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THE USE OF PLATELET-RICH FIBRIN MATRICES TO ENHANCE GROWTH FACTOR DELIVERY FOR CONNECTIVE TISSUE HEALING

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LANCE CHARLES VISSER

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# THE USE OF PLATELET-RICH FIBRIN MATRICES TO ENHANCE GROWTH FACTOR DELIVERY FOR CONNECTIVE TISSUE HEALING

By

Lance Charles Visser

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## ABSTRACT

## THE USE OF PLATELET-RICH FIBRIN MATRICES TO ENHANCE GROWTH FACTOR DELIVERY FOR CONNECTIVE TISSUE HEALING

By

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Growth factors are known to play a crucial role in the repair and regeneration of connective tissues. Research unraveling the basic biologic mechanisms of wound healing in a variety of tissues has led to the development of numerous exogenous and autologous growth factor products designed to enhance connective tissue repair. However, the optimal growth factor delivery method for tissue repair and regeneration remains unsettled. Autologous platelet-rich fibrin matrices (PRFM) may represent a promising method to deliver locally increased concentrations of platelet-derived growth factors and other bioactive molecules for a prolonged period of time. The goal of this thesis is to examine the role of a PRFM in enhancing growth factor delivery for connective tissue healing. In addition to providing a thorough review of the literature relevant to the PRFM, the growth factor elution kinetics and mitogenic capacity of PRFMs *in vitro* is examined. The role of a PRFM in enhancing and accelerating tendon healing in a canine model *in vivo* is presented. The use of PRFM-related technology to create a bioactive scaffold for tissue engineering applications is also presented. The results of this thesis supports the use of PRFMs to enhance growth factor delivery for connective tissue healing, particularly in biologically compromised or chronically injured tissues where a prolonged increase in growth factors may be particularly desired.

This work is dedicated to the two most important women in my life.

To my wife Blair:
Thank you for your continuous love, support, patience, and understanding.

To my Mom:
Thank you for teaching and showing me the true meaning of hard work.

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| | |
|---|---|
| bFGF | Basic fibroblast growth factor |
| BC | Blood clot |
| BMP | Bone morphogenetic protein |
| DMEM | Dulbecco's modified Eagle's medium |
| ECGF | Endothelial cell growth factor |
| ELISA | Enzyme-linked immosorbent assay |
| EGF | Epidermal growth factor |
| FBS | Fetal bovine serum |
| FGF | Fibroblast growth factor |
| GAG | Glycosaminoglycan |
| GFRP | Growth factor-rich plasma |
| H&E | Hematoxylin and eosin |
| HGF | Hepatocyte growth factor |
| IGF | Insulin-like growth factor |
| MMP | Matrix metalloproteinase |
| MK | Megakaryocyte |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphynyltetrazolium bromide |
| PT | Patellar tendon |
| PD-EGF | Platelet-derived epidermal growth factor |
| PDGF | Platelet-derived growth factor |
| PRFM | Platelet-rich fibrin matrix/matrices |

| | |
|---|---|
| PRFMatrix | Platelet-rich fibrin matrix |
| PRFMembrane | Platelet-rich fibrin membrane |
| PRP | Platelet-rich plasma |
| PSR | Picrosirius red |
| RT | Repair tissue |
| SEM | Scanning electron microscopy |
| TT | Tendon tissue |
| TGF-β | Transforming growth factor-beta |
| VEGF | Vascular endothelial growth factor |

## GENERAL INTRODUCTION

Growth factors play a vital role in connective tissue repair and regeneration. These polypeptides transmit signals that orchestrate the tissue repair process through a variety of mechanisms including either stimulating or inhibiting cell proliferation, migration, differentiation, or gene expression.(1) The use of autologous and recombinant growth factors to improve tissue repair and regeneration is rapidly growing and appears to hold great promise in a variety of fields in medicine. However, the optimal delivery method for making these factors available at a desired site remains unresolved. Bolus delivery of exogenous growth factors has been investigated as a potential method, yet questions regarding the proper selection and dosage of these recombinant proteins remain unanswered. The creation of biomaterials, constructs that incorporate growth factors with polymer or hydrogel scaffolds, designed to mimic the natural release kinetics of growth factors is an emerging field.(2) Although these technologies appear promising for the controlled release of growth factors, the optimal growth factor or combination of growth factors, dosage, timing of application, biosafety, and cost-effectiveness appear to be significant shortcomings.(3, 4)

Recently, the use of platelet-rich plasma (PRP) has been proposed as a potential method of delivering locally increased concentrations of a variety of bioactive autologous growth factors in an effort to optimize connective tissue healing.(5-12) Platelet-rich plasma has been defined as plasma that contains a platelet concentration above the "normal" physiologic level found in whole blood.(13) The increased concentration of platelets also yields an increase in the concentration of growth factors that are stored in platelet α-granules.(14, 15) These factors, such as platelet-derived growth factor (PDGF),

transforming growth factor-beta (TGF-β), basic fibroblast growth factors (bFGF), insulin-like growth factor-1 (IGF-1), and vascular endothelial growth factor (VEGF) have all been shown to be essential in the healing of connective tissues.(16) Because numerous *in vitro* studies have shown a direct dose-response influence of many growth factors on cell migration, proliferation and matrix synthesis,(17-20) it has been proposed that the local administration of increased concentrations of these growth factors through the use of PRP could optimize the local healing environment and thus enhance the ability of biologically compromised tissues to generate a repair response.(21) While PRP eliminates questions regarding growth factor selection and dosage, the relatively short half-life, rapid degradation, and diffusion/wash-out of most growth factors *in vivo* are potential significant limitations.(3) Therefore, the local administration of PRP alone may not be able to provide a prolonged release of chemotactic and mitogenic factors that may be particularly desired in connective tissue repair and regeneration.(22)

While platelets and their associated growth factors are important for initiating the healing cascade, of equal importance is the presence of a provisional fibrin scaffold.(23, 24) Fibrin provides a naturally-derived matrix, on which repair cells can adhere, migrate, proliferate, and deposit a more permanent extracellular matrix.(25, 26) Together with other plasma-derived proteins such as fibrinogen, fibronectin, and vitronectin, fibrin is able to bind to many growth factors present within platelet α-granules, thus creating a reservoir of growth factors(23, 24, 27) able to release growth factors over time.(26) Therefore, the ability to combine an increased concentration of platelets and their associated growth factors in plasma (i.e., PRP) within a fibrin scaffold may permit the sustained availability of increased concentrations of growth factors over time. This, in

turn, may provide an optimal environment for engineering biological substitutes for the body *in vitro* and/or for orchestrating tissue repair and regeneration *in vivo*.

Autologous platelet-rich fibrin matrices (PRFMs) represent a promising method to improve growth factor delivery by providing a locally available source of prolonged increased concentrations of growth factors in "normal" physiologic ratios within a dense provisional fibrin scaffold. However, there have been no published studies investigating the basic biologic mechanisms behind its proposed benefits. Therefore, the general purpose of this thesis was to determine if/how a PRFM is able to enhance growth factor delivery and, consequently, augment connective tissue healing. This was accomplished in three separate but related studies. First, however, a thorough review of the literature was conducted in order to become familiar with what is known regarding the topics relevant to the contents of this thesis and is detailed in chapter 1. Chapter 2 describes our first study where we sought to determine the elution kinetics and mitogenic activity of the growth factors eluted from a PRFM compared to a whole blood clot (BC) *in vitro*. In the second study detailed in chapter 3 we test the purported benefits of the PRFM *in vivo* using a canine tendon healing model. Chapter 4 contains our third and final study in which we attempt to create a bioactive scaffold using PRFM technology to enhance tissue regeneration *in vitro* for tissue engineering applications. Chapter 5 contains a concluding discussion and mentions future directions for further work related to the findings of this thesis.

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CHAPTER 1.

# Review of the Literature

## OVERVIEW OF CONNECTIVE TISSUE HEALING

Connective tissue healing is an exquisitely designed set of events involving three overlapping phases commonly referred to as the inflammatory, proliferative, and remodeling phases. Growth factors and cytokines play a vital role throughout all of the phases of wound healing. These bioactive molecules bind to transmembrane receptors on local and circulating cells which, initiates intracellular signaling that ultimately affects gene expression. Gene expression results in the production of additional growth factors as well as cytokines that regulate cell proliferation, cell migration, angiogenesis, cellular differentiation, further growth factor/cytokine expression, and extracellular matrix production.

### *Inflammatory Phase*

The inflammatory phase of tissue repair last for approximately 24 hours; it can be further divided into an early vascular (hemostatic) phase and a later cellular (inflammatory) phase.(1) The vascular phase begins immediately with vasoconstriction of the arterioles and is the first attempt to limit blood loss at the site of injury. Vasoconstriction is a transient process lasting for only a few minutes. It leads to platelet aggregation and to the formation of an unstable clot (platelet plug), which leads to the cessation of bleeding. Following hemostasis, vasodilation occurs, largely due to factors released from platelets and damaged endothelium. Factors such as kinins, histamine, prostaglandins, and leukotrienes increase vascular permeability leading to the leakage of blood components into the tissues surrounding the injury.(2) Meanwhile, platelets are exposed to the subendothelial layer of damaged blood vessels (primarily type IV

collagen), which causes them to adhere and aggregate. The coagulation cascade is simultaneously activated in response to the injured vessels via factors from the blood and the surrounding damaged tissues. Platelet aggregation and the polymerization of fibrin form what is known as the stable hemostatic clot.(3, 4) The formation of a clot serves many purposes. In addition to hemostasis, the clot acts as scaffold for many invading cells and as a bioactive reservoir of growth factors required throughout the healing process.(3-5) Platelets play a crucial role in the early phase of inflammation via release of the first wave of growth factors and cytokines from their α-granules.(3, 6) These growth factors and cytokines are known to play a role in signaling inflammatory cells, particularly macrophages, and later fibroblasts, to the injury site initiating the cellular phase of the inflammatory reaction. The cellular phase of inflammation typically begins first, by the influx of neutrophils and later, by the arrival of monocytes and macrophages. These inflammatory cells play a crucial role in phagocytosis of necrotic debris. Additionally, macrophages are particularly important for remodeling the previously formed clot, initiating the second wave of growth factors to recruit resident fibroblasts, and for initiating angiogenesis.(4, 5)

*Proliferative Phase*

The proliferative phase usually begins within a few days post-injury.(1) It is characterized by fibroblast and endothelial cell proliferation and migration into the wound. This is due in large part to growth factors and cytokines secreted mainly from platelets and macrophages.(3, 4) It is important that fibroblasts in the surrounding tissues become activated by growth factors in order to "awaken" from their dormant non-

replicative state.(4) Once activated, these fibroblasts secrete their own growth factors and migrate into the wound from the surrounding tissues with new capillaries lagging behind. The new capillaries arise from nearby intact vessels in response to angiogenic growth factors such as VEGF and PDGF.(4) Once the fibroblasts have entered the wound bed they begin to synthesize various components of the extracellular matrix, dominated by type III collagen which, at this point, remains disorganized and random. Furthermore, wound fibroblasts begin to acquire a contractile phenotype and become known as the myofibroblast, a cell type that plays a major role in wound contraction. The resulting wound connective tissue is more commonly referred to as granulation tissue due to the granular appearance of the many new capillary beds.

*Remodeling Phase*

The remodeling phase is characterized by the repair tissue slowly changing from cellular to fibrous. In the remodeling phase, continued collagen synthesis and reabsorption ensues. This phase is classically characterized by extracellular matrix remodeling, regression of blood vessels, and by the tissue regaining tensile strength. Overtime, type III collagen is replaced with the stronger type I collagen. As the tissue matures some mechanical strength is restored as type I collagen fibers are increasingly orientated along the direction of force through the tissue.(7) Applying controlled physiologic loads to the healing connective tissue stimulates cross-linking of type I collagen, granting additional strength to the tissue while allowing further orientation of type I collagen along the axis of tension.(8, 9) From a period of about ten weeks to a year the healing tissue continues to mature from a fibrous tissue to a scar beginning to

resemble the native tissue. During this time period the metabolism of the resident cells decreases and blood vessels within the scar gradually regress.

## PLATELET BIOLOGY

Traditionally known for their role in hemostasis, platelets are the first cells to respond to an injury through the process of adherence, aggregation, and degranulation. Platelets also play a crucial role in inflammation and tissue repair via cell-to-cell interactions and the release of soluble mediators liberated from activated platelets via the process of degranulation. Platelets circulate in the bloodstream for approximately 5 to 10 days where they survey the vasculature and respond to injury.(10, 11) Normal platelet concentrations in both human and canine blood range from approximately 150,000/μL to 350,000/μL. It is important to note that, although there are subtle differences, the basic biological properties of platelets are conserved across the mammalian species.(12, 13)

### *Platelet genesis*

Platelets are considered to be small (2 μm in diameter) subcellular fragments derived from megakaryocytes (MKs). MKs represent 0.1 to 0.5% of the nucleated cells in bone marrow.(14) Megakaryocytopoesis is stimulated by several cytokines (e.g., Interleukin (IL) -3, IL-6, and IL-11) and thrombopoeitin, and results in platelet formation.(15) MKs are located beneath the capillary sinuses in the bone marrow, where they follow a maturation program culminating in the conversion of most of their cytoplasmic material into multiple long processes known as proplatelets (Figure 1.1). One MK may protrude as many as 10-20 proplatelets.(15) These proplatelets begin as

blunt protrusions that are driven out away from the MK cell body by microtubule-based forces. Platelets are derived from bulges at the distal ends of the proplatelets, and as it matures, it receives granules and organelles delivered as a stream of individual particles from the MK cell body (Figure 1.1).(16) Once the platelet is saturated with the appropriate intracellular content, a single microtubule is rolled into a coil, and the platelet is then released into the bloodstream where it patrols the vasculature for injury for the remainder of its lifespan.

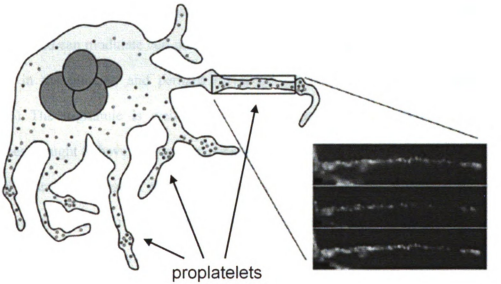


**Figure 1.1** Model of a Megakaryoctye (MK) depicting transport of α-granules during platelet formation. Granules are transported along microtubules from the MK cell body through extensions termed proplatelets. Platelets form as bulges at the distal end of these extensions. Contents of α-granules are shown in various colors, green = fibrinogen, red = von Willebrand factor. Adapted from ref. (16)

*Platelet Granules*

Platelets contain three major membrane-bound granules: lysosomal granules, dense granules, and alpha (α) –granules. Lysosmal granules contain reservoirs of hydrolases such as cathepsins D and E, elastases and other degradative enzymes.(17) The dense granules contain several bioactive molecules that play a significant role in tissue

repair including adenosine triphosphate (ATP), adenosine diphosphate (ADP), calcium, magnesium, serotonin, histamine, dopamine, and catecholamines. ADP plays a role in platelet aggregation, whereas ATP plays a role in the platelet-collagen interaction under flow.(3) Platelets take up and harbor circulating serotonin in the bloodstream and its release upon platelet degranulation causes vasoconstriction and increased vascular permeability. Interestingly, platelet-derived serotonin has recently been shown to play a vital role in liver regeneration.(18) Calcium has also demonstrated to play a central role in wound healing, as it is a necessary cofactor for platelet aggregation and fibrin formation and can modulate cell proliferation and differentiation.(19) The most abundant granule in the platelet, and perhaps the most well-known, is the α-granule (Figure 1.2).(16) The α-granule is well-known for harboring numerous growth factors and cytokines thought to have therapeutic potential for augmenting connective tissue healing, primarily through the use of platelet-rich plasma constructs.(3, 6) These granules contain a plethora of bioactive molecules including growth factors, adhesive proteins, coagulation factors, fibrinolytic factors, proteases and antiproteases, platelet-specific proteins, and membrane glycoproteins (Table 1.1).(16, 20) In addition to wound healing, many of the secreted α-granule proteins are thought to play critical roles in coagulation, inflammation, antimicrobial defense, and angiogenesis.(16)

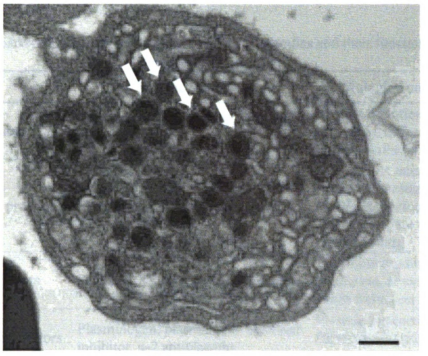

**Figure 1.2** Transmission election micrograph of a human platelet depicting numerous granules, including the α-granules indicated by the white arrows. Scale bar = 500 nm. Adapted from ref. (16)

