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The Use of Platelet Rich Fibrin in Pulpal and Periodontal Regeneration

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by

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When beginning my residency program, I knew I wanted to pursue the master thesis option, and finding an area of research that was both engaging and fulfilling for me was essential. After a few months of looking into current projects and bouncing ideas off faculty, I came to the decision to start a brand new project. This choice resulted in designing and executing a laboratory experiment as well as a randomized clinical trial. As my three years of study here in Lincoln, Nebraska come to a close, I am truly proud of the work my committee and I accomplished in this area of interest. I am profoundly grateful for the mentoring and guidance I received from not only my committee members, but many of the other university faculty and staff as well. I would like to thank them for their support over these past three years.

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University of Nebraska, 2016

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Purpose: Platelet rich fibrin (PRF) has been postulated to aid in regeneration. Therefore, the purpose of this study is to evaluate the inflammatory response of PRF *in vitro*, and analyze clinically and histologically the effectiveness of PRF on pulpal regeneration after reimplantation.

Materials and Methods: The *in vivo* experiment included 18 patients (69 teeth). Teeth were extracted and apicoectomy performed. The tooth was reimplanted and splinted after PRF was condensed into the apex. Control teeth received the same treatment with the exception of the PRF. After 3-11 months, vitality was checked, teeth were extracted and analyzed histologically.

For the *in vitro*, PRF was fabricated from two donors. A periodontal ligament (PDL) cell line was divided into four groups: A: PDL cells and PRF, B: PDL cells, PRF, and Lipopolysaccharide (LPS), C: PDL cells and LPS, and D: PDL cells only. After 24 h LPS was added to groups B and C. Supernatants were collected at 24, 48 and 72 h and an enzyme-linked immunosorbent assay was used to measure the concentration of IL-6.

Results: PRF had no effect on diagnostic tests or histological outcome. Out of mouth time lead to more post-operative non-vital responses. For the cell cultures, PRF synergized with LPS inducing higher IL-6 release.

Conclusions: PRF does not improve vitality responses *in vivo* and results in higher expression of IL-6 from PDLs.

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LIST OF ABBREVIATIONS

ANOVA	one-way analysis of variance
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EPT	electric pulp test
FBS	fetal bovine serum
FGF	fibroblast growth factor
DMEM	Dulbecco's modified eagle's culture medium
GLMM	generalized linear mixed model
H&E	hematoxylin and eosin
IL	interleukin
IGF	insulin-like growth factor
L-PRF	leukocyte and platelet-rich fibrin
L-PRP	leukocyte and platelet-rich plasma
LPS	lipopolysaccharide
Ml	milliliter
MMP	matrix metalloproteinase
MTA	mineral trioxide aggregate
OR	odds ratio
PDL	periodontal ligament
PDGF	platelet-derived growth factor
PG	picograms
PRF	platelet-rich fibrin

P-PRF	pure platelet-rich fibrin
PRP	platelet-rich plasma
P-PRP	pure platelet-rich plasma
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinases
TNF	tumor necrosis factor
TSP	thrombospondin
UNMC COD	University of Nebraska Medical Center College of Dentistry
VEGF	vascular endothelial growth factor

CHAPTER 1: INTRODUCTION

Pulpal necrosis after reimplantation, in either avulsion or autotransplantation, is a clinical complication. If the pulp tissue is not regenerated, or if endodontic therapy is not performed, the pulp space can become infected (Tronstad 1988). It has been suggested that treatment strategies should attempt to limit peri-radicular inflammation therefore promoting favorable cemental healing, pulpal revascularizaion, and limiting unfavorable osseous replacement (Trope, 2002).

To achieve regeneration of the dental pulp, three main elements are required. The first element is a source of cells that are capable of differentiating into the desired tissue (Hargreaves et al., 2013). Stem cells have been reported in both the dental pulp and the apical papilla (Nakashima & Akamine, 2005; Huang et al., 2008). The second element is the presence of growth factors and other tissue-inducing mediators. The third element is a scaffold, or a three dimensional tissue structure that regulates the release of the growth factors (Hargreaves et al., 2013). These characteristics drive the investigation for an ideal medium capable of promoting endodontic revascularization and eventually the potential for regeneration of avulsed or autotransplanted mature teeth. In a review article on pulpal regeneration of permanent teeth, Haregreaves et al. (2008) suggested that platelet concentrates may be a promising material for endodontics as they possess the characteristics required to achieve regeneration.

Platelet rich fibrin (PRF) is a second generation platelet concentrate that allows the clinician to obtain fibrin membranes enriched with platelets and growth factors from an anticoagulant-free blood harvest and without the addition of artificial biochemical modification (Dohan et al., 2006). PRF has demonstrated clinical relevance through its ability to stimulate cell proliferation of osteoblasts, gingival fibroblasts, and PDL cells. In addition, the PRF membrane includes VEGF, a potent stimulator of angiogenesis. These properties make it a favorable product for surgical procedures where revascularization is desirable (Tsai et al., 2009).

To date, no human studies have been performed evaluating the effectiveness of PRF on pulpal revascularization after reimplantation. Additionally, only histological evidence of the benefits of PRF has been demonstrated in animal models. Peters (2014) discussed the need for a broader spectrum of inclusion for cases attempting regeneration. Currently it is mostly limited to immature teeth with larger apical foramina. The article emphasizes the need to expand the evaluation of endodontic regeneration potential in mature teeth. Additionally, a further understanding of PRF's role in modulating inflammation is needed. A human trial evaluating PRF as an adjunct to endodontic regeneration could offer more insight on its potential for inducing pulpal regeneration of necrotic teeth. If PRF is capable of promoting regeneration in mature teeth, the potential benefits include limiting the need for endodontic therapy following tooth avulsion, potential for mature tooth transplantation in situations of congenitally missing teeth, and utilization of PRF in endodontic regeneration therapy of mature necrotic teeth.

The purpose of this study is to evaluate the inflammatory response of PRF *in vitro*, and analyze clinically and histologically the effectiveness of PRF on pulpal regeneration after reimplantation. The central hypothesis of this study is that PRF has anti-inflammatory properties that can aid in pulpal regeneration during reimplantation procedures or in cases of avulsion. The second hypothesis is that introducing PRF into the apical foramen during autotransplanation will enhance the regeneration of the pulpal tissue and improve clinical vitality results. Both hypotheses will be tested through the following aims:

1) Evaluate the PDL cell inflammatory response to LPS through measurement of IL-6 cytokine in the presence of PRF
2) Determine if PRF effects the clinical vitality of an extracted and reimplanted tooth by measuring responses to percussion, cold, and electric pulp test (EPT)
3) Evaluate histologically the inflammatory infiltrate and presence or absence of vascularity in the pulp chambers and canals of the teeth treated with or without PRF

CHAPTER 2: LITERATURE REVIEW

Regenerative endodontics: mature tooth avulsion

Dental trauma occurs frequently in children with an estimated 4-59% prevalence of dental trauma around the world. This oral trauma frequently leads to pulpal necrosis (Gelndor & Andreasen, 2007). Approximately 92% of avulsion cases of traumatized permanent teeth leads to pulpal necrosis (Borum & Andreasen, 2001). This necrotic tissue is at high risk for bacterial contamination. If the pulp tissue does not revascularize or if endodontic therapy is not performed, the pulp space can become infected (Tronstad, 1988). It has been suggested that treatment strategies should attempt to limit peri-radicular inflammation, therefore promoting favorable cemental healing, pulpal revascularizaion, and limiting unfavorable osseous replacement (Trope, 2002). Whole pulp regeneration is considered the reconstitution of viable tissue in a space that was previously avascular (Peters, 2014).

Regenerative endodontics is the formation and delivery of tissues to replace pulp that has been traumatized or is diseased or absent (Murray et al., 2007). Clinical endodontic regenerative treatment is still considered a challenge even for immature teeth, and for mature teeth is an area of endodontics where more research is needed. Regenerative endodontics has been mostly focused on immature teeth as they have a greater chance for pulpal regeneration; however, some case studies suggest potential to expand the practice to mature necrotic teeth. Payani & Kim (2013) published two case reports where a modified regenerative endodontic procedure was utilized in place of conventional endodontics for traumatized mature teeth. They observed a regression of the clinical symptoms and resolution of the apical radiolucency in both cases.

To achieve regeneration of the dental pulp, three main elements are required. The first element is a source of cells that are capable of differentiating into the desired tissue (Hargreaves et al., 2013). Stem cells have been reported in both the dental pulp and the apical papilla

(Nakashima & Akamine, 2005; Huang et al., 2008). The second element is the presence of growth factors and other tissue-inducing mediators. The third element is a scaffold, or a three-dimensional tissue structure that regulates the release of the growth factors (Haregreaves et al., 2013). These characteristics drive the investigation for an ideal medium capable of promoting endodontic revascularization and eventually the potential for regeneration of avulsed or autotransplanted mature teeth. In a review article on pulpal regeneration of permanent teeth, Haregreaves et al. (2008) suggests that platelet concentrates may be a promising material for endodontics as they possess the characteristics required to achieve regeneration.

Platelets and platelet concentrates: A history

Platelets, also referred to as thrombocytes, are a component of mammalian whole blood. The function of platelets is primarily hemostasis through aggregation at an injured portion of a blood vessel wall. Platelets are derived from bone marrow megakaryocytes, are discoidal anucleate cytoplasmic fragments, and are on average 2-3 μm in diameter. They are not actually true cells, in actuality they are circulating cell fragments. A normal platelet count for an adult human is 1.5-4 thousand per μL in peripheral blood, and the average lifespan of a platelet is approximately 8-10 days (Dohan et al., 2006). During tissue repair, platelets that have been activated will release two different types of granules. The dense granules have serotonin, adenosine triphosphotase, adenosine diphosphotase, and calcium (De Pascale et al., 2015). Platelets also contain α-granules, a few mitochondria, and two prominent membrane structures. The α-granules are spherical or oval structures with diameters ranging from 200-500 nm, each enclosed within a membrane. These α-granules activate signaling proteins, which ultimately result in gene expression for cellular proliferation, matrix formation, collagen synthesis, and osteoid production (Gasling et al., 2009). The growth factors present in these α-granules exhibit chemotactic and mitogenic properties which assist in promoting cell proliferation and modulating cell functions that promote tissue healing and regeneration (Anitua et al., 2007)

Platelet concentrations were first utilized in transfusion medicine for the prevention of hemorrhage induced by severe thrombocytopenia (Dohan et al., 2008). The benefit of these concentrates to assist in healing and stimulation of wound repair was initially explored through the surgical application of fibrin glues. These glues consisted of concentrated fibrinogen (Matras et al., 1970). The regenerative use for platelets was first described by Ross et al. in 1974. In this study, Ross et al. (1974) demonstrated that platelet-poor plasma is much less effective than blood serum in the proliferation of cultured monkey arterial smooth muscle. Addition of platelets to the platelet-poor plasma increased activity; therefore, indicating a potential role of the platelet in wound healing *in vivo*. Choukroun was the first to develop platelet rich fibrin, also termed leukocyte and platelet-rich fibrin (L-PRF). Most platelet concentrate protocols lead to a low-density fibrin gel, which has benefits in surgical application due to pliability and ease of use; however, it does not possess a true fibrin matrix. For the fibrin to be considered a biomaterial and have potential healing effects, it must be a high density fibrin network.

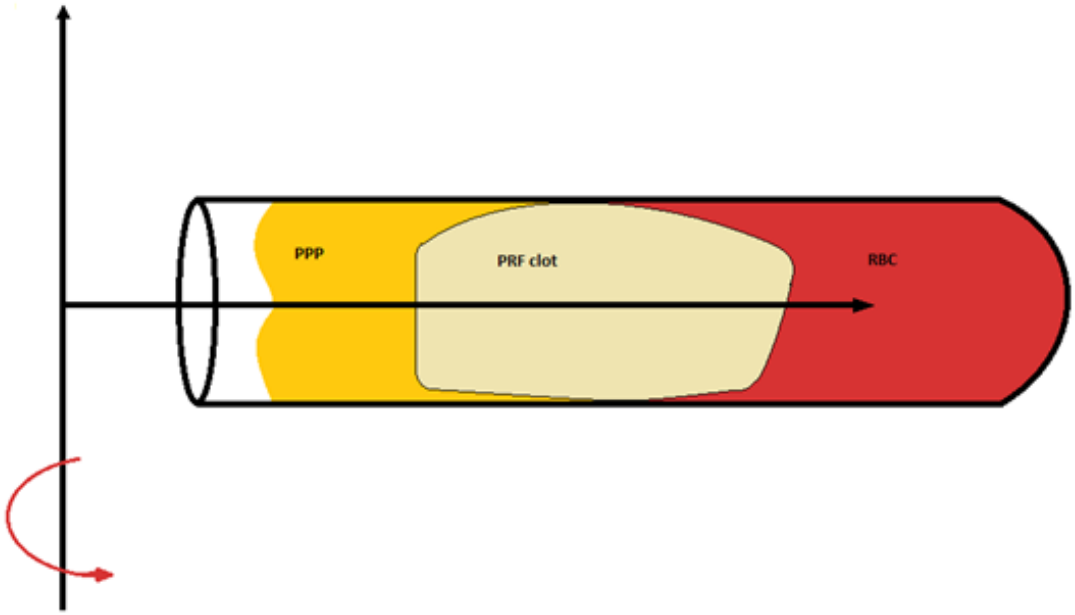
Depending on the methods after collection of the whole blood, the result can lead to four different platelet concentrate products. The concentrates that can be produced are pure platelet-rich plasma (P-PRP), leukocyte-rich platelet rich plasma (L-PRP), pure platelet-rich fibrin (P-PRF), and L-PRF (Dohan et al., 2009). The fabrication of P-PRP leads to a low platelet collection as it attempts to avoid the collection of leukocytes which are usually found along with platelets in the intermediate layer. The P-PRP procedure lacks consistency and the release of cytokines occurs in a shorter duration than with PRF. With L-PRP the leukocytes which were previously undesirable with fabrication of P-PRP are now included. The final product of L-PRP is rich in leukocytes; however, it disintegrates quickly. Additionally, the machinery and biologics required to fabricate L-PRP make it an expensive and time-consuming process (Dohan et al., 2009). P-PRF is leukocyte poor and has to be mixed with an anticoagulant and separation gel.

This process is both difficult to execute and the equipment needed is expensive. Finally, L-PRF (or Choukroun's PRF) is a simple and free technique that was developed to minimize blood handling and remove the need for additives.

Characteristics of L-PRF:

L-PRF, or from here simply referred to as PRF, derives from a natural and progressive polymerization occurring during the centrifugation of the blood without any additives (Choukroun et al., 2001; Dohan & Choukroun, 2006). Without anticoagulants, fibrin polymerization begins immediately after collection. This slow and natural polymerization process of the fibrin results in a 3-dimensional and homogenous matrix during the centrifugation process (Dohan et al, 2006). This polymerization and centrifugation leads to a strong, yet flexible and elastic fibrin matrix with platelet cytokine and glycan chains embedded within the fibrin meshes (Khiste & Tari, 2013). After centrifugation is complete, three layers are formed: the red blood cell or corpuscle layer, the acellular plasma layer on top and the fibrin clot in the middle. The strong fibrin matrix has a three-dimensional architecture where most of the platelets and leukocytes are concentrated (Dohan et al., 2009). Fabrication of the PRF involves drawing whole blood into two 10 ml glass tubes and immediately centrifuging at 2700-3000 rpm for 10-12 minutes. This process results in the three layers previously discussed. As the blood begins to coagulate during the collection process, rapid handling is essential (Gupta et al., 2011). The diagram below demonstrates the three different layers that are obtained after centrifugation.

Figure 1: PRF Layers After Centrifugation



There are numerous benefits to not discarding the leukocytes, as is done with P-PRF (Dohan et al. 2009). Firstly, the presence of leukocytes promotes the *in vivo* recruitment of new leukocytes aiding in inflammation and tissue repair (Zumstein et al., 2012). Regarding the obvious anti-infectious role, leukocytes and monocytes present in the PRF (or recruited from *in-vivo* activation) contain high levels of myeloperoxidase, which is toxic to bacteria (Everts et al., 2006). The combination of inductive and antimicrobial properties has made many advocate its use in non-healing wounds. Platelet concentrate antimicrobial activity against *Staphylococcus aureus* was shown to be equivalent to gentamicin and oxacillin and additionally was shown to have significant effects in reducing the growth of *Escherichia coli* (Bielecki et al., 2007). The synergistic antibacterial effect of both leukocytes and platelets yield supreme potential for antimicrobial activity. Platelet α-granules contain not only growth factors and antimicrobial peptides, but also catecholamines, serotonin, osteonectin, von Willebrand factor, proaccelerin, and other substances. (Anitua et al., 2005; Bielecki et al., 2006; Slater et al., 1995). These are released in high concentrations after platelet aggregation and may have antibacterial effects (Bielecki et al., 2007). Additional benefits to preserving leukocytes is that they produce large amounts of VEGF, which fosters healing and stimulates angiogenesis (Dohan et al., 2009; Nielsen et al., 2001; Werther et al., 2002). As a decrease in vascularity is seen with age, having a higher leukocyte count assists in higher levels of vascular endothelial growth factor (VEGF), which assists in the induction of new vessel growth (Zumstein et al., 2012). This angiogenesis potential is further promoted by the fibrin structure as fibrin induces healing after 4 days, in addition to cellular interactions and angiogenesis by replacement of the fibrin by capillaries (Butler et al., 2008; Laurens & Koolwijk, 2006).

PRF has been shown to be efficient in cell migration, proliferation, and cicatrisation (Bensaid et al., 2003; Laurens & Koolwijk, 2006). Using platelet concentrations in wound healing for either hard or soft tissue is mediated by a wide range of signaling proteins and intra and

extracellular events (Gasling et al., 2009). Activation and degranulation of the α-granules releases cytokines interleukin (IL)-1β, IL-6, TNF-α and growth factors TGF β1, PDGF, VEGF, EGF which initiate healing through stimulating cell migration and proliferation (Dohan et al., 2006). Dohan et al. (2006) suggests that through these cell mediators, PRF may act as an immune regulator and decrease some of the harmful effects of inflammation of surrounding tissues during wound healing. Below is a table adapted from information in Hotwani & Sharma (2014) describing the growth factors and cytokines identified in PRF and their role in inflammation and wound healing.

Table 1: List of cytokine/growth factors present in PRF

IL-1	**Stimulates T-helper cells, inflammatory mediator**
IL-6	**B cell differentiation, T cell activator, antibody secretion stimulation, inflammatory and remodeling mediator**
IL-4	**Proliferation and differentiation of activated B cells, moderates inflammation, increases fibroblast synthesis of fibrillary collagen**
TNF-α	**Monocyte activator, stimulate fibroblast remodeling, increase phagocytosis and neutrophil toxicity, modulates IL-1 and IL-6 expression**
VEGF	**Initiates angiogenesis**
TGF β1	**Significant synthesis of collagen and fibronectin**
PDGF	**Regulates migration, proliferation and survival of mesenchymal cell lineages, cicatrisation**
IGFs 1 and 2	**Mediator in apoptosis, chemotactic effects towards human osteoblasts**

These growth factors are released from platelets when activated by a stimulus or aggregated by activators (Kaplan et al. 1978). A brief review of the cytokines and growth factors present in a PRF membrane will demonstrate its influence in wound healing. IL-1, an inflammatory mediator, activates osteoclasts and inhibits bone formation. TNF-α stimulates fibroblasts and monocytes and modulates IL-1 and IL-6 (Gupta et al., 2011). IL-4 promotes healing by modulating inflammation, increasing collagen synthesis and inhibiting MMPs. Through the presence of IL-4, it is suggested that PRF prevents the production of IL-1β, TNF-α, and prostaglandins when presented with bacterial endotoxin. TGF-β1 is a strong fibrosis agent inducing collagen and fibronectin synthesis. PDGF assists in the production of cicatricial tissue as wound healing progresses. IGFs instigate osteoblast activation and mediate apoptosis (Gupta et al., 2011). VEGF is a powerful vascular endothelial growth factor, which initiates angiogenesis and is capable of directing and redefining the network growth (Dohan et al., 2006). In fact, the release of pro inflammatory cytokines IL-1β, IL-6 and TNF-α along with anti-inflammatory cytokine IL-4 from PRF additionally accelerate angiogenesis (Schmid et al., 1997).

