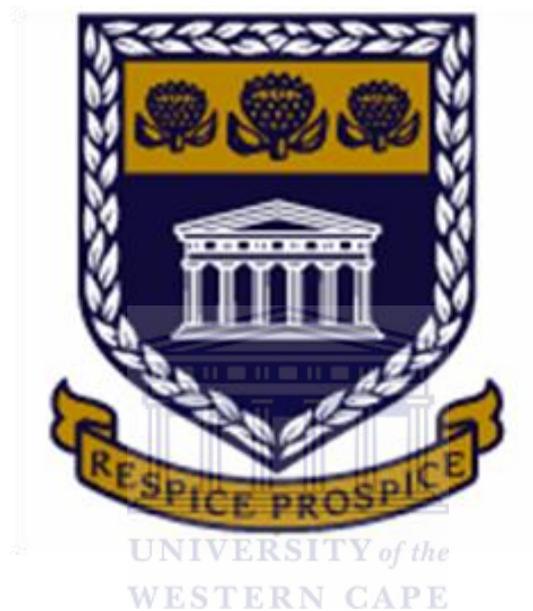


The effect of storage time on the platelet concentration of Choukroun's platelet rich fibrin (PRF)



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Keywords

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ABSTRACT

Wound healing is a complex process characterised by the repair and reconstitution of lost or damaged tissue. By the mid 1990s, several methods were proposed to enhance wound healing of surgical sites by introducing high concentrations of human platelets to these areas. In the early 21st century, Choukroun et al (2006b) introduced a new type of platelet concentrate that was devoid of any additives, and required no specialised equipment for its production. This concentrate was termed Platelet-rich fibrin (PRF) and although various aspects of this biomaterial had been studied, very little is currently known about its storage properties. **Aim:** To determine whether storage time had a significant effect on the platelet concentration of Choukroun's PRF **Method:** A total of 30 patients were enrolled into the study. Three blood samples of 10ml each were drawn from each patient. Two of the blood samples (Group A and Group B) were centrifuged to form PRF. The third sample was used to measure the baseline blood platelet concentration and was therefore not centrifuged. After PRF had formed in both test groups, it was removed from the test tubes at 2 different times i.e. immediately after centrifuge (Group A) or after 60 min of storage in the blood collecting tube (Group B). The remaining blood was then tested for platelet concentration and compared to each other and the baseline reading. **Results:** 14 males and 16 females participated in the study (average age 41.7 years). A mean blood platelet concentration of $282.8 \pm 58.3 \times 10^9/L$ was recorded for the baseline reading. Group A had a mean blood platelet concentration $7.9 \pm 3.03 \times 10^9/L$. Group B had a mean blood platelet concentration of $4.0 \pm 1.93 \times 10^9/L$. A statistically significant difference was seen between Groups A and B ($p < 0.0001$). **Conclusions:** Storage time has a significant effect of the platelet concentration of PRF. If stored over a period of 60 min, the platelet concentration of PRF increases. Further research is required to determine whether this finding is clinically significant.

DECLARATION

I hereby declare that “The effect of storage time on the platelet concentration of Choukroun’s platelet rich fibrin (PRF) “ is my own work, that it has not been submitted before for any degree or examination in any university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

M. Thabit Peck

July 2011

Signed:.....



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DEDICATION

To my parents and wife for their constant support, prayers and sacrifice

To my supervisors and support staff whose guidance, encouragement, help and support made this project possible



CONTENTS

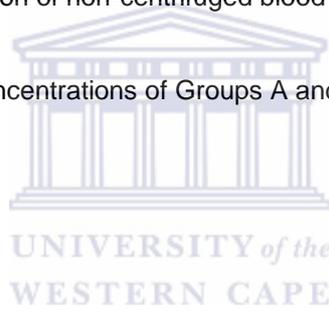
TITLE PAGE	I
TITLE PAGE 2	II
KEYWORDS	III
ABSTRACT	IV
DECLARATION	V
ACKNOWLEDGEMENTS	VI
DEDICATION	VII
LIST OF FIGURES	X
LIST OF TABLES	XI
CHAPTER 1: INTRODUCTION	1
CHAPTER 2: LITERATURE REVIEW	3
INTRODUCTION	3
THE PHYSIOLOGY OF HUMAN PLATELETS AND FIBRIN	3
PLATELETS	3
FIBRIN	7
THE DEVELOPMENT OF PLATELET-CONCENTRATES AND	
PLATELET-RICH-PLASMA (PRP)	7
PRP PREPARATION METHODS	9
SAFETY AND EFFICACY	10
JOSEPH CHOUROUN AND PLATELET RICH FIBRIN (PRF)	11
PRF: STRUCTURE, BIOLOGICAL PROPERTIES AND CLINICAL	
APPLICATIONS	12
PRF: CLINICAL APPLICATIONS	17
THE BIOLOGICAL STABILITY OF PRF	19
CURRENT CONTROVERIES REGARDING PRP AND PRF	19
IN SUMMARY	20
CHAPTER 3: AIMS AND OBJECTIVES	21
AIM	21



OBJECTIVES	21
NULL HYPOTHESIS	21
RATIONALE	21
CHAPTER 4: MATERIALS AND METHODS	22
STUDY DESIGN	22
SAMPLE SIZE	22
MATERIALS AND METHOD	22
DATA COLLECTION	24
DATA PROCESSING AND ANALYSIS	24
ETHICS	24
CHAPTER 5: RESULTS	25
STUDY PARTICIPANTS	25
THE PLATELET CONCENTRATION OF NON-CENTRIFUGED BLOOD	25
THE PLATELET CONCENTRATION OF RESIDUAL BLOOD AFTER REMOVAL OF CHOUKROUN'S PRF (GROUP A)	26
THE PLATELET CONCENTRATION OF RESIDUAL BLOOD AFTER REMOVAL OF CHOUKROUN'S PRF (GROUP B)	26
AN ANALYSIS OF THE DIFFERENCES IN PLATELET CONCENTRATIONS BETWEEN GROUPS A AND B	27
CHAPTER 6: DISCUSSION AND CONCLUSIONS	29
REFERENCES	33

LIST OF FIGURES

FIGURE 1	A-Acellular layer, B-PRF clot, C-RBC layer	13
FIGURE 2	PRF clot	13
FIGURE 3	PRF membrane	14
FIGURE 4	The PRF box (Toffler et al 2009)	16
FIGURE 5	Blood collecting tube	22
FIGURE 6	Tabletop centrifuge	22
FIGURE 7	Baseline concentration of non-centrifuged blood	25
FIGURE 8	Residual platelet concentrations of Groups A and B	27



LIST OF TABLES

TABLE 1	Age and gender of study participants	24
TABLE 2	Platelet concentration for the various groups tested	26



Chapter 1

Introduction

Wound healing is a complex process characterised by the repair and reconstitution of lost or damaged tissue. It involves a number of soluble chemical mediators as well as blood and parenchymal cells that interact with the extracellular matrix in three overlapping component phases. These phases are essentially controlled by the release of cytokines and growth factors (GFs) which stimulate cell migration and maturation required for tissue repair. Blood platelets are critical storage components of these chemical mediators and therefore play a crucial role in wound healing.

By the mid 1990s, several methods were proposed to enhance wound healing of surgical sites by introducing high concentrations of human platelets to these areas. It was assumed that platelets optimised wound healing by enhancing the amount of growth factors that were necessary for tissue growth. The most common type of platelet concentrate used in these procedures was platelet-rich-plasma (PRP). This concentrate, first described by Oz et al (1992) was an autologous blood-derived product that was formed from the patients' own blood and was initially intended to be used as a fibrin glue in patients undergoing cardiac surgery. However, further investigation revealed that it had enhanced wound healing properties and several researchers exploited this property in a variety of clinical procedures. Whitmen et al (1997) was the first to describe its use in the management of maxillofacial procedures and, in 1998, Marx et al described its use in bone augmentation procedures.