**Table 1.1**
Bioactive molecules found within platelet α-granules and their functions.
Adapted from ref. (10)

| Category | Specific Molecules | Biologic Function |
|---|---|---|
| Growth factors | TGF-β, PDGF, IGF-I&II, FGF, EGF, VEGF, ECGF | Cell proliferation, migration, differentiation, matrix synthesis |
| Adhesive proteins | Fibrinogen, fibronectin, vitronectin | Cell contact interactions, hemostasis, coagulation, ECM composition, chemotaxis, growth factor binding |
| Clotting factors | Factor V, XI, protein S, antithrombin | Thrombin production & regulation, & eventual fibrin formation |
| Fibrinolytic factors | Plasminogen, plasminogen activator inhibitor, α-2 antiplasmin | Plasmin production (fibrinolysis) and vascular remodeling |
| Proteases & Antiproteases | TIMP1-4, MMP-1,-2,-4,-9, α-1 antitrypsin, ADAMTS13 | Angiogenesis, matrix degradation |
| Anti-microbial proteins | Thrombocidins | Bactericidal & fungicidal |
| Basic proteins | Platelet factor 4, β-thromboglobulin, endostatins | Inhibit angiogenesis, platelet activation |
| Membrane glycoproteins | CD40 ligand, CD61, p-selectin, tissue factor | Platelet aggregation and adhesion, endocytosis, inflammation, thrombin generation, platelet-leukocyte interaction |

*Platelet Activation and Degranulation*

Resting (inactivated) platelets in circulation are disc-shaped, display a relatively smooth surface, and do not exhibit filopodia or microspikes typical of leukocytes. In contrast, platelet activation i.e., response to an *in vitro* or *in vivo* agonist(s), leads to a morphologic transformation into spiny spherical cells with numerous filopodia, in

addition to granule content secretion (degranulation), aggregation, and clot retraction.(20) Shape change occurs in response to most agonists and chilling via mechanisms regulated by G proteins, actin polymerization, and an increase in cytosolic calcium, and these mechanisms are reversible in some but not all cases.(20) Platelet degranulation occurs through a ligand-receptor interaction leading to an actin-myosin-governed cytoskeleton contraction. This calcium-mediated contraction culminates in the centralization of the α, dense, and lysosomal granules that then fuse with the platelet membrane canalicular system and release their contents into the extracellular environment. Release of granule contents further propagates platelet aggregation because many granular contents (i.e., ADP, thrombospondin, and fibrinogen) are themselves platelet agonists. It is important to note that platelet agonists are not all of equal potency and the response to different agonist may vary among species.(13, 21) However, across all species thrombin and collagen have consistently demonstrated to be the most potent platelet agonists.(13)

## FIBRIN AND CONNECTIVE TISSUE HEALING

As previously mentioned, platelets are involved in the first step in the process of hemostasis via formation of the so-called unstable platelet plug. This is usually adequate for the cessation of bleeding. In parallel with this process, the coagulation cascade is initiated and culminates with thrombin formation whereby the conversion of soluble fibrinogen to insoluble fibrin fibers ensues. This process stabilizes the primary platelet plug by forming a fibrin matrix that incorporates platelets via the binding of fibrin to the $\alpha_{IIb}\beta_3$ (CD61) receptors exposed on activated platelets.(22) The fibrin matrix not only helps to provide hemostasis, but also is a vital temporary extracellular matrix providing

an excellent provisional scaffold for repair cells such as fibroblasts and endothelial cells to adhere, migrate, proliferate, and synthesize the permanent extracellular matrix.

*Coagulation Cascade and Fibrin Formation*

The coagulation cascade is a series of several enzymatic reactions made up of two converging pathways known as the extrinsic and intrinsic pathways (Figure 1.3). The extrinsic pathway, probably the most important pathway *in vivo*, is initiated by exposed tissue factor on the subendothelial surface of blood vessels following injury.(23) Tissue factor binds to factor VII and leads to the subsequent activation of factors IX and X. The intrinsic pathway is not essential for coagulation *in vivo* and is initiated when factor XII comes in contact with negatively charged substances (e.g., collagen and glass).(24) Factor XII, which possesses the unique ability to change shape in response to negatively charged surfaces, stimulates the activation of factors XI, IX, VIII, and X.(23, 25) Despite each pathway's unique trigger, both converge at the activation of factor X, which subsequently converts prothrombin (factor II) to thrombin (factor IIa). Thrombin is a multipotent catalyst in the coagulation cascade as it converts soluble fibrinogen to insoluble fibrin polymers and initiates the entire cascade of events of platelets activation including shape change, aggregation, and degranulation, among others.(13, 24) The insoluble fibrin polymers form a network of fibrin fibers that is stabilized via a cross-linking mechanism due to a thrombin-activated factor XIII.(26) These cross-links not only provide stability, but also make the clot more resistant to enzymatic degradation.(27) It is important to note that most of the enzyme complexes of the coagulation cascade, including thrombin formation, are mediated through a calcium-phospholipid interaction

on the surface of activated platelets.(23) Hence, the chelation of calcium, usually via ethylenediaminetetra-acetic acid (EDTA) or citrate, is essential for keeping blood incoagulable prior to laboratory coagulation analysis.

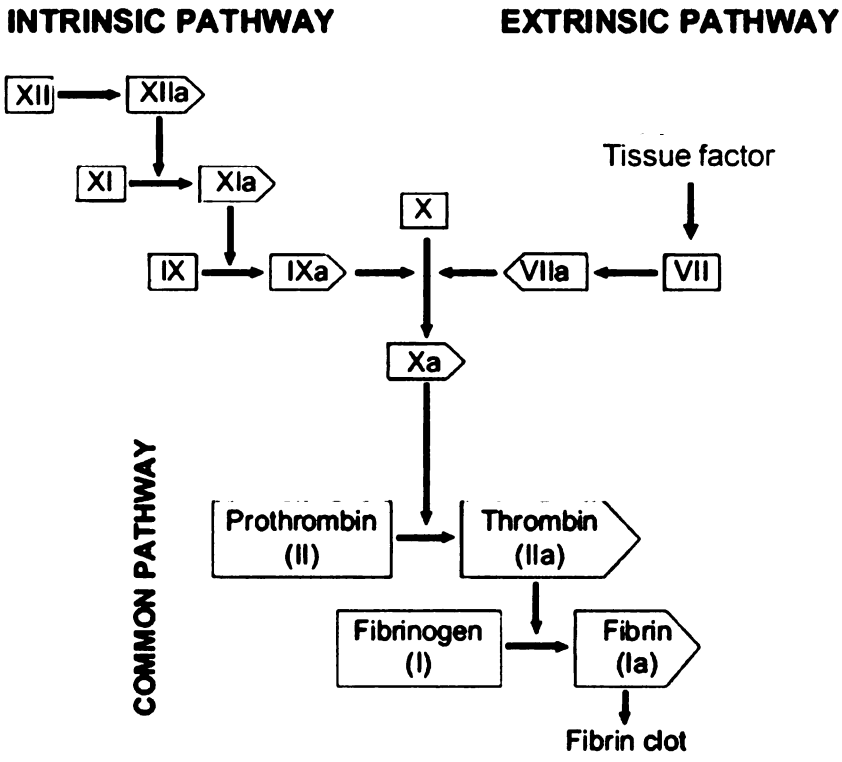


**Figure 1.3** Summary of the coagulation cascade and fibrin formation. Adapted from ref. (2)

*Fibrin Functions*

During the wound healing process, fibrin plays two major roles in addition to hemostasis. First, fibrin acts as a provisional scaffold providing a conducive surface for cell attachment, adhesion, and migration.(28) Fibrin, in conjunction with to the small adhesive proteins fibronectin and vitronectin, act as bridging molecules and support the invasion of key repair cells such as macrophages, endothelial cells, and fibroblasts at the

wound site in order to initiate tissue repair. Secondly, fibrin has been shown to directly and indirectly (via fibrinogen, fibronectin, and vitronectin) bind numerous growth factors including TGF-β, PDGF, bFGF, IGF, and HGF creating a growth factor reservoir within this scaffold and thus prolonging the biological activity of these factors.(29) Fibrin(ogen) plays a pivotal role in angiogenesis through the modulation of various pro-angiogenic factors such as bFGF and VEGF.(30) Further, both bFGF and VEGF have demonstrated to retain their activity when bound to the fibrin matrix.(31, 32) Fibrin and fibrinogen also act in concert by binding PDGF and TGF-β to stimulate fibroblast migration, proliferation, and matrix synthesis.(33, 34) Therefore, fibrin is a key director of connective tissue healing via acting and as a temporary scaffold and acting as a source of numerous growth factors. Moreover, the accumulating knowledge of fibrin's beneficial roles in tissue repair and its versatility as a biopolymer have made this molecular a popular adjunct for connective tissue engineering applications.(28, 35)

## GROWTH FACTOR DELIVERY METHODS

Contemporary research on the biology of connective tissue healing has led to the development of a variety of products designed to enhance connective tissue healing. The use of autologous products and exogenous products such as recombinant proteins, gene therapy and biomaterials for growth factor delivery are all rapidly growing in the orthopaedic and regenerative medicine –related fields and have shown promise in augmenting connective tissue healing. However, despite the excitement surrounding these products and their clinical use, it is clear that further study using more rigorous scientific standards is warranted.(36-38)

**Exogenous Growth Factor Delivery**

Although many recombinant growth factors and cytokines are now available for research purposes and some have also been tested in humans, the clinical experience thus far has been somewhat disappointing. For example, there has been extensive research performed on the use of bone morphogenetic proteins (BMPs) as therapeutic tools for the treatment of various orthopaedic disorders.(39) Despite the numerous BMPs discovered thus far, only recombinant human BMP-2 and BMP-7 (also Osteogenic protein-1) have been developed for clinical use. The former has been shown to be effective in fresh fractures(40) and interbony spinal fusion,(41) whereas the latter has shown efficacy in the treatment of non-union fractures.(42) However, the use of these recombinant BMPs has been associated with complications including heterotropic ossification and significant swelling.(39, 43) One explanation for the reason that few growth factors have been approved and commercialized for therapy in humans might lie in the manner in which these bioactive molecules are delivered: namely, bolus injection into the tissue of interest. Unfortunately, the rapid degradation and low local availability of growth factors delivered in a bolus manner likely do not meet the physiologic criteria to augment tissue repair. Hence, this might explain why several large-scale clinical trials utilizing bolus delivery methods have yielded unrewarding results.(44-46) Furthermore, it seems that the use of growth factors should attempt to mimic the natural tissue repair process as much as possible and thus should not be limited to a single growth factor. It seems that delivering a combination of key growth factors known to play a role in tissue repair at the proper physiologic ratio and in the proper spatiotemporal pattern should be the goal.(47)

In the search for better control over growth factor release, several innovative technologies are currently being explored, and the delivery of growth factors by means of 3D micro- or nanoparticles, injectable gels, composites, polymer scaffolds, or gene therapy are but a few examples. These so-called biomaterials appear promising for the controlled release of growth factors and previous studies have incorporated recombinant growth factors, transfected cells, or plasmid DNA with polymer or hydrogel scaffolds.(48) Vehicles for growth factor delivery take the physical form of porous scaffolds, microspheres, and micro- or nano-capsules. The release profile of the desired bioactive factor can be altered either by manipulating the polymer properties or by adjusting the physical and chemical properties such as porosity, pore size, degree of cross-linking, and degradation rate.(48, 49) Consequently, biomaterials can be designed to produce differential profiles of growth factor release, distinct spatial gradients of factors, and even the release of growth factors in response to specific cues from the cellular microenvironment.(50) Although these technologies appear promising for the controlled release of growth factors, the optimal growth factor or combination of growth factors, dosage, timing of application, biosafety, and cost-effectiveness appear to be significant limitations.(47, 51)

**Autologous Growth Factor Delivery: Platelet-Rich Plasma**

Platelet-rich plasma (PRP) is an example of an autologous product that has been used for decades and is currently being studied as a growth factor delivery method for connective tissue healing. PRP has been used extensively in oral and maxillofacial surgery to accelerate peri-implant soft tissue and bone healing,(52) and more recently has

been investigated in orthopaedics and general surgery.(53-57) PRP is becoming very popular in Sports Medicine and it is becoming more familiar to the general public,(36) in large thanks to a recent front page article in *The New York Times* describing the use of PRP to treat professional football players prior to the Superbowl.(58)

*Definition of Platelet-Rich Plasma*

Platelet-rich plasma has been defined as plasma that contains a platelet concentration above the "normal" physiologic levels found in whole blood, and PRP is considered to be, by definition, autologous.(59) As previously mentioned, normal platelet counts range from 150,000/μL to 350,000/μL whereas a platelet concentration of at least 1,000,000/μL in PRP has been associated with the enhancement of wound healing.(59) Lesser concentrations of platelets are thought not to be reliable to enhance wound healing and greater concentrations have yet to show further benefit.(59) The increased concentration of platelets also provides a 3-to 5-fold increase in the aforementioned platelet-associated growth factors. Throughout the literature several terms have been used to describe preparations that isolate and concentrate platelets. The term "platelet concentrate" is frequently used in the literature, but is not the same as PRP because this implies a solid composition of platelets without plasma, which would therefore not clot.(59) PRP can be thought of as nothing more than a concentration of platelets and their associated growth factors delivered in a normal clot. It is important to keep in mind that the clot also contains several bioactive molecules namely the cell adhesion molecules: fibronectin, vitronectin, and fibrin itself. As previously mentioned these molecules play a significant role in would healing, acting in concert both as a provisional

scaffold and a growth factor reservoir (due to their ability to bind growth factors). The term "platelet gel" is also used in the literature and is also incorrect terminology because the gel does not contain the cell adhesion molecules present in the clot.(59)

*Formulation of Platelet-Rich Plasma*

The increased use and increasing popularity of PRP has resulted in numerous proprietary and commercial methods of formulating PRP. However, three main formulation techniques exist: the gravitational platelet sequestration (GPS) technique (using centrifugation), standard cell separators, and autologous selective filtration technology (plateletpheresis).(53) Only the GPS technique will be discussed herein as this is the method used in the work of this thesis and is considered by many to be standard.(55, 59) PRP can only be made from anticoagulated blood, as it is impossible to concentrate platelets in clotted whole blood or serum. The formulation of PRP usually begins with adding whole blood to a collection tube pre-loaded with citrate. Anticoagulant citrate dextrose is considered the preferred anticoagulant in order to best support platelet viability.(59) Citrate reversibly binds to the ionized calcium and therefore inhibits the coagulation cascade. Next, two centrifugation steps must follow to truly concentrate the platelets.(59) The first centrifugation step, called the "hard spin" (higher *g*-force), separates the red and white blood cells from the plasma containing platelets and clotting factors. The second spin, called the "soft spin" (lesser *g*-force) further concentrates the platelets thus creating a platelet-rich and platelet-poor plasma component. The PRP must next be clotted in order to activate and degranulate the platelets.

Most PRP formulations use thrombin, a combination of thrombin and calcium, or collagen to induce clotting. The use of thrombin induces platelets to secrete approximately 70% of their stored growth factors within 10 minutes and close to 100% is secreted within the first hour.(59) Thereafter platelets may continue to synthesize small amounts of growth factors for the remainder of their lifespan (5-10 days) or until they are depleted.(59) Therefore, PRP should only be clotted when it is ready for use, as clotting too early may mitigate it beneficial effects. Of note, thrombin-activated PRP clots have demonstrated significant contraction whereas a recent study showed that collagen-activated PRP clots underwent much less contraction and were equally effective in stimulating growth factor secretion.(60)

The correct formulation of PRP is critical in order to achieve the greatest benefits from PRP.(61) Platelets are known to be fragile cells. They should be collected with large gauge needles and handled gently so as not to induce premature platelet activation. Some investigators have noted that the effectiveness of PRP seemed to vary among batches,(62) demonstrating the importance of the preparation methods. One investigator has proposed that studies that fail to show a significant benefit from PRP are often the result of improper preparation.(61)

*Rationale for the Use Platelet-Rich Plasma*

The main rationale behind the use of PRP arises from the many bioactive molecules present within the platelet's α-granules. Seemingly all of the growth factors present in platelet α-granules including PDGF, TGF-β, bFGF, VEGF, IGF-1, and EGF play a significant role in connective tissue healing (Table 1.2).(3, 5, 8, 52, 53, 55, 63)

Platelet-rich plasma is also appealing because safety concerns such as immune reactions and infectious disease transmission are theoretically nullified due to its autologous nature. Furthermore, PRP is relatively cost-effective and easily obtainable, especially compared to the aforementioned exogenous growth factor delivery methods. Not only does PRP provide increased concentrations of several growth factors and cytokines, it also provides these bioactive molecules in the "normal" physiologic proportions.(55, 59) Maintaining the physiologic proportional relationship between growth factors is thought to be important for optimal tissue regeneration.(64, 65) The growth factors that are used for exogenous delivery such as BMPs are produced by recombinant technology and are often delivered as a single factor in high or unpredictable doses using carrier vehicles. Due to the complexity of connective tissue healing, it is unlikely that a single growth factor delivery system will provide optimal benefits for connective tissue repair.