Characteristics of PRF: An emphasis on IL-6

The presence of IL-6 in PRF, and PRF's ability to induce IL-6 release from surrounding tissue, is of particular interest. IL-6 is a potent stimulator of inflammation, and therefore a good marker for determining a material's pro- and anti-inflammatory potential. IL-6 is a pleiotropic cytokine that provokes both cellular and physiological responses on a broad spectrum. These responses include, but are not limited to: inflammation, immune response, hematopoiesis, gene activation, and proliferation, survival, and differentiation (Hirano et al., 1990). IL-6, along with other pro-inflammatory cytokines like IL-1β, TNF- α, and TGF-β, are produced by many cell types in the body (Gabay, 2006). IL-6 is produced at the site of inflammation and is a key player in the acute phase response as well as the transition into chronic inflammation.

IL-6 expression can be stimulated by IL-1, bacterial endotoxin, TNF-α, and PDGF. IL-6 acts as a major amplifier for immune cells and leads to a combined response of destruction, inflammation, and remodeling (Dohan, 2006).

While IL-6 is produced by a variety of human cells, the IL-6 receptor (IL-6R) is expressed on few cells, namely hepatocytes, monocytes, B cells, a subset of T cells, and neutrophils (Barnes et al., 2011). IL-6 principally induces responses from fibroblasts, endothelial cells, and monocytes (Dohan 2006). At inflammatory sites the most important sources of these pro-inflammatory cytokines are monocytes and macrophages. IL-6 has dual roles as it can act as a defense mechanism by aiding in acute inflammation, but in chronic inflammation it is considered pro-inflammatory. (Gabay, 2006). IL-6 is important for the transition from acute inflammation into a chronic state by primarily changing the nature of the leukocyte infiltrate. This is accomplished by altering the neutrophil population as well as by inducing the differentiation of monocytes to macrophages (Mitani et al., 2000). By investigating the responses of these cells to IL-6, it becomes apparent that IL-6 is a major player in both acute and chronic inflammation.

Acute inflammation is a limited physiological response that requires resolution in a short period of time and in a localized area. If this is improbable, chronic inflammation will follow. During this phase, IL-6 can have a protective effect by suppressing pro inflammatory cytokine levels without compromising levels of anti-inflammatory cytokines (Xing et al., 1998; Kaplanski et al., 2003). It additionally stimulates IL-1 receptor antagonist, an anti-inflammatory mediator. Therefore, offering some prolongation of the acute phase (Gabay et al., 1997). Acute inflammation is represented by a leukocyte infiltrate that is primarily neutrophilic (Gabay, 2006). Endothelial cells (the epithelial lining of blood vessels) and fibroblasts (a connective tissue cell) do not express IL-6R, but rather, interact with IL-6 via trans-signaling (utilizing a soluble receptor) (Romano et al., 1997). IL-6 plays a role in angiogenesis through stimulation of fibroblasts. As a profibrogenic cytokine, it has been shown to increase fibroblast proliferation,

collagen production, tissue inhibitor of metalloproteinases-1 (TIMP-1), and regulate the expression of VEGF (Mihara et al., 1995). This can assist in modulating the amount of tissue destruction occurring during inflammation, as well as encourage healing and vessel proliferation. Activation of endothelial cells via trans-signaling leads to expression of various adhesion molecules, including intercellular adhesion molecule 1, which promote leukocyte migration to the site, and the release of chemokines like IL-8, which induces neutrophil migration. Additionally, IL-6 stimulates endothelial cells and fibroblasts to produce more IL-6, creating a positive autocrine feedback system. In this manner, IL-6 assists early on in promoting leukocyte, namely neutrophil, chemotaxis to the area of infection; however, as the process matures, IL-6 encourages a shift to chronic inflammation (Barnes et al., 2011).

Monocytes are white blood cells that can differentiate into macrophages or dendritic cells depending on cytokine stimulation (Mitani et al., 2000). Once inflammation has progressed to a chronic state, additional tissue damage begins occurring. After 24 to 48 hours the transition to chronic inflammation histologically reveals primarily monocytes and lymphocytes. Since IL-6 encourages end-stage B lymphocyte differentiation (into plasma cells), immunoglobulin secretion, and T lymphocyte proliferation and differentiation, it is therefore a key modulator in the progression to the chronic inflammatory state (Gabay, 2006). IL-6 has been demonstrated to favor the proliferation of Th1 cells (T helper cell that act primarily against bacteria and protozoa) and to contribute to IL-2 expression furthering chronic inflammation (Jones et al., 2005). By promoting the maturation of B lymphocytes into plasma cells, IL-6 assists in inducing the production of antibodies at the site of infection. This enhances lymphocyte migration to the area of inflammation, propagates the chronic inflammatory state, and ultimately leads to an increase in tissue destruction (Hirano, 1992). The length of this phase can also be influenced by IL-6 as it dictates apoptotic clearance of leukocytes like neutrophils, monocytes, and lymphocytes (Jones et al., 2005). IL-6 has been reported to induce neutrophil apoptosis while simultaneously rescuing

T-cells from apoptosis, therefore propagating the shift from acute to chronic stages of inflammation (Barnes et al., 2011). In this manner, IL-6 appears to be capable of dramatically influence the nature of the immune response through dictation of recruitment, activation, and apoptotic clearance of specific leukocyte subpopulations (Jones et al., 2005).

PRF application: Surgical benefits

During wound healing, endothelial cells, leukocytes, and platelets are important players in the formation of the initial platelet plug during hemostasis, and later in tissue regeneration (De Pascale et al., 2015). Historically, the first clinical application of a platelet-derived preparation was with collagen embedded with platelet proteins placed in chronic leg ulcers. This product stimulated vascularized connective tissue in the chronic wounds (Krupski et al., 1991; Ganio et al., 1993). Once fabricated, PRF can be compressed between two damp gauze yielding a strong membrane applicable in many surgical situations. It is thought that platelets are immediately activated upon application and that growth factors are massively released during the first hours after placement on the surgical site (Dohan et al., 2009). Unlike PRP, PRF does not dissolve within hours after application, but instead begins the slow remodeling process and disintegration of the fibrin complex. This slow resorption allows for a continuous release of the cytokines embedded within this fibrin matrix. Dohan et al. (2008) demonstrated that over the course of 1 week, PRF membrane sustains a very significant slow release of key growth factors. This suggests that the membrane stimulates its environment for a significant amount of time during its remodeling phase.

PRF has become a valuable adjunct to surgery in both the medical and dental fields through its combination of high efficiency platelet and leukocyte collection in a stable matrix, along with activation of growth factors and preservation of tissue repairing cytokines. Many studies advocate its use in rotator cuff and other orthopedic procedures for these very same

characteristics (Bielecki et al., 2007; Zumstein et al., 2012). In oral surgery, it has many recommended applications including: as a membrane, inclusion in implant placement, post enucleation of large periapical lesions, surgical removal of impacted third molars and impacted canines, and pre-prosthetic surgeries to enhance bone density and soft tissue healing (Saluja et al., 2011; Sunitha & Munirathnam, 2008). Kawamura and Urist (1988) demonstrated that PRF may act as a supportive matrix for bone morphogenetic protein as well. Chang et al. (2010) showed that PRF can be utilized to modulate the expression of osteoprotegerin and osteoblast proliferation. PRF has become a popular adjunct during bone grafting and other forms of reconstructive surgery.

PRF application: Use in regenerative endodontics

PRF was determined to be an ideal medium for revascularization of pulpal tissue due to the presence of major cytokines entrapped within the fibrin mesh. According to Dohan et al. 2006, an ELISA test was performed on PRF samples and the concentrations of five different cytokines was compared within the PRF clot and the blood serum. It is suggested that the fibrin mesh entraps these cytokines and as the fibrin degrades over time, it allows for slow release of these cytokines into the surrounding tissues. In a study by He et al. (2009) PRP and PRF in medium were evaluated to determine the release of TGF-β1 and PDGF over the course of 28 days. PRP was shown to release growth factors more rapidly but then concentrations declined. PRF demonstrated a gradual release in growth factors and their activity was kept up for a longer period of time compared to PRP. PRF exhibited a controlled and long-term release of growth factors where the highest amount was measured at day fourteen. In comparison, PRP had a more uncontrolled and short term release of the measured growth factors with its highest concentration on the first day. Due to this slow release, the investigators observed stimulation of rat calvaria osteoblasts from exudate of PRF but not PRP after day 14 (He et al., 2009). Additionally, Schar et al. (2015) observed that in comparison to PRP, PRF in medium had a higher release of TGF-β1, a

long-term release of growth factors, and was a stronger inducer of mesenchymal cell migration. PRF also had marginally higher release of VEGF and IGF-1.

The intrinsic incorporation of cytokines within the fibrin mesh allows for their progressive release over time (approximately 7-11 days), as the network of fibrin disintegrates around it (Huang 2010). Due to the solid consistency of the fibrin network, PRF membranes are slowly destroyed by a remodeling process, similar to a natural blood clot (Clark, 2001). The platelet cytokines that are gradually released as the matrix is resorbed leads to a sustained process of healing (Huang, 2010). The integration of this fibrin network into a site for regeneration facilitates cellular migration, particularly for endothelial cells necessary for neo-angiogenesis and vascularization (Dohan, 2006). Other benefits include the presence of leukocytes and cytokines in the fibrin network which can play a significant role in the self-regulation of inflammatory and infectious processes in areas of regeneration (Simonpieri, 2009). Additionally, the PRF membrane can offer a structural support and function similar to a fibrin bandage over a wound and accelerate the healing of the wound edges (Gassling, 2009).

Regenerative endodontics is based on the idea that despite a tooth being classified as necrotic, some pulpal tissue can survive in the apical region and if conditions are favorable, can be utilized to proliferate pulpal tissue and accomplish regeneration (Trope, 2010). In autotransplantation, replantation, and regenerative endodontic therapy, revascularization of the pulp chamber by ingrowth from the surrounding periapical tissues, passing through the apical foramen determines the success of these procedures (Laureys et al. 2013). While there is no standardized method for revitalizing a necrotic tooth, endodontic literature has investigated the benefits of PRF as an adjunct to apexification and pulpal revascularization. The sustained release of key growth factors, the trimolecular fibrin scaffold that allows for flexibility, and its ability to support cytokine enmeshment and cellular migration, are all reasons why PRF has recently gained attention as an ideal pulp capping material. Shivashankar et al. (2012), Khetarpal (2013), and

Rudagi & Rudagi (2012) published case reports on PRF in combination with mineral trioxide aggregate (MTA) in managing cases with open apexes and found accelerated periapical healing and apical bone formation. In a case report by Shivashankar et al. (2012), treatment of a 9-year-old boy with a fractured immature maxillary central incisor involved irrigation of the accessed canal with 5.25% sodium hypochlorite and .2% chlorhexidine followed by medicating the traumatized tooth with triple antibiotic paste for 21 days. After this time period, the paste was removed, whole blood was drawn from the boy's arm and used to fabricate PRF, via Choukroun's method. The PRF was packed into the canal to the level of the CEJ and MTA was placed over the top of it. A year later the tooth responded positively to vitality testing and demonstrated radiographic evidence of continued root lengthening and apex closure.

Shivashankar et al. (2012) cited *in vitro* studies with PRF and canine and human dental pulp cells as to why PRF is a choice material for pulpal revascularization. *In vitro* analysis with canine pulp cells has corroborated PRF's ability to promote chemotaxis and proliferation of the pulp cells and its ability to contribute to pulpal repair (Yang et al., 2013). When PRF was combined with human dental pulp cells derived from extracted third molars, it did not interfere with vitality and stimulated proliferation and differentiation. Additionally, it upregulated the expression of osteoprotegerin and alkaline phosphatase suggesting it may be beneficial in the formation of reparative dentin (Huang et al., 2010). Expression of these cytokines is usually regarded as a marker for odontoblastic differentiation. After regression of initial inflammation associated with an insult, the remaining dental pulp cells in the apical region differentiate into odontoblast like cells under the influence of Hertwigs Epithelial Root Sheath (Huang et al., 2010). In the case study by Shivashankar et al. (2012), since no intracanal bleeding was instigated, regenerated vital tissue can be attributed to the presence of the PRF.

Additionally, PRF has been shown to be compatible with PDL cells. PDL cells are fibroblast like cells that are characterized by collagen production and some osteoblastic features.

PDL cells produce numerous cytokines and chemokines in response to promoters of inflammation (Jönsson et al., 2011). Zhao et al. (2013) investigated PDL cells in combination with PRF to enhance periodontal healing in an avulsed tooth that has been reimplanted. This study demonstrated that PDL cells and PRF are compatible and that PRF and PDL cells may enhance the healing of avulsed teeth.

PRF application: Use in avulsion and tooth transplantation

The clinical utility of PRF and revascularization has also been demonstrated in several case studies of avulsion (Johns et al., 2013; Keswani et al., 2013; Mishra et al., 2013). These reports utilized a general protocol of minimally instrumenting the avulsed tooth. Johns et al. (2013) performed a 3 mm resection of the root apex followed by minimal instrumentation of the canal. Blood is then drawn from the patient and PRF is prepared via Choukroun's method. The PRF is then placed on the root surface and condensed into the canal. The tooth was then reimplanted, splinted, and the patient followed for up to 24 months. The tooth tested vital to thermal and percussion testing and radiographically did not show signs of replacement resorption or inflammation.

The reasoning for the enlargement of the apical foramen was discussed by Andreasen et al. (1990) in an autotransplantation study involving 370 premolars. It was determined that an apical foramen of at least 1 mm is required for adequate revascularization of autotransplanted teeth. It was demonstrated that an apical foramen smaller than 1 mm resulted in unpredictable revascularization of the pulp tissue and only 15% of mature teeth with closed apices had vital pulp after transplantation. While the benefits of enlargement of the apical foramen has been demonstrated, there does not seem to be any precise minimal apical foramen diameter. In a beagle study, Laureys et al. (2013) saw revascularization with an apical foramen of .32 mm. In a series of dog studies, Skogland's group showed that if the apical foramen is enlarged via an

apicoectomy, mature teeth can be revascularized with a high success rate (Skogland et al. 1981; Skogland et al. 1978; Skogland and Tronstad 1981). In their studies, control teeth that were transplanted without apicoectomies did not demonstrate revascularization when evaluated with a microangiographic technique. In a study by Laureys et al. (2010), 29 beagle teeth with root tip resections were reimplanted and followed for 90 days. The investigators observed that 80% of teeth with pulp tissue left in the canal showed 1/3-complete fill with viable pulp tissue, whereas the group where the pulp tissue was removed prior to reimplantation demonstrated a 79% loss of viability. The conclusion is that the pulp tissue in the canal assists in stimulating revascularization and tissue ingrowth through the apical foramen.

Additionally, PRF has been shown to reduce inflammation; an exaggerated inflammatory process sometimes causes deleterious ankylosis in traumatized teeth. Animal models evaluated PRF granules combined with human PDL stem cells placed on the root surface of freshly extracted canine teeth. The teeth were then reimplanted and evaluated histologically after a healing phase. A regeneration of the PDL-like tissue and reduction in ankylosis and inflammation were noted in animals with the PRF granules (Zhao et al., 2013). While these studies suggest that PRF may be a valuable additive in attempting pulpal revascularization after traumatic avulsion of the adult dentition, more information is needed on its capabilities and limitations. Further study of cytokine and growth factor components of PRF are needed to determine the proinflammatory and anti-inflammatory capabilities of PRF. Additionally, a controlled clinical trial in humans with the reimplantation model utilized by Skogland and Laureys would aid in understanding PRF's role in pulpal regeneration in auotransplantation or avulsion cases.

CHAPTER 3: MATERIALS AND METHODS

Both the *in vitro* and *in vivo* portions of the study were approved by the Institutional Review Board for Human Studies of the University of Nebraska Medical Center (# 443-14-FB)

Clinical Trial:

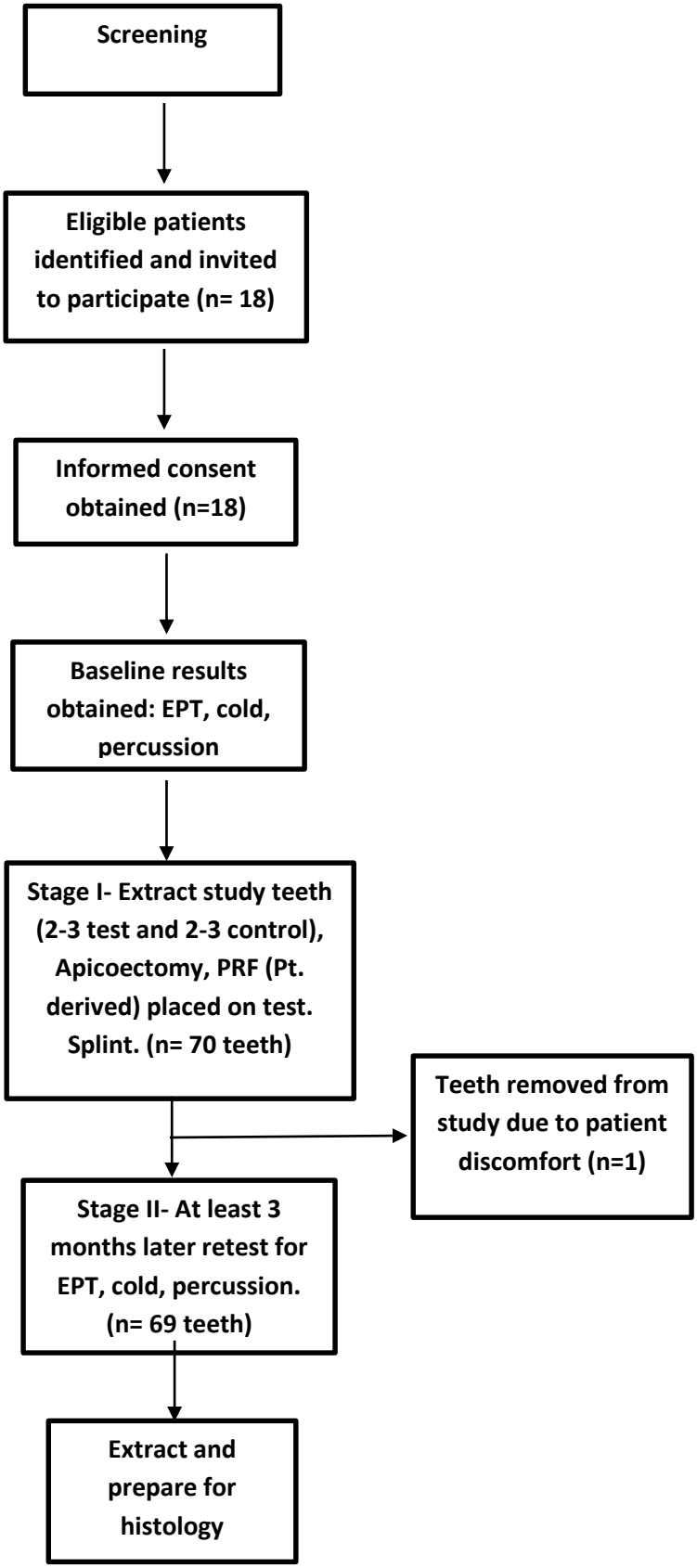
Investigator Calibration:

Investigator EE underwent a calibration exercise prior to the start of the study. EE collected 6 extracted healthy teeth with single roots from the College of Dentistry's Undergraduate Oral Surgery Department. EE performed an apicoectomy, enlarged the apical foramen to at least 1 mm (measured with a dental probe), and accessed the pulp chamber coronally to allow fixation of tissue in accordance to the study design. Teeth were then examined histologically to ensure that pulpal tissue could be properly fixed and stained. These teeth were used as baseline controls of ideal pulpal histology.