By the early part of the 21st century, the use of PRP to enhance bone augmentation in maxillofacial and dental implant procedures was commonplace, and several techniques had subsequently been developed to manufacture the product. However, the majority of these techniques required specialised equipment as well as animal-derived additives. Because of these disadvantages, researchers sought more tangible methods of producing PRP.

Choukroun et al (2006) introduced a new type of platelet concentrate that differed significantly from other platelet concentrates in that it was devoid of any additives, and

required no specialised equipment for its production. This new platelet concentrate was termed platelet-rich-fibrin (PRF). From its initial inception, PRF has extensively been studied and is now regarded as being a biological scaffold different to PRP. However, even though the use of PRF is starting to gain widespread acceptance, several questions regarding its biological stability and clinical efficacy remain unanswered. This study is an attempt to answer some of these questions.



Chapter 2

Literature Review

Introduction

Platelets and fibrin are essential components for normal haemostasis, and for many years it was assumed that this was their only function. However, recent evidence suggests that these elements have a much wider range of functions, and it is now accepted that they play a significant role in wound healing and microbial defence (Dohan Ehrenfest et al., 2010). These newly discovered properties have led to the development of several blood-derived products, consisting of concentrated platelets, that are used to enhance wound healing in both surgical and non-surgical procedures (Dohan Ehrenfest et al., 2009). Numerous techniques exist to prepare these concentrates, however, no single standardised method has been universally accepted. In order to further improve and enhance the efficacy of platelet concentrates, an essential understanding of platelets, fibrin, and their biological behaviour as concentrates, is required.

The physiology of human platelets and fibrin

Platelets

Platelets are anucleate, discoid shaped blood cells (actually cellular fragments) that are derived from megakaryocytes in the bone marrow. They enter the peripheral blood circulation where they circulate for about 7-10 days before ultimately being destroyed by the spleen and pulmonary vascular bed. The normal concentration of platelets varies amongst population groups, but on average is about $100-400 \times 10^9/L$ of circulating blood (Ezeilo et al, 2002).

Structurally, platelets consist of the following three identifiable zones:

- A *cytoskeleton or peripheral zone* containing the plasma membrane, receptors and the open canalicular system (OCS).
- A *sol-gel zone* that is centrally located and contains the cytoplasm and contractile proteins

- An *organelle zone* made up of granules, lysosomes, mitochondria and cytoplasmic organelles

The organelle zone has a high concentration of secretory granules, all of which play a part in platelet function. Among the three types of platelet secretory granules i.e. α -granules, dense granules, and lysosomes, the α -granule is the most abundant. There are approximately 50–80 α -granules per platelet, ranging in size from 200 - 500 nm (Blair and Flaumenhaft, 2009; Carlson and Roach Jr, 2002; Lacci and Dardik, 2010). The total α -granule membrane surface area per platelet is $14 \mu\text{m}^2$, about 8-times more than dense granules and approximately equal to that of the open canalicular system (OCS). The α -granules contain a variety of substances, including serotonin, which is a powerful vasoconstrictor. The remaining granules, the lysosomal granules, contain hydrolytic enzymes that dissolve phagocytosed debris. Platelet function is, therefore, directly related to the properties of their secretory granules (Blair and Flaumenhaft, 2009) .

The role of platelets in normal haemostasis

When a blood vessel is injured, it immediately contracts at the site of injury. At the same time platelets adhere to the site and form a temporary plug to stop blood loss. This plug formation is divided into 3 interrelated stages that includes platelet adhesion, a release reaction, and platelet aggregation.

Platelet adhesion is triggered by the exposure of injured collagen, secondary to tissue injury. This results in changes in platelet shape from normal discoid to spherical, with a number of protruding long projections known as filipodia, thereby allowing better adhesion to the underlying collagen.

The release reaction is characterised by the secretion of substances outside the platelet membrane. Although both α -granules and dense granules release proteins that are involved in coagulation, α -granules secrete polypeptides that play a role in both primary and secondary haemostasis. Another important function of the α -granules is the secretion of adhesive proteins (fibrinogen and von Willebrand factor). These are essential for platelet-platelet and platelet-endothelial interactions and are responsible for platelet aggregation (the third stage of platelet plug formation). Coagulation factors V, XI and XIII are also found in platelet granules, and are released upon platelet activation.

Platelet α -granules contain the inactive precursor of thrombin, i.e. prothrombin, and significant stores of high molecular weight kininogens, which augment the intrinsic clotting cascade. In addition, platelets release inhibitory proteases, such plasminogen activator inhibitor-1 (PAI-1) and α_2 -antiplasmin, which limit plasmin-mediated fibrinolysis.

Platelets also control the haemostatic process by secreting numerous proteins that antagonise the progression of coagulation. These include antithrombin, which cleaves activated clotting factors in both the intrinsic and extrinsic pathways, and C1-inhibitor, which degrades plasma kallikrein, factor XIa, and factor XIIa (Blair and Flaumenhaft, 2009).

Platelets and inflammation

Recent evidence also suggests that platelets may form an integral part of the inflammatory response (Blair and Flaumenhaft, 2009; Choukroun et al., 2006a). Platelet α -granules influence inflammation by secreting high concentrations of pro-inflammatory and immunomodulating factors. These mediators induce recruitment, activation, chemokine secretion, and differentiation of other vascular and blood cells. In some cases, these chemokines provide feedback to stimulate chemokine receptors on the platelet surface, thereby causing platelet activation, secretion, and perpetuation of the inflammatory cycle. Activated platelets bind to circulating immune cells in the blood, and surface-adherent platelets facilitate the recruitment, rolling, and arrest of monocytes, neutrophils, and lymphocytes to the activated endothelium. Platelets have also been shown to play an important role in the recruitment of neutrophils in certain diseases (Blair and Flaumenhaft, 2009; Tözüm and Demiralp, 2003).

Platelets as a host defence mechanism

Platelets protect the host against certain pathogenic microorganisms by directly interacting with viruses, bacteria, fungi, and protozoa (Blair and Flaumenhaft, 2009; Dohan Ehrenfest et al., 2009; Choukroun et al., 2006a). This antimicrobial effect is further enhanced by their ability to bind to erythrocytes infected with plasmodium, thereby killing the infecting parasite. Platelets secrete a group of microbicidal proteins collectively referred to as “platelet microbicidal proteins”, all of which have direct antimicrobial effects. Many of the cytokines that are released during the normal physiological function of platelets, are also

microbicidal and include diverse chemical chemokines such as CLCX7, CLCX4 and CLCX5. Other proteins that further enhance the antimicrobial effect of platelets include compliment and compliment binding proteins such as C3 and C4 precursors which participate in the compliment activation cascade (Blair and Flaumenhaft, 2009).

The role of platelets in wound healing

The wound healing potential of platelets is related to their ability to secrete a variety of growth factors (GFs) (Gassling et al., 2010; Jang et al., 2010; Kang et al., 2011; Lacci and Dardik, 2010). These are necessary for the recruitment of reparative cells to the affected area. Although these GFs are not unique to platelets, the rapidity with which platelets accumulate at sites of vascular injury, results in an accelerated rate of GF concentration.

The GFs associated with platelets have been identified as vascular endothelium growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), and transforming-growth-factor-beta (TGF-beta), all of which play a role in replacing lost tissue, resurfacing of the wound, and restoring vascular integrity (Dohan Ehrenfest et al., 2009, 2010; Dohan et al., 2006; He et al., 2009; Blair and Flaumenhaft, 2009). PDGF is chemotactic for several cells, including polymorphonuclear leukocytes, macrophages, fibroblasts and smooth muscle cells. It also stimulates the replication of stem cells associated with fibroblasts and endothelial cells (thereby promoting angiogenesis) as well as the production of fibronectin, a cell adhesion molecule required for proper cellular proliferation and migration during healing. PDGF is also known to induce osteogenesis and stimulate hyaluronic acid that is associated with wound contraction and remodelling. TGF- β , the other major growth factor secreted by platelets, stimulates fibroblast chemotaxis and the production of collagen and fibronectin by cells. It also inhibits collagen degradation and increases the concentration of protease inhibitors, thereby favouring fibrogenesis (Carlson and Roach Jr, 2002).