**Table 1.2**
Growth factors identified within PRP and their role in tissue repair.
Adapted from ref.(55)

| Growth Factor | Target Cell/Tissue | Function |
|---|---|---|
| PD-EGF | Endothelial cells, fibroblasts, and many other cells types | Cell proliferation, recruitment, differentiation, wound closure, cytokine secretion |
| PDGF A+B | Fibroblasts, smooth muscle cells, chondrocytes, osteoblasts, mesenchymal stem cells | Potent cell proliferation, recruitment, angiogenesis, growth factor secretion: matrix formation with BMPs |
| TGF-β1 | Blood vessel tissue, fibroblasts, monocytes | Potent collagen synthesis, chemotaxis, antiproliferative |
| IGF-I,II | Bone, blood vessel, skin, fibroblasts | Cell proliferation, differentiation, recruitment, collagen synthesis |
| VEGF, ECGF | Endothelial cells | Endothelial cell proliferation, chemotaxis, angiogenesis |
| bFGF | Endothelial cells, smooth muscle, skin, fibroblasts, others | Cell proliferation, chemotaxis, angiogenesis |

Numerous studies have demonstrated positive effects regarding the use of PRP to enhance the healing of connective tissues including bone,(66-68) cartilage,(69-72) tendon,(62, 73-80) and ligament.(80-84) Particularly well-documented is the positive effect of PRP on tendon and ligament cell proliferation, gene expression, and matrix synthesis. Several *in vitro* studies of human tenocytes cultured in the presence of PRP have documented that PRP increases cell proliferation, VEGF expression, and total collagen production.(73, 75, 76) Equine superficial digital flexor tendon and suspensory ligament explants cultured in PRP have shown enhanced gene expression of type I collagen, type III collagen, cartilage oligomeric matrix protein and a decrease in the gene expression of the catabolic matrix metalloproteinase (MMP) -3 and MMP-13.(79, 80) *In vivo* studies have also demonstrated positive results for tendon and ligament healing. Lyras *et al.* have shown evidence that PRP enhances and accelerates tendon healing via increased neovascularization in the early phases of tendon healing in rabbits.(85, 86) There is also evidence that PRP is able enhance the biomechanical properties of injured tendons in animal models, as increased tendon stiffness and strength following the use of PRP has been documented.(62, 87, 88) Furthermore, a recent study demonstrated that an injection of PRP into the patellar tendon injury site resulted in increased recruitment of circulation-derived cells to the injury site, with a concomitant increase in type I and type III collagen production.(78) Murray and colleagues have demonstrated that PRP in concert with a collagen scaffold has shown significant promise in enhancing anterior cruciate ligament healing in a variety of large animal models.(81-84, 89, 90)

*Clinical Applications of Platelet-Rich Plasma*

To date, there is abundant evidence documenting the safety and efficacy of PRP in a wide variety of medical fields. Numerous animal studies, cases reports, and small case series in oral and maxillofacial surgery,(91-93) plastic surgery,(94-97) cardiovascular surgery,(98, 99) and general surgery(100, 101) have examined the role of PRP in various clinical settings. In general these studies support the use of various PRP preparations in the clinical setting, suggesting an overall improvement in soft tissue healing. However, within the oral and maxillofacial surgery literature there are other small case reports that argue there is little benefit from using PRP to promote healing or osteogenesis.(102-104) In general, many of the human clinical studies claim excellent outcomes but are, at best, limited case series. Consequently, it is difficult to draw conclusions from these case reports that may or may not have controls, have small sample sizes, and do not define a standardized preparation of PRP; thus, it is hard to interpret and compare any of the results.(61) The nature of PRP may vary significantly from study to study and is somewhat "operator dependent". Therefore, one must pay careful attention to the specific method of PRP formulation when interpreting results.

Numerous applications for PRP in clinical orthopaedic medicine have been proposed including chronic (over-use) tendinopathy,(105-111) bone healing,(112-116) osteoarthritis,(117, 118) acute tendon/ligament repair,(119-121) muscle injury,(122) joint arthroplasty,(123, 124) rotator cuff repair,(125-128) and articular cartilage repair.(71, 72, 129) However, strong clinical evidence in support the use of PRP for these applications is currently lacking in peer-reviewed journals. To date, the majority of published clinical

orthopaedic studies have investigated the use of PRP for tendon and bone healing. A review of the clinical studies examining the role of PRP in tendon healing is discussed next.

*Elbow Tendinosis / Lateral Epicondylitis.* Lateral epicondylitis (more commonly referred to as "tennis elbow") is a common problem in individuals whose activities require strong gripping or repetitive wrist movements. It is often a chronic recurring problem that is refractory to current treatment regimens. Mishra and Pavelko were the first to publish a cohort study on the use of PRP for chronic severe elbow tendinosis.(108) Fifteen patients were given a PRP injection as an alternative to surgery and 5 patients served as controls. Individuals that underwent PRP treatment reported a 60% improvement in pain scores compared with 16% reduction in the controls at 8 weeks. However, 3 of the 5 controls dropped out of the study by 8 weeks to seek alternative treatment, which limited comparisons. At 6 months pain scores were reduced 81% in the PRP-group. At the final follow-up of 2 years PRP-treated patients reported 93% improvement in pain, and 94% return to sport and work. The authors attributed the significant reduction in pain to PRP and stated that this treatment should be considered prior to surgery. No adverse effects or complications were reported.

Recently, Peerbooms *et al.*(109) sought to determine the effectiveness of a single PRP injection compared to a corticosteroid injection for chronic lateral epicondylitis in a prospective randomized double-blind clinical trial. One hundred patients that previously failed nonoperative treatment (at least 6 months prior) met their inclusion criteria. Their results showed that 73% (37 of 51 patients) of the PRP-treated patients were considered to be successfully treated (defined as more than 25% reduction in pain and function

scores without reintervention after 1 year) compared to 51% (25 of 49 patients) in the corticosteroid group. The authors mentioned that the PRP-treated patients progressively improved with time whereas the corticosteroid group improved initially and then declined.

*Achilles Tendinopathy.* Often refractory to treatment and rehabilitation, chronic Achilles tendinopathy is a degenerative disorder thought to occur as a result of over-use of the tendon and is common in athletes and active individuals. Recently, a placebo-controlled, double-blinded, randomized controlled trial was conducted to determine if a PRP injection could improve pain and functional outcomes in individuals with chronic Achilles tendinopathy while also undergoing an eccentric exercise program (usual care).(105) Fifty-four individuals met the inclusion criteria and were randomly enrolled into the PRP and control groups (n = 27 per group). Although an improvement in pain and function scores was noted in the PRP group, this was not significantly different from the control group of eccentric exercises alone. The authors concluded that they do not recommend the use of PRP for chronic Achilles tendinopathy due to the lack of significant improvement in pain and activity witnessed in their study.

*Acute Achilles Tendon Repair.* In a case-control study, Sanchez *et al.*(119) evaluated the intraoperative effect of PRP in athletes that underwent Achilles tendon repair. The authors used both the PRP clot and supernatant (termed "preparation rich in growth factors") to augment open suture repair in 6 patients, which was retrospectively compared to 6 age-matched controls who underwent the conventional surgical procedure. Follow-up included physical examination and ultrasonic imaging at regular intervals up

to a year. The treated group had earlier range of motion, showed no wound complications, and took less time to return to gentle running.

*Rotator Cuff Repair.* Platelet-rich plasma appears to be an attractive adjunct to many shoulder surgeons, as its potential use for rotator cuff repair has been recently reviewed.(127) To date, there are no published clinical trials on the use of PRP for rotator cuff repair. However, the intraoperative use of PRP to augment rotator cuff repair has been gaining in popularity among shoulder surgeons.(55) A pilot study by Randelli *et al.*(128) is the only published clinical data on the use of PRP for rotator cuff repair, albeit without a control group. After arthroscopic repair of the torn rotator cuff, 14 patients received intra-operative autologous PRP combined with an autologous thrombin component. These patients were followed for 24 months and demonstrated a significant reduction in pain scores and a significant increase in function scores at 6, 12, and 24-month follow-ups compared to pre-operative scores. The authors mentioned that there were no adverse events related to the PRP augmentation procedure and concluded that the procedure was safe and effective.

*Patellar Tendinosis/Tendinopathy.* Chronic patellar tendinopathy, more commonly referred to as "jumper's knee", is a common problem of athletes who undergo repetitive jumping motions and is characterized by a localized tenderness of the patellar tendon at its origin on the inferior pole of the patella. To date, two small preliminary reports have evaluated the use of PRP for jumper's knee. A recent pilot study examined the use of PRP in 20 athletes with chronic patellar tendinosis that were prospectively evaluated at a 6 month follow-up.(107) No adverse effects were observed and significant improvements were noted in functional scores compared to the pre-treatment period.

Filardo *et al.*(106) conducted a second study on the use of PRP for jumper's knee incorporated a control group that underwent conventional therapy alone. Fifteen patients were treated with multiple PRP injections at 3 instances, 2 weeks apart and 16 patients were enrolled in the control group. The PRP injections caused significant improvements in pain and functionality scores and in sport activity level by 6 months.

*Platelet-Rich Plasma in Veterinary Medicine.*

The use of PRP in veterinary medicine is primarily limited to equine medicine. Experimental studies have examined the use of PRP for horse skin wounds(130, 131) and orthopaedic conditions.(79, 80, 88, 132, 133) Currently, there is one published clinical trial on the use of PRP for augmentation of suspensory ligament desmitis in horses,(110) despite its wide-spread use in equine sports medicine. Severe suspensory ligament desmitis is often a career-ending injury. Waselau *et al.* examined the effect of a single application of PRP in concert with a gradual exercise program (conventional therapy) in 9 Standardbred racehorses with moderate to severe suspensory ligament desmitis and compared them to the performance of healthy racehorses that raced during the same time period. They found that all of the horses in the PRP group not only returned to racing but also had comparable race records, in terms of number of starts, total earnings, and earnings per star, to healthy horses 3 years post-treatment. Thus, it appears PRP significantly contributed to the excellent prognosis for returning to racing in these equine athletes.

*Limitations of Platelet-Rich Plasma*

There are potential limitations regarding the use of PRP as a growth factor delivery method for connective tissue healing. One potential limitation may be the injection of liquid unclotted PRP, a commonly used clinical method of delivering PRP to desired tissues. Using this method there may be significant "washout" or rapid diffusion of PRP into the surrounding tissue. Therefore, this washout effect may make it difficult to determine how much PRP was actually delivered to the desired region; thus complicating the extrapolation of doses or concentrations of PRP from one study or clinical situation to the next. The injection method may also account for a lack of clinical benefit as mentioned in a large-scale clinical trial that used a PRP injection for Achilles tendinopathy.(105)

A second potential limitation of PRP may be a lack of a standardization protocol in the preparation of PRP, which (again) makes it problematic to extrapolate data from 1 study to the next. This lack in standardization protocol to produce and evaluate PRP in the literature may help to explain inconsistent and clinical and experimental results. In addition to a uniform PRP protocol, it has been proposed that future studies should be required to quantify platelet yields in both whole blood and the PRP preparation used, and use commercial assays to quantify growth factor concentration.(134)

**Autologous Growth Factor Delivery: Platelet-Rich Fibrin Matrix**

The platelet-rich fibrin matrix (PRFM) represents a new alternative autologous growth factor delivery method for connective tissue healing. Due to the relatively short half-life and rapid degradation of most growth factors,(47) the local administration of

PRP alone may not be able to provide a prolonged release of growth factors that may be particularly desired in biologically compromised connective tissues.(135) The PRFM system was therefore designed to prolong the release of growth factors for connective tissue healing.

*Formulation of Platelet-Rich Fibrin Matrices*

The PRFM essentially combines the benefits of PRP and a dense fibrin matrix and is created as follows (Figure 1.4). Whole citrated blood is collected and the blood cells are separated from the platelets and plasma proteins, during an initial low speed centrifugation step. Next, the supernatant (platelets and plasma proteins in plasma) is added to a second tube containing calcium chloride. This tube is then centrifuged at a higher speed. During the second centrifugation step fibrin polymerization ensues due to the addition of calcium chloride. Calcium chloride causes the conversion of autogenous prothrombin to thrombin thus initiating the fibrin polymerization process. This final centrifugation step permits the concomitant concentration of platelets and polymerization of fibrin resulting in a dense, pliable platelet-rich fibrin matrix that is suspended in a liquid serum component. The shape of the PRFM can be tailored into a flat circular membrane (termed, platelet-rich fibrin membrane [PRFMembrane]) or into a cylindrical construct (termed, PRFMatrix) depending on the shape of the tube/vial (Figure 1.4).

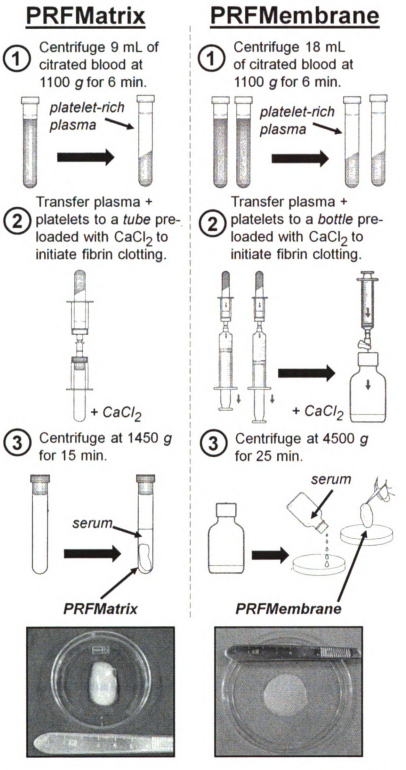


**Figure 1.4** Schematic of the creation of a platelet-rich fibrin matrix (PRFMatrix) and platelet-rich fibrin membrane (PRFMembrane).

*A Platelet-Rich Fibrin Matrix Is Not Platelet-Rich Plasma*

The PRFM is different from PRP in several ways. Most methods used to create PRP currently use calcium chloride and excess human or bovine thrombin. However, the use of excess thrombin may lead to premature platelet activation and degranulation, causing the immediate release of platelet-derived growth factors.(126) The PRFM system (Cascade Medical Enterprises, Wayne, NJ) employs a different strategy, where both platelets and fibrin are incorporated into a dense matrix without the use of exogenous thrombin. It is thought that the lack of excess thrombin in the PRFM system prevents premature platelet degranulation and subsequently intact growth factor-laden platelets become entrapped within the fibrin matrix allowing for a natural, gradual release of growth factors due to autologous activators present at the wound site.(126, 135)

*Rationale for the Use of Platelet Rich Fibrin Matrices*

The main rationale for the use of a PRFM is that it may prolong the release of growth factors and provide a growth factor reservoir in concert with a dense provisional fibrin scaffold that is potentially able to facilitate and enhance tissue repair. The PRFM may provide for a more natural mechanism to delay growth factor release. The prolonged release of growth factors may be particularly desired in biologically compromised(135) or chronically injured tissues, such as in rotator cuff tendon injury.(125-127, 136) Currently, there are few published studies in the peer-reviewed literature on the use of a PRFM for connective tissue healing. However, thus far, it has shown promise in assisting in the closure of chronic lower-extremity ulcers in small human pilot study.(135) An

experimental study in dogs found that the PRFM resulted in faster healing of tooth extraction sites.(137) Sarrafian *et al.* examined the use of a PRFM with a collagen scaffold for repair of acute Achilles tendon rupture in a sheep model.(138) The PRFM group showed complete bridging of the surgically-created gap and their findings supported the use of a PRFM to augment Achilles tendon repair. Additionally, a case report describing the use of a PRFM to augment rotator cuff repair in a 53 year-old man was recently published where clinical examination at 6 months post-operation showed good pain relief and a marked increased in range of motion.(136) Interestingly, several recent reviews on the biologic augmentation of rotator cuff tendon repair have noted that a prospective randomized patient-blinded clinical trial is underway investigating the efficacy of the PRFM in augmentation of arthroscopic rotator cuff repair.(125-127)

## PURPOSE AND HYPOTHESES

Based upon the above review of the literature, the general purpose of this thesis was to determine if a PRFM is able to enhance growth factor delivery and, consequently, augment connective tissue healing. The general hypothesis of this thesis was that a PRFM will enhance growth factor delivery and thus enhance connective tissue healing. This general hypothesis was evaluated in three separate but related studies. The specific purposes of each study were as follows.

**Study 1** (Chapter 2): To compare the kinetics and mitogenic activity of growth factors eluted from a PRFMatrix, a PRFMembrane and a whole blood clot (BC) over time.

**Study 2** (Chapter 3): To examine the effect of a PRFMembrane in enhancing and accelerating tendon healing in a canine central-third patellar tendon defect model.

**Study 3** (Chapter 4): 1) To create a bioactive scaffold using PRFM-related technology, termed growth factor-rich plasma (GFRP), for tissue engineering applications. 2) To evaluate the ability of GFRP-enriched scaffolds to induce tendon cell proliferation and matrix synthesis.

The specific hypotheses and specific aims of each study were as follows.

**Study 1** (Chapter 2): In study 1 our hypothesis was two-fold. $H_1$: A PRFMatrix and PRFMembrane will elute a significantly increased concentration of a sentinel growth factor (TGF-β1) when compared to a BC of similar volume, and $H_2$: The growth factors eluted from a PRFMatrix and PRFMembrane will significantly increase fibroblast proliferation *in vitro* over time when compared to the eluent from a whole blood clot of similar volume. Our specific aims were to quantify the amount of TGF-β1 eluted from a BC, PRFMatrix, and PRFMembrane over time *in vitro*. We also sought to compare the mitogenic activity of each construct over time *in vitro*.

**Study 2** (Chapter 3): In study 2 we hypothesized that the PRFMembrane would enhance the rate and quality of tendon healing in the PT defect when compared to empty contralateral defects (controls). Our specific aim was histoligcally compare the rate and quality of tendon healing in PRFMembrane-augmented tendons and controls using an *in vivo* canine central-third patellar tendon defect model.

