF-BB + β-TCP groups

Different trends were observed for the release of PDGF-BB in rhPDGF groups. No significant differences were observed in growth factor release at 1 hour or 8 hours. However, at 1 day and 3 days there was significant difference in PDGF-BB release in the

rhPDGF-BB + β-TCP and PRF + rhPDGF-BB + β-TCP groups. The PRF + rhPDGF-BB + β-TCP group demonstrated a gradual and steady release of PDGF-BB from 1 hour to 10 days, while the rhPDGF-BB + β-TCP group displayed a more marked early release of PDGF-BB from 8 hours to day 1 in the rhPDGF-BB + β-TCP group without significant additional growth factor release after day 3. There was no significant difference in growth factor release at 10 days.

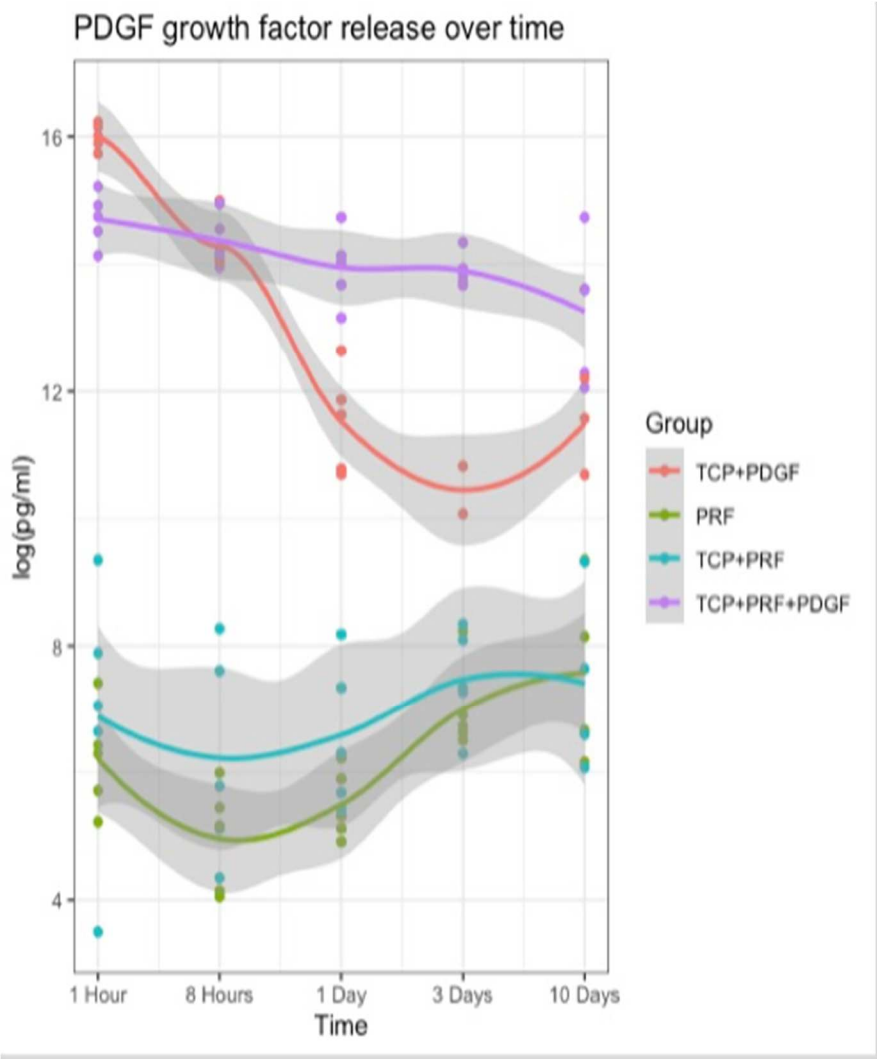

Figure 7: PDGF- BB release over time

Qualitative analysis

There was no difference in handling properties between PRF + β-TCP compared to PRF + rhPDGF-BB + β-TCP. Each sample was easy to handle and moldable into a desired shape.