Study Population and Design:

This study was designed as a prospective randomized, controlled clinical trial. A total of 18 patients were enrolled. Patients receiving treatment at the University of Nebraska Medical Center College of Dentistry (UNMC COD) that met the inclusion criteria were identified by investigator EE. The inclusion criteria included any adult (age 19 or older) with single rooted vital teeth previously treatment planned by UNMC COD prosthodontic faculty for extraction. These teeth were required to test positive to EPT but not necessarily to thermal. Exclusion criteria included: uncontrolled diabetics with a reported HBA1c level of 8 or above (taken within the past 3 months), pregnancy, or currently anyone taking or having a history of taking oral or IV bisphosphonates. A detailed informed consent was obtained from all subjects and all subjects were given the opportunity to ask questions before signing the consent form.

Figure 2: Flow of Clinical Study Design



Baseline Evaluations:

Baseline evaluations were recorded to determine the initial vitality status of each tooth. Current radiographs were evaluated for signs of pathology associated with teeth utilized in the study. Vitality testing included a yes or no response to percussion, a yes or no response to thermal testing with Endo Ice (tetrafluoroethane; manufactured by Coltene), and a numerical value for EPT. If no response was elicited from EPT the value was placed at 80 and teeth were not included in the study. A hard tissue exam of selected teeth was also performed and caries recorded. Investigator EE performed all initial baseline evaluations and teeth were analyzed based off of how many tested positive or negative to each variable (percussion and cold) and then the EPT changes for all teeth were evaluated.

PRF preparation:

Venous blood (20 mL) was collected from each participant, the PRF was prepared according to a protocol described by Choukroun by investigator EE. The blood samples were transferred into two sterile 10-mL glass tubes without anticoagulation and the tubes immediately centrifuged at 3200 rpm for 12 min in Clay Adams Compact II centrifuge. Three layers were obtained: the platelet poor plasma, platelet rich fibrin clot, and the red corpuscle layer. The fibrin clot was easily separated from the red corpuscles at the bottom and divided into thirds with a sterile 15 blade.

Figure 3: Fabrication and preparation of PRF

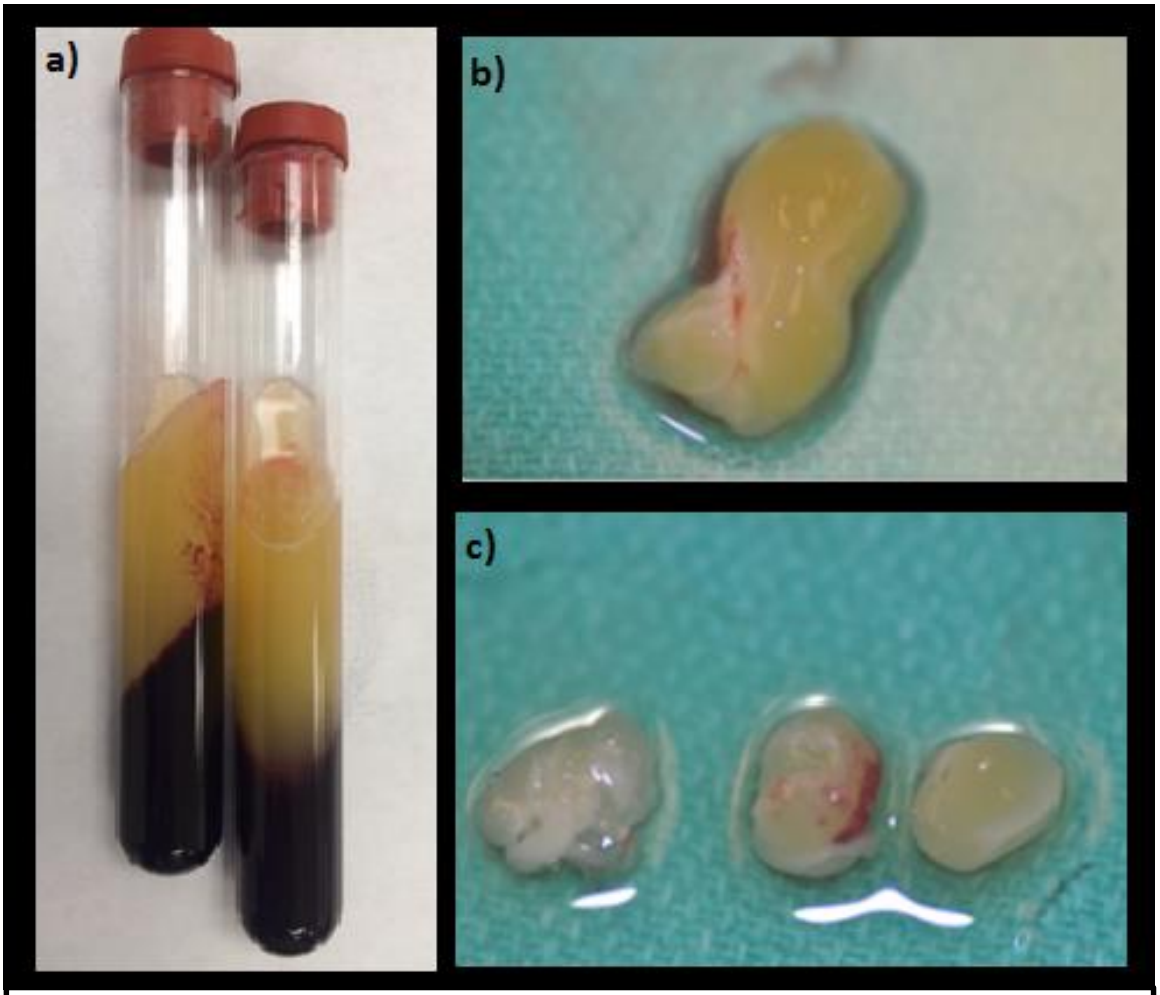


Figure Legend: a) Fabrication of PRF demonstrating the three layers: the platelet poor plasma, the PRF clot, and the red blood cell layer b) Separation of fibrin clot from other layers c) Dividing the clot into thirds prior to intraoral placement.

Surgical Protocol and Root preparation:

After obtaining informed consent, patients received 600 mg of clindamycin preoperatively. If needed, the teeth were initially debrided with a cavitron if visible plaque and calculus were present. Teeth were then atraumatically extracted by an oral surgeon (JB). After extraction, investigator EE performed an immediate root resection of 3 mm with a high-speed handpiece and a NS NTI super coarse diamond FG Axis (Dental Health Products). The resected root orifice was then enlarged with a ¼ round bur until the apical diameter was greater than 1 mm. Total out of mouth time was recorded for each tooth.

Application of PRF:

Experimental and control teeth were determined at random via coin toss (performed by surgical assistant Betty Shestak) during surgery and investigator EE was blind to which teeth received the PRF intervention. In each patient, between 2-4 teeth were utilized. The PRF was condensed into the canal and permitted to extrude from the apex. The socket was rinsed with saline and teeth reimplanted using light pressure. The teeth were splinted to adjacent teeth with Ribbond (bondable reinforced ribbon, Ribbond, Inc.) and flowable composite for a minimum of 6 weeks. Control teeth received all identical treatments with the exception of the PRF.

Figure 4: Apicoectomy, placement of PRF, and reimplantation with placement of splint

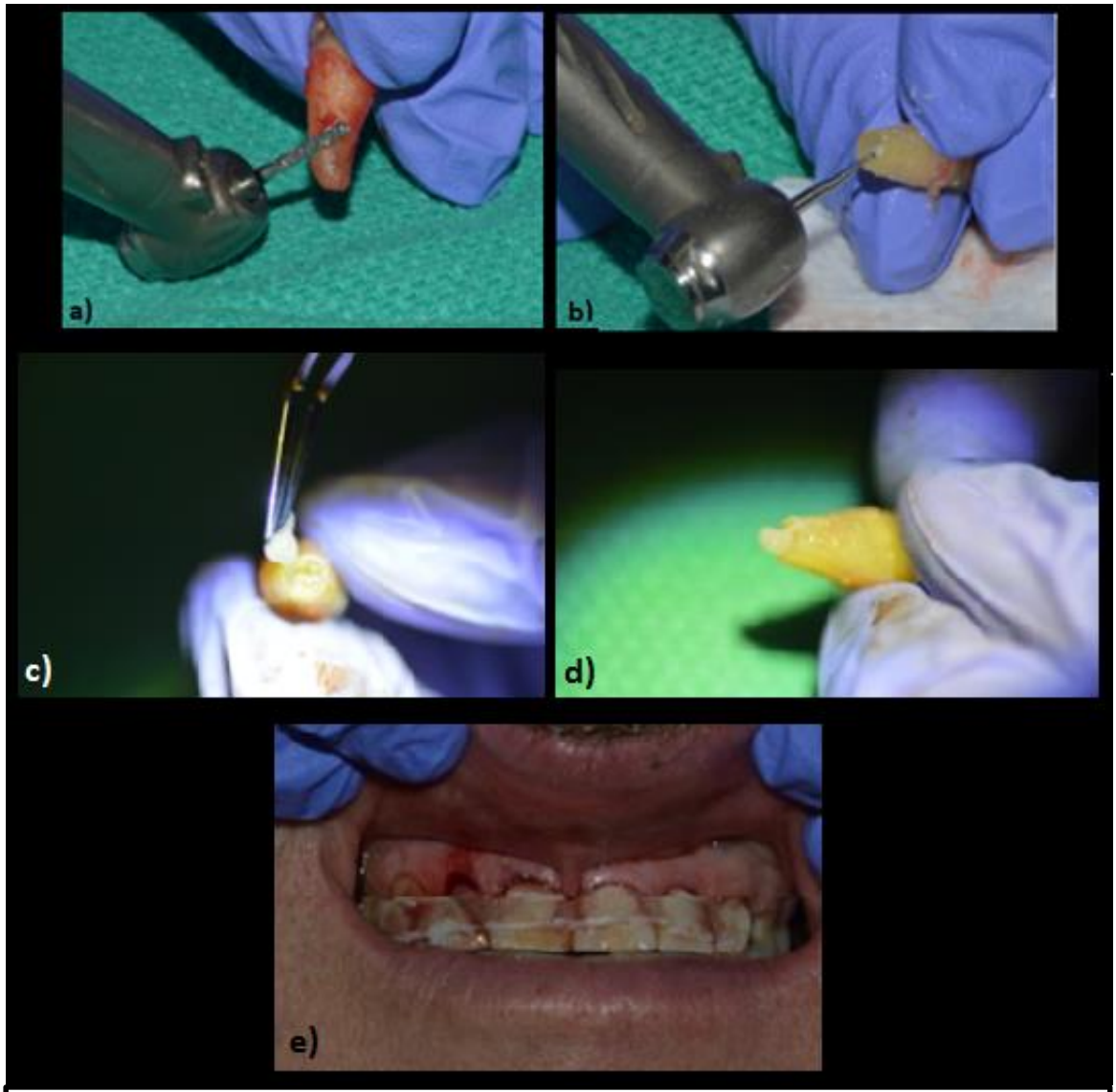


Figure Legend: a) Tooth preparing for apicoectomy b) Enlargement of apical foramen with ¼ round bur c) Placement of PRF into the apical foramen d) Tooth demonstrating PRF extruding from apex e) Teeth reimplanted and splinted.

Follow up:

Patients were followed for a minimum of 3 months and a maximum of 11 months while their final prostheses were fabricated by UNMC COD dental students. At the end of this period, teeth underwent the exact same diagnostic testing performed pre-operatively (percussion, thermal, and EPT) by investigator EE. Teeth were atraumatically extracted and coronal pulpal access immediately obtained with a 6 mm round bur prior to placing in 10% buffered formalin. The patients were then followed for a minimum of 2 weeks to ensure healing and completion of their predesigned prosthetic treatment plan.

Histological analysis:

To obtain a baseline comparison of the histological features of a healthy pulp, 6 freshly extracted healthy teeth (from the same patient) were obtained from the UNMC College of Dentistry Predoctural Oral Surgery Clinic. These teeth were utilized to determine the normal characteristics of a healthy pulp not receiving any study interventions, and to serve as beneficial comparisons throughout the histological analysis of study teeth.

Histological evaluation was performed by investigators PG and EE (both blind to research intervention). After extraction, teeth were allowed to remain in formalin for approximately 1 week. Teeth were then transferred to 5% formic acid for 2-4 weeks depending on tooth size and time needed to decalcify. Completion of the decalcification process was determined by cutting the tooth in half at the midline with a new scalpel blade. Halves were then processed with a Tissue-Tek VIP processor with an overnight program. They were then embedded in paraffin and sectioned to 6-7 micron cuts with an American Optical Microtome. Sections were then stained with a routine Mayer's hematoxylin and eosin (H&E) stain.

Sections were then evaluated microscopically by PG and EE and descriptive histology recorded for each tooth. Infiltrate (acute, chronic, or mixed), severity (mild, moderate or severe),

presence or absence of fibroblast nuclei indicative of vital tissue (yes or no), and presence or absence of blood vessels (none, few, many observed) were recorded for each tooth. Table 2 demonstrates the histological grading scale. If a determination could not be made from the slide, recuts were ordered until either: a determination of all four variables could be made, or the tooth was no longer able to be cut on the microtome. In the latter situation, the tooth was determined as inconclusive and was not utilized for statistical analysis. The histological examination was performed using a Nikon microscope and digital photomicrographs were obtained using a Nikon digital camera connected to a Zeiss Axiolab microscope. The system is designed to acquire high-definition digital images at 10x, 20x, and 40x magnifications.

Table 2: Histological grading scale

Degree	**Stage**	**Vital Tissue Observed**	**Blood Vessel Observation**
Mild	Acute	Yes	None observed
Moderate	Mixed	No	Observed in some slices
Severe	Chronic		Observed in several slices

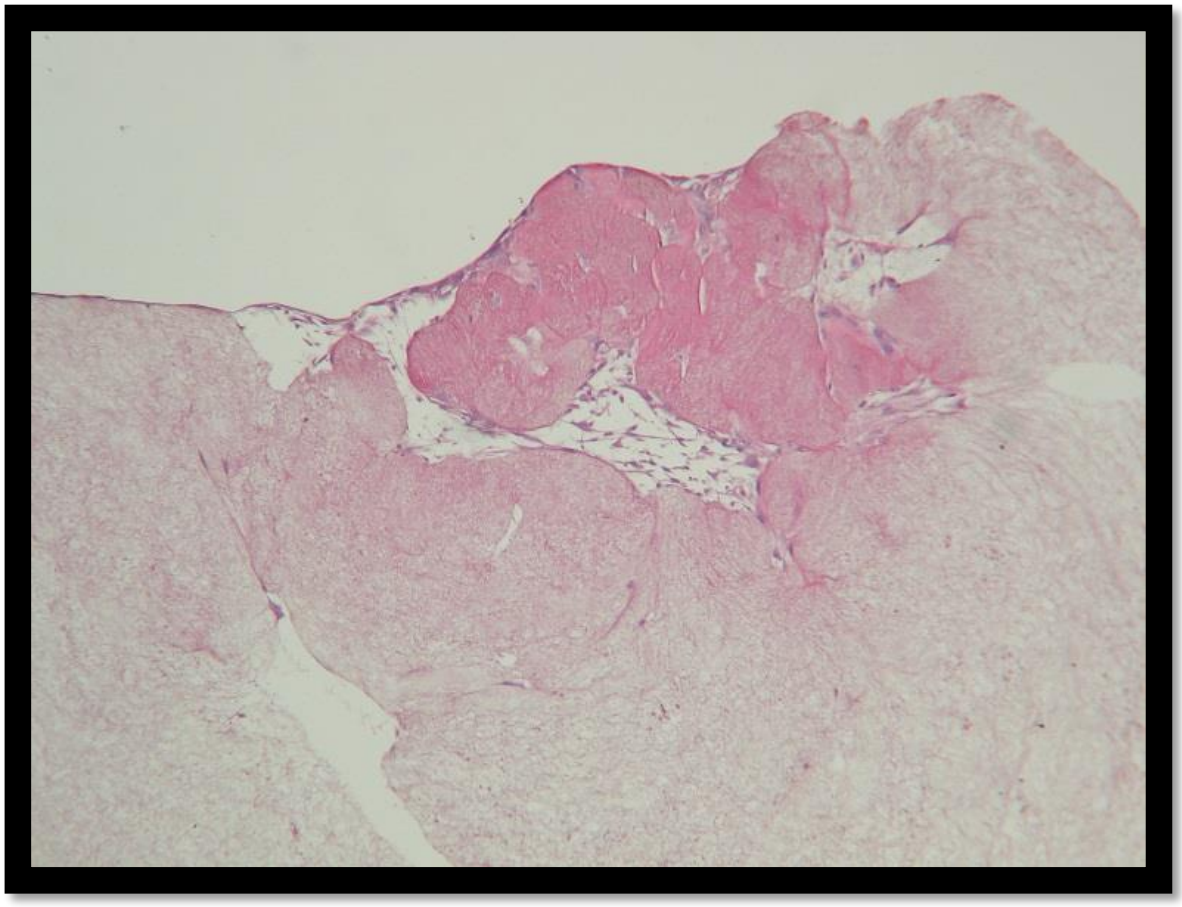
In vitro analysis of IL-6 and PRF:

Study design: group designation and cell culture technique

In the *in vitro* portion of the study, PDL cells were obtained from ScienCell (Carlsbad, CA) and grown in complete Dulbecco's modified Eagle's culture medium (DMEM) (Life Technologies, NY) supplemented with penicillin and 10% fetal bovine serum (FBS) at 37 °C in a humidified 5% CO_2, atmosphere incubator.

The following groups were analyzed: Group A consisted of PDL cells and PRF. Group B consisted of PDL cells, PRF, and LPS. Group C acted as the positive control with PDL cells and LPS. Group D served as the negative control and consisted of only PDL cells in medium. To ensure that the cells would adhere to the actual PRF membrane and not just around it on the sides and floor of the wells, an initial histological evaluation of the membrane was performed. Whole blood was obtained from one of the healthy subjects and a PRF membrane was fabricated and then cultured with PDL cells in accordance with the protocol. After culturing for 24 h the membrane was removed and fixed in formalin, sectioned, and then stained. Histological evaluation revealed fibroblast attachment directly to the surface of the PRF membrane.

Figure 5: PRF membrane with PDL cell adherence

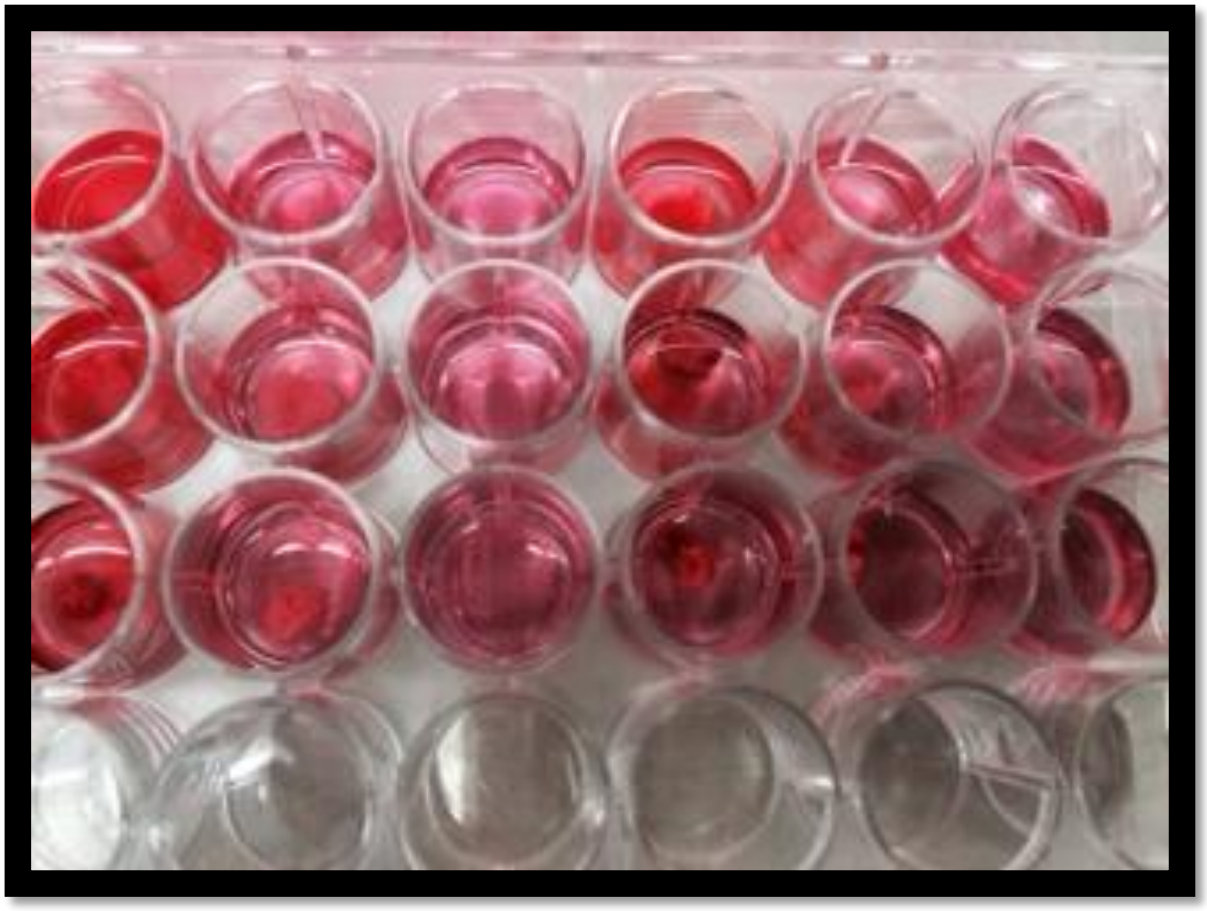


Study design: procedure

Cells between the 4^{th} and 8^{th} passages were used. Whole blood was obtained from two different healthy subjects and PRF was fabricated according to protocol. PRF was divided into equal segments and placed in wells of groups A and B of a 24-well plate. A total of 1×10^6 PDL cells were plated in 96-well plates and cultured overnight in complete DMEM medium to allow adherence to the surface.