**Study 3** (Chapter 4): In study 3 it was our hypothesis that GFRP-enriched scaffolds would significantly enhance cell proliferation and matrix synthesis over time when compared to serum-enriched scaffolds and scaffolds alone. Our specific aims were to combine a synthetic scaffold with GFRP, an autologous concentration of growth factors derived from a PRP preparation, and to compare cell proliferation and matrix synthesis *in vitro* in GFRP-enriched scaffolds, serum-enriched scaffolds, and scaffolds alone (control).

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CHAPTER 2.

# Platelet-Rich Fibrin Constructs Elute Higher Concentrations of TGF-β1 and Increase Tendon Cell Proliferation Over Time When Compared to Blood Clots: A Comparative *In Vitro* Analysis

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**Abstract**

The purpose of this study was to compare the concentration of a sentinel growth factor (TGF-β1) eluted from a platelet rich-fibrin matrix (PRFMatrix), a platelet-rich fibrin membrane (PRFMembrane), and a whole blood clot (BC) over time, and to compare the mitogenic effect of the eluents from each construct. PRFMatrices, PRFMembranes, and whole blood clots of similar volumes were created from each of 4 adult dogs. Each construct was placed in individual tissue culture wells containing media for 7 days. The media was collected and replenished with fresh media on days 1, 3, 5, and 7. The concentration of TGF-β1 eluted into the media from each construct at each time point was measured with an ELISA. Additional aliquots of the conditioned media were added to individual cultures of canine tendon cells and the amount of cell proliferation compared. At days 1 and 3, the conditioned media from both PRFM constructs contained significantly more ($p \leq 0.026$) TGF-β1 when compared to the media from the BC. The PRFMembrane conditioned media contained significantly more ($p \leq 0.05$) TGF-β1 than the PRFMatrix media at similar time points. Conditioned media from both the PRFMatrix and PRFMembrane produced a significant increase ($p \leq 0.044$) in cell proliferation compared to the BC at all time points examined except the PRFMatrix at day 7. The conditioned media from the PRFMembrane produced a significant increase ($p \leq 0.002$) in cell proliferation over both BC and PRFMatrix at all time points. These results demonstrate that both PRFM constructs are comprised of a dense fibrin scaffold that contains increased concentrations of TGF-β1 and are capable of increasing tendon cell proliferation over time when compared to a blood clot of similar volume. The sustained

increase in growth factor availability in PRFM constructs may be beneficial in the healing of biologically compromised tissues.

## Introduction

The ability to enhance the repair of connective tissues through the clinical application of bioactive factors represents an attractive adjunct to the orthopaedic surgeon.(1, 2) This is especially true in cases where the tissues in question may be biologically compromised due to the potentially chronic nature of the pathology, such as in rotator cuff injuries.(3) Recently, the use of platelet-rich plasma (PRP) has been proposed as a potential method of delivering locally increased concentrations of a variety of bioactive autologous growth factors in an effort to optimize connective tissue healing.(2, 4-7)

Platelet-rich plasma (PRP) has been defined as plasma that contains a platelet concentration above the "normal" physiologic level found in whole blood.(8) The increased concentration of platelets also yields an increase in the concentration of growth factors that are stored in the α-granules of platelets.(8) These factors, such as platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β), and vascular endothelial growth factor (VEGF) have all been shown to be essential in the healing cascade of connective tissues.(9) Because numerous *in vitro* studies have shown a direct dose-response influence of many growth factors on cell migration, proliferation and matrix synthesis,(10-13) it has been proposed that the local administration of increased concentrations of these growth factors through the use of PRP could optimize the local healing environment and thus enhance the ability of biologically compromised tissues to generate a repair response.(14) However, due to the relatively short half-life and rapid degradation of most growth factors,(15) the local administration of PRP alone may not be

able to provide a prolonged release of chemotactic and mitogenic factors that may be particularly desired in biologically compromised tissues.(16)

During the initial phase of wound healing, platelets interact with the fibrin clot to not only provide a hemostatic plug, but also create a provisional fibrin scaffold that supports and stimulates cell migration and proliferation.(17) The fibrin scaffold also serves as a reservoir for cytokines,(18) which are bound within the fibrin scaffold and are released over time.(17) Therefore, the creation of a dense, platelet-rich, fibrin construct may provide a reservoir of growth factors that permits the sustained elution of increased concentrations of growth factors over time.(16)

The purpose of this study was to compare the concentration of a sentinel growth factor (TGF-β1) eluted from a platelet-rich fibrin matrix (PRFMatrix), a platelet-rich fibrin membrane (PRFMembrane), and a whole blood clot (BC) over time. In addition, the mitogenic effect of the eluents from these constructs was also compared over time. We hypothesized that, $H_1$: A PRFMatrix and PRFMembrane will elute a significantly increased concentration of TGF-β1 when compared to a BC of similar volume, and $H_2$: The growth factors eluted from a PRFMatrix and PRFMembrane will significantly increase fibroblast proliferation *in vitro* over time when compared to the eluent from a whole blood clot of similar volume.

## Materials and Methods

*Preparation of Blood-Derived Products*

An overview of the processing techniques of each construct is shown in Figure 2.1. A PRFMatrix, PRFMembrane, and BC were created from each of four adult beagle

dogs. Each construct was created from whole blood collected via jugular venipuncture with a 20 gauge butterfly catheter. The PRFMatrix and PRFMembrane were created according to the manufacturer's instructions (Cascade Medical Enterprises, Wayne, NJ) using a double centrifugation process, where, during the second centrifugation step, fibrin polymerization ensues (due to the addition of calcium chloride) while the platelets are concentrated. The PRFMatrix was prepared from 9 mL of whole blood in a collection/separation tube containing a buffered tri-sodium citrate.(19) Following centrifugation for 6 minutes at 1,100 *g*, the blood was separated into a red and white blood cell component and a platelet-rich plasma (PRP) component as a result of the proprietary separation material. Next, the supernatant (PRP) was transferred to a new cylindrical tube containing calcium chloride, which, after a second centrifugation step (15 min. at 1450 *g*), resulted in a cylindrical-shaped PRFMatrix of approximately 2 mL in volume suspended in serum. The PRFMembrane was prepared from 18 mL of whole blood that was collected into two 9 mL tubes each containing trisodium citrate and the proprietary separator gel.(16) Both tubes were centrifuged at 1,100 *g* for 6 minutes creating the PRP supernatant, which was transferred to a 35 mm-diameter flat-bottom glass vial containing calcium chloride. The bottle was then centrifuged at 4,500 *g* for 25 minutes, which resulted in a flat, circular membrane (due to the radial centrifugation) approximately 2 mL in volume that was suspended in serum. Blood clots were created by allowing 4 mL of whole blood to clot in a cylindrical vacuum tube for 2 hours at room temperature creating a cylindrical-shaped clot the same volume (2 mL) as the PRFMatrix and PRFMembrane.

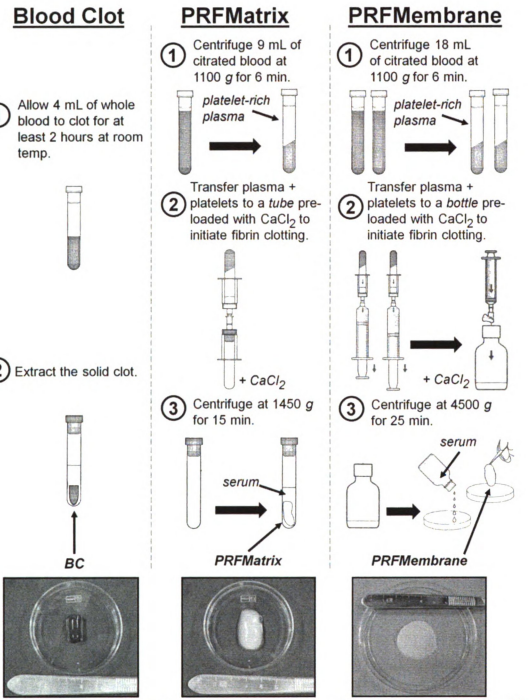


**Figure 2.1** Schematic flow chart illustrating the processing steps for the platelet-rich fibrin matrix (PRFMatrix), platelet-rich fibrin membrane (PRFMembrane) and whole blood clot (BC).

*Study Design*

Immediately following its creation, each construct was poured out of its respective container into a Petri dish and transferred to a 12-well tissue culture plate where it was submerged in 2 mL of serum-free Dulbecco's modified Eagle medium (DMEM) containing 1% antibiotic-antimycotic solution and incubated at 37°C in a humidified atmosphere containing 10% $CO_2$. The eluent (media containing the eluted factors) from each construct was collected in its entirety and replenished with fresh media after 24 hours of incubation and then every 48 hours thereafter. The eluent from each construct at days 1, 3, 5, and 7 was saved and stored at -80°C until either assayed for growth factor concentration or used for cell culture.

*Eluent Growth Factor Quantification*

TGF-β1 was used as a sentinel growth factor to compare the amount of eluted growth factor from each construct. The concentration of TGF-β1 was determined in triplicate aliquots of the collected eluent from each construct (n = 4/construct/time point) with a commercial sandwich enzyme-linked immunosorbent assay (ELISA) kit (MB100B, R&D Systems, Minneapolis, MN). Samples were processed according to the manufacturer's instructions and sample dilutions were determined empirically.

*Isolation of Tendon Cells*

Canine patellar tendon fibroblasts were harvested via primary explant cultures from adult mongrel dogs euthanized for reasons unrelated to this study. Cells were expanded to passage two in 75 $cm^2$ tissue culture flasks in 10% fetal bovine serum (FBS)

media (Dulbecco's modified Eagle medium (DMEM) containing 1% antibiotic-antimycotic solution, 0.02 mg/mL gentamicin, and 0.15 mg/mL ascorbate) incubated at 37°C in a humidified atmosphere containing 10% $CO_2$ (tissue culture conditions). Cells in each flask were cultured until sub-confluent and then detached by trypsinization for seeding. Only cells from the third passage were used for this study.

*Cell Culture Experiment*

Canine patellar tendon cells ($2.5 \times 10^4$) were seeded into wells of a 24-well tissue culture plate and maintained in 10% FBS media. After 24 hours under tissue culture conditions, media in each well was replaced with 2% FBS media, with (treatment) or without (control) eluent 50% (vol/vol) from each construct collected at each time point. After 48 hours under tissue culture conditions, representative live-cell images of each group were obtained and cell proliferation and viability was determined with the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay.

*MTT Cell Proliferation Assay*

Media from each well (n = 4/construct/time point) was replaced with DMEM without phenol red containing 0.5 mg/mL MTT (Sigma-Aldrich, St Louis, MO) and 2% FBS. Cells were then incubated at tissue culture conditions for 4 hours. Next, the MTT solution was discarded and acidic isopropanol containing 1% Triton-X 100 was added to each well. After 40 minutes of mixing on an orbital shaker, all the purple formazan crystals were dissolved. The colorless isopropanol solution changed to varying degrees of a purple color and the degree of color change is directly proportional to the degree of cell

proliferation and viability. Aliquots from each well were transferred to a 96-well plate in triplicate and then read immediately at 570 nm in a scanning multiwell spectrophotometer (Bio-Tek Instraments, Winooski, VT).

*Histological Evaluation*

Additional blood clots, PRFMatrices, and PRFMmembranes were created and fixed in 10% buffered formalin. Histological sections were cut and stained for fibrin using phosphotungstic acid-hematoxylin (PTAH) or processed for immunohistochemical staining for TGF-β1. Detection of TGF-β1 was performed using the anti-human polyclonal TGF-β1 antibody (Lifespan Biosciences, Seattle, WA, dilution 1:50) after heating in TRIS EDTA buffer, pH 9.0 three times for 5 min each for antigen retrieval. The primary antibody was incubated overnight at 4°C. Subsequently, the PowerVision poly HRP anti rabbit IgG (ImmunoLogic, Duiven, The Netherlands) was applied as secondary system followed by detection with Diaminobenzidine (DAB, brown staining). Sections were counterstained with hematoxylin.

*Statistical Analysis*

TGF-β1 concentration and MTT optical density values were analyzed with a one-way ANOVA within each time point followed by a Tukey's post hoc test. All data are displayed as mean ± standard error (SE). The threshold of statistical significance was set to $p \leq 0.05$ for all comparisons.

## Results

*Growth Factor Concentration*

The day 1 and 3 eluent from both the PRFMatrix and PRFMembrane contained a significantly greater ($p \leq 0.026$) concentration of TGF-β1 compared to the day 1 and 3 BC eluent. The day 1 and 3 PRFMembrane eluent contained a significantly greater ($p \leq 0.05$) concentration of TGF-β1 than the PRFMatrix (Figure 2.2). There were no significant differences in the concentration of TGF-β1 between the eluents from the PRFMatrix, PRFMembrane, and whole blood clots at either 5 or 7 days.

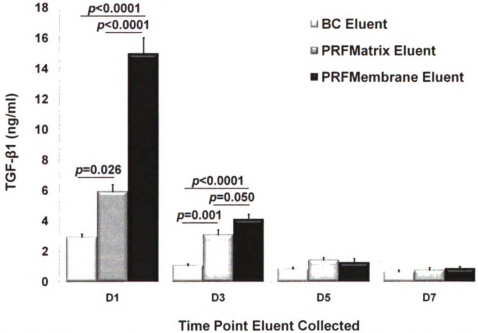


**Figure 2.2** Mean concentrations of TGF-β1 ± SE ($n = 4$) eluted from a blood clot (BC), platelet-rich fibrin matrix (PRFMatrix), and platelet-rich fibrin membrane (PRFMembrane). Statistical significance was not reached ($p > 0.05$) where $p$-values for comparisons are not shown.

*Cell Proliferation*

Cells that were subjected to each construct's day 1 eluent for 48 hours under tissue culture conditions demonstrated obvious differences in cell density (Figure 2.3). Cell density was greatest in the PRFMembrane eluent group followed by the PRFMatrix eluent. The cell density of the BC eluent appeared similar to controls. All cells exhibited a similar morphology typical of tendon cells (spindle-shaped fibroblast-like appearance).

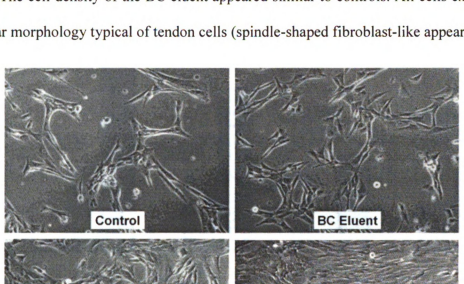


**Figure 2.3** Photomicroscopic live-cell images of representative tendon cell proliferation results after 48 hours of exposure to day 1 eluents from the respective constructs (Phase contrast, 100x original magnification).

The results of the MTT assay performed on the tendon cells subjected to the eluent from each construct at each time point are shown in Figure 2.4. Mean optical density values, indicating relative cell proliferation and viability, from the eluents of both PRFM constructs were significantly greater ($p \leq 0.044$) than the BC eluent at each time point except at day 7. The PRFMembrane eluent consistently induced a significant

increase (at least 2-fold) in cell proliferation compared to the PRFMatrix ($p \leq 0.002$) and BC ($p \leq 0.0001$) at each time point the eluent was collected.

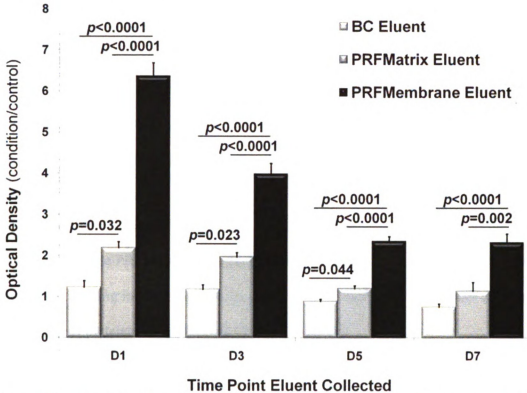


**Figure 2.4** Mean optical density values displayed as *n*-fold difference over control ± SE ($n = 4$) of tendon cells subjected to eluent from the blood clot (BC), platelet-rich fibrin matrix (PRFMatrix), or platelet-rich fibrin membrane (PRFMembrane) from the various time periods. Statistical significance was not reached ($p > 0.05$) where *p*-values for comparisons are not shown.

*Histological Evaluation*

Histological evaluation of the relative fibrin content revealed a marked increase in the density of fibrin in both the PRFMatrix and PRFMembrane constructs when compared to the blood clot (Figure 2.5A, B, and C). Clusters of platelets appeared trapped among the interconnecting strands of fibrin in the PRFMatrix (Figure 2.5B). The fibrin content of the PRFMembrane appeared subjectively increased and more compact than that of the PRFMatrix (Figure 2.5C). This increase in fibrin density made it difficult to delineate the presence of platelets within the PRFMembrane using PTAH staining.

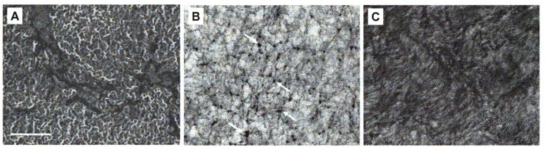


**Figure 2.5** Photomicroscopic images of a representative blood clot (A), platelet-rich-fibrin matrix (B), and platelet-rich fibrin membrane (C) stained for fibrin with a phosphotungstic acid hematoxylin (PTAH) stain. Fibrin strands are not visible among the red blood cells in the whole blood clot (A). "Nests" of platelets (arrows) can be seen among the dense fibrin network of the platelet-rich fibrin matrix (B). The dense compaction of the fibrin in the platelet-rich fibrin membrane is obvious (C). Scale bar = 50 μm and applies to each image.