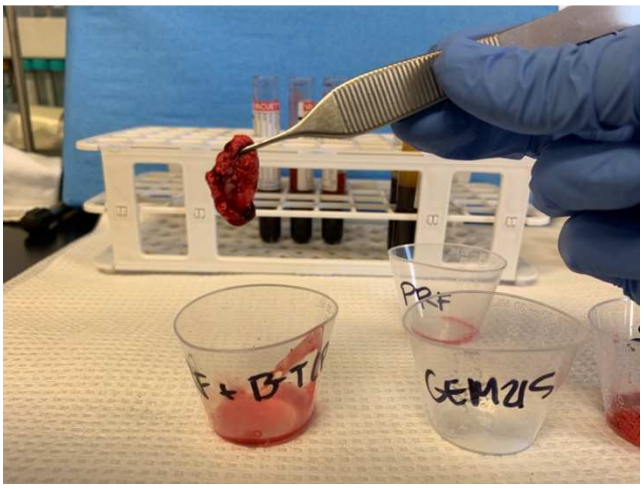

Figure 8: PRF + β-TCP sample

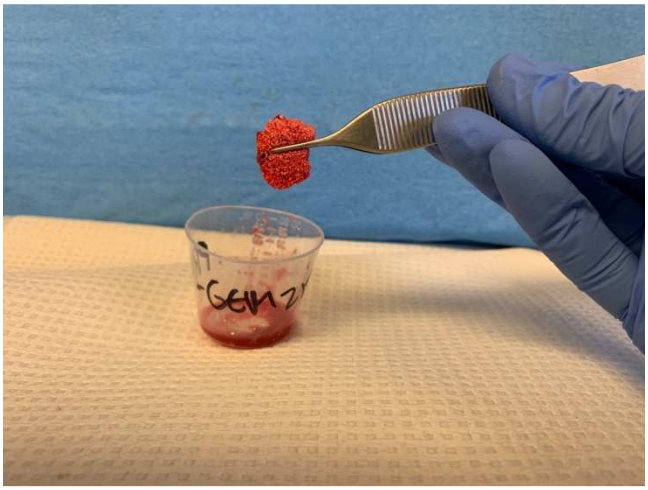
Figure 9: PRF + rhPDGF-BB + β-TCP

DISCUSSION

The aim of this study was to compare the rate of release of PDGF-BB from PRF alone, PRF + β-TCP, rhPDGF-BB + β-TCP, and PRF + rhPDGF-BB + β-TCP. A qualitative analysis was also performed to determine if the addition of rhPDGF-BB to β-TCP prior to making "sticky bone" altered the physical handling properties the graft.

The results from this investigation show that samples with PRF alone and PRF + β-TCP had significantly lower release of PDGF-BB at all time points compared to the samples containing rhPDGF-BB. The samples containing PRF appeared to exhibit slower release of PDGF-BB over time; however, after 10 days the samples with added rhPDGF still contained significantly greater PDGF-BB than the samples with PRF only. The rationale for the use of PRF in bone grafting is in the fact that the platelet alpha granules are a reservoir for a number of growth factors, including PDGF-BB. However, it is important to note that the amount of PDGF-BB found in PRF in this investigation was at a significantly lower level than in a commercially available product with rhPDGF-BB alone. Further, due to the autologous nature of PRF, it cannot be standardized and there can be significant inter-individual variation with PRF. Also, although PRF may enhance wound healing, the effects on bone formation are less clear as previous research demonstrated that it did not possess osteoinductive or osteogenic capabilities.[72]