After 24 h, the non-adherent cells were removed from the plates by aspiration and fresh complete culture medium was added. Cells were then stimulated with 25 ng/mL of LPS (Sigma) and incubated for 24, 48, and 72 h in 5% CO_2 at 37°C and throughout the experiment an equal volume of fresh medium was added back to each well after collection. All collected culture supernatants were stored at –80°C before ELISA analysis.

Figure 6: PRF membranes in culture medium



Cytokine expression analysis:

Supernatants were analyzed for IL-6 using standard ELISA techniques. ELISA kits utilized the quantitative sandwich technique. ELISA kits were purchased from eBioscience (Human IL-6 ELISA Ready-SET-Go! Kit, eBioscience, San Diego, CA, USA). All assay procedures were completed by one individual (EE). The ELISA tests were performed according to the manufacturer's instructions and protocol. Supernatants were allowed to thaw at room temperature.

100 µL of the standard or supernatant sample was pipetted into each well. Wells were pre-coated with Anti-Human IL-6 antibody. Samples and standards were then covered with a plate sealer and allowed to incubate overnight at 4 °C. All wells were then aspirated and washed a total of 5 times with wash buffer (1x PBS, 0.05% Tween-20). Following the wash, 100 µL of IL-6 conjugate was added to each well. The samples were again covered and incubated for 1 h at room temperature. Aspiration and washing was again performed 5 times. 100 µL of enzyme solution (Pre-titrated Avidin-HRP) was added to each well. The samples were again covered and incubated for 30 minutes at room temperature. Aspiration and washing was again performed 7 times. 100 µL of substrate solution was added to each well. The samples were then covered and incubated at room temperature for 15 minutes and protected from light. 50 µL of stop solution was added to each well and gently agitated until uniform color was obtained. Absorbances were read using a microplate reader ELx808 (BioTek). The plate was read at a 450 nm wavelength and corrected for optical imperfections using 570 nm wavelength readings subtracted from the 450 readings. All readings were completed within 30 minutes of the addition of the stop solution.

Standard calibration curves were generated using computer software and a regression analysis. Concentrations were then calculated and expressed as total weight of molecules by picograms/milliliter (pg/ml) for IL-6. For each experiment, means and standard deviations were

calculated and statistical significance between groups was analyzed. The minimum detectable concentration for the ELISA was less than 0 pg/mL and the maximum detectable concentration was 45,635.75 pg/mL. All samples were analyzed separately and in duplicate. The standard curve was used to estimate the concentration of each sample. If a sample tested at a level lower than the minimum detectable concentration, it was reported as 0 pg/mL.

Figure 7 ELISA samples demonstrating IL-6 identification

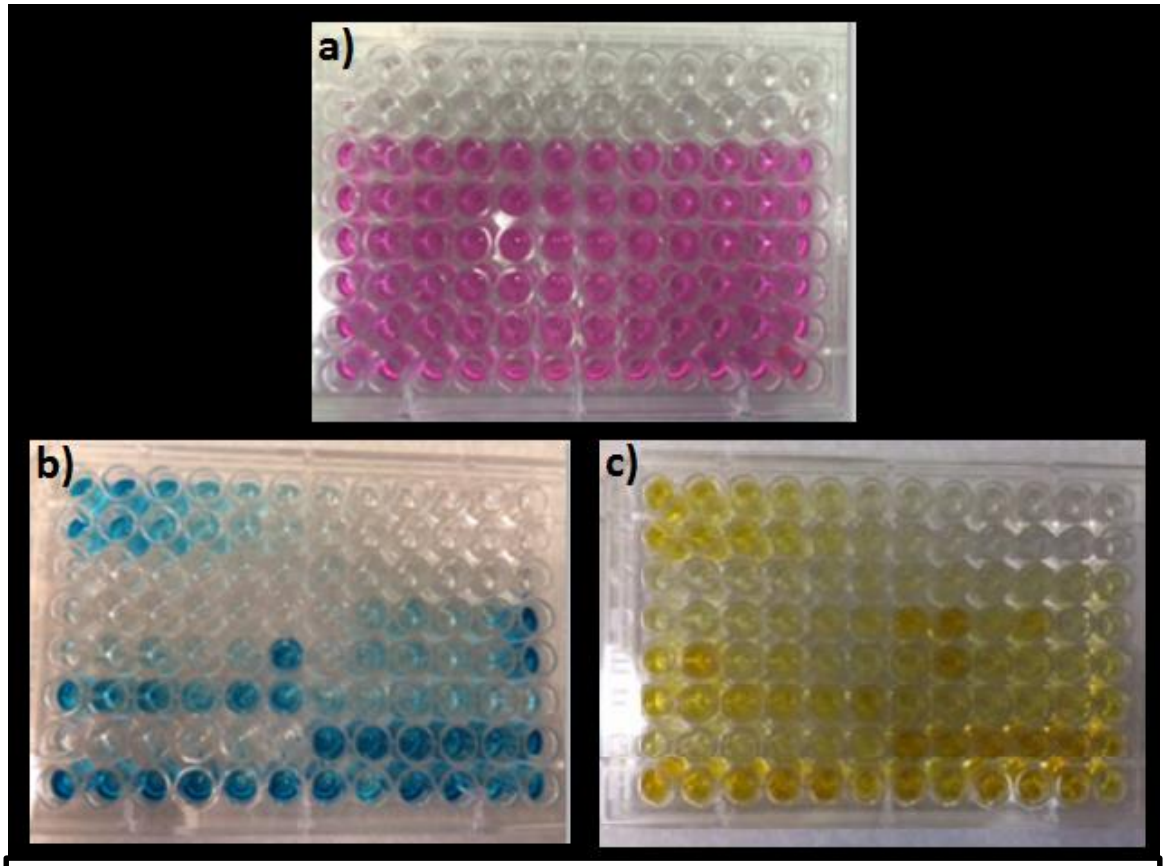


Figure Legend: a) Samples placed in wells. b) Samples and standards after substrate was added c) Samples and standards after stop solution.

Analysis of IL-6 expression in PRF membrane:

In an effort to minimize error, the release of IL-6 from the PRF membrane itself was analyzed. After fabrication, the membrane itself was separated into 12 pieces approximately 0.5 cm x 0.5 cm and placed in ELISA well plates. From another membrane, liquid was expressed immediately after fabrication and pipetted into 12 wells and the ELISA performed to analyze PRF independent release of IL-6 so that any background release by the membrane could be considered in group analysis.

Statistical analysis

Before initiation of the study, a power analysis was completed. Based on previous studies, it was anticipated that 50% of control teeth would be classified as vital and 79% of test teeth would be classified as vital. Using these criteria, power analyses were performed, and a proposed sample size of 20 patients with 4 teeth each (80 teeth) was determined. We expected 20 subjects to enroll in the study, with 4 reimplanted teeth per person, 2 PRF and 2 control teeth (giving a total of 80 teeth, 40 randomized to PRF and 40 randomized to control). With 80 teeth we can detect a difference of 32% in vitality (82% vitality in the PRF group vs. 50% vitality in the control group) with 90% power and an alpha level of 0.05. The test statistic used for the power calculation is the two-sided Z test (unpooled). The sample size calculation was performed using NCSS and PASS.

Patient characteristics were tabulated using frequencies and percentages for categorical variables and median and range for age. Linear mixed models were used to look at the effect of treatment on EPT pre-treatment, post-treatment and at changes over time. Compound symmetry structure was used for the covariance matrix, based on Akaike information criteria. This model allows for correlation due to multiple teeth examined in each person. Generalized linear mixed models (GLMM) were used to look at the proportion positive for thermal and percussion tests as

outcome variables both pre-treatment, post-treatment and at changes over time. For the models considering treatment and time, interactions were considered. Multivariate models were also examined looking at the treatment effect after adjusting for age, gender, race, smoking status, diabetes and duration of treatment. SAS software version 9.3 was used for statistical analysis (SAS Institute Inc., Cary, NC).

CHAPTER 4: RESULTS

Clinical Trial:

Patient characteristics:

A total of 18 subjects were enrolled in the study with a total number of 70 teeth. One tooth was lost to the study due to the need for early extraction for patient comfort. The median number of treated teeth per person is 4 and ranged from 2 to 6. Half of the teeth were randomized via coin flip to PRF treatment and half to no PRF treatment. To prevent imbalances between treatment groups and improve the power for the small sample size, patient stratified randomization was utilized. Patients were followed for a minimum of 3 months and a maximum of 11 months. Throughout the treatment period, 5 out of 18 patients (28%) required a single post-operative week of clindamycin (300 mg taken three times daily for 7 days) upon presenting with signs of infection. During the course of the study one patient reported an increased HBA1c from previously under 8 (in accordance to inclusion requirements) to 10.7. It was decided to not exclude this patient from the study, but to include this in the statistical considerations. Patient characteristics are shown in Table 3 below.

Table 3: Patient characteristics at baseline

		N (%) (n=18)
Age	Median Range	58.5 41-76
Gender	Male Female	9 (50%) 9 (50%)
Race	White Hispanic Native Hawaiian/ Other Pacific Islander	16 (89%) 1 (5.5%) 1 (5.5%)
Smoking status	Non-smoker Smoker	13 (72%) 5 (28%)
Diabetes	No Controlled Uncontrolled	14 (78%) 3 (17%) 1 (5.5%)
Number of teeth treated	2 4 6	2 (11%) 15 (83%) 1 (5.5%)
Post-Operative Clindamycin	Yes No	5 (28%) 13 (72%)

There was no statistical significance between groups for baseline diagnostic tests including EPT, thermal, and percussion. Table 4 depicts the mean diagnostic tests for both the control and test groups.

Table 4: Pre-treatment tooth characteristics

		Control	**PRF treated**	
		Mean (SE)	**Mean (SE)**	**p-value**
Pre EPT		33.1 (3.0)	34.0 (3.0)	0.74
Pre Thermal	Proportion +	0.939 (0.048)	0.852 (0.089)	0.25
Pre Percussion	Proportion +	0.092 (0.056)	0.121 (0.066)	0.70

Clinical outcomes:

There was no statistically significant difference between groups for any of the vitality readings post operatively. EPT, thermal, and percussion means per group are depicted in Table 5.

Table 5: Post-treatment tooth characteristics

		Control	PRF treated	
		Mean (SE)	Mean (SE)	p-value
Post EPT		55.4 (4.5)	57.0 (4.5)	0.64
Post Thermal	Proportion +	0.584 (0.102)	0.553 (0.104)	0.80
Post Percussion	Proportion +	0.038 (0.033)	0.062 (0.046)	0.62

During the initial intervention PRF treated teeth were out of the mouth for a longer amount of time in comparison to the control teeth and this difference in out of mouth time was statistically significant (p=0.035). Table 6 shows the mean out-of-mouth time and its statistical significance.

Table 6: Out of mouth time per treatment group

		Control	PRF treated	
		Mean (SE)	Mean (SE)	p-value
Time out of mouth	minutes	1.62 (0.11)	1.88 (0.11)	**0.035**

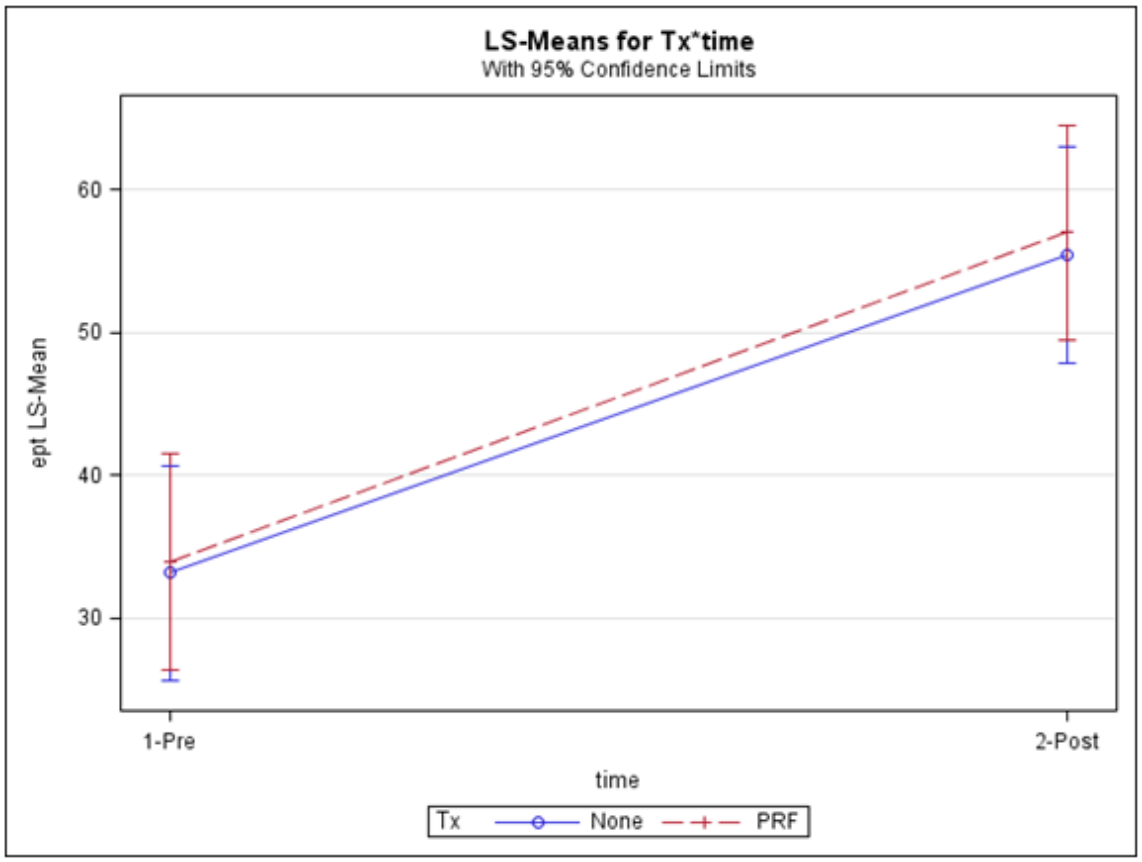
Diagnostic test: EPT

Changes in EPT over time (pre vs post) were evaluated by treatment group, using a linear mixed effects model. There was not a significant interaction between treatment and time (p=0.86) indicating that the effect of treatment did not differ over time. It was also noted that on average EPT did not differ by treatment vs. control (p=0.56). Both groups saw a significant increase in EPT from pre- to post-treatment (p<0.0001). On average, the post-treatment EPT is 22.7 higher than the pre-treatment EPT. Table 7 shows the diagnostic EPT values for both groups and Figure 8 depicts these changes.

Table 7: Changes in EPT over time

				interaction	time	tx
Tx	**time**	**Estimate**	**SE**	**p-value**	**p-value**	**p-value**
None	1-Pre	33.15	3.81	0.86	<0.0001	0.56
	2-Post	55.45	3.81			
PRF	1-Pre	33.97	3.81			
	2-Post	57.00	3.79			

Figure 8: Changes in EPT over time by group



A multivariate model of changes in EPT over time was utilized. Again, interactions between treatment and time were not significant; therefore, it was excluded from the model. As seen in Table 8, the multivariate model showed females have lower levels of EPT on average by 16.7, and a significant increase in EPT over time. The interaction between treatment and time out of the mouth was not significant (p=0.11). Time out of the mouth significantly increases EPT on average by 6.1 (p=0.008).

Table 8: Multivariate model of changes in EPT over time.

		Mean Estimate	SE	p-value
Intercept		63.94	23.34	
Age	1 year increase	0.08	0.32	0.81
Gender	Female	-16.72	5.68	**0.0041**
	Male	Ref		
Smoker	no	-9.14	6.50	0.16
	yes	Ref		
Diabetic	no	-3.24	7.28	0.66
	yes	Ref		
Duration of study	1 month increase	-2.15	1.48	0.15
Time out of mouth	1 minutes increase	6.14	2.28	**0.0082**
Clindamycin use	No	3.78	6.88	0.58
	Yes	Ref		
time	1-Pre	-22.65	4.69	<0.0001
	2-Post	Ref		
Tx	None	0.17	2.06	0.93
	PRF	Ref		

Diagnostic test: thermal

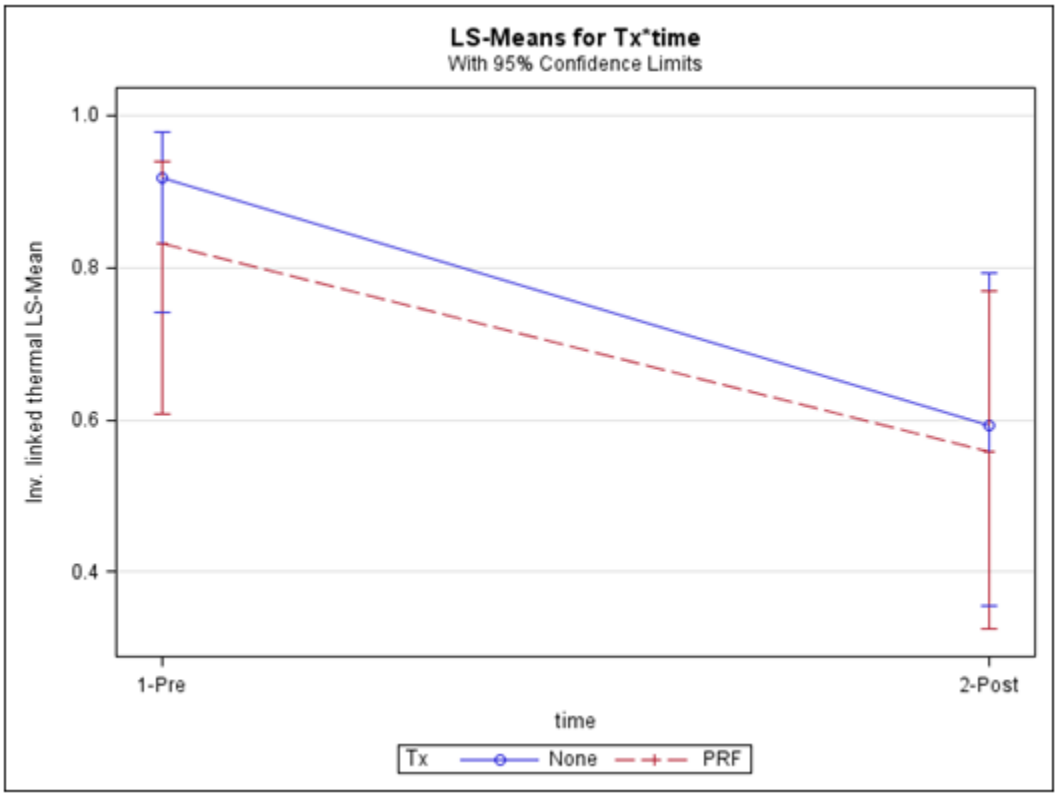
Changes in thermal over time (pre vs post) by treatment group were evaluated, using a GLMM. The link function was legit, but results were backtransformed to the original scale and can be interpreted as proportion positive. Show in Table 9 and depicted by Figure 9, there was not a significant interaction between treatment and time (p=0.45) indicating that the effect of

treatment on thermal did not differ over time. It was also noted that on average, thermal did not differ by treatment vs. control (p=0.30). Both groups saw a significant decrease in proportion positive for thermal from pre- to post-treatment (p=0.014). On average the post-treatment thermal proportion is 0.30 lower than the pre-treatment proportion.