Fibrin

Fibrin is the activated form of fibrinogen, and plays an integral role in platelet aggregation during haemostasis. It is present in plasma and in platelet α -granules, and is derived from fibrinogen by the enzymic action of thrombin. The initial step in this conversion from fibrinogen to fibrin, is the formation of soluble fibrin monomers that appear as loose fibrin strands. These are stabilised into insoluble fibrin polymers (via hydrogen bonds) by Factor XIIIa in the presence of calcium ions. This insoluble polymerised structure acts as a biologic glue capable of consolidating the initial platelet cluster and constitutes the first cicatricial matrix of the injured site (Ezeilo, 2002). This reinforces the haemostatic ability of the platelets and results in a protective wall along vascular breaches of the endothelium (Dohan et al., 2006). Fibrin therefore plays a significant role in the ability to concentrate platelets and their associated GFs.

The development of platelet-concentrates and platelet-rich-plasma (PRP)

The role of fibrin in haemostasis led to the development of many commercially available fibrin glues, or gels that are still currently used. These agents were initially described in the early 1970s and were designed to act as natural adhesives in the management of wound bleeding. Throughout the past four decades they have successfully been used in various surgical procedures including cardiothoracic surgery, plastic surgery and oral and maxillofacial surgery (Whitman et al, 1997).

The principle of fibrin glue production is the polymerisation of fibrinogen in the presence of thrombin and calcium. They were originally produced using donor plasma, but due to the low concentration of fibrinogen in human plasma, the stability and quality of the fibrin glue that was produced, was low (Raja and Munirathnam, 2008). Modern fibrin glues have sought to improve these properties, but have often been criticised for their high cost, requirement for donors, the use of animal-derived products, and the ability to transmit disease (Dohan et al., 2006).

Mehmet Oz and the “new autologous glue”

Due to the various disadvantages associated with the initial preparations of fibrin glue, researchers sought ways to improve the product during the early 1990s. In 1992, Oz et al published a seminal paper in which a new method of fibrin glue preparation was introduced.

This method was unique in that it involved the use of platelet concentrates together with thrombin and calcium to form what the authors called “new autologous glue”. The platelet-concentrate used in this method was derived from platelet-rich-plasma, which was readily available and routinely used to control haemorrhage for patients undergoing cardiac surgery. The platelet-rich-plasma was prepared by collecting whole blood from the patient and centrifuging it at 3000 rpm for 15 minutes, after which the red blood cells would be separated from the platelet-rich-plasma. Oz et al (1992) and colleagues then extracted 20 ml of the platelet-rich-plasma and added calcium chloride and bovine thrombin to it in an effort to stimulate clot formation. After various trials involving both humans and animals, the authors concluded that their new method of autologous glue formation had several advantages over traditional methods of preparation. These included:

1. Minimal cost was associated with the procedure due to the fact that platelet-rich-plasma was readily available
2. The risk of viral transmission from the use of infected blood was eliminated
3. The source material was easily obtained
4. The fibrin glue was autologous

Even though the new method promised a simpler way of obtaining fibrin glue, the authors cautioned against using it in patients with an allergy to bovine thrombin.

The birth of platelet-rich-plasma (PRP)

A year after Oz et al (1992) published his article, Hood et al (1993) and later Hill et al (1993), presented the findings in the Annual Proceedings of the American Academy of Cardiovascular Perfusion of a “newly developed autologous platelet-concentrated fibrin glue” that had wound healing properties. This was the first time wound healing properties had been linked to platelet concentrated gels. Four years later, in 1997, Whitman et al introduced the use of platelet concentrated gels to enhance wound healing in various maxillofacial procedures. These procedures included mandibular reconstruction, adjuvant implant augmentation procedures, sinus lifts, reparation of Schnidarian membrane tears, the reconstruction of oral-antral fistulas and the control of haemorrhage. The platelet gel used in this case was similar to that previously described by Oz et al (1992), with minor modifications made to the preparation protocol.

In 1998, Marx and colleagues introduced the term “platelet-rich-plasma” or “PRP” to describe platelet concentrated gels. This term, still in use today, refers to the platelet concentrated gel or modifications thereof that was initially proposed by Oz et al (1992). However, it must be borne in mind that the use of the term “platelet-rich-plasma”, was already in use and referred to platelet extracts used to control haemorrhage during surgery. Ultimately, this has led to confusion in the literature, with Dohan Ehrenfest et al (2009) proposing a standardised nomenclature to denote various platelet concentrates. This, however, has still not gained universal acceptance.

By the year 2000, PRP had gained widespread acceptance as a viable treatment option for various maxillofacial and other extra-oral procedures. Although research has focused on PRPs ability to enhance bone healing, it has been used successfully for the reparation of dural tears, during and after facial plastic surgery, as a haemostatic and lymphostatic agent in radical neck dissection, enhancement of knee and lumbar fusions, the management of chronic wounds such as diabetic ulcers, the treatment of burns, snake and spider bites, in the treatment of various cardiothoracic procedures, in major and minor vascular surgery, and in other areas such as ophthalmology and urology (Jameson, 2007).

By 2007, several theories of how PRP contributed to accelerated wound healing were advanced. Central to all of them was the ability of a high concentration of platelets to release several growth factors and other mediators to stimulate the preferential migration of undifferentiated stem cells to the wound site. Long-term clinical research has yet to verify whether these theories are justified.

PRP preparation methods

Since the original description of PRP preparation by Oz et al (1992), several modifications of this technique have been explored, and, even though minor differences in preparation techniques exist, common to all of them are the following:

- 1) Blood is collected with anticoagulant just before or during surgery and is immediately processed by centrifugation. The time for platelet concentrate preparation is variable, but is always completed within an hour. A first centrifugation step is designed to separate the blood into three layers, red blood cells (RBCs) are

found at the bottom, acellular plasma (PPP, platelet-poor plasma) is in the supernatant and a 'buffy coat' layer appears in between, in which platelets are concentrated.

- 2) The next steps vary among the numerous protocols, but are an attempt to discard both the RBC layer and the PPP to collect only the 'buffy coat' layer.
- 3) Finally, the residual platelet concentrate is applied to the surgical site with a syringe, together with thrombin and/or calcium chloride (or similar factors) to trigger platelet activation and fibrin polymerization (Dohan Ehrenfest et al., 2009).

This variation in preparation protocol has led to the non-standardisation of PRP with the result that potential clinical differences may be seen with the various preparations used (Dohan Ehrenfest et al., 2009).

Safety and efficacy

The preparation of PRP is not purely autologous and the addition of various chemicals, some of which are animal-derived, are common to most of the preparation techniques employed today. These chemicals, often include bovine thrombin and calcium chloride and are added to stimulate platelet activation and GF release. Other agents include acid citrate dextrose-A (ACD-A) and citrate phosphate dextrose (CPD), both of which are used to support the metabolic needs of platelets in anticoagulated blood. The addition of these chemicals and other agents during the preparation of PRP has drawn criticism by various authors for their potential adverse effects (Dohan Ehrenfest et al., 2009).

Critics of PRP preparation techniques highlight the fact that several studies show that exposure of surgical patients to bovine thrombin preparations may result in the generation of anti-bovine antibodies that cross-react with certain human coagulation factors (Ofosu et al., 2009). The generation of these antibodies is well documented and may also be seen in patients treated with human plasma-derived, or recombinant therapeutic proteins. Furthermore, the purity of bovine thrombin preparations have recently been questioned since they differ markedly in concentration (with the bovine thrombin content varying from >90% to <30% for various different preparations) (Ofosu et al., 2009).