Immunohistochemical staining for TGF-β1 revealed positive staining of the fibrin network in both the PRFMatrix and PRFMembrane (Figure 2.6A and B). Localized areas of increased staining intensity in both the PRFMatrix and PRFMembrane were associated with "nests" of platelets trapped within the fibrin network.

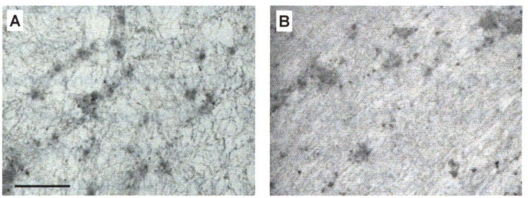


**Figure 2.6** Photomicroscopic images of a representative platelet-rich fibrin matrix (A), and platelet-rich fibrin membrane (B) immuno-stained for TGF-β1. In both constructs there is positive staining of the fibrin strands and localized areas of increased staining intensity which likely represents "nests" of platelets trapped within the fibrin network. Scale bar = 50 μm and applies to both images.

## Discussion

Fibrin has been shown to be an excellent provisional scaffold providing a conducive surface for cell attachment, adhesion, and migration during the initial phase of the healing process.(20) In addition, fibrin has been shown to indirectly bind cytokines creating a growth factor reservoir within this scaffold which, in turn, prolongs the biological activity of these factors.(21) Therefore, the ability to increase the concentration of growth factors as well as fibrin density within a polymerizing fibrin scaffold through the creation of platelet-rich fibrin constructs may prolong growth factor activity and availability when compared to a naturally occurring whole blood clot.

In the current study, the addition of platelet-rich plasma, created by the initial centrifugation of whole citrated blood, to a second tube containing calcium chloride permitted the activation of endogenous prothrombin to thrombin. This, in turn, initiated the natural fibrin polymerization cascade.(16, 19) The concurrent centrifugation of this mixture during the polymerization process resulted in a marked increase in fibrin density in the platelet-rich fibrin constructs when compared to a naturally occurring whole blood clot. Increasing the speed and duration of the centrifugation process further increased the fibrin density in the PRFMembrane when compared to the PRFMatrix.

The PRFMatrix and PRFMembrane demonstrated the ability to elute significantly increased levels of a sentinel growth factor (TGFβ-1) over a three day period when compared to a whole blood clot of similar volume. In addition, eluents from both the PRFMatrix and PRFMembrane were able to significantly enhance tendon cell proliferation over 7 days when compared to the eluent from whole blood clots of similar volume. The rationale for using a single sentinel growth factor (TGFβ-1) to document the

increase in growth factor concentration achieved with the PRFM constructs was based on the significant association demonstrated between platelet levels and TGF-β1 concentrations.(22, 23) While the concentration of specific mitogenic factors such as platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) normally stored in platelet α-granules were not measured in the current study, the increase in tendon cell proliferation observed following the addition of the eluents at various time periods indirectly suggests that these mitogenic factors are also likely increased.(24)

The current study was not able to determine if the increase in the duration of growth factor availability from both the PRFMatrix and PRFMembrane was due to a persistence of platelet activity within the constructs or merely as a result of prolonged diffusion kinetics resulting from the increased fibrin density of the constructs. Immunohistochemical evaluation demonstrated positive TGFβ-1 staining of fibrin as well as what appeared to be clusters of platelets trapped within the fibrin network of both PRFM constructs. However, this histological assessment could not delineate between intact and activated (degranulated) platelets.

Several investigators have examined the use of platelet-rich fibrin constructs to enhance connective tissue healing.(5, 16, 19, 25-27) However, the precise biological mechanism(s) by which the addition of these fibrin constructs may enhance connective tissue repair has yet to be elucidated. While the concept of adding increased levels of growth factors to enhance cellular proliferation and matrix synthesis has strong basic science support,(10-13) the clinical benefits of increased growth factor concentrations on connective tissue healing in association with surgical repair are less clear.(2, 28) Previous

studies have suggested that the addition of an exogenous fibrin clot provides the needed mitogenic and chemotactic factors, as well as the provisional scaffold, needed to initiate and support a regenerative response in avascular regions of the meniscus.(29, 30) Therefore, the significance of an autologous fibrin matrix in wound repair cannot be underestimated.

The healing response of connective tissues is an exquisitely designed continuum of events that is initiated by bleeding and the interaction of platelets and the polymerizing fibrin clot to create a growth factor-laden provisional fibrin scaffold on which reparative cells can migrate, adhere, proliferate, and begin to synthesize a matrix.(9) While this process of wound repair usually occurs without incident in normal healthy tissues, it may be inhibited in tissues that have compromised vascularity or limited cellularity such as chronic rotator cuff tears.(3) In these cases, vascular and cellular proliferation may be delayed and require prolonged availability of cytokines that signal such events.(16) Therefore, the ability of PRFM constructs to provide a bioactive fibrin substrate that elutes increased levels of active growth factors for longer periods of time, when compared to a naturally occurring fibrin clot, may represent a bioactive delivery vehicle for the optimization of healing in biologically compromised tissues. While a recent study has shown the ability of a PRFMembrane to induce healing in chronic lower extremity ulcers,(16) additional clinical studies are needed to determine if the use of platelet-rich fibrin constructs can enhance the repair of other biologically compromised tissues such as ligaments and tendons.

A potential limitation of this study may center around the use of canine blood. Purebred canine beagles were used in this study to minimize variability and permit

utilization of assays and immunohistochemical techniques that have been perfected in our laboratory. While the baseline levels of growth factors in canine whole blood (serum) are slightly different from that of humans,(24, 31-34) the same compliment of growth factors is present within the platelets of both species.(35, 36) In addition, the clotting cascade of canine blood is comparable to that of humans.(37) Therefore, we believe the use of canine blood in our *in vitro* culture model was a valid system in which to test our hypotheses regarding the ability of platelet-rich fibrin constructs to prolong growth factor activity and release when compared to a whole blood clot.

In conclusion, the results of the current study confirmed our hypothesis that platelet-rich fibrin constructs elute significantly higher concentrations of TGF-β1 compared to whole blood clots of similar volume. In addition, the results demonstrated that eluents from the platelet-rich fibrin constructs were able to significantly increase tendon cell proliferation over time when compared to whole blood clots of similar volume. While these results demonstrate the potential for prolonged delivery of increased concentrations of growth factors from a bioactive, fibrin scaffold *in vitro*, additional translational and clinical studies are needed to determine if the use of such platelet-rich fibrin constructs can enhance the repair of biologically compromised tissues *in vivo*.

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CHAPTER 3.

# The Use of an Autologous Platelet-Rich Fibrin Membrane to Enhance Tendon Healing: An Experimental Study in Dogs

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Visser LC, Arnoczky SP, Caballero O, Gardner KL. The use of an autologous platelet-rich fibrin membrane to enhance tendon healing: An experimental study in dogs. *Am J Vet Res*, in press.

**Abstract**

The purpose of this study was to examine the effect of an autologous platelet-rich fibrin membrane (PRFMembrane) in enhancing healing of a central-third patellar tendon (PT) defect in dogs. Bilateral central-third PT defects were created each dog. One defect was implanted with an autologous PRFMembrane and the contralateral defect left empty. Dogs were euthanized at 4 and 8 weeks post-operatively (n = 4/time period), and tendon healing was assessed grossly and histologically using a semiquantitative scoring system. Cross-sectional area of the PTs was also compared. Both treated and control defects were filled in with repair tissue by 4 weeks. There was no significant difference in the histologic quality of the repair tissue between control and PRFMembrane-treated defects at either time point. At both time points, the cross-sectional area of PRFMembrane-treated tendons was significantly ($P \leq 0.01$) greater (at least 2.5-fold) than that of control tendons. At 4 weeks the repair tissue consisted of disorganized proliferative fibrovascular tissue originating predominantly from the fat pad. By 8 weeks the tissue was less cellular and slightly more organized in both groups. A PRFMembrane did not enhance the rate or quality of tendon healing. However, it did increase the amount of reparative tissue within the defect. This may be due to the increased concentration and duration of growth factor exposure provided by the membrane. These results suggest that a PRFMembrane may be more beneficial in biologically compromised tissues where increased concentrations of bioactive molecules may be needed for longer periods of time.

## Introduction

Tendon injuries are a common cause of morbidity in both human and veterinary medicine and their management poses a significant challenge to the clinician. The injured tendon is often refractory to treatment, slow to heal, and despite nature's best repair efforts, remains functionally inferior to an uninjured tendon.(1, 2) Contemporary research has revealed that numerous growth factors such as platelet-derived growth factor, TGF-β, insulin-like growth factor, basic fibroblast growth factor, and vascular endothelial growth factor play critical roles in tendon healing, including mitogenesis, chemotaxis, angiogenesis, and matrix synthesis.(3, 4) In addition, increasing growth factor levels above those normally found in serum have been shown to improve both the rate and quality of tendon healing.(5-8) Therefore, the use of growth factors as a potential treatment option to enhance connective tissue healing holds great promise.(9) However, questions regarding the most effective dose, choice/combination of growth factor(s), and delivery method to enhance tendon repair remain unanswered.

One potential method of delivering growth factors as well as other beneficial bioactive molecules to an injured tendon is through the use of PRP. Platelets are known to contain all of the aforementioned growth factors involved in tendon healing within their α-granules.(10) Recently, several experimental and pre-clinical studies have demonstrated promising results following the use of PRP to enhance tendon healing.(11-19) Platelet-rich plasma has been defined as autologous plasma that contains a platelet concentration above the baseline levels found in whole blood.(20) It is typically created from citrated whole blood using centrifugation to separate the platelets from the other blood cells in plasma and to concentrate the platelets in plasma.(20) In addition to

providing a convenient source of increased concentrations of autologous growth factors, PRP also maintains the "normal" physiologic ratios of growth factors and bioactive molecules,(20) demonstrated to be ideal for wound healing.(21, 22)

While platelets and their associated growth factors are important for initiating the healing cascade, perhaps of equal importance is the presence of a provisional fibrin scaffold.(23, 24) Fibrin provides a naturally derived matrix, on which repair cells can adhere, migrate, proliferate, and deposit matrix.(25) Together with other plasma-derived proteins such as fibronectin and vitronectin, fibrin is able to bind to many growth factors present within platelet α-granules thus creating a growth factor reservoir.(23, 24, 26) Therefore, the ability to combine an increased concentration of platelets and their associated growth factors in plasma (PRP), within a fibrin scaffold, may provide an optimal environment for tissue repair and regeneration.

The purpose of this study was to examine the effect of a PRFMembrane (Cascade Medical Enterprises, Wayne, NJ) in enhancing and accelerating healing of a central-third patellar tendon defect in a canine model. It was our hypothesis that the PRFMembrane would enhance the rate (based on percent of defect fill) and histologic quality of the repair tissue (based on a semiquantitative evaluation of cellularity, vascularity, collagen organization, and glycosaminoglycan content) in the PT defect when compared to empty contralateral defects (surgical controls).

## Materials and Methods

All procedures in this study were approved by the Institutional Animal Care and Use Committee at Michigan State University.

*Study Design*

A central-third PT defect was created in both hind limbs of eight adult male beagle dogs (average weight, 14.5 ± 1.9 kg) and an autologous PRFMembrane was implanted into one defect and the contralateral defect was left empty as a surgical control (sham). Four dogs at 4 and 8 weeks post-operatively were humanely euthanized using an intravenous overdose of pentobarbital.

*PRFMembrane Preparation*

The autologous PRFMembrane was created from each dog as per the manufacturer's instructions as follows (Figure 3.1).(27) Prior to the induction of anesthesia, 18 mL of whole blood was drawn via jugular venipuncture with a 20 gauge butterfly catheter into two 9 mL tubes containing trisodium citrate and a proprietary separator gel. The tubes were centrifuged at 1,100 *g* for 6 minutes to create a PRP supernatant. Using an 18 gauge needle and a 20 mL syringe, the PRP supernatant from both 9 mL tubes was carefully transferred to a 35 mm-diameter flat-bottom glass vial containing 1.0 M calcium chloride. The vial was immediately centrifuged at 4,500 *g* for 25 minutes while fibrin polymerization ensued. The result was a dense, flat, circular, fibrin membrane (due to radial centrifugation) suspended in a liquid serum component (Figure 3.1).

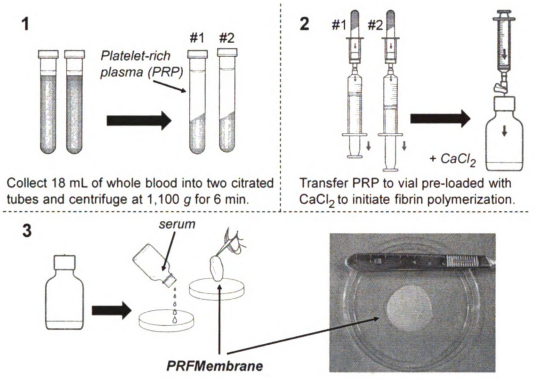


**Figure 3.1** Summary of the creation of the platelet-rich fibrin membrane (PRFMembrane) used in this study.

*Surgical Procedure*

After premedication with intramuscular morphine (0.6 mg/kg) and acepromazine (0.02 mg/kg), the dogs were anesthetized using intravenous thiopental (dosed to effect) and maintained with isoflurane in oxygen (0.5% to 3%). Next, preservative free Morphine (0.1 mg/kg) was injected into the epidural space for post-operative analgesia. With the animal placed in dorsal recumbency and using aseptic surgical technique, the patellar tendon was isolated and exposed utilizing a medial parapatellar approach (Figure 3.2A). After measuring the width of the patellar tendon, the central-third of the patellar tendon was sharply incised from the distal aspect of the patella to the tibial tuberosity and then resected (Figure 3.2B). Care was taken to separate the retropatellar fat pad from the

resected portion of the PT. In the treated leg, a bed of 4-0 polydioxanone suture was placed through the remaining patellar tendon and under the defect using a horizontal mattress pattern to act as caudal support for the PRFMembrane (Figure 3.2C). The PRFMembrane was rolled onto itself, placed into the defect and sutured to the remaining patellar tendon using a simple interrupted pattern of nonabsorbable 5-0 nylon suture (Figure 3.2D). The surgical site was closed in a routine layered manner. In the sham leg the surgical site was closed following the resection of the central-third of the patellar tendon. Carprofen (4 mg/kg, SC) was administered once during surgery and q 24 h as needed for post-operative analgesia.

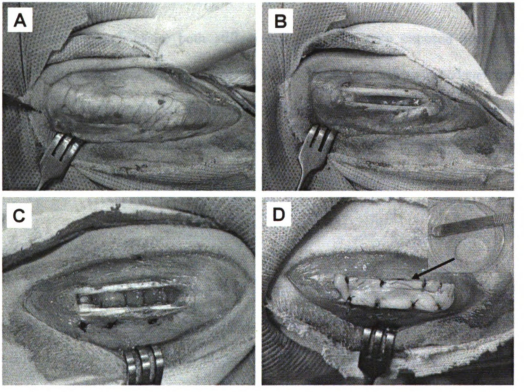


**Figure 3.2** Photographs of the surgical procedure. The patellar tendon was isolated and exposed (A). A central-third patellar tendon defect was created (B). A bed of sutures was created in the defect to act as caudal support (C). The autologous platelet-rich fibrin membrane was rolled onto itself and sutured to the defect (D). The contralateral limb consisted of only steps A and B.

*Post-operative Regimen*

After recovery, all animals were housed individually in 4 x 1.5 meter runs and allowed unrestricted activity. Tramadol (2-4 mg/kg, PO, q 8-12 h) was administered for a minimum of 3 post-operative days for analgesia. No post-operative bandage/immobilization was applied. The dogs were observed at least twice daily and general condition, temperature, pulse rate, respiratory rate, attitude, appetite, activity level, and degree of lameness was recorded daily. The incision sites were checked daily for signs of swelling, erythema, discharge and dehiscence until complete healing.

*Gross Evaluation*

Following euthanasia, both stifle joints and PTs were exposed and grossly evaluated. The patella-patellar tendon-proximal tibial complex was harvested *en bloc* and placed in 10% neutral-buffered formalin. Following formalin fixation, the tendons were bisected at their midpoint in the transverse plane and side-by-side digital photographs of each dog's sham and PRFMembrane-treated tendons were taken.

*Histologic Evaluation*

After routine histologic processing, 5-μm-thick sections of the proximal segments of 2 dogs PTs from each time period were sectioned in the coronal plane and the distal segments were sectioned in the transverse plane, and the vice versa was true for sectioning the PTs from the other 2 dogs from each time period. Digital photographs of the transverse hematoxylin and eosin (H&E) –stained histologic sections at the midpoint of each PT were taken and the cross-sectional area of each PT (excluding the fat pad) was

measured, where the pixels in the digital photographs were converted to area measurements using Scion Image (Scion Corporation, Frederick, MD) computer software (Figure 3.3). A modified version of a semiquantitative histologic tendon pathology scoring system(28) (Table 3.1) was used to compare tendon healing in the central-third defect, designated "repair tissue (RT)" and in the native patellar tendon, designated "tendon tissue (TT)". From 4 different sections of the same tendon, both the RT and TT were assigned an ordinal score based upon the following scoring variables: cellularity, vascularity, collagen organization, and glycosaminoglycan content. The histologic sections were evaluated by one of the authors (LCV) who was blinded to the identity of the animal and its treatment. The sum of the mean histologic scores for each variable was used to obtain a total histologic score for both the RT and TT from each patellar tendon. All sections were stained with H&E, except those evaluated for glycosaminoglycan content, where sections were stained with Alcian blue (pH 2.5)/periodic acid-Schiff. Collagen organization was evaluated using coronal sections under polarized light.