Table 9 Changes in thermal over time

				interaction	time	tx
Tx	**time**	**Estimate**	**SE**	**p-value**	**p-value**	**p-value**
None	1-Pre	0.919	0.052	0.45	0.014	0.30
	2-Post	0.592	0.118			
PRF	1-Pre	0.831	0.082			
	2-Post	0.559	0.120			

Figure 9 Changes in thermal over time by group



A multivariate model of changes in thermal over time was then evaluated, represented in Table 10, here on the logit scale. Again, interactions between treatment and time were not significant, therefore it was excluded from the model. Again, time out of mouth was found to be a statistically significant variable (p= 0.039). Proportional thermal change was significant over time, and decreases with time out of the mouth.

Table 10: Multivariate model of changes in Thermal over time (data on the logit scale)

		Mean Estimate	SE	p-value
Intercept		2.3892	3.9780	
Age	1 year increase	-0.0241	0.0550	0.66
Gender	Female	0.6250	0.9375	0.51
	Male	Ref		
Smoker	no	-0.0414	1.1066	0.98
	yes	Ref		
Diabetic	no	0.3308	1.1393	0.77
	yes	Ref		
Duration of study	1 month increase	0.1163	0.2413	0.63
Time out of mouth	1 minutes increase	-1.1204	0.5441	**0.042**
Clindamycin use	No	0.3746	1.1337	0.74
	Yes	Ref		
time	1-Pre	1.9248	0.7891	**0.021**
	2-Post	Ref		
Tx	None	0.0962	0.4843	0.84
	PRF	Ref		

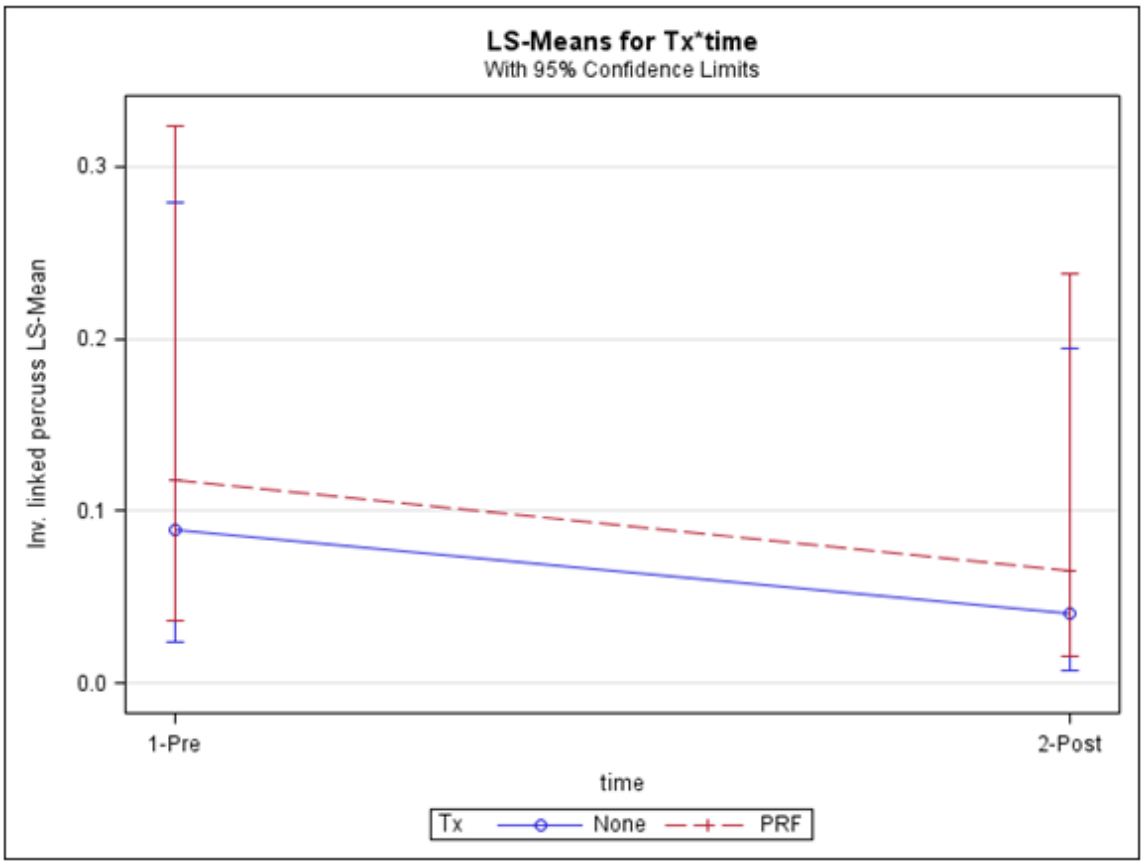
Diagnostic test: percussion

Next, changes in percussion over time (pre vs post) by treatment group were looked at using a generalized linear mixed effects model. The link function was logit, but results were back-transformed to the original scale and can be interpreted as proportion positive. Percussion did not differ significantly over time or by treatment as can be seen by Table 11 and depicted by Figure 10.

Table 11. Changes in percussion over time

				interaction	time	tx
Tx	**time**	**Estimate**	**SE**	**p-value**	**p-value**	**p-value**
None	1-Pre	0.089	0.056	0.88	0.38	0.53
	2-Post	0.040	0.034			
PRF	1-Pre	0.118	0.067			
	2-Post	0.065	0.046			

Figure 10. Changes in percussion proportion positive over time by group



A multivariate model of changes in percussion over time was then performed, here on the logit scale. Again, interactions between treatment and time were not significant; therefore, it was excluded from the model. Percussion did not differ significantly by any of the study variables as seen in Table 12.

Table 12: Multivariate model of changes in percussion over time (data on the logit scale)

		Mean Estimate	SE	p-value
Intercept		1.7943	6.9900	
Age	1 year increase	-0.0561	0.0792	0.48
Gender	Female	-1.3869	1.6532	0.40
	Male	Ref		
Smoker	no	1.1473	1.9108	0.55
	yes	Ref		
Diabetic	no	1.8869	2.0792	0.37
	yes	Ref		
Duration of study	1 month increase	0.1929	0.3034	0.53
Time out of mouth	1 minutes increase	-1.2193	0.9907	0.22
Clindamycin use	No	-3.0390	1.8391	0.10
	Yes	Ref		
time	1-Pre	1.0229	1.0448	0.34
	2-Post	Ref		
Tx	None	-1.0256	0.8874	0.25
	PRF	Ref		

Histological outcomes:

Comparison of histological grading:

After removing teeth that were determined to be inconclusive histologically, histology data was available on 62 teeth. There was no statistically significant difference between PRF and the control group for inflammatory type, grade, evidence of vitality (nuclei in pulpal tissue), or presence of blood vessels. Table 13 has basic chi-square tests at the tooth level demonstrating these results.

Table 13. Post-treatment tooth characteristics – histology (at the tooth level)

		Tx		
	Frequency Col Pct	None (n=29)	PRF (n=33)	p-value
Inflammation Type	Acute	11 37.93%	10 30.30%	0.82
	Chronic	2 6.90%	3 9.09%	
	Mixed	6 20.69%	5 15.15%	
	None	10 34.48%	15 45.45%	
Inflammation Grade	Mild	8 27.59%	12 36.36%	0.36
	Mixed	1 3.45%	0 0.00%	
	Moderate	6 20.69%	5 15.15%	
	None	10 34.48%	15 45.45%	
	Severe	4 13.79%	1 3.03%	
Blood vessels Noted	NONE	18 62.07%	18 54.55%	0.83
	YES-FEW	5 17.24%	8 24.24%	
	YES-MANY	6 20.69%	7 21.21%	
Evidence of Vitality	No	15 51.72%	15 45.45%	0.80
	Yes	14 48.28%	18 54.55%	

Next, the multivariate model GLMM was utilized to evaluate evidence of vitality. Table 14 presents odds ratios (OR) and 95% confidence intervals. Vitality was not associated with any patient characteristics. Older age was marginally associated with evidence of vitality

Table 14: Multivariate model of evidence of vitality

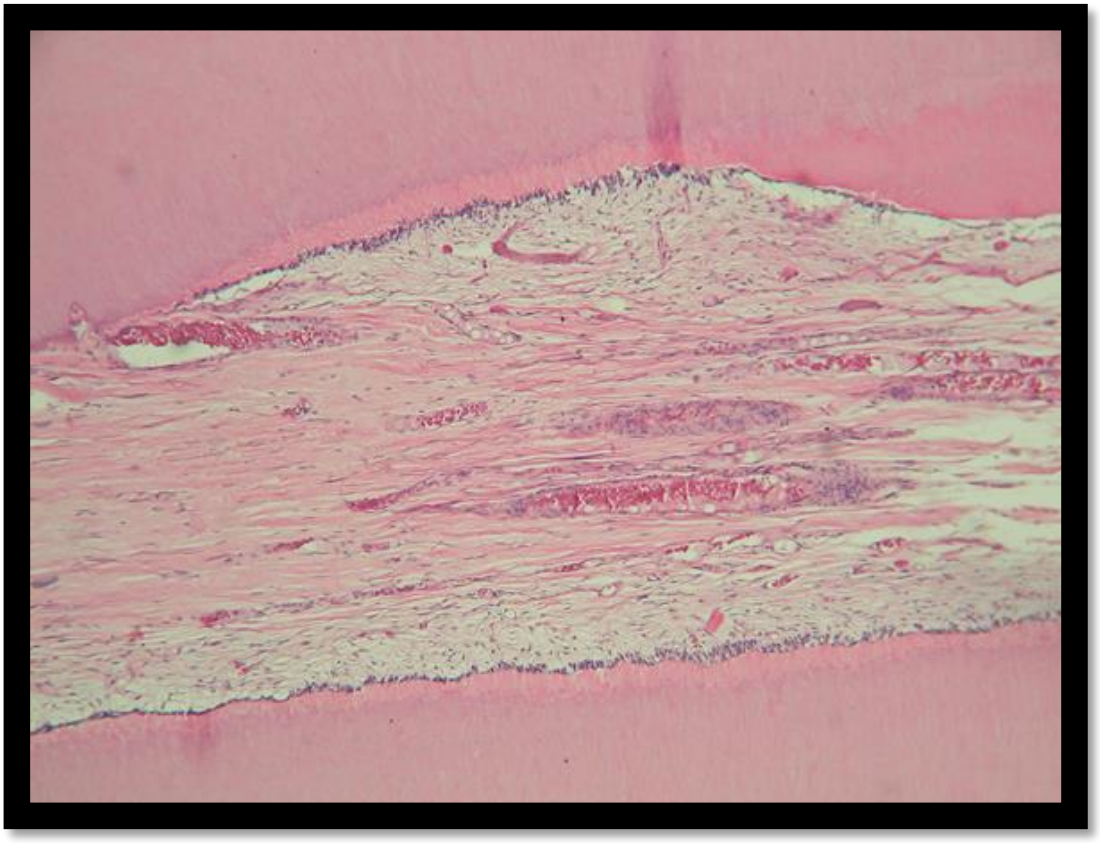
		OR	95% CI	p-value
Age	1 year increase	1.14	0.99-1.31	0.075
Gender	Female vs. Male	0.18	0.02-2.20	0.18
Smoker	No vs. yes	1.13	0.09-14.27	0.92
Diabetic	No vs. yes	13.2	0.53-325.5	0.11
Duration of study	1 month increase	0.70	0.36-1.34	0.27
Time out of mouth	1 minutes increase	1.68	0.34-8.30	0.52
Clindamycin use	No vs. Yes	0.69	0.04-13.34	0.80
Tx	PRF vs. None	1.23	0.32-4.76	0.76

The following section includes the descriptive histology that was observed for the healthy control teeth not involved in the study, teeth not receiving PRF (the control group), and teeth receiving PRF (the test group).

Healthy control teeth from patients not receiving study interventions:

The vessels were primarily centrally located and ran in a longitudinal direction from the enlarged apical foramina to the coronal pulp chamber. A well-vascularized, cell-rich connective tissue was observed in the apical areas of the pulp chambers. The larger blood vessels were centrally located and surrounded by smaller intermediate sized vessels. Histologically, a normal pulp tissue with a regular odontoblastic layer was observed.

Figure 11: Example of vital pulp from a healthy freshly extracted tooth not having received study interventions (10x)



Descriptive histological results for teeth without PRF (Control) and teeth with PRF (test):

There were teeth that exhibited signs of vitality and teeth that were apparently necrotic in both groups. The teeth that appeared to be necrotic exhibited significantly less signs of normal vital pulp including a generalized lack of vascularity, less organization of pulpal tissue, disorganized collagen, tissue without apparent nuclei, and a greater inflammatory infiltrate. Some tissues were either poorly stained or so minimal in quantity that it was not discernable. Frequently, only fragments of cells and necrotic collagen were found. When an infiltrate was observed, the majority of teeth exhibited an extensive neutrophilic presence with occasional signs of chronic inflammation distinguishable by the presence of plasma cells and lymphocytes.

The teeth that were determined to be vital had the following characteristics. These teeth were seen to generally have more organized collagen throughout the chamber with vitality evident through the presence of cellular nuclei. Areas of vascularization were mostly located in the apical region of the pulp canal for the majority of teeth; however, in many teeth the blood vessels had proliferated throughout the pulp chamber. The area of vascularized pulp tissue varied in size depending on the tooth. When blood vessels were observed in the chamber, they resembled the control teeth examined prior to the study. The vessels were usually primarily centrally located and ran in a longitudinal direction from the apical foramina to the coronal pulp chamber. A well-vascularized, cell-rich connective tissue was observed throughout the pulp chambers. The larger blood vessels were centrally located and surrounded by smaller intermediate sized vessels. Histologically, a normal pulp tissue with a regular odontoblastic layer was usually observed. In some cases, a mild infiltrate was observed. In teeth that were determined to be vital, there was most frequently either no infiltrate or acute inflammation distinguishable by neutrophils. Figures 12 and 13 depict the variety of histological that were observed with both control and test teeth respectively.

Figure 12: Histological images of teeth not treated with PRF (Control) representative of sample of n=34

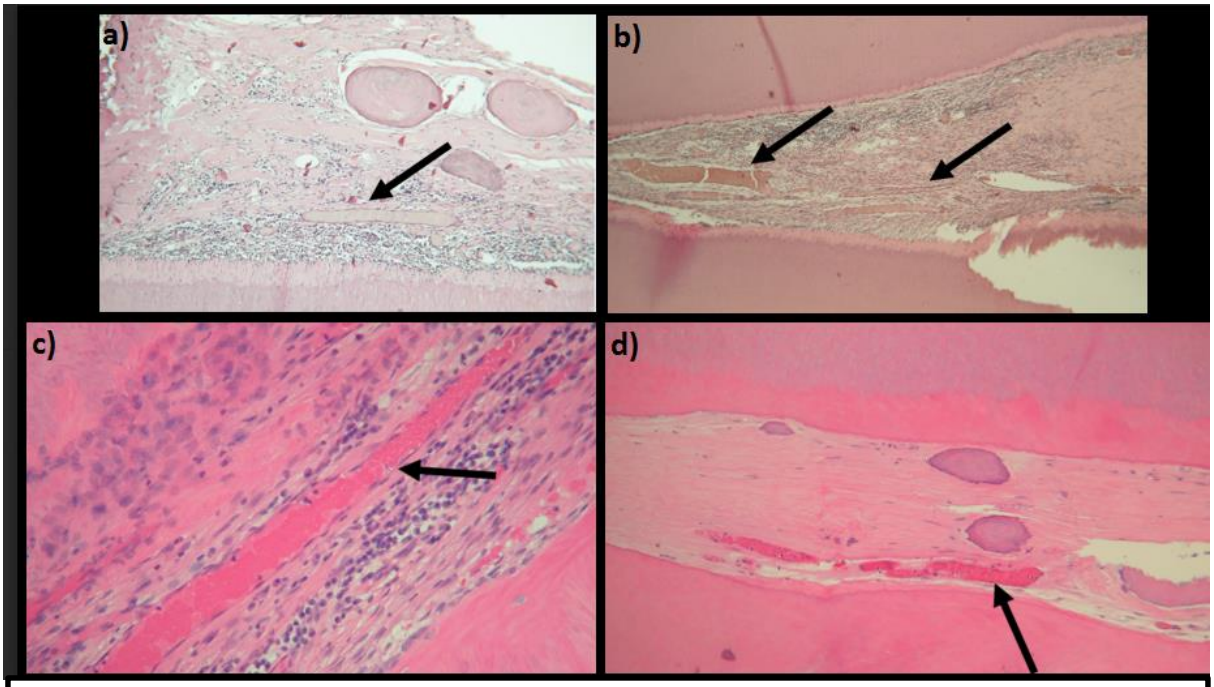


Figure Legend: a) Control tooth demonstrating pulp stones and blood vessels in a specimen present in the mouth for 3 months (10x). b) Control tooth demonstrating blood vessels in a specimen present in the mouth for 3 months (10x). c) Control tooth demonstrating blood vessels, nuclei, and local infiltrate, in a specimen present in the mouth for 3 months (10x). d) Control tooth demonstrating blood vessels, nuclei, and pulp stones in a specimen present in the mouth for 3 months (10x).

Figure 13: Histological images of teeth treated with PRF (Test) representative of sample of n=35

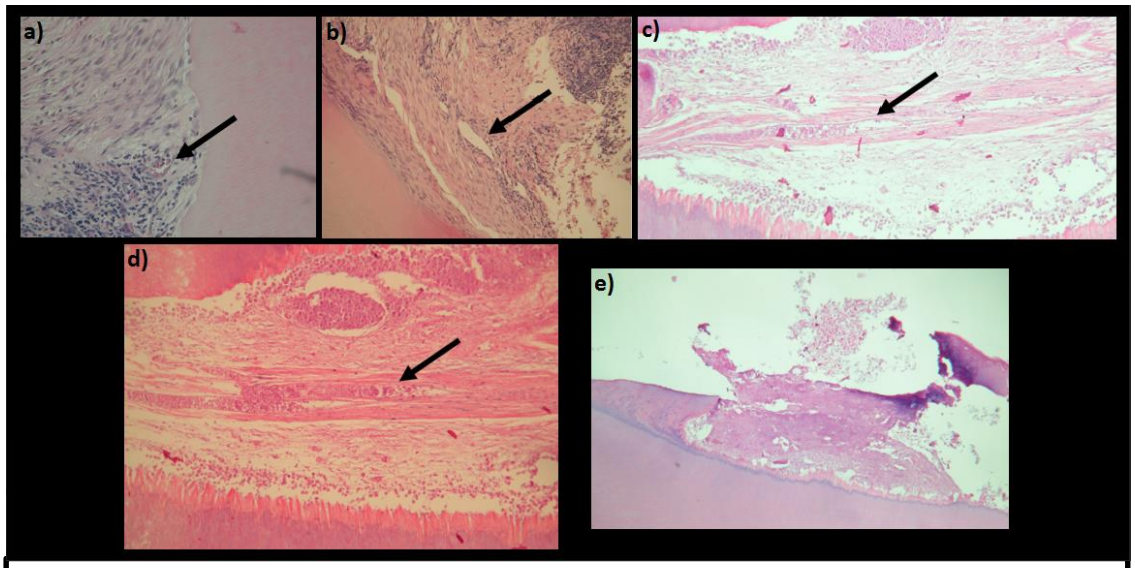


Figure Legend: a) Test tooth demonstrating vital pulp tissue in a specimen present in the mouth for 3 months (40x). b) Test tooth demonstrating endothelial lined blood vessels in a specimen present in the mouth for 11 months (20x). c) Test tooth demonstrating vital blood vessels in the pulp chamber of a specimen present in the mouth for 5 months (10x). d) Test tooth demonstrating vital blood vessels in the pulp chamber of a specimen present in the mouth for 5 months (40x). e) Test tooth demonstrating necrotic pulpal debris of a specimen present in the mouth after 4 months (5x).