Clinically, the adverse effects of using bovine-derived products range from acute and delayed allergic reactions to coagulative and bleeding reactions, and both these have been

documented with the use of PRP (Wei et al., 2003). Although a decline in the adverse events associated with the use of these agents have been noted during the past decade, researchers attribute this decline to underreporting and a lack of adequate research data (Clark et al., 2010).

The potential adverse effects of using bovine or donor-derived thrombin is, therefore, a significant consideration in the preparation of platelet concentrates and has led to researchers seeking alternative means of creating viable preparation methods for these products.

Joseph Choukroun and platelet-rich-fibrin (PRF)

In the late 1990s, Joseph Choukroun (cited by Dohan et al, 2006) successfully developed a new technique to concentrate blood platelets for surgical use. The unique feature of this new preparation method was that it allowed for the development of a platelet concentrate without the use of any animal additives or additional chemicals. Choukroun referred to the platelet concentrate as “platelet-rich-fibrin (PRF)” and the new method of preparation, as “Choukroun’s PRF”. The nomenclature was chosen to distinguish this platelet concentrate from other current forms of PRP. The term Choukroun’s PRF, PRF and “PRF clot” have been used interchangeably in the literature and this trend has been continued in this thesis. Different from traditional methods of producing PRP, Dohan et al (2006) described PRF preparation as follows;

- 1) Venous blood is collected from the patient in standard 10-ml anticoagulant-free blood collecting tubes (either glass tubes or silica coated plastic tubes). According to Dohan et al (2006), any type of dry glass tube (Terumo® Venoject® 10 mL) or glass-coated plastic tube (Terumo® Venosafe® 10 ml, Becton Dickinson Vacutainer® 10 ml or Greiner® Vacuette® 9 ml) may be used.
- 2) The collected blood is immediately centrifuged in any standard tabletop centrifuge at a relative centrifugal force of 400 x g for 10-12 minutes (Dohan et al., 2006). The absence of anticoagulant implies the activation of most platelets of the blood sample in contact with the tube walls and the release of the coagulation cascades within a few minutes. Fibrinogen is initially concentrated in the upper part of the tube, before the circulating thrombin transforms it into fibrin. A fibrin clot is then obtained in the

middle of the tube, just between the red corpuscles at the bottom and acellular plasma (PPP) at the top (**Figure 1**). Platelets are theoretically trapped in large numbers in the fibrin meshes (Gassling et al., 2010).

- 3) This platelet concentrated clot, together with the upper part of the red blood cell layer, may then be separated for clinical use (10 ml blood typically yields 1 ml PRF) (Dohan Ehrenfest et al., 2010b) (**Figure 2**).
- 4) The PRF clot may also be compressed to form an “autogenous membrane” (Dohan Ehrenfest et al., 2010b) (**Figure 3**).

When compared to traditional PRP, PRF is easier to prepare, requires no specialized equipment, uses no added “activators”, is free of animal additives, is less expensive, and requires less time before it can be clinically applied (Dohan et al., 2006; Dohan Ehrenfest et al., 2010). In addition, PRF can be easily manipulated into a membrane for use in a variety of surgical procedures.

PRF: Structure, biological properties and clinical applications

Once removed from the blood collecting tube, PRF appears as a clot that is composed of two main areas: a yellow portion constituting the main body, and a lower red portion constituting the upper most part of the red blood cell layer (**Figure 2**). Biological analysis shows that the yellow portion is made up of three major components, i.e. platelets, leukocytes, and fibrin. It is thought that the fibrin acts as a vehicle for these cells and other cytokines during the process of wound healing (Choukroun et al., 2006b; Dohan Ehrenfest et al., 2010a, 2010b; Gassling et al., 2010; Kang et al., 2010).

Dohan Ehrenfest et al (2010b) noted that PRF had a distinct architecture with a specific cellular distribution. Rather than the cells being evenly distributed throughout the PRF clot, it was shown that the highest concentration of platelets and leukocytes were found in the yellow area just above the red blood cell layer, with the concentration and homogeneity of cells, becoming progressively less as the distance from the red blood cell layer increased (Dohan Ehrenfest et al., 2010b).

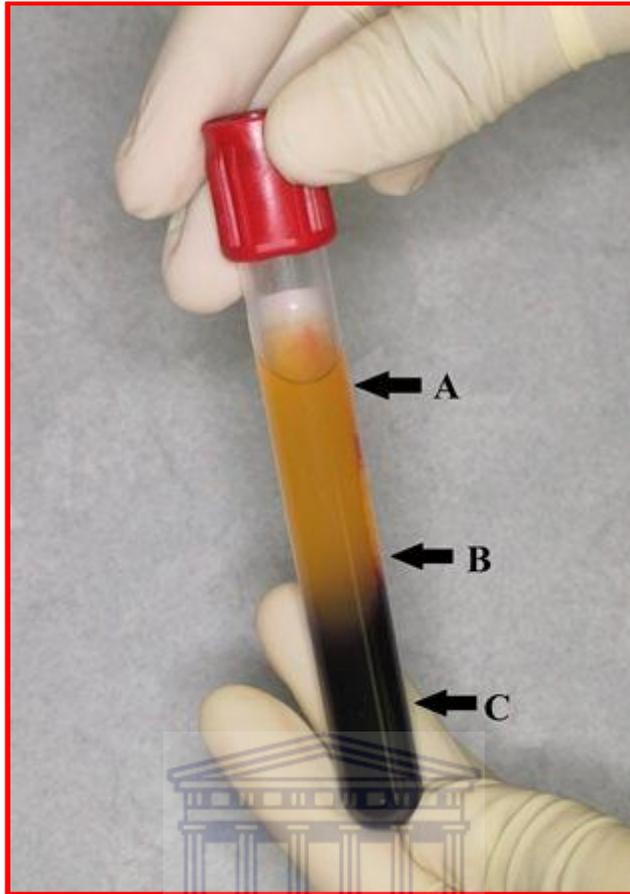


Figure 1: A-Acellular layer, B-PRF clot, C-RBC layer

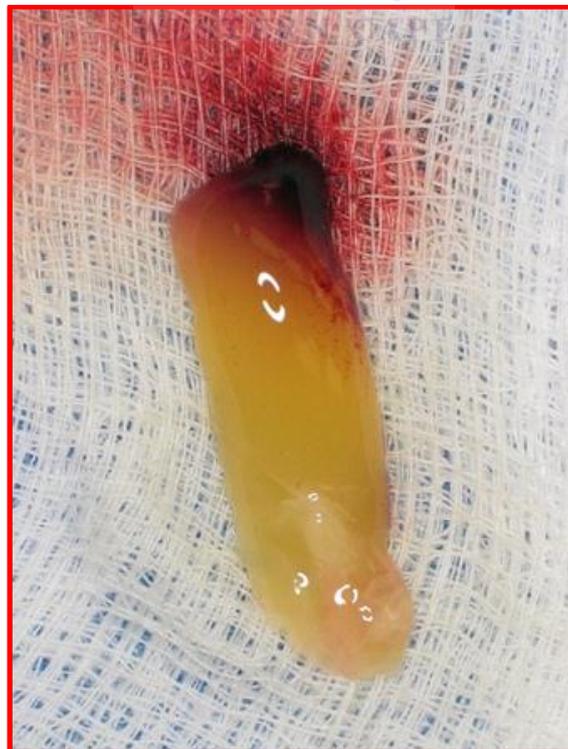


Figure 2: PRF clot



Figure 3: PRF membrane

This physical distribution of platelets is therefore specific for this type of platelet concentrate. For this reason, it has been suggested that PRF membranes be overlapped during its clinical application in order to maximise its efficacy.

A recent analysis of the concentration of platelets found in PRF preparations showed that it trapped 97% of the total platelets available (Dohan Ehrenfest et al., 2010b). Different to other platelet concentrates, PRF is also designed to retain a high concentration of leukocytes. These leukocytes are thought to undergo degranulation during clot formation, thereby activating a number of cytokines that protect the wound from infection (Dohan et al., 2006). Up to 50% of available leukocytes can be trapped in a platelet concentrate using the Choukroun PRF preparation method (Dohan Ehrenfest et al., 2010b).