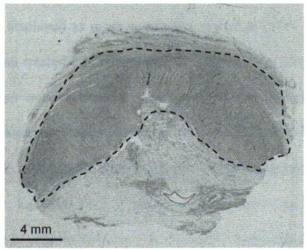


**Figure 3.3** Photograph of a patellar tendon after histologic processing mounted in cross-section representing an example of how the cross-sectional area (area within the dotted line) data were gathered in this study. The number of pixels within the dotted line was converted to area measurements using imaging software.

**Table 3.1**
**Semiquantitative Histologic Scoring System**

| Variable | Score | | | |
|---|---|---|---|---|
| | 0 | 1 | 2 | 3 |
| Cellularity | <100 cells/HPF[a] | 100-199 cells/HPF | 200-299 cells/HPF | >300 cells/HPF |
| Vascularity | Normal, vessels parallel to collagen fibers | Slight increase, transverse vessels in tendon tissue | Moderate increase within the tendon tissue | Severe increase, including clusters |
| Collagen Organization[b] | Organized, uniform, linear, parallel fibers, coarse even crimp | Moderately organized, >50% linear & uniform, fine even crimp | Slightly organized, 20-50% linear & uniform, <50% crimp | Disorganized, non-linear, complete disarray, no crimp |
| GAG content[c] | No alcianophilia | Slight alcianophilia between collagen fibers | Moderate alcianophilia | Severe alcianophilia forming blue lakes |

[a]High-power field (HPF) = 200x original magnification, [b]coronal sections assessed with polarized light, [c]alcian blue (pH 2.5)/periodic acid-Schiff staining, GAG = glycosaminoglycan. Adapted from ref. (28)

*Statistical Analysis*

All data are displayed as mean ± standard deviation (SD). Mean cross-sectional area data of sham and treated PTs within each time point were compared using a paired Student's *t*-test. Mean cross-sectional area data of sham and treated PTs were compared over time with an unpaired Student's *t*-test. Means from the histologic scoring system were compared with a Wilcoxon signed-rank test using a statistical software program (GraphPad Software, La Jolla, CA). A power analysis determined that 4 dogs per time period would suffice in order to detect a 33% difference in the quality of tissue repair at a confidence level of 95% and a statistical power of 0.8. Values of $P \leq 0.05$ were considered significant for all comparisons.

## Results

The post-operative period was uneventful and all of the animals were ambulatory and weight-bearing following the recovery period. There were no major clinical complications related to the surgical procedures, and lameness was not observed beyond the first post-operative week.

*Gross Healing Assessment & Cross-sectional Area*

Gross examination of the PTs immediately following euthanasia revealed that after 4 weeks, the central-third PT defect in both groups was filled in with repair tissue, which appeared contiguous with the retropatellar fat pad. Hyperemia was evident within the surrounding PT tissue by 4 weeks but was less obvious by 8 weeks. At both time points the PRFMembrane-treated tendons exhibited a more abundant healing response than the controls (Figure 3.4). The cross-sectional area of the PRFMembrane-treated tendons was at least 2.5-fold greater than the sham tendons at both time points (Figure 3.5). At the 4-week time point, the cross-sectional area of the PRFMembrane-treated tendons ($182 \pm 48$ mm$^2$) was significantly greater ($P = 0.001$) than the sham tendons ($73 \pm 47$ mm$^2$). Similarly, at the 8-week time point, the cross-sectional area of the PRFMembrane-treated tendons ($216 \pm 51$ mm$^2$) was significantly greater ($P = 0.012$) than the sham tendons ($62 \pm 28$ mm$^2$). The cross-sectional area of the treated and sham tendons did not significantly differ ($P = 0.383$ and $P = 0.711$, respectively) with time.

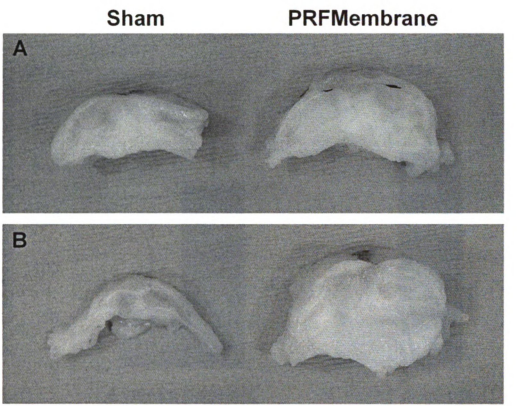


**Figure 3.4** Representative photographs of cross-sectional tissue sections of a sham and PRFMembrane-treated patellar tendons at 4 (A) and 8 (B) weeks after surgery. Note the difference in size of the PRFMembrane-treated tendons compared to its surgical control (sham).

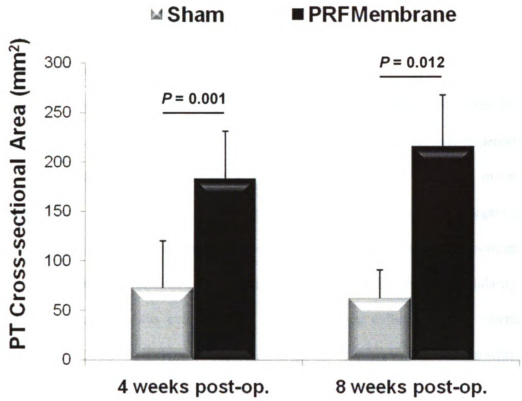


**Figure 3.5** Mean cross-sectional area of the sham and PRFMembrane-treated patellar tendons 4 and 8 weeks post-operation ± SD (n = 4).

*Histologic Healing Assessment*

When comparing the sham and PRFMembrane-treated tendons at each time point, they appeared histologically similar (Figure 3.6). After comparing the sham and PRFMembrane-treated tendons using the semiquantitative tendon pathology scoring system, there was no significant difference ($P > 0.05$) in any of the individual scoring variables (cellularity, vascularity, collagen organization, or glycosaminoglycan content) or the total histologic score (sum of the individual variables) in the RT or TT at either time point (Table 3.2). Histologic analysis confirmed the gross observations that by 4 weeks the defects in both groups had filled in with a hypercellular fibrovascular repair

tissue. The origin of the RT in both groups appeared to be predominantly from a proliferative response arising from the retropatellar fat pad and, to a lesser extent, the paratenon (Figure 3.7). In addition to repair cells, it appeared that the fat pad contributed a significant amount of the blood supply to the RT within the central-third defect of both groups at 4 weeks and to a lesser extent at 8 weeks. At 4 weeks the RT was hypercellular with a whorled fibroblastic appearance, and blood vessels were abundant in several planes in each group. Although there was some evidence of collagen organization (moderate linearity and crimp) at 4 weeks, overall, the collagen in the RT of both groups was disorganized and immature. By 8 weeks both groups exhibited less cellularity and vascularity, and there was evidence of a more organized collagen pattern (increased linearity and crimp). An increase in glycosaminoglycan staining was also noted with time in the RT of both groups. There was evidence of fibrous metaplasia within the retropatellar fat pad of both groups by 4 weeks. This finding persisted in the 8 week specimens as well.

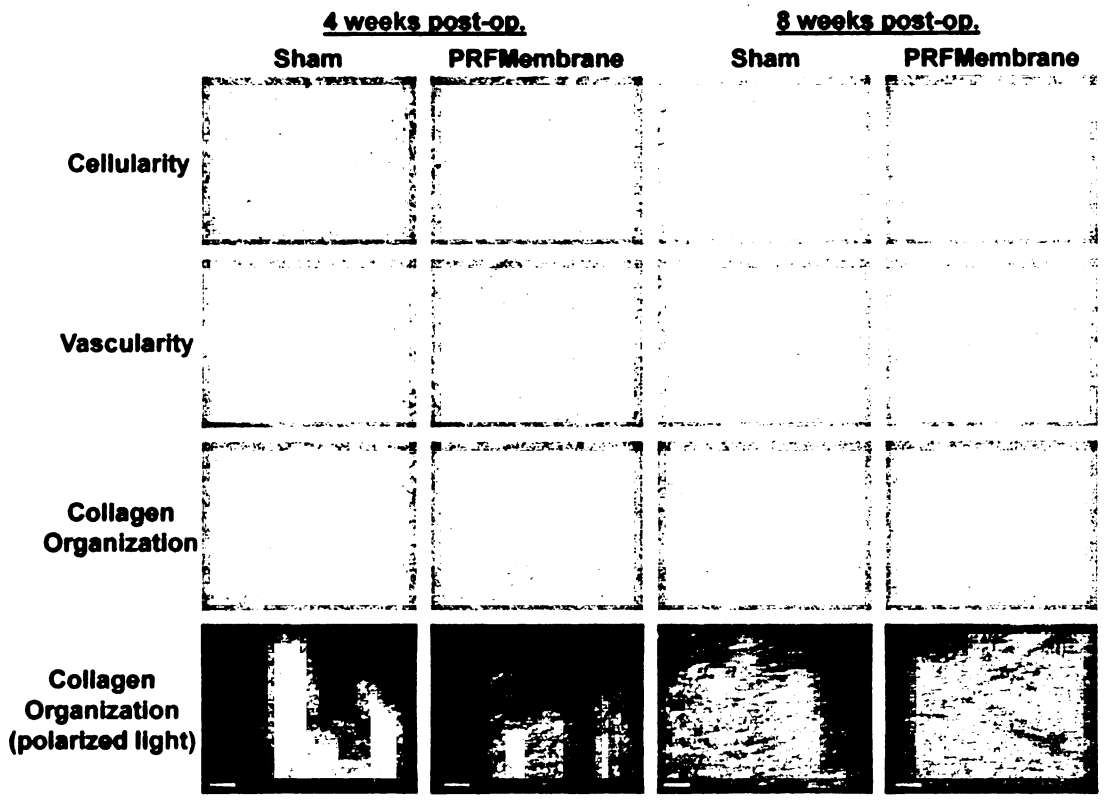


**Figure 3.6** Photomicrographs of representative H&E-stained sections of the repair tissue (RT) within the central-third patellar tendon defect of the sham and PRFMembrane-treated patellar tendons at each time point. The cellularity, vascularity, collagen organization, and glycosaminoglycan content (images not shown) of the PRFMembrane-treated tendons were similar when compared to the sham tendons at each time point. The white arrows denote blood vessels. Scale bar = 100 μm.

**Table 3.2**

**A**

**4 weeks post-op.**

| | *Repair Tissue (RT)* | | | *Tendon Tissue (TT)* | | |
|---|---|---|---|---|---|---|
| Variable (0-3) | Sham | PRFMem | *P*-value | Sham | PRFMem | *P*-value |
| Cellularity | 2.7 ± 0.1 | 2.6 ± 0.4 | >0.99 | 1.6 ± 0.9 | 1.9 ± 0.5 | 0.50 |
| Vascularity | 2.1 ± 0.5 | 2.6 ± 0.6 | 0.25 | 1.9 ± 0.7 | 2.0 ± 0.7 | 0.50 |
| Collagen | 2.8 ± 0.3 | 2.3 ± 0.4 | 0.10 | 1.1 ± 0.4 | 1.2 ± 0.4 | 0.85 |
| GAGs | 0.8 ± 0.4 | 1.1 ± 0.3 | 0.50 | 0.9 ± 0.5 | 1.1 ± 0.5 | 0.85 |
| Total (0-12) | 8.3 ± 0.5 | 8.6 ± 0.8 | 0.63 | 5.2 ± 2.3 | 6.2 ± 1.5 | 0.63 |

**B**

**8 weeks post-op.**

| | *Repair Tissue (RT)* | | | *Tendon Tissue (TT)* | | |
|---|---|---|---|---|---|---|
| Variable (0-3) | Sham | PRFMem | *P*-value | Sham | PRFMem | *P*-value |
| Cellularity | 1.5 ± 0.5 | 1.8 ± 0.4 | 0.50 | 1.4 ± 0.3 | 1.8 ± 0.4 | 0.50 |
| Vascularity | 1.9 ± 0.8 | 2.0 ± 0.5 | >0.99 | 1.8 ± 0.4 | 1.4 ± 0.5 | 0.50 |
| Collagen | 2.4 ± 0.1 | 2.3 ± 0.4 | 0.77 | 0.9 ± 0.3 | 1.3 ± 0.8 | 0.50 |
| GAGs | 1.4 ± 0.8 | 2.3 ± 0.7 | 0.13 | 0.7± 0.4 | 1.3 ± 0.5 | 0.25 |
| Total (0-12) | 7.2 ± 1.1 | 8.4 ± 1.1 | 0.50 | 4.7 ± 0.3 | 5.7 ± 2.1 | >0.99 |

**Table 3.2** Results of the semiquantitative histologic scoring system used to assess tendon healing at 4 (A) and 8 (B) weeks post-operation. The ordinal data is displayed as mean score ± SD (n = 4). An unoperated ("normal") tendon would have a perfect score of 0. Repair tissue (RT) refers to the regenerated healing tissue within the central-third patellar tendon defect and tendon tissue (TT) refers to the native patellar tendon tissue. GAGs = glycosaminoglycans, PRFMem = platelet-rich fibrin membrane.

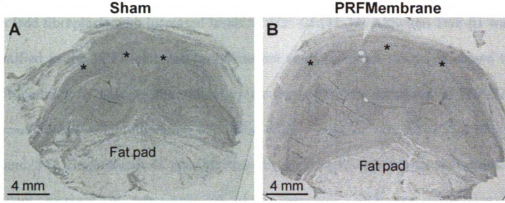


**Figure 3.7** Representative photographs of the sham (A) and PRFMembrane-treated (B) patellar tendons 4 weeks after surgery mounted in cross-section. Note that the origin of the repair tissue was primarily from a proliferative response arising from the fat pad and paratenon (asterisks) in both groups, albeit more abundant in the PRFMembrane group.

## Discussion

The results of the current study did not support the hypothesis that a PRFMembrane would enhance the quality and rate of healing of a central-third patellar tendon defect when compared to control defects. While both the control and PRFMembrane-treated defects healed with a fibrovascular repair tissue of similar histologic quality, the cross-sectional area of the PRFMembrane-treated tendons was significantly greater (at least 2.5-fold) than that of the controls at both 4 and 8 weeks.

The increase in cross-sectional area in the PRFMembrane-treated defects may be associated with the prolonged presence of increased levels of growth factors, including TGF-β1. Transforming growth factor-β1 plays a major role in the early phases of wound healing where it is involved in the recruitment of inflammatory cells and fibroblasts and, at later stages, promotes collagen production.(29) Transforming growth factor -β1 is known to be secreted from platelets as a latent precursor where continuous latent activation is thought to occur up to 14 days post-injury.(29) Platelets may provide a long-term source of TGF-β1 activity, as they are thought to activate only a small fraction of

the latent TGF-β1they release.(30) Moreover, numerous studies have associated increased fibroplasia with increased levels of TGF-β1.(31-33) Although TGF-β1 was not quantified in the PRFMembrane in the current study, a recent study has shown that a PRFMembrane is able to elute significantly increased concentrations of TGF-β1 and enhance tendon cell proliferation over time when compared to a blood clot of similar volume.(34) Therefore, it is possible that the significant increase in fibroplasia (and resultant increase in cross-sectional area) associated with the application of a PRFMembrane in the current study could be the result of increased levels (and a prolonged duration) of TGF-β1available to the repair cells.

In addition to the chemotactic and mitogenic stimuli provided to tendon cells by increased levels of various growth factors in PRP,(11, 14, 18, 35) it has been suggested that the addition of PRP in the initial period following acute tendon injury could actually exacerbate inflammation and pain.(10) A recent study using PRP to treat acute skin wounds of horses found that the PRP led to the development of excess (granulation) tissue and actually slowed wound healing.(36) The authors suggested that the development of excess granulation tissue could be due to a prolonged expression of TGF-β1 from PRP via two mechanisms. First, although platelets secrete approximately 95% of their growth factors within 1 hour after activation, they have demonstrated to continuously synthesize small amounts of growth factors for the remainder of their lifespan (5-10 days).(20, 37) Secondly, it was noted that TGF-β1, unlike other growth factors, can regulate its own production by monocytes and activated macrophages in an autocrine manner, resulting in a persistent expression at the wound site following a single exogenous application.(38) These results suggest that because of the increased

concentrations of growth factors present in PRP, such preparations may be better suited for more chronic wounds where a fresh exogenous source of growth factors would be more beneficial.(36)

A recent experimental study(39) on the spatiotemporal expression of TGF-β1 after an acute PT injury (transverse incision of the medial-half of the mid-body patellar tendon) may also help explain the abundant repair tissue witnessed. The study found that the expression of TGF-β1 propagates out and away from the wound site to the nearby uninjured tendon tissue with time and as healing progresses. Interestingly, the authors also noted that the expression of TGF-β1 is transiently enhanced over the entire length of the patellar tendon as early as 7 days post-injury.(39) Therefore, the reported spatiotemporal mechanism of TGF-β1expression, coupled with the prolonged elution of increased concentrations of TGF- β1 from the PRFMembrane may, help explain the abundant healing tissue witnessed in the current study.