The results from this study also show that on days 1 and 3 there was a statistically significant difference in the release of PDGF-BB in PRF + rhPDGF-BB + β-TCP

compared to rhPDGF-BB + β-TCP, but no significant difference at 1 hour, 8 hours, or 10 days. The greatest amount of PDGF-BB was released at the 1 hour interval for both of these samples. From 8 hours to day 3, PRF + rhPDGF-BB + β-TCP demonstrated a more gradual and steady release of PDGF-BB compared to an earlier release of PDGF-BB in the rhPDGF-BB + β-TCP samples over the initial 24 hours of this study. The proposed reason for a slower release of PDGF-BB over time in the PRF + rhPDGF-BB + β-TCP samples may be due to the PRF fibrin matrix holding the growth factor within its fibrin network. As the PRF slowly degrades over time through remodeling and hydrolysis, in a similar way to a blood clot, the PDGF-BB is gradually released. By day 10, there was a no significant difference in the release of PDGF-BB between the two groups.

There was no difference in the handling properties of rhPDGF-BB + PRF + β-TCP compared to PRF + β-TCP. Both sets of these materials were easy to handle and able to be molded into the desired shape. The addition of rhPDGF-BB to β-TCP prior to making sticky bone with PRF did not negatively affect the handling properties of the graft material. Because physical properties were not affected in this study, the addition of rhPDGF-BB to the bone graft prior to making sticky bone with β-TCP and PRF, may allow clinicians to improve post-surgical healing and bone regeneration with adequate initial and ongoing levels of PDGF-BB at the surgical site and also reap the benefits of PRF and its structural stability to improve graft handling, graft stability, cohesiveness, and space maintenance in the defect.

An effective carrier for recombinant proteins is critical for the optimal release of the growth factor over a desired period of time.[73] It is also important for such a carrier to exhibit characteristics such as space maintenance and the ability to act as a scaffold for

bone ingrowth and vascularization.[73] Other characteristics that are critical include: biocompatibility, ability to bind to the growth factor, osteoconductive surface for osteoid deposition, and ability to resorb at a rate of compatible with new bone formation and remodeling.[73] Experiments have demonstrated the feasibility of using rhPDGF-BB with β-TCP as its carrier.[74] The greatest limitation reported of β-TCP as a carrier is the rapid clearance *in vivo*, resulting in a potential reduction in therapeutic concentrations of PDGF-BB over time.[74]

The addition of PRF to make "sticky bone" has several advantages in clinical practice, including making the bone graft materials easier to handle and creating some stability of the graft at the surgical site. These are important qualities that can aid in the success of a bone graft. PRF is also able to deliver autologous growth factors that are present during physiologic healing simultaneously, which cannot be achieved using recombinant growth factors. As an autologous blood product, PRF includes growth factors present in the patient's platelets, such as TGF-β, PDGF, VEGF, EGF, and IGF.[71] Findings from this study also demonstrate that the addition of PRF to the preparation of rhPDGF-BB + β-TCP may potentially improve the growth factor release of PDGF-BB for at least several days. More data is needed to determine if such an extension of PDGF-BB release would be beneficial to bone formation *in vivo*; however, these unique properties may suggest that a combinatorial approach that utilizes PRF and bone replacement graft materials as a carrier for growth factors could be beneficial to clinical outcomes. There is no high-level evidence that supports PRF alone improves bone formation, so using it in combination of other materials or growth factors may allow for more optimal clinical outcomes and should be of great interest.[72]

To our knowledge, this is the first investigation of growth factor release that evaluated the use of PRF in combination with β-TCP and/or rhPDGF. While clinicians may use these materials in combination in their clinical practice, little published data exists on the efficacy of such composite graft/growth factor materials for hard and soft tissue regeneration. Understanding the ideal indications for these materials and the optimal use of such materials in practice is critical for enhancing patient outcomes.