When compared to traditional PRP, PRF shows a higher concentration of growth factors, including platelet-derived growth factor-BB (PDGF-BB) and tissue growth factor- β -1 (Gassling et al., 2010; He et al., 2009; Kang et al., 2010). Because no additional activators are used during the preparation of PRF, the material undergoes a slower polymerization reaction than other forms of PRP, thereby allowing it to trap a number of key cytokines that would otherwise be lost. These cytokines, essential for healing, have been shown to be released sustainably (over a longer time) than in other PRP preparations, in so doing optimizing healing over a longer period. In a study by He et al (2009), the effect of PRF and PRP was tested on rat osteoblasts over a period of 28 days. It was found that PRF released

autologous growth factors gradually over this period and had a significantly greater ability to induce mineralization when compared to PRP.

Several researchers have studied the clinical and biological effects of PRF on wound healing. The grafting potential of PRF was researched by Jang et al (2010) when he studied the effect of combining PRF with silk powder to treat peri-implant bone defects. The results from this animal study indicate that PRF in combination with silk powder could successfully be used to manage these defects. Shen et al (2008) studied the effect of PRF on cultured periodontal cells and concluded that it had the ability to inhibit epithelial cell growth, enhance osteoblast proliferation, and increase gingival fibroblast proliferation.

Choukroun et al (2006b) evaluated the potential of PRF in combination with freeze-dried bone allograft (FDBA) to enhance bone regeneration in lateral sinus floor elevation. Nine sinus floor augmentations were performed with 6 sinuses receiving PRF + FDBA particles (test group) and 3 sinuses receiving FDBA without PRF (control group). Four months after implantation (test group) and 8 months later (control), bone specimens were harvested with a 3-mm diameter trephine needle during implant insertion. Histologic evaluations revealed the presence of residual bone particles surrounded by newly formed bone and connective tissue. At 4 months, the histologic maturation of the test group appeared identical to that of the control group after a period of 8 months, with the quantities of newly formed bone equivalent between the two protocols. The use of PRF in combination with FDBA to perform sinus floor augmentation seemed to accelerate bone regeneration.

The PRF membrane

Unlike traditional PRP, PRF can be manipulated into a usable surgical membrane without the addition of animal derivatives (Dohan Ehrenfest et al., 2010b; Gassling et al., 2010; Aroca et al., 2009). Two methods to produce membranes from PRF have been described. These include;

1. Compression with sterile gauze

With this method, the PRF clot, once removed from the blood collecting tube, is compressed with hand/finger pressure between two sterile pieces of gauze on a flat surface thereby

driving out most of the fluid until a yellow-white membrane is produced (Toffler et al., 2009; Dohan Ehrenfest et al., 2010b).

2. The use of a PRF Box®

The PRF Box® (Process Ltd., Nice, France), first described by Toffler et al (2009), is a commercially available metal box designed to form PRF membranes in less than one minute **(Figure 4)** The design is based on a perforated metal tray onto which the freshly prepared PRF clot is placed. The clot is then compressed with the lid, driving out most of the fluids and resulting in a yellow-white membrane for clinical use. The exudate collected at the bottom of the box may be used to hydrate graft materials, rinse the surgical site, and store autologous grafts (Toffler et al., 2009, Dohan Ehrenfest et al., 2010c).

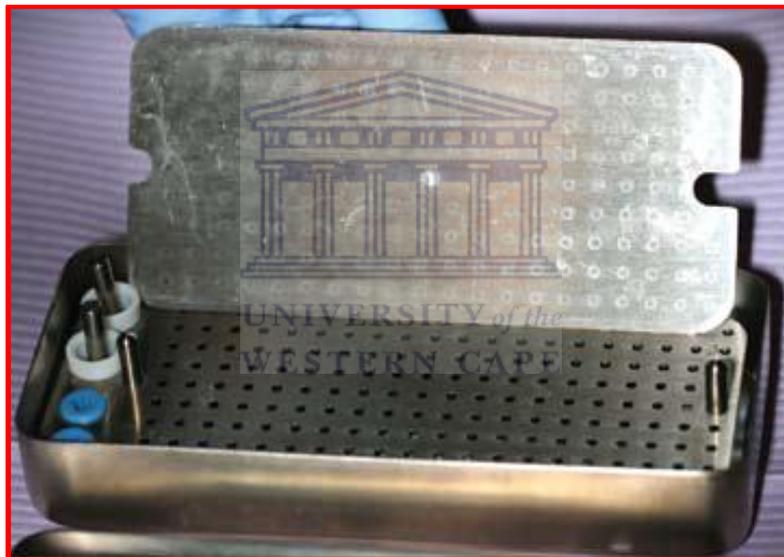


Figure 4: The PRF Box® (Toffler et al., 2009)

A recent morphological assessment of the PRM membrane produced the following results (Dohan Ehrenfest et al., 2010b);

- The PRM membrane was similar to the PRF in cellular distribution
- Its configuration consisted mostly of dense fibrin arranged in parallel chords
- The cellular composition was assumed to be the same as the PRF clot
- The cellular distribution showed that platelets and leukocytes were concentrated near the red blood cell interface

PRF: Clinical applications

In a case report by Anilkumar et al (2009), a PRF membrane was used in conjunction with a lateral sliding flap to successfully restore buccal soft tissue recession on a single anterior lower incisor tooth. The patient initially presented with gingival recession of 5 mm. After the surgical site was prepared, a PRF membrane derived from the patient's own blood was used to cover the exposed root area. This was then closed with a pedicle flap gained from the adjacent tooth. A 6-month clinical follow up showed complete root coverage with excellent colour and gingival contour.

Del Corso et al (2009) confirmed this clinical application when he published several case reports showing the successful use of PRF membranes in the management of both single and multiple gingival recession defects. Using multiple PRF membranes, instead of connective tissue grafts, Del Corso et al (2009) was able to restore the lost soft tissue successfully by layering the PRF membranes over each other thereby increasing gingival height and thickness. The clinical results were maintained successfully for at least one year (Del Corso et al., 2009).

In a clinical study over six months, Mazor et al (2009) showed that PRF could be used as the sole grafting material in sinus augmentation procedures (Mazor et al., 2009). In a similar study, Diss et al (2008) evaluated the radiographic changes in the apical bone levels of 20 patients treated with 35 microthreaded implants placed using the Osteotome Mediated Sinus Floor Elevation technique. PRF was the sole grafting material used. Despite a limited residual subantral bone height (RSBH) of 4.5 to 8 mm, a healing period of 2-3 months was found to be sufficient to resist a torque of 25 N-cm applied during abutment tightening. One implant failed during the initial healing, but at one year, 34/35 implants were clinically stable and the definitive prostheses were in function, resulting in a survival rate of 97.1%. The mean sinus bone gain was 3.2 mm with radiographic documentation of apical displacement of the sinus floor (Diss et al., 2008). Other authors have had similar results using similar protocols (Simonpieri et al., 2011, Simonpieri et al., 2009b, Toffler, et al., 2010).

Using radiographic analysis, Mazor et al (2009) tested the efficacy of using PRF as barrier membranes to close windows created in the lateral maxillary sinus wall after sinus

augmentation procedures. In the 9 cases where bone biopsies were harvested in the area of the window, no connective tissue invagination was observed, and bone samples were all very dense. This indicated that PRF was able to successfully protect the sinus graft area from the ingress of soft tissue. From the results of this study, Mazor et al (2009) concluded that PRF may be used to replace xenogenic and collagen membranes that are commonly used for this procedure.