Statistical analysis of histological results and clinical diagnostic tests:

Again a univariate GLMM model was utilized to evaluate evidence of vitality, looking at associations with pre diagnostic tests and vitality. Vitality was associated with Pre EPT but not with any other variable prior to the study. A one-unit increase in EPT is 5% more likely to have evidence of vitality (p=0.054).

Table 15: Evidence of vitality based on pre-diagnostic tests

		OR	95% CI	p-value
Pre EPT	1 unit increase	1.05	1.0-1.11	**0.054**
Pre Thermal	Pos vs. neg	0.29	0.04-1.93	0.19
Pre Percussion	Pos vs. neg	1.15	0.18-7.15	0.88

Lastly, the univariate GLMM model was again utilized while looking at the associations with post diagnostic tests and vitality. Vitality was not associated with post diagnostic tests.

Table 16: Evidence of vitality based on post-diagnostic tests

		OR	95% CI	p-value
Post EPT	1 unit increase	0.98	0.95-1.01	0.19
Post Thermal	Pos vs. neg	2.36	0.66-8.47	0.18
Post Percussion	Pos vs. neg	0.57	0.040-8.36	0.68

***In vitro* analysis of IL-6 and PRF outcomes:**

ELISA results between groups Group A (PDL+PRF), Group B (PDL + LPS +PRF), Group C (PDL + LPS), and Group D (PDL only), at three time points. Table 17 expresses the means and standard deviations of each group at time points 24, 48, and 72h.

Table 17: ELISA data in pg/ml of IL-6

Time	Group	N	Mean	SD
24	Group A (PDL +PRF)	5	7116.0	10946.1
	Group B (PDL + LPS +PRF)	6	37494.6	8755.1
	Group C (PDL + LPS)	6	11462.8	1553.6
	Group D (PDL only)	6	-1447.4	276.4
48	Group A (PDL +PRF)	5	2688.6	3754.0
	Group B (PDL + LPS +PRF)	6	36572.7	11298.1
	Group C (PDL + LPS)	6	3471.2	2175.7
	Group D (PDL only)	6	-822.8	360.1
72	Group A (PDL +PRF)	5	2693.4	631.6
	Group B (PDL + LPS +PRF)	6	29680.6	14941.5
	Group C (PDL + LPS)	6	793.4	994.1
	Group D (PDL only)	5	217.6	374.7

At 24 hours there is an overall significant difference between the groups by Kruskal-Wallis test (p=0.0004). Wilcoxon rank sum test was used to conduct pairwise comparisons. Table 18 contains the pairwise comparison p-values between the groups at 24 h, adjusted for pairwise comparisons with Bonferroni's method. The high standard deviation for Group A is likely due to laboratory error. Figure 14 graphically represents these comparisons.

- PDLs concentration is significantly lower than the group with LPS+PRF and the LPS only group.
- The LPS only group concentration is significantly different (lower) than the group with LPS+PRF

Table 18: 24-hour culture group IL-6 production comparison

	Group A	Group B	Group C	Group D
Group A (PDL +PRF)				
Group B (PDL + LPS +PRF)	0.082			
Group C (PDL + LPS)	1.0	**0.031**		
Group D (PDL only)	0.13	**0.03**	**0.03**	

Figure 14: 24-hour mean pg/ml concentrations PDL cells

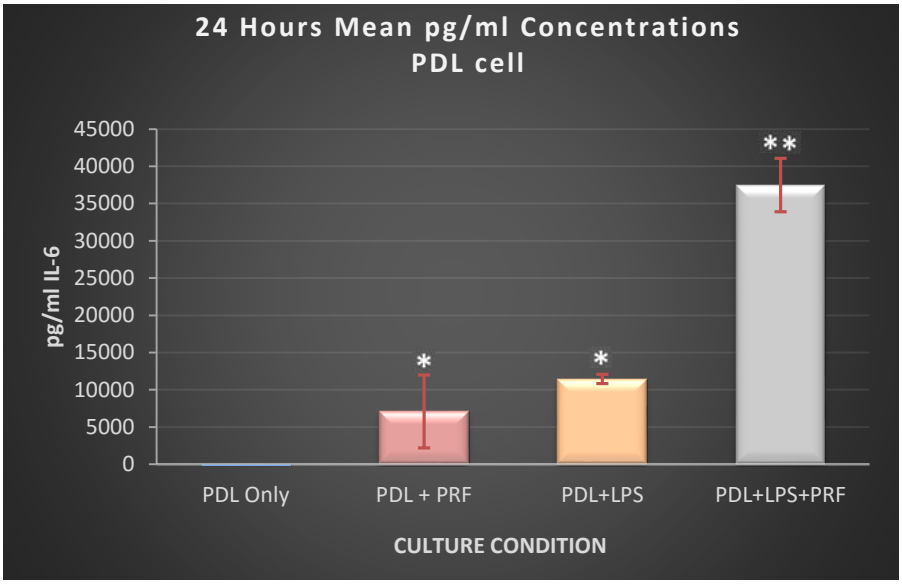


Figure legend: Same number of * indicates no difference between groups. Different number of * indicates statistical significance between groups. Between PDL+LPS+PRF and PDL+LPS ($p<0.031$). Between PDL+LPS+PRF and PDL ($p<0.03$). Between PDL+LPS and PDL ($p<0.03$).

At 48 hours there is an overall significant difference between the groups by Kruskal-Wallis test ($p=0.0003$). Wilcoxon rank sum test was used to conduct pairwise comparisons. Table 19 contains the pairwise comparison p-values between the groups at 48 h, adjusted for pairwise comparisons with Bonferroni's method. Figure 15 graphically represents these comparisons.

- The PDLs concentration is significantly lower than all other groups
- The LPS only group concentration is significantly different (lower) than the LPS+PRF group.
- The PRF only group concentration is significantly different (lower) than LPS+PRF

Table 19: 48-hour culture group IL-6 production comparison

	Group A	Group B	Group C	Group D
Group A (PDL +PRF)				
Group B (PDL + LPS +PRF)	**0.049**			
Group C (PDL + LPS)	1.0	**0.031**		
Group D (PDL only)	**0.049**	**0.031**	**0.031**	

Figure 15: 48-hour mean pg/ml concentrations PDL cells

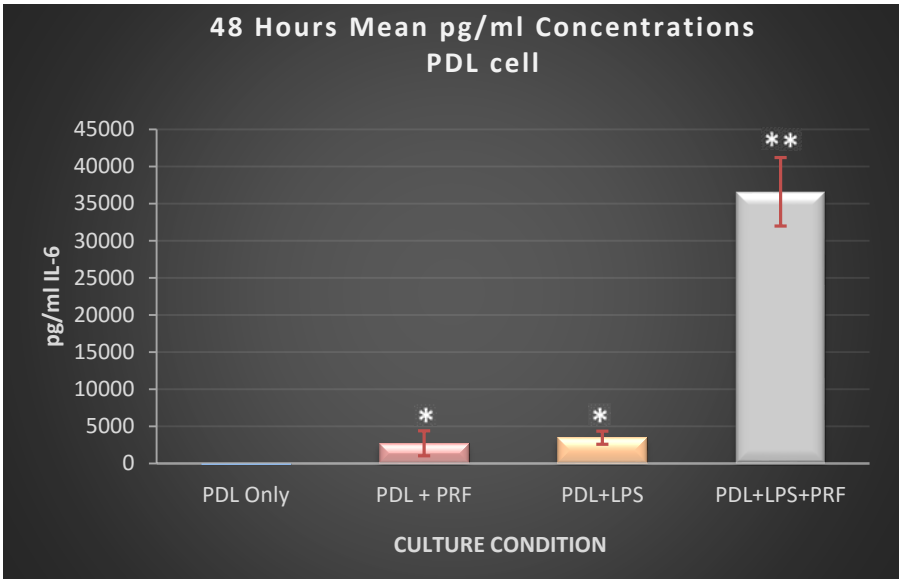


Figure legend: Same number of * indicates no difference between groups. Different number of * indicates statistical significance between groups. Between PDL+LPS+PRF and PDL+PRF ($p<0.049$) and PDL and PDL+LPS ($p<0.031$). Between PDL+PRF and PDL ($p<0.049$). Between PDL + LPS and PDL ($p<0.031$).

At 72 hours there is an overall significant difference between the groups by Kruskal-Wallis test (p=0.0006). Wilcoxon rank sum test was used to conduct pairwise comparisons. Table 20 contains the pairwise comparison p-values between the groups at 72 h, adjusted for pairwise comparisons with Bonferroni's method. Figure 16 graphically represents these comparisons.

- The LPS+PRF group concentration is significantly different (higher) than all other groups.

Table 20: 72-hour culture group IL-6 production comparison

	Group A	Group B	Group C	Group D
Group A (PDL +PRF)				
Group B (PDL + LPS +PRF)	**0.049**			
Group C (PDL + LPS)	0.14	**0.031**		
Group D (PDL only)	0.073	**0.049**	1.0	

Figure 16: 72-hour mean pg/ml concentrations PDL cells

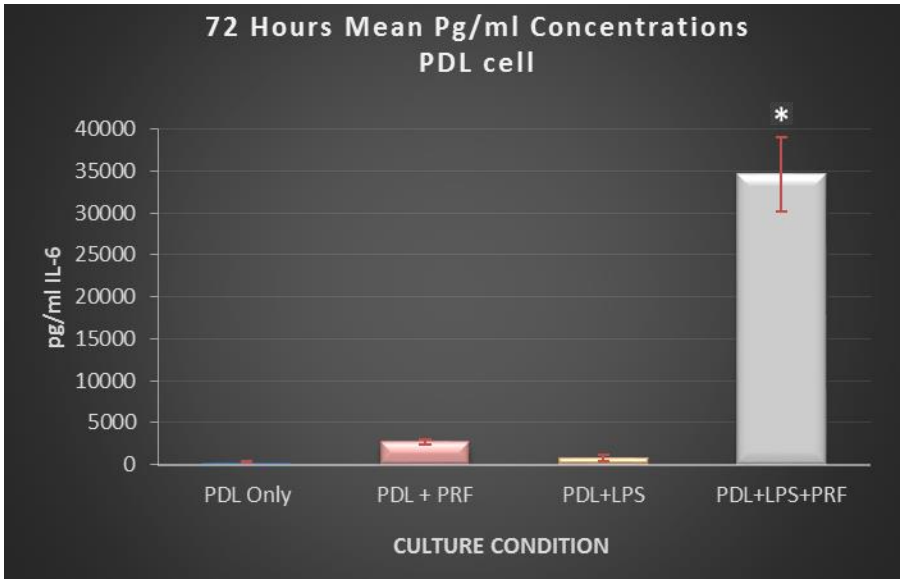


Figure legend: Same number of * indicates no difference between groups. Different number of * indicates statistical significance between groups. Between PDL+LPS+PRF and PDL+PRF and PDL ($p<0.049$). Between PDL+LPS+PRF and PDL+LPS ($p<0.031$).

The averages over time show an overall significant difference between the groups by Kruskal-Wallis test ($p<0.0001$). Wilcoxon rank sum test was used to conduct pairwise comparisons. Table 21 contains the pairwise comparison p-values between the groups, adjusted for pairwise comparisons with Bonferroni's method. Figure 17 graphically represents these comparisons.

- All groups are significantly different from each other, except for the groups with only PRF and only LPS.

Table 21: Average 24-72 hour culture group IL-6 production comparison

	Group A	Group B	Group C	Group D
Group A (PDL +PRF)				
Group B (PDL + LPS +PRF)	<0.001			
Group C (PDL + LPS)	1.0	<0.001		
Group D (PDL only)	0.0018	<0.001	<0.001	

Figure 17: Average pg/ml of IL-6 from 24-72 hours

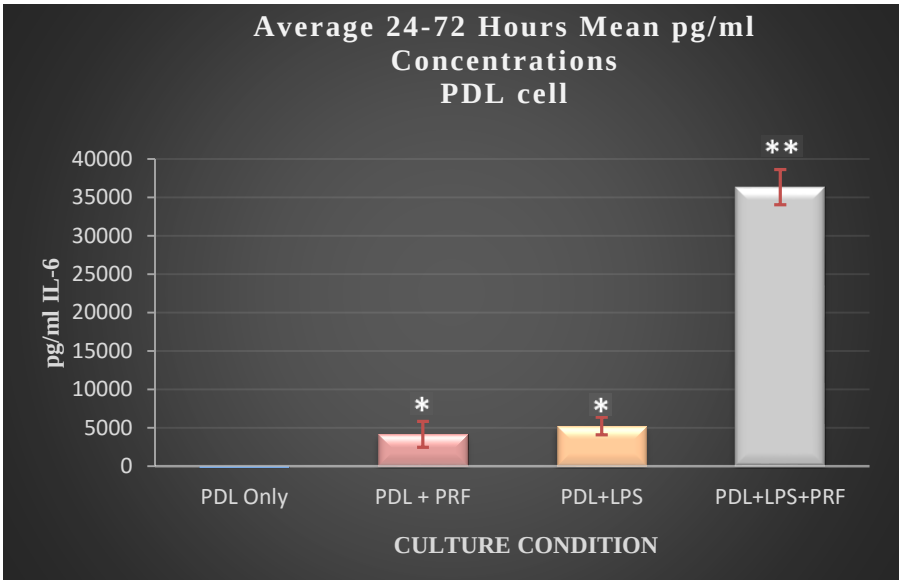


Figure legend: Same number of * indicates no difference between groups. Different number of * indicates statistical significance between groups ($p<0.001$).

Evaluating each individual group's change over time showed that the PRF only group did not statistically change over time ($p=0.83$) by using the Kruskal-Wallis test. The LPS+PRF group also did not significantly change over time ($p=0.13$). The LPS only group decreased over time ($p=0.0011$), and the PDLs increased over time ($p=0.0017$). Figure 18 graphically depicts these group comparisons.

Figure 18: Averages of groups in pg/ml of IL-6 over 24-72 hours

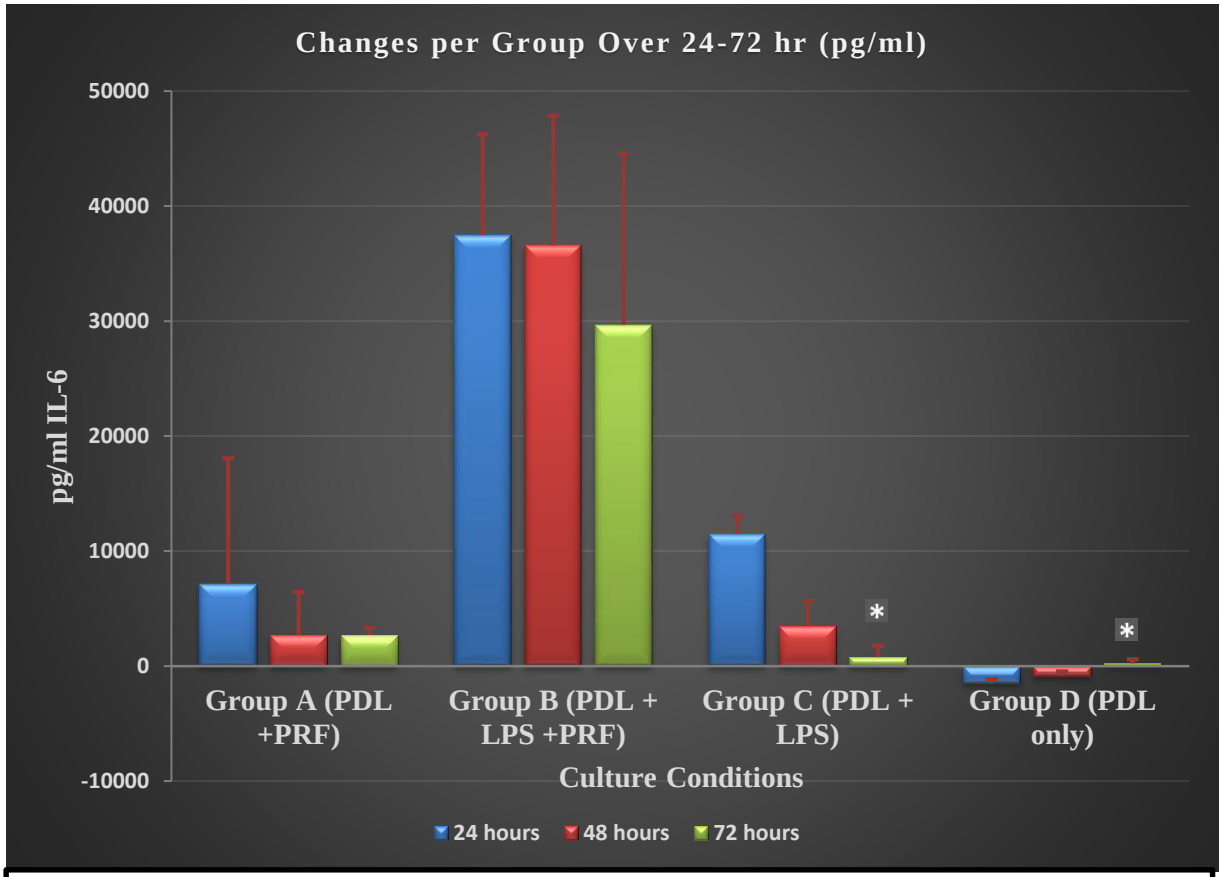


Figure legend: The PRF only group and the LPS+ PRF groups did not significantly change over time. The LPS only group decreased over time. The PDLs increased over time. The * indicates a statistically significant change between the 24-72 hour periods.

Evaluation of the PRF membrane and expressed liquid revealed that IL-6 release within 24 h of membrane preparation was negligible and therefore, did not interfere with group concentrations.

CHAPTER 5: DISCUSSION

This single-blinded randomized controlled clinical trial evaluated the effect of PRF on pulp vitality after atraumatic extraction, root resection, and immediate reimplantation for a period of 3-11 months. Every precaution was taken to eliminate any bias by compartmentalizing the various aspects of the study protocol as follows: blind examiner (EE) collected baseline and final diagnostic data, fabricated PRF, performed the root resection, and recorded histological results dictated by pathologist. Clinician (JB) performed extractions and randomization of PRF application, but was blind to diagnostic data and histological findings until study completion. Investigator (PG) evaluated histological specimens while blind to any patient information, diagnostic data, or tooth treatment. While patient selection, and furthermore tooth selection within those patients, was severely limited by the poor condition of their oral health, teeth were selected that permitted little to no baseline differences between treatment groups. These differences resulted in no statistical significance in baseline parameters. Pre EPT values were 33.1 (+/- 3.0) and 34.0 (+/- 3.0) for control and PRF teeth respectively. Pre thermal values demonstrated that 93.9% (+/- 4.8) of control and 85.2% (+/- 8.9) of PRF treated teeth responded positively to cold. During the study, 28% of patients required a post-operative week of clindamycin due to symptoms of infection; however, the multivariate analysis did not find any correlation between any other variable.

We found that the addition of PRF did not significantly impact any measured variable when compared to the non-PRF controls. There are numerous points to consider while interpreting these results. Firstly, vitality of control teeth was not an unexpected outcome. In Skoglund's research, the series of studies showed that if an apicoectomy procedure is used to enlarge the apical foramen, revascularization of mature teeth after transplantation is possible and has a high success rate. Therefore, the intention of the present study was to evaluate if PRF enhanced these results in mature teeth. The present study showed similar histological results for both groups as

did the apicoectomy histology of the Skoglund series, emphasizing that enlargement of the apical foramen may be enough to encourage regeneration. An additional finding in the Skoglund series suggests a possible reasoning for the decreasing trend in vitality parameters observed in the present study. Skoglund et al. (1981) discussed that widening of the apical foramen with a bur is not as successful as the method they chose in their study, which was enlargement with a wire cutter. As the methods in the present study involved a bur for the apicoectomy, it is possible that this encouraged more inflammatory response than the wire cutter in Skoglund's study. This may be one reason why both groups saw a significant increase in EPT (average of 22.7 EPT points higher post-treatment) and decrease in thermal response (30% lower) from pre to post vitality recordings.