One limitation of this study is the variability in growth factor content among the samples. This wide range of growth factor release between the participants may be due to a patients age, gender, or the time spent between blood draw and centrifugation of the blood. Variation in growth factor release has been noted based upon patient and platelet concentrate preparation protocols.[75-78] There are currently no studies that have assessed if the size of a PRF membrane affects the growth factor content. However, one study found that females and older patients produced larger PRF membranes.[79] This study also found a variability in PRF membrane size from the duration between blood draw and the start of centrifugation. The authors theorized that over time it is more difficult to transiently separate layers, which consequently results in smaller PRF membranes and potentially leads to fewer cells and less growth factors.[79] Other investigations have also identified macroscopic and microscopic structural differences between PRF membranes based upon preparation protocols.[15] Given the differences in structure and fibrin organization, these differences could also have a significant impact on growth factor release over time. Based upon the potential variability, the findings of this study may be limited to patients who were recruited in this study and to this particular platelet concentrate protocol.

Additional larger scale *in vitro*, animal, and/or human studies with a wider variety of inclusion/exclusion criteria may be beneficial to determine the optimal candidates for the use of a combined approach with PRF and β-TCP + rhPDGF. Further, due to the costs associated with the addition of adjunctive growth factors, identification of site and patient-specific clinical indications for such an approach may inform clinical decision-making for practitioners in the future. Additional future studies should be performed to see if optimizing the release of PDGF-BB over several days using PRF has positive effects *in vivo*. Optimizing growth factor release over time may allow for more rapid bone formation or maturation, which could be explored in future translational or clinical research.

CONCLUSIONS

There has been a growing interest in methods to accelerate wound healing and enhance hard tissue regeneration in complex alveolar reconstruction procedures. rhPDGF-BB is one of the most studied growth factors used in periodontics and oral surgery. It has been shown to be a powerful stimulant of angiogenesis and significantly increases the proliferation and migration of osteoblasts and other cells of the periodontium, which are all advantageous qualities when attempting to achieve alveolar bone regeneration.[9] The results of this study show that the release kinetics of PDGF-BB may be prolonged by using PRF with β-TCP as a carrier for rhPDGF-BB. The addition of rhPDGF to "sticky bone" made with PRF and β-TCP also did not impact the desirable handling properties. Thus, the clinical utility of a composite bone graft material with PRF + rhPDGF-BB + β-TCP may provide enhanced handling capabilities and clinically impactful growth factor release that may positively impact clinical outcomes. With such a composite graft, it may be possible to obtain the benefits of both rhPDGF-BB to improve post-surgical healing and bone regeneration as well as the benefits of PRF to improve graft handling, graft stability, cohesiveness, and space maintenance in the defect. Further research is necessary to determine the benefits of a longer release of PDGF-BB when used to promote bone regeneration and healing following dental surgery. Further research should also explore alternative carriers for growth factors that may allow for extended release of growth factors in dentistry.

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APPENDIX: IRB APPROVAL LETTER

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Office of the Institutional Review Board for Human Use

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University of Alabama at Birmingham Institutional Review Board
Federalwide Assurance # FWA00005960
IORG Registration # IRB00000196 (IRB 01)
IORG Registration # IRB00000726 (IRB 02)
IORG Registration # IRB00012550 (IRB 03)

19-Jul-2021

IRB-300005939
IRB-300005939-004
Release Kinetics of rhBMP-2 using E-PRF as an Autologous Carrier: An In Vitro Analysis

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The IRB reviewed and approved the Revision/Amendment submitted on 16-Jul-2021 for the above referenced project. The review was conducted in accordance with UAB's Assurance of Compliance approved by the Department of Health and Human Services.

	Expedited
	2, 4
Determination:	Approved
	19-Jul-2021
Expiration Date:	11-Jan-2022

- REVISION/AMENDMENT EFORM

To access stamped consent/assent forms (full and expedited protocols only) and/or other approved documents:
1. Open your protocol in IRAP.
2. On the Submissions page, open the submission corresponding to this approval letter. NOTE: The Determination for the submission will be "Approved."
3. In the list of documents, select and download the desired approved documents. The stamped consent/assent form(s) will be listed with a category of Consent/Assent Document