In this study the healing response in both the control and PRFMembrane-treated defects appeared to arise predominantly from the retropatellar fat pad. An increase in cross-sectional area in conjunction with a significant amount of repair tissue originating from the fat pad has been documented by numerous investigators using similar central-third patellar tendon defect models.(40-45) The retropatellar fat pad is thought to significantly contribute to PT healing due to their close proximity and shared blood supply.(46, 47) In addition to contributing a blood supply, it is possible that the fat pad also contributes reparative progenitor cells to the patellar tendon healing process, as the fat pad has been recently demonstrated to be a significant source of highly proliferative

adipose-derived mesenchymal stem cells potentially able to respond to tendon injury.(48, 49)

A previously published study using a similar defect model reported healing of the defects at three months post-operation.(42) However, contrary to our hypothesis, the PRFMembrane did not accelerate the healing or enhance the quality of the repair tissue in a canine central-third PT defect at the earlier time periods examined in the current study. This may have occurred because the central-third PT defect in this model was not of critical size as the control defect was filled in with a similar quality repair tissue at the earliest time point examined in this study. The results of the current study suggest that since the retropatellar fat pad appears to provide an adequate supply of repair cells, vasculature, and growth factor stimulus in acute PT defects, the PRFMembrane is not a significant adjunct to the healing process in these situations. It is possible that the PRFMembrane may be of more benefit in biologically compromised or chronically injured tissues where a fresh source of bioactive molecules may be needed for longer periods of time in order to enhance the repair process. A similar conclusion was reached in a study comparing the use of PRP in the healing of acute cutaneous wounds.(36) In that study, the histologic quality of the control and PRP-treated defects was also not significantly different.(36)

The outcome metrics in the current study were limited to the semiquantitative histologic assessment of the repair tissue using a previously published grading scale.(28) While only 4 animals in each group were evaluated at each time period, an *a priori* power analysis revealed that this number would be sufficient to detect a 33% difference in repair tissue quality, which was felt to be clinically relevant. The absence of any functional

assessment, such as biomechanical analysis of the healed tendons, could also be considered a limitation of this study. Since the animals did not show any obvious untoward clinical signs (i.e., abnormal activity or gait) related to the surgical procedure, a clinically relevant impact on joint function was not apparent. Finally, the 4 and 8 week time points used in the current study did not permit any assessment of the long-term impact of PRFMembrane augmentation on the remodeling of the repair tissue.

In summary, the PRFMembrane did not enhance the rate and quality of healing in central-third PT defects at either 4 or 8 weeks post-operation. While the PRFMembrane produced an increased amount of fibroblastic repair tissue, as determined by a significant increase in cross-sectional area, this tissue was not significantly different, with respect to cellularity, vascularity, collagen organization, and glycosaminoglycan content from the naturally occurring repair tissue. It is possible that a PRFMembrane may be of more benefit in larger defects where a naturally occurring provisional fibrin scaffold may not adequately fill the defect or in biologically compromised tissues where the prolonged release of growth factors may be required to induce and sustain a repair response from adjacent tissues. Further basic science and experimental studies are warranted to help determine the precise role of PRP preparations in tendon healing.

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CHAPTER 4.

# Growth Factor-Rich Plasma Increases Tendon Cell Proliferation and Matrix Synthesis on a Synthetic Scaffold: An *In Vitro* Study

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**Abstract**

Numerous scaffolds have been proposed for use in connective tissue engineering. Although these scaffolds direct cell migration and attachment, many are biologically inert and thus lack the physiological stimulus to attract cells and induce mitogenesis and matrix synthesis. In the current study, a bioactive scaffold was created by combining a synthetic scaffold with growth factor-rich plasma (GFRP), an autologous concentration of growth factors derived from a platelet-rich plasma preparation. *In vitro* tendon cell proliferation and matrix synthesis on autologous GFRP-enriched scaffolds, autologous serum-enriched scaffolds, and scaffolds alone were compared. The GFRP preparation was found to have a 4.7-fold greater concentration of a sentinel growth factor (TGF-β1) compared to serum. When combined with media containing calcium, the GFRP produced a thin fibrin matrix over and within the GFRP-enriched scaffolds. Cell proliferation assays demonstrated that GFRP-enriched scaffolds significantly enhanced cell proliferation over autologous serum and control groups at both 48 and 72 hours. Analysis of the scaffolds at 14, 21, and 28 days revealed that GFRP-enriched scaffolds significantly increased the deposition of a collagen-rich extracellular matrix when compared to the other groups. These results indicate that GFRP can be used to enhance *in vitro* cellular population and matrix deposition of tissue-engineered scaffolds.

## Introduction

Scaffolds play an essential role in engineering biological substitutes for the body *in vitro* and/or orchestrating tissue regeneration and remodeling *in vivo.*(1) To date, a variety of scaffolds derived from both natural and synthetic materials have been proposed for use in connective tissue engineering.(2) While these scaffolds provide a conductive surface for cell attachment and migration, many are biologically inert and thus lack the ability to induce chemotaxis, stimulate cell proliferation, and coordinate matrix synthesis on their own. The addition of bioactive factors to such materials could result in more rapid cellular repopulation and matrix synthesis *in vitro* and, in turn, optimize scaffold incorporation *in vivo*. Previous studies have incorporated recombinant growth factors, transfected cells, or plasmid DNA with polymer or hydrogel scaffolds in an attempt to increase the bioactivity of these scaffolds.(3) Although these techniques appear promising for the controlled release of specific growth factors, questions regarding the choice and concentration of growth factors to be utilized have yet to be answered.(4, 5)

One potential method for providing the entire complex of growth factors involved in tissue repair and regeneration might be through the use of platelet-rich plasma (PRP). Platelet-rich plasma is defined as an autologous concentration (above baseline) of platelets and their associated growth factors.(6) Several recent studies have demonstrated the effectiveness of PRP in the repair and regeneration of a variety of tissues including bone,(7-9) cartilage,(10-12) tendon,(13-18) and ligament.(19-22) The use of PRP for tissue engineering applications is appealing because it is safe (autologous), easily obtainable, and a cost-effective source of bioactive molecules.(23, 24) Most importantly, PRP not only provides an increased concentration of several growth factors, it also

maintains the physiologic proportions of individual growth factors to each other.(6) Maintaining the physiologic proportional relationship between growth factors is thought to be important in tissue regeneration.(25, 26) Combining the increased concentration of growth factors found in PRP with a synthetic scaffold could create a bioactive construct that is able to optimize cellular repopulation and matrix synthesis onto the scaffold *in vitro.*

Therefore, the purpose of this study was to evaluate the ability of growth factor-rich plasma (GFRP) -enriched scaffolds to induce tendon cell proliferation and matrix synthesis compared to serum-enriched scaffolds and scaffolds alone. Growth factor rich plasma was created by degranulating the platelets in a standard PRP preparation by a freeze-thaw process thereby liberating all the growth factors contained in the platelets. It was our hypothesis that GFRP-enriched scaffolds would significantly enhance cell proliferation and matrix synthesis when compared to serum-enriched scaffolds and scaffolds alone.

## Materials and Methods

*Scaffold Composition and Preparation*

The scaffold used in this study was a synthetic biodegradable poly-L-lactic acid (PLLA) scaffold constructed in a woven pattern that is used for tendon and connective tissue repair (Synthasome Inc., San Diego, CA). Eight-mm-diameter disks were isolated from a larger scaffold sample with a heated biopsy punch (Figure 4.1). Scaffold-disks were soaked in a 70% ethanol solution, rinsed with phosphate buffered saline (PBS), and then air-dried overnight under UV light to sterilize.

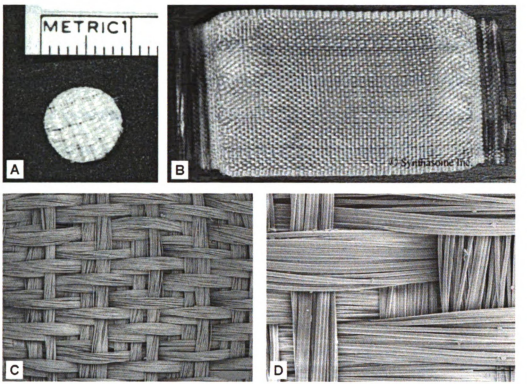


**Figure 4.1** Photographs of the synthetic biodegradable polymeric scaffold used in this study showing an 8-mm-diameter scaffold-disk (A) isolated from a larger sample of the scaffold (B). Scanning electron photomicrographs of the scaffold at 20x (C) and 100x (D) magnification.

*Preparation of Growth Factor-Rich Plasma (GFRP) and Serum*

GFRP was prepared from 18 mL of canine whole blood as follows. Blood was collected via jugular venipuncture with a 19G butterfly catheter in 9 mL increments in two separate vacuum tubes containing trisodium citrate. Red blood cells and plasma containing platelets (supernatant) were separated by centrifugation for 6 minutes at 1,100 *g*. The supernatant from both tubes was then transferred to one 15 mL test tube. Platelets were concentrated in the plasma with a second centrifugation step at 3,000 *g* for 15 minutes. A platelet pellet was evident and the platelets were resuspended in 2 mL of plasma. The resultant PRP was snap-frozen in liquid nitrogen and then allowed to thaw to

room temperature. The freeze-thaw process was repeated to ensure total platelet activation and thus release the maximum amount of growth factors available from the platelets.(27) The resulting preparation was termed growth factor-rich plasma and was always used immediately following its creation. Serum was obtained from 9 mL of whole blood at the same time and from the same animal used to create GFRP. The collection method was similar, except blood was drawn into a plain vacuum tube, allowed to coagulate for at least 2 hours at room temperature, and then centrifuged for 15 minutes at 3,000 *g*. Samples of both blood products were separated into aliquots and stored at -80°C until assayed for growth factor concentration.

*Isolation of Tendon Cells*

Canine patellar tendon fibroblasts were harvested via primary explant cultures from adult mongrel dogs euthanized for reasons unrelated to this study. Cells were expanded to passage two in 75 $cm^2$ tissue culture flasks in Dulbecco's modified Eagle medium (DMEM) containing 1% antibiotic-antimycotic solution, 0.02 mg/mL gentamicin, and 0.15 mg/mL ascorbate (DMEM media) containing 10% fetal bovine serum (FBS) incubated at 37°C in a humidified atmosphere containing 10% $CO_2$. Cells in each flask were cultured until sub-confluent and then detached by trypsinization for scaffold seeding. Only cells from the third passage were used for this study.

*Scaffold Enrichment and Cell Culture*

Scaffolds were divided into three treatment groups and 50 μL of either: 1) DMEM media containing 2% FBS (control), 2) serum, or 3) GFRP was pipetted onto the surface

of the scaffolds in a 24-well tissue culture plate. Scaffolds were allowed to soak in their respective treatments for approximately 10 minutes. Next, $10^5$ canine patellar tendon fibroblasts suspended in DMEM media containing 2% FBS were drip-seeded onto the scaffolds and maintained in DMEM media containing 2% FBS (total volume per well was 2 mL) at 37°C in a humidified atmosphere containing 10% $CO_2$. Upon the addition of the culture media, the calcium in the media triggered the conversion of pro-thrombin to thrombin and the clotting cascade.(28) The subsequent polymerization of fibrin produced a thin coating of fibrin over the surface and within the interstices of the scaffolds (Figure 4.2).

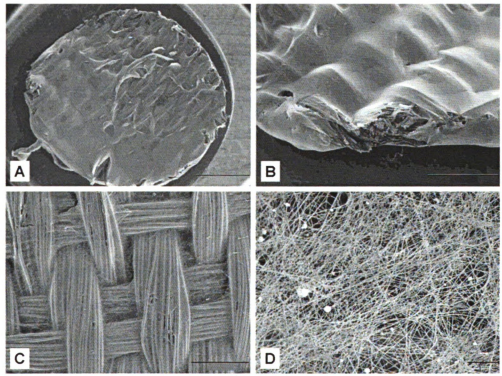


**Figure 4.2** Scanning electron photomicrographs of a GFRP-enriched scaffold after combining with cell culture media and prior to cell-seeding at 20x (A), 50x (B), 80x (C), and 3500x (D) magnification. Notice the thin fibrin-matrix coating throughout the scaffold surface that results from fibrin polymerizing around the scaffold when combined with culture media containing calcium.

After 72 hours, the scaffolds were maintained in DMEM media containing 10% FBS and the media was replenished every other day throughout the remainder of the study. Scaffolds were transferred to new plates once per week to avoid cell confluence around the scaffolds in the bottom of the wells. Care was taken to maintain the orientation of the scaffolds; i.e., the cell-seeded side of the scaffold was designated "surface."

*TGF-β1 Enzyme-Linked Immunosorbent Assay*

TGF-β1 was used as a sentinel growth factor to document the extent of growth factor enrichment in GFRP compared to serum. The concentration of TGF-β1 in aliquots of GFRP and serum from the same dog was compared using a commercial sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (MB100B, R&D Systems, Minneapolis, MN). Sample dilutions were determined empirically.

*DAPI and Rhodamine Phalloiden Staining*

To compare cell density on the scaffolds 24 hours post-cell seeding, cells on the scaffolds (n=3/group) were fixed in 10% neutral-buffered formalin for 20 minutes. The cells were rinsed with PBS and then incubated in 10% sucrose for 15 minutes. After a second rinsing with PBS, cellular actin filaments were stained with rhodamine phalloidin (50 U/mL) (Invitrogen, Carlsbad, CA) for 20 minutes. Scaffolds were mounted cells-up on a glass slide following an additional PBS rinse. Vecta-mount with DAPI (4',6-diamidino-2-phenylindole) (Vector, Burligame, CA) was added to counter-stain the

nuclei of cells on each scaffold. Scaffolds were photographed through a #1 coverslip on an inverted microscope (Zeiss, Oberkochen, Germany).

*MTT Assay*

Tendon cell proliferation and viability throughout the scaffolds was compared using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay.(29) After 48 and 72 hours, scaffolds (n=5/group/time point) were transferred to a 24-well tissue culture plate prior to the assay. DMEM without phenol red and containing 0.5 mg/mL MTT (Sigma-Aldrich, St Louis, MO), and 2% FBS was added to each well, and the plates were incubated at 37°C in a humidified atmosphere containing 10% $CO_2$ for 4 hours. Next, the MTT solution was discarded and acidic isopropanol containing 1% Triton-X 100 was added to each well. After 40 minutes of mixing on an orbital shaker, aliquots from each well were transferred to a 96-well plate in triplicate and then read immediately at 570 nm in a scanning multiwell spectrophotometer (Bio-Tek Instraments, Winooski, Vermont).

*Histological Evaluation*

After 14, 21, and 28 days in culture, scaffolds (n=4/group/time point) were processed for histology to compare tissue neogenesis on the scaffolds and cellular invasion into the scaffolds. All samples were fixed in 10% neutral-buffered formalin for at least 3 days. Samples were transected in half, processed for routine histology, and cut at 6-μm increments in cross-section. The sections were stained with hematoxylin and eosin (H&E) or picrosirius red (PSR).

Cross-sectional tissue thickness on the surface of the scaffolds was measured using Scion image software (Scion corp., Frederick, MD) at three separate locations from representative photomicrographs of H&E-stained sections from all of the scaffolds. All images were obtained with the 20x objective using a Zeiss Axioscope.

*Scanning Electron Microscopy*

To examine the morphology of GFRP-enriched scaffolds prior to cell-seeding, a representative sample was obtained and processed for scanning electron microscopy (SEM). Additionally, after 14, 21, and 28 days in culture, scaffolds (n=2/group/time point) were processed for SEM. All SEM samples were fixed in 4% glutaraldehyde buffered with 0.1M phosphate buffer (pH 7.4) for 36 hours at 4°C. After rinsing in the buffer, samples were dehydrated in a graduated ethanol series. Samples were then dried in a critical point drier (Balzers CPD, Lichtenstein), mounted, and osmium coated (Pure Osmium Coater, Neoc-An, Meiwa Shoji Co., Japan). Prepared samples were examined using a Jeol 6400V scanning electron microscope.

*Hydroxyproline and Dimethyl-Methylene Blue Assay*

Hydroxyproline and dimethyl-methylene blue assays were performed to compare the total collagen and glycosaminoglycan (GAG) content respectively amongst the scaffolds. The samples were digested with Proteinase K at 60°C and aliquots were taken for the hydroxyproline assay or dimethyl-methylene blue assay. To determine total collagen, the Proteinase K digest was hydrolyzed by heating with an equal volume of 12M HCl at 107°C for 18 hours, dried, and the hydrolysates were assayed for

hydroxyproline using a colorimetric procedure.(30) Total sulfated-GAG content was determined using the dye 1,9-dimethyl methylene blue.(31)

*Statistical Analysis*

MTT optical density values, surface tissue thickness, total collagen, and total GAG content were each analyzed with a one-way ANOVA within each time point followed by a Tukey's post hoc test. All data are displayed as mean ± standard error (SE). The threshold for statistical significance was set to $p < 0.05$ for all comparisons.

## Results

*Growth Factor-Rich Plasma*

The concentration of the sentinel growth factor (TGF-β1) in the GFRP preparation (315 ng/mL) used in the study was approximately 4.7-fold greater than the concentration of TGF-β1 in serum (67 ng/mL).