Because of the biological properties of PRF, several authors have attempted to use it in combination with bone substitutes to enhance healing during GBR procedures. Simonpieri et al (2009a), reported on a new technique for maxillary reconstruction using freeze-dried bone allograft (FDBA), PRF membranes and 0.5% metronidazole solution. PRF fragments were mixed with the graft particles, thereby functioning as a “biological connector” between the different elements of the graft. Using the reported protocol, they observed a high degree of gingival maturation after healing with a thickening of keratinized gingival tissues. In addition, they emphasized that the use of PRF seemed to reduce postoperative pain and oedema, and limited minor infections (Simonpieri et al., 2009a).

Toffler et al (2009) using a similar PRF-bone graft mixture to successfully augmented a narrow alveolar ridge for future implant placement. In this GBR procedure, the PRF membrane was fragmented to mix easily with the bone graft material and further PRF membranes were placed over the graft material to isolate the wound.

Alveolar ridge preservation or socket preservation is the term given to procedures that attempt to preserve bone at sites of tooth extraction. Several techniques exist, the most common being the placement of a bone grafting material in a freshly extracted tooth socket, followed by covering the socket with a collagen membrane or connective tissue graft. In a technique described by Toffler et al (2009), a PRF membrane can be compressed into a “plug” using the PRF Box® and used as a grafting material to fill recently extracted tooth sockets. The authors claim that this can be used to expedite soft tissue healing and allow for ideal implant placement. Other case reports have recently confirmed this phenomenon (Peck et al., 2011)

PRF membranes have been used to repair perforations of sinus membranes after injury during sinus augmentation procedures. Choukroun et al (2006b) showed that sinus

membrane perforations were successfully treated with PRF membranes thereby allowing sinus augmentation procedures to be completed successfully. In a similar study, Mazor et al (2009) showed that these perforations were completely sealed after sixth months and no adverse effects were noted during the healing process. Mazor et al (2009), therefore, concluded that the use PRF membranes was a viable treatment option in the management of iatrogenic sinus perforations (Mazor et al., 2009).

The biological stability of PRF

In order to gain maximum clinical efficacy from the use of PRF, its biological and structural properties requires stability over a period of time. Su et al (2009) proposed several techniques to improve the properties of PRF, including using it immediately after formation as well as using an impermeable material rather than cotton gauze to construct the PRF membrane. Other authors have suggested using a PRF box as the ideal storage medium to preserve the properties of PRF. This is based on the opinion that when PFR is stored in its centrifuging tube, it disintegrates after 15 minutes and becomes unusable as a biological material (Dohan Ehrenfest et al, 2010c). However, no known research has been published verifying this phenomenon.

Current controversies regarding PRP and PRF

Although widely used in a variety of clinical fields, the ability of PRP to enhance wound healing has recently been questioned (Wang et al, 2008; Trombelli et al, 2008; Plachkova et al, 2008). A systematic review of more than 100 papers concluded that significant evidence existed for the use of PRP in periodontal defects. However the same study concluded that the effect of PRP on sinus augmentation was less than 10% when compared to controls and suggested that no substantial evidence existed for its use in other dental procedures (Plachkova et al, 2008). The heterogeneity in studies that examine PRP is regarded as a major cause for the lack of conclusive evidence available for its use (Wang et al, 2007).

PRF is a new form of platelet concentrate which has features that are distinctly unique when compared to PRP and therefore cannot be regarded as the same biomaterial. Although as mentioned previously the material has shown beneficial effects in a number of clinical case reports, it however suffers from the same scientific weaknesses as PRP in that the product

currently lacks long term controlled trials that would endorse its routine use in surgical procedures. Recently, 5-year follow up reports have been published which demonstrates the clinical safety and efficacy of using this new biomaterial (Simonpieri et al, 2011). We await further such reports.

In Summary

PRF is reported to have a wide range of clinical applications. This is thought to be related to its biological properties that allow it to act as both a membrane as well as a platelet-concentrate. Its preparation requires neither specialised equipment nor the addition of animal-derived products. However, questions remain regarding the best form of storage for freshly prepared PRF, and further research is required to better understand the ideal mechanism to retain its reported biological properties.



Chapter 3

Aims and Objectives

Aim

To determine whether storage time had a significant effect on the platelet concentration of Choukroun's PRF.

Objectives

To determine the:

- a) platelet concentration of non-centrifuged blood
- b) platelet concentration of the residual blood immediately after Choukroun's PRF formation and subsequent removal from the blood collecting tube
- c) platelet concentration of the residual blood after removal of Choukroun's PRF from the blood collecting tube 60 minutes after it has been formed
- d) differences in platelet concentration between (b) and (c)

Null hypothesis

Storage time has no significant effect on the platelet concentration of Choukroun's PRF.

Rationale

Once prepared, Choukroun's PRF is often stored for a variable period of time before it is used. If the concentration of platelets in PRF remains constant during that time, then the potential for variation in clinical outcomes during a surgical procedure may be minimised.

Chapter 4

Materials and Methods

Study design

A cohort analytical study was used to conduct the research described in this thesis.

Sample size

A total of 30 patients were enrolled in the study. For each volunteer, three (3) blood samples of 10 ml each, were obtained from an antecubital vein by a professional nurse. Two (2) of these tubes were used as test samples and the remaining sample was used to measure baseline platelet concentration of the patients. A total of 90 independent freshly acquired human blood samples was thus analysed. The test samples were randomly divided into equal groups of 30 samples each (using a simple coin toss), designated Group A and Group B, respectively.

Materials and Method

1. After informed consent had been obtained, blood samples were collected at the Dental Faculty, University of the Western Cape, Tygerberg Hospital Campus from 30 healthy volunteers (age range: 20 to 70 years, mean age: 45 years) who had no acquired or congenital defects that might interfere with normal coagulation. The blood samples were divided into groups as mentioned above. Sixty (60) silica-coated plastic tubes (Vacuette® 10 ml serum tubes with Z Serum Clot Activator, Greiner Bio One International AG, Germany) were used to collect the test samples (**Figure 5**). Thirty (30) ethylenediaminetetraacetic acid (EDTA)-coated tubes (BD Vacutainer® K2EDTA 10 ml) were used to collect the baseline samples.
2. After blood collection, the test samples were centrifuged at 400 X g for 12 minutes, using a standard tabletop centrifuge (PLC-03, Hicare international, Taiwan) (**Figure 6**).
3. For all samples of Group A, the resultant PRF clot was immediately removed from the tube using sterile tweezers and separated from the red blood cell layer base using surgical scissors.



Figure 5: Blood collecting tube



Figure 6: Tabletop centrifuge

4. For all samples of Group B, the resultant PRF clot was removed only after 60 minutes of storage in the original collecting tube.
5. Immediately after removal of their respective PRF clots, the serum platelet concentrations of both Groups A and B were measured using an electronic automated cell counter (Advia 2120, Siemens AG, Erlangen, Germany).
6. The platelet concentrations of the baseline samples were measured as described above.

Data collection

Data were collected and entered into a spreadsheet (Microsoft Office 2010 Excel, Microsoft Corporation, Washington).

Data Processing and Analysis

The results were compared and analysed using SPSS[®] Version 13 for Windows.

For each group, the mean and standard deviation of platelet concentration was calculated.

The following statistical tests were applied:

- multiple analysis of variance
- a multiple comparison test to determine the significance of differences among the means at a significance level of $p = 0.05$.

Ethics

The study was conducted under the principles outlined by the “World Medical Association Declaration Of Helsinki, Ethical Principles for Medical Research Involving Human Subjects” of 2008. Ethical approval was obtained from the Ethics Committee of the University of the Western Cape (Registration number 11/4/29). All patients were fully informed of the research protocol and had to sign a declaration of informed consent before being allowed to participate in the study. All significant results obtained from this study will be submitted for publication in a relevant dental or medical journal.