Next, a discussion on subject complications is necessary. Subject recruitment proved to be challenging due to the specific requirements of the study and the general nature of the dentition of many patients presenting to the dental school in need of full or partial dentures. Therefore, the original N of 20 subjects with 80 study teeth was not attainable in the restricted time for the study. Instead, an N of 18 subjects with 69 teeth were evaluated. This 14% reduction may have kept some of the measurement parameters from achieving statistical significance. Additionally, 7 of these teeth were determined 'inconclusive' histologically and could not be utilized for histological evaluation.

Next, one must consider the state of the teeth and periodontium of the subjects in this study. Autotransplantation can be highly successful especially in teeth without fully developed roots (ideally no more than two thirds of its final length) and if it is moved atraumatically to a suitable receptor site with minimal out-of-mouth time. The receptor site should be wide enough and free of inflammation to enhance results (Laureys et al., 2013). In the present study, due to the limited patient pool as well as the nature of the dentition in most patients electing full dentures, the teeth and corresponding periodontium were not ideal. Laureys et al. (2013) notes that absence of

inflammation can improve the results of autotransplantation; however, most of the subjects in the present study had significant inflammation, minor to severe carious lesions, significant occlusal wear, and periodontal disease limiting the support for the reimplanted teeth.

In addition to the initial substandard condition of the subjects' dentition, it became apparent that in many cases the state of occlusion would be impossible to manage. During the healing process, it is important that the tooth is not traumatized as occlusal forces can expand the inflammatory response and lead to fibrous tissue formation in the pulp tissue (Hasegawa et al., 2007). This could be a contributing factor in some subjects whose teeth maintained less vitality than others. As these patients were undergoing the process of denture fabrication, many of them were in a state of considerable malocclusion and had few remaining teeth; therefore, attempts were made to minimize occlusal discrepancies but ideal was not always achievable. In many cases due to loss of vertical dimension, malocclusion, or limited remaining teeth for mastication, the subjects continued to function on the study teeth and the teeth were unable to be removed from traumatic occlusion.

A second occlusal issue that arose was patients with nocturnal parafunctional habits such as bruxing and clenching. It was observed that these patients experienced considerably more post-operative discomfort than other study patients, and likely resulted in more trauma to the study teeth throughout the observation period. The results from the histological analysis further pointed to the importance of minimal occlusal trauma. Many of the individuals who had more than one non-vital tooth (either control or test) at the end of the study were patients that presented with multiple post-operative complications due to nocturnal parafunctional habits. One individual had six teeth that were utilized for the study and all six, regardless of treatment, responded non-vital to diagnostic testing and were all observed to be necrotic histologically. This individual had the most post-operative appointments and reported significant pain while sleeping and had a history

of bruxing and clenching. Additionally, patients that had only one arch of dentition remaining, therefore no opposing dentition, were observed to have fewer complications throughout the study.

A factor found to be more critical than the application of the PRF was the amount of time it took to complete the intervention to the tooth and therefore, the amount of time the tooth was out of the mouth. In this study, the average out of mouth time for control teeth and PRF treated teeth was 1.62 (+/-.11) and 1.88 (+/- .11) minutes respectively; this was statistically significant (p= 0.035). Out-of-mouth time is another important feature to consider in the success of the reimplantation (Andreason et al., 1981; Andreason et al., 1990). The out-of-mouth time should be kept at a minimum through quick handling and efficient execution of the procedure steps (Laureys et al., 2013). In the present study, the out-of-mouth time was longer for teeth receiving the intervention. This resulted in a statistically significant relationship with post EPT (p=.0090) and thermal values (p=.0039). Better results for PRF treated teeth may have been observed if out-of-mouth time for PRF treated teeth and control teeth had been similar. When investigating pulpal revascularization and vitality, it may benefit future studies to focus more on the out of mouth time for tooth transplantations than on the different interventions to the root surface.

In addition, there was also subject bias that came into play regarding the accuracy of the results. Due to the nature of the diagnostic testing it is difficult to determine a simple yes or no vitality reading. Percussion is suggestive of pressure in the PDL or possibly traumatic occlusion, but does not give a clear answer regarding vitality. Thermal testing can be misleading as many individuals have vital teeth that all test negative to cold. EPT can allow the investigator to evaluate trends, but it is difficult to determine complete loss of vitality. Additionally, with regeneration, vitality diagnostic testing is not always predictable. Peters (2014) noted that it is unlikely there will be a positive response to cold or electrical stimuli in all cases of regeneration. This was verified histologically, as there was no correlation found between post-operative diagnostic testing and histological evidence of vitality; however, there was a correlation with pre-

diagnostic EPT tests (p=0.054). This suggests that teeth with high EPT values to start with had a higher chance of being vital at the end of the study. This was difficult to evaluate; however, as subjects were frequently not consistent in when they informed the investigator (EE) they felt the stimulus. For example, sometimes they would inform upon experiencing any response to stimulus and then would not respond unless it was to a certain 'strength' or 'discomfort' level they were expecting. It also became apparent that subjects may be withholding honest answers in efforts to sway the results in one direction or the other. Even though investigators did not inform the subjects on what responses were positive or negative, it was apparent to the investigator that some desired a successful result and tried to provide that. This may have led to some bias in reporting the diagnostic test results, therefore, it is crucial that histology was also available for examination.

For these reasons, it was beneficial that the study additionally included evaluation of the histological state of each study tooth. Some difficulties were encountered in this aspect of the study as well. As many of the teeth were not in ideal condition and many were small mandibular incisors, it was frequently difficult to obtain a clean view of the pulp chamber. If pulp tissue was not seen on initial cuts, recuts requesting deeper sections were ordered. After multiple recuts and sometimes reorientation of the teeth, the pulp chambers could still not be found. This led to 7 teeth (10% of samples) being labeled as inconclusive and histological data could not be submitted for statistical analysis on these teeth. From the histological patterns noted, it appears that repair of the pulp occurs through the proliferation and migration of cells from the recipient bed in the periapical region and moves upward towards the coronal aspect of the pulp canal. This is in accordance with other autotransplantation studies (Skoglund et al., 1998; Laureys et al., 2010).

Despite the teeth having been in the mouth for a period of at least 3 months, if there was inflammatory infiltrate present it was frequently observed as acute or mixed (52% of samples). This may have been related to the IL-6 present in the PRF membrane; given that IL-6 extends the period of acute inflammation before finally assisting in the transition to chronic inflammation

(Gabay et al., 1997). While 40% of the samples presented with no observable inflammatory infiltrate and only 8% demonstrated chronic inflammation, it is plausible that the PRF was aiding in prolonging the acute inflammatory response. As the PRF was placed based off of a coin flip, the teeth receiving the PRF may not have been very far apart and therefore, the benefits of the PRF may have extended to the control teeth which is why no observable difference was noted between groups for either diagnostic testing or histological analysis of regeneration. A split mouth design may not be reasonable when utilizing a material with the potential to measurably affect the inflammatory response so drastically. It is unlikely it remained localized to only the tooth apex to which it was applied. Regardless, after evaluation of both the diagnostic test results as well as the histologic data, the initial clinical hypothesis must be rejected. Ultimately, PRF did not enhance the regeneration of teeth in the treatment group over the control group.

Regarding the IL-6 *in vitro* results, it was observed that in contrast to the initial hypothesis, PRF synergized with LPS in stimulation of IL-6 from human PDL cells. A one-way analysis of variance (ANOVA) test was conducted. An ANOVA has 4 assumptions: independence, normality, linearity, and equal variance between groups. Because model assumptions were violated with the *in vitro* data (normality and equal variance) and transformations did not fix the violations, non-parametric tests were used for comparisons. Analysis was done of the data at each time point and for all the time points combined. A Kruskal-Wallis test was used to look at overall group differences. Pairwise comparisons were conducted with the Wilcoxon rank sum test and were adjusted for multiple comparisons using Bonferroni's method. SAS software version 9.3 was used for statistical analysis (SAS Institute Inc., Cary, NC).

Throughout the experiment from 24-72 h, the PRF+LPS group maintained a statistically significant ($p<.001$) higher concentration of IL-6 when compared to all other groups. The PRF only group behaved essentially like the LPS only group and demonstrated no statistical significance throughout the observed time period indicating that PRF and LPS are similar in their

abilities to stimulate the production of IL-6 from PDL cells in culture. Several considerations must be taken into account in interpreting these results. Firstly, PRF liquid contained negligible IL-6, which is in accordance with published literature on initial cytokine release from a PRF membrane (Dohan et al., 2009). Dohan et al. (2009), demonstrated that the initial extracted amounts of growth factors soon after membrane preparation was always significantly lower than the released total amounts from the membrane itself when tested after 7 days of incubation. Therefore, through determining the initial release of IL-6 from the PRF membrane to be negligible, it can be inferred that the IL-6 expression observed in the present study is a product of stimulation from the PDL cells in culture.

While there was no IL-6 detectable after 24 h in the PRF membrane or the liquid immediately expressed from the membrane, it is plausible that there may have been additional release over an extended period of time. In a study by Dohan et al. (2009), PRF membranes and the liquid expressed from them shortly after fabrication were analyzed for 4 main cytokines via ELISA. Over 200 h, cytokines TGFβ1, VEGF, PDGF-AB, and TSP-1 were evaluated in time intervals, and all but VEGF demonstrated similar release patterns with a quick release during the first 24 h and then a slow down around day 2 and finally a steady state of slow release up until day 5. VEGF showed an increased release during the initial 4 h and then followed the trends of the others. This demonstrates that PRF does release cytokines into the environment, even without provocation.

From the results of the present study it is evident that IL-6 does not follow a similar quick 24-hour release; however, we do see a similar trend (though not statistically significant) in the amount of IL-6 expressed by PDL cells at 24-72 h suggesting that the pro-inflammatory cytokines released from the PRF membrane evokes IL-6 production by the PDLs which peaks early on and then holds a relatively steady state with minor decline over the next two days. This demonstrates the known slow steady release of cytokines previously shown with PRF; had the study been

extended, there may have been a larger drop observed later. In a study by Su et al. (2009), the growth factor concentrations of PRF were analyzed and shown to increase in concentration in solution from 5 to 300 minutes. While the investigators did not analyze IL-6, it is plausible that its expression from the PRF membrane also increases over time. This slow sustained release of IL-6 and other pro-inflammatory cytokines evoking IL-6 release is a plausible reason for the observed increase in this cytokine over the course of 72 h in the present study. When considering an *in vivo* model, this may become an active player between 1-2 weeks after application. If the period of study had been extended, a continued decreasing trend in the production may have been observed.

Additional considerations for these results must include a discussion on the quantity of IL-6 observed in the laboratory environment versus the differences in the biological environment of the human mouth. Dohan et al. (2009) states that immediately after application of PRF, the platelets are activated and drastically evoke cytokine and growth factor release from neighboring cells during the first few hours after application. It is likely that the time frame of the current study captured this massive release of cytokines that elicited IL-6 production from the PDL cells, which initially ramped up cell inflammation but then began to taper off, and likely a steady state would have been reached. In the present study's closed system, this trend is straightforward; however, when utilized in a human body the multifactorial nature of these interactions become complicated. One such consideration involves the leukocyte concentration of the PRF. Choukroun's PRF is a leukocyte-rich form of PRF, meaning that leukocytes are not separated out during the fabrication process. The presence of leukocytes in the formulation has been suggested to influence the observed pro-inflammatory effects through the proteases and acid hydrolases contained in white blood cells (Anitua et al., 2007). This factor also may have perpetuated the release of IL-6 in the closed PDL cell environment in the present study; however, the leukocytes may have added many additional benefits that outweigh their pro-inflammatory characteristics in

an *in vivo* model. The presence of these leukocytes may have assisted in resolving the inflammation for the 40% of teeth that histologically demonstrated no inflammatory infiltrate.

It has to be acknowledged that the behavior of the leukocytes and the PRF membrane itself in a less diluted medium may also be different. Dohan et al. (2009) points out that by placing the PRF membranes in DMEM, it creates a massive call for cytokine release due to dilution related concentrations. Therefore, it is possible that in a physiological situation, the actual release profile in the biological environment may differ. In addition to this, aside from the actual cytokine release, one must also consider how the biological environment may respond to other components of the PRF fibrin mesh. Hotwani & Sharma (2014) indicate that there are several cytokines within the PRF matrix that modulate inflammation and act as anti-inflammatory counterparts to the pro-inflammatory cytokines present. IL-4, for example, assists in reducing inflammation through decreasing the production of cells that are major players in tissue destruction and propagation of the inflammatory response. Simonpiere et al. (2009) note that platelet cytokines PDGF, TGF-α, IGF-1 are gradually released as the fibrin matrix is resorbed, which allows a perpetual process of healing through neoangiogenesis and the formation of connective tissue components. Therefore, the nature of the inflammatory process and the complexity of the interactions occurring *in vivo* must be taken into account when interpreting the synergistic effect of PRF and LPS on IL-6 release.

A certain amount of inflammation is necessary and required for the healing process, therefore some stimulation of IL-6 by PRF can actually be a benefit in a biological environment. The anti-inflammatory properties of IL-6 can assist in extending the acute inflammatory period (Barnes et al., 2011). This, along with other anti-inflammatory cytokines released from PRF, may assist *in vivo* in reducing the tissue destruction during remodeling after tooth avulsion or transplantation. Additionally, the angiogenic properties of IL-6 through stimulation of VEGF from fibroblasts, as well as prevention of collagenolysis through the upregulation of TIMP-1,

may make IL-6 a valuable presence for the promotion of tissue healing (Mihara et al., 1995; Jones et al., 2005). Therefore, when considering both the beneficial aspects of IL-6 along with the PRF membrane's release of anti-inflammatory cytokines and ability to evoke other anti-inflammatory modulators from cells in the surrounding tissues, the other components within the membrane may counteract this seemingly large amount of IL-6 evoked by PRF and LPS in this study. IL-6 may have been a benefit in this present study's clinical arm where it may have helped to prolong the acute inflammatory response for all teeth in the area, both test and control, as well as promote new vessel growth and regeneration.

Therefore, from the results of the present study, it is apparent that the initial hypothesis must be rejected. Rather than reducing the inflammatory response, it can be concluded that PRF unequivocally elicits stimulation of IL-6 from PDL cells. Taking the previously mentioned factors into consideration it is likely that IL-6 concentrations in this cell culture study are inflated from what may actually be occurring when a PRF membrane is utilized by a dentist to promote intraoral healing or by a physician to overcome a patient's chronic non-healing ulcer.

CHAPTER 6: CONCLUSIONS

The addition of PRF does not improve the diagnostic tests (EPT, thermal, or percussion) or the histological evidence of vitality of an autotransplanted tooth when compared to apicoectomy and foramen enlargement alone. However, more benefit may be seen with the use of PRF in cases where trauma from occlusion can be ideally managed. Future studies may benefit from evaluating the effects of out of mouth time in regards to autotransplanted teeth and vitality testing. Additionally, PRF was found to synergistically enhance IL-6 production from PDL cell cultures when combined with LPS. The overall effects of this production may be diminished *in vivo* when considering the other anti-inflammatory cytokines produced in response to IL-6. Evaluating multiple pro and anti-inflammatory cytokines in the PRF membrane may be beneficial for future studies.

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Appendix A: IRB consent form

University of Nebraska Medical Center

Institutional Review Board (IRB)

What Do I Need To Know Before Being In A Research Study?

You have been invited to be in a **research study**. Research studies are also called "clinical trials" or "protocols." **Research** is an organized plan designed to get new knowledge about a disease or the normal function of the body. The people who are in the research are called **research subjects.** The **investigator** is the person who is running the research study. You will get information from the investigator and the research team, and then you will be asked to give your **consent** to be in the research.

This sheet will help you think of questions to ask the investigator or his/her staff. You should know all these answers before you decide about being in the research.

What is the **purpose** of the research? Why is the investigator doing the research?

What are the **risks** of the research? What bad things could happen?

What are the possible **benefits** of the research? How might this help me?

How is this research different than the care or treatment I would get if I wasn't in the research? Are there other treatments I could get?

Does **everyone** in this research study get the same treatment?

Will being in the research **cost** me anything extra?

Do I have to be in this research study? Will the doctor still take care of me if I say no?

Can I **stop** being in the research once I've started? How?

Who will look at my **records**?

How do I reach the investigator if I have more **questions**?

Who do I call if I have questions about being a **research subject**?

Make sure all your questions are answered before you decide whether or not to be in this research.

Academic Research & Services Building 3000 / 987830 Nebraska Medical Center / Omaha NE 68198-7830
402-559-6463 / FAX 402-559-3300 / Email: irbora@unmc.edu / http://www.unmc.edu/irb

University of Nebraska Medical Center

Institutional Review Board (IRB)

THE RIGHTS OF RESEARCH SUBJECTS

AS A RESEARCH SUBJECT AT THE NEBRASKA MEDICAL CENTER YOU HAVE THE RIGHT ...

... to be told everything you need to know about the research before you are asked to decide whether or not to take part in the research study. The research will be explained to you in a way that assures you understand enough to decide whether or not to take part.

... to freely decide whether or not to take part in the research.

... to decide not to be in the research, or to stop participating in the research at any time. This will not affect your medical care or your relationship with the investigator or the Nebraska Medical Center. Your doctor will still take care of you.

... to ask questions about the research at any time. The investigator will answer your questions honestly and completely.

... to know that your safety and welfare will always come first. The investigator will display the highest possible degree of skill and care throughout this research. Any risks or discomforts will be minimized as much as possible.

... to privacy and confidentiality. The investigator will treat information about you carefully, and will respect your privacy.

... to keep all the legal rights you have now. You are not giving up any of your legal rights by taking part in this research study.

... to be treated with dignity and respect at all times

The Institutional Review Board is responsible for assuring that your rights and welfare are protected. If you have any questions about your rights, contact the Institutional Review Board at (402) 559-6463.

University of Nebraska Medical Center

Adult Consent Form-Part I

Title of this Research Study

The use of platelet rich fibrin in pulpal and periodontal regeneration in mature teeth

You are invited to participate in this research study. Participation is completely voluntary. This study is trying to determine if the addition of Platelet Rich Fibrin (PRF) (derived from the patients own blood) can help revascularize a tooth that has been separated from its socket. If this is shown to be successful it may be utilized to prevent the nerve of the tooth from dying during traumatic incidents or tooth transplantations. The purpose of this part of the research is to determine if PRF can decrease inflammatory markers of tooth cell cultures.

We would like to obtain a one time blood sample from you of 4tsps.

The risks of the blood draw include swelling, pain at collection site, hematoma (bruise), fatigue (feeling tired), syncope (losing conscious, fainting). You are not expected to benefit from participating but If this is shown to be successful it may be utilized to prevent the nerve of the tooth from dying during traumatic incidents or tooth transplantations. You can choose not to participate or withdraw at any time. Choosing to not participate or withdraw will not impact your relationship with the institution or the investigator.

We will not record any identifying information about you. All research data will be securely stored on password protected computers.

You have rights as a research subject. These rights have been explained in this consent form and in The Rights of Research Subjects that you have been given. If you have any questions concerning your rights or complaints about the research, you can contact any of the following:

- The investigator or other study personnel
- Institutional Review Board (IRB)
    - Telephone: (402) 559-6463.
    - Email: IRBORA@unmc.edu
    - Mail: UNMC Institutional Review Board, 987830 Nebraska Medical
    - Center, Omaha, NE 68198-7830
- Research Subject Advocate
    - Telephone: (402) 559-6941
    - Email: unmcrsa@unmc.edu

Documentation of informed consent

You are freely making a decision whether to be in this research study. Signing this form means that:

- You have read and understood this consent form.
- You have had the consent form explained to you.
- You have been given a copy of The Rights of Research Subjects
- You have had your questions answered.
- You have decided to be in the research study.
- If you have any questions during the study, you have been directed to talk to one of the investigators listed below on this consent form.

You will be given a signed and dated copy of this consent form to keep.