*Cell Proliferation*

Histologic examination of the scaffolds at 24 hours after cell-seeding using DAPI and rhodamine phalloidin staining demonstrated an obvious increase in cell density on the GFRP-enriched scaffolds compared to the other two groups (Figure 4.3). In the control and serum groups, cells appeared to be orientated along the axes of the polymer fibers (Figure 4.3A and B) while cells in the GFRP group appeared less restricted and were spread out over the thin fibrin matrix coating (Figure 4.3C).

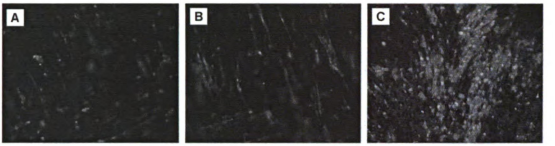


**Figure 4.3** Representative photomicrographs of the cell-seeded surface of a control (A) serum-enriched (B) and GFRP-enriched (C) scaffold stained rhodamine phalloidin and DAPI after 24 hours in culture. Note the dramatic increase in cell density of the GFRP-enriched scaffold compared to the other groups.

The results of the MTT assays performed on the scaffolds after 48 and 72 hours in culture are shown in Figure 4.4. Optical density values indicating relative cell proliferation and viability were significantly higher for the GFRP-enriched scaffolds compared to serum-enriched scaffolds ($p \leq 0.029$) and controls ($p < 0.0001$) at both time points examined. At 72 hours the GFRP-enriched scaffolds had a 7.8-fold higher optical density value compared to the controls. Serum-enriched scaffolds also induced a significant ($p \leq 0.013$) increase in cell proliferation compared to controls at both time points.

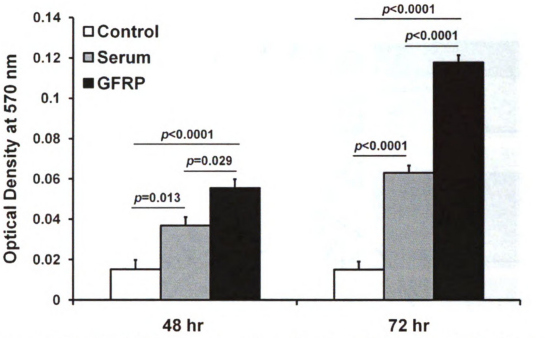


**Figure 4.4** Results of the MTT assays performed on the scaffolds after 48 and 72 hours in culture. Optical density values indicate relative cell proliferation. Bars represent mean optical density value ± SE (n = 5).

*Tissue Morphology and Morphometry*

Tissue neogenesis appeared most abundant on the surface of the GFRP-enriched scaffolds compared to the other groups at each time point examined (Figure 4.5). Figure 4.6 shows a comparison of the scaffolds in each group after 21 days in culture with H&E and picrosirius red (PSR) staining and SEM. Histological evaluation of the H&E-stained scaffolds showed that cells were able to invade into the scaffolds equally well in all three groups as cells were noted amongst the polymer fibers throughout the depths of the scaffolds in each group. Examination of sections stained with picrosirius red revealed that a large proportion of the extracellular matrix on the surface of the scaffolds was composed of collagen. Analysis of the scaffolds with SEM confirmed the observations found with histology.

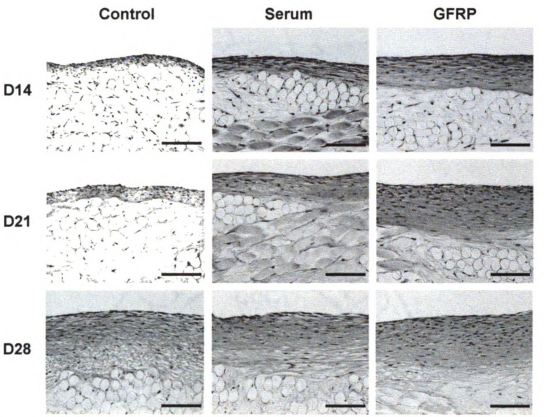


**Figure 4.5** Photomicrographs of representative transected scaffolds stained with hematoxylin and eosin (H&E) from each group after 14, 21, and 28 days in culture. Note the relative differences in the surface tissue thickness at each time point. Scale bar = 100 μm.

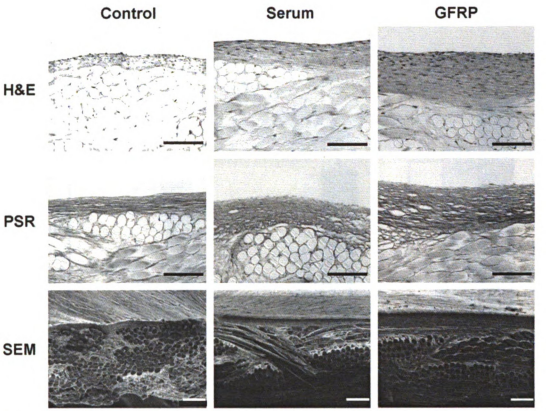


**Figure 4.6** Photomicrographs of representative transected scaffolds from each group after 21 days in culture. Tissue neogenesis on the surface of the scaffolds appeared most abundant on the GFRP-enriched scaffolds. Hematoxylin & Eosin (H&E) -staining (top row) showed that cells were able to invade into the scaffold equally well in all three groups. Picrosirius red (PSR) -staining (middle row) suggested that the majority of tissue generated was collagen. The bottom row shows scanning electron micrographs of the scaffolds in each group. Scale bar = 100 μm.

Surface tissue thickness of the GFRP-enriched scaffolds was significantly greater than controls ($p \leq 0.038$) at all three time points and significantly greater than serum-enriched scaffolds ($p < 0.0001$) at days 14 and 21 (Figure 4.7). Surface tissue thickness of serum-enriched scaffolds was significantly greater ($p \leq 0.017$) than controls at day 14.

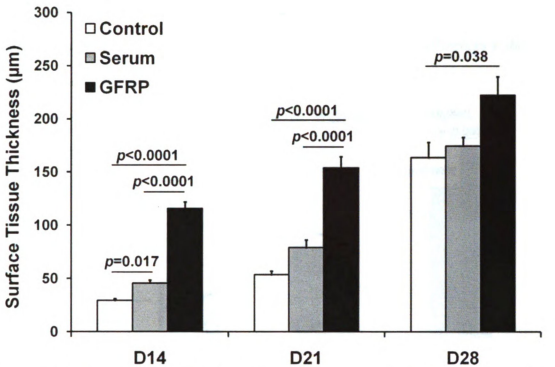


**Figure 4.7** Cross-sectional tissue thickness measurements on the surface of the scaffolds after 14, 21, and 28 days in culture. Bars represent the mean surface tissue thickness ± SE (n = 4). Statistical significance was not reached ($p \geq 0.05$) where $p$-values for comparisons are not shown.

*Total Collagen and GAGs*

The total amount of collagen in each group increased with time (Figure 4.8A). Both the serum-enriched and GFRP-enriched scaffolds had significantly ($p \leq 0.026$) greater amount of total collagen than the control scaffolds at each time point. Collagen content was significantly greater ($p < 0.0001$) on the serum-enriched scaffolds compared to the GFRP-enriched scaffolds at days 14 and 21. However, the GFRP-enriched scaffolds had a significantly greater ($p < 0.0001$) amount of collagen than the serum-enriched scaffolds at day 28.

The total sulfated-glycosaminoglycan (S-GAG) content remained unchanged with time (Figure 4.8B). At the day 14 time point, both the serum-enriched and GFRP-enriched scaffolds contained significantly more ($p < 0.0001$) S-GAGs than the controls,

and the serum-enriched scaffolds contained significantly more ($p < 0.0001$) S-GAGs than the GFRP-enriched scaffolds. There were no statistically significant differences at the other two times points examined.

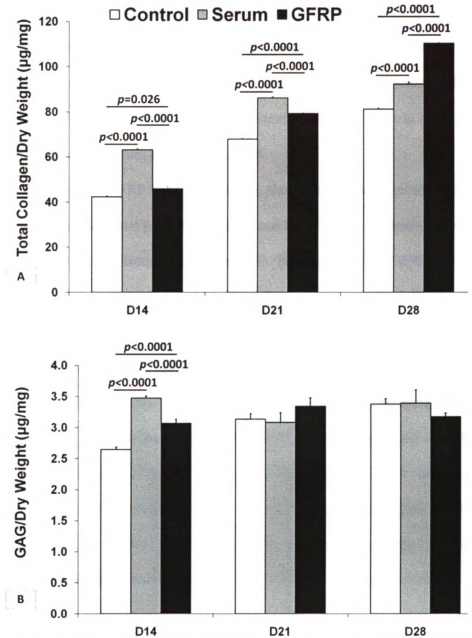


**Figure 4.8** Total collagen (A) and GAG (B) deposition by tendon cells seeded onto plain scaffolds (control), serum-enriched scaffolds, or GFRP-enriched scaffolds after 14, 21, and 28 days in culture. Bars represent the mean total collagen (A) or GAG (B) content among the scaffolds ± SE (n = 4). Statistical significance was not reached ($p \geq 0.05$) where $p$-values for comparisons are not shown.

## Discussion

Combining growth factors known to stimulate tissue repair and regeneration with scaffolds has the potential to significantly enhance host incorporation of scaffolds and more efficiently regenerate tissues.(3) The results of the current study suggests that GFRP-enriched scaffolds significantly enhanced early cell proliferation and collagen matrix synthesis on a synthetic scaffold when compared to serum-enriched scaffolds and scaffolds alone. However, GFRP failed to consistently enhance GAGs in the matrix over time when compared to serum-enriched scaffolds.

The creation of GFRP from platelet-rich plasma by the addition of a freeze-thaw step was done to ensure total platelet activation and thus release the maximum amount of growth factors available from the platelets.(27) This produced a GFRP solution that contained a 4.7-fold increase in the concentration of a sentinel growth factor (TGF-β1) compared to autologous serum. The rationale for using only a single growth factor to document the increase in concentration achieved by the creation of the GFRP is based on the significant association that has been demonstrated between platelets and TGF-β1 concentrations.(32, 33) Similar results have been reported regarding the ability to concentrate TGF-β1(10, 17, 33-35) in addition to other growth factors such as platelet-derived growth factor (PDGF)(17, 33, 34, 36) and vascular endothelial growth factor (VEGF)(34, 36) in various PRP preparations compared to other blood-derived components. While the concentration of specific mitogenic growth factors (e.g., PDGF, insulin-like growth factor (IGF) -1, and basic fibroblast growth factor (bFGF)) normally stored in platelet α-granules were not measured in the current study, the increase in cell proliferation observed as a result of the addition of GFRP indirectly suggests that these

mitogenic factors might also be increased. Thus, it is likely that, similar to platelet-rich plasma (PRP), GFRP provides the entire compliment of growth factors present in serum but in significantly higher concentrations.(6)

Several investigators have examined the ability of individual growth factors or various combinations of growth factors to increase tendon cell proliferation and matrix synthesis in a dose-dependent manner *in vitro*.(37-40) The results of the current study are consistent with other investigators who examined the ability of PRP preparations to increase tendon cell proliferation and matrix synthesis over time.(34, 36, 41) Most importantly, PRP not only provides an increased concentration of several growth factors, it also maintains the normal relative proportions of individual growth factors to each other.(6) Maintaining the normal proportional relationship between growth factors is thought to be important in encouraging tissue regeneration over scar formation.(25, 26)

The ability of GFRP-enriched scaffolds to significantly increase cell density within 24 hours of cell-seeding could be related to an increase in chemotactic growth factor concentration as well as the creation of an optimal surface for cell attachment by way of a fibrin coating on the polymer surface. As a result of combining GFRP with media containing calcium, the clotting cascade was initiated creating a thin fibrin matrix coating throughout the scaffolds. Fibrin has been shown to be an excellent provisional scaffold in a variety of applications providing a naturally-derived matrix for cell adhesion/attachment and migration.(42) Several elegant studies have demonstrated that fibrin coating of synthetic substrates resulted in an increase in initial cell adherence which, in turn, led to an increase in cellular proliferation.(43-46) Therefore, it is possible that the fibrin coating of the polymeric scaffold created by the plasma component of the

GFRP enhanced initial cell adherence and thus contributed to the increased cellular proliferation observed in this study.

While fibrin provides an excellent conductive surface for cells, fibrin itself (in the absence of growth factors) does not induce cell proliferation and promote cell viability.(47, 48) Additionally, the concentration of adhesion proteins such as fibronectin is not increased in fresh frozen plasma when compared to serum.(49) Therefore, the significant increase in cell and tissue proliferation seen in the GFRP group in the current study may not be a result of the fibrin coating alone but rather the combination of increase initial cell adhesion as well as the increased concentration of mitogenic growth factors present in the fibrin scaffold.(33, 34, 38, 40) Fibrin has been shown to indirectly bind growth factors, potentially creating a growth factor reservoir and prolonging their biological activity.(50) While the potential elution of growth factors from the precipitated fibrin matrix was not evaluated in the currently study, it is possible that a sustained release of mitogenic agents could occur. Additional studies are needed to evaluate this possibility.

In conclusion, the results of this study suggest that growth factor-rich plasma can enhance the bioactivity of a synthetic scaffold *in vitro*. The increase in growth factor concentration of GFRP over normal serum levels as well as the fibrin coating of the polymeric surfaces created by the interaction of the GFRP and the calcium within the culture media contributed to enhanced cell adhesion and proliferation as well as enhanced matrix synthesis on the surface of a bio-inert scaffold when compared to serum-enriched scaffolds and controls. While GFRP was able to significantly increase cell proliferation and collagen matrix deposition over serum-enriched controls, the *in vivo* benefit of this

effect has yet to be proven. Additional studies are needed to determine the ability of GFRP to accelerate *in vivo* host incorporation and remodeling of tissue-engineered scaffolds.

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CHAPTER 5.

# Concluding Discussion

## Concluding Discussion

The goal of this thesis was to investigate the role of an autologous platelet-rich fibrin matrix (PRFM) in enhancing growth factor delivery for connective tissue healing. Combining platelets and their associated growth factors with a fibrin scaffold represents a promising method to enhance growth factor delivery for tissue repair and tissue engineering applications. The work presented in this thesis supports the hypothesis that a PRFM will enhance growth factor delivery for connective tissue repair and regeneration. However, as evident in chapter 3, the benefit of a PRFM may be situation-dependent and be of greater benefit in chronically injured or biologically compromised tissues.

The results of the study 1 presented in chapter 2 of this thesis demonstrated that two different formulations of the PRFM (PRFMatrix and PRFMembrane) were able to elute a significantly greater concentration of a sentinel growth factor (TGF-β1) over time when compared to whole blood clots of similar volume. The eluents from the platelet-rich fibrin constructs were also able to significantly increase tendon cell proliferation over time when compared to whole blood clots of similar volume. This sustained increase in growth factor elution from the PRFM *in vitro* supports the idea that a PRFM may be beneficial for connective tissue repair *in vivo*, particularly in tissues where a prolonged increase in increased concentrations of growth factors is desired.

Study 2 presented in chapter 3 examined the use of a PRFM, specifically the PRFMembrane, *in vivo*. The PRFMembrane did not enhance the rate and quality of healing in central-third patellar tendon defects at either 4 or 8 weeks post-operation. While the PRFMembrane produced an increased amount of fibroblastic repair tissue, the quality of this tissue was not significantly different from the naturally occurring repair

tissue when assessed histologically using a semiquantitative scoring system. The results of study 2 suggest that a PRFMembrane may be of more benefit in larger defects where a naturally occurring provisional fibrin scaffold may not adequately fill the defect or in biologically compromised tissues where the prolonged release of growth factors may be required to induce and sustain a repair response from adjacent tissues.

In chapter 4 a bioactive scaffold using PRFM-related technology, termed growth factor-rich plasma (GFRP), for tissue engineering applications was created. The GFRP-enriched scaffolds significantly enhance early cell proliferation and the deposition of a collagen-rich extracellular matrix over time compared to serum-enriched scaffolds and controls. The results of this study indicate that a PRFM-related preparation (i.e., GFRP) can be combined with a synthetic scaffold to create a bioactive scaffold. This scaffold can potentially be used for tissue engineering applications to enhance cell proliferation and matrix synthesis *in vitro* and optimize scaffold incorporation *in vivo*.

## Future Directions

A number of important questions remain about the basic biologic mechanisms of the PRFM and represent potential areas of future study:

- What are the diffusion kinetics of other growth factors (in addition to TGF-β1) eluted from PRFMs?
- Are viable (unactivated platelets) trapped within the PRFM following the current preparation methods? Is the prolonged growth factor availability a result of sequential degranulation of viable platelets over their 5-10 day half-life or merely

the result of prolonged diffusion kinetics from the increased fibrin density of a PRFM?

- What are the optimal platelet and growth factor concentrations in a PRFM? Are more growth factors necessarily better?
- Given that the fibrin scaffold may act as a growth factor reservoir, how important is the contribution of the fibrin scaffold to the proposed benefits of the PRFM?
- Would the PRFM be of greater benefit in biologically compromised tissues, such as in chronic tendinopathies or desmopathies or patients with endocrinopathies e.g., Cushingoid or diabetic patients with delayed wound healing
- When is the best time to intervene with a PRFM following an injury?

Although autologous PRFM constructs appear promising, further *in vitro* characterization and experimental studies are necessary as the study of these constructs has merely just begun. Moreover, just as with platelet-rich plasma, well-control prospective randomized clinical trials under rigorous scientific scrutiny are necessary prior to the wide-spread clinical use of these products.