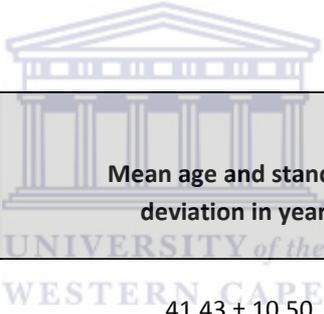
Chapter 5

Results

Study participants

A total of 30 patients (16 females and 14 males) were enrolled into the study. The mean age of the participants was 41.7 years, with male subjects being slightly younger than their female counterparts (41.3 years for males vs 42 years for females). The youngest participant was 24 years old and the oldest 58 years old (**Table 1**).

Table 1: Age and gender of study participants



	Number of participants	Mean age and standard deviation in years	Age range in years
Male	14	41.43 ± 10.50	27.00 - 58.00
Female	16	41.94 ± 10.25	24.00 - 55.00
Total	30	41.70 ± 10.19	24.00 - 58.00

The platelet concentration of non-centrifuged blood (baseline concentrations)

Platelet concentrations obtained from haematologic analysis of all 30 samples fell within the normal laboratory reference range of $170\text{-}400 \times 10^9/\text{L}$ of circulating blood and no participants displayed any significant haematological disease (**Figure. 7**). The mean blood platelet concentration was $282.8 \times 10^9/\text{L}$, the median $282 \times 10^9/\text{L}$, and the standard deviation was $58.3 \times 10^9/\text{L}$. The 95% confidence interval estimate for the mean was (261.0 to 304.6) (**Table 2**).

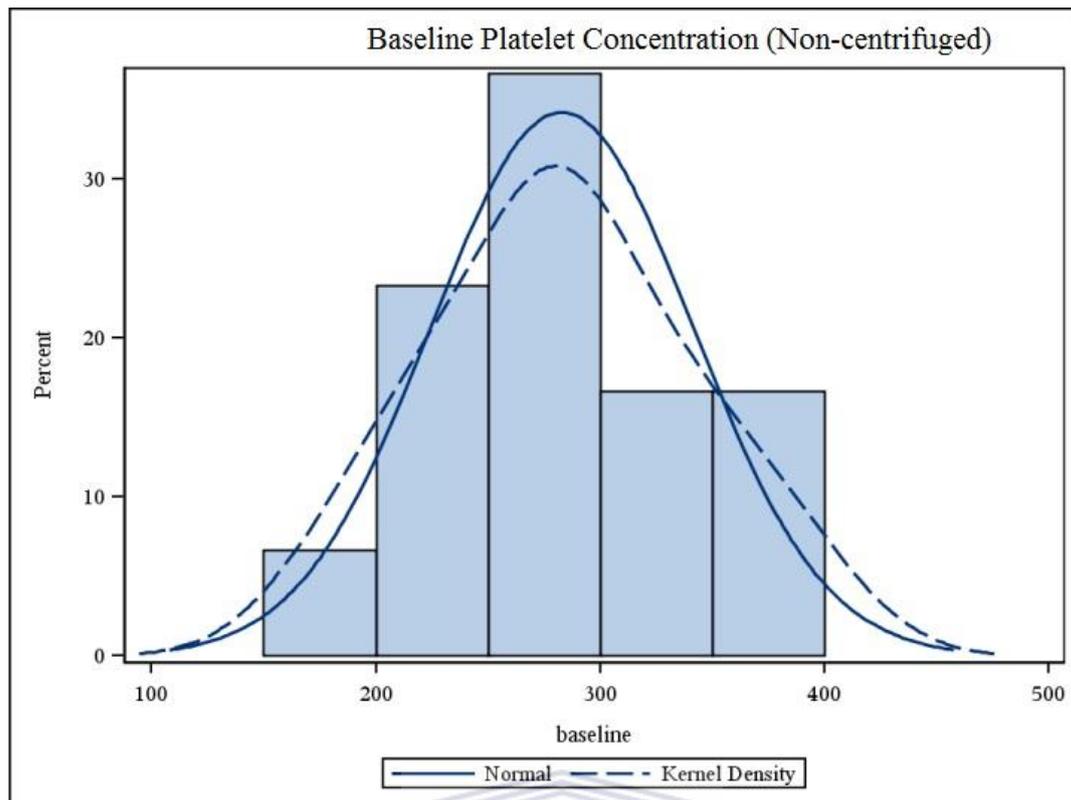


Figure 7: Baseline concentration of non-centrifuged blood

The platelet concentration of residual blood after removal of Choukroun's PRF (immediately after formation, Group A)

After removal of Choukroun's PRF, the residual blood yielded minimal concentrations of platelets. The mean concentration of platelets obtained was $7.9 \times 10^9/L$ with a median of $8.0 \times 10^9/L$ and a standard deviation of $3.03 \times 10^9/L$. The 95% confidence interval estimate for the mean was (6.76 to 9.32) (**Table 2**).

The platelet concentration of residual blood after removal of Choukroun's PRF (60min after formation, Group B)

As in Group A, blood platelet concentrations were minimal after removal of Choukroun's PRF. The mean concentration of the platelets was $4.0 \times 10^9/L$ with a median of $4.0 \times 10^9/L$ and a standard deviation of $1.93 \times 10^9/L$. The 95% confidence interval estimate for the mean was (3.27 to 4.72) (**Table 2**). The mean platelet concentration of Group B was higher than that determined for Group A.

Table 2: Platelet concentrations for the various groups tested

Groups	Number	Mean and standard deviation in cells $\times 10^9$ /L of blood	Confidence interval
Baseline	30	282.8 \pm 58.27	261.04 to 304.56
Group A (0 Min)	30	7.90 \pm 3.33	6.76 to 9.03
Group B (60 Min)	30	4.00 \pm 1.93	3.27 to 4.72
Difference between Group A and Group B	30	3.90 \pm 3.13	2.73 to 5.07

Analysis of the differences in platelet concentrations of Group A and Group B

From the descriptive analysis, it is clear that Group B yielded lower platelet concentrations than Group A. The mean difference in platelet concentrations between the 2 groups was 3.90×10^9 /L with a median of 3.5×10^9 /L and standard deviation of 3.13×10^9 /L. The 95% confidence interval estimate for the mean was 2.73 to 5.06) (**Table 2**).

Using a non-parametric Signed Rank Test, the statistical significance of the differences in platelet concentrations between the Groups A and B was analysed (**Figure 8**). The difference between the 2 groups was statistically significant ($p < 0.0001$) with a point estimate of the amount of difference of 3.9 units and a 95% confidence interval of 2.7 to 5.1. From the statistical analysis we, therefore, concluded that there is a significant difference in the mean concentration of PRF between time 0 and time 60 minutes.