Signature of Subject _____
Date _____

My signature certifies that all the elements of informed consent described on this consent form have been explained fully to the subject. In my judgment, the subject possesses the legal capacity to give informed consent to participate in this research and is voluntarily and knowingly giving informed consent to participate.

Signature of Person obtaining consent _____
Date _____

Authorized Study Personnel

Principal
Easley, Elisabeth
phone: 402-472-6770
alt #: 530-524-6020

ADULT CONSENT - CLINICAL BIOMEDICAL

Title of this Research Study
The use of platelet rich fibrin in pulpal and periodontal regeneration in mature teeth

Invitation
You are invited to take part in this research study. You have a copy of the following, which is meant to help you decide whether or not to take part:

- Informed consent form
- "What Do I need to Know Before Being in a Research Study?"
- The Rights of Research Subjects

Why are you being asked to be in this research study?
You are at least 19 years of age and have single rooted teeth treatment planned to be extracted (taken out) to meet your prosthetic or orthodontic needs. If you are pregnant or plan to become pregnant during this study, you may not be included in this study.

What is the reason for doing this research study?
This study is trying to determine if the addition of Platelet Rich Fibrin (PRF) (a substance from your own blood) can help bring blood vessels back to a tooth that has either been taken out or fallen out due to an accident. If this is shown to be successful it may be used to prevent the nerve of the tooth from dying during trauma accidents or dental procedures like tooth transplantations.

What will be done during this research study?
We will use the four teeth you are having removed as part of your care for this study. The teeth will be x rayed as determined by your original treatment plan. Thermal testing (placing cold) on the tooth will be done to determine if the tooth is alive, there are no adverse effects associated with this test. For the research, 2 teaspoons of blood will be taken from a vein in your arm and used to make the material being placed on your teeth.

The four teeth will be extracted and the study intervention performed (removing a section of the root, using instruments to clean the nerve canal and placing the PRF in and around the root) to two of them, while the other 2 remain un-treated. All four teeth will then be put back in the sockets and secured with a wire and tooth colored filling splint. 4 months after, the research requires the teeth will be x-rayed and extracted and this will be the completion of the study participation.

The first visit will be a consult to discuss the study, determine if you are eligible, and review the consent forms. This can be combined with your other dental appointments and should not exceed 45 minutes. The second visit will involve the blood draw and first extractions and should not exceed 3 hours. The third and final visit will involve x rays of the 4 teeth used in the study, a clinical exam, and the final extraction and this visit should not exceed 3 hours.

What are the possible risks of being in this research study?
Blood draw- swelling, pain at collection site, bruising, feeling tired, losing conscious, fainting

Surgical Procedures (extractions, reimplantations, use of local anesthetics)- bleeding, swelling, discomfort/pain, infection, damage to nerve, adjacent teeth, sinus exposure, allergic reaction (from anesthetic), heart palpatations or increased heart rate.

Endodontic necrosis and symptoms (nerve pain associated with the teeth utilized in the study)- few if any complications have been reported in case reports in 2-3 months of avulsion (where teeth have been lost in traumatic accidents) models

Radiation- additional radiographs are of a very small amount radiation
It is possible that other rare side effects could occur which are not described in this consent form. It is also possible that you could have a side effect that has not occurred before.

What are the possible benefits to you?
You are not expected to get any benefit from being in this research study.

What are the possible benefits to other people?
If this is shown to be successful it may be used to prevent the nerve of the tooth from dying during traumatic incidents or during tooth transplantations.

What are the alternatives to being in this research study?
Instead of being in this research study, you can choose not to participate.

What will being in this research study cost you?
There is no cost to you to be in this research study except for the cost of gas to drive to 2 additional appointments, which can be combined with other appointments already in your treatment plan.

You or your insurance company will be responsible for all standard of care procedures including the first extraction. The second extraction and all other procedures done solely for the research study will be covered by the research funds.

Will you be paid for being in this research study?
You will be compensated at $12.50/hour for each clinic visit given in gift cards to local department
stores.

Additionally, you will be compensated for driving time based on the googlemaps distance and time from your home address to the address of the UNMC dental school. The time will include both driving to the dental school and driving home.

Who is paying for this research?
This research is being paid for by University of Nebraska Medical Center surgical specialties dept

What should you do if you are injured or have a medical problem during this research study?
Your welfare is the main concern of every member of the research team. If you are injured or have a medical problem as a direct result of being in this study, you should immediately contact one of the people listed at the end of this consent form. Emergency medical treatment for this injury or problem will be available at the Nebraska Medical Center. If there is not sufficient time, you should seek care from a local health care provider.

The Institution has no plans to pay for any required treatment or provide other compensation. If you have insurance, your insurance company may or may not pay the costs of medical treatment. If you do not have insurance, or if your insurance company refuses to pay, you will be expected to pay for the medical treatment.

Agreeing to this does not mean you have given up any of your legal rights.

How will information about you be protected?
You have rights regarding the protection and privacy of your medical information collected before and during this research. This medical information is called "protected health information" (PHI). PHI used in this study may include your medical record number, address, birth date, medical history, the results of physical exams, blood tests, x-rays as well as the results of other diagnostic medical or research procedures. Only the minimum amount of PHI will be collected for this research. Your

which you are entitled.

What will happen if you decide to stop participating once you start?
You can stop participating in this research (withdraw) at any time by contacting the Principal Investigator or other research personnel listed at the end of the consent form.

Deciding to withdraw will otherwise not affect your care or your relationship with the investigator or this institution. You will not lose any benefits to which you are entitled.

For your safety, please talk to the research team before you stop doing any home care recommended by the research team that is related to the procedures. They will advise you how to care for your mouth after the surgeries. You may be taken off the study if you do not
follow instructions of the investigator or the research team.

You may also be taken off the study if you fail to keep surgical or post operative appointments.

Will you be given any important information during the study?
You will be informed promptly if the research team gets any new information during this research study that may affect whether you would want to continue being in the study.

What should you do if you have any questions about the study?
You have been given a copy of "What Do I Need to Know Before Being in a Research Study?" If you have any questions at any time about this study, you should contact the Principal Investigator or any of the study personnel listed on this consent form or any other documents that you have been given.

What are your rights as a research participant?
You have rights as a research subject. These rights have been explained in this consent form and in The Rights of Research Subjects that you have been given. If you have any questions concerning your rights or complaints about the research, you can contact any of the following:

- The investigator or other study personnel
- Institutional Review Board (IRB)
    - Telephone: (402) 559-6463.
    - Email: IRBORA@unmc.edu

Subject's Initials_____

- Mail: UNMC Institutional Review Board, 987830 Nebraska Medical Center, Omaha, NE 68198-7830
- Research Subject Advocate
  - Telephone: (402) 559-6941
  - Email: unmcrsa@unmc.edu

Documentation of informed consent

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- You have been given a copy of The Rights of Research Subjects
- You have had your questions answered.
- You have decided to be in the research study.
- If you have any questions during the study, you have been directed to talk to one of the investigators listed below on this consent form.
- You will be given a signed and dated copy of this consent form to keep.

Signature of Subject _____
Date _____

My signature certifies that all the elements of informed consent described on this consent form have been explained fully to the subject. In my judgment, the subject possesses the legal capacity to give informed consent to participate in this research and is voluntarily and knowingly giving informed consent to participate.

Signature of Person obtaining consent _____
Date _____

Authorized Study Personnel

Principal

Easley, Elisabeth
phone: 402-472-6770
alt #: 530-524-6020
degree: DMD, MPH

Secondary

Bavitz, J
phone: 402-472-1314

Johnson, Paul
phone: 402-472-6770

alt #: 402-472-6205
degree: DDS

degree: DDS

Subject's Initials_____

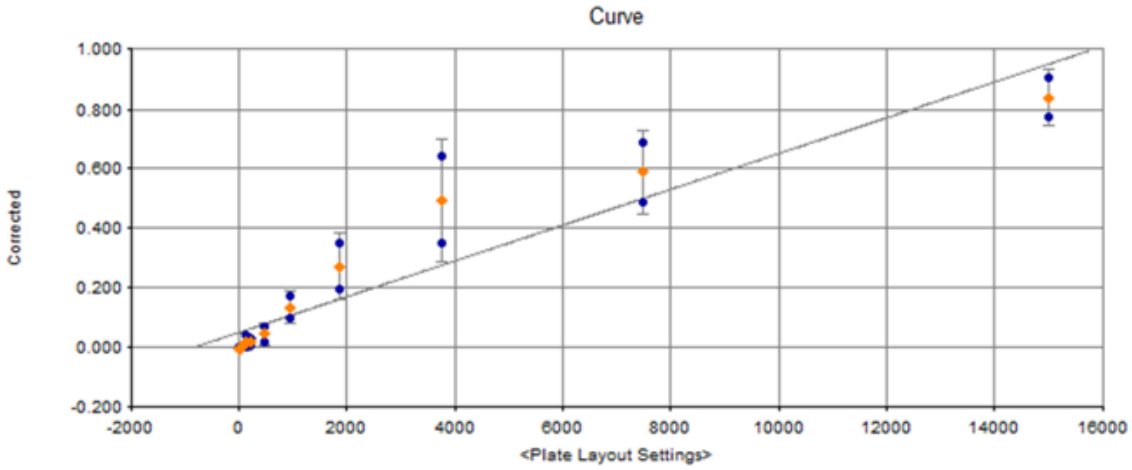
Appendix B: pg/ml concentrations of 24-72 hour collections

PDL ELISA Pg/ml Conversion Plate A (Experiment 4)

	1	2	3	4	5	6	7	8	9	10	11	12
C	-1415.47	-1810.56	-1595.06	-1415.47	-1475.34	-972.50	-912.64	-780.94	-1475.34	-733.05	-613.33	-421.77
D	-194.30	-14.71	69.09	643.77	583.90	X	24001.76	X	1661.41	12280.86	-1188.00	-1176.03
E	8353.94	X	547.99	4726.33	-278.10	93.04	3122.04	X	3277.68	3014.29	1829.03	2224.11
F	10772.35	12448.47	12855.53	10389.24	9239.89	13071.04	3852.35	2260.03	1074.77	2631.17	3588.96	7420.10
G	200.79	356.43	-74.57	512.07	1110.69	2655.12	43349.04	35375.47	41924.33	42534.92	41194.02	20589.65
H	44294.85	23247.51	42271.53	45635.75	43049.73	20936.85	40152.43	17704.32	41098.24	39841.15	34274.02	5013.66

Samples X disposed of due to outliers

Appendix C: Standard curve with R value



R value: .916

Appendix D: Patient demographics

Patient	Gender	Age	Race	Smoker	Diabetic (controlled-under 8.0)	Diabetic (uncontrolled-over 8.0)
Patient 1	F	41	White			
Patient 2	M	55	White	X		
Patient 3	M	61	White		X	
Patient 4	F	57	White	X		
Patient 5	F	72	White			
Patient 6	F	50	White			
Patient 7	M	47	White			
Patient 8	M	66	White			
Patient 9	F	60	White		X	
Patient 10	M	54	White			
Patient 11	F	52	White	X		
Patient 12	F	56	White	X		
Patient 13	M	67	Native Hawaiian/ Other Pacific Islander			
Patient 14	F	76	White		X	
Patient 15	M	61	Hispanic/Latino			X (approx 10.7)
Patient 16	M	69	White			
Patient 17	M	66	White			
Patient 18	F	55	White	X		

Appendix E: Diagnostic tests pre- and post- patients 1-9

Patient	Pre- Percussion	Pre-Thermal	Pre-EPT	Surgery Time out of Mouth (Minutes)	Duration of Study	Post-Percussion	Post-thermal	Post-EPT	Tx	Post Operative Clindamycin Needed?
Pt 1										X
7	-	+	5	3:54	4 months	-	-	68	none	
10	-	+	13	2:37	4 months	-	-	41	PRF	
8	+	+	26	3:30	4 months	-	-	80	PRF	
9	-	+	23	1:57	1 month	-	-	Removed	none	
Pt 2										X
8	-	-	42	2:12	11 months	+	+	60	PRF	
11	+	+	2	1:37	11 months	+	+	30	none	
13	-	+	17	1:43	11 months	+	-	66	none	
20	-	+	28	2:28	11 months	-	+	71	PRF	
Pt 3										X
9	-	+	45	1:46	3 months	-	-	80	none	
10	-	+	36	2:11	3 months	-	+	71	PRF	
Pt 4										
23	-	+	23	1:27	3 months	-	+	40	PRF	
24	-	+	38	1:39	3 months	-	-	58	None	
25	-	+	38	1:47	3 months	-	-	54	PRF	
26	-	+	34	0:58	3 months	-	+	47	none	
Pt 5										
7	-	+	7	1:42	5 months	-	+	30	PRF	
8	-	+	1	2:13	5 months	-	-	80	PRF	
9	-	+	1	1:38	5 months	-	+	80	None	
10	-	+	19	1:08	5 months	-	-	52	None	
Pt 6										
7	-	+	37	1:35	3 months	-	-	5	PRF	
8	-	+	34	1:49	3 months	-	-	29	PRF	
9	-	+	34	1:15	3 months	-	+	7	None	
10	-	+	32	1:25	3 months	-	+	6	None	
Pt 7										
7	+	+	32	1:12	5 months	-	+	42	None	
23	+	+	43	1:19	5 months	-	+	38	None	
24	+	+	50	2:50	5 months	-	+	74	PRF	
25	+	+	49	1:28	5 months	-	+	60	PRF	
Pt 8										
8	-	+	31	1:01	3 months	-	+	28	None	
7	+	+	45	1:50	3 months	-	+	24	PRF	
11	-	+	41	1:34	3 months	-	+	24	None	
26	-	+	43	2:10	3 months	-	+	23	PRF	
Pt 9										X
6	-	+	29	1:13	4 months	-	+	53	None	
8	+	+	19	2:02	4 months	-	-	58	PRF	
20	-	+	48	2:42	4 months	-	-	46	PRF	
9	-	-	24	1:45	4 months	-	-	48	None	

Appendix F: Diagnostic tests pre- and post- patients 10-18

Patient	Pre- Percussion	Pre-Thermal	Pre-EPT	Surgery Time out of Mouth (Minutes)	Duration of Study	Post-Percussion	Post-thermal	Post-EPT	Tx	Post Operative Clindamycin Needed?
Pt 10										
21	-	+	36	1:21	5 months	-	+	80	PRF	
22	-	+	39	2:03	5 months	-	-	80	PRF	
27	-	+	29	2:11	5 months	-	-	80	None	
28	-	+	45	1:39	5 months	-	-	80	None	
20	Unavailable	Unavailable	Unavaila	1:22	5 months	-	+	80	None	
29	Unavailable	Unavailable	Unavaila	1:46	5 months	-	-	80	PRF	
Pt 11										
8	-	+	35	2:13	3 months	-	+	74	None	
9	-	+	31	1:16	3 months	-	+	80	PRF	
10	-	+	28	2:06	3 months	-	-	52	None	
11	-	+	41	1:13	3 months	-	+	54	PRF	
Pt 12										
20	-	+	30	1:27	3 months	-	-	80	None	
21	-	+	39	1:24	3 months	-	+	36	None	
28	-	+	36	1:34	3 months	-	+	78	PRF	
29	-	+	36	2:01	3 months	-	-	80	PRF	
Pt 13										
7	-	-	48	1:11	4 months	-	+	51	None	
8	-	-	54	3:25	4 months	-	+	73	None	
9	-	-	46	1:36	4 months	-	+	59	PRF	
10	-	-	49	1:25	4 months	-	+	57	PRF	
Pt 14										
8	-	+	27	1:51	3 months	-	+	41	PRF	
9	-	+	32	1:16	3 months	-	+	58	None	
Pt 15										
7	-	-	30	2:12	3 months	-	-	46	PRF	
20	-	+	39	1:16	3 months	-	-	54	None	
22	-	+	25	1:23	3 months	-	-	79	None	
29	-	-	43	1:52	3 months	-	+	57	PRF	
Pt 16										
24	-	+	54	1:38	3 months	-	-	78	None	
23	-	+	33	1:13	3 months	+	-	52	PRF	
25	-	+	42	2:02	3 months	-	-	50	None	
26	-	+	65	1:36	3 months	-	-	73	PRF	
Pt 17										
6	-	+	36	1:07	3 months	-	+	80	None	
10	-	-	42	1:53	3 months	-	-	80	PRF	
11	-	-	42	1:23	3 months	-	+	80	PRF	
27	-	-	75	1:52	3 months	-	+	80	None	
Pt 18										X
10	+	+	36	1:06	3 months	-	+	30	None	
20	-	+	10	1:31	3 months	-	+	55	PRF	
21	-	+	28	1:01	3 months	-	+	50	None	
28	-	+	8	1:30	3 months	+	-	29	PRF	

Appendix G: Histological grading patients 1-9

Patient	Tx	Inflammat	Inflammation Grade	Blood vessels Noted	Evidence of Vitality
Pt 1					
7	none	Acute	Severe	No	No
10	PRF	Acute	Mild	No	No
8	PRF	Acute	Mild	Yes-few	Yes
9	none	Removed from study			
Pt 2					
8	PRF	Acute	Mild	Yes-few	yes
11	none	Mixed	Severe	No	no
13	none	Acute	Moderate	No	no
20	PRF	Mixed	Mild	Yes-Many	yes
Pt 3					
9	none	inconclusive			
10	PRF	Acute	Severe	None	no
Pt 4					
23	PRF	Acute	Mild	Yes-many	Yes
24	None	Acute	Severe	No	No
25	PRF	Acute	Mild	No	No
26	none	inconclusive			
Pt 5					
7	PRF	None	None	Yes-many	Yes
8	PRF	Acute	Moderate	Yes-few	Yes
9	None	Acute	Mild	No	No
10	None	None	None	No	No
Pt 6					
7	PRF	Acute	Moderate	No	No
8	PRF	None	None	Yes-many	Yes
9	None	None	None	Yes-many	Yes
10	None	None	None	Yes-many	Yes
Pt 7					
7	None	None	None	No	No
23	None	Inconclusive			
24	PRF	None	None	No	No
25	PRF	None	None	Yes-many	Yes
Pt 8					
8	None	Mixed	Mild	Yes-many	Yes
7	PRF	Mixed	Mild	Yes-few	Yes
11	None	Chronic	Moderate	Yes-few	Yes
26	PRF	Chronic	Mild	Yes-Many	Yes
Pt 9					
6	None	Acute	Mild	No	No
8	PRF	Mixed	Mild	No	No
20	PRF	inconclusive			
9	None	Mixed	Moderate	No	No

Appendix H: Histological grading patients 10-18

Patient	Tx	Inflamma	Inflammation Grade	Blood vessels Noted	Evidence of Vitality
Pt 10					
21	PRF	None	None	No	No
22	PRF	None	None	No	No
27	None	Acute	Mild	No	No
28	None	None	None	No	No
20	None	None	None	No	No
29	PRF	Acute	Mild	No	No
Pt 11					
8	None	Mixed	Moderate	Yes-many	Yes
9	PRF	Mixed	Moderate	No	No
10	None	Acute	Moderate	No	No
11	PRF	None	None	No	No
Pt 12					
20	None	None	None	No	No
21	None	Chronic	Mild	Yes-few	Yes
28	PRF	None	None	No	No
29	PRF	Chronic	Moderate	No	No
Pt 13					
7	None	None	None	Yes-many	Yes
8	None	inconclusive			
9	PRF	None	None	Yes-few	Yes
10	PRF	None	None	Yes-many	Yes
Pt 14					
8	PRF	None	None	Yes-few	Yes
9	None	None	None	No	No
Pt 15					
7	PRF	None	None	No	Yes
20	None	Mixed	Mild	No	Yes
22	None	Acute	Mild	Yes-few	Yes
29	PRF	Acute	Moderate	No	No
Pt 16					
24	None	Acute	Mixed	Yes-few	Yes
23	PRF	None	None	No	Yes
25	None	Mixed	Mild	Yes-many	Yes
26	PRF	Mixed	Mild	No	Yes
Pt 17					
6	None	Acute	Moderate	No	Yes
10	PRF	Chronic	Mild	Yes-few	Yes
11	PRF	None	None	Yes-few	Yes
27	None	Acute	Severe	No	Yes
Pt 18					
10	None	None	None	Yes-few	Yes
20	PRF	None	None	No	No
21	None	inconclusive			
28	PRF	inconclusive			