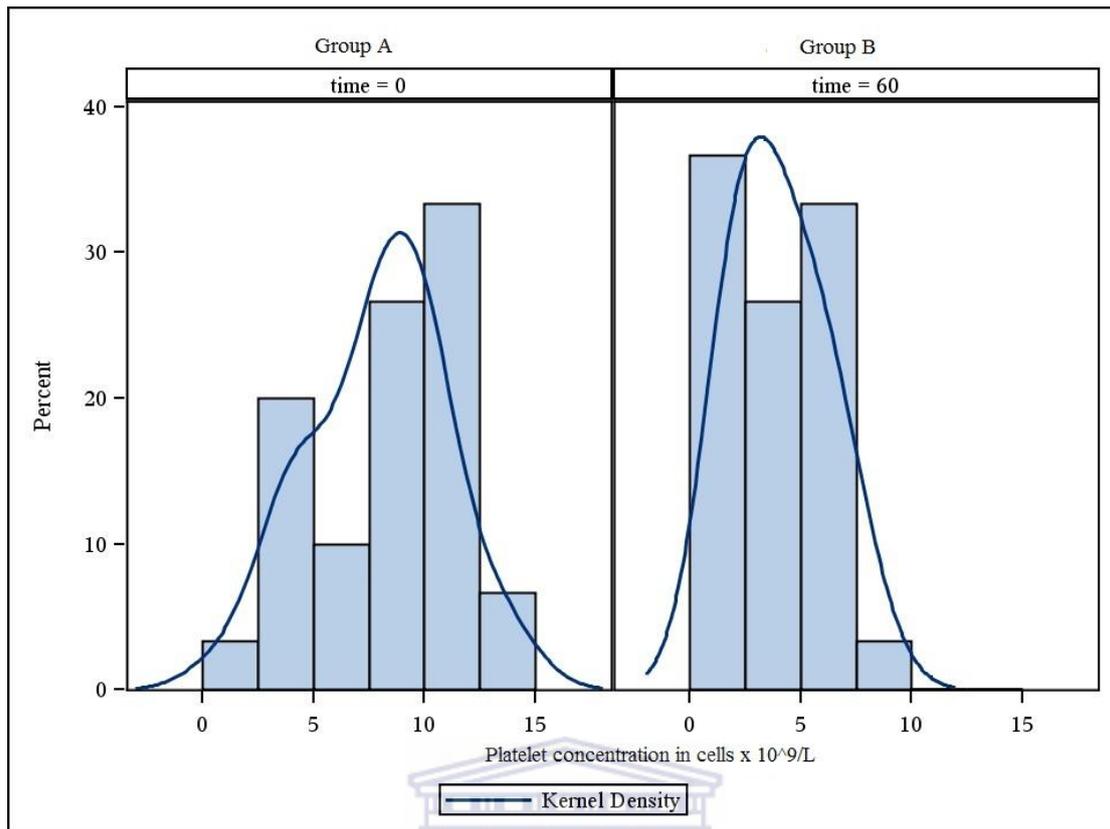


Figure 8: Residual platelet concentration of Groups A and B

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The difference in initial and residual platelet concentration indicates that more than 90% of the available platelets were trapped in the PRF after the blood was centrifuged.

Chapter 6

Discussion and Conclusions

The purpose of this study was to determine whether storage time had any significant effect on the platelet concentration of Choukroun's PRF. Because the structural properties of the PRF clot does not allow for a direct measurement of its platelet concentration, we used the residual platelet values left in the blood tube after removal of the PRF clot, as an indirect measurement of the platelet concentration in the PRF clot. Higher residual values indicate that less platelets were contained in the removed clot whereas lower residual values would indicate that more platelets were transferred to the clot. A similar study was carried out by Dohan Ehrenfest et al (2010b).

From the platelet counts obtained in both test groups it was clear that a significant amount of platelets had been removed from the blood after extraction of the PRF clots. In fact, when differences between the test groups and the baseline blood sample were compared, it was evident that more than 97-98% of the platelets were concentrated in the PRF clot. This observation is similar to results published for previous studies (97%) and thus validates the method of PRF production used in the experiment (Dohan et al, 2006). The natural process of fibrin and clot formation that occurs in the blood collecting tubes results in the entrapment of the majority of the available platelets in a fibrin matrix. This may act as a reservoir for a concentration of growth factors required in the initial stages of wound healing.

Although the physiology of Choukroun's PRF has been studied extensively, very few reports adequately document the ability of this platelet concentrate to be stored. Previous research has indicated that storing Choukroun's PRF under certain conditions may affect its ability to clinically yield positive results. In fact it is not recommended to store the platelet concentrate in its blood collecting tube since it is assumed that it may disintegrate into an unusable form after about 15 minutes of storage time (Dohan Ehrenfest et al, 2010). Instead, several authors recommend storing the agent in a metal dish or a propriety designed storage box. Data regarding the maximal storage time and ideal storage temperature of PRF are largely lacking.

In our study, we used standard blood collecting tubes with clot activators to store PRF for at least 60 minutes at room temperature. This particular length of time was chosen based on the average time of typical periodontal surgical procedures at our facility. The results of the study indicate that by using Choukroun's protocol for platelet concentrate preparation, it was possible to concentrate more than 97% of the available blood platelets into a readily usable form. This study also showed that by using the blood collecting tubes as a storage medium, that there was no detrimental effect on the platelet concentration of Choukroun's PRF. Indeed, storing the concentrate for 60 minutes in these tubes resulted in a form of Choukroun's PRF that had significantly higher concentrations of platelets compared to their non-stored counterparts. The reason for this phenomenon is unknown, but may be related to the "clot activators" that line these tubes. Clot activators are often silica based and are used in plastic tubes to mimic the clotting effect of glass based blood tubes. As a result, a longer contact time between blood and these activators may encourage fibrin activation and clot formation, with subsequent platelet entrapment.

Another reason for variation in platelet concentration seen in this study may be due to the fact that the recommended centrifuge time is too short to allow for complete clot formation to take place. Therefore, allowing the PRF clot to remain in the tube for a period longer than the recommended time, may result in a more complete physiological reaction taking place.

The ability of Choukroun's PRF to sustain its platelet concentrate over the tested time may have significant clinical implications. Rather than drawing blood at least 15 minutes before the PRF is required in a surgical procedure, it allows for blood to be drawn before the start of the procedure, thereby improving patient comfort and operator time. The ability to store the PRF in the same tubes in which it was formed, negates exposure to other environmental factors that may contaminate the sample. It is also cost-effective, since no specialised equipment or storage facilities are required. This may be significant in resource poor settings.

PRF has been recognised as a biomaterial that includes living cells (Dohan et al.,2006). In order to sustain cell viability over time, an isotonic solution is required for their storage. The blood collecting tubes, although not designed to store blood, act as containers for the PRF and the remaining cells and serum. Consequently, by storing PRF in the blood that it was

derived from, the remaining serum, after PRF has formed, acts as a natural isotonic solution that sustains cell survival.

The release of growth factors is a significant property of blood platelets (Kang et al., 2011). Previous studies indicate that several of these factors play an essential role in osteogenesis and periodontal regeneration (Shen et al., 2008; Gassling et al., 2010). By using PRF, the release of these growth factors appears to be constant, and over a longer period of time when compared that seen by PRP (He et al., 2009). In a direct comparison between the two, PRP was shown to have an initial larger release of growth factors after activation. However, these high concentrations were not stable and decreased over time. On the other hand, PRF releases less growth factors initially, but sustains this release for a longer duration (He et al., 2009). A number of authors speculate that this may be due to the fibrin clot that forms a network and acts as a reservoir for the trapped platelets (Dohan et al., 2006; Dohan Ehrenfest et al., 2010b; Toffler et al., 2009). In the present study, it was shown that the platelet concentration of PRF improved over a period of 60 minutes of storage. We assume that this may be due to prolonged fibrin clot maturation. It is therefore reasonable to speculate that if PRF is stored until optimal fibrin formation is achieved, then higher concentrations of growth factors may be available from the PRF during wound healing. Whether this has any clinical significance requires further investigation.

Temperature may affect the storage potential of PRF and criticism has been levelled towards storing it in near-freezing temperatures (Del Corso et al., 2009). In this study, all the samples were stored at room temperature. From the data collected, it was clear that storage under these conditions has no detrimental effect on the platelet concentration of the PRF clot.

Although the present study showed statistically significant differences between the two groups tested, several limitations were also evident. These include the limited number of study participants as well as the inability to directly measure the platelet concentration of PRF. Other factors include the small difference between the platelet concentrations of the groups tested. Although statistically significant, a mean difference of only 3.9×10^9 cells/L may not be clinically relevant and therefore warrants further research to validate the clinical significance of these findings.

Conclusions

The viability of storing PRF in its own blood collecting tubes has not yet been reported. This study showed that by using Choukroun's method of platelet preparation it is possible to concentrate more than 97% of the available platelets into a PRF clot. The study also indicated that, unlike previous reports, clot disintegration does not take place after 15 minutes of storage, but rather remains stable over a period of at least 60 minutes and may in fact increase in platelet concentration during that time. The null hypothesis of this study has therefore been rejected and it may be concluded that storage time does have a significant effect on the platelet concentration of PRF. This was an unexpected finding, and further investigations are required to elicit whether this has any clinical significance.



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